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Awards

Outstanding Achievement Award

Addressing the bottlenecks in gene therapy through synthetic biology and de novo vector design

Thierry VandenDriessche

Department of Gene Therapy & Regenerative Medicine - Free University of Brussels

The advancement in gene transfer technologies has greatly benefited the field of gene therapy. Nevertheless, several bottlenecks have been identified that undermine the efficacy and/or safety of the gene delivery vector, hampering clinical translation. We have gained a better understanding of how to improve the efficacy and safety of gene therapy using different synthetic biology approaches. To achieve this, we explored different strategies based de novo vector design principles, each tackling a specific bottleneck in gene therapy, either at the level of genomic integration, transgene expression or the immune response.

(i) Genomic integration: For the past 25 years, development of non-viral vectors for stable gene transfer into human CD34+ hematopoietic stem/progenitor cells (HSCs) has been unsuccessful. We have overcome this bottleneck by using de novo designed hyperactive Sleeping Beauty (SB) transposase, which led to unprecedentedf stable gene transfer efficiencies in CD34+ HSC. Consequently, xeno-transplantation of these transposon-marked CD34+ HSCs into immune deficient mice yielded long-term engraftment and hematopoietic reconstitution paving the way for new transposon-based gene therapy applications.

(ii) Transgene expression: To improve transgene expression for tissue-directed gene therapy, we validated a data-mining algorithm that led to the identification of combinations of regulatory elements, associated with high tissue-specific expression. Incorporation of these de novo designed, synthetic regulatory elements in viral and non- viral vectors enhanced transcriptional targeting in heart or liver. Up to a 100-fold enhancement in tissue-specific expression could be achieved, depending on the promoter used, while retaining high cardiac and liver selectivity. Vector performance could be enhanced further by using synthetic codon-optimized and/or hyperactive therapeutic genes. The de novo design of these regulatory elements and synthetic transgenes improved the therapeutic index of viral and non-viral gene delivery vectors.

(iii) Immune response: One of the main limitations of using AAV-based vectors is that they can evoke an AAV capsid-specific, T cell -mediated immune response. To reduce the risk of T cell-mediated immune rejection of AAV-transduced cells, we have now generated and characterized a novel "immune stealth" AAV vector that was specifically designed to inhibit antigen presentation. We demonstrated that T-cell activation and T- cell mediated recognition of liver cells transduced with this AAV "immune stealth" vector was substantially reduced which has broad implications for AAV-based gene therapy.

Acknowledgments: I want to thank all of our collaborators and team-members for their valuable contributions. This work was supported by grants from FWO, EHA, Bayer, IWT, VUB GOA, STK, EU FP6 INTHER, EU FP6 CLINIGENE, EU FP7 PERSIST.

Young Investigator Award winners

Restoration of vision by photoreceptor transplantation

Dr Rachael A. Pearson

Department of Genetics, UCL Institute of Ophthalmology, University College London, 11-43 Bath Street, London

Retinal degenerations culminating in photoreceptor loss are the leading causes of untreatable blindness in the Western world. Clinical treatments are of limited efficacy, at best slowing disease progression. As such, there is a clear need for new therapeutic approaches. Gene therapy is effective in the treatment of inherited retinal disease. However, such strategies will be ineffective once degeneration has occurred. Photoreceptor transplantation offers a complementary approach that could not only halt the progression of blindness but also potentially reverse it. In two landmark studies published in Nature, we have demonstrated that, by using donor cells from the early postnatal retina, photoreceptor cell transplantation is possible. The adult retina is capable of integrating trans-

planted cells and these cells develop unambiguous characteristics of mature rod photoreceptors. Moreover, we demonstrated that the cells that possess this capacity to migrate and functionally integrate are post-mitotic rod photoreceptor precursors, rather than stem or progenitor cells. Most importantly, we have provided definitive evidence of restoration of functional rod-mediated vision in adult mice that have congenitally-absent rod function. Together, these findings demonstrate the feasibility of photoreceptor transplantation as a therapeutic strategy for restoring vision following retinal degeneration.

AAV8 gene delivery for organic acidemias: A promising therapy for two orphan diseases

Randy J. Chandler

National Human Genome Research Institute, National Institutes of Health Bethesda Maryland, 20892 USA

Methylmalonic acidemia (MMA) and propionic acidemia (PA) are inherited disorders of propionate metabolism that have significant morbidity and can be fatal despite early detection by newborn screening and medical management with dietary restriction and co-factor supplementation. New treatment options are needed for patients who cannot be managed optimally with current therapies. MMA and PA patients with residual enzyme activity often have better clinical outcomes than patients with no enzyme activity, indicating that gene therapy may be a viable treatment option for these disorders. To test the efficacy of gene therapy, an AAV serotype 8 (AAV8) with an established tropism for the liver was engineered to deliver the transgene because a substantial amount of propionate metabolism occurs in the liver and liver transplantation has been reported to stabilize patients with MMA and PA. A single dose of the AAV8 rescued both the murine models of MMA and PA from neonatal lethality. The treated mice exhibited significant transgene expression and reduced disease related metabolites, and they also appeared to be otherwise healthy. Although there is a significant therapeutic effect, the disease related metabolites are not normalized in mice indicating this treatment is not curative. However, the AAV8 gene therapy developed for MMA and PA can provide increased levels of enzyme activity and metabolic stability for long periods in mice. Therefore, while AAV8 gene therapy may not be curative, it could be of great benefit to patients.

Assessing the Integration Profile of Lentiviral Vectors in Gene Therapy for X-Adrenoleukodystrophy

Cynthia C. Bartholomae[1], Nathalie Cartier[2,3], Salima Hacein-Bey-Abina[2,4], Ina Kutschera[1], Bruno l'Homme[2], Alain Fischer[2,4], Marina Cavazzana-Calvo[2,4], Patrick Aubourg[2,3], Manfred Schmidt[1], Christof von Kalle[1]

[1]German Cancer Research Center and National Center for Tumor Diseases, Heidelberg, Germany; [2]University Paris-Descartes, Paris, France; [3]Hopital Saint-Vincent de Paul, Paris, France; [4]Hopital Necker-Enfants Malades, Paris, France

Lentiviral vectors have been successfully used for the treatment of monogenic disorders by gene therapy. We performed an in depth integration site (IS) analysis of patient samples from the first clinical trial to treat a monogenetic cerebral disease using autologous hematopoietic stem cell transplantation with a HIV-1 based lentiviral SIN-vector. So far, the correction of hematopoietic stem cell has not been accompanied by signs of clonal dominance or even premalignant disproportional distribution of cellular contributions in the 4 treated patients. Large scale IS analysis performed on ex vivo transduced cells prior to reinfusion and on engrafted cells by LAM-PCR and 454 pyrosequencing showed a polyclonal hematopoietic reconstitution, revealing >18.000 unique IS. Downstream bioinformatics analysis revealed the characteristic insertion profile reported for lentiviral vectors, showing gene coding regions as preferred targets for lentiviral vector integration (P1: 74%; P2: 74%; P3: 72%; P4: 71%). Accordingly, a favored integration on chromosomes harboring gene dense regions and regions described for lentiviral clustering, such as KDM2A, PACS1 and HLA genes, has been found. A successful ex vivo transduction of early hematopoietic progenitors is indicated by the presence of identical IS identified in myeloid and lymphoid lineages in P1, P2 and P3 (analysis is ongoing for P4). Lentiviral gene therapy shows to be safe and effective, the cerebral disease has been stabilized in 2 out of 4 patients, the follow-up being too short in the 4th patient to draw any conclusion of clinical efficacy.

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Invited Speakers

INV001

Gene Transfer Strategies: Adenovirus

Florian Kreppel

Department of Gene Therapy, Ulm University, Ulm, Germany

Up to date adenovirus vectors are the most frequently used vectors in gene transfer approaches. Applications range from genetic vaccination to tumor therapy and classic gene therapy.

Starting with basic adenovirus biology this lecture will give an introduction to the different types of adenovirus vectors and will outline their potential as well as their limitations, which define current research strategies to improve this vector type.

INV002

Gene transfer using retrovirus-derived vectors: Current technology and applications

Cecilia Frecha

International Agency for Research on Cancer, Infections Biology Group, Lyon, France

Vectors derived from retroviruses such as lentiviruses and onco-retroviruses are among the most suitable tools to achieve a long-term gene transfer since they allow stable transgene integration and maintain persistent gene expression. Of these, lentiviral vectors (LVs) are considered among the preferred gene delivery vehicles since they have the ability to transduce non-proliferating and differentiated cells such as lymphocytes, dendritic cells or neurons. Moreover, the vast flexibility in the design of the expression cassette and their ability to accommodate heterologous envelope proteins allow tailoring of gene transfer tools with enhanced efficiency and improved biosafety. Features such as tissue-specific promoters, envelope engineering, inducible expression cassettes, integration defective LVs, or site-specific integration account for their increasing use in a multitude of gene therapy applications and clinical trials. Here, we will discuss the current state of the art of LV technology, its impact on biomedical research and implications for human clinical trials.

INV003

Cancer virotherapy, the oncolytic virus approach to treat tumours

Alberto Epstein

Centre de Génétique et Physiologie Moléculaires et Cellulaires, Lyon, France

Oncolytic viruses selectively replicate in and kill cancer cells and spread within the tumour mass while preserving healthy tissues. The notion of preferential killing of tumour cells with viruses is first encountered at the beginning of the 20th century. The idea of cancer virotherapy was explored more thoroughly during the 1950s and 1960, concomitantly with the development of various virus vaccines, when many different virus types, including rabies, mumps, measles, adenovirus, Sendai virus and others, were tested in experimental settings and in humans, in order to investigate their safety profile and therapeutic potential. Unfortunately, not only efficacy of virotherapy during that infancy period was very limited, but also some of these viruses induced severe side effects that ultimately terminated those early trials, and the interest on the oncolytic approach subsequently declined. The attention on cancer virotherapy only rekindled in the 1990s, with the identification of more naturally occurring oncolytic viruses, such as reovirus or parvovirus, or the genetic engineering of others, such as herpes simplex virus type 1 (HSV-1) or adenovirus (AdV). Owing to the current knowledge on the functional organisation of viral genomes, virotherapy represents today an actively researched scientific field, and is becoming a remarkable treatment option alongside surgery, chemo- and radiation therapy. Of all the oncolytic viral vectors under study at present, HSV-1 and adenovirus recombinant viruses are probably furthest along in their development and testing for cancer virotherapy. This talk with focus on HSV-1-derived oncolytic viruses as a paradigmatic model allowing (i) to exemplify how a virus can be engineered to become oncolytic, (ii) to illustrate the different approaches that are currently being developed, (iii) to describe the recent evolution of this field, and (iv) to explain the basic principles of this relatively novel but clearly valuable therapeutic strategy and its current convergence with immunotherapy approaches.

INV004

Non viral gene delivery techniques

Daniel Scherman[1,2]

[1]*Laboratoire de Pharmacologie Chimique et Génétique, U640 INSERM – UMR 8151 CNRS ;, Faculté de Pharmacie, Paris, France,* [2]*Ecole Nationale Supérieure de Chimie de Paris, Paris, France*

Plasmid DNA for in vivo gene therapy or ex vivo cell delivery can be administered into cells by using either chemical vectors or physical delivery techniques.

Chemical vectors: Chemical vectors are generally cationic. They are used: 1) to compact the DNA for a better cellular penetration and protection from nucleases; 2) to promote the cell membnrane adhesion of DNA which, because of its anionic charge undergoes an electrostatic repulsion from the plasmatic membrane which is itself anionic; 3) to promote the DNA transfer through the plasmatic or endosomal membrane; 4) eventually to promote DNA nuclear uptake, when trying to transfect quiescent cells.

Strikingly, in vitro is not predictive of in vivo gene transfer by cationic lipids. In particular, cationic lipids are strongly inhibited

by serum components. Solutions to this problem will be presented.

New avenues concerning the delivery of SiRNA will be presented.

Gene delivery by physical techniques. Electrotransfer

Several techniques have been investigated to deliver genes by physical techniques. They include: 1) Naked DNA; 2) Gene gun; 3) Hydrodynamic (tail vein, arterial or leg vein injection); 4) Electrotransfer; 5) Focused ultrasound mediated gene delivery; 6) Laser induced plasmid delivery. After a brief description of these different techniques, the lecture will focus on electrotransfer.

Gene delivery to skeletal muscle and to tumors is a promising strategy for the treatment of muscle disorders or cancer, and for the systemic secretion by muscle of therapeutic proteins. We and others had reported very efficient plasmid DNA transfer in muscle fibers using square-wave electric pulses of low field strength and of long duration. This intramuscular «electrotransfer» method increases reporter and therapeutic gene expression by several orders of magnitude in various muscles and species, and decreases inter-individual variability. The four following aspects will be reviewed: 1) Mechanism.; 2) Sustained plasmatic protein secretion. 3) I.M. electrotransfer of EPO encoding plasmid in beta-thalassemic mouse; 4) Non viral gene therapy by electrotransfer of hTNF-α soluble receptor-I variants and its application to the treatment of experimental arthritis.

INV005

Controlling vector tropism by transductional targeting

Els Verhoeyen

Université de Lyon; Inserm, EVIR, U758, Human Virology Department; Ecole Normale Supérieure de Lyon; Université Lyon 1, 69007 Lyon, France

Transductional targeting relies on the modification of the vector surface by incorporation of foreign envelope glycoproteins that have a natural restricted tropism. The vector specificity is than determined at the level of cell entry and therefore only cells carrying the specific receptor will be transduced.

The process, which alters the tropism of the vector, is called pseudotyping. Very often, the G protein of vesicular stomatitis virus (VSV-G) is used to pseudotype lentiviral vectors (LVs) because it is highly stable and it recognizes a receptor, which is ubiquitously expressed in mammalian cells.

Selective tropisms were achieved by using surface glycoproteins derived from viruses like Ebola virus or Influenza virus for pseudotyping, which proved useful for studies of viral entry or vaccination. Importantly, several viral gps target LVs to the central nervous system. Pseudotyping of the LVs with the E1 and E2 glycoproteins from the hepatitis C virus demonstrated HCV-specific selectivity for hepatocytes. Cat and baboon retroviral glycoprotein pseudotyped LVs proved efficient for HSC, T and B cell gene transfer. Interestingly, LVs incorporating the measles virus envelope (MV-LVs) transduced resting T- and B-cells, which are not permissive for polytropic VSV-G-LVs. Also an improved transduction of immature DCs was established with the MV-LVs. Thus, these novel MV-LVs represent excellent tools to study T- and B-cell and DC functions and make T- and B-cell and DC gene therapy and immunotherapy applications more feasible.

The objective is to demonstrate the power of transductional LV targeting for very diverse applications.

INV006

Exploiting microRNA Regulation for Genetic Engineering

Bernhard Gentner

San Raffaele Telethon Institute for Gene Therapy, Milano, Italy

RNA interference (RNAi) has been a landmark discovery in science. Making use of the tissue-, lineage-and differentiation stage-specificity of some endogenous microRNAs (miRNA), RNAi can be exploited to negatively regulate transgenes tagged with artificial miRNA target sequences. This has significantly expanded the regulatory potential of gene transfer vectors, and several specific applications for miRNA-regulated vectors/viruses have been proposed for basic research, gene- and virotherapy. Hematopoieis can be regarded as a paradigmatic example in which miRNA expression dynamically changes during lineage specification and differentiation. This presentation will outline how microRNA activity can be harnessed to negatively regulate transgene expression in distinct hematopoietic subpopulations and – in combination with appropriate transcriptional control elements – achieve tissue- and lineage restricted expression profiles with unprecedented specificity. We constructed "HSC-off" vectors lacking expression in hematopoietic stem cells as opposed to differentiated cells, thus allowing the delivery of a transgene into HSC without altering their proteome, while benefitting from sustained multi-lineage expression in the progeny. The utility of the "HSC-off" approach will be discussed for 2 genetic diseases: globoid leukodystrophy and chronic granulomatous disease, examples for overt and suspected transgene toxicity upon de novo expression of the corrective cDNAs in HSC, respectively.

INV007

Retroviral and lentiviral Integration sites study

Christof von Kalle

National Center for Tumor Diseases, Heidelberg, Germany

See additional abstracts section.

INV008

Clonality studies in gene therapy

Christopher Baum

Institute of Experimental Hematology, Hannover Medical School, Carl-Neuberg-Str.1, Hannover, Germany

New generations of integrating gene vectors promise increased potency in the genetic modification of transplantable cells ex vivo and for direct application in vivo. Retrovirus-based vectors (including alpharetroviral, gammaretroviral, lentiviral and spumaviral families) and transposon-based vectors (including Sleeping Beauty and Piggybac, among others) share the property of efficient but untargeted integration of the genetic cargo into cellular chromosomes, however with significant differences of the integration pattern depending on the nature of the integrase/transposase used. The untargeted integration profile introduces a delicate genetic mosaic in the target cell population, which is highly useful for clonality studies but might also drive functional heterogeneity with the extreme and highly undesirable end product of malignant transformation. This educational

talk addresses some of the burning questions in clonality studies related to the field of gene therapy: What are the benefits and risks associated with the untargated integration profile of integrating gene vectors? How can we measure clonality and what does it tell us? Which experimental limitations have to be considered in the interpretation of clonality data? What is the importance of secondary events in clonal progression towards malignant transformation? How frequent are dangerous mutational events related to vector integration and what are the consequences for the development of preclinical safety assays? And finally, which factors might prevent the uncontrolled expansion of single clones?

INV009

Methods for iPS Cell Generation and Quality Control Assesment

Olivier Féraud, Emilie Gobbo, Dominique Divers, Noufissa Oudrhiri, Frank Griscelli, Annelise Bennaceur-Griscelli

« ES-Team » Paris-Sud/Inserm U935, Ingestem Consortium, Campus CNRS de Villejuif, 7 rue Guy Moquet, 94800, Villejuif, France

The first generation of induced pluripotent stem (iPS) cells has been developed mainly by viral delivery of the reprogramming genes into the somatic target cell. However, viral integrations into the host genome are mutagenic and can induce unpredictable side effects. Moreover, a lack of extinction of the viral sequences is often observed, leading to ectopic expression of the transgene along differentiation. The development of non-integrative strategies for reprogramming represent a major step in iPS technology, not only for the safety but also for the quality of the cell lines developed. Now the use of iPS cells for cell-based therapies could be reasonably considered, provided that we develop highly efficient differentiation protocols and relevant quality controls. We will discuss (1) the various methods available for the generation of iPS cell lines, particularly as a function of the nature of the somatic target cell, (2) the quality controls required before and after reprogramming.

INV010

Application of induced Pluripotent Stem Cells in Hematological Genetic Diseases

José C. Segovia

Differentiation and Cytometry Unit. Hematopoiesis and Gene Therapy Division. Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT) and Centro de Investigación Biomédica en Re, Madrid, Spain

New advances in cell and gene therapies are speeding up regenerative medicine. Due to their acquired pluripotency, induced-Pluripotent Stem Cells (iPSCs) are a promising source of autologous cells for regenerative medicine. These cells show unlimited self-renewal while retain the ability to differentiate into any cell type of the human body. Since Yamanaka et al first reported the generation of human iPSC in 2007 a big effort has been made to understand the reprogramming process and to develop clinically safe iPSC-derived progenitor cells. The generation of patient specific iPSC has improved the understanding of the biological basis of several human diseases. Moreover, the gene correction of these cells might allow their clinical application in future. Nevertheless, available technologies to generate iPSCs have also non-desired effects. The main bottlenecks for the clinical application of this technology are the difficulties for generating functionally differentiated cells, and also the genetic instability associated to the reprogramming process. The use of non-integrative systems to avoid genotoxic effects of cell reprogramming constitutes a relevant approach that should improve the safety of cell reprogramming. Additionally, the self-renewal and maintenance of the pluripotency ability of iPSCs has opened the development of strategies to allow specific gene editing by means of homologous recombination (HR), avoiding potential side effects associated to conventional gene therapy. In this respect, recent reports have shown targeted gene correction in iPSCs generated from patients with different genetic diseases. The use of DNA-Nucleases to improve the efficacy of HR at specific loci of the human genome is now opening new possibilities for the application of HR-based gene therapy approaches in the clinics. Nevertheless, also in this case, undesirable mutagenesis due to integration in off-target loci can take place. All these aspects will be discussed.

INV011

ADIPOA project: adipose derived stroma cells for osteoarthritis.

Christian Jorgensen

Inserm, Montpellier, France

Osteoarthritis (OA) is a degenerative joint disease, with loss of matrix, fibrillation, formation of fissures, and ultimately complete loss of the cartilage surface, for which no efficient therapy is available. OA is a clinical syndrome of joint pain accompanied by varying degrees of functional limitation and reduced quality of life, with strong impact on quality of life of healthy aging population in European countries. Cartilage is known to be populated by chondrocytes but also in a limited number of cells with characteristics of mesenchymal stromal cells and side population. Culture expanded adipose derived stromal cells CD73/CD90/CD105 triple positive subpopulation had multipotency for chondrogenic, osteogenic and adipogenic differentiation. We have shown that stromal cells, in particular adipose derived stromal cells have an immuno-modulatory and anti-fibrotic activity, protect cells for oxidative stress through secreted growth factors. We have shown that Intra-articular injections of mesenchymal stromal cells prevent the development of osteoarthritis in two large animal models, but the mechanism are unknown, and the role of endogenous stem cells present in the cartilage is elusive.

The **objectives** of this collaborative FP7 project are to establish stem cell based regenerative medicine in Rheumatology. For this, we have performed preclinical experiments and toxicology studies using increasing dose of ADSC and we propose to validate new concepts for OA therapy in open phase 1 dose escalading clinical trials using autologous ASC focusing on symptomatic OA according to EMEA guidelines. We demonstrate that autologous ASC are optimal candidates to stimulate the regeneration of injured cartilage.

INV012

No abstract available

David Klatzmann[1,2]

[1]UPMC Univ Paris 06, CNRS, UMR 7211, INSERM, UMR_S 959, Immunology-Immunopathology-Immunotherapy (I3), Paris, France, [2]Hôpital Pitié-Salpêtrière, Biotherapy, Paris, France

No abstract available

INV013

Weal and woe of anti-AAV immune responses

Hildegard Büning[1]

[1]Center for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, Germany, [2]Department I of Internal Medicine, University Hospital Cologne, Cologne, Germany

AAV was initially identified as a satellite virus of adenovirus some 50 years ago. Since then, adeno-associated viruses (AAV) have become one of the most widely used tools for gene delivery in human clinical trials, the AAV vector system. As expected of a virus that requires helper virus function for progeny production, AAV remains hidden in the absence of adenovirus or herpes virus co-infection and thus induces only weak or transient innate immune responses. Two innate immune sensors of the Toll-like receptor (TLR) family, namely TLR-2 and −9, have been recently identified that induce inflammatory responses upon sensing the viral capsid and genome, respectively. Although nearly all cell types in the human body express innate immune sensors, they differ in their specific repertoire and level of expression. Immune sensing of AAV therefore occurs only in certain cell types involving distinct signaling pathways. Nevertheless, innate immune responses induced by AAV are sufficient to activate adaptive immune responses as indicated by pre-existing neutralizing antibodies and memory T cells that pose a continuous challenge for its clinical application. Strategies under development to overcome this obstacle will be discussed.

In gene therapy approaches vector induced immune responses are unwanted. In contrast, gene-delivery based vaccines rely on the induction of specific immune responses. The use of AAV vectors has recently been expanded to this area. We will briefly visit the area of vaccine development focusing in particular on strategies exploited to enhance the efficacy of AAV vector-based strategies.

INV014

Immune responses to recombinant adenovirus

Karim BENIHOUD[1,2]

[1]Uniiversity Paris-Sud, Orsay, France, [2]CNRS UMR 8203, Villejuif, France

Adenoviruses have been largely used as vectors for gene transfer in preclinical studies and clinical trials. However, their high immunogenicity constitutes a major limit to their use in gene therapy. Indeed, adenovectors are able to trigger a complex immune response, both innate and adaptive, that leads to the destruction of transduced cells and reduces the duration of transgene expression but also impairs efficient virus re-administration. In addition, the high seroprevalence of neutralizing antibodies to several adenovirus serotypes preclude any efficient gene transfer in humans.

Engineering the virus to reduce (early gene deletion) or to abrogate (helper-dependent adenovirus) viral gene expression led to a reduction of specific cellular immune responses with only minor effects on humoral and innate immune responses. Whereas the use of adenovectors for treatment of genetic diseases awaits further progress in clinical-grade less-immunogenic (devoid of all viral genes) vectors, adenovectors deleted for early genes are currently evaluated in cancer treatment and vaccine clinical trials.

The presentation will review recent advances in molecular and cellular mechanisms involved in adenovirus-mediated innate immune responses. In addition, we will present approaches that have been developed to avoid anti-adenovirus neutralizing antibody response. Finally, the interest of these findings for vaccination strategies will be discussed.

INV015

Safety Testing of High Titre Adenovirus

Daniel Galbraith

BioOutsource, Glasgow, UK

Gene Therapy trials in humans cannot proceed without adequate safety testing to ensure that the product is well characterised and is free from contaminating bacteria or viruses. The testing profile of a product is governed by a number of regulations in Europe and also the USA. The type of virus vector, cells used in production and the production methodology itself all contributes to the risk factors of a product and these are reflected in the choice of tests. Today many virus products are used at much higher titres of infectious virus than in the past, sometimes 2–3 logs higher. This can put a considerable burden on the testing methods used. The talk will consider Adenovirus as an example and will used some case studies of actual products to provide an insight into the pro's and cons of testing methodologies to provide a dossier which will be acceptable from a regulatory perspective. Moving forward there are a number of newer methods which might provide a faster and more cost effective means of achieving a safe product. In addition there is the risk of novel viruses being discovered which will need to be monitored.

INV016

Dealing with regulatory authorities

Nicolas FERRY

ANSM, Saint Denis, France

Making a new treatment available to patients is the ultimate goal of many young scientists in the field of gene and cell therapy. However the road from bench to be dside is long and rough. Interacting with regulatory authorities is often seen as a compulsory but sometimes devastating process which could annihilate years of scientific efforts. The aim of this talk is to give scientists some insights to abetter understanding of the rationale and the organisation of European regulation. The aim of a regulatory body is to protect the health of the population and to evaluate carefully the benefit/risk ratio of any new treatment. Any innovative medicinal product will have to go through a number of steps before being granted a marketing autHorisation. This includes, pre-clinical studies, biosafety studies, and a number of clinical trials from first-in-man adminisntration to large phase 3 efficiency studies. Gene and cell therapy products have some particularities and a specific regulation has been

created at the European level for these advances in therapeutic medicinal products (ATMPs). Because of the specific nature of these products that are at the cutting edge of medical science, regulation is striving to improve in parallel with scientific discovery. Therefore it is important to understand that discussions with regulatory authorities should be part of the development process right from the very beginning.

INV017

The clonal repertoire of gene modified cells in humans

Christof von Kalle

National Center for Tumor Diseases Heidelberg and German Cancer Research Center, Department of Translational Oncology, Heidelberg, Germany

Over the last decade hematopoietic stem cell (HSC) gene therapy was shown to be a promising and successful therapeutic option for certain congenital diseases such as X-linked severe combined immunodeficiency, adenosine deaminase deficiency, chronic granulomatous disease, or Wiskott-Aldrich syndrome. However, this progress has been accompanied by reports on malignant transformation following retroviral gene transfer into HSCs in several clinical gene therapy trials. Five patients of an otherwise successful X-linked severe combined immunodeficiency (SCID-X1) gene therapy trial acquired leukaemia due to insertional activation of cellular proto-oncogenes. To assess the clonal repertoire of these genes in modified human cells we have developed and performed qualitative and quantitative in vivo monitoring tools. However, the challenge that remained was the analysis of this vast amount of sequence information. Therefore, we have developed automated bioinformatics analysis tools which rapidly process and annotate vector integration sites. We were able to investigate the clonal situation in multiple preclinical trials and four clinical gene therapy trials as well as in one gene marking study and detected different possible clonal repertoires ranging from subtle and not clinically overt effects to clonal dominance and leukemogenesis. Unequivocally clonal dominance has been shown in a clinical trial of ADA-SCID and in successful CGD gene therapy trial. Further, we investigated the clonal repertoire in two SCID-X1 trials. While in one study, individual integrants in or near LMO2 were found five times, in the second trial only one integrant in LMO2 was detected. In a gene therapy trial for chronic granulomatous disease (CGD), we also have observed insertional side effects resulting in activation of MDS1/EVI1, PRDM16 or SETBP1 five months after therapy. Furthermore, we have intensively studied the integration site profile in nine of ten patients enrolled in a clinical gene therapy trial for WAS. A highly polyclonal reconstitution of modified HSCs was detected by the analysis of more than 70000 integration sites. A clustering in the proto-oncogenes MDS1-EVI1, LMO2, CCND2 and PRDM16 could be elucidated. Four patients developed T-ALL 16 months to 5 years after gene therapy harbouring an integration site within or close to the proto-oncogene LMO2. Repertoire studies were performed in new vector families (ZNF, TALEN) and for the analyses of DSB from any course.

One decade after a first draft of the human genome by the "Human Genome Project" the combination of highly sensitive PCR-based techniques with new sophisticated sequencing technologies and an optimized bioinformatics high-throughput integration site analysis pipeline enable us to intensively in depth study the clonal inventory and pharmacokinetics in clinical gene therapy studies.

INV018

In vivo genome editing as an approach to the treatment of genetic disease

Xavier M. Anguela[1,2], Rajiv Sharma[1], Hojun Li[1], Virginia Haurigot[1], Anand Bhagwat[1], Robert Davidson[1], Shangzhen Zhou[1], Yannick Doyon[3], Philip D. Gregory[3], Michael C. Holmes[3], Katherine A. High[1,2]

[1]*The Children's Hospital of Philadelphia, Philadelphia, PA, USA,* [2]*Howard Hughes Medical Institute, Philadelphia, PA, USA,* [3]*Sangamo Biosciences, Richmond, CA, USA*

AAV-F9 gene transfer has been successful in hemophilic adults, converting severe to mild hemophilia. However, AAV episome-derived expression is likely to diminish in the growing liver of an infant patient. With site-specific genome modification, correction is stably introduced into the genome and is passed to all daughter cells. We have previously shown that ZFN-mediated genome editing can correct hemophilia in a neonatal mouse model where the proliferation of hepatocytes may promote genome editing through homology directed repair. More recently we sought to determine whether ZFN-mediated genome editing is feasible in adult animals with predominantly quiescent hepatocytes. I.V. injection of the AAV-ZFN and AAV-Donor, containing a promoterless factor IX insert flanked by homology arms to the target site, into adult mice resulted in stable (>30wk) circulating F.IX levels of ~30% of normal, whereas mice receiving ZFN alone or AAV-Luciferase and AAV-Donor exhibited F.IX levels below detection (<15 ng/mL). Importantly, treated mice lacking the h*F9*mut gene averaged less than 100 ng/mL, suggesting that F.IX expression derived from on-target genome editing. PCR analysis of liver genomic DNA from treated mice revealed evidence of targeted gene addition by both homology directed repair and non-homologous end-joining. Induction of liver regeneration following hepatectomy did not decrease hF.IX expression. Finally, activated partial thromboplastin time, a measurement of clot formation, was corrected to wild-type levels in ZFN+Donor treated hemophilic mice. These findings substantially expand the potential of ZFN-mediated genome editing as a therapeutic modality, and suggest that further study of the mechanism(s) of ZFN-mediated genome editing is warranted.

INV019

Targeted gene correction in a mouse disease model

Toni Cathomen

Laboratory of Cell and Gene Therapy, Center for Chronic Immunodeficiency, University Medical Center Freiburg, Freiburg, Germany

Severe combined immunodeficiencies (SCID) are the most severe form of inherited blood disorders. Failure of the adaptive immune response is due to the absence of T and/or B/NK cells. The NOD.SCID mouse is a valuable model for human SCID in general and for radiosensitive SCID in particular. The T^-/B^- immunophenotype is caused by a point mutation in exon 85 of the *prkdc* gene, which encodes for the DNA repair factor DNA-PKcs. To correct the underlying mutation, we generated zinc-finger nucleases (ZFNs) that target intron 84 of the *prkdc* locus with the aim to restore DNA-PKcs function by integrating a donor DNA encompassing a splice acceptor followed by exons 85/86 and a selection cassette. Genetically corrected NOD.SCID fibroblasts revealed restored DNA-PK dependent signaling and reduced

sensitivity to DNA damage. Next, we generated NOD.SCID-derived induced pluripotent stem cells (iPSCs). Selected clones contained an intact karyotype and gave rise to all three germ layers in teratoma assays. Upon ZFN-based correction, ~90% of iPSC clones showed targeted integration of exon 85/86. In T cell differentiation assays, both corrected and uncorrected iPSC clones could be differentiated to DN2-stage T cells (CD44+/CD25+) *in vitro*, but only corrected cells gave rise to double-positive CD4+/CD8+ T cells, suggesting that DNA-PK dependent T cell receptor recombination was reestablished. In conclusion, our findings demonstrate that ZFN-based genome engineering can be applied to correct the RS-SCID phenotype in iPSCs by restoring DNA-PK dependent signaling and hence may present a paradigm for the generation of patient-derived T cell therapeutics.

INV020

Gene therapy for primary immunodeficiencies

Alessandro Aiuti

HSR-TIGET, San Raffaele Scientific Institute and University of Rome Tor Vergata, Rome, Italy

Primary immune deficiencies (PID) are inherited disorders of the adaptive and innate immune system characterized by severe infections, autoimmunity and high risk of cancer. Hematopoietic stem cell (HSC) transplantation from allogeneic donors is the treatment of choice, but in the absence of an HLA compatible donor, its outcome can be limited by complications and suboptimal reconstitution. In the past 15 years, over 75 patients affected by PID have received HSC gene therapy using gammaretroviral vectors, resulting in immunological reconstitution and clinical benefit in the majority of patients. However, the occurrence of insertional oncogenesis in SCID-X1, chronic granulomatous disease (CGD) and Wiskott-Aldrich Syndrome (WAS) trials has led to the development of approaches based on self-inactivating integrating vectors that can achieve more robust correction with less risk of insertional mutagenesis. Cooperative efforts at the European level, also in the context of the CELL PID consortium are contributing substantially to the development of novel technology and the implementation of new clinical trials. Monitoring vector insertions in these patients is providing crucial information not only on the safety of gene transfer but also on the biology of HSC. Here I will discuss the experience and perspectives of gene therapy for ADA-SCID and WAS conducted at HSR-TIGET and novel approaches of gene therapy for X-CGD which include transcription and post-transcriptional regulation of the transgene.

INV021

Gene therapy for ADA-deficient severe combined immune deficiency

Donald Kohn[1], Denise Carbonaro[1], Kit Shaw[1], Xiangyang Jin[1], Sabine Geiger[1], Suparna Mishra[1], Aaron Cooper[1], Satiro DeOliveira[1], Rob Sokolic[2], Fabio Candotti[2]

[1]*University of California, Los Angeles, Los Angeles, California, USA,* [2]*National Human Genome Research Institute, NIH, Bethesda, Maryland, USA*

We performed a Phase I trial of gene therapy for ADA-deficient SCID, using two different γ-retroviral vectors (RV) to transduce bone marrow CD34+ cells. Four subjects did not receive cytoreductive conditioning, remained on PEG-ADA enzyme replacement therapy (ERT), and realized minimal engraftment of gene-corrected cells. Six subjects received busulfan (65–90mg/m2) and ERT was discontinued prior to transplant. Higher levels of gene corrected cells were observed (PBMC > granulocytes) and several have protective immune reconstitution. Parallel studies in the ADA gene knock-out mouse indicated a critical role for non-myeloablative conditioning for successful immune reconstitution, but no adverse effects from continuing PEG-ADA ERT for 1–4 months after gene therapy. In a Phase II trial, we treated eight ADA-deficient SCID subjects, using only the MND-ADA γ-RV with 90mg/kg busulfan. Most of these subjects were infants and immune reconstitution was more robust than in older subjects. We have observed oligo-polyclonal integration sites, with no increase in common integration sites when comparing CD34+ cells prior to transplant and PBMC over the first two years. A next trial will use the EFS-ADA lentiviral vector developed by HB.Gaspar and A.Thrasher at University College, London. Pre-clinical studies showed highly effective ADA gene transfer to bone marrow CD34+ cells, with ADA expression in human T cells produced in transplanted NSG mice at levels similar to the MND-ADA γ-RV. In vitro insertional mutagenesis assays yielded significantly reduced colonies formed by EFS-ADA transduced murine lin- cells (none), compared to γ-RVs.

INV022

Development of Gene therapy for HLH due to perforin deficiency and for XLP1

Bobby Gaspar[1], Marlene Carmo[1], Paritha Arumugam[2], Maria Alonso-Ferrero[1], Axel Schambach[3], Christopher Baum[3], Kimberly Risma[2], Punam Malik[2], Michael Jordan[2], Christine Rivat[1], Claire Booth[1], Adrian Thrasher[1]

[1]*UCL, London, UK,* [2]*CCHMC, Cincinnati, USA,* [3]*MHH, Hannover, Germany*

Gene therapy has now been developed and implemented in clinical trials for a number of specific monogenic immunodeficiencies. We now report the development of lentivral mediated stem cell gene therapy for two disorders where there is a significant abnormality of effector lymphocyte funtion.

HLH is a devastating disorder arising from defects in T and NK cell cytotoxic function. Mutations in the perforin gene account for approximately 40% of all cases. Using a mouse model that recapitulates accurately the human manifestations of the disease we demonstrate that following lentiviral vector mediated perforin gene transfer into murine progenitors, transplanted recipient mice show recovery of cytoxic function in both T and NK cells. Furthermore, when challenged with LCMV, these mice are able to resist the onset of HLH demonstrating in vivo reconstitution. Introduction of perforin in murine stem cells did not adversely affect lymphocyte development.

In XLP1 mutations in the gene encoding the intracellular adaptor molecule SAP leads to defects of T and NK cell function, NKT cell development and of T dependent humoral responses. In a murine model of the disease, we have been able to demonstrate that lentiviral vector mediated stem cell therapy leads to recovery of NK cells, restoration of NK cell cytotoxicity and also following vaccinaton with T dependent antigens, the restoration of specific humoral responses with appropriate developement of splenic germinal follicles.

These studies demostrate for the first time proof-of-principle for corrective gene therapy in these two immunodeficiency diseases and allow further development towards clinical implementation.

INV023

Phase 1 gene therapy trial for pancreatic cancer: from bench to bedside

Louis Buscail

Department of Gastroenterology, INSERM UMR 1037 & Center For Clinical Investigation Integrated in Biotherapy CIC BT511, CHU Rangueil, Toulouse, France

Pancreatic adenocarcinoma is the fifth leading cause of cancer related deaths in Western countries. The sole curative treatment is surgical resection. Unfortunately, curative surgery is possible in only 10 to 15 % of cases, the remaining patients can be treated with palliative chemotherapeutics such as Gemcitabine and Folfirinox in locally advanced and metastatic tumors, respectively. These treatments may ameliorate some clinical parameters but modestly impact on median survival. It is therefore extremely urgent to develop new therapeutic strategies for this cancer. In this context, we are currently performing a unique clinical trial at the forefront of research and medicine that may revolutionize the therapeutic management of patients with advanced pancreatic cancer.

We have established a consortium between academic groups (INSERM, CHU of Toulouse) and Cayla-InvivoGen company. We previously demonstrated that combining sst2 (encoding for somatostatin receptor subtype 2) and DCK::UMK (encoding for enzymes that phosphorylate gemcitabine at the intracellular level) gene transfer in pancreatic cancer models (in vitro and in vivo) strongly impairs the proliferation of cancer cells, sensitizes tumor cells to chemotherapy, inhibits tumor angiogenesis and metastasis, and induces tumor cell death by apoptosis, with significant regression of very aggressive primary pancreatic tumors. This proof of concept led us to conduct pre-clinical studies with assessment of in vitro / in vivo biological effects, toxicology, and biodistribution of the pre-GMP and GMP experimental gene therapy product (a lyophilize medication of complexed therapeutic plasmid: CYL-02) developed by Cayla-InvivoGen. Following these pre-clinical studies, a pilot Phase I clinical trial of gene therapy for pancreatic cancer was started. (http://clinicaltrials.gov/ct2/show/NCT01274455). Patients with unresectable pancreatic adenocarcinoma were included for evaluation of feasibility, safety of transgastric or transduodenal intra-tumoral gene delivery (in escalating doses) guided by endoscopic ultrasound combined with standard gemcitabine treatment. We are currently completing the inclusion of patients and the preliminary results obtained so far demonstrate the excellent safety and tolerability profile of the gene therapy product. Analysis of disease progression, progression free survival and biodistribution are in progress. In conclusion, the potential benefits for basic cancer research, medicine and public health of this translational research program are numerous. As existing treatment offer little benefit, THERGAP is the first gene therapy trial that may give therapeutic perspectives for the treatment of pancreatic cancer or other human solid tumors.

INV024

Oncolytic Vaccinia induces programmed necrotic cell death in ovarian cancer

Lynsey Whilding, Kyra Archibald, Daniel Oberg, Iain McNeish

Barts Cancer Institute, Queen Mary University of London, London, UK

Evasion of cell death is one of the hallmarks of cancer and multiple mechanisms have been postulated to explain this evasion and the consequences for chemotherapy effectiveness. A greater understanding of how any novel cancer therapy kills malignant cells, and how resistance might occur, should be a vital element in preclinical development. Oncolytic viruses, including Vaccinia, were long assumed to induce apoptosis. However, the events preceding cell lysis and the mechanisms by which vaccinia induces tumour cell death are poorly understood. We have evaluated various pathways of cell death in ovarian cancer cells following infection with both wild type vaccinia and modified vaccinia virus, Lister-dTK.

We show that Lister-dTK induces cell death in ovarian cancer that is accompanied by some features of apoptosis, including accumulation of sub-G1 DNA and phosphatidylserine externalisation. Evidence of aberrant autophagic activity is also present, although inhibition of both apoptosis and autophagy fails to attenuate virus-induced death. Our data indicate that vaccinia induces necrosis, marked by a decline in intracellular ATP and altered mitochondria metabolism, leading to membrane rupture and release of HMGB1. This necrosis appears in part programmed, as co-immunoprecipitation reveals a RIP1/caspase-8 complex associated with programmed necrosis. In addition, pharmacological inhibition of both RIP1 and MLK1, a substrate downstream of RIP3, significantly attenuates cell death.

These results suggest that vaccinia virus causes necrotic cell death in ovarian cancer cells, and may be mediated through a programmed series of events. This may have important implications on the design of combination therapies including oncolytic vaccinia.

INV025

A novel RNAi-based treatment for pancreatic cancer (PC): pre-clinical & clinical results

Talia Golan[3], Ayala Hubert[1], Amotz Shemi[2], Elina Zorde Khvalevsky[2], Racheli Gabai-Malka[2], Gili Focht[2], Zivia Brunschwig[2], Stephen Raskin[3], Nahum Goldberg[1], Eli Ben-David[1], Tamar Peretz[1], Rami Eliakim[3], Alan Dankur[5], Eithan Galun[1,2], Itzik Rachmur[2], Avi Domb[2,4], Yael Kopelman[1], Yael Hantz[1], Mor Lahav[1], Sagit Arbel-Alon[1]

[1]Hadassah Hebrew University Hospital, Jerusalem, Israel, [2]Silenseed Ltd, Jerusalem, Israel, [3]Sheba Medical Center, Tel Aviv, Israel, [4]Hebrew University, Jerusalem, Israel, [5]Shaare Zedek Medical Center, Jerusalem, Israel

The major challenge for oligonucleotides therapeutics is delivery. Local prolonged delivery potentially overcomes this hurdle. PC is an aggressive disease. Genetic alterations in KRAS signaling pathway are involved in 90% of PC cases, the majority of KRAS mutations are gain-of-function mutations at codon 12 ($KRAS^{G12D}$). The tumor is addicted to $KRAS^{mt}$. The goal of this study was to investigate the impact of *kras* oncogene silencing on PC. We developed a controlled regional drug delivery system by design of a miniature biodegradable polymeric matrix that encompasses anti$KRAS^{G12D}$ siRNA drug, named *siG12D LODER®* (sL). This LODER releases the drug regionally within a pancreatic tumor, during three months. Treatment of pancreatic cells with sL resulted in a significant inhibition of KRAS mRNA and protein levels. This was associated with a decrease in cell proliferation, reversing EMT and inducting cell death. *In vivo* the growth of human PC cell lines was retarded, in subcutaneous and orthotopic models. The survival of mice implanted with sL was significantly improved. Our data reveals: 1) the sL efficiently overcomes current siRNA delivery obstacles related to systemic

approaches. The LODER pharmaco-kinetics enables dose reduction by orders of magnitudes and eliminates toxicity. 2) the strategy of local *kras* targeting by sL can be effectively used. We have initiated a phase I study with sL implanted into patients with locally advanced PC. Twelve patients were included in this study. No major safety issues were found. We observe significant and promising results of tumor marker decrease and tumor shrinkage.

INV026

Gene therapy for Duchenne muscular dystrophy: microdystrophins, oligonucleotides and endonucleases

George Dickson

Royal Holloway University of London, London, UK

Muscular dystrophies refer to a group of inherited disorders characterized by progressive muscle weakness, wasting and degeneration. So far, there are no strongly effective treatments but new gene-based therapies are currently being developed with particular advances in using conventional gene replacement strategies, RNA-based approaches, or cell-based gene therapy and with a main focus on Duchenne muscular dystrophy (DMD). DMD is the most common and severe form of muscular dystrophy and current treatments are far from adequate. However, genetic and cell-based therapies, in particular exon skipping induced by antisense strategies, and corrective gene therapy via functionally engineered dystrophin genes hold great promise, with several clinical trials ongoing. Proof-of-concept of exon skipping has been obtained in animal models, and most recently in clinical trials; this approach represents a promising therapy for a subset of patients. In addition, gene-delivery-based strategies exist both for antisense-induced reading frame restoration, and for highly efficient delivery of functional dystrophin mini- and micro-genes to muscle fibres *in vivo* and muscle stem cells *ex-vivo.* In particular, AAV-based vectors show efficient systemic gene delivery to skeletal muscle directly *in vivo*, and lentivirus-based vectors show promise of combining *ex vivo* gene modification strategies with cell-mediated therapies. This research lecture will discuss, (i) Global significance of neuromuscular disease, (ii) Advances and clinical trials in antisense and RNA directed therapies, (iii) Advances in AAV vector and microdystrophin gene therapy for muscle diseases, (iv) Manipulating myostatin to counter muscle atrophy, and (v) Gene editing technology for targeted correction of the Duchenne muscular dystrophy gene.

INV027

RNA-modulating therapeutics for Duchenne Muscular Dystrophy

Judith van Deutekom

Prosensa Therapeutics, Leiden, The Netherlands

Over the last few years the field of antisense oligonucleotides as RNA modulating therapeutics has made significant progress. To date, several antisense drug candidates are in (pre)clinical development for Duchenne muscular dystrophy (DMD). Their length may vary between 15 and 40 nucleotides depending on the chosen chemistry, and their mechanism of action is based on highly sequence-specific binding to a target exon such that splicing regulatory factors and/or structures are interfered with. The resulting exon skipping is aimed at correction of the transcript's open reading frame that is disrupted by a mutation (in 70% of cases a deletion of one or more exons) in the DMD gene of DMD patients. This allows for expression of a novel dystrophin protein that despite an internal truncation carries the N- and C-terminal domains most essential for its structural and signaling functions at the muscle fiber membranes. The (semi)functionality of such truncated proteins is evident in Becker muscular dystrophy (BMD) patients that typically have a milder phenotype and disease progression. The exon skipping approach is mutation-dependent and although applicable to subpopulations of patients with grouped mutations in the area of the targeted exon, the development of multiple oligonucleotides will be needed to treat a majority of patients. This presentation will provide an overview of the current (pre)clinical status of Prosensa's candidates.

INV028

Gene therapy for spinal muscular atrophy: perspectives and problems

Thomas Voit, Martine Barkats

[1]*Univeristé Pierre et Marie Curie Paris 6, UM 76, INSERM U 974, CNRS UM 7215, Institut de Myologie, GH Pitié-Salpêtrière, Paris, France*

Gene therapy approaches for spinal muscular atrophy have long remained elusive because of the difficulty to transduce spinal α-motor neurons with sufficient efficacy. This scenario has changed with the discovery of efficient α-motor neuron transduction by scAAV9 after systemic application (Barkats, WO 2009013290). Applying this approach to transfer *smn1* into *smn*-deficient Δ7 mice resulted in an extension of mean survival from 12 to >160 days (Foust et al, 2010, Dominguez et al, 2010). Subsequently, direct delivery of either scAAV8-SMN or sc-AAV9-SMN into the cerebroventricular space (icv) has also shown dramatic rescue effects (Passini et al, 2010; Lorson et al, 2011). The key question was if maximum substitution to spinal α-motor neurons was the only essential determinant for survival in the mouse model. Using a systematic comparison of a fixed dose of 4.5 10e10vg scAAV9-SMNopti delivered either iv, icv, iv + icv a maximum survival is obtained through combined iv + icv delivery while at the same time spinal *smn* protein levels are lower than with icv delivery alone. These results point to an important role for SMN expression in the periphery, most likely in skeletal and cardiac muscle, for optimum survival. Optimization of the therapeutic approach is important in order to transfer this approach into a treatment strategy for spinal muscular atrophy in man.

INV029

Major Concepts for Cell and Gene Therapy

Jude Samulski

University North Carolina, Chapel Hill, USA

Adeno-associated virus (AAV) vector are demonstrating therapeutic effect in a number of phase I clinical trials. Although AAV has very broad tropism, most humans have neutalizing antibodies that restrict vector re-administration. To further enhance AAV vector tropism and identify capsid variants that escape neutralizing antibody, we have generated synthetic AAV2 capsid by replacing a hexapeptide sequence in a previously identified heparan sulfate receptor footprint with corresponding residues from AAV8. The AAV2/AAV8 chimera designated AAV2i8 has displayed an

altered antigenic profile, readily traversed the blood vasculature, and selectively transduced muscle tissues including the heart with high efficiency in animal models. More remarkable, this capsid variant reduced hepatic tropism. In a parallel study, we demonstrated that a single amino acid (aa) insertion of Threonine into AAV2 capsid would convey high muscle transduction and changed the capsid immune profile as well. To explore whether the single aa insertion tropism in AAV2 was distinct from AAV2i8 capsid we inserted aa at 265 (AAV2i8D), and injected AAV2i8D vector encoding the firefly luciferase transgene into mice via the tail vein. Compared to parental AAV2i8, liver tropism was rescued and muscle transduction was preserved. To address whether unique pharmacokinetic, pharmacodynamic, and antigenic features of these synthetic AAV variants studied in mice translated across species, we screened thirty-three gravid nonhuman primates for AAV antibodies and identified 6 for study. Pregnancies were monitored sonographically during gestation and newborns were delivered at term by cesarean-section. Three AAV/luc vectors (AAV9, AAV2i8, and AAV2i8D) were administered intravenously at birth (N = 2 per construct). Infants were raised in a nursery and transgene expression was monitored monthly by bioluminescence imaging (BLI) immediately after the intravenous injection of D-luciferin. Blood samples were also collected from a peripheral vessel monthly to monitor complete blood counts (CBCs) and clinical chemistry profiles. Animals remained healthy during the study period, to date, and two years post-vector administration health, growth, CBCs, and clinical chemistry panels were within normal limits for the age group. Vector transgene expression determined by BLI has persisted without significant decline over time. Our results suggest that rational designed AAV variants can induce identical long-term transgene expression without safety concerns across various species (mice to nonhuman primates). Further studies comparing species-specific vector transduction tropism and immune profiles after systemic injection will be discussed.

INV030

Meganucleases for genome engineering

Fayza Daboussi, Georges Silva, Frederic Cedrone, Jean Charles Epinat, Alexandre Juillerat, Julien Valton, Philippe Duchateau

Cellectis SA 8 rue de la croix Jarry, 75013, Paris, France

Rare cutting endonucleases have emerged as powerful tools for precise genome engineering. Very different types of proteins have been used as designable scaffolds to generate artificial endonucleases cleaving chosen sequences, including Zinc Finger Nucleases (ZFNs), natural meganucleases from the I-SceI family, and more recently, Transcription Activator-Like Nucleases (TALENs).

Natural meganucleases, also called Homing Endonucleases, are the most specific endonucleases in nature, and thus, should provide ideal scaffolds for the creation of new genome engineering tools. We have conducted a genome-wide study to characterize the potential of the meganuclease platform. Our results demonstrate that (i) efficacy of meganuclease-induced genome editing is locus-dependent, with epigenetic modifications heavily impacting the process (ii) cleavage by MNs can induce targeted mutagenesis (TM) or homologous gene targeting (HGT) with the HGT/TM ratio being very stable throughout the genome. Moreover, Endonucleases create chemically clean breaks that are often subject to precise repair, limiting the efficiency of targeted gene disruption, we thus have developed a new strategy to improve the efficiency of targeted gene disruption by coupling rare-cleaving endonucleases with DNA end-processing enzymes that can modify endonuclease-induced breaks prior to resolution by non-homologous end joining. Using this strategy, we are able to increase endonuclease-induced disruption rates over 30 fold in transformed cell lines as well as in primary cells. This technology has the potential to dramatically increase the utility of rare-cleaving endonucleases for genetic knockout applications.

Finally, the Transcription Activator-Like Nucleases (TALEN) holds great promises in the field of genome engineering as it makes the technology easy and affordable to any laboratory. We will present our recent advances in TALEN technology as tool for genome engineering.

INV031

Human embryonic stem cells and iPS for regenerative medicine: two real prospects

Marc Peschanski

I-Stem, Evry, France

Pluripotent stem cells (PSCs), because they are virtually immortal and capable of giving rise to any cell phenotype, potentially provide almost unlimited resources for cell therapy.

Clinical trials using embryonic stem cells have now started, and important quite positive information obtained already concerning the major issue formerly raised by the safety of cells that, at the undifferentiated stage -not the one to be used in the clinic- develop into benign though potentially dangerous teratomas. Several clinical trials are currently under examination by regulatory authorities, allowing for some standardization of the roadmap between basic science and clinical application in the field.

iPSCs are less well characterized at this point than embryonic stem cells and their clinical potential will depend of the validation of their overall intrinsic similarity to embryonic stem cells that represent the "gold standard" of physiologically determined pluripotent cells. Recent studies have underscored the presence of a number of genomic and epigenetic abnormalities in the iPS cell lines tested, clearly calling for a persistent effort for the optimization of those techniques. This often leads to statements that clinical trials using iPSCs are still quite remote. This, however, can be challenged because the iPS technology allows us to select the donors, i.e. the genotype. Autologous customized iPSC grafts do not seem either technically or economically sound but allogeneic alternatives are considered. iPSCs indeed potentially open the path for an original way to avoid allogeneic immune cell responses, by aiming at hemi-similarity match using cell lines from donors exhibiting homozygosity for each of the HLA-A, B and DR genes.

INV032

Mesenchymal Stromal Cells: A new Paradigm for Cellular Immune Modulation

Willem Fibbe

Leiden University Medical Center, Leiden, The Netherlands

Mesenchymal stromal cells (MSCs) are multipotent progenitor cells that have emerged as a promising therapeutic modality for tissue regeneration and repair. It has been difficult to prospectively isolate mesenchymal progenitors and MSCs are still characterized on the basis of their morphology, ability to adhere to plastic and immunophenotype by a combination of positive

(CD73, CD 105, CD90) and negative (CD34, CD45, CD14, CD31, CD80, CD86) markers.

The interest in MSC therapy has been raised by the observation that MSCs are able to modulate immune responses in vitro and in vivo. In vitro, MSCs suppress the proliferation of T cells induced by alloantigens and mitogens. *In vitro* data suggest that MSCs are capable of inducing regulatory T cells (Treg) through the production of soluble factors including HO-1. Soluble factors (IL-6, PGE2, IL-10) are also involved in the ability of MSCs to interfere *in vitro* with dendritic cell (DC) differentiation, maturation and function.

MSC are applied to suppress allo-immune responses in graft-versus host disease after allogeneic stem cell transplantation, in chronic kidney allograft rejection and in autoimmune disorders including multiple sclerosis and Crohn's Disease. A recent follow-up study performed in children transplanted in Pavia (Italy) and Leiden (the Netherlands) suggests that the interval between onset of GHVD and treatment with MSCs may be an important variable determining response to MSC treatment. These preliminary data may indicate that timing of MSC treatment is a critical determinant of efficacy and outcome.

INV033

Skeletal muscle stem cells in regenerative biology

Shahragim Tajbakhsh

Institut Pasteur, Paris, France

Identifying and characterising stem cells in the tissue are critical goals in order to exploit them in the clinic. For many tissues and organs, the properties and behaviour of resident tissue specific stem cells remains poorly defined. Adult stem cells can assume different cellular states, either quiescence or proliferating. Recent evidence has indicated that adult skeletal muscle stem (satellite) cells exhibit heterogeneity in behaviour. Using a variety of genetically modified mice, we have used extensive transplantation assays to assess their regenerative potential. In addition to this heterogeneity, we have shown that skeletal muscle stem cells from different muscles (head vs. trunk) emerge in distinct ways with under different genetic regulatory programmes. The role of critical transcription factors and signaling pathways that regulate muscle stem cell fate will be discussed.

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INV034

Engineering mammalian cells for therapeutic applications

Martin Fussenegger

ETH Zurich, Department of Biosystems Science and Engineering (D-BSSE), Mattenstrasse 26, CH-4058, Basel, Switzerland

Capitalizing on our latest advances in the design of heterologous mammalian transgene control systems we have designed the first prosthetic networks that sense, monitor and score (disease-) relevant metabolites, process peak-level concentrations and coordinate adjusted diagnostic, preventive or therapeutic responses in a seamless, automatic and self-sufficient manner. We believe that the design of synthetic gene networks, which process molecular signals with near digital precision, may provide novel therapeutic opportunities. Highlights will include our proof-of-concept studies on prosthetic networks enabling the treatment of gout, providing a new type 2 diabetes therapy based on light-triggered glucose homeostasis and the design of a treatment strategy for the metabolic syndrome.

INV035

The future of the retros

Christopher Baum

Institute of Experimental Hematology, Hannover Medical School, Carl-Neuberg-Str.1, Hannover, Germany

Over the past years and decades various forms of retrovirus-based vectors have been developed, mostly on the basis of gammaretroviral, lentiviral and spumaviral and alpharetroviral families. Lentiviral vectors with their ability to transduce many nondividing cells have dominated recent activities, but some of the initial promises remained difficult to fulfil with this platform. New developments exploiting spumaviral and alpharetroviral principles have contributed interesting features in the search for reduced genotoxicity. Other, more generally applicable elements are being developed to insulate the integrated expression cassette, to avoid epigenetic silencing, and to regulate transgene expression. Furthermore, a deeper analysis of the early steps of the retroviral life cycle has led to new principles for the display of cell-targeting ligands and the delivery of mRNA and proteins.

This presentation focuses on the utility of emerging retroviral technologies with the aim to mediate a high efficiency, accompanied with a low risk of genotoxicity, in the genetic modification of hematopoietic stem cells and induced pluripotent stem cells. Examples will be provided to reach the following conclusion: The future of the retros depends upon an improved understanding of the retroviral life cycle, increasing the freedom to modify the vectors' production platforms, targeting properties, integration pattern and genetic cargo. A more holistic approach covering the large arena of retroviruses in combination with synthetic designer elements is key to the further development of this important platform.

INV036

Integration site analysis in a clinical trial of lentiviral vector based hematopoietic stem cell gene therapy for metachromatic leukodystrophy

Eugenio Montini[1], Alessandra Biffi[1], Andrea Calabria[1], Luca Biasco[1], Martina Cesani[1], Fabrizio Benedicenti[1], Tiziana Plati[1], Simone Leo[2], Gianluigi Zanetti[2], Alessandro Aiuti[3], Christof von Kalle[3], Manfred Schmidt[1], Maria Sessa[1], Luigi Naldini[1]

[1]San Raffaele Telethon Institute for Gene Therapy, Milan, Italy, [2]Center for Advanced Studies, Research, and Development in Sardinia, Pula, Italy, [3]National Center for Tumor Diseases, Heidelberg, Germany

A self-inactivating lentiviral vector (LV) has been used in an ongoing hematopoietic stem cell (HSC)-based clinical trial for metachromatic leukodystrophy (MLD) in Milan. In 5 treated patients we obtained multi-lineage hematopoietic reconstitution with up to 80% vector marking. To assess the safety and efficacy

of gene transfer as well as the dynamics of hematopoietic reconstitution we studied the integration site profile LAM PCR and high-throughput 454-pyrosequencing in different cell lineages and time points after transplantation (1, 3, 6, 9 and 12 months) in the first 3 MLD patients.

Overall, >32000 unique integrations from the 3 analyzed patients were obtained. The LV integration profile in this trial is highly similar to the one described in the LV-based adrenoleukodystrophy clinical trial. Common Insertion Sites (CIS) were clustered at specific mega-base wide chromosomal regions and were enriched in chromatin remodeling and HLA genes, suggesting that these CIS may be originating by a viral integration bias rather than genetic selection.

Analysis of sequencing reads as a surrogate of the abundance of specific cell clones shows a variable contribution of multiple clones without evidence of clonal dominance. Importantly, tracking of vector integrations across linages and time allow us to confirm HSC marking, the HSC contribution levels within committed hematopoietic lineages and to characterize distinctive dynamics hematopoietic reconstitution between myeloid and lymphoid lineages after transplantation.

Overall, our data show that in in MLD treated patients unprecedented high vector marking levels were achieved without overt evidence of genotoxicity.

EM and AB contributed equally.

INV037

Regulating dendritic cell signalling to improve vaccination

Mary Collins

University College London, London, UK

Until about 25 years ago, translational research into vaccines proceeded empirically without consideration of the dendritic cell network. Current problems in vaccine development include therapeutic vaccination to tackle persistent infectious disease or cancer, and prophylactic vaccination for highly variable pathogens such as HIV or influenza. In these cases a dominant neutralising antibody response is insufficient, and a T cell response will be crucial, requiring optimal vaccine interaction with dendritic cells.

Our approach has been to manipulate dendritic cell signalling pathways, using activator cDNAs or inhibitor shRNA, to stimulate a variety of immune responses. We have used lentiviral vectors to express antigens together with signalling regulators and demonstrated improved CD8+ and CD4+ T cell responses, or Treg expansion. We have shown efficacy of this approach in models of autoimmune disease or influenza infection. Our efforts to translate this work to the clinic involve safety and production improvements.

INV038

NK cells and innate immunity

Eric Vivier[1,2]

[1]1Centre d'Immunologie de Marseille-Luminy (CIML), Aix Marseille Université, UM2, Campus de Luminy, 13288, Marseilles, France, [2]INSERM, U 1104, 13288, Marseilles, France, [3]CNRS, UMR7280, 13288, Marseilles, France, [4]Assistance Publique des Hôpitaux de Marseille, Hôpital de la Conception, Marseilles, France

Natural killer (NK) cells are lymphocytes of the innate immune system with effector and regulatory functions. NK cells are involved in the elimination of stressed cells such as tumor cells and infected cells. They can kill their cellular targets via cytotoxic granule exocytosis and secrete cytokines such as interferon-g (IFN-g) that participate in the shaping of the adaptive immune response. NK cells express a wide range of surface molecules that include inhibitory and activating receptors. In humans, inhibitory receptors include MHC class I-specific receptors, and activating receptors comprise the natural cytotoxic receptors (NKp30, NKp44, and NKp46) and the Fcg receptor IIIA (CD16) which endows NK cells with antibody-dependent cell-mediated cytotoxicity properties.

Results will be presented in two emerging themes of research. First, it is increasingly clear that cells of the innate immune system have evolved various molecular mechanisms to sense their environment and react to alterations of self. NK cell are remarkably adaptable to changes in self. Second, the manipulation of NK cells against cancer leads to novel strategies of NK cell therapy.

INV039

Chimpanzee Ad vector technology platform for genetic vaccine applications

Stefano Colloca

Okairos Srl, Rome, Italy

Replication defective Adenovirus vectors based on the human serotype 5 (Ad5) have been shown to induce protective immune responses against several infectious diseases and cancer in animal models and to elicit potent, broad and long lasting cellular immunity in humans with a significant CD8 component. However, most humans have anti-Ad5, neutralising antibodies that can impair the immunological potency of the vaccine. We have demonstrated that viral vectors based on chimpanzee adenovirus strains are able to overcome the anti-hAd pre-existing immunity thus stimulating high levels of T-cell response against the encoded antigen in human clinical trials. The platform technology based on different non-cross reactive ChAd vectors was complemented by the parallel development of a novel packaging cell line (Procell-92) allowing propagation of ChAd vector to high titers. ChAd vectors carrying interfering transgenes were demonstrated to be genetically stable when serially propagated in Procell-92. In addition, we have showed that the introduction in the ChAd vector of MHC class II-associated invariant chain (human li) tethered to the antigen significantly increases CD8 T-cell response. Chimp Ad vectors, Procell-92 and the encoded adjuvant human li represent an integrated platform technology for prophylactic as well as therapeutic genetic vaccine development.

INV040

Transdifferentiation: Autologous cell replacement therapy

Irit Meivar-Levy, Vered Aviv, Dana Bernmann-Zeituni, Elad Chernichovsky, Naomi Litichever, Keren Ron, Tamar Sapir, Kfir Molakandov, Sarah Ferber

The Stem cells and Regenerative Medicine Institute Sheba Medical Ctr. Tel-Hashomer, and the department of Human Genetics and Molecular Medicine Sackler School of Medicine. Tel-Aviv University, Tel Aviv, Israel

Transdifferentiation is the conversion of one adult tissue/or cells into another type of cell, with distinct function. The process is artificially induced by ectopic expression of tissue specific transcription factors acting as organogenesis master regulators.

Transdifferentiation does not require insertion of ectopic genes and is mediated only by epigenetic modifications in the DNA

and chromatin structures. This relatively safe developmental process involves neither increased oncogene expressions nor increased adult cells proliferation.

The presentation will disclose the major characteristics of the process, and its implementation in generating autologous cell replacement therapy for numerous degenerative diseases. Using Diabetes as a model for degenerative disease we discuss the advantages and the challenges of using our own adult organs for autologous cell replacement therapy for diabetic patients.

Therapeutic significance: Generating new functional tissues from adult organs is a fundamental concept in regenerative medicine. The activation of the pancreatic lineage and function in adult human liver or skin cells allows the diabetic patient to be also the donor of his own insulin producing tissue. Using cells from adult organs to replace ablated insulin production overcomes both the limited supply of tissues from cadaveric donors and the need for anti-rejection treatment.

INV041

Reprogramming through the pluripotente state as a new paradigm for human cell rejuvenation

Jean Marc Lemaitre

Institute of Fuctional Genomics, Montpellier, France

Direct reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) provides a unique opportunity to derive patient-specific stem cells with potential application in autologous tissue replacement therapies and without the ethical concerns of Embryonic Stem Cells (hESC). However, cellular senescence, which contributes to aging and restricted longevity, has been described as a barrier to the derivation of iPSCs, suggesting that aging might be an important limitation for the derivation of iPSCs for therapeutic purposes from elderly individuals. Recently, we developed an optimized 6 factor based-reprogramming protocol that may cause efficient reversing of cellular senescence and reprogramming into iPSCs. We demonstrated that iPSCs derived from senescent and centenarian fibroblasts have reset telomere size, gene expression profiles, oxidative stress and mitochondrial metabolism, and are indistinguishable from hESC. Finally, we further demonstrate that redifferentiation, led to rejuvenated cells with a reset cellular physiology, defining a new paradigm for human cell rejuvenation. These results provide new insights into iPSC technology and pave the way for regenerative medicine for aged patients.

INV042

Direct generation of functional dopaminergic neurons from human fibroblasts

Vania Broccoli[1], Massimiliano Caiazzo[1], Maria Teresa Dell'Anno[1], Sebastiano Curreri[2], Damiana Leo[2], Raul Gainedtinov[2], Alexander Dityatev[2]

[1]San Raffaele Scientific Institute, Milan, Italy, [2]Italian Institute of Technology (IIT), Genoa, Italy

Having access to human neurons for regenerative therapies and understanding diseases has been prohibited for long time. However, genetic technologies of cell reprogramming have widen this possibility by *in vitro* differentiation of fibroblast-derived iPS cells. Recently, we found a minimal set of three transcription factors (Mash1, Nurr1 and Lmx1a) able to efficiently convert mouse and human fibroblasts into functional dopaminergic neuronal (iDAN) cells. Molecular and transcriptome studies showed iDAN cells to recapitulate gene expression of their brain homolog neurons to large extent while lacking expression of other monoaminergic neuronal subtypes markers. Transgene expression is necessary for at least 6 days to achieve a stable cell conversion sustained by activation of the endogenous genes of the three reprogramming factors. Strikingly, iDAN cells showed spontaneous electrical activity organized in regular spikes consistent with the pacemaker activity featured by brain DA neurons. Furthermore, iDAN cells express D2 autoreceptors and their activity is regulated by the D2/3R agonist quinperole. The three factors were able to elicit DA neuronal conversion in prenatal or adult fibroblasts from healthy donors and Parkinson's disease patients. Mouse iDAN cells integrated in orthotopic brain side after transplantation in the forebrain structures and matured into functional neurons. Importantly, when transplanted in rats unilaterally lesioned with 6-OHDA, iDAN cells were able to rescue drug-induced locomotor impairment over long time. iDAN cell technology and its future developments might have significant implications in studies of neural development, disease *in vitro* modeling and cell replacement therapies.

INV043

13 years of experience with gene therapy of primary immune deficiencies

Alain Fischer

Hôpital Necker, Paris, France

No abstract available

INV044

Development of a gene therapy pipeline for inherited retinal dystrophies

Robin Ali

UCL Institute of Ophthalmology, Dept of Genetics, London, UK

There are currently 120 genes that have been identified that lead to various forms of inherited retinal dystrophy. More than 100 of these genes can carry loss-of-function mutations and could therefore be targets for gene supplementation therapy. In order to decide which gene defects are the most suitable targets for the first gene therapy approaches, various parameters must be considered. These include disease severity and prevalence, the availability and suitability of animal models of disease, and the efficacy of the treatment in proof-of-concept studies in animals. Severity of disease in particular is an important consideration, as on the one hand a rapid loss of photoreceptor cells severely limits the window of opportunity for treatment, but on the other hand allows an efficient read-out of treatment efficacy in a clinical trial. Over the past decade, we have developed gene therapy protocols in over a dozen different animal models of retinal dystrophy and we have conducted a clinical trial of RPE65 gene therapy for Leber congenital amaurosis (LCA) type 2. Here I will discuss our pipeline of therapies. Our strategy is to begin our initial studies in rare, but probably most amenable disorders, such as RPE65-deficiency and AIPL1-deficiency and move towards more common, but more complicated disorders for which to develop treatments, culminating in the development of gene therapy for X-linked RP caused by defects in RPGR.

INV045

CART19 induces sustained functional CAR T cell persistence and B cell aplasia

Carl June[1], Michael Kalos[1], Bruce Levine[1], David Porter[1], Timothy Macatee[1], Irina Kulikovskaya[1], Minnal Guptaq[1], Erica Suppa[1], Saar Gill[1], Stephan Grupp[2]

[1]*University of Pennsylvania, Philadelphia, PA, USA,* [2]*Children's Hospital of Phildelphia, Philadelphia, PA, USA*

We reported initial clinical data on CAR T cells targeting CD19 expressed on normal and malignant B cells (CART19 cells) (Porter NEJM 2011; Kalos Sci Trans Med 2011). 10 patients with relapsed, refractory disease have been treated to date: 9 adults w/CLL and one child w/pre- B cell ALL. 4 pts (3 CLL, 1 ALL) had achieved CR at the primary endpoint (30 days post infusion) which is sustained and ongoing in all patients (range 2–24 months). 2 CLL patients had a partial response (PR) lasting 3 and 5 months, while 3 patients did not respond (NR) and 1 is not yet evaluable. In all patients with CR, robust in vivo expansion of CART19 cells was observed. Long term peripheral blood persistence of CART-19 cells and CAR19 surface expression was observed in all patients with CR in both CD3+/CD8+ and CD3+/CD4+ subsets. In patients with CR, elimination of peripheral B cells was observed at the time of CART19 in vivo expansion. Ongoing B cell aplasia has been documented in each CR patient in both peripheral blood and marrow by flow cytometry. Adoptive transfer of CART19 cells can result in long-term functional persistence of CART19 cells accompanied by ongoing complete clinical responses and long-term B cell aplasia in a substantial fraction of patients with advanced, refractory and high risk CLL and relapsed refractory ALL.

INV046

Stable Factor IX Activity Following AAV-mediated Gene Transfer in Patients with Severe Hemophilia B.

Amit Nathwani[1,2]

[1]*University College London, London, UK,* [2]*Royal Free Hospital, London, UK*

We are evaluating the safety and efficacy of gene transfer with serotype-8 pseudotyped self-complementary adeno-associated virus (scAAV) vector expressing a codon-optimised coagulation factor IX (FIX) transgene (scAAV2/8-LP1-hFIXco) in subjects with severe haemophilia B in the context of a phase I/II clinical trial. Highly promising but early safety and efficacy data from six subjects treated with our novel gene transfer approach has previously been reported (Nathwani et al, NEJM 365:2357–65, 2011). We will report longer follow-up of these participants, as well as data from two additional participants recently enrolled at the high dose level.

INV047

Cell type specific gene delivery in the vascular system

Christian Buchholz

Paul-Ehrlich-Institut, Langen, Germany

The endothelium lining the inner surface of the vascular system comprises an attractive target for genetic modifications towards both, a better understanding of the physiological functions of specialized endothelial cells and novel therapeutic options for genetic diseases affecting blood factors. As the endothelium cannot easily be genetically modified ex vivo, endothelial cell-specific vectors suitable for in vivo gene transfer are needed. State of the art lentiviral vectors (LVs) are currently equipped with the pantrophic VSV-G envelope protein, which does not discriminate between certain cell types. Cell entry based targeting vectors may overcome these technical limitations. We previously demonstrated specific gene transfer to various cell types such as lymphocytes, hematopoietic stem cells or neurons, ex vivo or upon local in vivo injection. Here we focus on the in vivo application of vectors targeted to the endothelial cell surface marker CD105 (endoglin), a cofactor in the TGF-β signaling pathway which is highly conserved between man and mouse. Target specificity of LVs specific for mouse and human CD105 has been investigated. Interestingly, the mouse CD105-specific vector turned out to be highly specific and effective in genetic modification of liver sinusoidal endothelial cells also when applied systemically. Cell type specificity of the human CD105 vector upon systemic gene delivery was investigated in xenograft mouse models reconstituted with human endothelial cells. The data suggest that CD105 targeting offers endothelial cell specific gene delivery with unprecedented specificity.

INV048

Genetically-targeted therapies for heart failure: Steps to clinical practice

Patrick Most

Center for Molecular & Translational Cardiology, Department of Cardiology, UniversityHospital Heidelberg, Heidelberg, Germany

This paper will discuss key steps of the translational strategy underlying the development of genetically-targeted therapeutic strategies against heart failure. A focus will be on molecular factors that control the cardiomyocyte's calcium cycle and emerged as promising therapeutic targets in failing cardiomyocytes. Development of tailored DNA-based therapeutics, therapeutic proof-of-concept studies using AAV-vectors and ultimate translation into human-relevant heart failure models will be shown. The paper will conclude by addressing potential solutions for currently unmet needs including more efficient cardiac application technology, regulatable expression systems and vector technology with optimized human cardiomyocyte tropism.

INV049

Regenerative medicine: Transforming health care solutions

Andre Terzic

Mayo Cinic, Rochester, Mn, USA

Regenerative medicine has begun to define a new perspective of future clinical practice. The U.S. Department of Health and Human Services report "2020: A new Vision" highlights that regenerative medicine is the vanguard of 21st century healthcare. Patients and society increasingly expect that regenerative medicine will lead to repair of diseased organs, injured tissues or congenital anomalies. Without the contribution of personalized products and services emerging from regenerative medicine technology, experts caution that healthcare will face an escalation in inefficient treatments and a rising global cost. Aimed on functional restoration of damaged tissues, not a mere abatement or

moderation of symptoms, regenerative medicine offers a "disruptive innovation" strategy uniquely poised to add value and transform healthcare by providing tailored, curative solutions for the unmet needs of our patients. Indeed, the rapidly developing regenerative medicine armamentarium promises significant human health benefit with tangible outcomes for increased quality of life and improved patient care building on breakthroughs. Maximizing potential return mandates, however, an integrated roadmap across the translational continuum of discovery-development-regulation-use to ensure optimal application of regenerative medicine algorithms in medical and surgical practice.

INV050

T-cell engineering for adoptive immunotherapy using TAL-effector nucleases (TALENs)

Andrew Scharenberg[1,2], Julianne Smith[1], Laurent Poirot[1], Cecile Schiffer-Mannioui[1], Roman Galetto[1], Isabelle Chion[1], Diane LeClerre[1], Celine Lebuhotel[1], Letitia Lemaire[0]

[1]*Cellectis therapeutics, Paris, France,* [2]*Seattle Children's Research Institute, Seattle, WA, USA*

Adoptive T-cell immunotherapy offers great potential for treating a variety of types of malignancy. However, present adoptive immunotherapy methods are limited by the need for complex, time consuming, manipulation of autologous patient T-cells, and the use of cytotoxic lymphodepletion regimens to promote engraftment. With the goal of circumventing these limitations through nuclease-based engineering, we have developed methods for efficient T-cell engineering using TALEN mRNA electroporation. Use of these methods to engineer T-cells to enable an off-the-shelf therapeutic T-cell platform will be presented.

INV051

TCR gene editing to treat malignancies

Chiara Bonini

San Raffaele Scientific Institute, Milano, Italy

Adoptive T cell therapy is a novel therapeutic modality aimed at providing effective and long-lasting tumor-reactive T cells to cancer patients. The significant advances in gene transfer technologies developed in the last decade, offer new tools to enforce natural T cells, enabling to generate high numbers of genetically modified tumor-reactive T cells from virtually every cancer patient. Recently, T cells have been manipulated *ex vivo* with viral vectors coding for Chimeric Antigen Receptors, exogenous T Cell Receptors or "suicide" genes to potentiate their efficacy and to minimize possible side effects. Our group developed a novel genetic approach that allows for the first time the complete editing of T cell specificity, by combining the disruption of the endogenous alfa and beta TCR chain genes with the transfer of a tumor-specific TCR. We designed Zinc Finger Nucleases promoting the disruption of both endogenous TCR b and a chain genes. ZFN-treated lymphocytes lacked CD3/TCR surface expression and were stable in culture with IL-7 and IL-15. Upon lentiviral transfer of an engineered TCR for the WT1 tumor antigen, these TCR-edited cells expressed the new tumor-specific TCR at high levels, were easily expanded to near-purity and proved highly effective and specific in recognizing and killing leukemic cells. Challenges and advances recently made to improve the potency and specificity of adoptive immunotherapy with genetically modified lymphocytes to treat cancer will be discussed.

INV052

Modifying the HIV-1 integration machinery to yield safer vector integration

Diana Schenkwein[1,3], Vesa Turkki[1,3], Mervi Ahlroth[4], Seppo Ylä-Herttuala[1,2]

[1]*Department of Biotechnology and Molecular Medicine, A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, Kuopio, Finland,* [2]*Department of Medicine, University of Eastern Finland, Kuopio, Finland,* [3]*Ark Therapeutics Oy, Kuopio, Finland,* [4]*Finnish Rheumatism Association, Helsinki, Finland*

Integrating vectors are essential tools for gene therapists as they can promote long-term gene expression after single vector administration. Detailed information has been collected about the integration characteristics of different vectors, and the risks related to unfavorable integration sites are well acknowledged. As an attempt to reduce vector-induced genotoxicity, different methods have been developed to affect the characteristic integration site selection of both viral and non-viral vectors. Also, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and meganucleases have shown great promise for accurate transgene integration through targeted genome cleavage and subsequent homology directed repair (HDR). Because the nuclease based systems often rely on DNA transfection methods that are difficult to translate into whole organisms, new vector chimeras have been developed to improve their cellular uptake.

We have studied the potential of lentivirus vectors as protein transducing particles by generating different fusions to the HIV-1 integrase protein (IN). Our experience has shown that IN-fusions are well tolerated from the perspective of vector production, and that relevant amounts of fusion proteins are transported to transduced cells' nuclei, where they can elicit specific cellular functions ranging from the induction of apoptosis to catalyzing DNA cleavage. Moreover, we found that vector-incorporated IN-fusion proteins are able affect the integration site selection of third generation lentivirus vectors. This talk will describe our findings related to IN-modified lentivirus vectors and their potential applications, and review the recent advances in lentivirus vectorology that aim to improve the safety of transgene integration.

INV053

No abstract available

Alessandra Biffi

HSR, Milano, Italy

No abstract available

INV054

Hematopoitic Stem Cell Gene Therapy for X-linked Adrenoleukodystrophy

Nathalie Cartier[1,2], Salima Hacein-Bey-Abina[3,4], Cynthia Bartholomae[6], Manfred Schmidt[6], Ina Kutschera[6], Michel Vidaud[1], Ivan Bièche[1], Ingrid Laurendeau[1], Céline Bellesme[2], Muriel Audit[8], Pierre Bougnères[2], Christof Von Kalle[6], Alain Fischer[4], Marina Cavazzana-Calvo[3,4], Patrick Aubourg[1,2]

[1]*Inserm UMR745, University Paris-Descartes, 75279, Paris, France,* [2]*Department of Pediatric Endocrinology and Neurology, Hôpital*

Bicêtre, Paris, France, [3]Department of Biotherapy, Hôpital Necker-Enfants Malades, 75743, Paris, France, [4]Inserm UMR768, University Paris-Descartes, 75743, Paris, France, [5]Clinical Investigation Center in Biotherapy, Groupe Hospitalier Universitaire Ouest, APHP, Paris, France, [6]Center for Tumor Diseases and German Cancer Research Center (DKFZ), 69120, Heidelberg, Germany, [7]Department of Pediatric Immuno-Hematology, Hôpital Necker-Enfants Malades, 75743, Paris, France, [8]Genosafe, 91002, Evry, France

X-linked adrenoleukodystrophy (X-ALD) is a severe genetic demyelinating disease of the central nervous system. In X-ALD, cerebral demyelination can be stopped or reversed within 12–18 months by allogeneic HSC transplantation. The long-term beneficial effects of HCT transplantation are due to the progressive turn-over of brain macrophages (microglia) derived from bone-marrow cells. We have developed a gene therapy protocol based on autologous transplantation of CD34+ cells corrected with a lentiviral vector. Four children, candidate for hematopoietic stem cell transplantation, but with no compatible donor received infusion of autologous corrected CD34+ cells. Stable correction of peripheral leukocytes was demonstrated. Integration of the vector was polyclonal was demonstrated up to the last follow up as suggested by extensive integration profil analysis and high throughput sequencing of integration sites. In the two first treated patients with the longest long-term follow-up, HSC gene therapy resulted in stabilization of the demyelinating lesions and neurological effects comparable with allogeneic HSC transplantation. Long-term results and perspectives will be discussed.

INV055

Gene Therapy for CNS Disorders

RG Crystal

Weill Medical College of Cornell University, New York, USA

The blood-brain barrier restricts the diffusion of blood-borne molecules to the CNS, providing a significant challenge to developing CNS therapies. We have 2 programs focused on using gene therapy to treat CNS disorders: direct administration of gene transfer vectors to the CNS to treat CNS lysosomal storage disorders, and liver-directed gene transfer to generate monoclonal antibodies that will block addictive drugs from reaching the CNS. First, using late infantile neuronal ceroid lipofuscinosis (LINCL, a neurodegenerative, autosomal recessive lysosomal storage disorder caused by mutations in the CLN2 gene, resulting in tripeptidyl peptidase deficiency) as a model, we have developed a strategy to treat the CNS manifestations of this disorder using direct CNS administration of an adeno-associated virus (serotype AAVrh.10) vector coding for the deficient lysosomal enzyme. We will provide an update on murine and non-human primate efficacy and toxicology assessment of CNS administration of AAVrh.10, as well as data regarding an ongoing clinical trial to treat children with LINCL, including new quantitative biomarkers to assess CNS therapy. Second, we have used the AAVrh.10 vector to modify the liver to produce and secrete a monoclonal antibody directed against nicotine on a persistent basis. With a single administration, mice vaccinated with this vector are completely blocked from responding to systemic administration of nicotine, providing a novel strategy to treat a serious addiction with major medical consequences.

INV056

Therapy for schwannomas using AAV-P0-caspase and exosome-mediated delivery of therapeutic RNA/protein

Xandra Breakeifeld[1], Shilpa Prabhakar[1], Miguel Sena-Esteves[2], Okay Saydam[3], Gary Brenner[4]

[1]Massachusetts General Hospital, Boston, MA, USA, [2]University of Massachusetts Medical School, Worcester, MA, USA, [3]Medical University of Vienna, Vienna, Austria, [4]Massachusetts General Hospital, Boston, MA, USA

Schwannomas form by proliferation of dedifferentiated Schwann cells along peripheral and cranial nerves and are common in patients with neurofibromatosis 2 (NF2) and schwannomatosis. Although typically benign, these tumors can cause extreme pain and compromise hearing, vision and motor functions. Surgical resection, the main treatment modality, can be problematic due to tumor inaccessibility and risk of nerve damage. We have explored two modes of therapy for schwannomas in a model in which immortalized human NF2 schwannoma cells (expressing a fluorescent protein and luciferase) are implanted within the sciatic nerve of nude mice. Direct injection of an adeno-associated virus (AAV) serotype 1 vector encoding caspase-1 (ICE) under the Schwann-cell specific promoter, P0 led to regression of these tumors and resolution of tumor-associated pain, with essentially no vector-mediated neuropathology or loss of neuronal function. We also used cell-derived extracellular vesicles (EVs) to deliver pro-drug activating RNA/protein to these tumors. Genetically engineered EVs were prepared by expressing high levels of the suicide gene cytosine deaminase (CD) fused to uracil phosphoribosyltransferase (UPRT) in donor cells. EVs isolated from conditioned medium were injected into schwannomas. Tumors regressed upon systemic treatment with the prodrug (5-fluorocytosine), which is converted within tumor cells to 5-fluorouracil - an anticancer agent. Both AAV1-P0-ICE vectors and "physiologic liposomes" armed with therapeutic RNA/protein provide clinically compatible means to reduce tumor size and normalize neuronal function in these non-malignant tumors.

Supported by funding from NIH/NINDS NS24279.

INV057

A non-replicating oncolytic viral particle as a novel therapeutic tool against cancers

Yasufumi Kaneda

Osaka University, Suita, Osaka, Japan

Cancers are still difficult targets despite recent advances in cancer therapy. Due to the heterogeneity of cancer, a single-treatment modality is insufficient for the complete elimination of cancer cells. Therapeutic strategies from various aspects are needed. We developed a novel gene delivery vector based on UV-irradiated non-replicating Sendai virus particle, called HVJ (hemagglutinating virus of Japan; Sendai virus) envelope vector (HVJ-E). While we treated various cancer models in mice using HVJ-E, we discovered that HVJ-E itself had anti-tumor activities. One of the activities is the generation of anti-tumor immunity by the enhancement of NK and CTL against cancers and the suppression of regulatory T cells. Another is the induction of apoptosis selectively in cancer cells by the introduction of viral RNA fragments into cancer cells. should be equipped with intrinsic anti-cancer activities. Clinical trials to treat melanoma and prostate cancer are

being performed in Japan using the clinical grade HVJ-E. In future, using therapeutic molecules incorporated HVJ-E, multi-modal therapeutic strategies will be achieved in cancer treatment.

INV058

ColoAd1 a group B oncolytic adenovirus: pre-clinical assessment of potency, safety and selectivity

Sam Illingworth[2], Ying Di[1], Dan Cochrane[2], Jeetendra Bhatia[2], Ashvin Patel[2], John Beadle[2], Tamara Nicolson[2], Terry Hermiston[3], Len Seymour[1], Kerry Fisher[1,2]

[1]*Oxford University, Oxford, UK,* [2]*PsiOxus Therapeutics, Oxford, UK,* [3]*Bayer, California, USA*

ColoAd1 is a chimeric Ad11p/Ad3 adenovirus, discovered by bio-selection from a library of chimeric adenoviruses for the ability to replicate and exit rapidly from tumour cells. The virus is active against a broad range cancer cell lines demonstrating a shorter time-to-lysis than either wild type Ad11p, Ad3 or Ad5. In normal cells, ColoAd1 is highly attenuated and shows little or no activity by either cytotoxicity or by quantitative PCR. The mechanism of tumour cell lysis is independent of apoptosis pathways and ColoAd1 readily kills drug resistant cells. *In vivo*, ColoAd1 shows efficacy in a range of subcutaneous and orthotopic metastatic tumour models following intratumoral, intravenous and intraperitoneal injection. When subpopulations of cells are isolated from tumour samples, sphere forming cells (with a self-renewing phenotype) were shown to be disproportionately killed by ColoAd1. The virus capsid is entirely derived from Ad11p for which there are limited circulating levels of neutralising antibodies in the general population. ColoAd1 associates with blood cells reversibly and unlike group C based oncolytic viruses, can efficiently kill tumour cells under clinically relevant conditions in undiluted human blood. Replication and lysis has been demonstrated in primary patient samples and efficacy has been demonstrated in metastatic orthotopic models. Like most adenoviruses, ColoAd1 can be grown to high titres under cGMP conditions and the robust structure is stable at 4°C for over a year. A full panel of safety studies has now been complete ahead of clinical trials later this year.

INV059

Prostate cancer gene therapy in Japan

Yasutomo Nasu

Okayama University Hospital, Okayama, Japan

Prostate is an ideal target organ for the gene therapy. It has advantages as follows : prostate is not a life keeping organ, can be approached easily by ultrasound as a routine clinical technique and PSA is a potent tumor marked for the evaluation of clinical response.

Many clinical gene therapy trials for prostate cancer with Herpes Simplex Virus Thymidine Kinase (HSV-tk) gene therapy have been conducted in many institutes showing clinical safety and efficacy including Japanese trial (Molecular Therapy 15:834, 2007). Also immunomodulatory *in situ* gene therapy using adenoviral Interleukin-12 gene is under way in Japan.

As a preclinical study, new therapeutic gene REIC/Dkk3 has been proved to be potential therapeutic gene for prostate cancer as it has induces local apoptosis and systemic immune activation. (Cancer Research 65:9617, 2005, Cancer Gene Therapy 14:765,2007, Int J Oncol 34:657,2009, J Bio Chem 284:14236,2009, Int J Mol Med 24:789, 2009). With positive experimental results, a phase I/II study of adenovirus-mediated REIC/Dkk-3 gene therapy for prostate cancer was initiated from January 2011 in Okayama university hospital and on going with positive safety profile and clinical response.

In this session ongoing development of *in situ* gene therapy for prostate cancer in Japan will be reported. I would like to discuss about the present problem and future direction for the development of prostate cancer gene therapy in Japan including Asia.

INV060

Targeted oncolytic and immunotherapeutic vaccinia JX-594: clinical proof-of-concept for systemic efficacy

Caroline J Breitbach[1], Tae-Ho Hwang[2], Steve Thorne[3], David H Kirn[1]

[1]*Jennerex Inc, San Francisco, CA*
[2]*Pusan National University Hospital, Busan, S.Korea*
[3]*University of Pittsburgh, Pittsburgh, PA*

Targeted oncolytic and immunotherapeutic viruses are an emerging multi-mechanistic therapeutic platform designed to induce both acute tumor debulking as well as chronic suppression of tumor outgrowth. Cancer-specific viral replication and immunostimulatory transgene expression (e.g. GM-CSF) result in direct cytolysis followed by tumor-specific humoral and cellular immunity. Product activation is driven by commonly activated genetic pathways in cancer. JX-594 is an oncolytic vaccinia virus derived from the Wyeth vaccine strain and has been engineered for 1) enhanced cancer targeting by TK disruption and 2) has been armed with the transgene of granulocyte-macrophage colony stimulating factor (GM-CSF) to augment oncolysis-induced anti-tumoral immunity. JX-594 replication within tumors, coupled with tumor'specific expression of GM-CSF, creates a pro-inflammatory microenvironment and exposes tumor antigens resulting in immune response induction to the patient's endogenous tumor antigens. Recent preclinical and clinical results demonstrate convincingly that products from this therapeutic class can achieve highly selective and potent cancer destruction systemically through a multi-pronged MOA, including tumor-specific immunity. Given recent clinical validation, we expect this therapeutic class of personalized yet off-the-shelf active immunotherapeutics to expand rapidly.

INV061

BioKnife,™ a uPA activity-dependent fusogenic Sendai virus, as a new class of oncolytic bio-device to treat malignancies

Yoshikazu Yonemitsu

Kyushu University Graduate School of Pharmaceutical Sciences, Fukuoka, Japan

Recently, we have designed an optimized and dramatically powerful oncolytic recombinant SeV vector via genetic modifications of viral genes; namely, truncation of 14-amino acid residues of the cytoplasmic domain of F protein (Fct14) resulted in dramatic enhancement of cell-killing activities of oncolytic SeV, and the combination with replacement of the trypsin cleavage site with the new uPA (urokinase type plasminogen activator: uPA)-sensitive sequence (-SGRS-), named as 'BioKnife™' (Kinoh H, et al. *Gene Ther* 2009;16:392–403). This BioKnife vector led a variety of human tumors to extensive death via massive cell-to-

cell spreading without significant dissemination to the surrounding non-cancerous tissue *in vivo*. BioKnife vector demonstrated dramatically high oncolytic activities and therapeutic effects against orthotopic lethal models of glioma and malignant mesotheliomas *in vivo* than as expected. Importantly, such therapeutic effects were determined by the unique cellular sensor system against *paramyxoviridae*, namely retinoic acid-inducible gene-I (RIG-I) helicase dependent activation of interferon responsible factor (IRF) as well as of nuclear factor-kB (NF-kB), resulted in the extensive cell death by IFN-b/fusion synergy (Hasegawa Y, et al. *Mol Ther* 2010;18:1778–86) as well as uPA/fusion synergy (Morodomi Y, et al. *Mol Ther* 2012;20:769–77). These biological features of BioKnife vector would be expected as a new and hopeful tool fighting intractable malignancies.

INV062

High-throughput identification of antigen-specific TCRs by TCR gene capture

Ton Schumacher

The Netherlands Cancer Institute, Amsterdam, The Netherlands

The transfer of T cell receptor (TCR) genes into patient T cells is a promising approach for the treatment of both viral infections and cancer. While efficient methods exist to identify antibodies for the treatment of these diseases, comparable strategies for TCRs have been lacking thus far. We have developed a high-throughput DNA-based strategy to identify TCR sequences, by capturing and sequencing of genomic DNA fragments encoding the TCR genes. This strategy allows the parallel handling of many samples. We exemplify its value by assembly and validation of a library of Cancer/Germline tumor-antigen reactive TCR genes for potential use in TCR gene therapy of cancer. Furthermore, we demonstrate that, due to its quantitative nature, TCR gene capture can also be utilized to correctly describe the TCR repertoire of oligoclonal T cell populations, through frequency-based matching of TCR α- and β-chains. This ability to dissect TCR $\alpha\beta$ repertoires without a need for single cell isolation opens new possibilities for the assessment of TCR repertoires.

INV063

No abstract available

John Gribbon

Barts and the London NHS Trust, London, UK

No abstract available

INV064

Advancing HSC and T lymphocyte therapies by manipulating cell metabolism

Naomi Taylor

Institut de Génétique Moléculaire de Montpellier, Montpellier, France

The success of hematopoietic stem cell transplantations (HSCT) as well as T lymphocyte-based immunotherapies is highly dependent on the cytokine milieu. As such, administration of exogenous cytokines has been used to support cell therapies. Notably though, the importance of nutrient transport and metabolism has only recently been recognized. The bioenergetic and biosynthetic requirements of HSCs and T cells are met by essential "fuels", which include glucose and glutamine. However, the interplay between these molecules and the specific energy source(s) that are critical for hematopoietic cell differentiation and activation have yet to be elucidated. We will present data showing that HSC and T lymphocyte differentiation are regulated by alterations in nutrient transporter expression and cell metabolism.

INV065

Cancer epigenetics: new paradigms and drugs

Frank Lyco

Division of Epigenetics. German Cancer Research Center (DKFZ). 69120, Heidelberg, Germany

No abstract available

INV066

Polycomb complexes co-associate with a specific RNA polymerase II variant inES cells

Inês de Santiago[1,2], Emily Brookes[1], Kelly J. Morris[1], Tom Carroll[1], Sheila Q. Xie[1], Daniel Hebenstreit[3], Boris Lenhard[1], Jiannis Ragoussis[4], Sarah A. Teichmann[3], Ana Pombo[1]

[1]Genome Function Group, MRC Clinical Sciences Centre, Imperial College School of Medicine, Hammersmith Hospital Campus, Du Cane Road, London, UK, [2]Gulbenkian Institute of Science, R. Quinta Grande, 6-2780, Oeiras, Portugal, [3]Computational Genomics Group, MRC Laboratory of Molecular Biology, Hills Road, Cambridge, UK, [4]Genomics Research Group, Wellcome Trust Centre for Human Genetics, 7 Roosevelt Drive, Oxford, UK

Polycomb repressor complexes are important chromatin modifiers fundamentally implicated in pluripotency and cancer. Polycomb-mediated gene silencing in embryonic stem cells is accompanied by active chromatin marks. Chromatin immunoprecipitation (ChIP) on a small cohort of PRC-repressed genes identified the presence of an unusual form of RNA polymerase II (RNAPII) at promoters and through coding regions, in the absence of active elongation marks (Stock et al. 2007, Nat Cell Biol 9, 1428).

To investigate the relationship between Polycomb and RNAPII genome-wide in ESCs, we mapped several markers of Polycomb repression and four different states of RNAPII activity across the ESC genome using ChIP-seq. We found that PRC-bound genes exhibit a variety of RNAPII states.

Firstly, we identify a large cohort of silent developmental Polycomb targets which are bound by unproductive RNAPII (S5p+S7p-S2p-), confirming the earlier analyses. We find that RNAPII-S5p+ transcribes through coding regions of Polycomb targets without productive expression of functional mRNAs. Based on sequential ChIP between RNAPII and Polycomb components, Ring1B-depletion analyses and genome-wide correlations, we unequivocally show that Polycomb complexes and RNAPII-S5p physically bind to the same chromatin at the same time and functionally synergise.

Secondly, our genome-wide data enable the identification of an unexpected group of active PRC targets, which have roles in metabolic control and development, and suggest that these genes switch between active and PRC-repressed states within the ESC population.

INV067

Epigenetic Regulation of VEGF expression

Seppo Yla-Herttuala, MD, PhD, FESC

A.I.Virtanen Institute, University of Eastern Finland, Kuopio, Finland

Vascular Endothelial Growth Factors (VEGFs) are important regulators of vascular growth and maintenance. VEGFs specifically stimulate proliferation and migration of endothelial cells but they have also other effects of gene expression in other types of cells. Expression of VEGF-A is regulated by hypoxia, inflammation and several cytokines and growth factors whereas some other VEGFs are not stimulated by hypoxia. Since excessive growth of blood vessels and angiogenesis can also be deleterious, regulation of VEGFs takes place at several levels (transcription, translation, mRNA stability and posttranslational regulation). Recently it was recognized that expression of some VEGFs is also regulated by epigenetic mechanisms, includeing a new class of promoter-targeted si/miRNAs (Turunen M et al, Circ Res 2009:105:604–609). These mechanisms will be discussed in the presentation together with new results about the possibilities to regulate VEGF expression with epigenetic mechanisms in vitro and in vivo.

INV068

Process Development in Single use to Facilitate cGMP in Gene Therapy

Manuel Carrondo

IBET-Instituto de Biologia Experimental e Tecnológica, Oeiras, Portugal

Process development for gene therapy is in its infancy, especially when compared to the protein biopharmaceuticals like Monoclonal Antibodies. Given the ample possibilities for treatment and the need to achieve many "first in man" clinical trials as the field gets ever more successful, flexible production facilities are required to allow campaign production of different viral vectors under cGMP rules. Single use technologies (SUT) both for upstream and downstream, are becoming available) in some cases even targeting complex enveloped viruses.

Some design concepts and specific tools for production and purification as developed at IBET, for enveloped and non-enveloped viruses, will be presented and data comparison with classical technologies discussed.

INV069

Large scale AAV production for clinical trials for the treatment of neuromuscular diseases

Otto Merten

Genethon, Evry, France

Gene therapy of neuromuscular diseases such as Duchenne muscular dystrophy by systemic administration of AAV vectors requires production of clinical-grade vector batches at very large scale. As an example, a phase-I/II clinical trial for a local-regional treatment of DMD using the AAV8-exon skipping approach would require 1-4x10E16 total vector genomes (vg). Traditional, cell transfection-based technology is inadequate to produce such large amounts of vector. The implementation of the Sf9/baculovirus technology is a highly efficient means for the large scale production of AAV vectors. Our standard production process at a 200L scale permits the production of up to 5x10E15 vg per run, meaning that several runs will be required for the manufacturing of the AAV necessary for the phase I clinical trial. In parallel to this development and scale-up, the required analytical and QC methods have been developed and/or adapted to this specific technology for releasing of large scale GMP-compliant vector batches. The talk will present some approaches to intensify the actual production scheme.

INV070

Lentiviral vectors for *in vivo* clinical applications: current and future production strategies

James Miskin

Oxford BioMedica (UK) Ltd, Oxford, UK

Oxford BioMedica (OXB) has pioneered the development of lentiviral vectors for *in vivo* clinical applications, and has progressed four products into first-in-man studies; ProSavin® for Parkinson's disease, RetinoStat® for age-related macular degeneration, StarGen™ for Stargardt disease and UshStat® for Ushers type 1B.

A GMP-compliant lentiviral vector manufacturing process has been developed by OXB utilising a transient upstream production process in adherent HEK293T cells, coupled with a downstream purification and concentration process to produce drug product for direct administration. The strategy was to devise a process that was scalable and that incorporated extensive use of disposable technologies. The production process enabled initiation of the first *in vivo* clinical trial worldwide of a lentiviral vector. The ProSavin® trial, which was completed in April 2012 and utilised direct intra-striatal administration of vector to treat the symptoms of Parkinson's disease, enrolled 15 patients in France and the UK. This process was adapted to produce three ocular products; trials are underway in US and France, with more than 10 ocular patients treated to-date. To-date the platform has shown an excellent safety profile, coupled with some encouraging signs of clinical benefit.

This manufacturing process is now in continued improvement/further development in preparation for the later stages of clinical evaluation and market supply. To facilitate this, a manufacturing facility has been acquired and is now licensed for GMP production by the MHRA. The focus is to ensure robust Phase III-ready process(es) that are appropriate to the clinical indication in terms of supply chain and cost of goods.

INV071

Applications of Induced Pluripotent Stem (iPS) Cells for the Pathogenesis of Lyosomal Storage Diseases (LSD)

Yoshikatsu Eto[1], Shiho Kawagoe[1], Takashi Higuchi[1], Jully Matsumoto[1], Reimi Hirayama[1], Hiyoyuki Ida[2], Yota Shimada[3], Hiroshi Kobayashi[3,2], Toya Ohashi[3,2], Mahito Nakanishi[4]

[1]Department of Genetics & Genome Science, Tokyo Jikei University School of Medicine, Tokyo, Japan, [2]Department of Pediatrics, Tokyo Jikei University School of Medicine, Tokyo, Japan, [3]Department of Gene Therapy, Tokyo Jikei Univeristy School of Medicine, Tokyo, Japan, [4]Biotherapeutic Research Team, AIST, Wako, Saitama, Japan

Induced pluripotent stem cells (iPS) technology is now widely applied for the elucidation of pathophysiology of various genetic diseases. However, few studies using iPS technology have been studied for the understanding of pathogenesis of lysosomal storage diseases. Currently, numbers of procedure to generate iPS cells have been carried out by retroviral/lentiviral transduction with two, three or four reprogramming factors or by plasmid/Sendai virus/chemical transduction. We generated iPS cells from various murine /human models of LSD such as Gaucher Fabry, MPS VII(Sly disease) and Pompe diseases, using Yamanaka's three or four factors with retrovirus/Sendai virus vector and studied about pathophysiology of these LSD. These iPS cells could successfully differentiate into cardiomyocytes from Fabry mice and human patient, neuronal cells from MPS VII mice and Gaucher disease, skeletal muscle cells from Pompe mice. The partial lethality of MPS VII mice in utero could be explained by abnormal embryonic development with MPS VII iPS studies. Morphological studies of iPS on EM picture demonstrated massive characteristic inclusions already in iPS cells from human Fabry, Gaucher, and MLD. Furthermore, we could also differentiate into skeletal muscle cells derived from murine Pompe iPS which exhibited marked staining of acid phosphatase and other typical lysosomal storages. These iPS technologies could be widely used for studying various pathogenesis and possible treatment strategies with gene therapy for lysosomal storage diseases.

INV072

Generation of Disease-free Fanconi Anemia iPSC-derived Hematopoietic Progenitors with ZFN-mediated targeted addition of *FANCA* to the *AAVS1* locus

P Rio[1], R Baños[1], A Lombardo[2], Z Garate[1], O Quintana[1], L Alvarez[1], Y Torres[3], P Genovese[2], S Navarro[1], JC Segovia[1], E Samper[3], P Gregory[4], M Holmes[4], L Naldini[2], JA Bueren[1]

[1]CIEMAT/CIBERER, MADRID, Spain, [2]San Raffaele Telethon Institute for Gene Therapy, MILANO, Italy, [3]Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III, MADRID, Spain, [4]SANGAMO BioSciences, RICHMOND,CA, USA

Fanconi anemia (FA) is a rare genetic disease mainly characterized by bone marrow failure (BMF) due to a hematopoetic stem cell (HSC) defect. Thus, the possibility of generating gene corrected HSCs from non-hematopoietic tissues through the combination of homologous recombination and cell reprogramming strategies could have particular importance in the correction of FA. In this study, h*FANCA* was targeted to the *AAVS1* safe harbor locus using an adenoviral vector carrying ZFNs and a donor IDLV vector containing a promoterless *EGFP* and the PGK-*FANCA*, flanked by sequences with homology to the *AAVS1* locus. Using FA-A patients' fibroblasts, we obtained between 0.2 and 4.5% EGFP positive cells following ZFN and donor IDLV delivery. Subsequent transduction of these $EGFP^+$ cells with an excisable reprogramming lentiviral vector generated 6 iPSC clones in which the h*FANCA* was integrated within the *AAVS1* locus. The stemness of three iPSC clones was analyzed both by immunofluorescence and qPCR for markers of pluripotency and by the generation of teratomas *in vivo*. Detailed cytogenetic studies and analyses of FANCD2 foci formation (in the presence and absence of a genotoxic drug) in one representative iPSC clone confirmed that these cells had a normal karyotype and were disease-free. The hematopoietic differentiation of these gene corrected iPSC clones generated populations containing up to 7% of $CD45^+CD34^+$ cells, which supported differentiation into erythroid and granulo-macrophage colonies in semisolid cultures. Our data demonstrates the feasibility of obtaining disease-free HSC from non-hematopoietic human FA cells using combined strategies of genome editing and cell reprogramming.

INV073

What we learnt from the gene therapy for ADA deficiency

Masafumi Onodera

National Center for CHild Health and Development, Setagaya-ku, Tokyo, Japan

An accumulation of the toxic metabolites of purine nucleotides, dATXP, due to an inherent deficiency of adenosine deaminase (ADA) causes one form of severe combined immunodeficiencies (SCID) by impairing proliferation of immature T lymphocytes in the thymus severely. ADA-SCID is also categorized as an inherent metabolic disease because the toxic metabolites are accumulated in the whole body resulting in serious organ failure of the brain, liver, or gastrointestinal tract, so on. Therefore, the enzyme replacement therapy (ERT) using the polyethylene glycol-conjugated ADA (PEG-ADA) is an effective therapeutic option for the disorder. So far, more than 160 patients with ADA-SCID have received PEG-ADA as the cumulative total number and approximately 90 are still being treated with the ERT. On the other hand, the stem cell gene therapy has been done for 36 patients all over the world and most of the cases have shown curative effects without any severe adverse effects.

We also performed T-cell directed gene therapy for an ADA-SCID patient in 1995 and stem cell gene therapy for two patients in 2004. Our stem cells gene therapy without myeloablative reagents showed importance of bone marrow niche in stem cell gene therapy and yielded the partial recovery of their clinical symptoms, resulting in a requirement of the restart of ERT at 6 years after gene therapy. In this seminar, I would like to introduce what we learnt from the gene therapy for ADA-SCID and think about the directional movement of stem cell gene therapy in Japan.

INV074

Toward the treatment of Huntington's disease with RNAi

V Drouet[1,2], M Ruiz[1,2], D Zala[3,4], M Feyeux[6,7], G Auregan[1,2], K Cambon[1,2], S Aubert[6,7], F Bourgois-Rocha[6,7], R Hassig[1,2], N Dufour[1,2], F Saudou[3,4], A Perrier[6,7], P Hantraye[1,2], N Déglon[1,8]

[1]CEA, Institute of Biomedical Imaging (I2BM) and Molecular Imaging Research Center (MIRCen), Fontenay-aux-Roses, France, [2]CNRS URA2210, Fontenay-aux-Roses, France, [3]Institut Curie, 91405, Orsay, France, [4]CNRS UMR3306, 91405, Orsay, France, [5]Inserm U1005, 91405, Orsay, France, [6]Inserm U861, I-STEM, AFM, Evry, France, [7]UEVE U861, I-STEM, AFM, Evry, France, [8]8Lausanne University Hospital (CHUV), Department of Clinical Neurosciences (DNC), Laboratory of Cellular and Molecular Neurotherapies (LNCM), Lausanne, Switzerland

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder resulting from polyglutamine expansion in the huntingtin (htt) protein. Although suppression of both wild-type and mutant htt expression by RNA interference is a

promising therapeutic strategy for this incurable pathology, a selective silencing of mutant htt represents the ultimate and safest approach preserving wild-type htt expression and functions.

In the present study, we tested small hairpin RNA targeting single nucleotide polymorphisms (siSNP) covering the majority of HD patients. We showed that lentiviral-mediated infection of 293T cells resulted in efficient and selective in vitro silencing for most siSNP. Importantly, the defect in huntingtin function in the vesicular transport of BDNF along microtubules was corrected in HD neural stem cells. By developing a rat model of HD based on the expression of mutant htt expressing the various SNPs, we found that the siSNP efficiently degraded htt mRNA and prevented the apparition of neuropathology only when the fully matched sequences were expressed. The silencing of mutant allele in transgenic BACHD mice further demonstrated the potential for allele-specific silencing and supported the therapeutic potential of RNAi for HD.

INV075

Re-directing vector fate by capsid engineering

Hildegard Büning[1,2]

[1]*Center for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, Germany,* [2]*Department I of Internal Medicine, University Hospital Cologne, Cologne, Germany*

Adeno-associated viral (AAV) vectors are widely used for *in vivo* gene transfer into post-mitotic tissue for which AAV serotype 2 (AAV-2) or the more recently developed serotypes (AAV-1, −4, −5, −6, −8 and −9) have tropism. Vector tropism is dependent on physical parameters such as the accessibility of the target cells within the human body and biological factors like availability of receptors that mediate cell entry and induce a cellular program that leads to nuclear delivery of vector genomes. Native tropism can be either expanded or restricted by modifying the viral capsid (cell surface targeting). Numerous strategies have been developed for this purpose in recent years; of which the most recent contributions will be discussed.

Modifying the viral capsid directly impacts on the vector-host-interaction: Non-genetic coupling of ligands or incorporation of receptor-binding peptides at the spikes, the major immunogenic region of the capsid, allows cell transduction despite the presence of wild-type neutralizing antibodies. Furthermore, targeting of novel cell surface receptors is frequently accompanied by improved intracellular processing allowing intracellular barriers to cell transduction by natural occurring serotypes to be overcome. By increasing the efficacy and specificity of viral gene delivery the vector dose required to achieve therapeutic efficacy may be reduced and anti-AAV immune responses may be diminished.

INV076

Modulation of adenovirus vector - host interactions

Florian Kreppel

Department of Gene Therapy, Ulm University, Ulm, Germany

Attempts to modify adenovirus vector-host interactions have for a long time been based on genetic capsid alterations. Such modifications allowed for example for the introduction of novel receptor binding motifs, targeting, and the ablation of blood coagulation factor X binding. However, systemic vector delivery in vivo is still hampered by a multitude of different interactions of the vector capsids with various cellular and non-cellular host components, and this makes more extensive capsid modifications mandatory to achieve safe and efficient delivery.

In this presentation we will show recent data from our laboratory on how the liver tropism of adenovirus serotype 5-based vectors can be modulated by combining genetic and chemical capsid modifications. Here, a special focus will be put on the evasion from Kupffer cell scavenging after intravenous vector delivery. Furthermore, data on geneti-chemically modified adenovirus vectors with a significantly reduced binding to human erythrocytes will be presented.

INV077

Solving the T-cell problem: generation of immune stealth AAV

Thierry VandenDriessche[1], Frederico Mingozzi[2], Nisha Nair Nisha Nair[1], Kathy High[2], Marinee K.L. Chuah[1]

[1]*Department of Gene Therapy & Regenerative Medicine - Free University of Brussels & University of Leuven, Brussels, Belgium,* [2]*Division of Hematology, the Children's Hospital of Philadelphia, Pennsylvania, USA*

Adeno-associated viral vectors (AAV) represent some of the most promising vectors for in vivo gene therapy. Nevertheless, clinical progress with AAV in gene therapy is severely hampered by the patient's immune response. One of the main limitations of using AAV-based vectors is that they can evoke a vector dose-dependent, AAV capsid-specific, major histocompatibility complex class I (MHC-I)-restricted CD8+ cytotoxic T cell (CTL)-mediated immune response. Consequently, this CD8+ CTL response results in the elimination of gene-engineered cells and the concomitant loss of transgene expression. This cellular immune response accounted for the rapid loss of FIX expression in a gene therapy trial for hemophilia B and the inability to establish a cure for hemophilia "B". To reduce the risk of T cell-mediated immune rejection of AAV-transduced cells, we have generated and characterized novel "immune stealth" AAV nanoparticles that were specifically designed to inhibit antigen presentation in cis. We validated this new concept in vitro and demonstrated that T-cell activation and T-cell mediated recognition of liver cells transduced with the AAV2 "immune stealth" vector was substantially reduced compared to when they were transduced with conventional AAV2 vectors. This type of vector engineering has implications for AAV-based gene therapy and may potentially overcome the limiting T-cell mediated immune rejection of AAV-transduced cells that is frequently observed in human clinical trials.

Supported by: EUFP7 (PERSIST), FWO, IWT, Bayer-Schering Hemophilia Award.

INV078

Genetic Engineering of Human Hematopoiesis for Treating Inherited Diseases and Cancer.

Luigi Naldini

HSR Tiget, Milano, Italy

No abstract available

INV079

Neural Plasticity and Neuronal Diversity in the Adult Mammalian Brain

Fred H Gage

Laboratory of Genetics, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA, USA

The first part of the talk will focus on evidence supporting the birth and maturation of new neurons in the adult dentate gyrus of the hippocampus in the mammalian brain. The mechanism by which the cells integrate and become functional will be discussed. In addition, the potential functional significance for adult neurogenesis in the context of the normal function of the hippocampus will be discussed. In the second part of the talk I will focus on the recent finding that LINE-1 (Long Interspersed Nucleotide Elements-1 or L1) retroelements are active in somatic neuronal progenitor cells (NPCs) providing an additional mechanism for neuronal diversification. Together with their mutated relatives, retroelement sequences constitute 45% of the mammalian genome with L1 elements alone representing 20%. The fact that L1 can retrotranspose in a defined window of neuronal differentiation, changing the genetic information in single neurons in an arbitrary fashion, allows the brain to develop in distinctly different ways. This characteristic of variety and flexibility may contribute to the uniqueness of an individual brain. However, the molecular mechanism that regulates L1 expression in NPCs is not completely understood. L1s are likely silenced in neural stem cells due to Sox2-mediated transcription repression. Down-regulation of Sox2 accompanies chromatin modifications, such as DNA demethylation and histone acetylation, which in turn may trigger neuronal differentiation. The characterization of somatic neuronal diversification will not only be relevant for the understanding of brain complexity and neuronal organization in mammals, but may also shed light on the differences in cognitive abilities.

INV080

Pulp Fictions

David Klatzmann[1,2]

[1]UPMC Univ Paris 06, CNRS, UMR 7211, INSERM, UMR_S 959, Immunology-Immunopathology-Immunotherapy (I3),UPMC Univ Paris 06, CNRS, UMR 7211, INSERM, UMR_S 959, Immunology-Immunopathology-Immunotherapy (I3, Paris, France, [2]Hôpital Pitié-Salpêtrière, Biotherapy, Paris, France

No abstract available

INV081

Ultra-rare orphan diseases and need for platform gene delivery

Jude Samulski

University of North Carolina School of Medicine, Chapel Hill, NC, USA

No abstract available

INV082

Ethical and regulatory issues in cell and gene therapy

Jean-Hugues Trouvin

Universite Paris Descartes, Paris, France

Since January 2008, date of entering into force of the European Regulation 1394/2007 on Advanced Therapy Medicinal Products (ATMP), the regulatory landscape is getting more and more clear for this new class of medicinal products. The Committee in charge of these products (CAT) and its working parties have elaborated a certain number of guidelines or concept papers which highlight the current requirements to be fulfiled during the development of an ATMP. This presentation will highlight the main points to be considered in the development and at the time of the marketing authorisation application.

INV083

Overlapping Ethical Issues in Cell and Gene Therapies

Nancy M King

JD, Wake Forest School of Medicine, Winston-Salem, NC, USA

Gene transfer research has addressed many bioethics issues from which "cell therapy" research can learn. Some ethical issues that arise in both fields represent long-unresolved debates in research ethics; others are unfolding as the science develops. Researchers and oversight bodies can make important contributions to addressing these issues collaboratively with bioethics scholars. Relevant questions include: translation from preclinical studies to research with human subjects; how to measure success and failure; describing research interventions when therapeutic misconception is likely; how to discuss research participation, especially when promising preliminary results may influence decision-making; and how to ensure adequate follow-up when adverse events are rare but serious and long-term efficacy data are needed. Research involving both gene transfer and cell-based interventions has developed rapidly in a climate of great enthusiasm, based on the promising logic of the science and its potential application to serious conditions for which few alternatives exist. The causes and consequences of overoptimism in research are well understood but difficult to remedy, and it is essential to consider whether novel biotechnologies like gene transfer and cell-based intervention research have altered our understanding of the nature and goals of first-in-human trials. Research development and design decisions must take account of the balance between clinical urgency and scientific thoroughness, by ensuring that the trajectory of research is focused on learning as much as possible while minimizing the risks of harm to patient-subjects. Responsible science moves carefully along the research trajectory, proceeding from stage to stage only when enough information has been gathered to justify a step forward; and responsible ethics requires that responsible science precede informed consent. Researchers and oversight bodies in gene transfer and cell-based interventions can lead the way in addressing these critical and contested matters, as the science in these overlapping fields continues its rapid development.

INV084

Multicenter international gene therapy clinical trials for Wiskott-Aldrich syndrome

Anne Galy

Genethon, Evry, France

The transplantation of gene-corrected autologous hematopoietic stem cells is a potential treatment for inherited diseases. Some patients with Wiskott-Aldrich syndrome (WAS), a rare X-linked

primary immunodeficiency (PID), could potentially benefit from this approach. Lentiviral vectors are efficient tools for gene transfer into hematopoietic stem cells. We have developed a lentiviral vector for the hematopoietic gene therapy of WAS. Encouraging preclinical efficacy and safety results with this vector, and the development of robust clinical-grade manufacturing processes at Genethon have supported the initiation of several phase I/II gene therapy studies in WAS. Ongoing international efforts in London, Paris and Boston to coordinate trials of gene therapy for the WAS will be presented and may provide a model for the expedited development of new treatments for other PIDs.

INV085

An exon-skipping strategy using AAV8-U7snRNA vectors for the treatment of Duchenne muscular dystrophy - results in GRMD dogs and clinical perspectives

Caroline LE GUINER[1,2], Marie MONTUS[2], Laurent SERVAIS[3], Luis GARCIA[3], Yves FROMES[3,5], Jean-Yves HOGREL[3], Pierre CARLIER[3], Yan CHEREL[4], Philippe MOULLIER[1,2], Thomas VOIT[3], and the "AFM-sponsored Duchenne Consortium"[2]

[1]INSERM UMR 1089, Nantes, France, [2]GENETHON, Evry, France, [3]Institut de Myologie, Paris, France, [4]INRA UMR 703, Nantes, France, [5]Centre de Boisbonne/ONIRIS, Nantes, France

In Duchenne Muscular Dystrophy (DMD), the selective removal by exon skipping of exons flanking an out-of frame mutation in the dystrophin messenger can result in shorter in-frame transcripts that are translated into functionally active Dystrophin. Our goal is to develop a clinical product for the treatment of DMD. It was defined as a rAAV8-U7snRNA vector specific for the exon skipping of the dystrophin transcript. The mode of delivery chosen is the locoregional intravenous injection in a forelimb.

Using the GRMD dog model, we determined the therapeutic dose of the product, established safety of the treatment and developed a functional read-out, including electrophysiology and NMR imaging. Six groups of GRMD dogs were exposed to different rAAV8-U7snRNA doses (from 2.5E12vg/kg to 2.5E13vg/kg, diluted in two different volumes).

In all cases, the treatment was well tolerated. Results demonstrated high of dystrophin expression level (up to 80%) in dogs injected with 2.5E13vg/kg. Lower levels of dystrophin expression (~35%) and (~10%) were observed in dogs injected with a dose 5 or 10 times lower, respectively. Strength improvement was demonstrated in muscles with more than 40% dystrophin expression. We also observed a dose dependant correction of RMN pathological indexes.

How to transfer these observations into a clinical study protocol will be discussed.

This project is supported by AFM (Association Française contre les Myopathies) and by ADNA (Advanced Diagnostics for New Therapeutic Approaches), a program dedicated to personalized medicine, coordinated by Institut Mérieux and supported by research and innovation aid from the French public agency, OSEO.

INV086

Human pluripotent stem cells for HD cell therapy: hope or hype?

Camille Nicoleau[1], Aurore Bugi[4], Pedro Viegas[1,2], Romina Aron-Badin[3], Christine Varela[4], Philippe Hantraye[3], Marc Peschanski[1,2], Anselme L. Perrier[1,2]

[1]INSERM U861, I-Stem/AFM, EVRY, France, [2]UEVE U861, I-Stem/AFM, EVRY, France, [3]CEA, Institute of Biomedical Imaging, Molecular Imaging Research Center, Fontenay-aux-Roses, France, [4]CECS, I-Stem/AFM, EVRY, France

Pluripotent stem cell (PSC) technologies are becoming a key asset for developing new treatments against many neurodegenerative disorders, such as Huntington's disease (HD). Unrestricted access to human PSCs has drastically changed the outlook of the field towards applying cell therapy to HD. HD is a hallmark striatal neurodegenerative disease; it the genetic disorder that involves progressive neuronal loss in the striatum, the cortex and the globus palidus. The relative spatial selectivity of the first neuronal lesions explains why surgical approaches using fetal striatal tissue are effective for the functional reconstruction of rodent and monkey with drug-induced striatal lesions. In the human, the efficacy of HD cell therapy is today not conclusively proven but this approach is nevertheless the only therapeutic for which a successful clinical outcome has been reported. A recent study has demonstrated that hESC-derived striatal grafts could integrate into the host neural circuitry and correct motor deficits in a rodent model of striatal neurodegeneration. Here, I will describe how human PSCs can be differentiated into striatal neurons population both in vitro and in vivo and how the therapeutic potential as well as the potential adverse effects of such cells can be assessed and possibly managed.

INV087

Restoration of vision in the pde6ß-deficient dog, a large animal model of rod-cone dystrophy

L Petit.[1], E Lhériteau.[1], M Weber.[2], G Le Meur.[2], JY Deschamps.[3], N Provost.[1], A Mendes Madeira.[1], L Libeau[1], C Guihal[1], MA Colle[4], Y Cherel[4], P Moullier[1,5], F Rolling[1]

[1]INSERM UMR1089, Institut de Recherche Thérapeutique 1, Université de Nantes, Nantes, France, [2]CHU-Hôtel Dieu, Service d'Ophtalmologie, Nantes, France, [3]Emergency and Critical Care Unit, ONIRIS, Nantes-Atlantic College of Veterinary Medicine Food Science and Engineering, Nantes, France, [4]UMR703 PAnTher INRA/ONIRIS, Nantes-Atlantic College of Veterinary Medicine Food Science and Engineering, Nantes, France, [5]Department of Molecular Genetics and Microbiology, College of Medicine, University of Florida, Gainesville, Florida, France

Defects in the β subunit of rod cGMP phosphodiesterase 6 (PDE6β) are associated with autosomal recessive retinitis pigmentosa (RP), a childhood blinding disease with early retinal degeneration and vision loss. To date, there is no treatment for this pathology. The aim of this preclinical study was to test recombinant AAV-mediated gene addition therapy in the rod-cone dysplasia type 1 (*rcd1*) dog, a large animal model of naturally occurring PDE6β deficiency that strongly resembles the human pathology. A total of eight *rcd1* dogs were injected subretinally with AAV2/5RK.cpde6β (n=4) or AAV2/8RK.cpde6β (n=4). *In vivo* and *post-mortem* morphological analysis showed a significant preservation of the retinal structure in transduced areas of both AAV2/5RK.cpde6β- and AAV2/8RK.cpde6β-treated retinas. Moreover, substantial rod-derived ERG signals were recorded as soon as 1 month postinjection (35% of normal eyes) and remained stable for at least 21 months (the duration of the study) in treated eyes. Rod-responses were undetectable in untreated contralateral eyes. Most importantly, dim light vision was restored in all treated *rcd1*

dogs. These results demonstrate for the first time that gene therapy effectively restores long-term retinal function and vision in a large animal model of autosomal recessive rod-cone dystrophy, and provide great promise for human treatment.

INV088

No abstract available

Fatima Bosch

Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain

No abstract available

INV089

Pancreatic Beta Cell Development: Experimental Models to Transfer Data from Rodent to Human

Raphael Scharfmann

INSERM U845, Paris, France

Pancreatic beta cells develop from endodermal pancreatic progenitors that proliferate and next differentiate into functional insulin-producing cells. This is a complex process, each step being controlled by specific signals. Theoretically, beta cell mass can be enhanced by: i) activating the proliferation of pancreatic progenitors; ii) activating their differentiation into beta cells; iii) activating the proliferation of beta cells themselves.

During the past years, we developed bioassays based on rodent models to search for signals controlling each step of beta cell development. With this bioassay, we screened and characterized a number of signals regulating pancreatic progenitor cell proliferation and differentiation. We also developed models of human pancreatic development, allowing the transfer to human of data generated in rodent models. Such models were instrumental for the development of functional human beta cell lines.

Such different approaches, aiming at better dissecting signals regulating functional beta cell mass in rodent and human will be presented.

INV090

Helper-dependent adenoviral vectors for inborn errors of liver metabolism.

Nicola Brunetti-Pierri[1,2]

[1]*Telethon Institute of Genetics and Medicine, Naples, Italy,* [2]*Department of Pediatrics, Federico II University of Naples, Naples, Italy*

Treatment for inborn errors of liver metabolism has focused on dietary, drug, and cell therapies. However, significant morbidity and mortality still remain and alternative and adjunctive strategies are needed. The goal of liver-directed gene therapy is to deliver therapeutic genes to hepatocytes, where the majority of metabolic reactions occur, and to provide long-term treatment for these diseases.

Helper-dependent adenoviral (HDAd) vectors are attractive for hepatocyte gene therapy because they can provide efficient and long-term transgene expression in the absence of chronic toxicity. Following intravenous injections, hepatocyte transduction is hampered by Kupffer cells and liver sinusoidal endothelial cells. Efficient hepatocyte transduction by intravenously injected HDAd vector requires high vector doses, which result in dose-dependent activation of acute, potentially lethal toxicity. To address this problem, we investigated physical and pharmacological methods to achieve preferential HDAd-mediated hepatocyte gene transfer. We developed in non-human primates a minimally invasive balloon occlusion catheter-based method to achieve efficient and long-term hepatocyte transduction with low vector doses. We next investigated HDAd vector uptake by liver non-parenchymal cells, with the goal of evading these barriers to further improve hepatocyte gene transfer. Blood-borne HDAd particles interact with scavenger receptor A (SR-A) and scavenger receptor expressed on endothelial cells I (SREC-I), both in Kupffer cells and endothelial cells and blocking these receptors increases the efficiency of hepatocyte transduction and thus, the vector therapeutic index. Ultimately, the combination of physical methods and pharmacological agents, preventing vector uptake in non-parenchymal cells, may improve efficiency and safety of HDAd vectors for clinical applications.

INV091

No abstract available

Philippe Leboulch

Brigham and Women's Hospital, Boston, USA

No abstract available

INV092

When insulation is not a solution: post-transcriptional deregulation of gene expression induced by vector integration

Fulvio Mavilio

Genethon, Evry, France

Gene transfer vectors derived from oncoretroviruses or lentiviruses are widely used in human gene therapy. Integration of these vectors in the human genome may, however, have genotoxic effects, caused by de-regulation of gene expression at the transcriptional level. "Insulating" a vector may in part reduce the impact of integration on normal gene regulation. However, integration of retroviral vectors within transcribed genes may interfere with their regulation also at post-transcriptional level, by interfering with splicing and polyadenylation of primary transcripts. Traditional insulator elements have no effect on post-transcriptional gene deregulation. The identification of constitutive and cryptic splice and polyadenylation signals within a vector provides a strategy for recoding vector backbones and transgenes and reducing their potential post-transcriptional genotoxicity.

INV093

New chromatin insulators for gene therapy

George Stamatoyannopoulos

University of Washington, Seattle Washington, USA

Chromatin insulators blocking the interaction between regulatory elements of viral vectors and neighboring oncogenes are expected to decrease the risk of clinical genotoxicity. The best studied chromatin insulator is the DNAse I hypersensitive site 4 (cHS4) of the chicken beta globin locus. This powerful insulator has been shown to block the interactions between enhancers and promoters in several mammalian experimental systems and it

has been used in clinical gene therapy. However cHS4 has two important disadvantages: its large size (1.2Kb), and the substantial decrease in viral titers when it is incorporated in gamma retroviral and lentiviral vectors. We therefore focused on the discovery of new chromatin insulators that do not have the disadvantages of cHS4.

We developed a new genomic approach that allowed us to mine the human genome for potential chromatin insulators. We have identified several hundred sequences in different chromosomal locations that could be potential chromatin insulators. We cloned 27 of these and, using functional assays, we showed that all 27 function as enhancer blocking insulators. 18 of the 27 are more powerful enhancer blockers than cHS4. One is about 6 times more powerful than cHS4. Importantly, the size of these elements is about 150 to 200 nucleotides. Several do not affect titers or have only small effects on the titers of lentiviral vectors.

These insulator elements, and others to be identified with our continuing mining of the human genome, will help decrease the risk of genotoxicity of integrating viral vectors.

INV094

Glial cell regulation of hematopoietic stem cell hibernation in the bone marrow niche

Hiromitsu Nakauchi[1,2]

[1]*Division of Stem Cell Therapy, Center for Stem Cell Biology and Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan,* [2]*Japan Science and Technology Agency (JST), ERATO, Nakauchi Stem Cell and Organ Regeneration Project, Tokyo, Japan*

Most hematopoietic stem cells (HSCs) in the bone marrow (BM) niche are in a hibernating state. We recently demonstrated that lipid raft clustering is a key event in the regulation of HSC hibernation. Freshly isolated $CD34^{-}c\text{-}Kit^{+}Sca\text{-}1^{+}$Lineage marker $(Lin)^{-}$ ($CD34^{-}KSL$) HSCs from the BM niche lacked lipid raft clustering, resulting in muted signals (including those of the AKT-FOXO pathway). By contrast, lipid raft clustering induced by cytokines augmented signals and appeared essential for HSC re-entry into the cell cycle. Conversely, inhibition of lipid raft clustering caused sustained nuclear accumulation of FOXO transcription factors and induced HSC hibernation ex vivo. These findings established a critical role of lipid rafts in regulating the cell cycle, survival, and apoptosis of HSCs and uncovered a striking similarity between HSC hibernation and C. elegans dauer formation.

We then hypothesized that a niche factor regulates the lipid raft clustering of HSCs in vivo. Screening of candidate niche signals revealed that transforming growth factor-b (TGF-b) efficiently inhibited cytokine-mediated lipid raft clustering and induced HSC hibernation ex vivo, establishing TGF-b as a candidate niche signal in the control of HSC dormancy. Given that TGF-b is produced as a latent form by a variety of cells, we searched for cells that express activator molecules for latent TGF-b Non-myelinating Schwann cells in BM, proved responsible for activation. These glial cells ensheathed autonomic nerve, expressed HSC niche-factor genes and were in contact with a substantial proportion of HSCs. Autonomic-nerve denervation reduced the number of these active TGF-b producing cells and led to rapid loss of HSCs from BM. We propose that glial cells are constituents of a bone marrow niche and maintain hematopoietic stem cell hibernation by regulating activation of latent TGF-b.

INV095

Antisense based therapeutic approaches in Hutchinson-Gilford Progeria

Annachiara De Sandre-Giovannoli[1,2], Claire Navarro[1], Fernando Osorio[3], Catherine Bartoli[1], Danielle Depetris[1], Racha Fayek[1], José M. P. Freije[3], Carlos Lopez-Otin[3], Nicolas Levy[1,2]

[1]*Aix-Marseille Université, Inserm UMR_S910 : Medical genetics and Functional genomics, Faculté de Médecine de Marseille, Marseilles, France,* [2]*AP-HM, département de génétique Médicale, Hôpital Timone, Marseilles, France,* [3]*Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Instituto Universitario deOncología, Universidad de Oviedo, Oviedo, Spain*

Hutchinson-Gilford Progeria (HGPS) is an extremely severe and rare segmental premature aging syndrome. Since the discovery that a recurrent point mutation of the LMNA gene encoding Lamins A/C is responsible of the development of the disease through the production of Progerin, an ubiquitously accumulating Lamin A toxic derivative, our team endeavors to find treatments for the disease. Recently, in collaboration with Carlos Lopez Otin's team (Oviedo, Spain), we developed the $Lmna^{G609G/G609G}$ knock-in mice (LAKI), faithfully recapitulating the clinical phenotype as well as reproducing the molecular defects underlying the disease. Using these mice, we have obtained the first preclinical, in vivo, proof of principle that the reduction of Progerin levels can be achieved through an antisense "gene therapy" systemic approach, with beneficial effects on several disease parameters, including lifespan extension. This amelioration was achieved in mice after intravenous delivery of the antisense modified (vivo-morpholino) oligonucleotides. In order to test less invasive therapeutic interventions, foreseeing a future translation of preclinical studies into a clinical trial and potentially life-long treatments in children, we have designed an assay using lipid micelles microemulsions (Aonys®) allowing to deliver antisense oligonucleotides by per-mucosal transfer (oral or rectal administration routes), with potentially high biodistribution and bioavailability. These studies on $Lmna^{G609G/G609G}$ mice will be exposed and are ongoing in order to confirm or infirm the feasibility and efficacy of the per-mucosal delivery approach before longer-term studies are undertaken.

INV096

Effect of ageing and muscle origin on human satellite cells

Vincent Mouly[1,3], Anne Bigot[1,2], Aurore Wielgosik[1,2], Elisa Negroni[1,2], Capucine Trollet[1,2], Teresa Gidaro[1,2], Marei-Catherine LeBihan[1,2], Gillian Butler-Browne[1,2]

[1]*UPMC Université Paris 6, UM76, Institut de Myologie, Paris, France,* [2]*INSERM U 974, Institut de Myologie, 47 bd de l'Hôpital, Paris, France,* [3]*3 CNRS UMR 7215, Institut de Myologie, Paris, France*

Skeletal muscle ageing or sarcopenia is characterized by a decrease in muscle mass and function, and a decrease in its regenerative capacity after trauma. Muscle regeneration in aged subjects is much slower, which can be partly explained by a severe decrease in the number of satellite cells expressing markers such as N-CAM, M-CAD or Pax7, but there is a lack of information regarding their functionality in humans. We have investigated in vitro the potential of human myoblasts to proliferate and differentiate, as well as to signal to other cells, and compared these

data between young and old adult subjects. The differentiation program of myoblasts is perturbed when they approach proliferative senescence, which in humans is caused not only by telomere shortening, but primarily by triggering of the p16 stress pathway, at least in vitro. The secretome was investigated in differentiating myoblasts, a situation corresponding to early steps of regeneration, and was found perturbed in several pathways when they approach senescence, such as inflammatory cytokines, IGF-1 or matrix remodeling. The proliferative capacity of myoblasts isolated from young or old sedentary or active subjects was measured in vitro, and found similar in all cases. This was confirmed by telomere measurements and the only difference we observed concerned an earlier activation of the p16 pathway, although at levels that do not seem to hamper their proliferative capacity in elderly subjects. However, it is reduced in myoblasts isolated from patients suffering from muscular dystrophies.

A project supported by AFM, ANR In-A-Fib, and EU (MYOAGE).

INV097

Successful AAV-mediated gene therapy of rod-cone and cone-rod dystrophies in dogs: great promise for the treatment of rapid degenerative photoreceptor defects

Fabienne Rolling

INSERM UMR 1089, Nantes, France

Our team has recently provided the first demonstration of AAV-mediated restoration of rod function and vision in Pde6ß-deficient dogs, a large animal model of a degenerative rod-cone disorder. In parallel, we have recently observed AAV-mediated restoration of both cone function and vision in Rpgrip1-deficient dogs, a large animal model of a degenerative cone-rod disorder.

Previous successful gene therapy study on photoreceptor-defects in dogs was demonstrated in 2 canine models of stationary disorder (Cngb3-achromatopsia).

The fact that both degenerative cone-rod and rod-cone disorders can be successfully treated by gene therapy in dogs, is new and constitutes a major advance for the treatment of degenerative retinal diseases originating in photoreceptors.

INV098

Optogenic vision restoration strategy

Jens Duebel

Institut de la Vision, Paris

No abstract available

INV099

Transplantation of ES cell-derived photoreceptors

EL West

UCL Institute of Ophthalmology, London, UK

Retinal degeneration leading to a loss of photoreceptors is a major cause of untreatable blindness. Inherited retinal dystrophies affect 1 in 3,000 of the population, and age-related macular degeneration (AMD) affects 1 in 10 people over 60 yrs. Currently no treatments restore lost photoreceptor cells and visual function and thus there is a need for new therapeutic approaches. As new photoreceptors need only make short, single synaptic connections to the inner retinal circuitry to contribute to visual function, retinal repair by photoreceptor transplantation represents one of the most feasible types of CNS repair.

We have established that photoreceptor precursors at the correct ontogenetic stage are able to migrate and functionally integrate into the degenerate adult retina (MacLaren et al., 2006; Pearson et al., 2012). To translate this therapeutic approach we need to establish a renewable source of correctly staged photoreceptor precursors. Therefore, we have investigated ES cell-derived retinal differentiation and the integration competence of ES cell-derived photoreceptor precursors. Our initial investigations generated photoreceptor precursors by the stepwise treatment of ES cells with defined factors, using an adherent 2-dimensional culture system. We optimized retinal progenitor cell differentiation and further cultured these cells to generate sufficient numbers of retinal cells for transplantation. With the recent publication of a 3-dimensional retinal differentiation culture system (Eiraku et al., 2011), we have also investigated this method of retinal differentiation to generate correctly staged photoreceptor precursors for transplantation. This approach provides a highly promising strategy for the treatment of degenerative retinal disease.

INV100

In vitro generation of Red Blood Cells: perspectives for transfusion medicine

Luc DOUAY[1,2]

[1]Université Pierre et Marie Curie, Paris, France, [2]Hopital St Antoine, Paris, France, [3]EFS Ile de France, Créteil, France

Transfusion of red blood cells (RBCs) is now considered as a well settled and essential therapy. However, some difficulties and constraints still occur,. RBCs can be now cultured in vitro from human hematopoietic, human embryonic or human induced pluripotent stem cells. The highly promising hiPSC technology represents a potentially unlimited source of RBCs and opens the door to the revolutionary development of a new generation of allogeneic transfusion products. We draw here some futuristic, but realistic scenarios, regarding potential applications for alloimmunized patients and those with a rare blood group. We retrospectively studied a cohort of 16,486 consecutive alloimmunized patients, showing 1 to 7 alloantibodies with 361 different antibody combinations. We showed that only 3 hiPSC clones would have been sufficient to match more than 99% of the 16,486 patients in need of RBC transfusions. The study of the French National Registry of People with a Rare Blood Phenotype/Genotype (10-year period) showed that 15 hiPSC clones would have covered 100% of the needs in patients of Caucasian ancestry. In addition, one single hiPSC clone would have met 73% of the needs in alloimmunized patients with sickle cell disease, for whom rare cryopreserved RBC units were required. As a result, we may consider that a very limited number of RBC clones would be able not only to provide for the need for most alloimmunized patients and those with a rare blood group, but also to efficiently allow for a policy for alloimmunization prevention in multiply transfused patients.

INV101

Large scale, high efficiency transduction of human CD34+ cells with lentiviral vectors for β-Thalassemia and ccALD clinical applications

Gabor Veres

bluebirdbio Inc, Cambridge, MA, USA

Efficient transduction of hematopoietic stem/progenitor cells (HSPCs) ex vivo with lentiviral vectors is of clinical importance for gene therapy applications. HIV-based vectors provide advantages over γ-retroviral vectors, including the possibility to transduce non-dividing cells. The currently used HIV based vectors provide an efficient and safe gene transfer systems by minimizing the use of viral genes, preventing the risk of recombination with split-genome design and the risk of replication with self-inactivating (SIN) design. Large scale production of recombinant HIV vector at high quality is critical to transduce human CD34+ cells efficiently as measured by percent of transduced cells and vector copy number (VCN) in CD34+ cells and its progeny.

We have optimized the transduction of mobilized CD34+ cells at large scale demonstrating transduction efficiency of 70–80% and VCN over 2 copies in CD34+ cell both in short and long term progeny using LentiGlobin vector intended to be used in β-Thalassemia clinical trial and a LentiD vector expressing the human ABCD1 cDNA to be used in childhood cerebral Adrenoleukodystrophy (ccALD) clinical trial. We also demonstrate efficient transduction of CD34+/CD38- early progenitor population.

INV102

Progress towards the cGMP production of hESC derived RBCs

Joanne Mountford[1,2]

[1]*University of Glasgow, Glasgow, UK,* [2]*Scottish National Blood Transfusion Service, Scotland, UK*

Blood Transfusion has become a mainstay of modern medical practice. However problems persist both nationally and internationally in maintaining adequacy of supply, managing the risk of transmission of infectious agents and immune incompatibility between donor and recipient. Human embryonic and induced pluripotent stem cells (hESCs & iPSC) have unique properties in that they can be maintained indefinitely in culture in an undifferentiated state and yet retain the ability to form all the cells and tissues within the body. They therefore offer a potentially limitless source from which to generate red cells (RBCs) for use in clinical transfusion. Initially *in vitro* RBCs may have particular utility for patients who receive regular transfusions such as those with haemoglobinopathies. Within the project we have the capability to generate hESC to cGMP grade in compliance with UK regulatory requirements for eventual clinical use. We are able to differentiate these to form haematopoietic progenitor cells (HPC) and to subsequently differentiate these down the erythroid lineage with high efficiency in a stromal free, suspension based culture system. I will present our progress in this project and discuss the scientific, clinical and regulatory challenges for this work.

INV103

The sodium iodide symporter (NIS) and its role as a theranostic gene

Christine Spitzweg

University Hospital of Munich, Department of Internal Medicine II, Munich, Germany

No abstract available

INV104

Imaging brain activity by monitoring Po_2 transients in capillaries

Alexandre Parpaleix, Yannick Goulam Houssen, Serge Charpak

INSERM U603, Laboratory of Neurophysiology and New Microscopies, Université Paris Descartes, Paris, France

Two-photon phosphorescence lifetime microscopy (2PLM) has been recently used for depth-measurements of the oxygen partial pressure (Po_2) in the rodent brain. In capillaries of olfactory bulb glomeruli, 2PLM has also allowed simultaneous measurements of Po_2 and blood flow, and revealed the presence of erythrocyte-associated transients (EATs), i.e. Po_2 gradients associated with individual erythrocytes. We investigated here the extent to which EAT properties in capillaries report local neuronal activity. We find that at rest, Po_2 at EAT peaks overestimates the mean Po_2 by 35 mm Hg. Po_2 between two EAT peaks is at equilibrium with, and thus reports, Po_2 in the neuropil. During odor stimulation, a small Po_2 decrease is detected prior to functional hyperemia, demonstrating that the controversial Po_2 initial dip is present at the level of capillaries. We conclude that imaging oxygen dynamics in capillaries provides a unique and non-invasive approach to finely map neuronal activity.

INV105

Multiparametric spinal MRI in degenerative motor neuron diseases

Pierre-François Pradat[1,2], Mohamed-Mounir El Mendili[2], Julien Cohen-Adad[3], Serge Rossignol[3], Habib Benali[2]

[1]*Departement of Neurology, Pitie-Salpetriere Hospital, Paris, France,* [2]*UMR-678, INSERM-UPMC, Pitie-Salpetriere Hospital, Paris, France,* [3]*Montreal University, Montreal, Canada*

In neurodegenerative diseases of the spinal cord, standard spinal MRI is limited to the differential diagnosis. New developments in neuroimaging allow better investigating spinal cord and provide new avenues to develop biomarkers. To increase the sensitivity and specificity of this method, we developed a multiparametric approach which combines several MRI metrics. These metrics associate measures of spinal cord atrophy, parameters derived from diffusion tensor imaging (fractional anisotropy, axial diffusivity, radial diffusivity) and magnetisation transfer ratio. We have shown that such an approach was sensitive to detect abnormalities in amyotrophic lateral sclerosis and spinal muscular atrophy patients compared to controls. In amyotrophic lateral sclerosis, we observed correlations with clinical index of disease severity. Cervical spinal cord atrophy correlated with muscle weakness at the same metameric level (spinal cord atrophy at the C5 spinal cord level correlated with deltoid muscle strength and spinal cord atrophy at the C8 spinal cord level correlated with abductor pollicis brevis strength). We also observed a correlation between fractional anisotropy and a functional score (ALSFRS-R scale). It suggests that spinal cord multiparametric MRI is promising to provide surrogate markers in therapeutic trials testing new drugs as well as gene therapy approaches.

INV106

New avenues in non viral gene delivery and therapy

Pascal Bigey, Aurore Burgain, Virginie Escriou, Corinne Marie, Mickael Quiviger, Anne Schlegel, Daniel Scherman

Laboratory of Chemical and Genetic Pharmacology and of Imaging, Université René Descartes – Chimie ParisTech, UMR 8151 CNRS - U1022 Inserm, Paris, France

Clinical gene therapy most recent successes have been obtained by using viral vectors such as lenti- or adenovirus derived vectors. However, given the broad scope of gene therapy, it is considered that both viral and non viral vectors will find their own niche of excellence and application.

For instance, "naked DNA" vaccination is now widely used in the veterinary field for prophylactic purposes, such as salmon vaccination against infectious hematopoietic virus in Canada (Novartis), horse vaccination against the West Nile virus infection (Ft Dodge Animal Health), and for an immunotherapeutic treatment of melanoma in dogs in the USA (Merial). Another non viral gene therapy product enables the sustained expression of growth hormone-releasing hormone (GHRH) to avoid fetal loss in swine (VGX Animal Health).

The recent progresses obtained both in our laboratory and by others will be presented, including:

- new biosafe plasmids such as minicircles or plasmids free of antibiotic resistance markers (pFAR) for both genetic immunization and gene therapy
- improved physical techniques such as electrotransfer for passive and active immunotherapy and for vaccination
- the use of another physical technique, hydrodynamic liver delivery, for the treatment of both systemic and brain metabolic disease.
- the use of chemical vectors for SiRNA delivery.

These recent progresses give hope for a rapid extension of non viral gene delivery for both cognitive and translational science at the laboratory level, and also for human or animal clinical applications.

INV107

Update on the UK CF Gene Therapy Consortium Multidoes, non-viral, gene therapy trial

EW Alton[1,7], AC Boyd[2,7], SH Cheng[3], S Cunningham[4,7], JC Davies[1,7], DR Gill[5,7], U Griesenbach[1,7], TE Higgins[1,7], SC Hyde[5,7], JA Innes[2,7], GD Murray[1,7], DJ Poteous[2,7]

[1]Department of Gene Therapy, Imperial College, London, UK, [2]Medical Genetics Section, Centre for Molecular Medicine, MRC Institute of Genetics and Molecular Medicine, University of Edinburgh, Western General Hospital, Edinburgh, UK, [3]Genzyme Corporation, Framingham, MA, USA, [4]Department of Respiratory and Sleep Medicine, Royal Hospital for Sick Children, Edinburgh, UK, [5]Gene Medicine Group, Nuffield Dept of Clinical Laboratory Sciences, University of Oxford, John Radcliffe Hospital, Oxford, UK, [6]Centre for Population Health Sciences, University of Edinburgh, Edinburgh, UK, [7]On behalf of The UK CF Gene Therapy Consortium, London, Oxford, Edinburgh, UK

The UK CF GTC has been working for several years to determine the clinical benefit of *CFTR* gene therapy. To achieve clinical benefit, we hypothesised that repeated administration would be required, therefore limiting us to a non-viral approach. We demonstrated that GL67A (Genzyme Corp) complexed with a plasmid, pGM169, depleted of pro-inflammatory CpG motifs and driven by the EF1a promoter produced optimal expression. A longitudinal observational study (Run-in) provided key data on the optimal outcome measures, as well as the patient population (FEV1 50–90% predicted) which would be optimal for the trial. The former include a) primary outcome (FEV_1), b) secondary efficacy outcomes (lung clearance index, CT scan parameters, Quality of life questionnaire [CFQ-R], exercise capacity and activity, and selected sputum and serum inflammatory markers), and c) safety measures (clinical findings, exacerbation rate, gas transfer, sputum culture, serum inflammatory markers, renal and hepatic markers).

130 patients, aged 12 years, and above are being randomised in a 1:1 fashion to active treatment or placebo and will receive the nebulised agent at monthly intervals for 12 doses at a dose determined from our recently completed single dose (Pilot) study. The group size was determined on the basis of a 6% relative improvement in FEV_1. Mechanistic substudies will assess nasal and bronchial PD and mRNA. The double-blinded nature of the trial means that final outcome data will only be available upon completion of the study. The trial was initiated in April 2012.

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INV108

A holistic approach towards restoring vision in retinitis pigmentosa, between prosthetics, optogenetics and rehabilitation

José-Alain SAHEL[2,1]

[1]Institut de Vision, Paris, France, [2]UPMC Univ Paris VI, Paris, France, [3]INSERM, Paris, France, [4]CNRS, Paris, France, [5]Centre Hospitalier National des Quinze-Vingts, Paris, France, [6]Fondation Opthtalmologique Adolphe de Rothschild, Paris, France, [7]Institute of Ophthalmology University College of London, London, UK

In retinitis pigmentosa, the loss of light-adapted visual responses is the key event leading to blindness. Preservation or partial restoration of impaired cone function offers very promising perspectives based on neuroprotection, prosthetics or optogenetics. In this presentation we shall describe the potential strategies to protect or restore cone function, our clinical experience in high resolution imaging of these cells, and methods to assess reliably and enhance the impact of such innovative therapies in daily life.

Awakening retinal neurons : Recent experience from retinal prostheses establish that reactivation of degenerated retinal tissue in blind patients leads to restoration of useful visual function, after a structured process of rehabilitation. Recently, work conducted by Botond Roska and our group showed that in advanced cases, cone cell bodies of dormant cones can be reactivated by vectorization of halorhodopsins, i.e. chloride-pumps activated by light, thus restoring cone function through adequate stimulation.

Patient-centred rehabilitation: Both these approaches will benefit from advances in visual information processing and stimulation, and more importantly from the involvement of patients in the assessment and the optimization of the rehabilitation pattern. In order to evaluate and document such functional outcomes, we have developed novel tools for assessing reproducibly visual impairment and restoration, as well as palliative aids and associated training protocols. These include the development of virtual simulators, the construction of versatile

environments reproducing daily life situations (e.g. apartment, street, shop, obstacles …) and the implementation of monitoring tools (captors, cameras, multiparametric modelling).

INV109

ZFN-edited CD4+ T cells for HIV/AIDS Therapy: Phase 1 Trials of SB-728-T in HIV-infected Subjects

Philip Gregory

Sangamo BioSciences Inc, Richmond, California, USA

Zinc finger nucleases (ZFNs) provide a platform technology for the targeted engineering of the human genome. We have previously shown that ZFNs targeting CCR5, an obligate co-receptor for entry of R5 HIV, render modified CD4 T cells resistant to HIV analogous to cells from subjects homozygous for a naturally occurring CCR5 mutation (CCR5Δ32). Here we report data from Phase I studies evaluating infusion of an autologous CCR5-modified T-cell product (SB-728-T) in HIV-infected subjects. We show that infusion was well tolerated, resulted in marked and prolonged enhancement of total circulating CD4 T-cell numbers, and prolonged engraftment of SB-728-T in peripheral blood and key CD4 T-cell trafficking sites, such as the gut-associated lymphoid tissue. Importantly, following SB-728-T engraftment, a small cohort of treated HIV-infected subjects underwent experimental HAART interruption (TI), providing the first evidence of an impact of SB-728-T on circulating HIV load and prompting confirmatory clinical trials.

INV110

The role of partnerships in regenerative medicine and gene therapy – a pharma perspective

Jason Gardner

GlaxoSmithKline, PA, USA

Discovering and developing medicines based on new modalities beyond small molecules and biopharms will require expertise in multiple areas. This presentation will discuss representative partnerships that GlaxoSmithKline has established in regenerative medicine, stem cell gene therapy and oligonucleotides as examples of the synergies that work in these alliances.

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Selected Oral Presentations

OR001

Lentiviral vector mediated gene therapy for the treatment of Wiskott-Aldrich Syndrome

S Scaramuzza[1], F Ferrua[2], S Giannelli[1], MC Castiello[1], MP Cicalese[2], C Evangelio[2], A Assanelli[2], L Biasco[1], M Casiraghi[2], M Bosticardo[1], A Finocchi[3], A Metin[4], PP Banerjee[5], JS Orange[5], A Biffi[1,2], F Ciceri[6], A Villa[1,7], MG Roncarolo[2,8], L Naldini[1,8], A Aiuti[1,3]

[1]*San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Milan, Italy,* [2]*Pediatric Immunology, Scientific Institute HS Raffaele, Milan, Italy,* [3]*University of Rome "Tor Vergata", Rome, Italy,* [4]*Ankara Dispaki Children's Hospital, Ankara, Turkey,* [5]*Dep. of Pediatric, Children's Hospital, Philadelphia, USA,* [6]*Div. of Hematology, Scientific Institute HS Raffaele, Milan, Italy,* [7]*IRGB-CNR, Milan, Italy,* [8]*Università Vita-Salute San Raffaele, Milan, Italy*

Wiskott-Aldrich Syndrome (WAS) is an X-linked immunodeficiency characterized by thrombocytopenia, infections, autoimmunity and lymphomas. Gene therapy with *ex vivo* transduced hematopoietic stem cells (HSC) could represent a valid therapeutic option for patients lacking an HLA-identical donor. We designed a phase I/II clinical trial based on infusion of autologous transduced $CD34^+$ cells and reduced intensity conditioning with anti-CD20, busulfan and fludarabin; ATG is included in case of autoimmune manifestations. Three patients were treated with autologous bone marrow (BM) $CD34^+$ cells transduced with highly purified lentiviral vector. The first patient received also mobilized peripheral blood (PB) transduced $CD34^+$ cells to reach an adequate cell dose. Transduction of clonogenic progenitors was highly efficient ($94.3 \pm 5.3\%$), with a mean VCN/genome in bulk $CD34^+$ cells of 2.1 ± 0.6. Robust multilineage engraftment was observed at 1 year in BM myeloid lineages (VCN range: 0.29–0.78), including BM clonogenic progenitors ($38.7 \pm 11.6\%$), and in PB granulocyte (VCN: 0.56 ± 0.15) and lymphocytes (VCN range: 1.05–2.29) at the latest follow-up. WASp expression was observed in PB platelets, monocytes and at higher levels in lymphoid cells. Moreover, proliferative responses to anti-CD3, NK cell cytotoxic activity and Treg suppressive function were normalized after gene therapy. All patients are currently clinically well, independent from platelet transfusions, free from eczema and severe infections. In conclusion, the unprecedented level of gene transfer resulted in robust engraftment of transduced HSC even when combined to reduced intensity conditioning. Further studies will be required to asses the long-term safety and efficacy of LVV gene therapy for WAS.

OR002

Correction of the X-CGD phenotype by a self-inactivating alpharetroviral vector in human and murine models

Kerstin B. Kaufmann[1], Christian Brendel[1], Julia D. Suerth[2], Linping Chen-Wichmann[1], Joachim Schwaeble[1], Hana Kunkel[1], Axel Schambach[2], Christopher Baum[2], Manuel Grez[1]

[1]*Georg-Speyer-Haus, Frankfurt, Germany,* [2]*Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany*

Alpharetroviruses provide characteristics that make them highly attractive for gene therapy: Comparative integrome analysis have revealed an almost random integration profile with no strong preferences for integrations close to transcription start sites or into active genes as it has been reported for gammaretro- and lentiviruses, respectively. Replication deficient, self-inactivating (SIN) alpharetroviral vectors recapitulate this neutral integration profile and, in addition, have revealed a significantly lower genotoxicity than gammaretro- and lentiviral vectors. We chose X-linked chronic granulomatous disease (X-CGD) as a model to evaluate the potential of this recently developed SIN-alpharetroviral vector system for gene therapy of monogenic diseases. Therefore, we combined the vector backbone with the physiological elongation factor-1α short (EFS) promoter to drive transgene (gp91phox) expression and evaluated it in murine and human XCGD models.

Transduction of the human myelomonocytic cell line X-CGD PLB985 revealed no marked difference to the lentiviral equivalent in terms of titer, expression and functional activity. Reconstitution of superoxide production was also observed in primary cells in vitro and in a murine X-CGD transplantation model. Finally, studies on transduced human primary XCGD CD34+ cells have shown efficient gp91phox expression and functional activity upon differentiation as well as gene marking of NSG repopulationg cells at low vector copy number. Taken together, we provide the first proof of principle that SIN-alpharetroviral vectors functionally restore a disease phenotype. This and the vector system's lower genotoxicity not only emphasize its application in X-CGD but also highlight its potential for future gene therapy approaches in general.

OR003

Gamma-retroviral gene therapy for X-CGD: Differential outcome of single MDS1-Evi1 integration vs double MDS1-Evi1/STAT3 integration

J Reichenbach[1], U Siler[1], A Paruzynski[2], H Holtgreve-Grez[3], E Renner[7], U Köhl[4], C Alhan[5], AA van de Loosdrecht[5], E Kouzmenko[1], K Kühlcke[6], M Schmugge[1], T Güngör[1], G Notheis[7], M Schmidt[2], M Grez[8], RA Seger[1]

[1]Division of Immunology/Haematology/BMT, University Children's Hospital and Children's Research Centre, Zurich, Switzerland, [2]Department of Translational Oncology, National Center for Tumor Diseases and German Cancer Research Center, Heidelberg, Germany, [3]Institute of Human Genetics, University Hospital Heidelberg, Heidelberg, Germany, [4]Paediatric Haematology, Oncology and Haemostaseology, University Medical School, Frankfurt, Germany, [5]Cancer Centre Amsterdam, Department of Haematology, VU University Medical Centre Amsterdam, Amsterdam, The Netherlands, [6]EUFETS AG, Idar-Oberstein, Germany, [7]Departments for Paediatric Haematology/Oncology and Infection/Immunity, Dr. von Haunersches Kinderspital, Ludwig-Maximilians-University, München, Germany, [8]Institute for Biomedical Research, Georg-Speyer-Haus, Frankfurt, Germany

Gene-modified autologous haematopoietic stem cells (HSC) can provide significant clinical benefit to subjects suffering from X-linked chronic granulomatous disease (X-CGD), a rare inherited immunodeficiency characterized by recurrent, often life-threatening bacterial and fungal infections. Here we report on the cellular and molecular events observed in two boys with X-CGD treated by γ-retroviral gene therapy in a Swiss-German clinical phase I/II trial in 2005 and in 2007. After the initial resolution of life-threatening fungal infections, one child developed insertional activation of ecotropic viral integration site 1 (EVI1) and of signal transducer and activator of transcription 3 (STAT3). This particular clone displaced all other EVI1 expressing clones and dominated gene marked hematopoiesis from month 22 after GT onwards leading to a myelodysplastic syndrome (MDS) with monosomy 7, resistant to allogeneic HSC transplantation (allo-HSCT) with myeloablative conditioning. The other child did not develop MDS despite expansion of an MDS1 clone and was definitively cured by allo-HSCT. Our data revealed that overexpression of EVI1 and STAT3 in human cells cooperates in the development of monosomy 7, and clonal progression toward myelodysplasia.

OR004

Efficient generation of gene-corrected and reprogramming factor-free iPS cells from SCID-X1 patients

Angelo Lombardo[1], Claudia Firrito[1], Andrew R. Gennery[2], Michael C. Holmes[3], Philip D. Gregory[3], Luigi Naldini[1,4]

[1]San Raffaele Telethon Institute for Gene Therapy, Milan, Italy, [2]Newcastle Upon Tyne Hospital, Newcastle Upon Tyne, UK, [3]Sangamo BioSciences Inc., Richmond, CA, USA, [4]Vita-Salute San Raffaele University, Milan, Italy

Patient-derived, gene-corrected induced Pluripotent Stem Cells (iPSCs) have great potential for the treatment of human genetic diseases for which isolation, *ex vivo* expansion and/or genetic correction of autologous cells is technically challenging. To date the most efficient approaches to replace malfunctioning genes and derive iPSC exploit randomly integrating vectors, which entail the risks of insertional mutagenesis, unregulated transgene expression and reactivation of the reprogramming genes. To overcome these hurdles we have established a strategy that allows for targeted correction of the endogenous malfunctioning gene and safe reprogramming of patient-derived cells. Using improved Zinc Finger Nucleases and an optimized donor template vector for homologous recombination-mediated correction of the *IL2RG* gene, which is mutated in SCID-X1 patients, we knocked-in a functional *IL2RG* cDNA downstream of its endogenous promoter in fibroblasts from normal male donor and SCID-X1 patients with high efficiency. To select for the gene corrected fibroblasts, which do not physiologically express *IL2RG*, we included downstream of the *IL2RG* cDNA a loxP-flanked selector cassette, and efficiently reprogrammed the selected cells to iPSC by using a single-copy, Cre-excisable lentiviral vector (LV) expressing the human OCT4, KLF-4 and SOX2 cDNAs plus the miRNA 302B.C.A.D-367 cluster. Transient Cre delivery resulted in near complete excision of the reprogramming LV and the selector cassette from the iPSC genome. The hematopoietic differentiation potential of these cells is under investigation. Overall, we demonstrate here the feasibility of generating gene-corrected and reprogramming factor-free iPSC from SCID-X1 patients, paving the way to the development of safer iPSC-based strategies for regenerative medicine.

OR005

High-level clustering of integration sites in proto-oncogenes and leukemogenesis in gamma-retroviral WAS gene therapy

Anna Paruzynski[1], Kaan Boztug[2], Christian J. Braun[3], Paul Theodor Pyl[4], Claudia Ball[1], Ali Nowrouzi[1], Anne Arens[1], Christina Lulay[1], Maximilian Witzel[5], Marie Dorda[5], Sonja Naundorf[6], Klaus Kühlcke[6], Rainer Blasczyk[7], Irina Kondratenko[8], Laszló Maródi[9], Hanno Glimm[1], Wolfgang Huber[4], Christoph Klein[3], Christof von Kalle[1], Manfred Schmidt[1]

[1]Department of Translational Oncology, National Center for Tumor Diseases and German Cancer Research Center, Heidelberg, Germany, [2]Department of Pediatrics and Adolescent Medicine, Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria, [3]Dr. von Hauner Children's Hospital, Ludwig-Maximilians-University, Munich, Germany, [4]Department of Computational Biology and Genomics, European Molecular Biology Laboratory, Heidelberg, Germany, [5]Department of Pediatric Hematology/Oncology, Hannover Medical School, Hannover, Germany, [6]EUFETS AG, Idar-Oberstein, Germany, [7]Institute for Transfusion Medicine, Hannover Medical School, Hannover, Germany, [8]Department of Clinical Immunology, Russian Clinical Children's Hospital, Moscow, Russia, [9]Department of Infectious and Pediatric Immunology, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary

9/10 patients showed efficient engraftment of gene modified hematopoietic stem cells in the German WAS clinical gene therapy trial. Comprehensive analysis of integration sites (IS) showed a highly polyclonal reconstitution for all patients early after gene therapy and allowed to detect >70.000 unique IS so far. The comparative analysis of common integration sites (CIS) showed the highest clustering in the proto-oncogenes MDS1-EVI1, LMO2, CCND2 and PRDM16. Strikingly those CIS were clustered in sub-gene regions comprising just 100–300 kb. Further analysis revealed that MDS1-EVI1 and PRDM16 CIS are mainly found in the myeloid fraction while LMO2 and CCND2 CIS are mainly found in the lymphoid fraction. 4 patients developed T-ALL 16 months to 5 years after gene therapy. For all patients the malignant clone harbors an IS within or close to the proto-oncogene LMO2 and RT-PCR analysis showed an up-regulation of this gene. Whole

genome sequencing allowed the detection of a TCR translocation in all patients, LOH on chromosome 9 for 3 patients affecting the tumor suppressor genes CDKN2A/B and a large deletion on chromosome 6 for one patient affecting more than 50 genes, 10 cancer-associated genes and one tumor suppressor gene. The comparison of our data with recently published data showed that aberrations in 37 of 67 T-ALL genes could be detected. We could further find aberrations in 17 potential new candidate genes. The frequent monitoring of the patients together with whole genome and transcriptome sequencing may help to better understand the sequential progression of T-ALL.

OR006

Safety and efficacy of repeated infusions of CELYVIR in children with metastatic neuroblastoma.

J Garcia-Castro[1], G Melen[0,2], D Ruano[0,2], I Mirones[1], E Rincon[1], MA Rodriguez[1], F Casco[0,2], M Ramirez[0,2]

[1]*Instituto de Salud "Carlos III", Madrid, Spain,* [2]*Hospital Infantil "Niño Jesús", Madrid, Spain*

Metastatic and refractory solid tumors carry a dismal prognosis and new therapies are much needed for this situation. We published a preliminary experience infusing CELYVIR, autologous mesenchymal cells carrying an oncolytic adenovirus in children with neuroblastoma (CGT 2010, 17: 476). We have extended this compassionate-use program in a new group of children with metastatic tumors. Seven kids with NB and one with rhabdomyosarcoma, refractory to at least 3 lines of therapies, received weekly doses of CELYVIR, minimum 6 and maximum 31. The single maximal dose was 5×10^6 cells per kilo. The maximal accumulated dose in a single patient was 1200×10^6 cells, corresponding to $2{,}4\times10^{13}$ viral particles. The treatment was very well tolerated, with autolimited fever as the most common toxicity event. Patients were evaluated for clinical responses after 6 doses, with 5 progressive diseases (PD), 1 partial response (PR), 1 stable disease (SD) and 1 complete remission (CR). We found several changes in immune cells both at peripheral blood (PB), primary tumor and metastasis, related with the therapy. These, and other results, indicated an immune-related effect of the treatment with CELYVIR, in agreement with the known immune stimulating capacity of oncolytic viruses. Our results confirm that CELYVIR is a safe therapy in patients with metastatic and refractory tumors, and may be of benefit through an oncolytic and an immune-based mechanism of action.

OR007

Lentiviral vector-based insertional mutagenesis identifies new liver cancer genes and molecular networks that have a pivotal role in human hepatocarcinogenesis

Marco Ranzani[1], Daniela Cesana[1], Cynthia Bartholomä[3], Francesca Sanvito[4], Mauro Pala[5], Fabrizio Benedicenti[1], Michela Riba[8], Pierangela Gallina[1], Lucia Sergi Sergi[1], Stefano Annunziato[1,2], Stefania Merella[1,8], Alessandro Bulfone[5], Claudio Doglioni[4], Christof von Kalle[3], Yoon Jun Kim[6], Elia Stupka[8], Manfred Schmidt[3], Giovanni Tonon[7], Luigi Naldini[1,2], Eugenio Montini[1]

[1]*San Raffaele Telethon Institute for Gene Therapy, Milan, Italy,* [2]*Vita-Salute San Raffaele University, Milan, Italy,* [3]*National Center for Tumor Diseases, Heidelberg, Germany,* [4]*Department of Pathology, San Raffaele Hospital, Milan, Italy,* [5]*Bio))flag Ltd., Piscinamanna, Italy,* [6]*Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, Seoul, Republic of Korea,* [7]*Division of Molecular Oncology, Functional Genomic of Cancer Unit, San Raffaele Scientific Institute, Milan, Italy,* [8]*Center for Translational Genomics and BioInformatics, San Raffaele Scientific Institute, Milan, Italy*

We devised and validated a novel insertional mutagenesis strategy based on replication-defective Lentiviral Vectors (LV) specifically tailored to efficiently induce hepatocellular carcinoma (HCC) in three different mouse models of cancer with or without chronic liver injury. Retrieval of LV integrations from 30 HCCs allowed the identification of 4 Common Insertion Sites (CIS) targeting *Braf*, *Fign*, *Sos1* and *Rtl1* (within the imprinted *Dlk1-Dio3* region). We validated *in vivo* the causative role of these genes in hepatocarcinogenesis by forced expression in mouse liver. Moreover these genes are relevant in human disease as they are deregulated and/or amplified/deleted in human HCC, and their expression levels predict patients' survival.

Gene expression analysis of the LV-induced HCCs indicated that CIS gene deregulation shaped the tumor transcriptome generating distinctive gene expression signatures. Integrations within the *Dlk1-Dio3* region induced *Rtl1* overexpression and a peculiar upregulation of oxidative phosphorylation genes. Intriguingly, 2 other CIS genes converged to deregulate the genes within the *Dlk1-Dio3* region. Indeed, *Braf* and *Fign* LV-mediated overexpression induced the upregulation of maternally expressed microRNAs encoded within the *Dlk1-Dio3* region, a result that we could also reproduce in human hepatocytes. We obtained functional evidence that a complex interplay between *Rtl1* and maternally expressed microRNAs is involved in the regulation of metabolism and hepatocarcinogenesis, suggesting a central role of the *Dlk1-Dio3* region in these processes.

By this approach we discovered new liver cancer genes that represent novel prognostic markers and candidate therapeutic targets for human HCC and identified molecular networks that figure prominently in human hepatocarcinogenesis.

OR008

Redirection of T_H17 cells with an ICOS-based CAR enhances function, antitumor activity and persistence of T_H17 cells

Sonia Guedan, Carmine Carpenito, Shannon McGettigan, Matthew Frigault, John Scholler, Yangbing Zhao, Carl June

University of Pennsylvania, Philadelphia, PA, USA

Adoptive transfer of T_H17 cells polarized and expanded in vitro holds great potential for the treatment of cancer. CD278, the inducible costimulator (ICOS), has been shown to be critical for the sustained expansion of human T_H17 cells after their primary activation. We analyzed whether incorporation of the ICOS intracellular domain in a chimeric antigen receptor can promote T_H17 phenotype after antigen priming and enhance the antitumor activity of engineered T cell therapies. T_H17 polarized cells were engineered to express a single-chain variable fragment that binds mesothelin (SS1) fused to the T cell receptor- ζ signal transduction domain in tandem with the CD28, CD137 (4-1BB) or CD278 (ICOS) intracellular domains. After antigen stimulation in vitro, T_H17 cells redirected with SS1-ICOS-z showed increased IL17-A secretion and CD161 expression, consistent with a predominant Th17 phenotype. By contrast, T_H17 cells expressing SS1-28-z secreted higher amounts of IL-2, TNF-α and IFN-γ, and showed enhanced proliferation. When transferred into NSG mice

with large vascularized pre-established tumors, T_H17/T_C17 cells redirected with SS1-ICOS-z mediated enhanced antitumor responses, with 70% of mice showing complete remission. Importantly, incorporation of the ICOS intracellular domain in the CAR significantly increased T_H17 cell persistence after infusion when compared with the incorporation of CD28 or 4-1BB, although T_C17 cell persistence was similar in all groups. Our studies indicate that redirection of T_H17 cells with ICOS-based CARs is critical for obtaining potent T_H17 cells with enhanced function and persistence. The design of novel ICOS-based CARs has the potential to augment antitumor effects in clinical trials.

OR009

Lymfactin™ (LX-1101): an adenovirally-delivered VEGF-C gene therapy in combination with lymph node transfer for the treatment of patients with secondary lymphoedema associated with breast cancer

AM Saaristo[1], KM Honkonen[2], CA Carrington[3], AK Boyd[3], R Rautsola[3]

[1]*Turku University Central Hospital & University of Turku, Turku, Finland,* [2]*A.I.V Institute of Molecular Sciences, University of Eastern Finland, Kuopio, Finland,* [3]*Laurantis Pharma, Turku, Finland*

Lymfactin™ (LX-1101), is a vascular endothelial growth factor C (VEGF-C) in an adenoviral vector, being developed for the treatment of secondary lymphedema that occurs following treatment of breast cancer. There are no approved pharmacological based therapies for the treatment of lymphedema. Preclinical studies have shown that adenovirally-delivered VEGF-C is the optimal growth factor for a lymphedema therapy. In a porcine model of lymphedema, the effects of adenovirally-delivered VEGF-C were compared to controls (LacZ or NaCl). Seroma formation, lymphatic vessel function and lymph node morphology and histology were studied. Two months after transplantation, VEGF-C-treated lymph nodes were larger than NaCl treated controls (32.6 ± 14.7 cm3 v 8.0 ± 6.0 cm3) and more lymph vessels had been formed (16 ± 5 v 3 ± 1) (n = 4 in this experiment). The immunohistochemistry and structural analysis of the lymph nodes and assessment of atrophy grade also supported the positive effect of VEGF-C on the growth and structural integrity of the transplanted lymph nodes.

The therapy with Lymfactin will involve a surgical operation where a lymph node flap will be harvested from the patient's lower abdominal wall and injected with Lymfactin, which leads to the transient presence of the adenovirus containing the VEGF-C gene. The lymph node is then transferred to the axillary (armpit) region. Lymfactin addresses the underlying cause of lymphedema, the dysfunctional lymphatic system, and consequently has the potential to become the first disease-modifying therapy for secondary lymphedema. It is expected to start a phase I/II study in patients with breast cancer associated lymphedema during 2013.

OR010

Monitoring by serum miRNA of a gene transfer treatment in a g-sarcoglycanopathy mouse model

Marie Montus[1], Fatima Amor[1], Angélique Duvallet[1], Laetitia Van Wittenberghe[1], Guillaume Tanniou[1], Nicolas Guerchet[1], Alban Vignaud[1], Simon Jimenez[1], Emilie Bretil[1], Christophe Gorger[1], Bernard Gjata[1], Yves Fromes[1], David Israeli[1]

[1]*Genethon, Evry, France,* [2]*ONIRIS/Centre de Boisbonne, Nantes, France*

LGMD-2c is an incurable disease caused by autosomal recessively mutations in the γ-sarcoglycan gene. A recent phase I clinical trial studied the intramuscular delivery of an AAV1 vector encoding for the γ-sarcoglycan protein to LGMD-2c patients, and concluded to an appropriate expression of the transgene product with no serious adverse effects (Herson et al, Brain. 2012).

The present preclinical study aimed at defining efficacy and dose-responses of intravenous delivery of AAV-8 encoding for the γ-sarcoglycan, in the sgcg-/- mouse model for this pathology.

AAV8 expressing the human gamma-sarcoglycan gene under the desmin promoter (AAV8-des-hgSCG) was administrated intravenously sham or with 3 escalating doses of vector into Sgcg-/- mice. Functional analyses were performed 4 weeks after gene delivery, blood was drawn for serum miRNA analysis and necropsy was completed by histological studies.

Human g-sarcoglycan protein was expressed in all studied muscles and for all tested doses, showing a direct dose-response correlation. Similarly, whole body force development was proportional to therapeutic dose. In contrast, no simple dose-response was found for serum biomarkers. Normalization of the levels of creatine kinase and of a set of serum miRNAs was obtained only at the highest viral dose. At the lower and intermediate doses biomarkers presented dysregulated levels similar or higher than untreated mice. These results suggest a threshold level for the stabilization of myofibers. The long-term consequences of application of the non-integrative AAV for muscle degenerating pathologies need to be further investigated. Profiling serum miRNA provides a new important monitoring tool of systemic gene transfer.

OR011

rAAV9-mediated gene transfer in the spinal cord of a feline model of motor neuron degeneration

T Bucher[1], M Maquigneau[1], L Dubreil[2,3], D Briot-Nivard[1], E Wakeling[4], J Fyfe[4], P Costiou[3], P Moullier[1,5], M Barkats[6], MA Colle[2,3], B Joussemet[1]

[1]*INSERM UMR1089, Nantes, France,* [2]*UMR 703 PAnTher INRA, Nantes, France,* [3]*Ecole Nationale Vétérinaire, Agroalimentaire et de l'Alimentation Nantes-Atlantique (ONIRIS), Nantes, France,* [4]*Department of Microbiology and Molecular Genetics, Michigan State University, Lansing, Michigan, USA,* [5]*Department of Molecular Genetics and Microbiology, College of Medicine, University of Florida, Gainesville, Florida, USA,* [6]*UMR974 INSERM-UPMC-CNRS-AIM, Institut de Myologie, Paris, France*

Among viral vectors used in pre-clinical studies, self-complementary serotype 9 adeno-associated virus (scAAV9) has been shown to induce unprecedented levels of gene transfer in the spinal cord. This transduction efficiency is particularly usefull for developing gene therapy of inherited motor neuron (MN) diseases such as spinal muscular atrophy (SMA) or amyotrophic lateral sclerosis. Indeed, recent studies have demonstrated significant lifespan increase of a severe murine model of SMA after gene replacement mediated by neonatal intravenous injection of scAAV9. Nevertheless, adverse effects induced by off-targets transduction following systemic administration must be considered. The purpose of this study is to determine the efficacy and the safety of a scAAV9-mediated therapeutic gene transfer into MNs of a unique large animal model of cats exhibiting inherited lower MN degeneration. Intrathecal injections – especially

intracisternal (IC) in cats – should reduce the vector peripheral diffusion while improving the dose-related efficacy. Three different transgenes were thus administrated by scAAV9 IC injections in neonates or adult cats. The efficacy of the gene transfer were assessed considering (i) the presence of the transgene in the feline spinal cord by qPCR (ii) the expression of this transgene by RTqPCR, by immunohistochemistry and by ELISA and (iii) a potential phenotypic improvement. Our results showed a strong transgene expression in almost all MNs following IC injection both in neonates and adults cats together with a limited untargeted transduction representing a key safety issue. These results may finally impact on defining clinical trials applied to human MN disorders.

OR012

Human mesoangioblasts can elicit alloreactive T-cell immune responses: implications for allogeneic cell therapy of DMD

Maddalena Noviello[1], Francesco Saverio Tedesco[1,2], Attilio Bondanza[1], Rossana Tonlorenzi[1], Mattia Gerli[1,2], Sarah Marktel[1], Sara Napolitano[1], Maria Pia Cicalese[1], Fabio Ciceri[1], Giuseppe Peretti[1], Giulio Cossu[1,2], Chiara Bonini[1]

[1]*San Raffaele Scientific Institute, Milan, Italy,* [2]*University College London, London, UK*

Duchenne muscular dystrophy (DMD) is a severe and incurable genetic disorder leading to disability and death early in life. Recently different approaches have been proposed to ameliorate disease progression. Among these, cell therapy with donor mesoangioblasts (MAB) produced significant functional improvements in animal models of muscular dystrophy. Since immune-mediated rejection of infused cells might limit the efficacy of mesoangioblast-based cell therapy, we characterized the immunological properties of MAB. In vitro isolated and expanded human MAB proved poorly immunogenic in resting conditions and intrinsically resistant to T-cell killing. However, upon exposure to gINF or differentiation in myotubes, MAB acquire the ability to expand alloreactive T cells and become sensitive to T-cell killing. In a phase I-II clinical trial based on the systemic infusion of MAB isolated from HLA-identical siblings to patients affected by DMD, we longitudinally analyzed T-cell dynamics. Patient samples were harvested before the beginning of treatment, prior to each infusion and bimonthly for one year. Despite standard corticosteroid plus cyclosporine continuous treatment the numbers of circulating lymphocytes, the relative proportion of naïve/memory T-cell subset and of Tregs were comparable to that measured before treatment. Donor-specific T-cell responses were detectable by gIFN ELISpot in two out of three treated patients, at frequencies significantly lower than frequencies of viral specific T cells. Altogether these results suggest that hypoimmunogenic MAB become immunostimulatory in the inflammatory milieu encountered in dystrophic muscles, justifying and recommending the use of immunosuppressive and/or anti-inflammatory drugs in allogeneic cell therapy of DMD.

OR013

AAV-mediated gene replacement therapy for X-linked myotubular myopathy

Martin K Childers[1], Thibaud Jamet[2], Romain Joubert[2], Mark E Furth[1], Melanie Holder[1], Robert W Grange[3], Michael W Lawlor[4], Karine Poulard[2], Carole Masurier[2], Samia Martin[2], Alban Vignaud[2], Nadia Messaddeq[5], Xuan Guan[1], Melissa Goddard[1], Erin Mitchell[1], Jane Barber[1], Nathalie Danièle[2], Philippe Moullier[2,6], Alan H Beggs[4], Anna Buj-Bello[2]

[1]*Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Winston-Salem, North Carolina, USA,* [2]*Généthon, Evry, France,* [3]*Virginia Polytechnic and State University, Blacksburg, Virginia, USA,* [4]*The Manton Center for Orphan Disease Research, Children's Hospital Boston, Harvard Medical School, Boston, Massachusetts, USA,* [5]*Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France,* [6]*INSERM U649, Laboratoire de Thérapie Génique, Nantes, France*

Mutations in the myotubularin gene (*MTM1*) result in X-linked myotubular myopathy (XLMTM), a fatal pediatric disease of skeletal muscle characterized by small myofibers with frequent central nuclei and abnormal mitochondrial accumulations. Patients with XLMTM typically present with severe hypotonia, muscle weakness and respiratory failure. Previous local studies in *Mtm1*-mutant mice demonstrated potential efficacy of gene therapy to treat the disease. We now report the first results of intravenous delivery of an adeno-associated virus serotype 9 (AAV9) vector expressing myotubularin under the muscle-specific desmin promoter in mouse models of the disease. Myotubularin was rapidly and persistently expressed in muscles scattered throughout the body, including the diaphragm, and this translated into robust improvement of skeletal muscle pathology and contractile force, and normalized motor activity of treated mutant mice. Importantly, the lifespan was prolonged from under two months to at least one year. In addition, we also report results for the intramuscular delivery of an AAV8 vector carrying the canine *MTM1* gene (c*MTM1*) into the cranial tibialis muscle of the recently characterized dog model of the disease. We observed an improvement in muscle mass and myofiber size and decreased pathological features at 6 weeks post-injection. Moreover, the muscular strength of treated limbs increased markedly, approaching that of normal littermates. Our findings provide proof-of-principle that AAV-mediated myotubularin replacement is highly efficient in rescuing the muscular phenotype of both small and large animal models of myotubular myopathy.

OR014

Sustained stimulation/expansion of regulatory T cells treat autoimmune disease without impairing effector immune responses to infection, vaccination and cancer.

Guillaume Churlaud[1,2], Veronica Jimenez Cenzano[3], Jesus Ruberte[3], Martin Amadoudji[1], Gaelle Gottrand[1], Benedicte Lambrecht[4], Bertrand Bellier[1], Eliane Piaggio[1], Fatima Bosch[3], David Klatzmann[1,2]

[1]*UPMC Univ Paris 06, CNRS, UMR 7211, INSERM, UMR_S 959, Immunology-Immunopathology-Immunotherapy (I3), Paris, France,* [2]*AP-HP, Hôpital Pitié-Salpêtrière, Biotherapy, Paris, France,* [3]*Center of Animal Biotechnology and Gene Therapy, Edifici H, Universitat Autonoma de Barcelona, 08193 Bellaterra (Barcelona), Spain,* [4]*Veterinary and Agrochemical Research Center (VAR), brussels, Belgium*

Interleukin-2 (IL-2) plays a key role in the immunobiology of regulatory T cells (Tregs). It has been recently reported that low-dose IL-2 administration safely expands/stimulates Tregs and likewise improve autoimmune conditions in humans. The further developement of IL-2 in autoimmune diseases (AID) will likely require chronic IL-2 administration to maintain a proper Treg/effector T cells (Teff) balance, i.e. tipped towards Tregs. However,

little is known about how this could affect beneficial effector immune responses. In the current study, we used a recombinant adeno-associated virus (AAV) expressing IL-2 to permanently release IL-2 and assess its effect on immune responses during vaccination, infection, cancer developement and pregnancy. We used single injections of AAV IL-2 at 2 doses. 10^9 virus particles (v.p.) increased CD25 and Foxp3 expression in Tregs, had minimal effects on Treg numbers and delayed but did not prevent the occurrence of type 1 diabetes (T1D) in NOD mice; 10^{10} v.p. enabled sustained stimulation and expansion of Tregs without inducing Teff activation and prevented T1D in NOD mice. After several weeks of IL-2 production at these two doses, mice could normally (i) eradicate a viral challenge with flu, (ii) mount immune responses to vaccination, and (iii) have normal pregnancies with pups that developed normally. They also had no change in occurrence and growth of chemically induced tumors, as well as in growth of transplanted tumors. Altogether, chronic low doses IL-2 treatment appears safe and does not affect usefull effector immune responses.

OR015

AAV gene therapy for Alzheimer disease: Consequences of AAV-mediated CHOLESTEROL 24-HYDROXYLASE overexpression in THY-TAU22 mouse model.

Marie-Anne Burlot[1], David Blum[3], Jérôme Braudeau[1], Sophie Ayciriex[2], Ivan Bieche[1], Jennifer Varin[1], Benoit Gautier[1], Laëtitia Troquier[3], Nadège Zommer[3], Raphaëlle Caillerez[3], Laurent Pradier[4], Patrick Aubourg[1], Olivier Laprévote[2], Nicolas Auzeil[2], Luc Buée[3], Nathalie Cartier[1]

[1]INSERM UMR_745, Paris, France, [2]Paris Descartes University EA 4463, Paris, France, [3]INSERM UMR_837, Lille, France, [4]Sanofi-Aventis, Paris, France

Alzheimer's disease (AD), the major cause of dementia, is characterized by two hallmarks, amyloid pathology and neurofibrillary tangles of hyperphosphorylated Tau protein. We recently described the therapeutic effect of cerebral cholesterol 24-hydroxylase (CYP46A1) overexpression on amyloid pathology of APP23 mice (Hudry et al.).

The aim of this work was to investigate the consequences of CYP46A1 overexpression on the Tau component of AD.

CYP46A1 overexpression was induced in the THY-Tau22 transgenic mouse model by stereotactic injection of an AAV vector carrying the human CYP46A1 gene in cortex and hippocampus. An AAV vector coding a mutated inactive CYP46A1 enzyme was used as a control. Injected animals were compared to normal control littermates.

In contrast to THY-Tau22 mice treated with inactive CYP46A1, THY-Tau22 mice overexpressing functional CYP46A1 enzyme showed normal memory abilities in two behavioral tasks (Morris Water Maze and Y-maze). Thus CYP46A1 overexpression rescues the main cognitive dysfunction of this model.

Hyperphosphorylation of Tau and astrogliosis that characterize this THY-Tau22 model were not modified by CYP46A1 overexpression. Lipidomic analysis evidenced modified lipid profile in THY-Tau22 mice. CYP46A1 overexpression allowed complete restoration of this profile and increased expression of genes implicated in cholesterol synthesis in both hippocampus and cortex.

Mechanisms that underlie the neuroprotective effect of CYP46A1 overexpression in THY-Tau22 mouse are under investigation. Overall, phenotypic improvement of both APP23 and THY-Tau22 mice suggest that CYP46A1 is a relevant therapeutic target on AD.

OR016

Exon skipping gene therapy for Dystrophic Epidermolysis Bullosa

Sandrina Turczynski[1], Matthias Titeux[1], Laure Tonasso[2], Alain Hovnanian[1,3]

[1]INSERM U781, University Paris Descartes and Imagine Institute of genetic diseases, Paris, France, [2]CNRS UMR5288, Toulouse, France, [3]Department of genetics, Necker hospital for sick children, Paris, France

Dystrophic Epidermolysis Bullosa (DEB) is a group of genetic skin disorders inherited in a dominant (DDEB) or recessive (RDEB) manner and characterised by severe skin and mucosae blistering after mild traumas. DEB is caused by mutations in *COL7A1* encoding type VII collagen that assembles into anchoring fibrils forming key dermo-epidermal adhesion structures. To date, there is no specific treatment for DEB. Exon skipping strategy consists in modulating the splicing of a pre-messenger RNA to induce the skipping of a mutated exon. Exons 73, 74 and 80 of *COL7A1* are of particular interest because they carry several recurrent mutations and their excision preserves the open reading frame. We first demonstrated the dispensability of these exons for type VII collagen function in an *in vivo* xenograft model using RDEB cells transduced with retroviral vectors containing *COL7A1* cDNAs deleted of the sequences of these exons. We then transfected primary RDEB keratinocytes and fibroblasts with antisense oligoribonucleotides (AONs) targeting key splicing regulatory elements (exonic splicing enhancers and/or acceptor sites) to induce efficient skipping of these exons (50% up to 90%). Western blot and immunocytochemistry analyses demonstrated significant collagen VII re-expression in cells from two RDEB patients, one homozygous for a nonsense mutation in exon 80, the other compound heterozygous for frameshift mutations in exon 73 and 80. We now aim to demonstrate the feasibility of this approach *in vivo* using animal models. If successful, this approach would offer potential for treating both RDEB and DDEB patients using local or systemic administration of AONs.

OR017

SMN rescue by using oligonucleotides of tricyclo-DNA to induce exon 7 inclusion in SMN2 mRNA

Valerie ROBIN[1], Damian ITTIG[2], Thomas VOIT[1], Christian J. Leumann[2], Luis GARCIA[1]

[1]Institut de Myologie. UPMC Um76 / Inserm U974 / CNRS UMR 7215, Paris 75013, France, [2]University of Bern, Department of Chemistry & Biochemistry, Bern, Switzerland

Spinal muscular atrophy is a recessive disease caused by mutations in the SMN1 gene, which encodes a protein (SMN) involved in RNA processing whose absence dramatically affects the survival of motor neurons. In Man, the severity of the disease correlates with the SMN2 gene copy number, which varies from individual to individual.

SMN2 differs from SMN1 by 5 nucleotides, only one of which is in the coding sequence: it is a silent C to T change located at the sixth nucleotide of exon 7. Thus, SMN2 encodes the same SMN protein as SMN1. However, the single nucleotide change affects the definition of exon 7 during splicing such that about 90% of SMN2 mRNAs lack this exon.

Here, we show that SMN activity can be restored in SMA cells by using tricyclo-DNA (Tc-DNA) antisense oligonucleotides

annealing either the exon 7 terminal stem loop (TSL) or a nearby intron 7 splice silencer (ISS) of the SMN2 pre-mRNA. RT-PCR showed that about 30% and 60% of SMN2 mRNAs were rescued after treatment with Tc-DNA analogues annealing the TSL and ISS, respectively. For Tc-DNA[ISS], SMN levels were close to normal in Western blot analysis. Finally, immuno-staining revealed that rescued SMN was properly located in nuclear gems.

OR018

Lentiviral vectors designed for liver-directed gene therapy do not display detectable genotoxicity in sensitive *in vivo* assays

Marco Ranzani[1], Alessio Cantore[1,2], Monica Volpin[1,2], Francesca Sanvito[3], Pierangela Gallina[1], Cynthia Bartholomä[4], Fabrizio Benedicenti[1], Stefano Annunziato[1,2], Lucia Sergi Sergi[1], Christof von Kalle[4], Claudio Doglioni[3], Manfred Schmidt[4], Luigi Naldini[1,2], Eugenio Montini[1]

[1]San Raffaele Telethon Institute for Gene Therapy, Milan, Italy, [2]Vita-Salute San Raffaele University, Milan, Italy, [3]Department of Pathology, San Raffaele Hospital, Milan, Italy, [4]National Center for Tumor Diseases, Heidelberg, Germany

We developed and validated sensitive genotoxicity assays based on *Cdkn2a*$^{-/-}$ or wild type (WT) mice to assess the biosafety of lentiviral vectors (LV) designed for liver gene transfer. As a positive control of insertional mutagenesis, we used a LV carrying Enhanced Transthyretin enhancer/promoter (ET) cloned in the Long Terminal Repeats (LV.ET.LTR) which induced hepatocellular carcinoma (HCC) in 30% of *Cdkn2a*$^{-/-}$ mice ($p < 0.01$) and 75% of WT mice in combination with CCl_4 treatment ($p < 0.01$).

We exploited these mouse models to test the safety of Self-Inactivating LVs developed for the therapy of haemophilia B which express factor IX under the control of ET (SINLV.ET.FIX). Systemic administration of SINLV.ET.FIX did not induce HCCs neither in tumor prone *Cdkn2a*$^{-/-}$ ($N = 39$) nor in WT mice + CCl_4 ($N = 23$). We retrieved a total of 9215 unique insertion sites from LV.ET.LTR- and SINLV.ET.FIX-treated mice. None of the previously validated cancer genes recurrently targeted by the oncogenic LV.ET.LTR was hit by SINLV.ET.FIX-integrations. However, SINLV.ET.FIX-insertions clustered at different Common Insertion Sites (CIS). Importantly, we found that: 1) SINLV.ET.FIX-CIS had a lower CIS power compared to LV.ET.LTR-CIS ($p < 0.01$); 2) SINLV.ET.FIX-CIS were represented by a lower percentage of sequence reads compared to LV.ET.LTR-CIS ($p < 0.001$); 3) SINLV.ET.FIX-CIS did not show any skewing towards genes involved in cancer; 4) SINLV.ET.FIX-CIS were embedded in large genomic areas with high LV integration incidence. These findings indicate that SINLV.ET.FIX-CIS are the result of LV-intrinsic integration biases rather than the result of selection.

Our data indicate that SINLV.ET.FIX represents a safe vector design for hepatocytes gene transfer for different gene therapy applications.

OR019

High-throughput monitoring of bone marrow clonality in pre-clinical and clinical gene therapy studies

FA Giordano[1], S Gerdes[2], JU Appelt[3], I Röder[2], C von Kalle[3], M Grez[4], M Schmidt[3], S Laufs[3]

[1]Department of Radiation Oncology, University Medical Center Mannheim, University of Heidelberg, Mannheim, Germany, [2]Institute for Medical Informatics and Biometry, Medical Faculty Carl Gustav Carus, Dresden University of Technology, Dresden, Germany, [3]Department of Translational Oncology, National Center for Tumor Diseases and German Cancer Research Center (DKFZ), Heidelberg, Germany, [4]Institute for Biomedical Research, Georg-Speyer-Haus, Frankfurt, Germany

Clonal dominance and leukemia are potential side effects of hematopoietic stem cell gene therapy. High-throughput methods enable fast identification of individual stem cell-derived clones. Quantitative real-time PCR (QRT-PCR) has been shown to be an accurate method to quantify individual transduced cell clones. However, due to frequently scarce target material large scale clonal analysis of multiple clones by QRT-PCR is hardly achievable.

The aim of this study was to explore the potential of highly sensitive techniques for vector insertion site analysis (LAM-PCR, nonrestrictive LAM-PCR) to describe clonal compositions in gene therapy compared to QRT-PCR. Therefore, we designed artificial insertion sites (*arIS*) of different sizes which were mixed for mimicking defined clonal situations in clinical settings ranging from balanced clonality to monoclonality in an *in vitro* setting. We subjected all *arIS* mixes to either linear amplification-mediated PCR (LAM-PCR) or nonrestrictive LAM-PCR (nrLAM-PCR) both combined with 454 sequencing compared to QRT-PCR. By performing this important first technical comparison we showed that nrLAM-PCR/454-based clonal assessment is in the same range as the results obtained with QRT-PCR. To prove whether nrLAM-PCR/454 can prospectively facilitate clonality analyses in clinical gene therapy studies, we followed clonal kinetics of two clones detected in a patient enrolled in a clinical trial using both, nrLAM-PCR/454 and QRT-PCR. In line with the previous *in vitro* data, nrLAM-PCR/454 has shown to tightly reflect QRT-PCR-measured clonal contributions. We show for the first time a feasible high-throughput strategy to reliably monitor clonality in large-scale analyses of gene-marked cells in clinical gene therapy trials.

OR020

Tracking T-memory stem cells in humans by retroviral tagging

Serena Scala[1,2], Luca Biasco[2], Cristina Baricordi[2], Nicoletta Cieri[1,3], Andrea Calabria[2], Eugenio Montini[2], Chiara Bonini[3], Alessandro Aiuti[2,4]

[1]Università Vita-Salute San Raffaele, Milano, Italy, [2]Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Milano, Italy, [3]San Raffaele Scientific Institute, HSR, Milano, Italy, [4]University of Rome "Tor Vergata", Roma, Italy

Vector integrations in ADA (adenosine deaminase) deficient-SCID patients treated with hematopoietic stem cells (HSC) or mature lymphocytes (PBL) gene therapy (GT) introduce a univocal tag in each infused target cell. These studies allow tracking of single T-cell clones and studying the survival potential and hierarchical relationships of naive and memory subpopulations directly in vivo in humans. We analyzed at high-throughput level the integrome of sorted T cell subtypes from PBL-GT patients years after last infusion of gene corrected lymphocytes. Strikingly, we found that transduced T cells with an apparent naïve phenotype (CD45RA+/62L+) share the highest percentage of insertions (41.2%) with other T subpopulations while still surviving in vivo 10 years after infusion. A novel T-cell type (T memory stem cell, TSCM) with long-term survival capacity coupled with naive-like plasticity, has been recently identified as CD45RA+/62L+/CD95+. Interestingly, we found that the vast

majority of phenotypically naive cells in PBL-GT patients are CD95+ (92.5%) differently from HSC-GT (37.2%). Our preliminary data show that TSCM could be generated in vitro following PBL-GT transduction protocol. Additionally, the proportion of TSCM within CD8 compartment was higher in PBL-GT (13.0%) and HSC-GT (9.2%) as compared to bone marrow transplanted patients (3.2%) and healthy donors (4.4%) suggesting an in vivo selection for TSCM during immune reconstitution after GT. Therefore, ADA-SCID PBL-GT represents a unique human model to study at single clone level the fate and plasticity of marked T cells and could provide information on whether TSCM gene transfer is applicable for future T-cell based therapies.

OR021

Identification of a domain responsible for the dose-dependent antiproliferative and apoptotic effects of *Sleeping Beauty* transposase

Melanie Galla[1], Zsuzsanna Izsvak[2], Christine S. Falk[1], Kathrin Thomay[1], Gudrun Göhring[1], Zoltan Ivics[3], Axel Schambach[1], Christopher Baum[1]

[1]*Hannover Medical School, Hanover, Germany,* [2]*Max Delbrück Center for Molecular Medicine, Berlin, Germany,* [3]*Paul Ehrlich Institute, Langen, Germany*

The *Sleeping Beauty* transposase (SB) and its newly developed hyperactive variant, SB100X, are of increasing interest for genome modification in experimental models and gene therapy. Potential off-target effects of transposases require careful assessment, as other DNA-modifying enzymes have been associated with dose-dependent cytotoxicity. We compared retrovirus-based approaches for delivery of mRNA (RMT), episomal DNA or integrating DNA, and found that overexpression of SB may trigger a substantial premitotic cell cycle arrest followed by apoptosis in different cell types. This cytotoxic phenotype was strictly dose-dependent and occurred even in the absence of a co-transfected transposable element. Induction of p53 and c-Jun as well as increased numbers of γH2AX foci suggested genotoxicity as the underlying mechanism. However, inactivation of SB's catalytic domain only slightly abrogated cytotoxicity, indicating that direct DNA damage inflicted by SB is not responsible for the observed cytotoxicity. This promoted studies in which we introduced serial deletions of individual SB (sub-)domains to determine their impact on cell cycle arrest, apoptosis, double strand breaks and stress signaling. We were thus able to identify a small domain comprising 16 amino acids that is largely responsible for the cytotoxic effects. Further fine-mapping identified 8 out of the 16 amino acids to be involved in cytotoxicity, which will hopefully allow us to generate variants with a greater "therapeutic index". When using conventional SB or SB100X, we recommend reducing the level and duration of transposase expression via RMT or other transient expression methods to avoid the previously described overproduction inhibition and the newly discovered cytotoxicity.

OR022

Engineering lentiviral vectors to target dendritic cells: the Nanobody display technology

Cleo Goyvaerts[1], Kurt De Groeve[2], Sandra Van Lint[1], Carlo Heirman[1], Jakob Reiser[3], Kris Thielemans[1], Patrick De Baetselier[2], Geert Raes[2], Karine Breckpot[1]

[1]*Vrije Universiteit Brussel, Brussels, Belgium,* [2]*VIB, Brussels, Belgium,* [3]*Louisiana State University Health Sciences Center, New Orleans, Louisiana, USA*

Antigen-presenting cells (APC), such as dendritic cells (DCs) and macrophages, are targets for immunotherapy. Lentiviral vectors (LVs) have been used to modify APC *in vivo*. However, their broad tropism hampers their clinical use. As selective targeting will improve safety and efficacy, we developed the "Nanobody (Nb) display technology". Herein, LVs are pseudotyped with a fusion-competent but binding-defective envelope together with an APC-specific (DC1.8 or DC2.1) or control Nb (BCII10). We demonstrated incorporation of Nbs on the viral surface and production of high titer LVs. Flow cytometry revealed that BCII10 LVs didn't mediate infection *in vitro*, whereas DC1.8 and DC2.1 LVs selectively transduce primary DCs (DC1.8) and macrophages (DC2.1). Broad tropism LVs additionally transduced T and B cells. We further evaluated the biodistribution of the LVs by *in vivo* bioluminiscence imaging and flow cytometry demonstrating a similar transduction profile *in vivo*. Currently we are evaluating the potential of Nb displaying LVs encoding the antigen ovalbumin (OVA) to induce OVA-specific immunity. We show the induction of immune responses even with non-infectious BCII10 LVs. The latter was scrutinized and was attributed to non-specific packaging of OVA proteins. Our data further indicate qualitative differences in $CD4^+$ and $CD8^+$ T cell responses, which is reflected in the therapeutic efficacy of the LVs in anti-cancer therapy. In conclusion, we report on the Nb display technology to target LVs to APC, a strategy that can be exploited for fundamental research exploring the stimulatory capacity of APC-types and to facilitate the translation of LV-vaccines from bench to clinic.

OR023

Gene transfer in Naive-derived memory stem T-cells: a novel promising platform for cancer immunotherapy

Nicoletta Cieri[1,2], Barbara Camisa[1], Fabienne Cocchiarella[3], Mattia Forcato[3], Elena Provasi[1], Attilio Bondanza[1], Claudio Bordignon[2,4], Jacopo Peccatori[1], Maria Teresa Lupo Stanghellini[1], Fabio Ciceri[1], Fulvio Mavilio[3], Anna Mondino[1], Silvio Bicciato[3], Alessandra Recchia[3], Chiara Bonini[1]

[1]*San Raffaele Scientific Institute, Milan, Italy,* [2]*University Vita-Salute San Raffaele, Milan, Italy,* [3]*University of Modena and Reggio Emilia, Modena, Italy,* [4]*Molmed S.p.A., Milan, Italy*

The genetic transfer of tumor specific TCRs or chimeric antigen receptors into T cells is a promising approach, currently tested in clinical trials to treat cancer patients. However, suboptimal persistence of transferred cells is a major limitation. Long-living memory stem T cells (T_{SCM}) with the ability to self renew and the plasticity to differentiate into potent effectors could thus be valuable weapons in adoptive T-cell therapy against cancer. Here we show that it is possible to differentiate in vitro, expand and gene modify in clinically compliant conditions $CD8^+$ T_{SCM} lymphocytes starting from naïve precursors. Requirements for the generation of this T-cell subset, described as $CD62L^+$ $CCR7^+$ $CD45RA^+$ $CD45R0^+$ IL-7Rα^+ $CD95^+$, are CD3/CD28 engagement and culture with IL-7 and IL-15. In accordance T_{SCM} accumulates early after hematopoietic stem cell transplantation. The gene expression signature and functional phenotype define this cell population as a distinct memory T lymphocyte subset, intermediate between naïve

and central memory cells. When transplanted in immunodeficient mice, gene-modified naïve-derived T_{SCM} prove superior to other memory lymphocytes for the ability to expand and differentiate into effectors able to mediate a potent xenogeneic GvHD. Furthermore, gene-modified T_{SCM} are the only T-cell subset able to expand, and mediate GvHD upon serial transplantation, suggesting self-renewal capacity in a clinically relevant setting. Together these findings provide novel insights into the origin and requirements for T_{SCM} generation and pave the way for their clinical rapid exploitation in adoptive cell therapy.

OR024

In vitro and in vivo modulation of AAV capsid CD8+ T cell responses with IgG-derived MHC class II epitopes

Daniel Hui[1], Etiena Basner-Tschakarjan[1], Yifeng Chen[1,3], Gary Pien[1], Anne de Groot[2], Katherine High[1,2], Federico Mingozzi[1]

[1]*Children's Hospital of Philadelphia, Philadelphia, PA, Philadelphia, PA, USA,* [2]*EpiVax, Inc., Providence, RI, USA,* [3]*Howard Hughes Medical Institute, Philadelphia, PA, USA*

Adeno-associated virus (AAV) vectors are among the most efficient tools for *in vivo* gene transfer. Results from clinical gene transfer studies indicate that the human immune system represents an obstacle to successful gene transfer, particularly when high doses of AAV vector are delivered systemically. To overcome the issue of AAV vector immunogenicity, the use of pharmacological immunosuppression has been proposed, with all the limitations of the approach. Here we tested a novel strategy for the immunomodulation of AAV capsid-driven T cell responses using MHC class II peptide ligands identified within the human IgG Fc fragment (Blood 2008;112:3303), which have been shown to expand regulatory T cells (Tregs). Restimulation of human peripheral blood mononuclear cells *in vitro* with AAV capsid antigen in the presence of these peptides resulted in suppression of capsid-specific $CD8^+$ T cells and in the expansion of $CD4^+CD25^+FoxP3^+$ Tregs. We next tested the efficacy of this strategy *in vivo* in mice. Co-expression of the AAV capsid protein and the immunomodulatory peptides, or a scramble control, in muscle resulted in a decrease in frequency of capsid-specific $CD8^+$ T cells responses after adenoviral challenge. We conclude that peptide-mediated inhibition of capsid-specific $CD8^+$ T cell responses may represent an alternative to pharmacological immunosuppression to overcome the limitation of immune responses to AAV vectors.

OR025

Incorporation of antigens into viral capsids augments efficacy of adeno-associated viral (AAV) vector-based vaccines

A Nowag[1], J Rybniker[1], H Janicki[1,2], K Demant[1], S Winter[1], P Hartmann[1], H Büning[1,2]

[1]*University Hospital Cologne, Cologne, NRW, Germany,* [2]*Center for Molecular Medicine Cologne, Cologne, NRW, Germany*

Genetic modification of AAV capsids has previously been exploited to re-direct viral tropism. We hypothesized that capsid-modified vectors may also be used as prime-boost vaccines to outperform conventional vaccination strategies. For this proof of principle study we designed recombinant adeno-associated viral (rAAV) vectors encoding the mycobacterial antigen Ag85a. In addition to Ag85a expressed as a transgene, viral capsids of AAV serotype 2 were engineered as scaffolds for antigen display by fusion of Ag85a to the VP2 capsid protein. This "prime-boost vaccine", Ag85a-AAV:Ag85a, is supposed to function by priming the immune response by an early contact to the capsid bound antigen and boosting by the later expression of the antigen expressing cassette. Balb/c mice vaccinated intramuscularly with Ag85a-AAV:Ag85a developed antigen-specific humoral immune responses significantly faster and with higher IgM and IgG antibody titers than mice treated with conventional vectors encoding Ag85a but with a wildtype AAV2 capsid (AAV:Ag85a) or with the recombinant protein rAg85a. Remarkably, IgG antibody titers in "prime-boost vaccine"-treated mice reached levels after only four weeks that were not obtained in the AAV:Ag85a-vaccinated animals until eight weeks post immunization. Our findings demonstrate that combining antigen incorporation into the AAV capsid with overexpression of the antigen after cell transduction dramatically enhances the antigenic potential of AAV-based vaccines. In addition to eliciting higher antibody titers, humoral immune responses were induced much more rapidly than with conventional vaccination strategies therefore offering earlier protection. Antigen-VP2 fusion is conceivable for a variety of applications such as vaccination with multiple antigens using a single construct.

OR026

iPSC-based gene therapy of congenital erythropoietic porphyria

A Bedel[1,2], M Taillepierre[2], V Guyonnet-Duperat[3], P Dubus[4], T Thibaud[1,2], B Cardinaud[1,2], C Ged[1,2], H de Verneuil[1,2], F Moreau-Gaudry[1,2]

[1]*Université, Bordeaux, France,* [2]*INSERM U1035, Bordeaux, France,* [3]*Vectorology platform INSERM, Bordeaux, France,* [4]*Université EA 2406, Bordeaux, France*

Congenital erythropoietic porphyria (CEP) is due to a deficiency in the UROS enzymatic activity leading to porphyrin accumulation and that results in skin lesions and hemolytic anemia. CEP is a candidate for retroviral-mediated gene therapy but recent reports of insertional leukemogenesis underscore the need for safer systems. The discovery of induced pluripotent stem cells (iPSCs) has opened up new horizons in gene therapy since it may overcome the difficulty of obtaining sufficient amounts of autologous hematopoietic stem cells for transplantation and the risk of genotoxicity. In this study, we isolated keratinocytes from a CEP person and generated iPSCs with two excisable lentiviral vectors. Gene correction of CEP-derived iPSCs was obtained by lentiviral transduction of a therapeutic vector containing UROS cDNA under the control of an erythroid-specific promoter shielded by insulators. One iPSC clone, free of reprogramming genes, was obtained with a single proviral integration of the therapeutic vector in a genomic safe region. Metabolic correction of erythroblasts derived from iPSC clones was demonstrated by disappearance of fluorocytes. This study reports for the first time the feasibility of gene therapy using iPSCs for porphyria.

OR027

Impaired epithelial differentiation of induced pluripotent stem cells from Ectodermal Dysplasia EEC patients is rescued by APR-246/PRIMA-1^{MET}

Daniel Aberdam[1,2], Ruby Shalom-Feuerstein[1,2], Klas Wiman[3], Hans van Bokhoven[4], Isabelle Petit[1,2]

[1]INSERM, Nice, France, [2]INSERTECH, Haifa, Israel, [3]Karolinska Institutet, Stockholm, Sweden, [4]Radboud University, Nidjeden, The Netherlands

Ectodermal dysplasia is a group of congenital syndromes affecting a variety of ectodermal derivatives. Among them, ectrodactyly, ectodermal dysplasia and cleft lip/palate (EEC) syndrome is caused by single point mutations in the *p63* gene, which controls epidermal development and homeostasis. Phenotypic defects of the EEC syndrome include skin defects and limbal stem cell deficiency. In this study, we designed a novel cellular model that recapitulated major embryonic defects related to EEC. Fibroblasts from healthy donors and EEC patients carrying two different point mutations in the DNA binding domain of p63 were reprogrammed into induced pluripotent stem cell (iPSC) lines. EEC-iPSC from both patients showed early ectodermal commitment into $K18^+$ cells but failed to further differentiate into $K14^+$ cells (epidermis/limbus) or $K3/K12^+$ cells (corneal epithelium). APR-246 ($PRIMA\text{-}1^{MET}$), a small compound that restores functionality of mutant p53 in human tumor cells, could revert corneal epithelial lineage commitment and reinstate normal p63-related signaling pathway. This study illustrates the relevance of iPSC for p63 related disorders. This unique modelserves to characterize the abnormal molecular circuitry of this disease and paves the way for future therapy of EEC.

OR028

Generation of Disease-free iPS Cells from Fanconi Anemia Mice with a Hypomorphic Mutation in *Brca2*.

V Moleiro[1], S Navarro[1], ML Lozano[1], R Chinchon[1], E Almarza[1], O Quintana-Bustamante[1], P Rio[1], G Mostoslavsky[6], T Maetzig[2], B Schiedlmeier[2], U Modlich[2], M Galla[2], E Samper[5], JC Segovia[1], A Raya[7], G Güenechea[1], C Baum[2], JC Izpisua-Belmonte[3,8], JA Bueren[1]

[1]Hematopoietic Gene Therapy Division. Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT) and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain, [2]Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany, [3]Center of Regenerative Medicine in Barcelona, Barcelona, Spain, [4]Centro de Investigación Biomédica en Red de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Barcelona, Spain, [5]Department of Regenerative Cardiology, Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III, Madrid, Spain, [6]Center for Regenerative Medicine, Boston University School of Medicine, Boston, Massachusetts, USA, [7]Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain, [8]Gene Expression Laboratory, Salk Institute for Biological Studies, La Jolla, California, USA

Upon correction of the genetic defect, induced pluripotent stem cells (iPS) from Fanconi anemia (FA) patients and FA mouse models can be efficiently generated and differentiated, *in vitro*, into hematopoietic progenitors. As mutations in the *BRCA2/FANCD1* gene have specific defects in homology directed recombination, here we have investigated the generation of iPS cells from mouse fibroblasts with a hypomorphic mutation in Brca2 ($Brca2^{\Delta 27/\Delta 27}$). Defects in *Brca2* implied that the reprogramming process was extremely inneficient. However, when $Brca2^{\Delta 27/\Delta 27}$ MEFs were gene-complemented with a *BRCA2*-lentiviral vector (LV) and then transduced with a reprogramming excisable polycistronic lentiviral vector, several iPSC clones were generated. iPSC clones were then transduced with an integration-deficient LV carrying the Cre-recombinase to excise the reprogramming cassette. Gene-corrected $Brca2^{\Delta 27/\Delta 27}$ iPSC clones efficiently expressed the hBRCA2 transgene and did not reveal the characteristic FA phenotype. Cells from the hematopoietic lineage could be generated from these iPSC clones, even though with a lower efficacy compared to mWT or mES cells. Additionally, no engraftment in irradiated $Brca2^{\Delta 27/\Delta 27}$ recipients could be demonstrated after IV infusion of $Brca2^{\Delta 27/\Delta 27}$ iPSC-derived hematopoietic grafts. Genetic analyses performed by aCGH in these samples showed the presence of genetic abnormalities which might account for the limited engraftment ability of these cells. Our studies evidence the efficacy of the cell reprogramming technology to generate disease-free iPSCs, and also reveal the limitations existing nowadays to generate safe and functional iPSC-derived hematopoietic stem cells to be used in cell therapy approaches.

OR029

Mitotic correction of trisomy 21 by disomic cell segregation occurred in human pluripotent stem cells from Down Syndrom

M Valentin[1,2], L Tosca[1,3], O Féraud[1], N Oudrhiri[1], E Gobbo[1], F Griscelli[1,4], D Luton[2], M Mitjavila-Garcia[1], G Tachdjian[1,3], A Bennaceur-Griscelli[1,5]

[1]INSERM UMR-S 935, ESTeam Paris-Sud, Villejuif, France, [2]Hôpital Beaujon, APHP, Service of gynecology, Paris, France, [3]Hôpitaux Universitaire Paris-Sud, APHP Service of histology and embryology, Clamart, France, [4]Université Paris Descartes, Paris, France, [5]Hôpitaux Universitaires Paris-Sud, APHP, Service of Hematology, Villejuif, France

Down Syndrome (DS) is a common aneuploïd genetic disorder and only 1–2% of DS harbor a germinal or somatic post-mitotic mosaÏcism with less severe phenotypic manifestations. Autologous cell-replacement therapies after correction of trisomy 21 would be a promising therapeutic strategy. We have generated DS-iPSC from three amniotic fluid cell donors harboring a complete trisomy 21 without mosaic. Trisomy 21 was confirmed by karyotype and FISH. PloÏdy was followed in cultured iPSC by CGHa. We observed upon iterative passages (>30), that one DS-iPSC line has completely lost a surnumerary chr. 21 and a disomic iPSC has been derived (PB6.1). This event was reproducible in 3 independent culture experiments. Microsatellites of chr.21 in PB6, PB6.1 and parental AFC were identical which eliminate the hypothesis of a contamination with an unrelated iPSC. Presence of chr.Y in PB6.1 by FISH eliminate a maternal contaminating cells in the initial AFC sample. SNP on ch21p confirm that persistent chr.21 originate from maternal and paternal genome. There is no heterozygosity loss. CGHa (135K) in PB6.1 compared to PB6 revealed a minor new CNV with a Dup 12q24.33 277,55Kb (including POLE, PXMP2, PGAM5, ANKLE2, GOLGA3, CHFR, ZNF605 genes) and a DupYp11.32 167,11Kb (SHOX gene).

This study, is the first demonstration that IPSC can reproduce *in-vitro* a spontaneously correction of trisomy 21 by a post-mitotic chromosome mis-segregation mechanism. Generation of related isogenic disomic iPSC should rescue the phenotype of disease and will allow to evaluate consequences of DS phenotypic reversion in by a potential autologous normal substitution cell therapy.

OR030

Intracerebral gene therapy for Sanfilippo syndrom

Michael Hocquemiller, Amina Zinaï

Lysogene, Paris, France

Sanfilippo syndrome type A or mucopolysaccharidosis type IIIA (MPSIIIA) is a rare lysosomal storage disease affecting approximately 1 in 100.000 births, belonging to the group of lysosomal storage diseases. MPSIIIA is caused by an autosomal recessive genetic defect resulting in the deficiency of the lysosomal enzyme N-sulphoglucosamine sulphohydrolase (SGSH). SGSH deficiency results in accumulation of heparan sulfate (HS) oligosaccharides with primary pathological expression occurring in the central nervous system (CNS). Neurological features are characterized by neurodegeneration, progressively leading to severe cognitive impairment, mental retardation, and invasive behavioral disorders such as hyposomnia and hyperactivity. The patient's life expectancy is usually reduced to less than two decades.

There is currently no available treatment for patients affected with Sanfilippo syndrome. However, major advances have been obtained over the past ten years, demonstrating the feasibility of AAV-mediated stable gene transfer into the brain resulting in extended intra-cerebral distribution of enzyme in animal models of Sanfilippo and correction of the major clinical manifestation.

Lysogene's intracerebrally administrated AAV-based investigational medicinal product, SAF-301, aims at treating Sanfilippo syndrome type A. It entered into the clinical stage in August 2011. The open-label, single arm, monocentric, phase I/II clinical study is primarily designed to evaluate the tolerance and the safety. It is also designed to evaluate exploratory neuropsychological, radiological and biological endpoints in the view of future efficacy clinical studies. To date, all planned 4 patients have received the treatment. This phase I/II study will be completed June 2013.

OR031

Cancer regression and neurologic toxicity following anti-MAGE-A3 TCR gene therapy

Richard A. Morgan, Alena Gros, Paul F. Robbins, Zhili Zheng, Steve A. Feldman, James C Yang, Richard M. Sherry, Giao Phan, Marybeth S. Hughes, Udai S. Kammula, Akemi Miller, Crystal Hessman, Ashley Stewart, Nicholas P. Restifo, Steven A Rosenberg

Surgery Branch, National Cancer Institute, Bethesda, MD, USA

Nine cancer patients were treated with adoptive cell therapy using autologous anti-MAGE-A3 TCR engineered T cells. Five patients experienced clinical regression of their cancers including two on-going responders. Beginning 1–2 days post-infusion, three patients (#'s 5, 7, and 8) experienced mental status changes, and two patients (5 and 8) lapsed into comas and subsequently died. MRI analysis of patients 5 and 8 demonstrated periventricular leukomalacia, and examination of their brains at autopsy revealed necrotizing leukoencephalopathy with extensive white matter defects associated with infiltration of CD3+/CD8+ T cells. Patient 7, developed Parkinson-like symptoms, which resolved over 4 weeks and fully recovered. Immunohistochemisrty staining of patient and normal brain samples demonstrated rare positively staining neurons with an antibody that recognizes multiple MAGE-A family members. The TCR used in this study recognized epitopes in MAGE-A3/A9/A12). Molecular assays of human brain samples using Q-RT-PCR, Nanostring quantization, and deep-sequencing indicated that MAGE-A12 was expressed in human brain (and possibly MAGE-A1, MAGE-A8, and MAGE-A9). This previously unrecognized expression of MAGE-A12 in human brain was possibly the initiating event of a TCR-mediated inflammatory response that resulted in neuronal cell destruction and raises caution for clinical applications targeting MAGE-A family members with highly effective immunotherapies.

OR032

Self-Inactivating Lentiviral Vectors for Correction of Rag1 Severe Combined Immunodeficiency

K. Pike-Overzet[1], M. Rodijk[1], Y.Y Ng[1], M.R.M Baert[1], C Lagresle-Peyrou[2], A Schambach[3], F Zhang[4], R.C Hoeben[5], S Hacein-Bey-Abina[2], A.C Lankester[6], R.G.M Bredius[6], G.J.A Driessen[7], A.J Thrasher[4], C Baum[3], M Cavazzana-Calvo[2], J.J.M van Dongen[8], F.J.T Staal[1]

[1]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, 2333 ZA, Leiden, The Netherlands, [2]Institut National de la Santé et de la Recherche Médicale (INSERM) and Department of Biotherapy, Centre d'Investigation Clinique intégré en Biothérapies, Hôpital Necker-Enfants Malades, Assistance Pub, Paris, France, [3]Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany, [4]Molecular Immunology Unit, Institute of Child Health, University College London, London, UK, [5]Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands, [6]Department of Pediatric Immunology, Hemato-Oncology, Bone Marrow Transplantation and Auto-immune Diseases, Leiden University Medical Center, Leiden, The Netherlands, [7]Department of Pediatrics, Erasmus MC University Medical Center, Rotterdam, The Netherlands, [8]Department of Immunology, Erasmus University Medical Center, Rotterdam, The Netherlands

Severe combined immunodeficiency (SCID) patients with an inactivating mutation in recombination activation gene 1 (RAG1) lack B- and T lymphocytes due to the inability to rearrange immunoglobulin (Ig) and T-cell receptor (TCR) genes. Gene therapy is a valid treatment option for RAG1-SCID patients, especially for patients lacking a suitable bone marrow donor. Hematopoietic progenitor cells from Rag1-deficient mice were transduced using lentiviral SIN vectors containing human RAG1sequences and transplanted into lethally irradiated Rag1-deficient mice. Treatment resulted in the appearance of B- and T lymphocytes in peripheral blood, albeit at a lower frequency than in wild-type controls. The development of B- and T cells was phenotypically confirmed in central and peripheral lymphoid organs. Serum Ig levels and TCR Vβ as well as C_μ and C_γ gene-segment usage was comparable to wild-type controls, indicating that RAG-mediated rearrangement took place. Upon stimulation of the B-cell receptor or the TCR, spleen cells showed a robust proliferative response and cytokine production. In addition, in vivo challenge resulted in production of antigen specific Igs. The qualitative regeneration of the B- and T-cell compartment provides proof-of-principle for therapeutic RAG1 gene-transfer in Rag-deficient mice using lentiviral SIN vectors. Our current efforts are aimed at lentiviral RAG1 gene transfer into RAG1-SCID CD34+ hematopoietic stem/progenitor cells and to see correction of the phenotype in CD34+ cells from RAG1-SCID patients transplanted into the murine NSG xenograft model.

OR033

A pRb-responsive, RGD-modified, and hyaluronidase-armed canine oncolytic adenovirus as a therapeutic veterinary agent and a model for human virotherapy

Eduardo Laborda[1], Cristina Puig-Saus[1], Alba Rodriguez[1], Rafael Moreno[1], Manel Cascallo[3], Josep Pastor[2], Ramon Alemany[1]

[1]*Institut d'Investigació Biomèdica de Bellvitge-Institut Català d'Oncologia, Barcelona, Spain,* [2]*Universitat Autònoma de Barcelona, Barcelona, Spain,* [3]*VCN Biosciences SL, Barcelona, Spain*

Human and canine cancer share similarities including genetically and molecular aspects, biological complexity, tumor epidemiology, and targeted therapeutic treatment. We have developed ICOCAV17, a CAV2-based canine adenovirus with the characteristics of our previously published ICOVIR17: expression of E1a controlled by E2F sites, deletion of the pRB-binding site of E1a, insertion of an RGD integrin-binding motif at the fiber Knob, and expression of hyaluronidase under the MLP/IIIa splicing acceptor. Preclinical studies show increased RGD and E2F-associated citotoxicity in tumor cells and strong hyaluronidase activity. Dogs with different types of tumors have been treated, including two osteosarcomas, one adenocarcinoma and one mastocitoma. All of them show tumor lysis after day 4 of treatment. No virus-associated adverse effects have been observed, but toxicity associated to tumor lysis including disseminated intravascular coagulation and systemic failure has been found in one case. Additional preclinical and clinical studies are warranted to validate ICOCAV17 as a potent and safe virus for the treatment of cancer in dogs.

OR034

LMO2 perturbs human T cell development *in vivo* by two different mechanisms

Anna-Sophia Wiekmeijer, Karin Pike-Overzet, Martijn Brugman, Wim Fibbe, Frank Staal

Department of Immunohematology and Bloodtransfusion, Leiden University Medical Centre, 2333 ZA, Leiden, The Netherlands

Background: In a gammaretrovirus-based gene therapy trial for X-linked SCID, insertional mutagenesis has caused the development of T-ALL in 5 out of 20 patients. Insertional mutagenesis caused the overexpression of genes by enhancer effects of the viral long terminal repeat (LTR). LMO2 was the most prominent amongst the activated genes and was most frequently hit by insertional mutagenesis. LMO2 is known for its role in development of T-ALL. Here we study the effect of LMO2 overexpression on human T cell development and the underlying mechanisms in the NSG xenograft model.

Methods: $CD34^+$ hematopoietic stem/progenitor cells were isolated from umbilical cord blood and transduced with a gammaretrovirus virus containing the coding sequence of LMO2 and a GFP tag. Cells were transplanted into NSG mice. The untransduced cells were used as an internal control.

Results: Mice transplanted with cells overexpressing LMO2 show an increased percentage of T cells in their peripheral blood. Within the thymus we can see different effects of LMO2 overexpression; either causing several partial, consecutive arrests in development or an accelerated development with increased thymic output. Effects of LMO2 were confined to the T cell lineage, despite transplantation of LMO2 transduced HSCs.

Conclusion: Here, we show that the presented model is a valuable tool for studying human hematopoiesis and its molecular regulation. Surprisingly, we found that LMO2 overexpression can cause both a block and an accelerated development of T cells in the thymus. The latter mechanism might cause the observed overrepresentation of T cells within the periphery.

OR035

An alpharetroviral vector platform suitable for permanent and transient cell modifications

JD Suerth[1], T Maetzig[1], MH Brugman[2], N Heinz[1], M Galla[1], JU Appelt[3], KB Kaufmann[4], M Schmidt[3], M Grez[4], U Modlich[1], C Baum[1], A Schambach[1]

[1]*Hannover Medical School, Hannover, Germany,* [2]*Leiden University Medical Center, Leiden, The Netherlands,* [3]*National Center for Tumor Diseases and German Cancer Research Center, Heidelberg, Germany,* [4]*Georg-Speyer-Haus, Frankfurt am Main, Germany*

Retroviral vectors can be used to integrate therapeutic transgenes into the genome in a highly efficacious, dose-controlled, and cell-specific manner. However, their semi-random integration can be genotoxic. Because integrome analyses have highlighted alpharetroviral vectors with a comparatively neutral integration spectrum, we developed a self-inactivating (SIN) alpharetroviral vector system with an advanced split-packaging design, enabling high-titer vector production in human cells. In a mouse model of serial bone marrow transplantation we compared alpharetroviral, gammaretroviral and lentiviral SIN vectors and showed that all vectors allowed for long-term transgene expression in hematopoietic cells. Importantly, alpharetroviral SIN vector integrations were comparatively neutral with respect to e.g. transcription start sites, intragenic regions, and cancer genes. Furthermore, sensitive genotoxicity studies using the *in vitro* immortalization assay revealed a decreased incidence of immortalization.

The advanced split-packaging design not only facilitates the production of stably integrating vectors, but also of vectors capable of transiently delivering "intermediate products" of the retroviral life cycle, such as episomal DNA or protein. Besides allowing for life cell imaging, these "intermediate products" can be used to modify cell functions or transfer proteins, whose permanent expression would be cytotoxic. Introducing different modifications to the alpharetroviral Gag/Pol expression constructs, we were able to extend the alpharetroviral vector platform by episomal DNA and protein transfer vectors. In summary, we provide proof-of-principle for the applicability of the alpharetroviral SIN vector platform not only for the permanent modification of hematopoietic cells with a comparatively neutral integration pattern, but also for the transient delivery of episomal DNA and protein.

OR036

AAV based gene therapy rescues the murine cardiac phenotype associated with Friedreich ataxia

Morgane Perdomini[1], Brahim Belbellaa[1], Laurent Monassier[2], Nathalie Cartier[3], Patrick Aubourg[3], Hélène Puccio[1]

[1]*Institute of Genetics and Molecular and Cellular Biology, Inserm U596, CNRS UMR7104 and Université de Strasbourg, Illkirch, France,* [2]*Laboratoire de Neurobiologie et Pharmacologie*

Cardiovasculaire, INSERM E333, Faculté de Médecine, Strasbourg, France, [3]Inserm, U745, Paris, France

Friedreich ataxia (FRDA), a progressive neurodegenerative disorder associated with cardiomyopathy, is caused by severely reduced levels of frataxin (FXN), a mitochondrial protein involved in Fe-S cluster assembly. To date, no treatment exists for stopping or slowing FRDA. We have evaluated an AAV-mediated gene therapy approach in the conditional MCK mouse model that recapitulates the FRDA cardiac phenotype. The MCK mice develop a progressive cardiac hypertrophy and dilatation starting at 5–6 weeks of age leading to death at 76±10 days. The AAV10.CAG-FXN vector, encoding human frataxin, was injected intravenously at different age at the dose of 5.10^{13} vg/Kg. Treatment of presymptomatic animals (3 weeks) resulted in the complete rescue of survival of the treated mice over 35 weeks of ages (duration of the study). Longitudinal evaluation of cardiac function and morphology by echocardiography demonstrated a complete normalization of treated mice. Furthermore, a complete rescue of the cellular and biochemical hallmarks of the disease was observed. Considering the efficacy of the gene therapy approach in preventing the disease in presymptomatic mice, we sought to evaluate the potential of correction/stabilization of the phenotype in postsymptomatic animals. Treatment of MCK mice with advanced cardiac failure (7 weeks) resulted in a progressive improvement of cardiac function to reach a complete normalization by 12 weeks of age. Preliminary analyses suggest also a complete correction of the FRDA cellular and biochemical features in the heart. Our results show for the first time an efficient and promising therapeutic outcome for the FRDA cardiac phenotype in a mouse model.

OR037

Stimulation of transmural capillary endothelialization of small-diameter synthetic vascular grafts through local overexpression of a novel recombinant VEGFR2-ligand VEGF-A109

Jarkko Hytonen[1], Olli Leppanen[1], Jouni Taavitsainen[1], Petra Korpisalo[1], Svetlana Laidinen[1], David Bergqvist[2], Kari Alitalo[3], Tuomas Rissanen[1], Seppo Yla-Herttuala[1]

[1]A.I.Virtanen- Institute, University of Eastern Finland, Kuopio, Finland, [2]Uppsala University Hospital, Uppsala, Sweden, [3]University of Helsinki, Helsinki, Finland

Endothelialization of prosthetic vascular conduits through transmural capillarization is a theoretically appealing way to improve the patency rates of small-diameter prosthetic grafts. However, in contrast to non-human primates, in which high-porosity vascular grafts are consistently endothelialized through transmural capillarization, human results have been discouraging, which may be caused by inadequacy of angiogenesis. We hypothetized that transfection of perigraft tissues with VEGF-A at the time of graft implantation would augment transmural capillarization and luminal endothelialization of high-porosity PTFE grafts.

52 rabbits received 87 ePTFE carotid interposition grafts, and were randomized to local therapy with adenoviruses encoding VEGF-A165, novel recombinant VEGF-A109 or control protein (LacZ). At 6 or 28d after surgery contrast-enhanced ultrasound data were obtained and vessels were explanted for histology.

AdVEGF-A165 and AdVEGF-A109 dramatically increased perfusion in perigraft tissues at 6d, a time point of peak transgene expression (14.2±3.6 or 16.7±2.6 fold increase vs. baseline, $P<0.01$). At 28d the effect was attenuated but still significantly higher than baseline. At 6d no luminal endothelium was observed in any of the groups. At 28d, animals that received AdVEGF-A165 or AdVEGF-A109 displayed an increase in luminal endothelialization through transgraft growth (9.8±3.3% or 7.9±3.4% luminal endothelial coverage vs. 0% in controls, $P<0.01$). No signs of luminal stenosis were observed in the treatment groups as compared to LacZ controls.

This study suggests that local delivery of AdVEGF-A introduced to the surgical wound at the time graft implantation is a promising novel strategy to increase endothelialization of high-porosity synthetic vascular grafts.

OR038

Pluripotent stem cells carrying a mutated LaminA/C: a cell model of laminopathies for the search for new therapeutic targets.

AC Guénantin[1], M Bialic[1], A Bertrand[1], J Leschick[1], G Bonne[0,1], M Pucéat[1]

[1]INSERM University Paris Descartes UMR633, Evry, France, [2]INSERM University Pierre et Marie Curie Paris 6 UM76, CNRS, UMR7215 Institut de Myologie, Paris, France

Laminopathies are genetic disorders caused by mutations in Lmna which encodes nuclear lamins A/C. Among hundreds of mutations so far identified, LmnaH222P leads to an Autosomal Dominant Emery-Dreifuss Muscular Distrophy (AD-EDMD). AD-EDMD patients suffer of both muscle dystrophy and cardiomyopathy. The origin of the cardiomyopathy (i.e, impaired transcription, nuclear desorganisation and or lamin toxicity) remains elusive.

Herein, we investigated the effects of H222PLmna in cardiac development and function using wt and mutated mouse embryonic stem cells (mESC), human induced pluripotent stem cells (hIPCs) as well as mouse mutant embryos. H222PLmna impairs cardiogenesis of both mESC and hiPSC. Expression of mesodermal and cardiac genes (i.e., brachyury, MesP1, Nkx2.5, Mef2c, Isl1 …) in mESC derived embryoid bodies (mEBs) and in BMP2-induced cardiac progenitors from hIPCs was deficient in mutated cells.The formation of mesendoderm was not affected by H222PLmna. Cell contractility was impaired in mutated mEBs which correlated with a poor sarcomeric network visualised by cell immunostaining. Preliminary experiments to monitor by in utero echocardiography heart function of embryos suggest that the cardiomyopathy is established early in embryogenesis (E12.5-E14.5). Work in progress tests the hypothesis that H222P affects binding of Lmna to heterochromatin thus preventing its remodeling and the formation of transcriptional factories required for the epithelio-mesenchymal transition, a process required for the formation of the cardiac mesoderm. Thus, iPSCs are a powerful model to unravel mechanistic components of the laminopathies raising new ideas as to the gene therapeutic approach to use or to combine with a drug delivery to relieve the cardiomyopathy.

OR039

Retargeting of lentivector integration by LEDGF/p75 chimera functionally rescues a cell culture model for X-linked CGD

S Vets[1], J De Rijck[1], KE Ocwieja[3], C Brendel[2], M Grez[2], FD Bushman[3], Z Debyser[1], R Gijsbers[1]

[1]KU Leuven, Leuven, Flanders, Belgium, [2]Georg-Speyer-Haus, Goethe Universität, Frankfurt-am-Main, Germany, [3]University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA

Retrovirus-based vectors are frequently used as delivery vehicles to correct genetic diseases because of their precise integration mechanism. However, adverse events in which vector integration activated proto-oncogenes, leading to clonal expansion and leukemogenesis complicate their application. The host cell-encoded lens epithelium-derived growth factor/p75 (LEDGF/p75) targets lentiviral integration into active transcription units. We demonstrated that HIV-based vector integration can be retargeted by replacing the chromatin interaction domain of LEDGF/p75 with an alternative DNA-binding protein, such as CBX1, that binds heterochromatin and intergenic regions. Here, we show that mRNA electroporation in wild-type cells results in transient expression of the chimeric CBX1-LEDGF$_{325–530}$ protein and efficient retargeting of lentivector integration. Next, we employed this technology in human myelomonocytic PLB-985 cells, a cell culture model for X-linked chronic granulomatous disease (X-CGD). X-CGD is a primary immunodeficiency caused by mutations in the CYBB gene encoding the phagocyte NADPH-oxidase catalytic subunit, gp91^{phox}. Following electroporation with LEDGF-chimera mRNA, PLB-985 cells were transduced with a therapeutic lentivector encoding codon-optimized gp91^{phox}. Integration site analysis revealed integration away from genes and near CBX1-binding sites, in regions rich in epigenetic marks associated with gene silencing. Nevertheless, gp91^{phox} expression was stable and NADPH-oxidase activity was restored to normal levels as determined by superoxide production. Integration sites and rescue potential of monoclonal cell lines derived from these cells are currently analyzed. These results provide proof-of-principle that transient expression of engineered LEDGF-chimera determines lentiviral vector integration site selection and functionally rescues a disease phenotype. Altogether, these results open perspectives for safer gene therapy.

OR040

Developing engineered zinc finger transcriptional repressors of Huntingtin as a potential therapy for Huntington's disease

HS Zhang, B Zeitler, J Pearl, Q Yu, J Cherone, DE Paschon, JC Miller, L Zhang, M Mendel, J Laganiere, Y Santiago, BJ Vu, FD Urnov, EJ Rebar, PD Gregory

Sangamo BioSciences, Richmond, CA, USA

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease caused by CAG-trinucleotide repeat expansion in exon 1 of Huntingtin (Htt). Repeat lengths of ≤35 CAGs cause no pathophysiology, while those of ≥40 CAGs lead to HD. In rodent models of HD, reducing Htt levels can prevent or delay disease onset, however, the complete loss of wild-type Htt expression is embryonically lethal in mice. Thus, strategies that selectively reduce expression of the disease-causing form of Htt alone, represent an attractive strategy. Engineered zinc-finger protein transcription factors (ZFP-TFs), which can be designed to virtually any gene target, offer a novel approach to the therapeutic regulation of Htt expression. To this end, we designed a panel of ZFP transcriptional repressors to bind the proximal promoter of Htt (to regulate both wild type and mutant Htt alleles). In cell-based experiments, such pan-allele specific ZFP-TFs achieved 50–90% down-regulation of Htt mRNA and protein. To generate transcription factors capable of selectively repressing the mutant Htt allele - we designed ZFP DNA binding domains to recognize the CAG repeat, and asked whether Htt alleles of different repeat lengths could be differentially regulated. We show that ZFP repressors can be engineered to exhibit minimal repression of normal Htt alleles but drive ~90% repression of mutant Htt alleles in HD fibroblasts lines. Thus, both pan-allele and allele-specific repression of Htt can be achieved with ZFP-TFs. *In vivo* testing in HD models is warranted to determine the efficacy and safety of these approaches to the potential treatment of HD.

OR041

Correction of Hemophilia B Phenotype Following ZFN Mediated Genome Editing in Adult Mice

Rajiv Sharma[1], Xavier Anguela[1], Yannick Doyon[2], Sunnie Wong[2], Hojun Li[1], Virginia Haurigot[1], Anand Bhagwat[1], Robert Davidson[1], Shangzhen Zhou[1], Philip Gregory[2], Michael Holmes[2], Katherine High[1]

[1]Children's Hospital of Philadelphia, Philadelphia, PA, USA, [2]Sangamo BioSciences, Inc., Richmond, CA, USA, [3]Howard Hughes Medical Institute, Philadelphia, PA, USA

We have previously reported on AAV-mediated delivery of zinc finger nucleases (ZFNs) and donor template to achieve persistent and clinically meaningful levels of genome editing in neonatal mice. To expand the applicability of this approach, we investigated whether treatment of adult mice results in efficient gene correction. Tail vein injection of the AAV-ZFN and AAV-Donor, containing a promoterless wild type human factor IX (hF.IX) insert flanked by arms of homology to the target, into adult (8 week old) mice resulted in stable (>30wk) mean circulating hF.IX levels of ~30% of normal, a substantial increase over the levels observed in similarly treated neonatal mice (3–7% of normal). Liver regeneration following 2/3 partial hepatectomy, which is known to substantially reduce expression from non-integrated AAV genomes, had no significant impact on hF.IX expression, suggesting hF.IX expression in ZFN-treated mice is predominantly a result of stable correction at the intended target. PCR analysis of liver genomic DNA from treated mice revealed evidence of targeted gene addition by both homology-directed repair and non-homologous end-joining. Finally, we treated HB mice containing a copy of the defective human F.IX locus. Mice receiving both the ZFN and Donor vectors expressed levels of hF.IX in the range of 30% and showed a normalization of clotting times. In summary, our data suggest that highly efficient genome editing is achieved following AAV-mediated ZFN and donor delivery in adult mice. Furthermore, ZFN-driven genome editing in HB mice resulted in substantial levels of circulating hF.IX and a correction of clotting times.

OR042

De novo and *in silico* design of regulatory elements for targeted and robust tissue-specific expression in gene therapy

Marinee Chuah[1,2], Melvin Rincon[1,2], Inge Petrus[2,3], Mario Di Matteo[1,2], Pieter De Bleser[4], Janka Matrai[1,2], Caroline Le Guiner[5], Nathalie Ward[6], Dina Danso-Abeam[3], Mira Samara[1,2], Bing Yan[3], Veronique Blouin[5], Simon Waddington[6], Kathy High[7], John McVey[6],

Philippe Moullier[5], Frederico Mingozzi[7], Thierry VandenDriessche[1,2]

[1]*Department of Gene Therapy & Regenerative Medicine, Free University of Brussels (VUB), Brussels, Belgium,* [2]*Center for Molecular & Vascular Biology, University of Leuven, Leuven, Belgium,* [3]*Vesalius Research Center, Flanders Institute of Biotechnology (VIB), Leuven, Belgium,* [4]*Bioinformatics Core, Department for Molecular Biomedical Research (DMBR), Flanders Institute of Biotechnology (VIB) - Ghent University, Ghent, Belgium,* [5]*INSERM UMR U649, CHU-Hotel Dieu, Nantes, France,* [6]*Institute of Child Health, University College London, London, UK,* [7]*Children's Hospital of Philadelphia, University of Philadelphia, Philadelphia, USA*

The robustness and safety of gene therapy can be substantially improved by enhancing targeted tissue-specific expression of the therapeutic transgene. To improve transcriptional targeting for tissue-directed gene therapy we validated a data-mining algorithm that led to the identification of novel evolutionary conserved regulatory elements, associated with high tissue-specific expression. Incorporation of these de novo designed regulatory elements in viral and non-viral vectors enhanced tissue-specific gene expression in heart or liver. Up to a 100-fold enhancement in tissue-specific expression could be achieved, depending on the promoter used, while retaining high tissue-selectivity. Robust liver-specific expression of therapeutic genes encoding coagulation factor IX or factor VIII could be obtained in hemophilic mice at very low vector doses. This was confirmed using non-viral PiggyBac transposon-based vectors. The superior performance of these liver-specific elements was subsequently validated following AAV transduction in non-human primates (NHP) resulting in FIX expression levels up to 40% of normal levels without any notable side-effects. This study underscores the potential of *in silico* rational vector design to improve their robustness and safety in different target tissues with broad implications for gene therapy and regenerative medicine. This new approach may possibly reduce attrition rates of advanced therapy medicinal products (ATMPs) in clinical trials.

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OR043

MiR-155 silencing decreases neuroinflammation in Aβ-stressed astrocytes and microglia cells

Joana Guedes[1], Luís Pereira de Almeida[2,1], Maria Conceição Pedroso de Lima[3,1], Ana Luísa Cardoso[1]

[1]*Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal,* [2]*Laboratory of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra, Coimbra, Portugal,* [3]*Department of Life Sciences, Faculty of Science and Technology, University of Coimbra, Coimbra, Portugal*

MiRNAs are small non-coding RNA molecules that modulate gene expression at a post-transcriptional level and their role in the regulation of biological processes makes them an emerging class of therapeutic targets. Dysregulation of miRNA networks has been linked to neurodegeneration and immune dysfunctions and has become a research focus in the context of Alzheimer's disease (AD).

In this work, we demonstrate that miR-155 expression is increased in the brain of 3×Trg AD transgenic mice, prior to senile plaque formation, and is co-localized with intraneuronal APP accumulation in the cortex and hippocampus. In view of the pro-inflammatory functions of miR-155 and aiming at clarifying its role in AD, we evaluated the levels of miR-155 in Aβ-stressed astrocytes and microglia cultures, two brain-related cell types involved in neuroinflammation. We show that miR-155 expression levels are increased in astrocytes and microglia following activation with Aβ fibrils but not with Aβ oligomers. These findings correlate with the observed decrease in SOCS-1 expression, a miR-155 validated target, and with the increase in the production of TNF-α, IL-1β and IL-6 by these cells. Importantly, silencing of miR-155 using DLS (DOGS:DOPE) cationic liposomes complexed with anti-miR-155 oligonucleotides led to an increase in SOCS-1 expression and reduced the production of inflammatory mediators.

Overall, our results demonstrate the important role of miRNA-155 in AD and show that silencing of this miRNA may constitute an interesting and promising anti-inflammatory therapeutic strategy towards this disease.

OR044

Lentiviral delivery of human full-length wild-type Tau protein mediates progressive spatio-temporal and cortico-cortical propagated neurofibrillary degeneration in rat brain

M Colin[1,2], S Dujardin[1,2], K Lécolle[1,2], R Caillierez[1,2], S Bégard[1,2], N Zommer[1,2], N Dufour[3], G Auregan[3], P Hantraye[3], N Déglon[3], L Buée[3]

[1]*Inserm, UMR837, Lille, France,* [2]*Université Lille 2, Faculté de Médecine, IMPRT, JPARC, Lille, France,* [3]*Atomic Energy Commission (CEA), Institute of Biomedical Imaging (I2BM), Molecular Imaging Research Center (MIRCen), Fontenay-Aux-Roses, France,* [4]*Lausanne University Hospital (CHUV), Department of Clinical Neurosciences (DNC), Laboratory of Cellular and Molecular Neurotherapies (LMCN), Lausanne, Switzerland*

The abundant and abnormal accumulation of the hyperphosphorylated microtubule-associated protein, Tau, is a pathological feature of neurodegenerative diseases referred to as tauopathies. Recent studies define an extracellular form of Tau as a major player in the spatio-temporal evolution of neurofibrillary degeneration. Tau protein inclusions could develop in a stereotypical manner in particular brain regions from where they could spread to neighboring brain regions. To date, all of the works dealing with Tau propagation and spreading use mutant Tau proteins found in an autosomal dominant tauopathy. However, in more than 90% of cases, Tau-related pathology is a sporadic disease with a neurodegenerative process resulting from the aggregation of wild-type (WT) Tau. The goal of this study was then to address the ability of Tau proteins, either WT or mutant, to propagate Tau pathology throughout the brain. VSVG-pseudotyped lentiviral vectors (LVs) are suitable tools to mediate stable and specific gene expression in neurons. Tau pathology was initiated by intracranial injection of these vectors in a region that is prematurely affected by the neurodegenerative process in the most common tauopathy, Alzheimer's disease. It allowed us to design for the first time a rat model of trans-synaptic sporadic tauopathy spreading. Compared to mutant Tau WT Tau protein leads to a Tau pathology that spreads in the rat brain with higher efficiency and, with time, affects distant brain regions connected to the injected site. Finally, we demonstrate that the propagation of lesions is due to a neuron-to-neuron transfer of WT Tau protein.

OR045

Widespread vector distribution following intra-CSF delivery of AAV9 vectors in a large animal model.

Virginia Haurigot[1], Sara Marco[1], Albert Ribera[1], Miquel Garcia[1], Albert Ruzo[1], Pilar Villacampa[1], Eduard Ayuso[1], Sonia Añor[2], Anna Andaluz[2], Mercedes Pineda[3], Gemma Garcia Fructoso[3], Maria Molas[1], Luca Maggioni[1], Sandra Motas[1], Jesus Ruberte[1], Federico Mingozzi[4], Marti Pumarola[2], Fatima Bosch[1]

[1]cbateg, Universitat Autonoma De Barcelona, Bellaterra, Barcelona, Spain, [2]school Of Veterinary Medicine, Universitat Autonoma De Barcelona, Bellaterra, Barcelona, Spain, [3]hospital Sant Joan De Deu, Barcelona, Spain, [4]Children's Hospital of Philadelphia, Philadelphia, PA, USA

Therapeutic success of gene-based approaches to genetic diseases affecting the Central Nervous System (CNS), requires widespread distribution of the transgene product. We previously demonstrated in Mucopolysaccharidosis Type IIIA (MPSIIIA) mice that intracisternal (IC) delivery to the cerebrospinal fluid (CSF) of AAV9-sulfamidase vectors led to whole-body disease correction via transgene expression throughout CNS and liver. In an effort to translate MPSIIIA results to the clinic, we assessed the feasibility and tolerability of this approach in a large animal model. Widespread CNS transduction was observed following IC administration of AAV9-GFP vectors to naïve, healthy dogs, which resulted in vector dissemination throughout the CNS and transduction of 3–5% of hepatocytes, confirming results obtained in mice. Importantly, IC administration of AAV9 vectors encoding for human sulfamidase, currently under clinical development, led to increased sulfamidase activity in CSF. As an alternative route of CSF delivery we also tested intracerebroventricular (ICV) administration using a clinically-approved surgical procedure. Side-by-side comparison of IC versus ICV delivery yielded similar profiles of vector distribution, with a remarkable transduction of ependimocytes lining the ventricle wall in the latter case. Both approaches resulted in high anti-AAV antibodies titers in serum but not in CSF. Moreover, high-titer serum antibodies to AAV9 only partially blocked CSF-mediated gene transfer to the brain of pre-immunized dogs. Consistently, anti-AAV antibody titers were lower in CSF than in serum in healthy and MPSIIIA-affected children. These results support the clinical translation of this approach for the treatment of MPSIIIA and other lysosomal storage diseases (LSD) with CNS involvement.

OR046

Combining Oncolysis with genetic ImmunoRNase delivery as innovative strategy for cancer therapy

I Fernández-Ulibarri[1], M Arndt[2], DE Dorer[1], S Engelhardt[1], R Kontermann[3], J Krauss[2], DM Nettelbeck[1]

[1]DKFZ, Heidelberg, Germany, [2]NCT, Heidelberg, Germany, [3]Universidad de Stuttgart, Stuttgart, Germany

Oncolytic adenoviruses (OAds) are emerging therapeutics for cancer treatment which have shown a favorable safety profile but often limited clinical benefit. A powerful approach for improving the efficacy of virotherapy is arming OAds with therapeutic transgenes. In this study, we constructed a tumor selective OAds (Ad5/3-ONC-scFvEGFR) encoding a tumor-targeted ImmunoRNase, consisting of a recombinant single-chain antibody against EGFR (scFvEGFR) as targeting moiety and the amphibian RNase ONCONASE (ONC) as effector moiety. After optimization of the adenoviral expression of the toxic ImmunoRNase, ONC-scFvEGFR was efficiently expressed in a replication-dependent manner and without interfering with viral replication. We could show that the secreted ONC-scFvEGFR selectively binds to $EGFR^+$ tumor cells and that EGFR binding of ONC-scFvEGFR can be blocked by the EGFR-specific antibody Cetuximab but not by the control antibody Rituximab with specificity for CD20. Importantly, ONC-scFvEGFR induced bystander killing specifically in $EGFR^+$ cells in a dose-dependent manner and increased the cytotoxicity of the virus up to 1000x. Taken together, our results demonstrate that OAds can mediate an effective ImmunoRNAse transfer, that the secreted ImmunoRNAse is fully functional regarding its ability to bind to EGFR and to kill selectively $EGFR^+$ tumor cells and that armed OAds showed significantly improved killing of target cells. In conclusion, arming OAds with a targeted and secreted cell death-inducing protein represents a potent method to enhance antitumor efficacy of virotherapy. Furthermore, our system provides an attractive platform for treating a variety of tumors due to the great flexibility offered by the exchangeable targeting domain.

OR047

Oncolytic adenovirus with temozolomide induces autophagy, immunogenic cell death and antitumor immune responses preclinically and in cancer patients

I Liikanen[1], L Ahtiainen[1], MLM Hirvinen[1], S Bramante[1], V Cerullo[1], P Nokisalmi[1], O Hemminki[1], I Diaconu[1], S Pesonen[1], A Koski[1], L Kangasniemi[2], SK Pesonen[1], M Oksanen[1], F Zhao[3], L Laasonen[4], K Partanen[4], T Joensuu[4], A Kanerva[1], A Hemminki[1]

[1]Cancer Gene Therapy Group, Molecular Cancer Biology Program, Transplantation Laboratory and Haartman Institute, University of Helsinki, Helsinki, Finland, [2]Oncos Therapeutics, Ltd., Helsinki, Finland, [3]Advanced Microscopy Unit, Haartman Institute, University of Helsinki, Helsinki, Finland, [4]International Comprehensive Cancer Center Docrates, Helsinki, Finland

Tumor cell autophagy appears useful for immunogenic cell death and induction of antitumor immune responses. Oncolytic adenoviruses and alkylating chemotherapeutic temozolomide have been shown to induce autophagic cell death preclinically. We studied safety, efficacy and immunological effects of oncolytic adenovirus combined with low-dose pulse temozolomide. Metronomic low-dose cyclophosphamide was added to treatments to selectively reduce regulatory T-cells. Preclinically, combination therapy resulted in enhanced cytotoxicity and tumor-growth inhibition ($P < 0.01$). Electron microscopy and LC3-immunohistochemistry revealed increased autophagy in combination-treated tumor tissues. Furthermore, elevated calreticulin exposure, ATP release and HMGB1 secretion indicated immunogenicity of cell death. 42 combination treatments were given to 17 chemotherapy-refractory cancer patients in the context of an advanced therapy access program. Treatments were well-tolerated with mostly grade 1–2 fever, nausea, lymphopenia and anemia. We observed transient increases in anti- and proinflammatory cytokines, evidence of virus replication, and induction of neutralizing antibodies. LC3-stained ascites tumor cells showed increased autophagy post-treatment. 8/15 evaluable cases showed induction of antitumor T-cells by ELISPOT. Interestingly, HMGB1 release into serum increased in 60% of

treatments, and seemed to correlate with tumor-specific T-cell responses ($P = 0.0833$), suggesting a candidate predictive marker for antitumor immune responses. Objective evidence of antitumor efficacy was seen in 67% of evaluable treatments, with a trend for increased survival over matched controls treated with virus only (median OS 269 *versus* 170 days). In summary, the combination of oncolytic adenovirus with low-dose pulse temozolomide and metronomic cyclophosphamide increased tumor cell autophagy, elicited antitumor immune responses and showed promising safety and efficacy.

OR048

A ubiquitous chromatin opening element (UCOE) prevents transgene silencing in murine pluripotent cells and their differentiated progeny

M Ackermann[1], N Pfaff[1], N Lachmann[1], R Eggenschwiler[2], S Kohlscheen[3], C Brendel[4], T Maetzig[3], MN Antoniou[5], M Grez[4], A Schambach[3], T Cantz[2], T Moritz[1]

[1]*REBIRTH Research Group Reprogramming, Hannover Medical School, Hannover, Germany,* [2]*REBIRTH Research Group Stem Cell Biology, Hannover Medical School, Hannover, Germany,* [3]*Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany,* [4]*Institute for Biomedical Research, Georg-Speyer-Haus, Frankfurt, Germany,* [5]*Gene Expression and Therapy Group, King's College London School of Medicine, Department of Medical and Molecular Genetics, Guy's Hospital, London, UK*

Transgene-silencing constitutes a serious problem for iPSC-based gene therapy. As a defined 1.5kb ubiquitous chromatin opening element (A2UCOE) has been shown to prevent methylation-induced silencing in cell-lines and primary hematopoietic cells, we hypothesized a similar effect in pluripotent cells and their differentiated progeny. Proof-of-concept studies were performed in the context of lentiviral gene transfer of the drug-resistance gene cytidine deaminase (CDD), an approach to render hematopoietic cells resistant to chemotherapy. Here, in non-differentiated iPSCs the A2UCOE significantly enhanced CDD- and dTomato-transgene expression from housekeeping and viral promoters and completely inhibited the silencing of viral promoters. Even more important, upon hematopoietic differentiation the A2UCOE significantly reduced the rapid silencing of transgenes expressed from the truncated elongation factor-1α (EFS) promoter, resulting in robust transgene expression in $\sim$80% of CD41$^+$ hematopoietic progenitor cells (EFS-driven controls: 1–3%), as demonstrated by flow cytometry, western blot, qRT-PCR and functionally by significantly enhanced drug resistance of clonogenic progenitor cells (73 + /-15% vs. 11 + /-3%; $p < 0{,}01$; A2UCOE.EFS vs. untransduced control). Bisulfite sequencing revealed protection of the EFS-promoter from differentiation-induced CpG-methylation to be associated with the A2UCOE effect. Similar results were obtained for differentiation towards the neuronal and hepatic lineage. Again the A2UCOE effectively reduced silencing of the EFS-promoter allowing for sustained transgene expression in $\sim$97% (EFS-driven controls: $\sim$3%) of hepatically and $\sim$65% ($\sim$3%) of neuronally differentiated cells, respectively. Thus, our data demonstrate that UCOEs can efficiently prevent differentiation-induced transgene silencing and thereby offer a generalized concept to stabilize transgene expression during the generation of ESC/iPSC-derived transgenic cell therapy products.

OR049

Deciphering the Molecular Mechanisms of Reprogramming using miRNA screens

Nils Pfaff[1], Jan Fiedler[3], Thomas Thum[3], Thomas Moritz[1], Tobias Cantz[2]

[1]*REBIRTH Research Group Reprogramming, Hannover Medical School, Hannover, Germany,* [2]*REBIRTH Research Group Stem Cell Biology, Hannover Medical School, Hannover, Germany,* [3]*Molecular and Translational Therapeutic Strategies (IMTTS), Hannover Medical School, Hannover, Germany,* [4]*Institute for Experimental Hematology, Hannover Medical School, Hannover, Germany*

Induced pluripotent stem cells (iPSCs) can be generated by overexpression of the transcription factors Oct4, Sox2, and Klf4. Recently, the importance of microRNAs (miRNAs) for the induction of pluripotency was highlighted. To further elucidate the molecular mechanisms underlying transcription factor-mediated reprogramming and to increase the efficiency of this process, we have established a system which allows screening of large miRNA libraries during iPSC generation.

We transduced murine embryonic fibroblasts (MEFs) from OG2 mice (Oct4-GFP) with a polycistronic lentiviral construct expressing Oct4, Klf4 and Sox2 and a dTomato reporter. One day after transduction, we individually transfected murine miRNAs from a Pre-miR^{TM} Library (Ambion) containing 379 miRNAs into these MEFs. At day 7 to 10 after transduction, emerging GFP$^+$ colonies were counted and analyzed.

Using this system, we previously were able to identify a miRNA family consisting of miR-130b, miR-301b and miR-721, which strongly enhances iPSC generation, at least in part through repression of the homeobox transcription factor Meox2. Further analyses revealed several miRNAs, such as miR-132 and miR-212, which effectively inhibited iPSC generation when overexpressed. Accordingly, repression of these miRNAs by miRNA inhibitors led to increased reprogramming efficiencies. Putative downstream targets of these inhibitory miRNAs include major epigenetic modulators, such as Kdm5a, Arid1a, Arid1b, p300 or Hmga2.

In conclusion, by applying a full library miRNA screen we identified a novel miRNA family strongly enhancing iPSC generation from MEFs, partially through repression of Meox2. In addition, we identified several miRNAs, which strongly restrain iPSC-generation and inhibition of which in turn enhances reprogramming.

OR050

Lentiviral transduction of CD34 + cells induces genome-wide epigenetic modifications

Daniel Stockholm[1,2], Yamagata Yoshiaki[1,3], Véronique Paietti[1,2], Guillaume Corre[1,2], Catherine Poinsignon[1,4], Nizar Touleimat[5], Damien Delafoy[5], Céline Besse[5], Jorg Tost[5,6], Anne Galy[1,4], Andras Paldi[1,2]

[1]*Inserm, U951, Genethon, Evry, France,* [2]*Ecole Pratique des Hautes Etudes, UMRS_951, Evry, France,* [3]*Department of Obstetrics and Gynaecology, Yamaguchi University Graduate School of Medicine, Minamikogushi, Japan,* [4]*Université Evry Val d'Essonne, Evry, France,* [5]*Centre National de Génotypage, CEA – Institut de Génomique, Evry, France,* [6]*Fondation Jean Dausset- CEPH, Paris, France*

Lentiviral vectors (LV) transduction has become the method of choice for stable gene transfer into human CD34+ hematopoietic cells, but the effects of the ex vivo transduction process remain poorly characterized. While LVs permanently modify the genome of transduced cells at the sites of genomic integration of the expression cassette, it remains unknown if the transduction process also results in epigenetic modifications in the cellular population. Human CD34+ cells were therefore treated in vitro with cytokines followed by LV to measure epigenetic changes during a typical ex vivo transduction process. We have adapted an image cytometry method to screen known nuclear proteins associated with epigenetic changes. Analysis revealed an up-regulation of SIRT1 in response to cytokines, and further modulation in response to LV. Large-scale molecular analysis of genomic DNA methylation confirmed the extensive epigenetic effects of LV. A genome-wide increase in DNA methylation occurred in almost 700 genes in CD34+ cells as early as 24 hours following LV infection and was observed preferentially in regions undergoing methylation changes in response to cytokine pre-activation. Epigenetic modifications are therefore induced in CD34+ cells during ex vivo transduction resulting from the effects of cytokines and LV. These effects may constitute previously unsuspected sources of variability and of risk in gene therapy.

OR051

Plasmid DNA for indirect clinical applications - how much GMP is necessary?

Marco Schmeer, Ruth Baier, Martin Schleef

PlasmidFactory GmbH & Co. KG, Bielefeld, Germany

Amongst other applications, our plasmid DNA is used in the GMP-compliant production of recombinant viruses, antibodies and RNA, where these are the active pharmaceutical ingredients (API) used in clinical trials. Here, it is not always necessary to produce the plasmid DNA under GMP as well, in order to use it for such applications. An alternative is the so-called *High Quality Grade* plasmid DNA which is highly purified and well-characterised and, hence, meeting the requirements of most regulating agencies.

PlasmidFactory's new facility for the production of this high purity plasmid DNA is now operating successfully in new, modern labs. Plasmid DNA of the highest quality is being produced in the new premises in accordance with the EMEA guideline CHMP/BWP/2458/03. To ensure product safety, substances of animal origin are not used at any stage of the entire process, guaranteeing maximum possible product purity by reliable exclusion of contaminants such as bacterial chromosomal DNA or damaged plasmids. Only one plasmid is produced in each area - different plasmids are not produced in parallel in the same lab.

"High Quality Grade" plasmid DNA is produced in our facility based on a research cell bank (RCB) and the very effective, patented *ccc Grade* DNA technology. A number of quality controls both to the cell bank and to the plasmid DNA product, ensure that the final result is a product designed especially for the intended application and that complies with the appropriate regulatory standards.

OR052

Development of a Manufacturing Process for an Oncolytic Vaccine

Maria Fe C. Medina[1], Monique Marguerie[3], Uma Sankar[1], Natasha Kazhdan[2], Angelica Ortega[1], Dave Stojdl[4], John Bell[3], Brian Lichty[2]

[1]Robert E. Fitzhenry Vector Laboratory, McMaster University, Hamilton, Ontario, Canada, [2]McMaster Immunology Research Centre, McMaster University, Hamilton, Ontario, Canada, [3]Ottawa Hospital Research Institute, Center for Cancer Therapeutics, Ottawa, Ontario, Canada, [4]Apoptosis Research Centre, Children's Hospital of Eastern Ontario Research Institute, Ottawa, Ontario, Canada

A manufacturing process for an oncolytic vaccine based on Maraba virus (Family Rhabdoviridae) expressing the MAGE A3 (melanoma associated antigen 3) was developed. The purification method involved the use of suspension cells grown in chemically-defined, protein free medium, but infected in the presence of fetal bovine serum. Under these conditions, the titers of infected cell supernatants were 10^9 PFU/mL. In our current large-scale production protocol, the cell supernatants (40L) were clarified by depth filtration and the vaccine was purified by tangential flow filtration. The purified vaccine titer was up to 1.4×10^{10} PFU/mL, which was sufficient to enable initiation of toxicology testing in rodents and macaques. This was achieved in spite of the five-fold decrease in supernatant titer that was observed when infecting large volumes >30L. To further increase the vaccine yield, we are currently investigating the parameters that govern the titer of the infected cell supernatants during scale-up. In addition, we are transitioning the manufacturing process to clinical production and developing specifications for our first clinical lot. We will describe the results we have obtained to date.

OR053

Inverted terminal repeat read-through and reverse packaging: mechanisms of occurence and strategies to minimize DNA impurities in recombinant AAV.

Olga Zelenaia[1], Marina Sumaroka[1], Liza Tai[1], Bernd Hauck[1], J Fraser Wright[1,2]

[1]Center for Cellular and Molecular Therapeutics, The Children's Hospital of Philadelphia, Philadelphia, PA, USA, [2]Dept of Pathology and Laboratory Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA

Recombinant AAV vector based investigational products have shown dramatic therapeutic promise in several independent early phase clinical trials. Development of this emerging class of biologic product through advanced clinical development requires further improvements in vector manufacturing and characterization, including understanding parameters that affect impurity levels in purified clinical grade vectors. Removal of DNA impurities in AAV vectors is complicated by the fact that, even with efficient nuclease treatment to remove accessible nucleic acids during vector purification, fragments of DNA may be packaged and resistant to nuclease treatment. Here we studied the levels of residual plasmid DNA in a series of AAV2 vectors containing single stranded transgene expression cassettes that

ranged in size as follows: rAAV A: 2.7kb; rAAV B: 3.7kb; and rAAV C: 4.3kb. Multiple lots of each of the constructs were generated and purified using the same process. Residual plasmid DNA was determined by qPCR using primers and probes targeting the AmpR or KanR gene sequences, one relevant measure of impurity DNA species. This evaluation showed that rAAV A contained 164 pg/10E9 vg residual plasmid DNA; rAAV B contained 42.7 pg/10E9 vg; and rAAV C contained 14.0 pg/10E9 vg. PCR demonstrated that ITR read-through in the shorter expression cassettes accounted for the higher level of DNA impurities. These data support that recombinant AAV vectors with expression cassettes near the natural packaging capacity of AAV have lower plasmid DNA impurities. Expression cassette optimization, in conjunction with the use of oversized backbones, are effective strategies to minimize DNA impurities.

OR054

Lentiviral SGSH expression from the human CD11b promoter in transplanted haematopoietic stem cells fully corrects behaviour and neuropathology of Mucopolysaccharidosis IIIA mice

Alexander Langford-Smith[1], Ana Sergijenko[1], Fiona Wilkinson[1], Kia Langford-Smith[1], Ai Yin Liao[1], Simon Jones[2], J Ed Wraith[2], Robert Wynn[3], Brian Bigger[1]

[1]University of Manchester, Manchester, UK, [2]St Mary's Hospital, Manchester, UK, [3]Royal Manchester Children's Hospital, Manchester, UK

Mucopolysaccharidosis IIIA (MPSIIIA) is a lysosomal storage disorder caused by mutations in N-sulfoglucosamine sulfohydrolase (SGSH), resulting in accumulation of heparan sulphate and progressive neurodegeneration.

We have previously demonstrated improved neuropathology and behavioural correction of MPSIIIA mice using wild type haematopoetic stem cell transplants (HSCT) overexpressing SGSH driven by a viral promoter in a lentiviral vector (LV). After engraftment, monocytes traffic to the brain and engraft as microglial cells, cross-correcting diseased neurons. Using this vector we could not achieve behavioural correction with LV transduced MPSIIIA cells and we wanted to improve vector safety by restricting expression to monocyte/microglial lineages.

SGSH was thus codon optimised to increase expression and LV vectors under ubiquitous PGK or myeloid-specific promoters (CD11b) compared. LV-CD11b-eGFP gave significantly higher expression than LV-PGK-eGFP in CD11b+ or CD19+ peripheral blood cells. Subsequently MPSIIIA HSCs were transduced with LV-PGK-coSGSH or LV-CD11b-coSGSH vectors and transplanted into MPSIIIA mice.

At 6 months, all abnormal behaviours of MPSIIIA mice were fully corrected by LV-CD11b but unchanged with LV-PGK. At 8 months in the brain, both vectors mediated complete correction of glycosaminoglycan storage, secondary storage of GM2 gangliosides and presynaptic vesicles. However, LV-PGK only increased brain enzyme activity to 7% of WT levels whilst LV-CD11b was significantly better at 11%. Although both vectors significantly reduced neuroinflammation, this was only corrected by LV-CD11b.

LV-CD11b-coSGSH was the only effective vector in the context of LV-HSCT using MPSIIIA HSCs and mediates full correction of neuropathology and behaviour in MPSIIIA mice. We are preparing this vector for clinical trial.

OR055

AAV9 mediated gene therapy of MLD model mice

Noriko Miyake[1], Koichi Miyake[1], Atsushi Sakai[2], Motoko Yamamoto[1], Ayumi Endo[1], Hidenori Suzuki[2], Takashi Shimada[1]

[1]Department of Biochemistry and Molecular Biology, Division of Gene Therapy Research Center for Advanced Medical Technology, Nippon Medical School, Tokyo, Japan, [2]Department of Pharmacology, Nippon Medical School, Tokyo, Japan

The success of gene therapy for lysosomal storage diseases with neurological involvement such as metachromatic leukodystrophy (MLD) depends on the development of efficient delivery of lysosomal enzymes and/or vectors across the blood-brain barrier (BBB). In this experiment, we studied the utility of AAV9 in two different approaches, systemic intravenous (IV) and intrathecal (IT) injection, for treatment of MLD model mice. We generated AAV9 vector expressing human arylsulfatase A (AAV9/ASA) and IV injected into neonatal MLD mice. ELISA analysis showed that sustained expression of ASA was detected in the brain for more than one year. Alcian blue staining showed significant decrease of the amount of stored sulfatide in AAV9/ASA treated MLD mice. Furthermore, in the behavior test, AAV9/ASA treated mice showed a significant improvement in their ability to traverse narrow balance beams. In contrast, IV injection of AAV9/ASA into adult MLD mice resulted in no improvement of neurological symptoms. As an alternative approach, we examined the possibility of IT administration of AAV9/ASA for the treatment of adult MLD model mice (6 week-old). AAV9/ASA was IT injected via a suboccipital puncture and analyzed at age 1.3 year. Significant decrease of the amount of stored sulfatide and improvement of the behavior test were observed in IT injected mice. These data indicate that IT administration of AAV vector is useful to treat adult MLD mice. In conclusion, systemic IV injection of AAV9/ASA for neonates and IT injection for adult were promising strategies to treat neurological symptoms of MLD.

OR056

Neonatal intracerebral gene delivery to correct CNS pathology in a mouse model of Globoid Cell Leukodystrophy

Annalisa Lattanzi[1,3], Camilla Salvagno[1], Claudio Maderna[1], Sabata Martino[3], Aldo Orlacchio[3], Luigi Naldini[1,2], Angela Gritti[1]

[1]S. Raffaele Scientific Inst., Telethon Inst. for Gene Therapy (TIGET), Milano, Italy, [2]Vita-Salute S. Raffaele Univ. Medical School, Milano, Italy, [3]Dept. of Exp. Medicine and Biochemical Sciences, Univ. of Perugia, Perugia, Italy

Effective therapies for Globoid Cell Leukodystrophy require efficient delivery and distribution of a functional β-galactocerebrosidase (GALC) in CNS tissues to prevent demyelination and neurodegeneration. Injection of lentiviral vector (LV) coding for the *galc* gene (LV.GALC) in the external capsule (EC) of early symptomatic GALC-deficient Twitcher (Twi) mice allows CNS enzymatic correction and amelioration of neuropathology (Lattanzi et al., 2010). In this study we assessed whether LV-mediated GALC supply provided to asymptomatic Twi mice might delay the onset of symptoms, prevent neuropathology progression and prolong survival. We injected LV.GFP or LV.GALC in newborn

Twi mice and WT littermates and assessed biodistribution and persistence of transgene expression, short- and long-term toxicity (apoptosis, macrophage/microglia activation and astrogliosis). Despite a moderate loss of transduced cells mediated by transient activation of innate immunity, we detected rapid and persistent GALC activity in CNS tissues and cerebrospinal fluid (40–50% of the WT levels) of LV.GALC-injected Twi mice at 6 weeks post-injection (p.i.). Clearance of storage, amelioration of histopathology and improved survival indicated that the enzyme was functional. Importantly, we detected LV genome and transgene expression in CNS tissues at 6 months p.i., while LV genome was undetectable at any time p.i in peripheral organs. Preliminary results indicate a safe integration profile of LV in injected brain tissues. Overall we show that intraparenchymal neonatal LV injection is safe and effective in providing rapid and long-term GALC supply in CNS tissues of Twi mice. Biodistribution studies in non-human primates are planned to assess feasibility in larger brains.

OR057

A lentiviral vector pseudotyped with a Baboon retrovirus envelope glycoprotein outperforms VSV-G-LVs for gene transfer into hematopoietic stem cells and lymphocytes.

Anaïs Girard Gagnepain, Fouzia Amirache, Caroline Costa, Cecilia Frecha, Didier Nègre, Dimitri Lavillette, François-Loïc Cosset, Els Verhoeyen

Ecole Normale Supérieure de Lyon, Université de Lyon, Inserm, EVIR, U758, Human Virology Department, Lyon, France

HSC-based gene therapy holds promise for the cure of many inherited and acquired diseases. The two recent successful clinical trials, though, used high doses classical VSV-G pseudotyped lentivectors and very strong cytokine-cocktails compromising the 'HSC'-character. Thus, current VSV-G-LVs are unsatisfactory for gene therapy applications.

We generated here for the first time a LV pseudotyped with a BaEVgp derived from a baboon endogenous retrovirus. We evaluated these vectors for hCD34+-cell transduction under mild cytokine prestimulations (TPO, SCF or SCF+TPO) that allow better preservation of the 'HSC'-characteristics. After a single application, BaEVgp-LVs stably transduced up to 80–90 % of TPO+SCF stimulated hCD34+ cells, where VSV-G-LVs reached 5–10% transduction. Even more stricking was that these new BaEVgp-LVs allowed at low vector doses efficient transduction of up to 30 % of quiescent hCD34+-cells where VSV-G-LVs were inefficient. Importantly, reconstitution of NOD/SCID/gammaC-/- mice with BaEVgp-LV transduced hCD34+-cells (MOI=10) resulted in 60–70% transduction of all analyzed myeloid and lymphoid engrafted lineages including CD34+ lineage negative cells in the BM. In contrast, VSV-G-LVs (MOI=100) did not result in equivalent transduction levels of all hematopoietic lineages with a strong bias toward B-cell lineage.

Of importance, resting lymphocytes were also transduced effiently with this new BAEVgp-LVs, where VSV-G-LVs failed. Moreover, transduced thymocytes, T and B cells conserved their quiescent naive and memory phenotypes upon BAEVgp-LV mediated transduction.

Together, these results strongly suggest that the novel BaEVgp-LVs efficiently transduce true HSCs and T and B cells at high levels. This paves the way to multiple gene and immunotherapy applications.

OR058

MicroRNA-150-regulated vectors for cell type-specific transgene expression in hematopoietic gene therapy

S Brennig[1], N Lachmann[1], J Jagielska[1], D Heckl[2], N Pfaff[1], T Maetzig[2], U Modlich[2], T Cantz[3,4], B Gentner[5], A Schambach[2], T Moritz[1]

[1]REBIRTH Research Group Reprogramming, Hannover Medical School, Hannover, Germany, [2]Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany, [3]REBIRTH Research Group Stem Cell Biology, Hannover Medical School, Hannover, Germany, [4]Max Planck Institute for Molecular Biomedicine, Münster, Germany, [5]San Raffaele Telethon Institute for Gene Therapy, Division of Regenerative Medicine, Stem Cells and Gene Therapy, Milan, Italy

Cell type-specific off-target transgene toxicity has been encountered in hematopoietic gene therapy approaches. Incorporation of microRNA (miRNA) target sequences into gene transfer vectors to specifically repress transgene activity in cells expressing the respective miRNAs allows circumventing this problem. Here, we have investigated the potential of miRNA-150 target (miR150T) sequences to suppress transgene expression in murine lymphocytes *in vitro* and *in vivo* utilizing mono- (LV.SFFV.GFP+/-miR150T)- and bicistronic (LV.Bd.PGK.GFP+/-miR150T) SIN lentiviral vectors carrying the reporter genes GFP and mCherry. QRT-PCR analysis confirmed abundant miRNA-150 expression specifically in differentiated B and T lymphocytes, whereas minimal expression was detected in the stem- and progenitor cell and the differentiated myeloid compartment. *In vitro* studies employing LV.SFFV.GFP.miR150T demonstrated effective downregulation of transgene expression in murine $B220^+$ B and $CD3^+$ T cells with lin^- progenitor and differentiated $GR1^+$ myeloid cells virtually unaffected. Furthermore, in a murine bone marrow transplant/ gene transfer model, significantly reduced transgene activity was observed in $B220^+$ B and $CD4^+$ or $CD8^+$ T cells for the LV.Bd.PGK.GFPmiR150T lentiviral vector. Again, expression in $CD11b^+/GR1^+$ myeloid cells, lin^- progenitor cells or $lin^-/Sca1^+/c\text{-}kit^+$ stem cells remained almost unaffected. No lymphohematopoietic *in vivo* toxicity of miRNA-150 targeting was detected when peripheral blood counts and bone marrow cellularity were assessed in primary and secondary recipients. Therefore, our data demonstrate the suitability of miRNA-150 targeting to specifically prevent off-target transgene expression in the mature lymphoid compartment and further support the concept of miRNA targeting to improve the specificity and safety of gene therapy approaches.

OR059

Displaying high affinity ligands on adeno-associated viral vectors enables tumor cell-specific and safe gene transfer

Robert Münch[1], Hanna Janicki[2], Iris Völker[1], Anke Rasbach[1], Michael Hallek[2], Hildegard Büning[2], Christian Buchholz[1]

[1]Paul-Ehrlich-Institut, Langen, Germany, [2]University of Cologne, Cologne, Germany

Gene transfer vectors derived from the adeno-associated virus (AAV) have recently received increasing attention due to substantial therapeutic benefit in several clinical trials. Nevertheless, their great potential for *in vivo* gene therapy can only be partially exploited owing to their broad tropism. Current cell surface targeting strategies expanded vector tropism towards transduction

of cell types that are inefficiently infected naturally, but failed to restrict or fully re-direct AAV's tropism. Hypothesizing that this limitation can be overcome by equipping natural receptor blinded AAV vectors with high-affinity ligands, we displayed designed ankyrin repeat proteins (DARPin) as VP2 fusion protein on AAV capsids ablated for natural primary receptor binding. These second generation targeting vectors demonstrated an as of yet unachieved efficiency to discriminate between target and non-target cells in mono- and mixed cultures. Moreover, DARPin-AAV vectors delivered a suicide gene precisely to tumor tissue and substantially reduced tumor growth without causing fatal liver toxicity. The latter caused death in animals treated with conventional AAV vectors with unmodified capsids, which accumulated in liver tissue and failed to affect tumor growth. This novel targeting platform will be key to translational approaches requiring restricted and cell-type specific *in vivo* gene delivery.

OR060

Regulated lentiviral vector gene transfer for X-CGD

Valentina Capo[1], Giada Farinelli[1], Bernhard Gentner[2], Gigliola Di Matteo[1], Silvia Di Cesare[1], Samantha Scaramuzza[2], Lucia Sergi Sergi[2], Manuel Grez[3], Didier Trono[4], Andrea Finocchi[1], Paolo Rossi[1], Luigi Naldini[2,5], Maria Chiriaco[1], Alessandro Aiuti[1,2]

[1]*Department of Pediatrics, Children's Hospital Bambino Gesù and University of Rome Tor Vergata School of Medicine, Rome, Italy,* [2]*San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Scientific Institute HS Raffaele, Milan, Italy,* [3]*Georg-Speyer Research Institute, Frankfurt, Germany,* [4]*École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland,* [5]*"Vita-Salute" S. Raffaele University, Milan, Italy*

Chronic Granulomatous Disease (CGD) is caused by defective NADPH oxidase function in phagocytes leading to increased susceptibility to fungal and bacterial infections. Gene therapy with hematopoietic stem cells (HSC) may represent a valid alternative for gp91phox deficient patients (X-CGD). Our aim was to develop a novel gene transfer approach into HSC for safe and effective therapy of X-CGD based on SIN lentiviral vectors (LV) encoding gp91phox. To reduce possible toxic effects of gp91phox in HSC, we designed and tested different LV that incorporate: 1) a myeloid specific promoter (LV.MSP.gp91) for transcriptional regulation or 2) miR126 target sequences (LV.PGK.gp91_126T) to induce post-transcriptional downregulation in HSC expressing miRNA126. Human cell lines, murine Lineage- and human CD34+ cells were transduced and tested for specific expression and function of gp91phox. All vectors fully restored gp91phox expression and NADPH oxidase activity in human X-CGD myeloid cell lines. Transduction of X-CGD murine and human progenitors resulted in effective transgene expression and activity after differentiation. After short-term culture, gp91phox was detected ectopically in undifferentiated CD34+ cells transduced with LV.PGK.gp91 driving constitutive transgene expression. This ectopic expression was substantially reduced after transduction with LV.MSP.gp91 and LV.PGK.gp91_126T, but was maintained in differentiated cells. Preliminary results in the mouse model showed that transgene expression and activity was restored also in vivo. Both regulation systems showed their efficacy in restricting the expression of gp91phox in HSC while maintaining it in differentiated myeloid cells. Further studies will be required to assess their preclinical efficacy and safety before proceeding to clinical application.

OR061

Redifferentiation of Expanded Human Pancreatic β-Cell-derived Cells by Inhibition of the NOTCH Pathway

Yael Bar, Holger A. Russ, Elad Sintov, Leeat Anker-Kitai, Sarah Knoller, Shimon Efrat

Tel Aviv University, Tel Aviv, Israel

In-vitro expansion of β-cells from adult human pancreatic islets would overcome donor β-cell shortage for cell replacement therapy for diabetes. Using a β-cell-specific labeling system we have shown that β-cell expansion is accompanied by dedifferentiation resembling epithelial-mesenchymal transition, and loss of insulin expression. Epigenetic analyses indicate that key beta-cell genes maintain open chromatin structure in expanded beta-cell-derived (BCD) cells, although they are not transcribed. In the developing pancreas important cell-fate decisions are regulated by NOTCH receptors, which signal through the Hairy and Enhancer of Split (HES) 1 transcription regulator. We have reported that BCD cell dedifferentiation and proliferation in vitro correlate with reactivation of the NOTCH pathway. Inhibition of HES1 expression using small hairpin RNA (shRNA) during culture initiation results in reduced β-cell replication and dedifferentiation, suggesting that HES1 inhibition may also affect BCD cell redifferentiation following expansion. Here, we used *HES1* shRNA to downregulate HES1 expression in expanded human BCD cells, and show that HES1 inhibition is sufficient for inducing BCD cell redifferentiation, as manifested by a significant increase in insulin expression. Combined treatment with *HES1* shRNA, cell aggregation in serum-free medium, and a combination of soluble factors, further stimulated redifferentiation of BCD cells. In-vivo analyses demonstrated the ability of the redifferentiated cells to replace β-cell function in hyperglycemic immunodeficient mice. These findings demonstrate the redifferentiation potential of ex-vivo-expanded BCD cells, and the reproducible differentiating effect of HES1 inhibition in these cells. (Bar et al. J Biol Chem. 2012 May 18;287(21):17269–80)

OR062

Hepatic gene transfer of TFEB, a master autophagy regulator, results in clearance of mutant alpha-1-antitrypsin.

Nunzia Pastore[1], Keith Blomenkamp[2], Pasquale Piccolo[1], Fabio Annunziata[1], Rosa Maria Sepe[1], Francesco Vetrini[3], Donna Palmer[4], Philip Ng[4], Elena Polishchuk[1], Simona Iacobacci[1], Roman Polishchuk[1], Jeffrey Teckman[2], Andrea Ballabio[1,3], Nicola Brunetti-Pierri[1,5]

[1]*Telethon Institute of Genetics and Medicine, Naples, Italy,* [2]*Department of Pediatrics, Saint Louis University School of Medicine, Cardinal Glennon Children's Medical Center, Saint Louis, MO, USA,* [3]*Jan and Dan Duncan Neurological Research Institute, Texas Children's Hospital, Houston, TX, USA,* [4]*Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA,* [5]*Department of Pediatrics, Federico II University, Naples, Italy*

Classical form of α-1-antitrypsin is the most common genetic disease of the liver and is due to a missense mutation that alters folding in the mutant protein (ATZ). The ATZ accumulates in hepatocyte endoplasmic reticulum and causes liver injury and toxicity. The transcription factor EB (TFEB) is a master gene that regulates lysosomal function and autophagy, and promotes cellular clearance. We investigated the efficacy of hepatic gene

transfer of TFEB for clearance of hepatotoxic ATZ in the PiZ mouse model. PiZ mice were injected intravenously with a helper-dependent adenoviral vector expressing TFEB under the control of a liver-specific promoter (HDAd-TFEB) or, as controls, with a HDAd expressing an unrelated reporter gene (HDAd-AFP) or saline. Compared to controls, mice injected with HDAd-TFEB showed a dramatic reduction in hepatic ATZ globules and polymers. Ultrastructural studies revealed increased ATZ signals within autophagolysosomes in HDAd-TFEB injected mice. Taken together, these results demonstrate that hepatic gene transfer of TFEB reduces accumulation of ATZ by enhancement of hepatic autophagy. A marked reduction of hepatic monomeric ATZ was due to down-regulation of ATZ mRNA and was associated to a reduction of liver inflammation, as shown by decreased NFκB activation and hepatic IL-6 expression. PiZ mice injected with HDAd-TFEB showed significant decrease of hepatocyte apoptosis and hepatic fibrosis, which are key features of the hepatic disease. In summary, TFEB-mediated hepatocyte expression resulted in clearance of ATZ, improvement of the liver phenotype and therefore, is a novel and attractive gene-based strategy for the treatment of alpha-1-antitrypsin deficiency hepatic disease.

OR063

Optimization of liver gene transfer for hemophilia B

Xavier M. Anguela[1,2], Yifeng Chen[1], Robert J. Davidson[1], Shangzhen Zhou[1], Timothy C. Nichols[3], Mark A. Kay[4], Federico Mingozzi[1], Katherine A. High[1,2]

[1]Center for Cellular and Molecular Therapeutics, Philadelphia, PA, USA, [2]Howard Hughes Medical Institute, The Children's Hospital of Philadelphia, Philadelphia, PA, USA, [3]Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA, [4]Departments of Pediatrics and Genetics, Stanford University School of Medicine, Stanford, California, USA

AAV8 is the gold standard for liver-targeted gene therapy. However individuals with high neutralizing antibodies to the AAV8 capsid ($\sim$30% of the population) may not be eligible for enrollment in AAV8 gene transfer studies. Here, we screened AAV serotypes, highly efficient at transducing human hepatocytes in primary cultures, to develop an alternative treatment option for this population. First we compared chimeric AAV-DJ to AAV-2, -6, and 8 after intravenous administration in mice. Plasma human factor IX (hFIX) levels in AAV-DJ-treated animals were 60X and 30X higher than mice treated with AAV2 and 6, respectively. AAV8 was the most potent vector, with hFIX levels 3-4X higher than AAV-DJ. Liver transduction was also quantified in mice passively immunized with human intravenous immunoglobulin. AAV2 and AAV6 expression was reduced to almost undetectable levels whereas AAV-DJ and AAV8 maintained hFIX levels at about 25% and 50% of the original values, respectively. In a canine model of hemophilia B, AAV-DJ expression was significantly higher than AAV6 and about 2–3 times lower than AAV8. Next, we tested self-complementary (sc) AAV vectors. However, we were unable to detect significant differences in hFIX levels between scAAV-DJ and ssAAV-DJ vectors. Finally, we compared the Padua hFIX variant to its wild-type form. In hemophilia B mice, activity of the Padua hFIX was about ten times higher than wild-type. Hyperfunctional hFIX variants combined with continuous work to expand the AAV portfolio represent a viable strategy to extend the application of AAV therapeutics to broader patient cohorts.

OR064

Integration-defective lentiviral vectors expressing engineered hyperactive factor IX improve vector performance following hepatic transduction

Nisha Nair[1], Mario Di Matteo[1,2], Janka Matrai[1,2], Alessio Cantore[3,4], Luigi Naldini[3,4], Marinee Chuah[1,2], Thierry VandenDriessche[1,2]

[1]Free University of Brussels, Department of Gene Therapy & Regenerative Medicine, Brussels, Belgium, [2]University of Leuven, Center for Molecular & Vascular Biology, Leuven, Belgium, [3]San Raffaele Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milan, Italy, [4]Vita Salute San Raffaele University, Milan, Italy

Lentiviral vectors are attractive tools for liver-directed gene therapy because of their capacity for stable gene expression and the lack of preexisting immunity in most human subjects. However, the use of integrating vectors may raise some concerns about the potential risk of insertional mutagenesis. We have shown that this risk could be substantially reduced using integrase-defective lentiviral vectors (IDLVs) containing an inactivating mutation in the integrase (D64V). IDLV-mediated and hepatocyte-targeted coagulation factor IX (FIX) expression prevented the induction of neutralizing antibodies to FIX even after antigen rechallenge in hemophilia B mice and accounted for relatively prolonged therapeutic FIX expression levels (Matrai, Cantore, Bartholomae, Annoni et al., *Hepatology* 2011;53:1696–1707). Nevertheless, FIX transgene expression levels were reduced in comparison with their integrase-competent vector counterparts in contrast to other tissues. We now show that this limitation of IDLVs can be overcome by using synthetic codon-optimized FIX and hyper-functional codon-optimized FIX-R338L transgenes carrying a R338L amino acid substitution, designated as FIX-Padua, previously associated with clotting hyperactivity and thrombophilia. These changes allows reconstituting FIX activity to fully therapeutic levels, exceeding 100% of normal FIX activity. The extent of the increased expression and activity with these IDLVs was consistent with that observed with integrase-competent LVs encoding either human or canine codon-optimized FIX. To our knowledge, our current approach yielded the highest level of functionally active FIX to date using IDLV allowing the use of lower and thus potentially safer vector doses.

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OR065

HLA-universal platelets infusions prevent platelet refractoriness in a mouse model

Christiane Gras[1], Radiana Antarianto[1], Lilia Goudeva[1], Britta Eiz-Vesper[1], Kai Schulze[2], Carlos Guzman[2], Rainer Blasczyk[1], Constanca Figueiredo[1]

[1]Institute for transfusion medicine, Hannover Medical School, Hannover, Germany, [2]Department of Vaccinology and Applied Microbiology, Helmholtz Center for Infection Research, Braunschweig, Germany

Refractoriness to platelet transfusion caused by HLA alloimmunization constitutes a significant clinical problem. It would be desirable to generate platelet-units devoid of HLA. Previously, we showed that the generation of HLA class I-silenced (HLA-

universal) platelets from CD34+ cells using a shRNA targeting β2-microglobulin transcripts is feasible. Now, we assessed the functionality of HLA-silenced platelets and their ability to escape HLA antibody-mediated complement-dependent cytotoxicity (HLA-ACDC) *in vitro* and *in vivo*. Platelet-activation in response to ADP and thrombin were assessed *in vitro*. The immune-evasion capability of HLA-universal megakaryocytes and platelets was tested in lymphocytotoxicity-assays using anti-HLA antibodies. *In vivo*, $1x10^6$ HLA-silenced megakaryocytes were infused into NOD/SCID/IL-2Rγc$^{-/-}$ mice in presence or absence of anti-HLA antibodies. Platelet production was evaluated by flow cytometry using anti-CD42a and CD61 antibodies. HLA-universal platelets demonstrated to be functionally similar to blood-derived platelets. Lymphocytotoxicity-assays showed that HLA-silencing efficiently protects megakaryocytes against HLA-ACDC. 80–90% of HLA-expressing megakaryocytes, but only 3% of HLA-silenced megakaryocytes were lysed. *In vivo*, both HLA-expressing and HLA-silenced megakaryocytes showed to produce platelets (up to 0.5% within the platelet population) when anti-HLA antibodies were absent. However, in presence of anti-HLA antibodies HLA-expressing megakaryocytes were rapidly cleared from the circulation of mice, while HLA-silenced megakaryocytes escaped HLA-ACDC and human platelet production was detectable up to 11 days. Our data show that HLA-silenced platelets are functional and efficiently protected against HLA-ACDC. All mice tolerated well the HLA-universal Megakaryocytes infusions. Provision of HLA-universal platelet units may become an important component in the management of patients with platelet transfusion refractoriness.

OR066

CD34+ cells mobilized by Plerixafor treatment in thalassemic patients are enriched in stem/progenitor cells endowed with high reconstitution potential and gene transfer susceptibility

MR Lidonnici[1,4], A Aprile[1], M Frittoli[1,4], G Mandelli[1], B Gentner[1,2], L Bellio[5], E Cassinerio[3], L Zanaboni[3], S Rossini[5], MD Cappellini[3], F Ciceri[2], S Marktel[2], G Ferrari[1,4]

[1]O.San Raffaele-Telethon Institute for Gene Therapy (HSR-TIGET), Milan, Italy, [2]Hematology and Bone Marrow Transplantation Unit, San Raffaele Scientific Institute, Milan, Italy, [3]Università di Milano, Ca Granda Foundation IRCCS, Milan, Italy, [4]Università Vita-Salute San Raffaele, Milan, Italy, [5]Immunohematology and Transfusion Medicine Unit, San Raffaele Scientific Institute, Milan, Italy

Gene therapy of inherited blood diseases requires harvest of hematopoietic stem cells (HSCs) from patients and autologous transplantation of genetically modified cells. In order to achieve correction of the disease, high number of HSCs and previous conditioning of the host bone marrow (BM) are necessary. In the clinical application of gene therapy for adult thalassemic patients, the HSC source is a crucial issue since the minimal target dose poses a challenge for the use of steady state BM. Indeed, intrinsic characteristics of thalassemic patients (i.e. splenomegaly and thrombophilia) dictate caution in the use of G-CSF as mobilizing agent.

We have the unique opportunity to study and characterize stem/progenitor cells mobilized from the BM of adult thalassemic patients by treatment with Plerixafor (AMD3100), in the context of a Phase-I-II clinical trial. The objectives of this clinical protocol are to test safety and efficacy at obtaining an adequate yield of CD34+ cells and to evaluate these cells as a source of HSCs for gene therapy.

Purified CD34+ cells from leukoapheresis of 4 treated patients are analyzed for their biological and functional properties, subpopulations composition and expression profile. In vivo reconstitution potential and lymphomyeloid differentiation of CD34+ cells are tested following transplantation in NSG mice. The results indicate that cells mobilized by Plerixafor have a primitive phenotype with a high reconstitution potential and are efficiently transduced, thus being a suitable source of target cells for gene therapy.

OR067

Targeting CD133+ cells to improve long-term gene marking in Hematopoietic Stem Cell based Gene Therapies

Christian Brendel[1], Daniela Abriss[1], Benjamin Göbel[2], Sabrina Kneissl[3], Christian Buchholz[3], Manuel Grez[1]

[1]Georg-Speyer-Haus, Frankfurt, Germany, [2]Med. Clinic II, KGU, Frankfurt, Germany, [3]Paul-Ehrlich-Institute, Langen, Germany

Generally, CD34+ cells are used for genetic modification in gene therapy trials. CD34+ cells consist of a heterogeneous cell population with mostly limited long-term repopulating capabilities, resulting in low long-term engraftment levels in particular in those diseases, in which gene modified cells lack a proliferative advantage over non-modified cells. Therefore modifications in gene transfer vectors and gene transfer strategies are required to improve long-term clinical benefit in gene therapy patients. One particular attractive approach to solve this problem is the improvement of HSC based gene transfer by specifically targeting cells with long-term engraftment capabilities.

To this end we constructed lentiviral gene transfer vectors (LV) specifically targeting CD133+ cells, a cell population with recognized long-term repopulating capabilities. Targeting is achieved by incorporating the measles virus (MV) glycoproteins hemagglutinin, responsible for receptor recognition, and fusion protein into LVs. The hemagglutinin protein is blinded for its native receptors and displays a single-chain antibody specific for CD133 (CD133-LV).

Transduction of CD34+ cells with CD133-LV vectors resulted in stable gene expression and gene marked CD34+ cells could be expanded in vitro, while the number of VSV-G-LV transduced CD34+ cells declined over time. Competitive repopulation experiments in NSG mice showed a significantly improved engraftment of CD133-LV transduced HSCs. At 8 to 10 weeks post-transplantation gene marked hematopoiesis was dominated by the progeny of CD133-LV and not VSV-G-LV transduced cells.

In conclusions this new strategy may be promising to achieve long-term engraftment in patients treated by gene therapy.

OR068

Protection of hematopoietic stem cells from stress-induced functional impairment by very low-dose interleukin-1 stimulation

Jun Ooehara, Shigeru Kakuta, Yoichiro Iwakura, Makoto Otsu, Hiromitsu Nakauchi

Institute of Medical Science, University of Tokyo, Tokyo, Japan

Background: Hematopoietic stem cell (HSC) gene therapy is a treatment option that potentially provides life-long immune reconstitution for patients with primary immunodeficiency diseases. Virus-mediated gene transfer is the measure currently used to confer functionality on patients' blood $CD34^+$ cells

(HSPCs). Because this procedure typically requires *ex vivo* stimulation of HSPCs for 4–5 days, it is essential to optimize the cocktail of cytokines so that the transduced cells retain the best reconstitution capability. Here we sought to revisit the use of interleukin-1 (IL-1), which is the well-known cytokine, but its positive effects on highly purified HSCs remain doubtful.

Results: By analyzing IL-1 receptor knockout mice, we found impaired reconstitution ability in their HSCs, suggesting the positive effect of IL-1 at the stem cell level. We then tested *in vitro* effects of IL-1 in a 7-day serum-free culture and observed its dose-dependent biphasic effects on HSCs: while it forced HSC differentiation at the concentrations higher than ~10 ng/ml, it clearly enhanced proliferative response of the primitive cells at low concentrations around 0.05 ng/ml. Consistent with this, addition of IL-1 at a limited dose improved *in vivo* reconstitution capability of cultured HSCs in both primary and secondary recipients, and the positive effects became more evident with certain stress conditions including hypoxia and aging.

Conclusions: This study provides the possible revival of one of the oldest cytokine as a positive amplifier/protector of HSCs, potentially culminating in the improvement in long-term hematopoiesis that is relevant to clinical gene therapy trials.

OR069

Silencing HLA class I expression in human corneas to decrease the risk of graft rejection after keratoplasty

Constanca Figueiredo[1], Stefanie Vahlsing[1], Monika Valtink[2], Martin Börgel[3], Katrin Engelmann[2], Rainer Blasczyk[1]

[1]*Institute for Transfusion Medicine, Hannover Medical school, Hannover, Germany,* [2]*Institute for Anatomy, Technical University of Dresden, Dresden, Germany,* [3]*German Society for tissue transplantation, Hannover, Germany*

The variability of the human histocompatibility leukocyte antigen (HLA) complex significantly contributes to an immune response after allogeneic cornea transplantation. Thus, we permanently reduce HLA expression on cornea transplants to decrease graft rejection after keratoplasty. Lentiviral vectors encoding short hairpin RNAs (shRNA) targeting β2-microglobulin were used to downregulate HLA class I expression. Levels of β2-microglobulin mRNA or HLA class I expression were determined by Real-Time PCR and flow cytometry, respectively. Cell viability tests using 7-Aminoactinomycin (7-AAD) and Propidium iodide (PI) staining were performed after transduction. Light and fluorescence microscopic analysis were carried out to evaluate the integrity of the corneal endothelium. A mean of 92%±7% of the total cornea cells were transduced. In comparison to the non-engineered cornea, the delivery of the β2-microglobulin-specific shRNA caused a reduction by up to 95% in β2-microglobulin transcript levels as detected at day 5 and day 15 after cornea transduction. The reduction of β2-microglobulin levels induced a decrease HLA class I expression by up to 90%. Microscopic analysis of the cornea endothelium showed that this layer remained unaffected. Also, the absence of a positive signal upon 7-AAD/PI staining indicated the absence of off-target effects that might impair the endothelial function or viability. This data demonstrates the feasibility of silencing HLA class I expression in the original 3D-tissue-structure by RNAi-mediated nucleic acid targeting. As in vivo data in an allogeneic rat model showed a superior survival of RT1-silenced cells, silencing HLA expression has the potential to overcome alloimmunization and tissue rejection in allogeneic keratoplasty.

OR070

Use of a short hPDE6b promoter sequence to drive efficient gene expression specifically in rods in a model of severe retinal dystrophy, the *Rd10* mouse.

Yvan Arsenijevic[1], Corinne Kostic[1], Alberto Auricchio[2], JongEun Ihm[1]

[1]*Unit of Gene Therapy & Stem Cell Biology, Jules-Gonin Eye Hospital, Unil, Lausanne, Switzerland,* [2]*Telethon Institute of Genetics and Medicine and Dep. of Pediatrics, Napoli, Italy*

Gene therapy for severe retinal dystrophies directly affecting photoreceptor is still a challenge for clinical application. One of the main hurdles is to generate high transgene expression specifically in rods or cones. In the present study, we are investigating the possibility to drive hPDE6b expression in the *Rd10* mouse (which bears a mutation in the Pde6b gene resulting of rapid rod loss starting at P18 in our colony) using a specific sequence of the human PDE6b promoter.

Among the two 5′ flanking fragments of the human PDE6b gene analysed, the short 146 bp promoter (-93 to +53) showed the highest activity in the Y-79 cells, as described previously (Di Polo and Farber, 1995), and was used for further in vivo studies. AAV2/8-PDE6bp-EGFP was then produced and showed a rapid expression specifically in rods but not in cones. Five weeks post-injection of AAV2/8-PDE6bp-PDE6b in P9 *Rd10* mouse retina, ERG recordings revealed that 6 eyes out of 14 eyes presented an increased rod sensitivity of about 300 fold as well as a cone response to flicker stimuli. Furthermore OCT images and histological analyses revealed an increased ONL size and presence of PDE6b-positive outersegments following injection of AAV2/8-PDE6bp-PDE6b but not of control vector.

This promoter sequence is the shortest rod-specific (149 bp) sequence allowing efficient gene transfer into rods and has thus a great interest for AAV vector design. The proof of principle of such vector was demonstrated in the *Rd10* by transferring the human PDE6b sequence without using mutated capsids, nor self-complementary vectors.

OR071

Intravitreal delivery of AAV-NDI1 for the treatment of Leber Hereditary Optic Neuropathy.

Naomi Chadderton[1], Arpad Palfi[1], Sophia Millington-Ward[1], Mary O'Reilly[1], Oliverio Gobbo[1], Nora Overlack[2], Matthew Carrigan[1], Matthew Campbell[1], Carsten Ehrhardt[1], Uwe Wolfrum[2], Pete Humphries[1], Paul F Kenna[1], G Jane Farrar[1]

[1]*Trinity College Dublin, Dublin, Ireland,* [2]*Johannes Gutenberg University of Mainz, Mainz, Germany*

Leber Hereditary Optic Neuropathy (LHON) causes significant visual disability in approximately 1 in 30,000 individuals, representing a large unmet clinical need. LHON is a mitochondrially inherited eye disorder which involves mutations in genes encoding components of the mitochondrial respiratory NADH-ubiquinone oxidoreductase complex (complex I) (http://www.mitomap.org/MITOMAP). Development of gene therapies for LHON has been impeded by genetic heterogeneity and the need to deliver therapies to the mitochondria of retinal ganglion cells (RGCs), the cells primarily affected in LHON. The therapy under development involves intraocular injection of a nuclear yeast gene NADH-quinone oxidoreductase (NDI1) that

encodes a single subunit complex I equivalent and as such is mutation independent. NDI1 is imported into mitochondria due to an endogenous mitochondrial localisation signal.

In the present study intraocular delivery of NDI1 has been shown to protect RGCs, in a rotenone-induced murine model of LHON. Recombinant AAV serotype 2 (AAV2/2) expressing NDI1 from a CMV promoter (AAV-NDI1) was administered to mice using a single intravitreal injection. This route of administration is directly applicable to human patients and is routinely used clinically to administer drugs such as Avastin and Lucentis for the treatment of age-related macular degeneration (AMD). In this study, intravitreal injection of AAV-NDI1 significantly reduced RGC death and optic nerve atrophy and led to preservation of retinal function as assessed by manganese enhanced magnetic resonance imaging (MEMRI) and optokinetics (OKR). The gene therapy under development holds great therapeutic promise for a debilitating mitrochondrial disorder for which there is currently no cure.

OR072

Alterations of epithelial stem cell marker patterns in human diabetic corneas and effects of c-met gene therapy

Mehrnoosh Saghizadeh[1], Siavash Soleymani[1], Angel Harounian[1], Bhavik Bhakta[1,2], Sergey M. Troyanovsky[3], William J. Brunken[4], Graziella Pellegrini[5], Alexander V. Ljubimov[1,2]

[1]*Ophthalmology Research Laboratories, Cedars-Sinai Medical Center, Los Angeles, USA,* [2]*University of California Los Angeles, Los Angeles, USA,* [3]*Department of Dermatology, Northwestern University, Chicago, USA,* [4]*Departments of Ophthalmology and Cell Biology, State University of New York, Downstate Medical Center, Brooklyn, New York, USA,* [5]*Centre for Regenerative Medicine, University of Modena e Reggio Emilia, Modena, Modena, Italy*

Purpose: We have previously identified specific epithelial proteins with altered expression in human diabetic central corneas. Decreased hepatocyte growth factor receptor (c-met) and increased proteinases were functionally implicated in the changes of these proteins in diabetes. The present study examined whether limbal stem cell marker patterns were altered in diabetic corneas and whether c-met gene overexpression could normalize these patterns.

Methods: Cryostat sections of 28 ex vivo and 26 organ-cultured autopsy human normal and diabetic corneas were examined by immunohistochemistry using antibodies to putative limbal stem cell markers including ATP-binding cassette subfamily G member 2 (ABCG2), N-cadherin, ΔNp63α, tenascin-C, laminin γ3 chain, keratins (K) K15, K17, K19, β1 integrin, vimentin, frizzled 7, and fibronectin. Organ-cultured diabetic corneas were studied upon transduction with adenovirus harboring c-met gene.

Results: Immunostaining for ABCG2, N-cadherin, ΔNp63α, K15, K17, K19, and β1 integrin, was significantly decreased in the stem cell-harboring diabetic limbal basal epithelium either by intensity or the number of positive cells. Basement membrane components, laminin γ3 chain, and fibronectin (but not tenascin-C) also showed a significant reduction in the ex vivo diabetic limbus. c-Met gene transduction, which normalizes diabetic marker expression and epithelial wound healing, was accompanied by increased limbal epithelial staining for K17, K19, ΔNp63α, and a diabetic marker α3β1 integrin, compared to vector-transduced corneas.

Conclusions: The data suggest that limbal stem cell compartment is altered in long-term diabetes. Gene therapy, such as with c-met overexpression, could be able to restore normal function to diabetic corneal epithelial stem cells.

OR073

CD70 increases survival and avidity of cytotoxic T lymphocytes generated for adoptive cell immunotherapy strategies

Mohamad Hamieh[1], Pauline Maby[1], Jean-François Chatillon[2], Florence Bayeux[2], Anne-Bénédicte Duval Modeste[3], Emilie Fauquembergue[1], Aurélie Drouet[1], Philippe Musette[2,3], Thierry Frébourg[1], Jean-Baptiste Latouche[1]

[1]*Inserm U1079, Rouen University Hospital, Institute for Research and Innovation in Biomedicine (IRIB), North-West Cancéropôle, Rouen, France,* [2]*Inserm U905, Rouen University Hospital, Institute for Research and Innovation in Biomedicine (IRIB), Rouen, France,* [3]*Dermatology Department,Rouen University Hospital, Rouen, France*

Recent clinical trials have demonstrated the potential efficacy and safety of adoptive cell therapy using Artificial Antigen Presenting cells (AAPCs) to stimulate cytotoxic T lymphocytes (CTLs), which recognize antigen-derived peptides presented on HLA class I molecules. Our team has developed AAPCs expressing the HLA-A2.1 molecule, the three essential accessory molecules (CD54, CD58, CD80) and an A2.1-restricted peptide derived from MART-1, a melanoma-associated antigen. These AAPCs have been shown to efficiently stimulate CTLs from healthy donors and melanoma patients. To study the effect of CD70/CD27 signaling on anti-tumor CTLs, we constructed new AAPCs expressing CD70 with or without CD80 ($CD80^+$, $CD70^+$, $CD80^+CD70^+$ AAPCs). In this study, we compared CTLs obtained after co-culture of healthy donors' and melanoma patients' peripheral blood T cells with these three types of AAPCs. CD70 improved CTL expansion by increasing survival rate. Importantly, CD70 improved specific CTL cytotoxicity by increasing avidity and cytokine release (IFNγ, TNFα, IL-2). Moreover, CD70 increased specific CTL effector and central memory populations. In addition, CD70 did not lead to any regulatory T cell expansion. The beneficial effects of CD70 were associated with a more diverse Vβ subtype representation in the expanded specific CTL population. Altogether, our results show that CD70/CD27 interaction can replace, at least in part, CD80/CD28 signaling, and that combined expression of CD80 and CD70 results in a greater effect as compared to each molecule alone. These data strongly suggest that these novel CD80 and CD70 expressing AAPCs represent a promising tool to stimulate CTLs for adoptive cell therapy.

OR074

Co-expression of a Suicide Gene in CAR T cells Enables the Safe Targeting of CD44v6 for Leukemia and Myeloma Eradication

Monica Casucci[1], Benedetta Nicolis di Robilant[1], Laura Falcone[1], Barbara Camisa[1], Pietro Genovese[1], Bernhard Gentner[1], Claudio Bordignon[2], Barbara Savoldo[3], Fabio Ciceri[1], Luigi Naldini[1], Gianpietro Dotti[3], Chiara Bonini[1], Attilio Bondanza[1]

[1]*S. Raffaele Hospital, Milano, Italy,* [2]*Molmed SpA, Milano, Italy,* [3]*Baylor College of Medicine, Houston TX, USA*

Introduction: The recent successes of chimeric antigen receptor (CAR) T cells in B-cell tumors heralds a new frontier in cancer gene therapy. Targeted antigens are however limited to B-cell lineage markers. CD44v6 is widely expressed in cancer and plays a crucial role in chemoresistance and metastasis. Targeting CD44v6 with mAb is effective, but toxic due to non-restricted tumor expression.

Aim: To target CD44v6 safely by co-expressing a suicide gene in CAR T cells

Results: We constructed a novel, fully humanized CD44v6.CAR including a CD28 endodomain. The CAR was cloned in a LV carrying a bi-directional promoter for the co-expression of the suicide gene HSV-TK. After LV transduction, T cells acquired potent antitumor effects, leading to leukemia and myeloma eradication in xenografted NSG mice and could be effectively ablated by the administration of the prodrug GCV. Antitumor efficacy associated with in vivo expansion and persistence, which were dependent on previous activation with CD3/CD28-beads. Bead activation indeed uniquely enriched for central memory T cells expressing the self-renewal marker IL-7R. Since HSV-TK is immunogenic, we explored the humanized suicide gene iCasp9. CD44v6.CAR T cells co-expressing iCasp9 could be ablated as effectively as with HSV-TK, and much faster (>90% in 2 hrs).Suicidal CD44v6.CAR T cells spared CD44v6-negative HSC, but recognized CD44v6-positive mature monocytes. CD44v6 recognition could be completely switched off by suicide gene activation

Conclusions: Targeting CD44v6 with CAR T cells has the potential to cure leukemia and myeloma. Co-expressing a suicide gene is an effective tool for rescuing off-tumor recognition and toxicity.

OR075

Clinical-grade human myoblasts for cell therapy: a chromosomal stability analysis

Bisson Aurélie[1,2], Le Corre Stéphanie[1,3], Chambon Pascal[4,5], Joly-Helas Géraldine[4], Démoulins Laetitia[3], Jean Laetitia[1], Adriouch Sahil[1], Drouot Laurent[1], Giverne Camille[3], Roussel Françis[6], Jacquot Serge[1,3], Doucet Christelle[3], Michot Françis[7,8], Lamacz Marek[1], Frébourg Thierry[5,9], Flaman Jean-Michel[5,9], Boyer Olivier[1,3]

[1]Inserm, U905, Institute for Research and Innovation in Biomedicine, University of Rouen, Rouen, France, [2]Celogos, Paris, France, [3]Rouen University Hospital, Laboratory of biotherapy, Rouen, France, [4]Rouen University, Departement of cytogenetics, Rouen, France, [5]Inserm, U1079, Institute for Research and Innovation in Biomedicine, University of Rouen, Rouen, France, [6]Rouen University hospital, Departement of pathology, Rouen, France, [7]Inserm, U1073, Institute for Research and Innovation in Biomedicine, University of Rouen, Rouen, France, [8]Rouen University hospital, Departement of Digestive Surgery, Rouen, France, [9]Rouen University hospital, Departement of genetics, Rouen, France

The objective of this work was to investigate the phenotypic and safety features of human clinical-grade myoblasts.

Human myoblasts (n = 10) were prepared from a muscle biopsy under a clinical-grade protocol. After 3 cell culture amplifications, myoblasts were recovered at passage P3 (20–30 days). As expected, they expressed MyoD, Myf5, Myh3, desmin (RT-PCR) and CD56 (flow cytometry), and could differentiate *in vitro* into myotubes. Cellular senescence (growth arrest) occurred at day 47–88. Uncontrolled proliferation was never observed and *hTERT* expression (RT-PCR) was always negative. Karyotype on 30 metaphases followed by FISH revealed a low rate of chromosomal alterations (5/10 normal, 5/10 with 1-4/30 alterations). There was no recurrent anomaly, except chromosome 2 trisomy found in 2-3/10 P3 samples (1-2/30 metaphases and 2/100 FISH). These erratic abnormalities did not persist when prolonging cultures (P4, P8) and were never associated to a growth advantage *in vitro*. Molecular analysis of individual p53 transcripts did not reveal any tumorigenic mutation. CHG array (10 samples) and exome sequencing (1 sample) failed to detect copy number variations or accumulation of mutations, respectively. Clinical-grade myoblasts did not grow in soft agar nor *in vivo* after intramuscular injection to immuno-deficient NOD*scid* mice.

In conclusion, some erratic genomic alterations can be observed in a minority of clinical-grade myoblasts as an expected consequence of the cell culture process, but are never associated to a selective advantage. These results allow a prospective evaluation of the safety and efficacy of autologous myoblasts prepared with this technique for treating fecal incontinence (ClinicalTrials.gov NCT01523522).

OR076

Integrated bioprocesses for scalable production, purification and cryopreservation of iPSC-derived cardiomyocytes

Margarida Serra[1,2], Cláudia Correia[1,2], Marcos Sousa[1,2], Catarina Brito[1,2], Janne Jensen[3], Peter Bjorquist[3], Azra Fatima[4], Karsten Burkert[4], Jurgen Hescheler[4], Manuel Carrondo[2,5], Tomo Saric[4], Paula Alves[1,2]

[1]Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal, [2]Instituto de Biologia Experimental e Tecnológica, Oeiras, Portugal, [3]Cellartis AB, Cellectis Stem Cells, Gotemborg, Sweden, [4]Institute for Neurophysiology University of Cologne, Cologne, Germany, [5]Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Monte da Caparica, Portugal

Human pluripotent stem cells (hPSCs), with their ability for extensive proliferation and pluripotency, are renewable cellular resources with outstanding potential for Cell therapy and Drug Discovery applications. An imperative pre-requisite for the transition of hPSCs to these fields is the establishment of integrated bioprocesses capable to guarantee efficient cell expansion, differentiation, purification and cryopreservation of the desired hPSC-based product.

The laborious and time consuming 2D-cultures are difficult to control, present poor yields and often lack the required cell functionality. These characteristics can have severe consequences on robustness, reproducibility, scalability and relevance of the cell systems, hampering their possible application in Cell Therapy and Drug Discovery.

Our work has been focused on the development of 3D-culture systems for the production, purification and cryopreservation of clinically relevant hPSCs (embryonic and induced) and/or their derivatives (cardiomyocytes). Different 3D setting (cell aggregates, cells immobilized on microcarriers or microencapsulated in hydrogels) have been explored and combined with stirred tank bioreactor technology aiming at controlling hPSC's fate and culture outcome. For the cardiac differentiation step, different bioprocessing parameters have been evaluated and the results showed the importance of controlling pH, pO_2 and stirring profile to improve the final yields of functional cardiomyocytes. The incorporation of a perfusion system in the bioreactor is being

evaluated, providing a promising tool to facilitate and improve the purification of induced PSC-derived cardiomyocytes.

The knowledge of hPSC bioprocessing gained from our work provides important insights for the establishment of more robust production platforms, hopefully potentiating the implementation of novel hPSC-based therapies.

OR077

Positive molecular imaging of endogenous microRNA regulation in pathophysiological conditions in mice

S Ezzine[1,4], G Vassaux[2], B Pitard[3], B Barteau[3], P Midoux[1,4], C Pichon[1,4], P Baril[1,4]

[1]*CNRS UPR4301, Orléans, France,* [2]*Inserm U948, Nantes, France,* [3]*Inserm UMR915, Nantes, France,* [4]*Université d'Orléans, Orléans, France*

MicroRNAs (miRNAs) are small RNA regulatory molecules that play important roles in many physiological and pathophysiological processes. RNAi-based therapeutic reagents have tremendous potential in a wide variety of clinical applications but theirs exploitations are, in part, limited by the lack of sensitive *in vivo* imaging facilities. We recently developed a novel non-invasive imaging system based on the use of a homemade inducible expression vector designed to induce the expression of the luciferase reporter gene only when a specific miRNA is present in target cells. We validated our approach by visualizing in mice with high sensitivity, expression of the muscle-specific myomiRs-133, −206 and −1 and the liver-specific miRNA-122. *In vivo* bioluminescence experiments demonstrate robust, high specificity and relevant qualitative and quantitative data that correlate remarkably with the endogenous expression pattern of miRNA detected by conventional invasive method. We also found that this imaging system provides sharper insights and better trend analysis of miRNA regulation under pathophysiological conditions in mice. We demonstrate, indeed, that regulation of the miRNA-206 expression during the chronic phase of muscular atrophy is individual dependent, timely and finely regulated and more complex than information gathered through real time PCR performed at autopsy of several mice. Because of the unique nature of information generated, we are currently using this novel imaging system for image-guided gene therapy. Objective is to deliver pharmacological inhibitors at the expression peak of microRNAs using non-viral gene delivery vectors. We believe that our imaging system may have an impact in emerging field of personalized medicine.

OR078

Imaging of mesenchymal stem cell recruitment into the stroma of hepatic colon cancer metastases using the sodium iodide symporter (NIS)

Kerstin Knoop[1], Nathalie Schwenk[1], Patrick Dolp[1], Michael J Willhauck[1], Christoph Zischek[2], Guido Böning[3], Heidrun Zankl[3], Marcus Hacker[3], Burkhard Göke[1], Ernst Wagner[4], Peter J Nelson[2], Christine Spitzweg[1]

[1]*University Hospital of Munich, Department of Internal Medicine II, Munich, Germany,* [2]*University Hospital of Munich, Medical Policlinic, Clinical Biochemistry Group, Munich, Germany,* [3]*University Hospital of Munich, Department of Nuclear Medicine, Munich, Germany,* [4]*Ludwig-Maximilians-University, Department of Pharmacy, Center of Drug Research, Pharmaceutical Biotechnology, Munich, Germany*

The tumor-homing property of mesenchymal stem cells (MSC) has lead to their use as delivery vehicles for therapeutic genes. The application of the sodium iodide symporter (NIS) as therapy gene allows non-invasive imaging of MSC biodistribution and functional transgene expression by ^{123}I-scintigraphy or PET-imaging, as well as therapeutic application of ^{131}I or ^{188}Re. Based on the critical role of the chemokine RANTES/CCL5 secreted by MSCs in the course of tumor stroma recruitment and differentiation into cancer associated fibroblasts, use of the RANTES/CCL5 promoter allows tumor stroma-targeted expression of NIS after MSC-mediated delivery.

After establishment of the liver metastasis model by intrasplenic injection of the human colon carcinoma cell line LS174t in nude mice, we investigated the biodistribution and tumor recruitment of engineered MSCs expressing NIS driven by the RANTES/CCL5-promoter (RANTES-NIS-MSC). Five days after intrasplenic tumor cell injection RANTES-NIS-MSCs were injected via the tail vein three times followed by ^{123}I-scintigraphy and ^{124}I-PET imaging revealing active MSC recruitment and RANTES/CCL5-promoter activation in the multifocal liver lesions in 70% of mice as shown by tumor-selective iodide accumulation. Immunofluorescence analysis confirmed selective MSC accumulation in the stroma of liver metastases, while healthy liver tissue and non-target organs did not show MSC recruitment.

Taken together, our results convincingly demonstrate selective recruitment of MSCs stably expressing NIS driven by the RANTES/CCL5-promoter into liver metastases resulting in induction of tumor-specific iodide accumulation, opening the exciting prospect of NIS-mediated radionuclide therapy of metastatic cancers after MSC-mediated gene delivery.

OR079

Quantitative NMR 1H imaging and 31P spectroscopy evaluation of locoregional high venous pressure rAAV8-U7-ESE6-ESE8 exon-skipping therapy in the GRMD

JL Thibaud[1,2], C Wary[2], S Moullec[3], N Azzabou[2], C Le Guiner[4], L Garcia[5], M Montus[6], Y Chérel[7], Y Fromes[6], L Servais[2], S Roques[2], P Moullier[8], T Voit[2], PG Carlier[2]

[1]*ENVA, Maisons Alfort, France,* [2]*Institut of myology, Paris, France,* [3]*Boisbonnes center-ONIRIS, Nantes, France,* [4]*INSERM UMR 649, Nantes, France,* [5]*UPMC, UMR_S974, Paris, France,* [6]*Généthon, Evry, France,* [7]*INRA UMR703, Nantes, France,* [8]*INSERM, UMR1089, Nantes, France*

While replacement therapy of dystrophinopathies is most promosing, non-invasive quantitative tools are needed to demonstrate its potential benefits or side effects but also to determine the optimal protocol. Fifteen GRMD dogs were treated by unilateral high-pressure high-volume injection into the cephalic vein with a rAAV8-U7- ESE6-ESE8 solution. They were divided into 6 groups according to the dose of viral particles and the volume injected. Two dogs were injected with saline only. After high dose injection, some muscles displayed up to 80% of dystrophin positive fibers. Three months after injection, injected and non-injected arm were blindly evaluated with spectroscopy and NMR imaging. Indices of mock dogs were within the reference range of the GRMD population. Both spectroscopy and imaging indices showed differences between the two arms at the highest dose irrespective of volume injected and at the intermediate dose-high

volume combination. The putative identification of the treated arm proved systematically correct. Among the spectroscopy indices, Pi/PCr, PCr/ATP and PDE/(Pi+PCr) were the most sensitive and 31P NMR showed changes proportional to the number of AAV particles injected. With regard to imaging, the most relevant indices were: muscle heterogeneity in T2w images, the T1w/T2w signal ratio (SR), the T2w/PDw SR and the maximal relative signal enhancement after gadoteric acid injection. When high or intermediate doses were injected, indices actually decreased towards the normal range in both arms. This study demonstrated NMR ability to detect changes in dystrophic muscle structure and metabolism in response to exon-skipping therapy.

OR080

Changes in the progression of amyloid deposition in APP/PS mice after overexpression of different apoe isoforms through intraventricular injection of an adeno-associated virus serotype 4

Eloise Hudry[1], Jonathan Dashkoff[1], Allyson Lee[1], Robert M. Koffie[1], Tadafumi Hashimoto[1], Maria Scheel[2], Tara Spires – Jones[1], Brian J. Bacskai[1], Beverly Davidson[2], Bradley T Hyman[1]

[1]1. MassGeneral Institute for Neurodegenerative Disease, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA, USA, [2]2. Gene Transfer Vector Core, University of Iowa, Iowa, USA

The epsilon 4 allele of APOE is the most prevalent genetic risk factor associated with the sporadic form of Alzheimer's disease (AD), whereas inheritance of the rare APOE epsilon 2 allele reduces this risk by about a half. However, the mechanisms whereby apoE confers risk remain uncertain.

In order to decipher how the different apoE isoforms (apoEe2, -e3 and e4) impact the formation and stability of fibrillar amyloid plaques, AAV4 vectors coding for each of the isoforms were injected into the ventricle of 7 month-old APP/PS mice. Ependymal cells were transduced and stably secreted small amount of apoE into the interstitium. After 5 months, a significant increase of the number of plaques occurred with APOE4, whereas a relative decrease was observed with apoE2, despite the observation that only 1% of total apoE was derived from the transgene.

To distinguish between a change in the rate of new plaque formation and apoE-mediated clearance of plaques, we tracked populations of amyloid deposits at baseline and after exposure to apoE over a two-month interval using in vivo multiphoton imaging, thus allowing us to have a dynamic view of the progression of amyloidosis in a living animal. We observed that the kinetics of amyloid plaque deposition varies according to each isoform, so that APOE4 injected mice have a 38% increase in the density of senile plaques whereas mice treated with APOE2 present a 15% decrease compared to baseline after 2 months. Post-mortem analysis confirms these results and reveals the presence of human apoE proteins decorating amyloid plaques in the cortex, thus reflecting a large diffusion of the recombinant protein throughout the parenchyma and its focal accumulation where Ab peptides are deposited. Importantly, peri-plaque synapse loss was significantly exacerbated or attenuated around amyloid plaques when mice were injected with an AAV4-APOE4 or an AAV4-APOE2 respectively.

Overall, our data demonstrate that introduction of different apoE isoforms in the interstitial fluid is able to influence the progression of amyloid deposition and can modulate the extent of synapse loss.

OR081

Non viral gene transfer via *Sleeping beauty* transposon for Collagen VII delivery in human primary keratinocytes.

Maria Carmela Latella[1], Giandomenico Turchiano[1], Cocchiarella Fabienne[1], Zsuzsanna Izsvak[2], Zoltan Ivics[3], Fulvio Mavilio[1,4], Alessandra Recchia[1]

[1]Center for Regenerative Medicine, University of Modena and Reggio Emilia, Modena, Italy, [2]Max Delbruck Center for Molecular Medicine, Berlin, Germany, [3]Paul Ehrlich Institute, Langen, Germany, [4]Genethon, Evry, France

Autosomal recessive epidermolysis bullosa (RDEB) is a genetic skin adhesion defect caused by mutations in the type VII collagen gene (COL7A1). Although full-length type-VII collagen is successfully produced in human keratinocytes by retroviral vectors, genetic instability due to the large size (9kb) and the highly repeated nature of the gene sequence is a persistent problem. The *Sleeping-Beauty* (SB) transposon-based integration system can potentially overcome these issues by taking advantage of the hyperactive SB100X transposase in combination with the wild-type (pT2) transposon or the "sandwich" version (pSA) that showed robust transposition efficiency in human cells. We molecularly characterized the "sandwich" SB-mediated integrants in epithelial cell lines and in primary keratinocytes. Co-transfecting the transposase together with 10kb-transposon (pT2 or pSA) we observed up to 37% of transposition in HaCaT and in GABEB (generalized atrophic benign epidermolysis bullosa keratinocytes) cells with both transposons. Clonal analysis demonstrated that the transposition events occur with a minimal risk of rearrangements (<3%). Comparing the average copy number of pT2 and pSA transposons integrated in HaCaT cells we observed 1.5 and 5.2 copies/cell respectively. LM-PCR based bi-directional sequencing of the transposon-genome junctions shows genuine "cut and paste" activity of the SB hyperactive transposase.

Interestingly we observed up to 40% transposition efficiency of pT2 transposon carrying the functional COL7A1 cDNA in GABEB cells. Clonal analysis revealed no rearrangements for the COL7A1 transgene. Finally we will investigate the transposition efficiency and the restoration of type VII collagen expression and function in COL7A1-deficient keratinocytes from DEB patients.

OR082

In vivo mRNA introduction to central nervous system using polyplex nanomicelles

Keiji Itaka, Satoshi Uchida, Kazunori Kataoka

The University of Tokyo, Tokyo, Japan

Messenger RNA (mRNA) has high potentials to produce proteins or peptides for therapeutic purposes in a safe manner without any risks of random integration into genome. However, therapeutic approaches by in vivo mRNA introduction have been difficult due to the instability and immunogenicity of mRNA. In this study, a novel carrier, polyplex nanomicelle, was developed for in vivo mRNA introduction. This system is based on self-assembly of our original block copolymer composed of PEG and a functional polycation possessing the capacity of facile endosomal escape with minimal toxicity. Through its characteristic core-shell architecture with inner core of mRNA surrounded by PEG surface, the nanomicelle has strong potentials to function as an effective mRNA-containing carrier with high stability and

stealth property. By intrathecal injection of nanomicelle containing luciferase or acGFP-expressing mRNA, efficient protein expression was achieved chiefly in the meninges from brain to lumber lesion. The inflammatory responses after mRNA introduction were significantly decreased when using the nanomicelle, regardless of the modification of mRNA. By immunopathological analyses using transformants of HEK293 cells that stably expressed a specific TLR, the mRNA incorporated in nanomicelle was revealed to effectively avoid the recognition by TLRs localizing on the membrane of endosomes. Eventually, the nanomicelle containing secretory luciferase-expressing mRNA provided the protein secretion into the cerebrospinal fluid continuously for up to 3 days after the administration. Polyplex nanomicelle effectively holds mRNA in a stable manner and reduces the immune reactions, thus opening the door to a range of new therapeutic strategies using mRNA.

OR083

Enhancement of Zorro-LNA efficiency in Huntingtin gene silencing via combination with bisLNA

Eman M. Zaghloul[1], Pedro M.D. Moreno[1], Abdalla J. Mohamed[1], Jesper Wengel[2], Karin E. Lundin[1], C.I. Edvard Smith[1]

[1]*Karolinska Institutet, Stockholm, Sweden,* [2]*University of Southern Denmark, Odense, Denmark*

Huntingtin (HTT) is a ubiquitous protein that has a crucial function in embryonic development and other biological processes. Huntington disease (HD) is a severe neurodegenerative disorder caused by the increased number of CAG repeats leading to formation of a mutant HTT. We have recently applied the Zorro-LNA anti-gene technology for the potential treatment of HTT disease. Zorro-LNAs are oligonucleotide (ON)-based, Z-shaped constructs with the ability of strand invasion and specific binding to target sequences in duplex DNA. We designed Zorros for targeting several regions in the *HTT* gene. Some of the successful candidates were the pair of Zorros targeting a stretch in the polyA1 region. Combined transfections with 12.5 nM of each Zorro caused 50% down-regulation of *HTT* gene expression. Furthermore, we designed a bisLNA clamp type of ON to target a polypyrimidine/polypurine stretch just upstream to the polyA1 Zorro-target sites. Combinatorial treatment of bisLNA at 50 nM with the Zorros at 12.5 nM lead to a significant enhancement of HTT silencing reaching 80% reduction in *HTT*-mRNA. These effects were obtained after transfection of the ONs into Human Embryonic Kidney 293 cells for 2 days. In this study, we show for the first time, that bisLNA can be used to enhance the HTT silencing effect produced by Zorro-LNA, possibly providing a novel treatment strategy for HD.

OR084

Noninvasive imaging of pulmonary gene transfer: from pretty images to product development.

Peggy Richard-fiardo[1,3], Philippe Franken[1,3], Thierry Pourcher[1,2], Mathieu Mevel[4], Bruno Pitard[4], Georges Vassaux[1,3]

[1]*laboratoire TIRO, UMRE4320, CEA, Nice, France,* [2]*Centre Antoine Lacassagne, Nice, France,* [3]*Faculté de Medecine, université Nice Sophia antipolis, Nice, France,* [4]*Institut de Thorax, INSERM UMR915, IRT-UN, Nantes, France*

Pulmonary gene therapy has been proposed for the treatment of cystic fibrosis or cancer. However, clinical applications are limited by the delivery vectors. Viral delivery vectors are efficient but toxic. Non-viral, synthetic delivery vectors are non- toxic but inefficient. The classic route of product development in non-viral gene delivery vector has led to identification of transfection reagents very effective in vitro but completely inefficient in vivo. In this context, we have embarked, in close collaboration with chemists, in a screening programme to test directly new formulations in vivo, using an imaging of gene expression as an endpoint. For this, a new small-animal-dedicated SPECT/CT scanner is available in the TIRO laboratory (eXplore speCZT CT120, General Electric). Upon intratracheal administration of the gene delivery vector containing an expression plasmid encoding the Na/I symporter (NIS) and subsequent injection of a radiotracer that accumulates in NIS-expressing cells, the pattern of gene transfer is monitored using SPECT. First, we have validated the quantitative nature of the images produced by the scanner. We have now started our screen and our first round of tests has identified a non-viral formulation capable of transfecting the lungs in vivo that compares favourably in terms of gene transfer efficacy and inflammatory response with the gold standard in the field, the GL67A used in the current UK cystic fibrosis clinical trial. At the technological level, the relevance of an in vivo screen for the selection of non-viral gene delivery vectors to lung using SPECT/CT imaging has been validated.

OR085

In vivo expression of nuclear triggered PEGylated polyacridine peptide polyplexes

Kevin Rice, Sanjib Khargharia, Koby Kizzire, Mark Ericson, Nick Baumohover

University of Iowa, Iowa City, IA, USA

We have previously demonstrated the unique biological attributes of PEGylated polyacridine peptides such as $(Acr\text{-}Lys)_6$-Cys-PEG as carriers for DNA delivery in vivo, where acridine (Acr) is attached to the ε-amine of a Lys residues. This study established that a 1 μg pGL3 polyplex dose delivered in 50 μl in the tail of mice produce a high level of luciferase expression in the liver after applying HD-stimulation at times up to 1 hour post polyplex-dose.[1] Based on this lead structure, PEGylated polyacridine peptides were prepared to test the influence of increasing the number of Lys residues separating a fixed number of Acr residues. The results demonstrate a progressive increased circulation time, correlating with the production of maximal luciferase expression in liver when applying HD-stimulated at times up to 5 hours.[2] Comparison of polyacridine peptides with PEG linked to Cys or Pen with different linkages produced surprising and counter-intuitive results. Thiol-maleimide, Pen-Mal, and Pen-thiol linkages were all stable and nearly equivalent at mediating in vivo expression, however, PEG_{5kDa} with either a vinyl sulfone or thiol maleimide linkage, designed to be the most stable, were much less stable, producing lower expression due to rapid metabolism of DNA polyplexes. In conclusion, PEGylated polyacridine peptides are unique non-viral vectors that produce stable polyplexes in the circulation, that shift equilibrium to selectively trigger the release of plasmid DNA in the nucleus.

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2. Kizzire, K; et al. *Gene Therapy* (2012), Jul 12. doi: 10.1038/gt.2012.47

OR086

Transcription Activator-Like Effector Nucleases (TALENs) for targeted inactivation of Hepatitis B virus cccDNA

Kristie Bloom[1], Abdullah Ely[1], Claudio Mussolino[2], Toni Cathomen[2], Patrick Arbuthnot[1]

[1]*Antiviral Gene Therapy Research Unit, School of Pathology, Health Sciences Faculty, University of the Witwatersrand, Johannesburg, South Africa,* [2]*Laboratory of Cell and Gene Therapy, Center for Chronic Immunodeficiency, University Medical Center Freiburg, Freiburg, Germany*

Hepatitis B virus (HBV) continues to be a major global public health burden with over 350 million people chronically infected. Antiviral treatment is only partially effective, and complicating cirrhosis and hepatocellular carcinoma result in approximately 600,000 deaths annually. Treatment failure is largely as a result of the stability of the episomal viral cccDNA and difficulty with eliminating this HBV replication intermediate. This cccDNA minichromosome serves as a reservoir of HBV DNA and is capable of re-establishing viral replication following withdrawal of treatment. Currently, anti-HBV therapeutics target post-transcriptional viral processes without affecting cccDNA. Engineered DNA sequence-specific nucleases, such as Transcription Activator-Like Effector Nucleases (TALENs), therefore have useful therapeutic potential for inactivating HBV cccDNA. To explore the utility of TALENs against HBV, we have generated a panel of five sequence-specific TALEN dimers. Each TALEN dimer was designed to interact with two 18 bp targets separated by a 13 bp spacer within the *polymerase*, *core*, *surface* and *X* regions of HBV cccDNA. In vitro co-transfection with an HBV replication-competent and TALEN-expressing plasmids inhibited secretion of HBV surface antigen by up to 80%. A T7E1 or CELI endonuclease assay was employed to verify that cleavage and mutagenic non-homologous end joining occurred specifically at the intended HBV targets. Based on a sensitive MTT toxicity assay, the TALENs did not have any detectable adverse effects on cultured liver-derived Huh7 cells. These results indicate that TALENs designed to disable cccDNA have promising therapeutic potential.

OR087

ProSavin® a gene therapy approach for the treatment of Parkinson's Disease: a Phase I Clinical Trial Update

Stephane Palfi[3,1], Collin Watts[4], Gurruchaga Jean Marc[3], Buttery Peter[4], Ralph Scott[2], Deeley Sarah[2], Lepetit Helene[3], Miskin James[2], Iwamuro Hiro[3], Lavisse Sonia[1], Kas Aurelie[1], Ramelli Anne Laure[3], Tani Naoki[3], Fenelon Gilles[3], Brugie're Pierre[3], Naylor Stuart[2], Hantraye Philippe[1], Remy Philippe[1,3], Barker Roger[4], Mitrophanous Kyriacos[2]

[1]*CEA/I2BM MirCen, Fontenay aux Roses, France,* [2]*Oxford Biomedica Ltd, Oxford, UK,* [3]*Henri Mondor Hospital APHP, Creteil, France,* [4]*Cambridge centre for Brain repair, Cambridge, UK*

Parkinson s disease (PD) results in the damage of dopamine neurons and loss of motor control. The dopamine precursor L-Dopa provide the primary standard of care and demonstrate good therapeutic benefit in the early stages of disease. However, their long term use leads to severe motor side effects that are at least partially caused by the fluctuating nature of dopaminergic stimulation. Therefore, there is a need to develop a therapy that allows the continuous and local supply of dopamine.

ProSavin® is a gene therapy product that utilises a lentiviral vector to transfer three genes that are critical for dopamine biosynthesis. ProSavin® converts non-dopaminergic cells of the striatum, that do not degenerate in the disease, to dopamine-producing cells with the aim of restoring the dopamine loss and reversing the Parkinsonian symptoms.

Clinical evaluation of the safety and efficacy of ProSavin® in mid to late stage PD patients is currently ongoing. In the study fifteen patients have received ProSavin® in three dose cohorts. Nine patients have reached 12 months follow up. ProSavin® has been safe and well tolerated at all doses evaluated. There have been no serious adverse events related to Prosavin® or the administration procedure and no severe immunlogical responses. In terms of efficacy, an improvement in the primary endpoint, UPDRS Part III, has been observed in all cohorts relative to their baseline scores. An improvement has been maintained out up to 3 years for the earliest cohort.. An update on the trial and future plans for ProSavin® will be presented.

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Poster Presentations

P001

Differentiation of human adipose tissue derived mesenchymal stromal cells to the endothelial lineage in a glioblastoma tumor: implications for cell-based therapies

Maria Alieva[1], Juli R. Bago[0,2], Elisabet Aguilar[1], Nuria Rubio[1], Jeronimo Blanco[1]

[1]*Cardiovascular Research Center, CSIC-ICCC, Barcelona, Spain,* [2]*CIBER-BBN, Barcelona, Spain*

Lately human adipose tissue mesenchymal stromal cells (hAMSCs) have emerged as cellular vehicles for therapy of solid tumors, due to their ease of isolation and manipulation, and wound/tumor homing capacity. HAMSCs have been successfully used in suicide gene therapy, employing the prodrug activating system based on Herpes simplex virus type I thymidine kinase (HSV-TK)/ganciclovir (GCV). In the current study we demonstrate an effective model of glioblastoma therapy based on the use of genetically modified hAMSCs and in vivo monitoring of tumor and therapeutic cells. In addition, the bioluminescence imaging platform (BLI) allowed us to observe the behavior and differentiation state of therapeutic hAMSCs, offering insight into the therapeutic mechanism.

Continuous monitoring of tumor size by BLI showed that hAMSCs/GCV treatment resulted in a significant reduction (99.8% vs. control) of tumor cell number and prolongation of survival time. In addition, the combination of BLI and confocal microscopy analysis of therapeutic cells suggests that efficient tumor eradication results from hAMSCs homing to tumor vessels, where they differentiate to endothelial cell lineage, intensifying their cytotoxic effect by destroying tumor vasculature and negating nutrient supply. Close association between hAMSCs and gliomas stem cells integrated in the tumor vascular system seems to be essential for an effective tumor reduction. Besides, hAMSCs endothelial differentiation inhibition resulted in an unsuccessful therapeutic effect compared to the one obtained with normal hAMSC (64% vs 6% respectively).

We propose that genetically modified hAMSCs should be useful vehicles to deliver localized therapy to glioblastoma surgical borders following tumor resection.

P002

Development of gene activated matrices for tissue regeneration in osteoarthritis

Martina Anton[1], Maria Hillreiner[1], Youlia Kostova[1], Christian Koch[1], David Eglin[2], Cedric Sapet[3], Olivier Zelphati[3], Pascal Borget[4], Guy Daculsi[5,4], Mauro Alini[2], Christian Plank[1]

[1]*Institute of Experimental Oncology and Therapy Research, Klinikum rechts der Isar der TUM, Munich, Germany,* [2]*AO Research Institute Davos, Davos, Switzerland,* [3]*OZ Biosciences, Marseille, France,* [4]*Biomatlante SA, Vigneux de Bretagne, France,* [5]*INSERM Université de Nantes UMR U791 Faculté de Chirurgie Dentaire, Nantes, France*

Osteoarthritis (OA) is a degenerative disease of the joints that comprises cartilage degeneration, degeneration of the underlying bone and inflammation. The etiology is largely unknown, thus treatment is mainly symptomatic ranging from pain reduction and anti-inflammatory drugs to ultimately joint replacement.

Our aim is to develop a gene activated matrix (GAM) concept that allows for spatiotemporal control of growth factor expression involved in cartilage and bone regeneration. GAMs are biomaterial scaffolds comprising gene vectors. Cells growing on or into the matrix will become transfected/transduced by the immobilized or released vector and will consequently express the growth factor genes, resulting in local autocrine and paracrine stimulation of a desired differentiation process.

Mesenchymal stem cells (MSC) are multipotent cells that can differentiate into mesenchyme-derived cell types like osteoblasts, chondrocytes, and adipocytes. The EU funded project GAMBA aims at combing MSC with GAM that allow for spatiotemporal control of gene expression.

Here we show preliminary results on spatial control using a resorbable bone substitute material (MBCP+TM) and a thermoreversible hyaluronan based hydrogel as scaffolds, seeded with different non-viral vectors coding for reporter genes and mesenchymal stem cells. Temporal control of reporter gene expression is achieved using the Tet-on system in 2D cultures with non-viral as well as adenoviral vectors coding for reporter genes and growth factor genes such as BMP-2 and TGFbeta and the anti-inflammatory gene vIL-10.

Our results indicate that the concept of GAM might be feasible for regulated growth factor expression upon command and demand.

P003

Epicardially delivered adipose stromal cell sheets in dilated cardiomyopathy

Hadhami Hamdi[1], Solene Boitard[2], Valérie Planat-Benard[3], Julia Pouly[1,6], Hany Nematalla[1,6], Pierre Joanne[2], Marie-Cécile Perrier[4], Valérie Bellamy[1], Louis Casteilla[3], Zhenlin Li[5], Mathias Mericskay[5], Philippe Menasché[1,6], Onnik Agbulut[2]

[1]*Inserm U633, Paris, France,* [2]*Univ Paris Diderot, Sorbonne Paris Cité, Paris, France,* [3]*UMR 5273 UPS, CNRS, EFS, Inserm U1031, STROMALab, Toulouse, France,* [4]*INSERM U970, Paris, France,* [5]*UPMC Sorbonne Univ, Paris, France,* [6]*Hôpital Européen Georges-Pompidou, Paris, France*

Introduction: Few studies have assessed the effects of cell therapy in nonischemic cardiomyopathies which, however, contribute to a large number of cardiac failures. Assuming that such conditions are best suited for a global delivery of cells, we

assessed the effects of epicardially delivered adipose tissue-derived stroma cell (ADSC) sheets in a mouse model of dilated cardiomyopathy.

Methods: The function of the left ventricle (LV) was assessed by echocardiography. Twenty-nine mice were then randomly allocated to control (n=9, non-transgenic), sham (n=10, transgenic untreated) and treated (n=10) groups. In the treated group, 3×10^6 allogeneic ADSCs were cultured for 2 days onto temperature-sensitive polymers and the generated sheets were then grafted over the surface of the LV through a thoracotomy. Function, engraftment, and fibrosis were blindly assessed after 3 weeks, at which time PCR were also performed to screen a wide array of cardiac genes. All animals were ciclosporine immunosuppressed.

Results: In the untreated group, shortening fraction declined compared with baseline, whereas the sheet application resulted in its stabilization. This correlated with a lesser degree of LV remodeling as LV end-diastolic and end-systolic diameters did not differ from baseline values whereas they significantly increased in the untreated group. Many GFP^+ cells were identified in the epicardial construct and in the myocardium. Treated animals also displayed a reduced expression of the stress-induced genes. These protective effects were also accompanied by a reduction of myocardial fibrosis.

Conclusions: These results strongly suggest the functional relevance of epicardially-delivered cell-seeded biomaterials to nonischemic heart failure.

P004

Relevance of Fanca gene in mesenchymal stromal cells transformation

Rosa Yañez[1], Alberto Oviedo[1], Enrique Samper[2], Juan C Estrada[2], María Fernández[1], Juan A Bueren[1], María L Lamana[1]

[1]Hematopoiesis and Gene Therapy Division. CIEMAT/CIBERER, Madrid, Spain, [2]National Centre for Cardiovascular Research Foundation, Madrid, Spain

Fanconi anemia (FA) is a rare genetic disease associated with cancer predisposition. Because the in vitro culture of mesenchymal stromal cells (MSCs) constitute a good model for studying the molecular events involved in cancer, we have investigated the role of the FA pathway in these molecular events using adipose tissue-derived mMSCs from Fanca-/- and WT mice. Both MSC types showed similar growth kinetics, cell cycle status, immunophenotype and differentiation capacity. Replicative senescence was detected by SA-β-galactosidase staining mainly at passage P3-4 in both Fanca-/- and WT and MSCs, associated with evident morphological changes and a low telomerase activity. In spite of these similarities, Fanca-/- mMSCs had a significantly reduced clonogenic capacity and were highly sensitive to mitomycin C. Anchorage-independent colonies were observed in either type of mMSCs, although an increased number of colonies was apparent in Fanca-/- mMSCs. Additionally, cytogenetic analyses of both MSC types showed the presence of a mixed population of diploid and aneuploid cells at passages P3-P5, while at P20-P50 most MSCs were highly aneuploid. The frequency of chromosomal aberrations was, however, significantly higher in Fanca-/- mMSCs. In spite of these observations, no tumors were generated when either type of mMSCs was inoculated in nude or NOD/SCID mice. Our results show that Fanca deficiency in mMSCs does not result in a significant increased risk of tumorogenesis in vivo. Additionally, these results offer further evidence showing the reduced transformation risk of in vitro expanded MSCs, even in situations of a DNA repair deficiency such as the FA pathway.

P005

A comparison between grafting efficiencies of myoblasts and embryonic stem cells in a Mouse model of dilated cardiomyopathy

Cyril Catelain[1], Stéphanie Riveron[1], Aurélie Papadopoulos[1], Nathalie Mougenot[2], Adeline Jacquet[2], Karine Vauchez[1], Erica Yada[1], Michel Pucéat[3], Marc Fiszman[1], Gillian Butler-Browne[1], Gisèle Bonne[1,4], Jean-Thomas Vilquin[1]

[1]UPMC UM76, INSERM U974, AIM, CNRS UMR7215, IFR 14 CMV, AP-HP, Paris, France, [2]INSERM UMRS 956, UPMC, PECMV IFR 14, AP-HP, Paris, France, [3]INSERM UMR633, Evry, France, [4]UF Cardiogénétique et Myogénétique, Service de Biochimie métabolique, Paris, France

The therapeutic perspective represented by cell transplantation has been less explored in the setting of nonischemic dilated cardiomyopathy (DCM), than in the context of post-ischemic heart failure. We compared the integration and functional efficacy of two categories of progenitors, Myoblasts (Mb) and cardiac-committed embryonic stem cells (ESC), upon transplantation into the myocardium of $Lmna^{H222P/H222P}$ mice, a faithful genetic mouse model of laminopathy exhibiting a progressive and lethal DCM. Animals of matched ages and left ventricular fractional shortenings (LVFS) were randomized into three groups receiving medium alone, or 3.10^5 ESC or D7LNB1 myogenic cells at four sites on the anterior-lateral wall of the left ventricle. All animals were immunosuppressed using Tacrolimus. LVFS showed a stabilisation in Mb-transplanted mice two months after transplantation, when compared to ESC-transplanted and control-injected animals. Engrafted, differentiated Mb were consistently detected in myocardia of mice receiving Mb, while no cells were detected in hearts of mice receiving cardiac-committed ESC, excepted in two cases where teratoma formation was observed. These results suggest that the functional benefits of Mb transplantation might extend to nonischemic DCM while committed ESC failed to integrate in the myocardial niche of this specific model of non-ischemic DCM. The contrasting differences between the engraftment efficiency of ESC and Mb confirm the use of Mb as a standard tool for engraftment in experimental models of heart failure and it highlights the need for newinstruments to improve ESC integration and survival in dilated cardiac tissue.

P006

Cross-talk between human adipose-derived stem cells and natural killer cells modulate NK cell-mediated cytotoxicity

Olga DelaRosa[1], Beatriz Sánchez-Correa[2], Sara Morgado[2], Cristina Ramírez[1], Borja DelRío[1], Eleuterio Lombardo[1], Wilfried Dalemans[1], Raquel Tarazona[2], Javier G Casado[2,3]

[1]Tigenix S.A. R&D, Tres Cantos, Madrid, Spain, [2]Immunology Unit, Department of Physiology, University of Extremadura, Cáceres, Spain, [3]Stem Cell Therapy, Minimally Invasive Surgery Centre, Cáceres, Spain

The interest in the use of Human adipose-derived stem cells (ASCs) for treating inflammatory mediated diseases is growing. Although allogeneic ASCs are low immunogenic, these cells need

to cross the NK innate barrier to exert its immunomodulatory potential before being cleared by the host. We have studied the impact of the presence of ASCs over the phenotype and function of NK cells and whether NK cells are able to modify ASCs. In this work, the PBMCs cultured in the presence of ASCs displayed a significantly reduced expression of the activating receptors CD69, NKG2D and NCRs. With respect to the MSC phenotype, the ligands for activating/inhibitory receptors showed an absence or a very low expression of CD112, CD155 and MICA/B on ASCs. The ULBPs and NCR ligands were low or absent both in ASCs and BM-MSCs cells. When NK cells and ASCs were co-cultured, upregulation of HLA class I and II was found on ASCs both in contact and in transwell conditions. On the contrary, no significant change of HLA II expression was found when BM-MSCs were co-cultured with NK cells. The analysis of the culture supernatants demonstrated a IFN-gamma, TNF-alpha and TNF-beta release after NK-ASCs co-culture. In conclusion, the down-regulation of NK cell receptor expression upon contact with ASCs indicates that allogeneic ASCs impair, at least during a short period of time, the NK cell mediated cytotoxicity. Additionally, the lower expression of ligands for NK activating receptors on ASCs could be translated to their increased resistance to NK-mediated recognition.

P007

Influence of umbilical cord mesenchymal stem cells culturing *in vitro* on their characteristics

OG Deryabina[1,2], OO Maslova[1], NS Shuvalova[1,2], OM Sukhorada[1,2], SP Shpylova[1,2], LI Lykhacheva[1,2], SY Rymar[1], OK Toporova[1,2], VM Kyryk[1], MV Makarenko[3], DO Govseiev[3], VA Kordium[1,2]

[1]Institute of Genetic and Regenerative Medicine, Kyiv, Ukraine, [2]Institute of Molecular Biology and Genetics, Kyiv, Ukraine, [3]5th Kyiv Municipal maternity hospital, Kyiv, Ukraine

UC MSC are very perspective for cell therapy not only due to differentiation potential into different cell types, but also because they fit all ethical demands, and their isolation isn't connected with traumatic procedures for donor.

The aim of the present work was studying the stability of Wharton jelly MSC main characteristics during their cultivation *ex vivo.*

Source for MSC were human umbilical cords from term deliveries. Cells isolation and cultivation was carried out in stadard conditions. Morphological characterization was performed using confocal microscopy with fluorescent nuclei dye. Surface markers were determined in FACSAria with specific for MSC MbA, inner markers were deteced in RT-PCR. MSC differentiation into adipocytes and chondrocytes was identified by specific staining.

UC MSC *ex vivo* cultivation led to changes practically of all initial charactertics that increased with each next passage. Cell morphology altered from typical spindle-shaped form to cells with heterogeneous vacuolated cytoplasma and multilayer formations. Spontaneous differentiation into adipocytes and chondrocytes was confirmed with specific staining already at the 3rd passage.

Surface markers monitoring up to 6th passage indicated maintance of expression level of some of them and gradual intensity decrease of others.

Alterations recognized with immune system appeared at the 3rd passage. Adult blood mononuclears began to destroy MSC while at the level of 0–2nd passages such interaction was absent.

The data obtained testify the preservation of initial properties during 2 passages and the necessity of their regular control while UC MSC cultivating *ex vivo,* which is especially important for clinical cell application.

P008

Acquisition of biliary epithelial cells from hepatic progenitors

Noushin Dianat[1], Hélène Dubois Pot Shneider[0,2], Aurélien Raveux[1], Anne Dubart Kupperschmitt[1], Anne Corlu[0,2], Anne Weber[1]

[1]Inserm U972, Villejuif, France, [2]Inserm U991, Rennes, France

Cell therapy represents an alternative to liver transplantation in some cases like severe metabolic disorders. However, the shortage of organ donors implies the need to a new source of cells capable of differentiation into liver cells like pluripotent stem cells.

During embryonic development, the liver bud is composed of bipotent hepatic progenitor cells (hepatoblasts) that differentiate into mature and fetal hepatocytes and also into mature and fetal biliary epithelial cells (cholangiocytes). Cholangiocytes are responsible for various cholestasis as Alagille syndrome or certain primitive biliary cirrhosis. HepaRG cells represent a cell line with adult liver cell phenotype capable of being reverted into hepatoblasts. The purpose of this study was to evaluate the ability of HepaRG progenitors, and also hepatic progenitors derived from human embryonic stem cells (hESc) to differentiate into cholangiocytes.

For the differentiation of HepaRG cells into cholangiocytes, several factors and cytokines was tested. Some promote biliary differentiation with a significant induction of GGT, CK14 and Notch2 markers. The differentiation of hES cells was performed in two steps: acquisition of hepatoblasts under conditions already established in the laboratory, and then induction of the biliary pathway.

After testing various cytokines and mediums, we developed a protocol which induces the proliferation of cells co-expressing HNF6, CK19 and CK7. This expression is correlated with the disappearance of HNF4α and AFP. Various markers such as Notch,

CFTR and GGT was studied on hES-derived cholangiocytes. The 3D functionality tests made show that both the HepaRG- and ES-derived cholangiocytes are capable of forming the tubules.

P009

Consequences of in vivo inhibition of Cholesterol 24-hydroxylase function: insight into the mechanism of CYP46A1-linked genetic susceptibility to Alzheimer Disease

Fathia djelti[1], Eloise Hudry[1], jennifer Varin[1], Sophie Aycierex[0,2], Ivan Bieche[1], Nicolas Auzeuil[0,2], Patrick Aubourg[1], Nathalie Cartier[1]

[1]INSERM U745, Paris, France, [2]EA4463, Paris, France

High cerebral cholesterol content was linked to b amyloid pathology in Alzheimer disease. The major mechanism of excess cholesterol elimination in brain is its conversion into the 24-hydroxy-cholesterol catalyzed by cholesterol-24-hydroxylase encoded by CYP46A1. In late onset Alzheimer disease (LOAD), the neuronal CYP46A1 expression was decreased and different polymorphisms was associated with an increase of b amyloid

pathology and a TAU phosphorylation. But the link between CYP46A1 inhibition and the Alzheimer disease are not well understood. Here, we evaluated the consequence of *in vivo* neuronal cholesterol-24-hydroxylase inhibition in C57Bl/6 mice. The AAV5 vector encoding a CYP46A1-specific shRNA injection leads to a significant inhibition of CYP46A1 in hippocampal CA3a layer. This inhibition induces a neuronal loss 4 weeks after injection endings an atrophy of hippocampus. Just before the neuronal loss, an increase of cholesterol and APP content in membrane-raft-purified fraction is observed and is accompanied with the presence of endosomes and b CTF fragment of APP. Then, an endoplasmic reticulum stress, characterized by XBP1 and CHOP expression leadings to a TAU (AT180) phosphorylation, an astrocytes recruitment and caspase 9 and 3 activation. These effects were accelerated in APP23 mice models.

Together, our data suggest that the loss of function of CYP46A1 in hippocampus of C57Bl/6 mice could be responsible for severe impairment of cholesterol metabolism and neuronal toxicity. It gives insight into the predisposition to LOAD conferred by CYP46A1 gene.

P010

Allogenic Bone marrow derived Fibroblasts in the treament of Dystrophic Epidermolysis Bullosa: A pilot study

Mohamed El-Darooty, Hala Gabr, Marwa Fawzy, Zenab El-Maadawi

Faculty of medicine, Cairo University, Cairo, Egypt

Background: Patients with Dystrophic Epidermolysis Bullosa (DEB) have mutations in the type VII collagen gene, encoding a large collagenous protein that is the predominant component at the dermo-epidermal junction. Type VII collagen is synthesized by keratinocytes and fibroblasts. Based on the ability of bone marrow non-hematopoeitic stem cells (NHBMSC) to develop into fibroblasts that can induce collagen VII formation, we decided to investigate the use of NHBMSC in the treatment of RDEB. ***Methods***: This is a pilot study which included fourteen patients with recessive DEB (RDEB). Bone marrow was harvested from one of the parents, cultured to separate fibroblasts (CFU-F). CFU-F were separated and tagged using iron oxide particles and injected intravenously in patients in a dose of 2×10^6/ Kg. Patients were followed clinically for 12 months. Skin biopsies were done to evaluate collagen expression and to track injected cells using Prussian blue staining. ***Results***: The difference in the number of new blisters before and after treatment was statistically significant ($P=0.003$ and 0.004 respectively). The difference in the rate of healing of blisters before and after treatment was statistically significant ($P<0.001$). No major side effects were reported during the one year follow-up period; none of the patients showed any signs of rejection. ***Conclusion:*** Our findings highlight the safety and short term efficacy of allogenic bone marrow derived fibroblasts in the treatment of RDEB.

Keywords: epidermolysis bullosa, collagen, fibroblasts

P011

Mesenchymal stromal cells enhance engraftment of limited numbers of hematopoietic progenitors in syngenic transplants

María Fernández, María L Lamana, Paula Busto, Rebeca Sánchez-Domínguez, Antonio Rubio, Israel Ormán, Jose C Segovia, Juan A Bueren, Rosa Yañez

Hematopoiesis and Gene Therapy Division. CIEMAT/CIBERER, Madrid, Spain

Co-transplantation of human mesenchymal stromal cells (hMSC) has been reported to reduce the risk of graft failure in severe aplastic anemia, and in haploidentical or unrelated donor hematopoietic stem cell transplants (HSCT). Additionally, hMSCs enhance engraftment of NOD/SCID mice with human hematopoietic progenitor cells. However, non conclusive results have been obtained regarding the role of hMSC in the engraftment of patients transplanted with autologous HSCs. In this work we investigate whether MSCs enhance the hematopoietic engraftment of a limited number of HSCs using a mouse model of autologous transplantation with reduced conditioning. Lineage negative, Sca-1 positive, c-kit positive cells (LSK cells) from P3D2F1 (Ly5.1/Ly5.2) mice were purified and transplanted, either with or without mMSCs, into syngenic B6D2F1 (Ly5.2/Ly5.2) irradiated with a non myeloablative dose of 5Gy. Our results showed that the co-infusion of the MSCs significantly improved both the kinetics and the level of hematopoietic engraftment of donor LSK cells. This improvement was dependent both on the MSC-dose and on the co-infusion of MSCs together with LSK cells. After serial HSCT, improved levels of donor hematopoietic engraftment were also observed in secondary and tertiary recipients. These results suggest that MSCs may have a relevant role to prevent engraftment failure after the infusion of limited numbers of autologous HSCs, including gene-modified HSCs.

P012

Absorbable surgical sutures coated with mesenchymal stem cells improve wound healing through the release of different collagen types

Javier G Casado[1], Inmaculada Jorge[2], Mariano Ortega-Muñoz[2], Guadalupe Gómez-Mauricio[1], Verónica Álvarez[1], Antonio Bernad[3], Jesús Vázquez[2], Francisco Miguel Sanchez-Margallo[1]

[1]Stem Cell Therapy Unit, Minimally Invasive Surgery Centre Jesus Uson, Cáceres, Spain, [2]Cardiovascular Proteomics laboratory, Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain, [3]Translational Platform, Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain

The mesenchymal stem cells (MSCs) have demonstrated their efficacy for the repairment or regeneration of damaged tissues. These MSCs in combination with surgical sutures *could be somehow considered* as a scaffold to hold cells for clinical applications providing a biomechanical support to avoid the spreading of MSCs. In order to improve the strength adhesion of MSCs, absorbable sutures were pre-treated with gelatine, poly-L-Lysine and chemically modified with NaOH. The MSCs adhesion rates were quantified by CCK8 assays. The phenotypic analysis of MSCs cultured onto pre-treated sutures was performed by FACS. The secreted protein profile from MSCs was studied by proteomics strategies. The in vivo collagen secretion into the sutured tissues was quantified by hydroxyproline assay. The present study demonstrates that suture pre-treatments increase the MSCs adhesion to multifilament sutures without conferring any phenotypic modification. In particular, poly-L-Lysine pretreatment significantly improved the resistance to suture passages and MSCs adhesion strength. The microscopic observations demonstrated that, MSCs keep adhered and surrounding

the poly-Lysine treated sutures during the surgical procedure. The experimental in vivo evaluation demonstrated that MSCs-coated sutures provided an enhanced collagen deposition after suturing. Finally, the proteomics analysis from in vitro cultured MSCs allowed us to identify the different secreted collagen types. In conclusion, the implanting of MSCs in tissue via suturing may have beneficial effects on the wound healing, closure properties and tissue regeneration in the shortest time possible and may be applicable for those patients that tend to have difficulty healing.

P013

In vitro characterization of HNSCS derived from different neuraxis regions

Daniela Celeste Profico[1], Giada Sgaravizzi[1], Emilio Ciusani[3], Massimo Projetti-Pensi[1], Gianmarco Muzi[1], Lidia de Filippis[4], Laura Rota Nodari[4], Maurizio Gelati[1,2], Angelo Luigi Vescovi[2,4]

[1]*Azienda Ospedaliera Santa Maria, Terni, Italy,* [2]*Casa Sollievo della Sofferenza, San Giovanni Rotondo (foggia), Italy,* [3]*Istituto Neurologico "C.Besta", Milano, Italy,* [4]*Università Bicocca, Milano, Italy*

Soon after neural induction, neural stem cells acquire distinct identities and different fates depending on their position along the rostro-caudal (R-C) and dorso-ventral (D-V) axis of the neural tube. Although a large amount of literature about the homeobox genes in vivo expression pattern has been published little is know about the positional identity of in vitro cultured human neural stem cells. The aim of this study was to determine human Neural Stem Cell (hNSC) lines ability to maintain in vitro the same in vivo gene expression pattern and how this could influence their ability to grow, differentiate and their response to environmental stimuli (plasticity). hNSC lines have been obtained from 8–12 week human fetus according to an ethical committee approved procedure.

The identification of different anatomical source of hNSC lines has been pursued trough RT-PCR analysis of transcription factors encoding genes involved in the neural tube patterning. The presumptive positional identity has been put in relation with the ability to replicate (growth curve), to differentiate and with the amount of clones (neurosphere assay).

So far, data obtained show that cultured hNSCs maintain in vitro specific regional identity in terms of gene expression. Comparison of lines of different regional origin shows that lines from the anterior-ventral forebrain have different in vitro behaviour.

Particularly they form the larger number of clones and apparently grow faster than the others.

In conclusion, in vitro expansion seems to have no influence on gene expression pattern in hNSCs, furthermore cell lines seem to maintain their stemness after long term in vitro expansion.

P014

Modification of the T cell genome using TALENs for adoptive cell immunotherapies

Agnès Gouble, Stéphanie Grosse, Elsa Raban, Julianne Smith, Andrew Scharenberg

Cellectis Therapeutics, Paris, France

Reactivation of human cytomegalovirus (CMV) infection remains one of the most common infectious complications in patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT). This has led to the development of clinical protocols in which CMV-specific T cell clones are isolated from the transplant donor and transferred to the patient after transplantation. However, the patients at greatest risk of CMV infection are those with ongoing immune-deficiency, often mediated by therapeutic immune suppression to prevent graft-versus-host disease (GvHD) after HSCT. Since first-line treatment for GvHD is glucocorticoid therapy, these patients cannot be effectively treated with classic CMV-specific adoptive cellular therapies.To address this problem we have developed Transcription Activator-Like Effector Nucleases (TALENs) specific for the glucocorticoid receptor (GR) gene. TALENs are a novel class of sequence-specific nucleases created by the fusion of transcription activator-like effectors (TALEs) to the catalytic domain of an endonuclease. TALEN-mediated cleavage at a predetermined site in the GR gene, followed by repair of the break via an error-prone repair pathway, can result in the inactivation of the GR gene.We have produced TALENs specifically directed against the GR human gene and tested their efficacy in primary T cells. High throughput sequencing revealed up to 30% of targeted mutagenesis in the GR gene without apparent cell toxicity. In addition, following dexamethasone treatment, we were able to specifically select GR KO cells in a TALEN-transfected T cell population. This same approach will be applied to CMV-selected T cells in order to render them resistant to glucocorticoid treatment.

P015

Strategy for cell therapy evaluation in GRMD dog (model of Duchenne muscular dystrophy): quantitative proteomic analysis of skeletal muscle

Laetitia GUEVEL[1,2], Blandine GUEVEL[3,4], Florence ROBRIQUET[1,2], Mélanie LAGARRIGUE[3,4], Laurence DUBREIL[1], Thibaut LARCHER[1], Jack-Yves DESCHAMPS[1], Yan CHEREL[1], Charles PINEAU[3,4], Karl ROUGER[1]

[1]*INRA/ONIRIS-UMR703 PAnTher, F-44307 Nantes, France,* [2]*Université de Nantes, F-44035 Nantes, France,* [3]*Plateforme protéomique Biogenouest, F-35042 Rennes, France,* [4]*INSERM, U1085, F-35042 Rennes, France*

Duchenne Muscular Dystrophy (DMD), the most common form of inherited neuromuscular disorder, is caused by mutations in the dystrophin gene leading to the absence of the protein. Membrane permeabilization and subsequent alterations in signaling pathways and energy metabolism play important roles in muscle fiber necrosis. Here, using the clinically relevant Golden Retriever Muscular Dystrophy (GRMD) dog model, we employed quantitative proteomic, to compile changes in protein expression profiles of GRMD *versus* healthy dog muscles. We found that the set of over-expressed proteins was composed of factors involved in apoptosis, calcium signaling and myoblast development/differentiation. On the other hand, the set of under-expressed proteins appeared primarily composed of metabolic ones, many of which have been shown to be regulated by PGC-1α. Thus, for the evaluation of novel therapeutic approaches, it is essential to analyse the direct reversal of dystrophin lack and the associated consequences in treated muscles. In this context, we used isotope-coded protein labeling to study the global proteome after systemic delivery of MuStem cells, a progenitor cell population that showed its therapeutic efficacy after

systemic delivery. LC-MS/MS analyses performed on an ESI-LTQ-Orbitrap mass spectrometer led to the relative quantification of 750 proteins out of 1750 identified proteins. Moreover, same samples were analyzed at transcriptional level through a dedicated expression microarray to identify differentially expressed genes after treatment. The set of signature molecule identify by combined "*omics*" approaches allowed us to evaluate the MuStem cell based therapy.

P016

Development of a new immunotherapy strategy against melanoma based on the use of artificial antigen-presenting cells

Mohamad Hamieh[1], Jean-François Chatillon[2], Florence Bayeux[2], Anne-Bénédicte Duval Modeste[3], Emilie Fauquembergue[1], Aurélie Drouet[1], Philippe Musette[2,3], Jean-Baptiste Latouche[1]

[1]*Inserm U1079,Rouen University Hospital, Institute for Research and Innovation in Biomedicine (IRIB), North-West Cancéropôle, Rouen, France,* [2]*Inserm U905, Rouen University Hospital, Institute for Research and Innovation in Biomedicine (IRIB), Rouen, France,* [3]*Dermatology Department, Rouen University Hospital, Rouen, France*

Adoptive immunotherapy based on the use of cytotoxic T lymphocytes (CTLs) is a promising strategy to cure cancers. However, rapid expansion of numerous highly functional CTLs remains a challenge. Different systems have been developed, including artificial antigen presenting cells (AAPCs), to circumvent this difficulty. We constructed NIH/3T3 mouse fibroblast-based AAPCs stably expressing the essential molecules involved in CTL activation in the HLA-A2.1 context and an A2.1 restricted peptide derived from MART-1, an auto-antigen overexpressed in melanoma. Using these AAPCs, in a preclinical setting, we have defined the optimal conditions to expand MART-1-specific CTLs from healthy donors and melanoma patients without generating any xenoreactivity. After expansion, MART-1-specific CTLs were highly purified by magnetic bead-sorting and re-stimulated on AAPCs for amplification. This standardized procedure always led to rapid and major expansion of these specific CTLs, both in healthy donors and in patients. Flow cytometry analyses revealed that MART-1-specific CTLs had a suitable phenotype for immunotherapy, with effector and memory T cell features. These cells were highly cytotoxic and had the capacity of specifically targeting $A2.1^+$ $MART\text{-}1^+$ tumor cells. Vβ TCR repertoire and CDR3 sequence analyses revealed that MART-1-specific CTL responses were oligoclonal and restricted to some Vβ subfamilies, which were similar to those reported in the literature and obtained with autologous or allogeneic presenting cells. In conclusion, these "off-the-shelf" AAPCs represent a unique tool to rapidly and easily expand large numbers of highly functional CTLs for the development of efficient adoptive immunotherapy strategy against melanoma.

P017

Integrated characterization of human placental messecnchymal stem cells as unavoidable step prior their use in medical approaches

Elvira Herrero de Laorden, Maria del Carmen Turpin, Francisco Moreno Garzón, María Isabel Portero, Maite Iglesias Badiola

Francisco de Vitoria University, Madrid, Spain

Placenta tissue is studied as an important reservoir of stem cells with great potential in regenerative medicine and cell therapy because of its unique features: phenotypic plasticity, immunomodulatory properties and ease of isolation procedure. This application involves: removal of cells from a donor, cell expansion in culture and administration to the recipient. Culture of the cells *in vitro* is a tricky step since cells must overcome a "traumatic" situation preserving all its characteristics and properties. Our group has characterized the expanded human placenta messenchymal stem cells (phMSCs) further culture amplification from a genotypic, phenotypic and functional standpoint. We have monitored the proliferative capacity of phMSCs at 10 different passages and in two different cell culture conditions, low density (subconfluence) and high density (confluence); a marked difference in the proliferation ability was observed. An analysis of the genes related to self renewal and pluripotency Sox-2, Oct-4 and Nanog was performed by qRT-PCR without significant variation in their expression. A similar pattern of mesenchymal marker expression such as CD90, CD105 and CD44 was observed by flow cytometry analysis. We analyzed a possible relation between phMSCs doubling and growing conditions and their potential to differentiate into adipogenic and osteogenic lineages. Furthermore the immunogenic phenotype was addressed; the HLA-ABC and HLA-DR antigen expression and TGFb secretion were analyzed. Our data suggest a relationship between phMSCs culture conditions and their properties that should be taken into account in raising clinical applications.

P018

Low molecular weight dextran sulfate binds to human myoblasts and improves their survival after transplantation in mice

Thomas Laumonier, Pierre Hoffmeyer, Jacques Menetrey

Department of orthopedic surgery, Geneva university hospital, Geneva, Switzerland

Myoblast transplantation represents a promising therapeutic strategy in the treatment of several genetic muscular disorders including Duchenne muscular dystrophy. Nevertheless, such an approach is impaired by the rapid death, the limited migration and the rejection of transplanted myoblasts by the host. Low molecular weight dextran sulfate (DXS), a sulfated polysaccharide, has been reported to act as a cytoprotectant for various cell types. Therefore we investigated whether DXS could act as a "myoblast-protectant" both in vitro, and in vivo after transplantation in immunodeficient mice. In vitro, DXS bound human myoblasts in a dose-dependent manner and significantly inhibited staurosporine-mediated apoptosis and necrosis. DXS pretreatment also protected human myoblasts from natural killer cell-mediated cytotoxicity and is able to counteract the IFN(-induced upregulation of the MHC-II complex and ICAM-1. When human myoblasts engineered to express the renilla luciferase transgene were transplanted in immunodeficient mice, bioluminescence imaging analysis revealed that the proportion of surviving myoblasts was, 1 day and 3 days after transplantation, two times higher when cells were preincubated with DXS compared to control, ($77.9 \pm 10.1\%$ vs. $39.4 \pm 4.9\%$; $p = 0.0009$ and $38.1 \pm 8.5\%$ vs. $15.1 \pm 3.4\%$; $p = 0.01$, respectively). Immunofluorescence analyses assessing the expression of human lamin A/C in the grafted tissue corroborated the quantitative bioluminescence imaging analysis. Taken together, we provide

evidence that DXS acts as a myoblast protectant in vitro and is able in vivo, to prevent the early death of transplanted myoblasts.

P019

Personalized cellular adoptive immunotherapy strategy in microsatellite unstable colorectal cancers

Pauline Maby[1], Mohamad Hamieh[1], Aurélie Drouet[1], Emile Fauquembergue[1], Jacques Mauillon[2], Thierry Frébourg[1,2], Jean-Baptiste Latouche[1,2]

[1]*Inserm U1079, Rouen University Hospital, Institute for Research and Innovation in Biomedecine (IRIB), North-West Cancéropôle, Rouen, France,* [2]*Department of Human Genetics, Rouen University Hospital, Rouen, France*

Colorectal cancers (CRCs) with microsatellite instability (MSI) accumulate frameshift mutations that can lead to the synthesis of neo-antigens expressed only in tumor cells. Degradation of these neo-antigens can release immunogenic neo-peptides presented on HLA class I molecules and targeted by a specific cellular immune response.

Antitumor response strengthening could be exploited in MSI+CRC treatment. Therefore we preclinically developed a personalized cellular adoptive immunotherapy strategy based on the characterization of frameshift mutations in a given patient's tumor and the stimulation of this patient's T lymphocytes against neo-peptides derived from these mutations. To detect tumor mutations we designed 3 multiplex PCRs that amplify repeated coding sequences of 29 genes. To efficiently stimulate specific cytotoxic T lymphocytes *in vitro*, we constructed Artificial Antigen Presenting Cells (AAPCs) that can present a transgene-encoded antigen on the most frequently expressed HLA class I molecule, HLA-A2.1.

In one patient, most tumor cells harboured a single nucleotide deletion of *ASTE1/HT001, TGFβRII* or *TAF1B* gene, leading to neo-protein synthesis. Degradation of these neo-proteins potentially releases frameshift mutation-derived peptides (FSPs) predicted to have a high affinity for the HLA-A2.1 molecule, expressed by this patient. We cultured this patient's T lymphocytes with AAPCs expressing each one of these FSPs. After expansion, activated T lymphocytes were able to specifically kill cells presenting the relevant peptides, expressed only in this patient's tumor cells.

These preclinical data emphasize the pertinence of developing personalized cellular adoptive immunotherapy strategies, based on the use of our AAPCs, to treat MSI+CRCs.

P020

Cytokine-Induced Killer Cells effectively kill autologous Osteosarcoma and Soft Tissue Sarcomas including putative cancer stem cells.

D Sangiolo[1], G Mesiano[1], L Gammaitoni[1], V Leuci[1], M Todorovic[1], L Giraudo-Diego[1], MG Volpe[1], S Miano[1], I Ferrero[0,2], F Carnevale-Schianca[1], S Gallo[1], L D'ambrosio[1], T Venesio[1], A Pisacane[1], Y Pignochino[1], F Fagioli[0,2], G Grignani[1], M Aglietta[1]

[1]*Medical Oncology Division, IRCC Institute for Cancer Research and Treatment, Candiolo, (Turin), Italy,* [2]*Pediatric Onco-Hematology, Stem Cell Transplantation and Cellular Therapy Division, Regina Margherita Children's Hospital, Turin, Italy*

Metastatic osteosarcoma (mOS) and soft tissue sarcomas (mSTS) are currently incurable diseases.

We investigated the preclinical efficacy of immunotherapy with Cytokine-Induced Killer (CIK) cells against autologous mOS and mSTS, including the potential killing of putative sarcoma cancer stem cells (sCSC), considered responsible for relapses and drug resistance. CIK cells are ex-vivo expanded T lymphocytes with mixed T-NK ($CD3^+CD56^+$) phenotype, endowed with MHC-unrestricted antitumor activity.

We successfully expanded CIK cells from PBMC of 21 patients (median 172 fold, range 18–3968) with mOS (n=7) and mSTS (n=14). CIK cells from all patients efficiently killed sarcoma cell lines in-vitro (n=22). In 7 cases CIK cells efficiently killed autologous metastatatic cell lines generated from fresh biopsies (1 mOS and 6 mSTS). The specific killing was 83%, 76%, 74% 60% at 40:1, 20:1, 10:1, 5:1 effector/target ratio. To identify putative sCSC we developed a gene-transfer strategy based on transduction of bulk sarcoma cells with a lentiviral vector encoding the eGFP under control of the human OCT4 promoter; sCSC could be visualized exploiting their exclusive ability to activate the OCT4 promoter.

The average presence of $eGFP^+$sCSC within bulk sarcoma cells was 14.6±7.9% (n=5). CIK cells effectively killed $eGFP^+$ sCSC (82%, 72%, 71%, 60% of specific killing at 40:1, 20:1, 10:1, 5:1 effector/target ratio). We demonstrated the intense killing of CIK cells against autologous mOS and mSTS, including a putative subpopulation of sCSC. Our findings support CIK cells as promising candidates for effective immunotherapy strategies against currently incurable diseases and lead the basis for further clinical investigations.

P021

DNA Methylation and Histone Modifications are the Molecular Lock in Lentivirally-transduced Hematopoietic Stem Cells

Siew Ching Ngai[1], Rajesh Ramasamy[2], Rozita Rosli[2], Syahril Abdullah[2]

[1]*International Medical University, Kuala Lumpur, Malaysia,* [2]*Universiti Putra Malaysia, Selangor, Malaysia*

P022

Repair of striated anal sphincter by injection of syngeneic myoblasts to treat fecal incontinence in a rat model

Bisson Aurélie[1,2], Fréret Manuel[1], Drouot Laurent[1], Jean Laetitia[1], Le Corre Stéphanie[1,3], Gourcerol Guillaume[4], Doucet Christelle[2], Jacquot Serge[1,3], Michot Françis[4,5], Lamacz Marek[1], Boyer Olivier[1,3]

[1]Inserm, U905, Institute for Research and Innovation in biomedicine, University of Rouen, Rouen, France, [2]Celogos, Paris, France, [3]Rouen University Hospital, Laboratory of Biotherapy, Rouen, France, [4]Inserm, U1073, Institute for Research and Innovation in biomedicine, University of Rouen, Rouen, France, [5]Rouen University Hospital, Departement of Digestive Surgery, Rouen, France

Fecal incontinence (FI) remains a socially isolating condition with profound impact on quality of life for which autologous myoblast cell therapy represents an attractive treatment option. We developed an animal model of FI and investigated the efficacy of intra-sphincter myoblast injection.

Several types of anal cryo-injuries were evaluated on anesthetized Fischer rats receiving analgesics. The minimal injury yielding sustainable (D60) anal sphincter deficiency was 2x90° lesions at a 24h interval. Sphincter pressure was evaluated longitudinally by anorectal manometry under local electro-stimulation. Myoblasts were prepared from gastrocnemius muscles of syngeneic rats. They showed characteristic myoblasts features including expression of myogenic factors (RT-PCR). Isolated cells were transduced with a GFP-encoding lentiviral vector before intra-sphincter injection. Experimental groups were: uninjured controls; cryoinjured + PBS; cryoinjured + myoblasts ($1–3 \times 10^6$ cells).

Histological analysis at D60 showed sphincter repair with *in situ*-differentiated dystrophin$^+$ muscle fibers expressing GFP and some evidence of re-innervation. Sphincter pressures were significantly higher in grafted rats than in controls at D30 and D60, reaching almost normal levels, and there was a tendency to a cell dose-effect. Intra-sphincter injections at lesion borders were equally as effective as intra-lesion. Longitudinal follow-up showed stability of the therapeutic effect on sphincter function over a 6-month period. There was no extra-sphincter dissemination of grafted cells or detectable side effects.

In conclusion, we established the proof of principle of using myoblast cell therapy for treatment of FI in a rat model. This strategy is currently being evaluated in humans in a placebo-controlled clinical trial (ClinicalTrials.gov NCT01523522).

P023

TALEN-mediated inactivation of TCR-alpha and CD52 in primary T cells for cancer immunotherapy.

Laurent Poirot[1], Isabelle Chion-Sotinel[1], Diane Le Clerre[1], Kahina Oussedik[2], Frédéric Cedrone[2], Julianne Smith[1], Andrew Scharenberg[1]

[1]Cellectis Therapeutics, Paris, France, [2]Celectis SA, Paris, France

Encouraging data have emerged from adoptive T-cell therapies in advanced forms of cancer. Anti-tumor immunity is found in tumor infiltrating lymphocytes as well as engineered T cells where exogenous expression of a chimeric antigen receptor (CAR) confers cancer recognition on the cells. Despite these promising results, adoptive immunotherapy is currently based on autologous cell transfer. The availability of such a treatment using allogeneic cells is currently not possible due to poor persistence of the cells in the host and the potential for graft versus host disease (GvHD). To address these limitations Transcription Activator-Like Effector Nucleases (TALEN), a novel class of sequence-specific nucleases created by the fusion of transcription activator-like effectors (TALEs) to the catalytic domain of an endonuclease, can be used to inactivate one or several genes in primary T cells. For example, eliminating the expression of endogenous T cell receptors in donor T cells will prevent GvHD and allow the production of an "off the shelf" preparation that could be used to treat several patients. In addition, inactivation of CD52 in transferred cells will enable the use of Alemtuzumab for immunosuppression, allowing a longer time window for treatment. We will present our latest results on inactivation of TCR-alpha and CD52 in human T cells as well as the functionality of these genetically modified cells.

P024

Cell therapy for chronic ischemic disease and peripheral artery disease: optimization of the GMP manufacturing method to improve product quality

Marina Radrizzani[1], Viviana Lo Cicero[1], Sabrina Soncin[1], Sara Bolis[1], Daniel Sürder[1,2], Tiziano Torre[3], Francesco Siclari[3], Tiziano Moccetti[1,2], Lucia Turchetto[1]

[1]Cell Therapy Unit, Cardiocentro Ticino, Lugano, Switzerland, [2]Cardiology Service, Cardiocentro Ticino, Lugano, Switzerland, [3]Cardiac Surgery Service, Cardiocentro Ticino, Lugano, Switzerland

Our Cell Therapy Unit is authorized for the production of experimental advanced therapy medicinal products (ATMP).

The ATMP for the METHOD study ("Bone marrow derived cell therapy in the stable phase of chronic ischemic heart disease" - ongoing feasibility phase: 5/10 patients treated) consists of fresh mononuclear cells (MNC) isolated from autologous bone marrow through density gradient centrifugation on standard Ficoll-Paque™; cells are formulated in saline/HSA and tested for safety (sterility, endotoxin), identity/potency (CD45/CD34/CD133, viability) and purity (contaminant granulocytes and platelets).

The aim of the present work was to further develop the manufacturing process in order to reduce contaminants. Moreover, potency assays were introduced in view of the upcoming more advanced phases of the METHOD study and of a new trial in which the same ATMP will be administered to patients with peripheral artery disease.

A new manufacturing method (low density Ficoll-Paque™, low speed wash) was set-up and compared with the current one. It guarantees significantly higher granulocyte removal (97 vs 89%, $p < 0.01$, T-test) and platelet removal (95 vs 91%, $p < 0.01$), even though the MNC yield is lowered (13 vs 25%, $p < 0.01$); consequently, product purity is improved.

The frequencies of CD34 and CD133 cells are significantly higher (CD34: $6.53 \pm 2.29\%$ vs $2.54 \pm 1.10\%$, $p < 0.01$; CD133: $2.87 \pm 1.44\%$ vs $0.88 \pm 0.57\%$, $p < 0.01$) and the product potency is improved (CFC/1E6 cells: 11269 ± 4007 vs 5891 ± 2249, $p < 0.01$; CFU-F/1E6 cells: 34 ± 23 vs 25 ± 11, $p < 0.05$). These results allowed us to update product release specifications.

The new manufacturing method has recently been successfully validated for safety, identity, purity and potency aspects.

P025

Phenotypic characterization and myogenic potential of human MuStem cells, candidates for therapy of Duchenne Muscular Dystrophy

Florence Robriquet[1,2], Judith Lorant[1], Thibaut Larcher[1], Laurence Dubreil[1], Isabelle Leroux[1], Céline Zuber[1], Mireille Ledevin[1], Sabrina Viau[3], Lucie Chabrand[3], Bruno Delorme[3], Yan Chérel[1], Laetitia Guével[1,2], Karl Rouger[1]

[1]*INRA/Oniris, UMR703 PAnTher, F-44307 Nantes, France,* [2]*Université Nantes Angers Le Mans, F-44035 Nantes, France,* [3]*MacoPharma, F-59200 Tourcoing, France*

Over the last decade, adult stem cells have received attention for their potential use in neuromuscular diseases therapies. Using serial platings, we first isolated delayed adherent stem cells from healthy dog skeletal muscles, named MuStem cells, and showed their therapeutic potential after systemic delivery in Golden Retriever Muscular Dystrophy dogs (Rouger et al., 2011). MuStem are promising tool for cell-based therapy approach of muscle diseases. We recently isolated human MuStem (hMuStem) cells from Paravertebralis muscle biopsies of 9 to 15-year-old patients free of known muscular pathology. The aim of our study was to characterize hMuStem cells and investigate their myogenic behavior. In vitro, we established that these cells are defined by a high clonogenic potential and display ability to proliferate in suspension as revealed by the myosphere formation. By FACS analysis, we determined that they are positive for several perivascular cell and mesenchymal markers, that could suggest a common filiation. In return, they are negative for hematopoietic, endothelial markers as well as blood cell related surface and CD133 ones. Interestingly, we showed that they express markers of stemness and pluripotency such as the transcription factors Oct-3/4 and Klf4, using RT-PCR. Myogenic commitment was demonstrated by Myf5, MyoD, myogenin mRNA detection. In vitro, hMuStem cells differentiate into myosin heavy chain positive myotubes in fusion-promoting low serum conditions. After implantation into injured Tibialis anterior muscle of immunodeficient scid mice, we determined that they fuse with host fibers revealing their participation in muscle fiber regeneration.

P026

Hematopoietic stem cell transplantation improves the phenotype of mouse models of X-linked Adrenoleukodystrophy

Karelia Ruffert[1], Stéphane Fourcade[2,3], Isidre Ferrer[5,6], Manuel Portero-Otin[7], Reinald Pamplona[7], Bruno L'Homme[1], Ivan Bièche[1], Ingrid Laurendeau[1], Ornella Ahouansou[1], Françoise Fouquet[1], Sylvie Guidoux-Boralévi[1], Patrick Aubourg[1], Nathalie Cartier[1], Aurora Pujol[2,4]

[1]*INSERM U745, Paris, France,* [2]*IDIBELL, L'Hospitalet de Llobregat, Barcelona, Spain,* [3]*CIBERER, Valencia, Spain,* [4]*ICREA, Barcelona, Spain,* [5]*Neuropathology Institute, Hospital of Bellvitge, Barcelona, Spain,* [6]*CIBERNED, ISCIII, Madrid, Spain,* [7]*Experimental Medicine Department, University of Lieida-IRB-LLEIDA, Lieida, Spain*

Patients who lack ALDP, a peroxisomal protein, and therefore suffer from the neurodegenerative disease X-linked Adrenoleukodystrophy (X-ALD), are successfully treated by the autologous graft of hematopoietic stem cells (HSCs) corrected by a lentivirus. Beneficial effects of HSC transplantation in X-ALD are based on migration of the HSCs into nervous tissue and their differentiation into microglia/macrophages, as shown by graft of human HSCs into the mouse model of the non-demyelinating form of X-ALD (Abcd1-/- mice). The precise mechanism of the effect of microglia/macrophage replacement on the disease remains elusive, however microglia/macrophages could represent an important source of oxidative stress in nervous tissue which had been shown to be an important factor for the development of many neurodegenerative diseases. Abcd1-/- mice show oxidative stress in the spinal cord and antioxidative treatment ameliorates the motor performance of Abcd1-/- mice and Abcd1, 2-/- mice which lack a related peroxisomal protein. We therefore investigated whether transplantation of murine HSCs from wild-type donor into Abcd1-/- mice and Abcd1, 2-/- mice is able to improve motor performance 15 months post-graft (rotarod test). We also investigated modifications of oxidative stress markers or microgliosis/astrogliosis observed in untransplanted mice. We observed that replacement of microglia/macrophages by the transplantation of normal syngeneic hematopoietic stem cells leads to decreased oxidative stress level or a normalization of the number of microglia/astroglia and the restoration of motor performance in grafted mice.

P027

Cell and gene therapy unit (UTCG), Nantes University Hospital. Cell based therapeutics: From concept to manufacture

S Saiagh, S Bercegeay, MC Pandolfino, P Lemarre, T Zuliani, S Ponsolle, A Bellec, T Boutard, M Baud'huin, D Heymann, B Dreno

CIC Biotherapy 0503, Universtiy Hospital, Nantes, France

Established in 1994, UTCG is a manufacturing platform for the clinical investigation centre in biotherapies located within the Nantes University Hospital. UTCG produces cell therapy treatments for clinical trials and for routine medical care.

Because of its location in this environment of multidisciplinary skills, UTCG is strongly linked with research teams, closely related to clinical units in various medical fields. This situation promotes effective translational research and development of phase I/II clinical trials considered as the "proof of concept".

Since 2001, UTCG is certified ISO 9001 and since 2003 by the French Health Authorities for the manufacturing of human cells and products in compliance with the good practices for preparation, conservation, delivery and transfer.

The services, which offer UTCG, concerned all the development stages of the cellular product: from the concept to the preclinical process validation, safety and finally to the manufacturing for clinical use. UTCG also offers advice on legislation for academic research teams or private customers, to make transfer of their research projects to clinical applications easier.

Due to the high number and diversity of the projects managed by the facility and its longstanding experience in cell engineering, today the UTCG boasts know-how on a number of cell types, mainly used in immunotherapy, and recently acquired skills in cell types used in regenerative medicine.

That explains why 18 years after UTCG is considered as one of the most advanced academic manufacturing facility to meet the national and European requirements for the advanced cell therapy medicinal products.

P028

Validation of Cytokines for the Chemically Defined Manufacturing of Human CD34 Positive Hematopoietic Precursor Cells

Jenny Schroeder[1], Alexis Bossie[2], Jessica Carmen[2], Nicole Spottke[1], Stefanie Buesch[1], Tamara Grabeck[1], Alexandra Krumnow[1], Brad Morie[2], Jon A Rowley[2], Herbert Mueller-Hartmann[1]

[1]Lonza Cologne GmbH, Cologne, Germany, [2]Lonza Walkersville, Inc., Walkersville, MD, USA

Human CD34 positive hematopoietic precursor cells (hHPC) have been successfully used over the years to treat disorders of the blood and immune system. Although hHPC can be easily isolated form cord blood, bone marrow and peripheral blood, their *in vitro* expansion is challenging. For instance, using current methodology hHPC tend to lose their pluripotency during *in vitro* expansion.

Therefore the identification of an optimal, ideally chemically defined culture medium for the scalable expansion of high-quality hHPC according to GMP standards is an important task during the development of hHPC-based autologous or allogeneic cell therapies. Chemically defined media performance robustness is highly dependent on the quality of recombinant cytokines used for serum replacement.

In order to find appropriate suppliers of high quality recombinant proteins for chemically defined hHPC medium, recombinant human Flt3-ligand, stem cell factor and thrombopoietin from five different commercial suppliers were tested in combination with chemically defined medium containing only human proteins. Using hHPC from 3 independent donors, cell expansion rate was determined and cell identity was confirmed by flow cytometric analysis. Cell functionality after 7 days of *in vitro* expansion was monitored by analyzing the myeloid differentiation potential of the cells.

This report is an example of a best practice for qualifying primary and secondary suppliers of critical raw materials for cell therapy expansion media. The results demonstrate the importance of thoroughly qualifying recombinant proteins from different sources during process development campaigns.

P029

Silencing of endogenous T cell receptor (TCR) by RNAi reduces severe autoimmune reactions in TCR gene therapy caused by mispaired TCR

Wolfgang Uckert[1], Bunse Mario[1], Gavin Bendle[2], Carsten Linnemann[2], Ton Schumacher[2]

[1]Max Delbrück Center for Molecular Medicine, Berlin, Germany, [2]The Netherlands Cancer Institute, Amsterdam, The Netherlands

Transduction of TCR genes into T cells generates TCR-modified cells with new antigen specificity, which are used to treat viral diseases and cancer. These T cells express two TCRa and TCRb chains which form mispaired TCR, composed of endogenous and transferred chains. As consequence, therapeutic efficiency is diminished and adverse autoimmune reactions are possible. To overcome these deficits, we developed a replacement approach that simultaneously silences the endogenous TCR by RNA interference (RNAi) and expresses an RNAi-resistant therapeutic TCR.

We introduced artificial miRNAs (miR) targeting the mouse TCRa and TCRb chain into a γ-retrovirus vector encoding the LCMV-specific P14 TCR and determined TCR silencing. In transduced T cells the miR reduced endogenous TCR protein expression by more than 80%. Analysis of TCR-modified T cells demonstrated an unequal P14 TCRab chain expression if native or optimized P14 TCR genes were used. Strikingly, miR-TCR replacement balanced P14 TCRab chain expression and the miR did not negatively influence *in vivo* T cell function as shown by tumor growth suppression in an *in vivo* B16 melanoma model. In an autoimmune model, which mimics TCR gene therapy, we observed severe transfer-induced graft-versus-host-disease caused by mispaired TCR in all mice, which received T cells with native or optimized P14 TCR genes. In contrast, addition of miR increased survival of mice to over 60% for the group with native and over 80% for the group with optimized P14 TCR genes.

Our data indicate that silencing of endogenous TCR expression considerably improves the safety of TCR gene therapy.

P030

Evaluation of small interfering RNA delivery by nanoparticle-loaded tumoritropic cells.

Laura Wayteck, Stefaan De Smedt, Koen Raemdonck

Laboratory for General Biochemistry and Physical Pharmacy-Ghent University, Ghent, Belgium

In the context of cancer therapy, many different nanoformulations have been suggested for the delivery of small interfering RNA into tumor cells. Despite several efforts, the accumulation of nanoparticles in the tumor site after intravenous injection based on passive or active targeting is often insufficient. In this work, the aim is to anchor siRNA loaded nanoparticles to the surface of tumoritropic cells (e.g. cytotoxic $CD8^+$ T lymphocytes) which can again be released from these cells at the tumor site by an endogenous or exogenous trigger (e.g. glutathione). The selected RNAi target is signal transducer and activator of transcription3 (STAT3), that is often overexpressed in tumor cells and which upregulates genes crucial for survival, proliferation, angiogenesis and promotes further the expression of immune suppressive factors.

In a first set of experiments, the attachment of liposomes to OT-I T lymphocytes was evaluated. A pyridyl dithiopropionate (PDP) modified lipid was incorporated into the liposome composition to form a reducible disulfide binding with free thiol groups present at the cellular surface. After different optimization steps, we were able to attach an appropriate amount of liposomes to the surface, as revealed by flow cytometry data and confocal microscopy imaging. Because nanoparticles have to be internalized by cancer cells, we also focus on the detachment of nanoparticles from the surface of carrier cells. To allow this triggered detachment, aspecific binding should be avoided and is investigated for our nanoformulation.

P031

Development of neurofibrillary tangles in the rat brain following AAV-mediated gene transfer of wild type and mutant forms of Tau.

AP Bemelmans[1], K Cambon[1], G Auregan[1], N Dufour[1], MC Gaillard[1], M Colin[2], L Buée[2], P Hantraye[1]

[1]*URA2210 CEA / CNRS, Fontenay-aux-Roses, France,* [2]*U387 Inserm, Lille, France*

Neurofibrillary tangles (NFT) are a hallmark of Alzheimer's disease (AD) and tauopathies. They are composed essentially of hyperphosphorylated Tau protein. The mechanism underlying their toxicity is still elusive, although NFT numbers have been correlated with cognitive decline and synaptic loss, they may only reflect the final products of neuroprotective aggregative processes against soluble but toxic oligomeric forms of Tau. Our aim is to establish a fast-developing model of tauopathy in the rat brain to study the neurological symptoms associated with NFT independently of the ß-amyloid component of AD and validate MR/PET imaging biomarkers specific of this pathology. To reach this goal, we have constructed AAV vectors encoding wild type or mutant forms of Tau. In a first step, we optimized targeting of the hippocampus, one of the major site of neurodegeneration in AD patients, by stereotactic injections of AAV vectors encoding fluorescent reporter genes. Using the AAV5 serotype and ubiquitous promoters we were able to achieve a maximal level of transduction of granular neurons of the dentate gyrus, while transduction of pyramidal neurons of CA1 and CA3 regions was less intense. In a pilot experiment, we performed intra-hippocampal injections in rats with vectors encoding wild type or mutant Tau. Four weeks after gene transfer, histological analysis showed at the injection site a dense network of neurons containing hyperphosphorylated Tau, a step preceding NFT. Evolution of the lesion induced by AAV-mediated gene transfer of Tau at longer time points will be presented.

P032

DCLK3, a molecular marker of the striatum that protects against Nterminal domains of mutant huntingtin

Laetitia Francelle[1,2], Laurie Galvan[1,2], Marie-Claude Gaillard[1,2], Géraldine Liot[4,5], Michel de Chaldée[3], Martine Guillermier[1,2], Diane Houitte[1,2], Marion Chaigneau[1,2], Charlène Joséphine[1,2], Gilles Bonvento[1,2], Carole Malgorn[1,2], Fanny Petit[1,2], Caroline Jan[1,2], Noëlle Dufour[1,2], Jean-Marc Elalouf[3], Sandrine Humbert[4,5], Frédéric Saudou[4,5], Nicole Déglon[1,2], Emmanuel Brouillet[1,2]

[1]*CEA, DSV, I*^{2}*BM, Molecular Imaging Research Center, F-92265 Fontenay-aux-Roses, France,* [2]*CNRS, CEA URA 2210, F-92265 Fontenay-aux-Roses, France,* [3]*CEA, Institut de Biologie et de Technologies de Saclay (iBiTec-S), Service de Biologie Intégrative et Génétique Moléculaire (SBIGeM), F-91191 Gif-sur-Yvette Cedex, France,* [4]*Institut Curie, F-91405 Orsay, France,* [5]*CNRS UMR 3306, F-91405 Orsay, France*

Huntington's disease (HD) is an inherited neurodegenerative disorder caused by an abnormal polyglutamine expansion in the protein Huntingtin (Htt). Mutant Htt, despite its ubiquitous expression in the brain, leads to preferential neurodegeneration of the striatum through unknown mechanisms. Our working hypothesis is that gene products selectively expressed in the striatum could be involved in the high vulnerability of striatal neurons to mutant Htt. In the present study, we show that overexpression using lentiviral vectors of a newly identified "striatal" gene product, Doublecortin-like kinase 3 (DCLK3), is neuroprotective against mutant Htt in primary culture of striatal neurons and in the mouse striatum *in vivo*. Since the function of DCLK3 is totally unknown, we generated mutants and truncated fragments of DCLK3 and found that the protective effects of the protein are associated with its C-terminus part that contains a putative kinase domain. We obtained results from autophosphorylation experiments that show that DCLK3 is actually a functional kinase. We identified mechanisms through which DCLK3 could possibly modulate striatal degeneration: DCLK3 is cleaved in different cultured cells and in vivo. Its cleavage in transgenic BACHD mice (HD mouse model) is different from that found in WT mice. Recombinant DCLK3 is localized in the cytoplasm with a higher density in the perinuclear region, according to a web-like organization reminiscent of the cytoskeleton.

The identification of the neuroprotective mechanisms produced by DCLK3 could lead to novel potential therapeutic strategies for HD and possibly other disorders involving the striatum.

P033A

Mitogen and Stress-activated Kinase-1 deficiency and transcriptional dysregulation in Huntington's Disease.

Lara Moumné[1], Karen Brami-Cherrier[2], Sandrine Betuing[1], Elodie Martin[1], Stéphane Le Crom[3], Patrick Aubourg[4], Jean-Antoine Girault[2], Jocelyne Caboche[1]

[1]*UMRS 952, INSERM, UMR 7224, CNRS Université Pierre et Marie Curie - Paris-6, Paris, France,* [2]*Institut du Fer à Moulin, UMR-S 839, Inserm and Université Pierre et Marie Curie, 17 rue du Fer à Moulin, 75005, Paris, France,* [3]*École normale supérieure, Institut de Biologie de l'ENS, IBENS, Inserm, U1024, CNRS, UMR 8197, Paris, France,* [4]*INSERM UMR745, University Paris-Descartes, Paris, France*

Huntington's disease (HD) is a neurodegenerative disorder caused by an abnormal polyglutamine expansion in huntingtin protein (Exp-HTT) leading to neuronal dysfunction and death. Early damages are most marked in the striatum but, as the disease progresses, other areas of the brain including cortex are also affected. Among the multiple cellular dysfunctions observed in HD, transcriptional dysregulation has emerged as a pathogenic process that appears early in disease progression and has been recapitulated across multiple HD models. In recent years we have been studying the involvement of the Mitogen and Stress-activated Kinase-1 (MSK-1) in transcriptional dysregulation in HD. This striatum-enriched protein kinase acts on chromatin remodeling through histone H3 phosphorylation leading to activation of transcription. MSK-1 plays a dual role in gene transcription since it also activates the transcription factor cAMP-responsive element binding protein (CREB) in the striatum through its phosphorylation. We have previously shown that MSK-1 expression is reduced in the striatum of HD patients and model mice. Its overexpression in primary striatal cells as well as in a rat model of HD prevents neuronal dysfunction and death induced by Exp-HTT (Roze et al. 2008; Martin et al. 2011). To understand the mechanism by which MSK-1 exerts its protective effect in the striatum, it is important to identify its molecular targets. For this purpose we compared the expression of striatal genes between MSK-1 knock-out and wild type mice using a whole transcriptome shotgun sequencing (RNAseq). Among the

genes up or down-regulated in MSK-1 KO mice, we identified genes involved in several cellular processes including oxidative or excitotoxic stress protection and synaptic plasticity. Expression study of a set of these genes by quantitative RT-PCR showed that most of them are dysregulated in a cellular model (STHdh-Q111) and a mouse model (R6/2) of HD. These results suggest that MSK-1 down-regulation in HD is responsible for part of the transcription dysregulation observed in this disease and that restoring its expression could lead to reversion of these dysregulations. We have set up a strategy aiming at over-expressing MSK-1 in the striatum of HD mouse models to study its protective effect in vivo. For this we used an AAVrh10 viral vector that shows strong neuronal tropism in the striatum and associated brain structures.

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P033B

The "safety switch": a new system to ensure post-grafting safety of cell therapy products

R Aron Badin[1], A Bugi[2], M Guillermier[1], P Viegas[2], JM Helies[1], C Jan[1], D Carvallo[3], P Noguiez Helin[3], D Trubetskoy[4], F Lemoine[4], J Cohen[4], A Perrier[2], P Hantraye[1]

[1]MIRCen, URA CEA CNRS 2210, 18 Route du Panorama, 92265, Fontenay-aux-Roses, France, [2]Inserm UEVE 861, I-STEM, BP 118, 91030, Evry, France, [3]LTKFarma, Pépinière Générale d'Entreprises, 4 Rue Pierre Fontaine, 91000, Evry, France, [4]CNRS UPMC UMR7087, Hôpital Pitié Salpêtrière – Cervi, 83 Bd de l'Hôpital, 75013, Paris, France

Background & Objectives: Cell therapy is a potential therapeutic strategy but the difficulty in eliminating dangerous cells after grafting is a limiting factor. Our aim was to test the efficacy of gancyclovir (GCV) in eliminating over-proliferative monkey ESC-derived cells in an in vivo model of Huntington's disease in primates. The TK suicide-gene safety system can specifically sensitize cells to GCV by overexpressing the thymidine kinase enzyme before implantation.

Materials & Methods: 8 macaques received unilateral instrastriatal stereotaxic injections of quinolinic acid. Two weeks later they received intrastriatal allografts of overproliferative M.mulatta ES cells previously transduced with the TK suicide-gene system. All stereotaxic coordinates were determined on magnetic resonance imaging scans, and all animals were followed-up at 21, 35, 52, 63 and 70 days post-grafting. At 35 days post-grafting half of the animals were treated with 20mg/kg/day GCV and treatment lasted 33 days. Animals were perfuse-fixed and brains were recovered and processed for histology.

Results & Conclusions: MRI was used to assess the implantation site and survival of grafted cells and to calculate their proliferation rate over time. Although the initial size of individual grafts was heterogeneous, volumetric analysis suggests that GCV slows down or stops cell hyper-proliferation in treated animals compared to the untreated ones. Characterization of graft composition, differentiation and inflammation are currently ongoing.

Results suggest that embryonic stem cell allografts can survive and proliferate in the primate brain in the absence of peripheral immunosuppression in a primate model of HD and that the risk of overgrowth can be controlled using a TK-suicide gene system.

P033C

Beneficial effects of striatal restoration of CYP46A1 expression using AAVrh10 serotype in Huntington's disease mice model (R6/2)

Anabelle Planques[1], Amélie Hu[1], Françoise Piguet[2], Lara Moumné[1], Fathia Djelti[2], Philippe Mailly[1], Nathalie Cartier[2], Patrick Aubourg[2], Jocelyne Caboche[1], Sandrine Bétuing[1]

[1]UMR/CNRS7224/INSERM U952- Physiopathologie du Système Nerveux Central Université Pierre et Marie Curie -75252, Paris, France, [2]INSERM U745 Faculté de Pharmacie 75006, Paris, France

Huntington's Disease (HD) is a dominant hereditary neurodegenerative disease, that typically emerge in adulthood and results in motor, cognitive and behavioural abnormalities. HD has a single genetic cause (abnormal expansion of a CAG trinucleotide repeat into the gene encoding huntingtin) and a well-defined striatal neuropathology. There is no available treatment for HD. Recent findings emphasize the involvement of cholesterol metabolism in Huntington's disease (HD) pathogenesis. The concentration of the brain cholesterol metabolite, 24S-hydroxycholesterol, is decreased in the plasma of HD patients. Moreover cholesterol membrane accumulation has been shown in cellular and mouse models of HD. We thus investigate the role of CYP46A1, the enzyme responsible for the conversion of cholesterol into 24S-hydroxycholesterol, in HD pathogenesis. We showed that cyp46A1 mRNA expression is decreased into the striatum of the R6/2 mouse model from early stages (6 weeks) as well as in STHdh ^{Q111} cell line. Interestingly, CYP46A1 protein expression is also significantly decreased in HD patient putamen extracts. Furthermore, overexpression of CYP46A1 is neuroprotective in primary striatal neurons expressing polyQ-HTT. We thus aimed to restore CYP46A1 expression in the striatum of HD mouse models, using a new serotype (rh10) of AAV vector (AAVrh10). We characterized transduction and tropism of AAV rh10-GFP after stereotaxic injection in the striatum, and showed widespread expression of the transgene within the striatum (around 63% of the whole rostro-caudal extension of the striatum), along with the synaptically connected brain regions. As expected, the transgene showed a particular tropism for neuronal cells. We further evaluated the therapeutic potential of AAV rh 10-CYP46A1 on the phenotype of R6/2 mice based on the behavioral hallmarks of HD pathology. When compared to saline control groups, R6/2 mice injected with AAV rh 10-CYP46A1 exhibited lower clasping score as well as improvement of rotarod performance from 7 to 11 weeks of age. Ongoing experiments aim at deeply analyzing the beneficial effect of CYP46A1 restoration through neuropathological, biochemical and life span studies. Overall these studies will provide a body of preclinical information to propose a new target with potential efficacy of gene therapy in HD patients based on targeted overexpression of CYP46A1 enzyme.

P034

Intracisternal administration of AAV9-sulfamidase vector corrects brain and somatic pathology in MPSIIIA mice.

Sara Marco[1], Virginia Haurigot[1], Albert Ribera[1], Miquel Garcia[1], Albert Ruzo[1], Pilar Villacampa[1], Eduard Ayuso[1], Maria Molas[1],

Luca Maggioni[1], Sandra Motas[1], Jesus Ruberte[1], Federico Mingozzi[2], Fatima Bosch[1]

[1]*CBATEG, Universitat Autonoma de Barcelona, Bellaterra, Barcelona, Spain,* [2]*Children's Hospital of Philadelphia, Philadelphia, PA, USA*

For most lysosomal storage diseases (LSD), particularly those affecting the central nervous system (CNS), there is currently no cure. The blood-brain barrier (BBB) that limits bioavailability of drugs administered systemically, combined with the short half-life of certain lysosomal enzymes, hamper the development of effective therapies for LSDs. Direct injection of gene transfer vectors in the brain parenchyma can potentially overcome these obstacles, but the limited diffusion from the point of injection of both vector and transgene product requires invasive procedures. Mucopolysaccharidosis Type IIIA (MPSIIIA) or Sanfilippo A syndrome is an autosomic recessive LSD caused by deficiency in sulfamidase, a sulfatase involved in the stepwise degradation of the glycosaminoglycan (GAG) heparan sulfate. Here, we demonstrate that intra-CSF administration of an AAV9 vector encoding for the sulfamidase transgene mediates widespread correction of both CNS and peripheral pathology in a mouse model of MPSIIIA disease. Four months after intracisternal administration of AAV9-Sulfamidase, increased enzyme activity was detected throughout the brain of treated MPSIIIA mice, resulting in correction of GAG storage, lysosomal distention, cellular ultrastructure and neuroinflammation. In the periphery, high sulfamidase activity was detectable in the liver and serum with whole-body normalization of GAG accumulation and lysosomal pathology. Importantly, treated MPSIIIA mice had normal locomotor activity, showed correction of behavioral deficits, and had extended lifespan with a mean survival similar to that of healthy littermates. The results presented herein demonstrate the whole-body correction of the disease in a mouse model of MPSIIIA after intra-CSF administration of the viral vector AAV9-sulfamidase.

P035

In vitro testing of a self-complementary AAV9 construct expressing a codon-optimized *Mecp2* transgene for further use in a preclinical model of Rett Syndrome

Valerie Matagne[1,2], Martine Barkats[3], Laurent Villard[1,2], Jean-Christophe Roux[1,2]

[1]*Aix-Marseille University, Marseille, France,* [2]*UMR_S 910, INSERM, France,* [3]*Institut de Myologie, Univ Pierre et Marie Curie, Paris, France*

Rett syndrome (RTT) is an X-linked neurodevelopmental disorder primarily affecting CNS functions but also peripheral functions. There is currently no cure for the disease and availabletreatments are aimed at improving RTT symptoms. Most RTT cases are due to mutation in methyl CpG binding protein 2 (MECP2), whose main function is that of a global transcriptional repressor.

The recent findings that reactivation of *Mecp2* rescued adult diseased RTT mice (*Mecp2*-null mice) not only indicates that MECP2 is needed for normal adult function (Robinson et al, Brain, 2012) but also that gene therapy might be beneficial for RTT patients, even after the disease has started.

AAV9 has been shown to cross the blood brain barrier and infect brain cells after intravenous injection in both rodent and primates (Foust et al, Nat Biotech, 2009; Duque et al, Mol Ther, 2009; Gray et al, Mol Ther, 2011). Our aim was to generate an AAV9 expressing a codon-optimized version of *Mecp2* (MCO) under the regulation of a short mouse *Mecp2* promoter and to assess its efficacy in treating RTT mice. NIH/3T3 cells transfected with the MCO plasmid construct showed higher levels of MECP2 protein (2 fold increase) compared to cells transfected with a pCMV-Mecp2 plasmid. We will next use mixed neurons/glia primary cultures to test the infectivity of AAV9 viruses expressing MCO or GFP and determine the levels of transgene expression.

Future studies will be aimed at in vivo testing and, ultimately, the determination of a potential rescuing effect of scAAV9-MCO in RTT mice.

P036

Efficient Striatal Expression of *GDNF* from Integration-Deficient Lentiviral Vectors Ameliorates Rodent Parkinsonism

NB Lu-Nguyen, M Broadstock, RJ Yáñez-Muñoz

School of Biological Sciences, Royal Holloway-University of London, Egham, Surrey TW20 0EX, UK

Parkinson disease (PD), the second most common neurodegenerative disease, results from the loss of dopaminergic neurons in the substantia nigra and subsequent depletion of striatal dopamine. Trophic factor transgenes can be delivered by lentiviral vectors to protect against PD in animal models, but with an associated risk of insertional mutagenesis. We are developing integration-deficient lentiviral vectors (IDLVs), with much improved biosafety profile due to their reduced risk of causing insertional mutagenesis. Using transgenic *eGFP* expression we have compared transduction efficiency of IDLVs with the standard integration-proficient lentiviral vectors (IPLVs), noting some differences between vector types in both rat primary ventral mesencephalic cultures and 6-hydroxydopamine (6-OHDA)-lesioned rats. The neuroprotective effects of vector-mediated delivery of glial cell-line derived neurotrophic factor (*hGDNF*) were also investigated using IPLVs and IDLVs. Transduction of rat primary neurons significantly improved survival upon withdrawal of exogenous trophic factors. Vector-driven *hGDNF* expression succeeded in protecting dopaminergic neurons of rats against 6-OHDA toxicity. A long-term investigation of GDNF-induced neuroprotective effects mediated by both IPLVs and IDLVs in 6-OHDA-lesioned rats demonstrated the long-lasting efficacy of *hGDNF* vectors, with similar improvement in behaviour of IPLV- and IDLV-*hGDNF*-treated animals. The authors acknowledge financial support from the 6th EU Framework Programme (CLINIGENE, grant agreement no. 18933), the 7th EU Framework Programme (NEUGENE, grant agreement no. 222925) and Royal Holloway-University of London.

P037

Targeted gene correction of inherited liver defects using artificial endonucleases: Application to Crigler Najjar disease

Cécilia Abarrategui Pontes[1], Orlane Pineau[1], Françoise Schmitt[1], Angélique Fourrier[1], Laurent Tesson[1], Claudio Mussolino[2], Toni Cathomen[2], Guillaume Podevin[1], Ignacio Anegon[1], Tuan Huy Nguyen[0]

[1]*UMR 1064 Center of Research in Transplantation and immunology, University Hospital, 44093, Nantes, France,* [2]*Laboratory of Cell and Gene Therapy, Center for Chronic Immunodeficiencies, University Medical Center Freiburg, 79108, Freiburg, France*

Crigler-Najjar type 1 (CN1) disease is a liver disease due to the deficiency of UGT1A1 enzyme. We demonstrated a lifelong cure of the Gunn rat model of CN1, with lentiviral and AAV vectors.

As uncontrolled vector integration represents a potential risk of insertional mutagenesis, we aim at developing strategies of targeted vector integration.

Zinc-finger nucleases (ZFNs) and Transcription Activator-like Effector Nuclease (TALEN) can induce a locus-specific DNA double strand break (DSB). Then, DNA repair can be performed through homologous recombination (HR) with an exogenous donor DNA homologous to the DSB site. *In vitro*, it allows efficient knock-in and gene repair.

We constructed liver-specific lentiviral (LV) and AAV vectors encoding IgM-ZFNs, IgM-TALENs for a knock-in and UGT1A1-ZFNs to perform gene repair. As IgM is not expressed in hepatocytes, it represents an innocuous insertion site.

These constructs were shown to promote specific DSB in vitro after transfection using mutation detection assay with T7 endonuclease I. UGT1A1-TALENs were generated and are being tested.

We constructed DNA donors for both strategies that are being tested *in vitro.*

Gunn newborns were injected with AAV-UGT1A1-ZFNs. Up to 9% of alleles were cleaved in the liver. Newborns will be co-injected soon with both AAV-ZFNs and AAV-donor carrying wild-type UGT1A1, to evaluate the level of targeted gene repair.

In conclusion, we showed that endogenous UGT1A1 gene can be cleaved *in vivo*, paving the way to gene repair strategies. Such recent targeted strategies may offer safer therapeutic options to treat inherited monogenic diseases.

P038

Building Novel Ubiquitous Chromatin Opening Elements (UCOEs)

Omer Faruk Anakok, Thalia Vlachou, Michael Antoniou

King's College, London, UK

UCOEs are structurally defined as methylation free CpG islands spanning over the promoters of two closely spaced divergently transcribed House Keeping Genes (HKGs). The human HNRPA2B1-CBX3 locus (A2UCOE) has been tested within a SIN-LV (Self inactivating lentiviral vector) and shown to increase the number of cells that express transgenes at highly reproducible and stable levels in a variety of different cell lines and especially in vivo following ex vivo gene transfer to mouse bone marrow HSCs (Zhang et al. 2007). It has recently been shown that efficient and stable expression of transgenes from the A2UCOE is at least in part due to its resistance to DNA methylation-mediated silencing (Zhang et al. 2010). The A2UCOE can also confer stability of function on tissue-specific promoters (Brendel et al., 2012). However, the A2UCOE shows orientation bias in its function (Zhang et al. 2010; Brendel et al., 2012) and may not function uniformly in all cell types. The aim of this project is the identification and testing of novel dual divergent HKG promoters in the human genome and assess their activity and to build artificial UCOEs by linking single HKG promoters back to back in a divergent configuration. Database searches have allowed us to identify a number of good candidate HKG loci and their UCOE activity will be tested by determining if they are able to confer reproducibility and stability of expression on a linked SFFV promoter in P19 Murine Embryonic Teratocarcinoma cells (Zhang et al., 2010) both before and after differentiation into neurons.

P039

Genetic Correction of the Human ΔF508 CFTR Locus Using the Zinc-Finger Nuclease Technology

C Bednarski[1,2], K Sobczak[3], WM Weber[3], T Cathomen[1,2]

[1]*Center of Chronic Immunodeficiency, University Medical Center Freiburg, Freiburg, Germany,* [2]*Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany,* [3]*Institute of Animal Physiology, University of Muenster, Muenster, Germany*

Much effort has been undertaken to develop a sustainable gene therapy for Cystic Fibrosis. Most of these approaches are based on transfer of a normal copy of the *CFTR* gene. Another promising strategy is the targeted correction of the mutated *CFTR* locus by harnessing the homologous recombination (HR) pathway of the cell. Frequency of HR can be stimulated >1000-fold by introducing a DNA double strand break at the desired target locus using tailor-made zinc-finger nucleases (ZFN). In this study, we aimed at correcting the ΔF508 mutation using the ZFN technology in combination with an appropriately designed donor DNA to correct the mutated *CFTR* locus. Activity of the *CFTR* exon 10-specific ZFN was verified by employing cleavage assays *in vitro* and *in cellula*. Then, human CFBE41o$^-$ cells, which are homozygous for the ΔF508 mutation, were nucleofected with ZFN expression vectors and a donor DNA carrying a *CFTR* superexon encoding exons 10–24 and a puromycin resistance cassette. PCR-based genotyping was used to confirm the presence of correctly targeted cells in the selected cell population. Subsequent clonal analysis revealed a gene targeting frequency of 8%. All analyzed clones carried a monoallelic targeted integration of the superexon and showed donor specific mRNA transcripts. Currently, clones are analyzed for CFTR protein expression by Western blot and immunocytochemistry to confirm both correct processing and localization of the protein, respectively. Finally, corrected CFBE41o$^-$ clones will be evaluated for restored CFTR function via ion transport measurements in Ussing chamber experiments.

P040

Treatment of diabetes and long-term survival following AAV gene therapy

David Callejas[1,2], Christopher J. Mann[1,2], Eduard Ayuso[1,2], Ricardo Lage[1,2], Iris Grifoll[1,2], Carles Roca[1,2], Anna Andaluz[3], Rafael Ruiz-de Gopegui[3], Joel Montané[1,2], Sergio Muñoz[1,2], Tura Ferre[1,2], Virginia Haurigot[1,2], Shangzhen Zhou[5], Jesús Ruberte[1,4], Federico Mingozzi[5], Katherine High[5,6], Felix Garcia[3], Fatima Bosch[1,2]

[1]*Center of Animal Biotechnology and Gene Therapy, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain,* [2]*Department of Biochemistry and Molecular Biology, Universitat Autonoma de Barcelona, Bellaterra, Barcelona, Spain,* [3]*Department of Animal Medicine and Surgery, School of Veterinary Medicine, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain,* [4]*Department of Animal Health and Anatomy, School of Veterinary Medicine, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain,* [5]*Division of Hematology, Children's Hospital of Philadelphia, Philadelphia, PA, USA,* [6]*Howard Hughes Medical Institute, Philadelphia, PA, USA*

Diabetes is associated with severe secondary complications, caused largely by poor glycemic control. Treatment with exogenous insulin fails to prevent these complications completely, leading to significant morbidity and mortality. We previously demonstrated that it is possible to generate a "glucose sensor" in skeletal muscle through co-expression of glucokinase (Gck) and insulin (Ins), increasing glucose uptake and correcting hyperglycemia in diabetic animals. Here, we demonstrate long-term efficacy of this approach in a large animal model of diabetes. A one-time intramuscular administration of adeno-associated viral vectors of serotype 1 (AAV1) encoding for Gck and Ins in diabetic Beagle dogs resulted in normalization of fasting glycemia, accelerated disposal of glucose after an oral challenge, and no episodes of hypoglycemia during exercise for more than four years after gene transfer. This was associated with recovery of body weight, normal levels of glycosylated plasma proteins and lipid profile, and long-term survival without secondary complications. We further dissected the role of the two transgenes, showing that Ins provides for basal glucose uptake, while Gck acts synergistically with Ins in achieving a tight control when glycemia is high. This demonstration of long-term correction of diabetic hyperglycemia provides the first proof-of-concept in a large animal model for a gene transfer approach to treat diabetes. This work lays the foundations for the future translation of this approach to the clinic.

P041

Effective liver gene therapy of hemophilia B in mice and dogs by engineering lentiviral vectors with hyper-functional transgenes

Alessio Cantore[1,2], Patrizia Della Valle[3], Sara Bartolaccini[1], Lucia Sergi Sergi[1], Thierry VandenDriessche[4], Marinee Chuah[4], Dwight Bellinger[5], Armando D'Angelo[3], Timothy Nichols[5], Luigi Naldini[1,2]

[1]San Raffaele-Telethon Institute for Gene Therapy, Milan, Italy, [2]"Vita Salute San Raffaele" University, Milan, Italy, [3]San Raffaele Scientific Institute, Milan, Italy, [4]The Free University of Brussels, Brussels, Belgium, [5]University of North Carolina, Chapel Hill, USA

Lentiviral vectors (LVs) are attractive vehicles for liver gene therapy, thanks to a sizeable transgene capacity and to their ability to integrate in the genome. Moreover most human subjects have no humoral and cellular pre-existing immunity against vector components. We have developed microRNA 142-regulated LVs that stringently target transgene expression to hepatocytes and induce immune tolerance to transgene proteins. We have shown that these LVs can fully correct the disease phenotype in hemophilia B (hemoB) mice. We are currently evaluating this platform in a large animal model of the disease. Our results show long-term canine factor IX (FIX) expression up to 1% of normal levels and clinical improvement (almost complete prevention of spontaneous bleedings) in two hemoB dogs, with up to 3 years follow up. In order to improve these levels, we generated hyper-functional FIX transgenes carrying a R338L amino acid substitution, previously associated with clotting hyperactivity and thrombophilia. The hyper-functional FIX transgene and its codon-optimized version increased FIX activity reconstituted in the plasma of hemoB mice, without detectable adverse effects, allowing correction of the disease phenotype at low vector doses. The combined effect of codon-optimization with the hyper-activating mutation resulted in a robust 15-fold gain in potency, thus decreasing the required therapeutic dose of LV and improving the efficacy, feasibility and safety of our gene therapy strategy. We are now planning the treatment of a hemoB dog with LVs expressing the codon-optimized and hyper-functional canine FIX transgene. Overall these data position our platform for further pre-clinical development and translation.

P042

Pig liver gene therapy: a surgical model of whole watertight liver.

Omar A. Carreño[1], Luis Sendra[2,4], Eva M. Montalvá[1], María J. Herrero[2,4], Juan F. Orbis[1], Carlos Vila[3], Rafael López Andújar[1], Salvador F. Aliño[2,4]

[1]Unidad de Cirugía Hepatobiliopancreática y Trasplantes del Hospital Universitario y Politécnico La Fe, Valencia, Spain, [2]Departamento de Farmacología, Facultad de Medicina Universidad de Valencia, Valencia, Spain, [3]Servicio de Veterinaria. Instituto de Investigación Sanitaria del Hospital Universitario y Politécnico La Fe, Valencia, Spain, [4]Instituto de Investigación Sanitaria Hospital Universitario y Politécnico La Fe, Valencia, Spain

Introduction: The aim of this research is to develop a surgical technique in an *in vivo* pig model that allow us to achieve a whole watertight liver through a complete hepatic vascular exclusion.

Material and Method: Under general anesthesia a complete midline laparotomy is carried out.

The hepatic artery and suprahepatic cava vein were identified and referenced. Inferior vena cava, above renal veins, and extrapancreatic portal vein are dissected and referenced with two vessel loops, to create a closed segment to cannulate them. Then, we proceeded to clamp first hepatic artery, second portal vein and third infrahepatic vena cava and suprahepatic cava to obtain a total hepatic vascular exclusion. The cannulation is performed through portal and cava veins simultaneously.

Results: The technique to achieve gene transfer to whole watertight liver can easily be developed in less than 60 min. The period of total vascular exclusion was less than 7 min and during this period, the only relevant hemodynamic change observed was a decrease in arterial pressure (from 80/60 to 40/30), which was fully recovered in the first 5 min after vascular unclamping. The gene delivery was successfully achieved employing mild hydrodynamic conditions, since a volume of 200ml at 20 ml/s or 60 ml/s showed similar gene transfer efficacies (approx. 10^4-10^5 eGFP RNA copies/100ng total RNA) in all the lobes studied.

Conclusion: We describe an *in vivo* pig model that can be safely performed, to insert gene material into the liver with potential clinical application. Partially supported by AP-151-11 and SAF2011-27002.

P043

Biotherapy for achondroplasia

Stephanie Cassagnaud[1], Isabelle Gennero[2], Elvire Gouze[1]

[1]INSERM U1065, NICE, France, [2]INSERM U1043, Toulouse, France

Achondroplasia is a rare genetic disease characterized by abnormal bone development and early obesity. No cure is available. Achondroplasia is caused by a point mutation in FGFR3 leading to its constitutive activation following ligand binding. We have

developed a recombinant protein approach using a soluble form of FGFR3 to treat transgenic *Fgfr3*$^{ach/+}$ mice.

Recombinant sFGFR3 is produced by transient transfection in 293 cells. To determine the mechanism of action, several doses of sFGFR3 were incubated on ATDC5 and Erk phosphorylation was evaluated by in cell western. To determine treatment effect, two doses of sFGFR3 (0,5 and 5 ng) were injected every 2–3 days by subcutaneous injection on newborn *Fgfr3*$^{ach/+}$ mice. Animals were sacrificed at day 22 and bone growth was determined.

Our results show effective binding of FGFs to sFGFR3 and confirm competition with transmembrane endogenous FGFR3 as seen by a decrease in MAPK signalling in ATDC5 cells.

Skeletal growth was enhanced dose-dependently in *Fgfr3*$^{ach/+}$ and wt mice following sFGFR3 treatment. At sacrifice, *Fgfr3*$^{ach/+}$ mice treated gained 11 to 15% of growth compared to untreated *Fgfr3*$^{ach/+}$ mice. wt mice gained 3 to 6% of growth compared to untreated wt mice. At the highest dose, 0% of mice developed hind limb paralysis, compared to 10% in the low dose group and 25% in the untreated group. No side effects were observed following treatment as shown by morphological analysis of several organs and toxicological analyses of plasma.

These results show that soluble FGFR3 can be used as an effective treatment for achondroplasia.

P044

Diabetogenic response to streptozotocin varies among NOD and NOR mice

Sevim Kahraman[1,2], Cigdem Aydin[1,2], Gulsum Ozlem Elpek[3], Ercument Dirice[1,4], Ahter Dilsad Sanlioglu[1,2]

[1]*Human Gene and Cell Therapy Center of Akdeniz University Hospitals and Clinics, Antalya, Turkey,* [2]*Department of Medical Biology, Faculty of Medicine, Akdeniz University, Antalya, Turkey,* [3]*Department of Pathology, Faculty of Medicine, Akdeniz University, Antalya, Turkey,* [4]*Section of Islet Cell and Regenerative Medicine, Joslin Diabetes Center, Harvard Medical School, Boston, MA, USA*

Type 1 Diabetes (T1D) is an autoimmune disease characterized by selective destruction of pancreatic beta cells. While non-obese diabetic (NOD) mice, which spontaneously develop T1D, are the most frequently preferred animal models in T1D research, non-obese diabetes resistant (NOR) mice are defined as proper controls for NOD mice in non-MHC related studies. We found in a previous study that streptozotocin (STZ), which is a diabetes-accelerating agent that selectively destroys beta cells after uptake via the GLUT2 glucose transporter, had a more severe effect on NOR mice compared to NOD mice. In this study, we aimed to detect the differences in GLUT2 expression levels in beta cells to understand the relation between GLUT2 expression and STZ sensitivity. A single dose of STZ (150 mg/kg) was administered by i.p. injection to 10 week-old female NOD and NOR mice. NOR mice showed rapid rise in blood glucose levels compared to NOD mice shortly after STZ injection. Additionally, higher weight loss was apparent in NOR mice than NOD mice, and the survival was only 20% at day 7 while 100% in NOD mice. To assess the expression levels of GLUT2 in pancreatic beta cells, pancreatic sections were immunostained with GLUT2 antibody. Examination of the mean fluorescence intensities in the islets revealed no significant difference between the NOR and NOD groups. The severe effect of STZ application on NOR mice may be due to a somewhat higher toxicity of the agent on these mice, presumably through a higher exposition by a yet unknown mechanism.

P045

Embedding siRNA sequences targeting Apolipoprotein B100 in shRNA and miRNA scaffolds results in differential processing and *in vivo* efficacy

Piotr Maczuga[1,2], Jacek Lubelski[1], Richard van Logtenstein[1], Florie Borel[1,3], Bas Blits[1], Erwin Fakkert[4], Adalberto Costessi[4], Derek Butler[4], Sander van Deventer[2], Harald Petry[1], Annemart Koornneef[1], Pavlina Konstantinova[1]

[1]*uniQure, Amsterdam, The Netherlands,* [2]*Leiden University, Leiden, The Netherlands,* [3]*Academic Medical Center, Amsterdam, Amsterdam, The Netherlands,* [4]*Baseclear BV, Leiden, The Netherlands*

Overexpression of short hairpin RNA (shRNA) often causes cytotoxicity and using microRNA (miRNA) scaffolds can circumvent this problem. In the current study identical predicted small interfering RNA (siRNA) sequences targeting Apolipoprotein B100 (siApoB) were embedded in shRNA (shApoB) or miRNA (miApoB) scaffolds and a direct comparison of the processing and long-term *in vivo* efficacy was performed. Next generation sequencing (NGS) of small RNAs originating from shApoB- or miApoB-transfected cells revealed substantial differences in processing, resulting in different siApoB length, 5′ and 3′ cleavage sites and abundance of the guide or passenger strands. Murine liver transduction with adeno-associated virus (AAV) vectors expressing shApoB or miApoB resulted in high levels of siApoB expression associated with strong decrease of plasma ApoB protein and cholesterol. Expression of miApoB from the liver-specific LP1 promoter was restricted to the liver, while the H1 promoter-expressed shApoB was ectopically present. Delivery of $1x10^{11}$ genome copies AAV-shApoB or AAV-miApoB led to a gradual loss of ApoB and plasma cholesterol inhibition, which was circumvented by delivering a 20-fold lower vector dose. In conclusion, incorporating identical siRNA sequences in shRNA or miRNA scaffolds results in differential processing patterns and *in vivo* efficacy that may have serious consequences for future RNAi-based therapeutics.

P046

Threshold dose of factor IX expression in non-human primates following IV administration of AAV8 vectors

Hiroaki Mizukami[1], Jun Mimuro[0,2], Tomonori Tsukahara[1], Masashi Urabe[1], Akihiro Kume[1], Tsukasa Ohmori[0,2], Seiji Madoiwa[0,2], Yoichi Sakata[0,2], Keiya Ozawa[1]

[1]*Div. of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical University, Shimotsuke, Japan,* [2]*Div. of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, Shimotsuke, Japan*

Adeno-associated virus (AAV) vectors are suitable in gene therapy approaches especially for hemophilia. In an effort to find optimal vector dose for liver-mediated approach, we tested the efficacy of AAV8 vector into cynomolgus monkey. AAV8-based single stranded vector encoding macaque factor IX (with a single amino acid mutation for detection), driven by hAAT promoter with HCR enhancer was prepared. Vectors were injected at 3 different doses; high (5×10^{12} vg/kg), intermediate (2×10^{12} vg/kg) and low (5×10^{11} vg/kg). Vector was injected into saphenous vein of NAb-negative monkeys. In high dose monkeys, expression levels after 2 months were 15.4, 6.8 and 2.2 % of normal, with a mean value of 8.1 %. In intermediate dose group, 8.7 and 3.3 %

(mean value was 6.0 %) were observed. In low dose group, 1.4 and 0.1% were observed. All of the animals exhibited long-term expression at constant levels. Vector copy number in liver tissues, analyzed by biopsy, correlated well with the transgene expression levels. No adverse effects related to the vector administration were observed. Our results indicate that the optimum vector dose in monkey is 2×10^{12} vg/kg or above. This vector dose coincides with the "high dose group" in recently reported clinical trial in UK, suggesting the utility of cynomolgus monkey in preclinical study. This study was performed in collaboration with Tsukuba Primate Research Center, National Institute for Biomedical Innovation, and The Corporation for Production and Research of Laboratory Primates, Japan.

P047

Safe and efficacious delivery of the human clotting factor IX gene to non human primates using a recombinant AAV vector produced in a fully-scalable GMP-compliant production system.

Bart Nijmeijer, Harald Petry, Marc Sonnemans, Stuart Beattie, Florence Salmon, Hans Preusting, Arie Van Oorschot, Jaap Twisk

uniQure BV, Amsterdam, The Netherlands

Hemophilia B (HB) is caused by a lack of circulating factor IX (FIX). Recently, a recombinant adeno-associated viral (AAV-) vector encapsidating the codon optimized human FIX expression cassette (*hFIXco*) showed efficacy in patients with severe HB[1]. Commercial exploitation of such a vector however requires a scalable production platform. We have developed a proprietary baculovirus expression vector system (BEVS), which is scalable and GMP compliant. In this system we produced an AAV5-pseudotyped vector containing the *hFIXco* expression cassette. To evaluate the AAV5-hFIXco vector in a pharmacologically relevant model, 3 Rhesus monkeys were dosed with $5x10^{12}$ vector genome copies (gc) per kg body weight, by intravenous infusion. Blood was sampled weekly, from 4 weeks before dosing until sacrifice 12 weeks after dosing. Human FIX levels peaked to 7%–16% of normal human levels one week after infusion, and stabilized to 5–10% of normal human levels 4 weeks after infusion until sacrifice. Post mortem, (RT)-QPCR demonstrated homogeneous vector DNA delivery and transgene expression in the liver. No signs of adverse reactions were observed. Infusion was associated with slight and transient effects in plasma chemistry shortly after dosing, such as a brief increase of liver enzyme activity levels, consistent with infusion of a viral protein. Necropsy revealed no significant macroscopic or microscopic abnormalities. In conclusion, administration of AAV5-hFIXco vector resulted in therapeutically relevant hFIX levels and was well tolerated. The scalable production platform allows further nonclinical and clinical development.

[1]Nathwani et al, NEJM (2011); 365(25):2357–65.

P048

Direct hepatic injections of HDAd vector are more efficient and safer than systemic intravenous injections for liver-directed gene therapy of Crigler-Najjar syndrome type I.

Nunzia Pastore[1], Edoardo Nusco[1], Francesco Vetrini[2], Donna Palmer[2], Philip Ng[2], Libor Vitek[3], Nicola Brunetti-Pierri[1,4]

[1]*Telethon Institute of Genetics and Medicine, Naples, Italy,* [2]*Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA,* [3]*4th Department of Internal Medicine, 1st Faculty of Medicine, Charles University, Prague, Czech Republic,* [4]*Department of Pediatrics, Federico II University of Naples, Naples, Italy*

Crigler-Najjar syndrome type I is an inborn error of bilirubin metabolism due to mutations of the uridine diphosphoglucuronosyl transferase 1A1 (UGT1A1) gene. Affected patients have life-threatening increase in serum bilirubin and are often treated with liver transplantation. Life-long phenotypic elimination of hyperbilirubinemia has been achieved with a single intravenous injection of HDAd expressing UGT1A1 into Gunn rats, the animal model of Crigler-Najjar syndrome. However, high intravenous doses of HDAd can activate an acute inflammatory immune response with potentially lethal consequences. To overcome this obstacle, we investigated safety and efficacy of direct injections of low HDAd vector doses delivered into the liver parenchyma. HDAd expressing UGT1A1 was injected either intravenously or directly into the liver parenchyma of Gunn rats. Intraparenchymal injections were performed into three hepatic sites through a small laparotomy for direct visualization of the liver. While doses $\geq5\times10^{11}$ vp/kg resulted in complete correction of the hyperbilirubinemia by both routes of administration, a greater reduction of hyperbilirubinemia was achieved with 1×10^{11} vp/kg by intraparenchymal injections compared to intravenous injections of the same vector dose. Thrombocytopenia was observed in rats injected intravenously with 1×10^{12} vp/kg but not in animals receiving intraparenchymal HDAd injections. We conclude that direct injections into the liver parenchyma, which in humans may be performed by less invasive ultrasound-guided percutaneous approach, improve the therapeutic index of HDAd and may represent a safe and efficient approach for HDAd-mediated liver-directed gene therapy of Crigler-Najjar syndrome.

P049

Generation of a cost effective and a practical animal model for Type 2 Diabetes

Nil Guzel[1,2], Hale Tasyurek[1,2], Ahter Dilsad Sanlioglu[1,2], Mustafa Kemal Balci[1,3], Salih Sanlioglu[1]

[1]*Human Gene and Cell Therapy Center of Akdeniz University, Antalya, Turkey,* [2]*Department of Medical Biology and Genetics, Akdeniz University Faculty of Medicine, Antalya, Turkey,* [3]*Department of Internal Medicine, Division of Endocrinology and Metabolism, Akdeniz University Faculty of Medicine, Antalya, Turkey*

Background: Type 2 diabetes (T2D) is characterized by chronic insulin resistance and a progressive decline in beta cell function. Due to the induction of insulin resistance and obesity, fat-fed/STZ-treated animals simulate natural disease progression and metabolic characteristics typical of individuals at increased risk for developing T2D. Since commercially available high fat diets (HFD) are very expensive, we have prepared a cost effective and a practical recipe to induce insulin resistance, obesity and eventually beta cell loss in experimental animals.

Methods: C57BL/6J mice were used to develop Diet Induced Obesity (DIO) model for T2D. Insulin resistance is generated via

feeding mice with diets enriched in fat, and hyperglycemia is induced by a low dose of STZ injection.

Results: Mice fed with HFD gained more weight and consumed less water compared to mice fed with normal diet (ND). Intraperitoneal Glucose Tolerance Tests (IPGTT) revealed impaired beta cell function. HFD fed animals also displayed insulin resistance as demonstrated by an injection of a fast acting insulin analogue (Insulin Lispro, Humalog, Elli Lilly). A low dose of STZ delivery facilitated appearance of hyperglycemia as a result of by beta cell loss in C57BL/6J mice. T2D related pathologies were also revealed by molecular analysis.

Conclusion: We have successfully generated a fast and an economic DIO model of T2D to develop and test the efficacy of novel gene therapy approaches against T2D.

P050

Pancreatic acinar cells respond differentially to diabetic agents Streptozotocin and Cyclophosphamide

Sevim Kahraman[1,2], Ercument Dirice[3], Gulsum Ozlem Elpek[4], Cigdem Aydin[1,2], Mustafa Kemal Balci[5], Abdulkadir Omer[6], Salih Sanlioglu[1,2], Ahter Dilsad Sanlioglu[1,2]

[1]Human Gene and Cell Therapy Center, Akdeniz University, Antalya, Turkey, [2]Department of Medical Biology and Genetics, Akdeniz University, Antalya, Turkey, [3]Section of Islet Cell and Regenerative Medicine, Joslin Diabetes Center, Harvard Medical School, Boston, USA, [4]Department of Pathology, Akdeniz University, Antalya, Turkey, [5]Division of Endocrinology and Metabolic Diseases, Akdeniz University, Antalya, Turkey, [6]Department of Medicine, Department of Endocrinology and Diabetes, University of Massachusets, Worcester, Massachusets, USA

Purpose: Streptozotocin and Cyclophosphamide are frequently used as Type 1 Diabetes (T1D) inducing/accelerating agents. They have different diabetes-causing mechanisms, and affect various parameters in the living system as systemically applied molecules. Revealing the nature of these effects may provide additional information on the action mechanisms of these agents, as well as on development of T1D. TNF-Related Apoptosis-Inducing Ligand (TRAIL) is an important component of the immune system, with a proposed protective role in T1D. We found that pancreatic acinar cells of NOD mice respond differentially to Streptozotocin and Cyclophosphamide treatments, in terms of TRAIL ligand and receptor expressions.

Material and Methods: NOD mice received 150 mg/kg STZ or 200 mg/kg CY (n = 15/group). Pancreatic tissues isolated at different stages of disease were analyzed immunohistochemically.

Results: Acinar cells displayed increased expressions of all markers in the STZ group. TRAIL expression was prominently kept at minimum throughout disease development following CY application.

Conclusion: STZ is taken into pancreatic beta cells but not to acinar cells. Thus, increased expressions of all markers after STZ treatment may be outcome of a general stress exerted by this particular agent on the acinar cells. This confirms the specificity of our previous results showing increased expressions of only TRAIL and DcR1 within beta cells, possibly against the infiltrating leukocytes. Suppression of TRAIL in acinar cells following CY expression, as was observed previously in the beta islets, may be an additional implication on action mechanism of CY, which is already known to suppress regulatory T cells leading to T1D.

P051

Development of ZFN-based pre-clinical in vitro cell model in human embryonic stem cells for Wiskott-Aldrich syndrome

Miguel Toscano[1], Pilar Muñoz[1], Per Anderson[1], Karim Benabdellah[1], Marien Cobo[1], Philip Gregory[2], Michael Holmes[2]

[1]Genomics and Oncological Research Center, Granada, Spain, [2]Sangamo BioSciences Inc, Richmond, USA

The development of disease-specific stem cells is fundamental for the understanding of disease mechanisms and to study safety and efficacy of potential treatments. We have developed a human cellular model based on AND-1 (male) and H9 (female) human embryonic stem cells (hESC) of the primary immunodeficiency Wiskott-Aldrich Syndrome (WAS), by specific targeting the WAS gene (located in the X chromosome) using zinc finger nucleases (ZFN). Mutations in WAS cause the syndrome characterized by eczema, immunodeficiency and thrombocytopenia. WAS-deficient hESC lines (hESCWASKO) were developed by inducing homologous recombination (HR) with a donor DNA containing mutated exons 1 and 2 and a neomycin-resistant cassette into the first intron of the gene. We corroborated by PCR and sequencing the proper edition of the WAS locus achieving targeting of WAS in both X chromosomes in the H9 hESC cells. Upon differentiation of hESCWASKO cells into hematopoietic cells using the OP9 co-culture system, neither WAS mRNA nor WASP protein were detected. Generation of hemangioblasts (CD31 + CD34 + CD45 −), hematopoietic progenitors (CD34 + CD45 +) and mature hematopoietic cells (CD45 +) followed a similar pattern than the original hESCs. However, levels of expression of CD34 were significantly decreased in hECSWASKO. The present work validates the hESCWASKO as pre-clinical human cell model where to study the role of WASP during hematopoietic development and to assay gene and cell therapy strategies for the treatment of Wiskott Aldrich Syndrome.

P052

Lentivirus-mediated silencing of snail expression ameliorates experimental arthritis

Chao-Liang Wu[1], Shih-Yao Chen[1], Yuan-Tsung Li[1], Chrong-Reen Wang[2], Ai-Li Shiau[3]

[1]Department of Biochemistry and Molecular Biology, National Cheng Kung University Medical College, Tainan, Taiwan, [2]Department of Internal Medicine, National Cheng Kung Univeristy Medical College, Tainan, Taiwan, [3]Department of Microbiology and Immunology, National Cheng Kung University Medical College, Tainan, Taiwan

Rapid progression of joint destruction suggests aggressive characteristics of rheumatoid arthritis (RA) as resemblance to neoplastic disorders. Morphological characteristics of RA synovial fibroblasts (SFs) are similar to transformed cells. We hypothesized that epithelial-mesenchymal transition (EMT) regulated by snail may be involved in the transition of quiescent SFs into an invasive mesenchymal phenotype. We studied the pathogenic role of EMT and the regulation of cadherin-11 in SFs from RA patients and collagen-induced arthritis (CIA) rats. Our results showed that snail and cadherin-11 were expressed at high levels and positively correlated in the synovium of RA patients and CIA rats. Notably, overexpression of snail in SFs and joints of normal rats promoted EMT, including down-regulation of

epithelial markers, gain of mesenchymal markers, enhanced invasive capacity, and increased cadherin-11 expression. Moreover, intra-articular knockdown of snail ameliorated arthritis signs concomitantly with reduced cadherin-11 expression through the induction of mesenchymal-epithelial transition (MET) in CIA rats. Our results demonstrate that regulation of snail can alter cell morphology and gene expression between epithelial and mesenchymal phenotypes and modulate cadherin-11 expression in SFs. They also implicate that EMT and cadherin-11 expression regulated by snail may be further explored as a novel therapeutic strategy for RA.

P053

Integration-deficient lentiviral vectors as gene delivery vehicles for treatment of AMD

Anne Louise Askou, Marie Hebsgaard Holm-Nielsen, Thomas Gryesten Jensen, Lars Aagaard, Jacob Giehm Mikkelsen, Thomas Corydon

Department of Biomedicine, Aarhus University, Aarhus, Denmark

VEGF plays an essential role in ocular angiogenic diseases including the late-stage form of AMD, the primary cause of vision loss in the western world. Over-expression of VEGF leads to development of vasculature emanating from the choroid, invading the subretinal space through breaks in Bruch's membrane. Strategies leading to long-term suppression of inappropriate ocular angiogenesis are required.

We have evaluated the capacity of rAAV-encoded shRNAs or miRNAs to silence endogenous VEGF gene expression in mouse models. Levels of VEGF and sizes of the CNV were found to be significantly reduced following rAAV2/8-shRNA intramuscular and subretinal delivery, respectively. To further improve efficacy and biosafety, novel versatile vector constructs have been designed based on integration-deficient lentiviral vectors for elimination of adverse events caused by integration into the target cell genome. In these vectors, we combine RNA interference with overexpression of an anti-angiogenic inhibitor, PEDF. Furthermore, tissue-specific promoters together with miRNA target sites will be included to limit transgene expression to RPE cells. By the use of this strategy high efficiency of gene transfer and expression is possible in postmitotic tissue without a requirement for vector integration. Preliminary results show a silencing effect of lentivirally delivered anti-VEGF shRNAs both in vitro and in vivo.

P054

In vitro evidence of mutation repair by Spliceosome-mediated RNA *trans*-splicing in a cellular model of rhodopsin mutation.

Adeline Berger[1], Stéphanie Lorain[2], Mélissa Desrosiers[1], Peggy Fabre[1], Cécile Peccate[2], Thomas Voit[2], Luis Garcia[2], José-Alain Sahel[1], Alexis-Pierre Bemelmans[3,1]

[1]*Centre de recherche Institut de la Vision, Université Pierre et Marie Curie, Paris, France,* [2]*Institut de Myologie, INSERM, Paris, France,* [3]*MIRCen, URA2210 CEA/CNRS, Fontenay-aux-Roses, France*

To implement new gene therapy strategies for autosomal dominant Retinitis Pigmentosa, we applied Spliceosome-Mediated-RNA-*trans*-splicing to correct mutations of the rhodopsin (*RHO*) gene. This technology consists of a splicing reaction between a donor site on the endogenous mutated rhodopsin pre-mRNA and an acceptor site on an exogenous pre-*trans*-splicing molecule (*PTM*) devoid of mutation. This approach allows to preserve the endogenous expression level, and to repair any mutation present in exons 2 and following by promoting the *trans*-splicing event at the first intron. We tested fourteen different *PTM* targeting the first intron by transient co-transfection with wild-type or mutated *RHO* in HEK293 cells and quantified *trans*-splicing efficiency. We observed that a subset of PTM targeting a specific region of the endogenous mRNA were more efficient, suggesting the presence of a "*trans*-splicing hotspot" at this particular site. The most efficient PTM was one of them and reached a *trans*-splicing efficiency of 25%. The rate of *trans*-splicing was equivalent for wild-type and mutated alleles, confirming that *trans*-splicing occurred independently of the mutation. To quantify *trans*-splicing in more physiological conditions, we created, by lentiviral transduction, cellular models with stable expression of *RHO*. We observed an expression of *RHO* in more than 95% of the cells. We are currently characterizing the phenotype of the cells expressing wild type or mutated alleles and the consequence of *trans*-splicing on it. The next step will be to vectorize the best PTM into an AAV vector to test its efficiency *in vivo* in a mutated *RHO* mouse model.

P055

AON-mediated exon skipping restores ciliation in fibroblasts harboring the common Leber congenital amaurosis *CEP290* mutation.

Xavier Gerard[1,2], Isabelle Perrault[2], Sylvain Hanein[2], Eduardo Silva[3], Karine Bigot[4], Sabine Defoort-Delhemmes[5], Marlène Rio[2], Arnold Munnich[2], Daniel Scherman[6], Josseline Kaplan[2], Antoine Kichler[1,6], Jean-Michel Rozet[2]

[1]*GENETHON, Evry, France,* [2]*INSERM U781 and Department of Genetics, Université Paris Descartes-Sorbonne Paris Cité, Institut Imagine, Paris, France,* [3]*Center for Hereditary Eye Diseases, Department of Ophthalmology, University Hospital of Coimbra, Coimbra, Portugal,* [4]*Centre d'Exploration et de Ressources Thérapeutiques en Ophtalmologie, CERTO, Paris, France,* [5]*Service d'Exploration de la Vision et Neuro-Ophtalmologie de l'Hôpital Salengro du CHRU de Lilles, Lilles, France,* [6]*CNRS UMR 8151-Inserm U1022, Université Paris Descarte-Sorbonne Paris Cité, Chimie-Paristech, Paris, France*

Leber congenital amaurosis (LCA) is a severe hereditary retinal dystrophy responsible for congenital or early-onset blindness. The most common disease-causing mutation (>10%) is located deep in intron 26 of the *CEP290* gene (c.2991+1655A>G). It creates a strong splice donor site that leads to insertion of a cryptic exon encoding a premature stop codon. In the present study, we show that the use of antisense oligonucleotides (AONs) allow an efficient skipping of the mutant cryptic exon and the restoration of ciliation in fibroblasts of affected patients. These data support the feasibility of an AON-mediated exon skipping strategy to correct the aberrant splicing.

P056

Preclinical safety study of simian immunodeficiency virus-based lentiviral vector for retinal gene transfer in non-human primates

Yasuhiro Ikeda[1], Yoshikazu Yonemitsu[2], Masanori Miyazaki[1], Ri-ichiro Kohno[1], Yusuke Murakami[1], Toshinori Murata[3], Yoshinobu Goto[4], Yasuji Ueda[5], Mamoru Hasegawa[5], Tatsuro Ishibashi[1]

[1]Department of Ophthalmology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, [2]R&D Laboratory for Innovative Biotherapeutics, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan, [3]Department of Ophthalmology, Shinshu University School of Medicine, Matsumoto, Japan, [4]Department of Occupational Therapy, Faculty of Rehabilitation, International University of Health and Welfare, Okawa, Japan, [5]DNAVEC Corporation, Tsukuba, Japan

Purpose: Recently, we have demonstrated the efficient and stable retinal gene transfer mediated by the non-pathogenic simian immunodeficiency virus (SIV)-based lentiviral vector as well as the therapeutic outcome in some animal models of retinal degeneration using recombinant SIV vectors carrying human pigment epithelium-derived factor (hPEDF). Here, we report the systemic and local effects following intraocular administration of SIV-hPEDF in Macaca fascicularis, as a preclinical safety study.

Methods: Seven Macaca fascicularis were enrolled in this study. Approximately 20 μl of SIV-hPEDF (low titer: 2.5×10^7 transducing units [TU]/ml, n = 4 and high titer: 2.5×10^8 TU/ml, n = 3, respectively) were injected into subretinal space via a glass capillary tube. We undertook an ophthalmic examination, including slitlamp biomicroscopy, intraocular pressure (IOP) measurement, fundoscopic examination, fluorescein anigiography, and electroretinogram (ERG) measurement, and a systemic examination, including general body condition, vital sign, hematology, and blood and urine chemistry, during experimental course (5 years).

Results: Long-lasting hPEDF expression was detected in the aqueous humor at the end of this study. No serious local effect was observed. Functional evaluation via ERGs including multifocal ERGs revealed no remarkable change of the retinal functions. Neither dead animal nor serious side effect was found during experimental course.

Conclusions: The current study indicated the long-term systemic and local safety of intraocular administration of SIV-hPEDF. This long-term safety study using non-human primates is encouraging for clinical application of neuroprotective gene therapy for patients with retinitis pigmentosa.

P057

Efficient transfection of 10,000 pigment epithelial cells with PEDF using the *Sleeping Beauty* transposon system.

Sandra Johnen[1], Zsuzsanna Izsvák[2], Peter Walter[1], Gabriele Thumann[1,3]

[1]Department of Ophthalmology, RWTH Aachen University, Aachen, Germany, [2]Max Delbrück Center for Molecular Medicine, Berlin, Germany, [3]Department of Vitreoretinal Surgery, University of Geneva, Geneva, Switzerland

Age-related macular degeneration (AMD) is the major cause of blindness in the elderly population. Its neovascular form is characterised by increased expression of the pro-angiogenic vascular endothelial growth factor (VEGF) and decreased expression of the anti-angiogenic pigment epithelium-derived factor (PEDF), suggesting that inhibition of neovascularisation by higher PEDF levels will improve vision. Using the non-viral *Sleeping Beauty* transposon system (*SB100X*), we developed a protocol for the efficient transfection of 10,000 pigment epithelial cells and showed that transplantation of 15,000 PEDF-transfected cells reduces induced neovascularisation in two different animal models.

Cells were electroporated with two plasmids, one encoding the *SB100X* transposase and a second encoding the *PEDF* gene. Stability of *PEDF* expression was determined by RT-PCR and consistency of PEDF secretion was analysed by immunoblotting. PEDF-transfected cells were transplanted immediately following laser-induced choroidal neovascularisation and one week after chemical-induced corneal neovascularisation, respectively. Two weeks after transplantation, the area of neovascularisation was measured.

Using *SB100X*, the *PEDF* gene was efficiently delivered to 10,000 cells and integrated into the cells' genome, resulting in stable and continuous rPEDF secretion for longer than one year. Transplantation of as few as 15,000 PEDF-transfected cells reduced the area of choroidal and corneal neovascularisation by 48% and 36%, respectively.

SB100X ensures genomic integration and sustained expression of the *PEDF* gene in as few as 10,000 primary pigment epithelial cells, an essential step for the development of a protocol that comprises the isolation of autologous cells, followed by genetic modification and subretinal transplantation for the treatment of neovascular AMD.

P058

Human cellular models of retinal dystrophies, generated via patient iPSc, for preclinical gene therapy studies

Marie Pequignot[1], Nicolas Cereso[1], Lorenne Robert[1], Aude Conscience[1], Fabienne Becker[2], John de Vos[2], Christian Hamel[1], Vasiliki Kalatzis[1]

[1]INM Inserm U1051, Montpellier, France, [2]IRB Inserm U632, Montpellier, France

Clinical trials for retinal gene therapy are underway with positive results. Thus, the requirements for future preclinical studies will be less stringent, notably concerning the need for large animal disease models. However, a growing number of diseases lack even an appropriate small model, compromising their chances of one day reaching a clinical trial. In such cases, a viable alternative would be to perform preclinical studies on human cellular models of the pathogenic retina.

As a pilot project, we generated a human cellular model of choroideremia (CHM), a retinal dystrophy due to mutations in the *CHM* gene encoding REP1; mouse and zebrafish REP1-deficient models are lethal. We first reprogrammed patient skin fibroblasts into induced pluripotent stem cells (iPSc). We then differentiated the bona fide iPSc into a retinal pigment epithelium (RPE) monolayer that is polarised, expresses RPE-specific markers, has a characteristic subcellular organisation, has tight junctions, and is capable of active transport and phagocytosis. This human RPE model will allow disease modelling in a human system and testing of novel therapeutics. Along this line, we are currently evaluating the most efficient AAV serotype to transduce the human RPE in view of AAV-mediated *CHM* gene transfer studies.

The use of such innovative human disease systems represents an interesting alternative to animal models. Furthermore, preclinical studies on human cells may allow a transition to clinical trials for diseases lacking an appropriate animal model.

P059

An *ex vivo* assay to measure the mobility inside the eye of nanomedicines for retinal gene therapy

TF Martens[1,2], D Vercauteren[1,2], K Forier[1,2], H Deschout[1,2], K Remaut[1], J Demeester[1], SC De Smedt[1], K Braeckmans[1,2]

[1]*Laboratory of General Biochemistry and Physical Pharmacy, Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium,* [2]*Center for Nano-and Biophotonics (NB-Photonics), Ghent University, Ghent, Belgium*

Several retinal diseases have been linked to genetic defects of the retinal cells, which form interesting targets for gene therapy. To protect therapeutic nucleic acids from premature degradation, they are usually packaged into nano-sized particles by means of a gene vector. Here, biodegradable polymers were used as non-viral vectors of plasmid DNA (pDNA), which spontaneously form cationic complexes of approximately 100 nm diameter. Because the eye is isolated from systemic circulation, these gene nanomedicines should be administered directly in the ocular environment for them to reach the retinal target cells. Injection into the vitreous humour, the central part of the eye, appears to be a promising delivery method which should be feasible on a large scale, granted that the gene nanomedicines remain sufficiently mobile within this vitreous humour.

To aid in the rational design of these gene nanomedicines destined for intravitreal injection, we have optimized an *ex vivo* assay to quantify the intravitreal mobility of nanoparticles. Excised bovine eyes were disposed of extraocular material, anterior chamber and lens, exposing the membrane surrounding the vitreous. This provided us with a window for fluorescence microscopy, while preserving the fragile vitreal structure. With this assay, we have quantified the intravitreal mobility of model polystyrene nanoparticles of different sizes and surface functionalizations, and noticed that shielding of the cationic charge was necessary to retain mobility. Also, we evaluated the suitability of disulfide-containing poly(amido amine)s as gene vector for intravitreally injected nanomedicines, and noticed that PEGylation was again necessary to ensure mobility of the particles.

P060

Comparison of AAV 2/8 and 2/rh10 serotypes in the murine retina

Sophia Millington-Ward[1], Naomi Chadderton[1], Mary O'Reilly[1], Arpad Palfi[1], Marian H Humphries[1], Pete Humphries[1], Jean Bennett[2], Paul F Kenna[1], G Jane Farrar[1]

[1]*Trinity College Dublin, Dublin, Ireland,* [2]*University of Pennsylvania, Philadelphia, USA*

The development of efficient gene delivery systems for photoreceptors represents an important component of gene therapies for disorders such as Retinitis Pigmentosa involving these target cells. In the current study delivery of a rhodopsin gene to photoreceptors is explored using subretinally injected recombinant adeno associated virus (AAV) 2/8 and 2/rh10. AAV 2/8 has previously been shown to transduce photoreceptor cells efficiently making it an interesting gene delivery vehicle for therapeutics targeted to photoreceptor cells. However, gene delivery to photoreceptors has not previously been demonstrated using AAV 2/rh10. Following subretinal injection of AAV 2/8 and 2/rh10, rhodopsin expression levels from these viruses were compared in wild type mice. In addition, electroretinography (ERG) responses were compared in rhodopsin knockout (Rho-/-) mice subretinally injected with AAV 2/8 and AAV 2/rh10 expressing rhodopsin (employing a rhodopsin promoter sequence to drive expression). Rho-/- mice lack endogenous murine rhodopsin expression, do not elaborate rod photoreceptor cells and have no rod isolated ERGs. Relative efficiencies of gene delivery, levels of rhodopsin gene expression, retinal histology and ERG responses obtained in mice were compared between serotypes. Results providing a comparative profile of AAV 2/8 and 2/rh10 as potential viral vectors for mammalian photoreceptors will be presented.

P061

Laser photocoagulation enhances gene transfer mediated by Adenovirus-associated virus vector in mouse retinal tissue.

Tae Kwann Park[1,2], Si Hyung Lee[1], Tae Gon Lee[2], Heuiran Lee[3]

[1]*Soonchynhyang Univ. Bucheon Hospital, Bucheon/Gyeonggi-D0, Republic of Korea,* [2]*Kim's Eye Hospital, Seoul, Republic of Korea,* [3]*Ulsan University College of Medicine, Seoul, Republic of Korea*

Purpose: Adeno-associated virus (AAV) has shown great promise as a viral vector in ocular gene therapy, but method of administration which guarantees both safety and effectiveness is still under investigation. In this study, we used mouse retinal tissue to assess the changes in transduction of intravitreally administered self complementary adeno-associated virus (scAAV) after laser photocoagulation.

Methods: Experiments were conducted on 30 C57BL/6J mice. Laser photocoagulation was done in right eyes, and left eyes were untreated for control groups. Three different scAAV serotypes (scAAV1, scAAV5, and scAAV8) were injected in each eye after 48hours of laser photocoagulation. Whole cup fluorescent images and immunohistochemical analyses were used for quantifying and locating protein expression.

Results: All three serotypes revealed enhanced protein expression in whole cup images. Transverse retinal section images also revealed enhanced expression in laser photocoagulated eye with some differences between serotypes. scAAV2 group showed enhanced expression thoughout the whole laser of retina; retinal pigment epithelium (RPE), Müller cells, inner nuclear layer (INL), and retinal ganglion cells (RGC). scAAV5 revealed mainly infected RPE cells, whereas scAAV8 showed enhanced expression in RPE and Müller cells. Migrating RPE cells were also visible, as evidenced by colocalization of enhanced green fluorescent protein (EGFP) and cytokeratin.

Conclusions: Laser photocoagulation can promote transduction efficiency of scAAV-mediated gene transfer when intravitreally administered and target certain area for enhanced local gene expression.

P062

H_2O_2 modulates the expression of VEGF isoforms in retinal cells

S Simao[1], G Silva[1,2]

[1]*Centre for Molecular and Structural Biomedicine (CBME) / Institute for Biotechnology and Bioengineering (IBB, LA), University of Algarve, Campus Gambelas, Gambelas - Faro, Portugal,* [2]*Department of Biomedical Sciences and Medicine, University of Algarve, Campus Gambelas, Gambelas - Faro, Portugal*

Retinal diseases such as diabetic retinopathy - a debilitating consequence of diabetes - are the main causes of acquired blindness in developed countries. Hyperglycemia in diabetes generates reactive oxygen species and triggers angiogenic responses. Vascular endothelial growth factors (VEGF) are the major regulators of physiological and pathological angiogenesis.

By contrast, the $VEGF_{xxx}b$ isoforms have an anti-angiogenic effect. In pathological conditions, an imbalance between pro- and anti-angiogenic VEGF isoforms has been described. The purpose of this study was to evaluate the effect of H_2O_2 upon retinal cells and determine its influence on VEGF and $VEGF_{165}b$ expression. To this end, D407 cells, a human retinal pigment epithelial cell line, were exposed to H_2O_2 (0 – 500 μM; 12h) and cellular viability was assessed. Interestingly, the applied range of H_2O_2 concentrations induced proliferation of D407 cells. Additionally, we detected the presence of VEGF and $VEGF_{165}b$ in the cytoplasm of D407 cells in basal and treatment conditions. Several recent studies seem to point to the role of H_2O_2 as a signalling molecule, implicated in different cellular functions such as apoptosis and proliferation. Our results suggest that micromolar H_2O_2 concentrations induce changes in the expression of VEGF and $VEGF_{165}b$ in D407 cells. These results reinforce the role of H_2O_2 as a signalling molecule and intend to clarify the role of oxidative stress in retinal cells, particularly in the differentiated expression of pro- and anti-angiogenic factors classically involved in retinal diseases.

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P063

CFTR targeting by interference RNA to generate a cystic fibrosis phenotype in well-polarized human airway epithelial cells.

Jessica Bellec[1], Davide Losa[2], Marc Bacchetta[2], Marc Chanson[2], Tuan Huy Nguyen[1]

[1]Institute for Transplantation, Urology and Nephrology, Nantes, France, [2]Laboratory of Clinical Investigation III, Foundation for Medical Research, Geneva, Switzerland

Cystic fibrosis (CF) is an autosomal recessive disorder affecting approximately 70,000 individuals worldwide. Whereas the gastrointestinal symptoms are adequately managed, the airway defects remain the major cause of mortality in patients. The sequence of events occurring at the early onset of pulmonary infection and inflammation remains controversial. Because of the lack of natural animal models of CF, the absence of lung phenotype in mouse models of the disease and the poor quality of patients' biopsies, new strategies are needed to understand these mechanisms. We have developed a model of human airway epithelial cell (HAEC) differentiation at the air-liquid interface into polarized and mucociliated respiratory epithelium. We aim at engineering normal primary HAECs to conditionally disrupt the CFTR gene. This will allow studying the consequences of CFTR silencing with direct comparison to parental HAECs. Primary HAECs, human Calu-3 cells and BHK cells were infected with HIV-based lentiviral vectors expressing different shRNAs targeting the CFTR mRNA. These shRNAs were used either alone or in combination in non inducible and tetracycline inducible constructs. After FACS sorting and culture, these cells were monitored for CFTR protein knock-down using western blot, immunofluorescence and transepithelial resistance analysis. Primary HAECs have been successfully transduced, FACS sorted and differentiated on Transwell filters. Preliminary experiments using a shRNA against connexin 26 showed silencing of the protein expression within a week after transduction in primary HAECs. CFTR knock-down was confirmed in cell lines and is currently investigated in primary HAECs.

P064

Combined delivery of S1PLyase siRNA and rHMGB-1 box A peptide for acute lung injury using amphiphilic peptide carrier

Binna Oh, Minhyung Lee

Department of Bioengineering, College of Engineering, Hanyang University, Seoul, Republic of Korea

Acute lung injury (ALI) is an inflammatory disease, which is characterized by the increased lung vascular permeability alveolar flooding and cytokine release. S1Plyase modulates LPS-mediated pro-inflammation pathway. The delivery of the S1Plyase siRNA can inhibit LPS-mediated p38 MAPK and NF-kB and reduce the LPS-induced cytokines secretion. Meanwhile the HMGB-1 box A peptide has been reported to be an antagonist HMGB1, which was proved as a pro-inflammatory cytokine. Thus, the rHMGB-1 box A peptide can reduce the pro-inflammatory cytokine secretion and lead to anti-inflammatory effect. Also, HMGB-1 box A peptide is a cationic peptide and can form siRNA/rHMGB-1A complex. The R3V6 amphiphilic peptides were added to siRNA/HMGB-1 box A peptide complex to form tight ternary complex. The siRNA/HMGB-1 box A/R3V6 ternary complex has higher delivery efficiency than siRNA/HMGB-1 box A complex, due to its stability. MTT assay showed that siRNA/HMGB-1 box A peptide/R3V6 complex was non-toxic to the cells. In vitro cell study showed that the siRNA/HMGB-1 box A/R3V6 complex reduced the IL-6 and TNF-a levels, due to the anti-inflammatory effect of HMGB-1 A box. In addition, the S1PLyase siRNA/rHMGB-1 A box peptide/R3V6 complex reduced inflammation reaction and apoptosis in lipopolysaccharides mediated acute lung injury animal models. Therefore, S1PLyase siRNA/HMGB-1 box A peptide/R3V6 complex may be useful for the treatment of acute lung injury.

P065

Cumulative CFTR expression following repeated aerosol delivery of non-viral pGM169/GL67A formulation to mouse lung

EWFW Alton[1,5], CA Boyd[2,5], SH Cheng[3], JC Davies[1,5], LA Davies[4,5], A Dayan[1], DR Gill[4,5], U Griesenbach[1,5], T Higgins[1,5], SC Hyde[4,5], JA Innes[2,5], G McLachlan[2,5], DJ Porteous[2,5], IA Pringle[4,5], R Scheule[3], SG Sumner-Jones[4,5]

[1]Imperial College, London, UK, [2]University of Edinburgh, Edinburgh, UK, [3]Genzyme, Framingham, MA, USA, [4]University of Oxford, Oxford, UK, [5]The UK Cystic Fibrosis Gene Therapy Consortium, London, Edinburgh, Oxford, UK

A clinical trial of Cystic Fibrosis gene therapy, delivering CpG-free pGM169 plasmid DNA complexed with cationic lipid GL67A to the nose and lungs, demonstrated safety and molecular efficacy in CF patients. In preparation for evaluation of this formulation in a Multi-dose trial, the pGM169/GL67A formulation was aerosolised to mice at 2-week intervals for 0.5, 2 or 6hr. The lungs and non-target organs were harvested after one, six and 12 doses. pDNA and plasmid-specific mRNA were quantified. A significant positive correlation was observed between the quantity of pDNA present in the lungs 1 day after delivery of one, six and 12 doses and aerosol duration. pGM169 pDNA levels in non-target organs were orders of magnitude lower than in lung. Levels of pGM169-derived CFTR mRNA were low in lungs after

a single dose in Low- and Medium-dose groups, with increased signal in the High-dose group. After 12 doses, a cumulative treatment effect was noted with high levels of CFTR mRNA observed in all treatment groups. Robust mRNA levels were maintained for >21 weeks. This supports our clinical strategy to deliver multiple doses to maximise CFTR expression.

P066

Delivery of rAAV2/5 to fetal rather than neonatal murine airways allows successful readministration of viral vectors in adult life

D. Vidovic[1], R. Gijsbers[1], J. Dooley[3], B. Mori[1,2], J. Toelen[2], A. Liston[3], J. Deprest[2], C. Van den Haute[4], Z. Debyser[1], M. Carlon[1]

[1]*Laboratory for Molecular Virology and Gene Therapy, KU Leuven, Leuven, Flanders, Belgium,* [2]*University Hospital Gasthuisberg, Department of Gynaecology and Obstetrics, Leuven, Flanders, Belgium,* [3]*Laboratory of Genetics of Autoimmunity, KU Leuven, Leuven, Flanders, Belgium,* [4]*Laboratory for Neurobiology and Gene Therapy, KU Leuven, Leuven, Flanders, Belgium*

Background: Gene therapy of the fetus rather than the neonate may offer advantages for treatment of genetic pulmonary disorders such as cystic fibrosis (CF). Use of non-integrating vectors such as rAAV for CF gene therapy will require repeated administration. We reasoned that prenatal gene transfer into the lung may induce tolerance allowing repeated administration after birth. In this study we compared gene transfer efficiency of pre- and postnatal rAAV2/5 delivery to the murine airways. We compared immune responses and evaluated the feasibility to readminister rAAV.

Methods: rAAV2/5-β-gal and -fLuc were co-administered to fetuses by intra-amniotic injection or intranasal instillation to neonatal pups. Reporter gene expression was monitored till 3 months by BLI and X-gal staining. Immune response against vector or transgenes was assessed by transduction inhibition assay, ELISPOT, ELISA and FACS.

Results: Transient reporter gene expression was obtained in murine airways. Luciferase expression in lung declined less between 1 and 3 months after fetal (3-fold drop, $p < 0.01$) than after postnatal delivery (15-fold drop, $p < 0.001$). 3 months after gene delivery transduction efficiency of airways was 4% after fetal and 1.5% after postnatal delivery. Next, we compared readministration of rAAV at 3 months after first delivery. Four weeks after readministration, the BLI signal was 3-fold higher in lung and 7-fold higher in nose in the fetal than in the postnatal group. Analysis of serum neutralizing antibodies (nAb) demonstrated a 16-fold lower titer after fetal delivery. Our initial data indicate that fetal gene delivery outperforms postnatal gene delivery and allows readministration of rAAV.

P067

Sonic hedgehog stimulates skin angiogenesis by recruitment of endothelial progenitor cells

Agnieszka Lipiec[2], Karolina Podolska[2], Emilia Kuźnicka[2], Anna Stachurska[1,2], Agnieszka Zajkowska[1], Maciej Malecki[1,2]

[1]*Medical Uniwersity of Warsaw, Warsaw, Poland,* [2]*Centre of Oncology, Warsaw, Poland*

Searching for gene therapy products that deliver genes into the skin is associated with an extensive progress in methodology that is observed in the field of gene therapy, and at the same time is a consequence of needs to conduct studies on new medicinal products that can directly be used in research hospitals specialising e.g. in oncology or vascular medicine. Angiogenic gene therapy is a method based on an assumption that agiogenic genes can also be used to treat. It is already known that Sonic hedgehog gene (*SHH*) strongly participates in the formation of blood vessels. The main aim of this study was the investigation of angiogenic potency of plasmid SHH due to recruitment of endothelial progenitor cells (EPCs) to the transfected skin. Appropriate formulas were prepared for experiments, and they were applied to the skin of laboratory mice; after pre-determined time (3–30 days) mice were sacrificed, transfected skin specimens were collected and the presence of a pDNA sequence in samples as well an expression level of studied genes were analysed with qPCR. It was demonstrated that *SHH* gene administered to mouse skin as various formulations (pSHH, pSHH:aminoprenols, pSHH:PEI) recruits endothelial progenitor cells to the sites of injection. It was observed an increased expression of genes specific to endothelial progenitor cells as *CD34, CD44, CD133, KDR* and others. Experiments also indicate that SHH stimulates angiogenesis and proangiogenic effect depends on a plasmid dose and time of stimulation. This work was partially supported by a grant WND-POIG 01.03.01-14-036/09.

P068

Controlling the environment in order to induce eye-cup formation from embryonic stem cells

Sarah Decembrini, Yvan Arsenijevic

Jules-Gonin Eye Hospital, UNIL, Lausanne, Switzerland

Retinal degenerative diseases resulting in the loss of photoreceptors are a major cause of blindness. Recently, different groups validated the possibility to reactivate retinal circuits of degenerating retinas focusing on approaches aimed to create/replace new photoreceptors. Among them, photoreceptor replacement therapy may be feasible since transplanted photoreceptors, collected directly from the developing or the adult retina, have been shown to restore some visual function in degenerating retinas. Because the developing retina is not a suitable source of renewable photoreceptors, we focused on embryonic stem cells (ESCs) for their capacity to generate retinal progenitors and photoreceptor cells. In this study we examined a recently published protocol for its potential to give rise to 3D-optic cups from a three-dimensional culture of mouse ESC aggregates. RT-PCR, immunostaining, activation of a retinal progenitor-specific reporter, the *Rx-gfp* transgene, were used to assess the rate of optic cup formation. Since we observed that the *in vitro* self-formation of optic cups is a process completely out of control depending at least of ESC strain, batch of matrigel, dimension of manually isolated optic vesicles, rendering impossible to predict the number and rate of optic cups produced in each single experiment, we are developing a "bio-microfluidics" chip in order to form gradients of morphogens and acquire a tight and reproducible control of signalling responsible for the optic cup induction and development. We are currently testing bimodal delivery of morphogens to induce optic cup and retinal pigment epithelium formation.

P069

A heat-activated and ligand-dependent gene switch for the spatiotemporal control of regenerative transgenes

FM Martin-Saavedra[1,2], C Wilson[3], R Voellmy[4], N Vilaboa[1,2], R Franceschi[4]

[1]*Hospital Universitario La Paz-IdiPAZ, Madrid, Spain,* [2]*Networking Research Center on Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN, Madrid, Spain,* [3]*Periodontics & Oral Medicine, School of Dentistry University of Michigan, Ann Arbor-MI, USA,* [4]*HSF Pharmaceuticals S.A., La Tour-de-Peilz, Switzerland*

The development of methods for spatial and temporal control of therapeutic transgenes is a major challenge for the gene therapy field, yet such approaches can provide unique capabilities for controlling tissue regeneration. We report here a novel gene switch that can provide for spatial and temporal control of transgene expression in virtually any desired anatomical localization. This switch utilizes the strictly heat-regulated human HSP70B promoter to trigger expression of a rapamycin-activated transactivator that maintains its own expression by an autoregulatory loop and transactivates a target gene in the presence of rapamycin or its non-immunosuppressive analog, AP21967. To test the functionality of this genetic tool, we generated murine C3H10T1/2 cell lines stably expressing firefly luciferase (fLuc) or human Vascular Endothelial Growth Factor 165 (hVEGF165) under the control of the gene switch. Heat-activation of the cells in the presence of the inducers increased fLuc activity or VEGF secretion by several orders of magnitude, while the transgene activity induced by heat or drug alone was negligible. Engineered C3H10T1/2 cells were subcutaneously implanted on the backs of mice using an in situ-polymerizable fibrin scaffold. Bioluminescence imaging revealed strong activation of the fLuc-switch exclusively in implants subjected to heat-shock in the presence of rapamycin/AP21967. Similarly, implants seeded with cells harboring the hVEGF165-switch promoted a marked angiogenic response in adjacent host tissues subsequently to the activation of the gene circuit. These results highlight the potential of this therapeutic approach in scenarios of regenerative medicine where spatial and temporal bioavailability of growth factors plays a fundamental role.

P070

Effects of fucoidan on osteogenic differentiation of human adipose tissue-derived stem cells in cocultures with endothelaial colony-forming cells

Jessica Pereira[1], Sophie Portron[2], Isabelle Galy-Fauroux[1], Sylvia Colliec-Jouault[3], Anne-Marie Fischer[1,5], Pierre Weiss[2], Jérôme Gicheux[2], Dominique Helley[1,4]

[1]*UMR S765, INSERM, Université Paris Descartes, Sorbonne Paris Cité, Paris, France,* [2]*INSERM U791, Université de Nantes, LIOAD, Nantes, France,* [3]*IFREMER, Laboratoire de Biotechnologie et Molécules Marines, Nantes, France,* [4]*Université Paris Descartes, Faculté de Médecine, Paris, France,* [5]*AP-HP, Hôpital Georges Pompidou, Paris, France*

The aim of our study is to associate endothelial colony-forming cells (EPC) and human adipose tissue-derived stem cells (hATSC) to induce vascularization to promote a better bone matrix formation. To improve bone defect repair we used the fucoidan, a sulfated polysaccharide able to bind angiogenic growth factors, to enhance pro-angiogenic capacities of EPC and osteogenic differentiation of hATSC.

hATSC and EPC were pretreated in osteogenic medium (OM) or endothelial growth medium (EGM-2), respectively, with or without fucoidan (10 μg/mL). Then, hATSC, EPC or both (ratio 70/20) were cultured in OM, EGM-2 or OM + EGM-2 supplemented or not with fucoidan. Fucoidan increased the formation of calcium deposits at 7 days for hATSC ($p < 0.05$) and at 4 days for hATSC co-cultivated with EPC ($p < 0.05$). By immunofluorescence staining, we have shown that fucoidan induced the formation of mineralized nodules by hATSC associated with EPC at J4. To determine whether these effects on hATSC were due to factors synthesized by EPC, we used a second model of culture. hATSC were cultured in OM + EGM-2 or supernatants of EPC cultured or EPC-hATSC co-cultured. After 4 days of culture, the formation of nodules rich in calcium deposits were observed only when hATSC were cultivated in media of EPC culture or EPC-hATSC co-culture.

In conclusion, our data suggest that in presence of fucoidan, EPC are able to synthesize molecules, such as cytokines, which enhance osteogenic differentiation of hATSC. Identification and quantification of these cytokines are in progress.

P071

Potential of lentiviral transduced adipose tissue-derived stem/stromal cells toward hemophilia B using cell sheet technology

Natsumi Watanabe[1], Kazuo Ohashi[1], Inkyong Shim[1], Kohei Tatsumi[1], Rie Utoh[1], Kazuko Kanegae[1], Yuji Kashiwakura[2], Tukasa Omori[2], Yoichi Sakata[2], Makoto Inoue[3], Teruo Okano[1]

[1]*Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University (TWIns), Tokyo, Japan,* [2]*Research Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University School of Medicine, Tochigi, Japan,* [3]*DNAVEC Corporation, Ibaraki, Japan*

Hemophilia B is an inherited bleeding disorder, caused by a deficiency of biologically active coagulation factor IX (FIX). Here we report the potential of adipose tissue-derived stem/stromal cells (ADSCs) for a self-inactivating (SIN) simian immunodeficiency virus (SIV) vector-mediated human FIX (hFIX) gene transduction for a gene and cell therapy toward hemophilia B. Mouse ADSCs (mADSCs) were transduced with SIV-hFIX vector *in vitro* at multiplicities of infection (MOIs) from 1 to 60. hFIX protein production was increased in a MOI-dependent manner from MOI 1 to 30. However, cell-toxicity associated with the SIV vector was observed at MOIs of 30 and higher. We investigated hFIX secretion per cell and mADSC cell numbers at day 4, 8, 19 and 28 after transduction. Although hFIX production decreased to one-third of the levels at day 4, it is important to note that hFIX secretion were sustained for 4 weeks after transduction *in vitro*. It was found that mADSCs of MOI 10 produced the largest amount of hFIX protein at day 8 after transduction among the 4 time points, showing necessity of future challenges to keep protein expression level. We then conducted cell sheet-based tissue engineering approach using transduced mADSCs. Cell-sheets made of hFIX-transduced mADSCs were fabricated with temperature-responsive culture dishes, and were transplanted into the subcutaneous space of mice. Transplanted cell-sheets were successfully engrafted, resulting in a detection of hFIX protein in the recipient mice plasma. This study demonstrated the possibility of genetically modified ADSCs for cell and gene therapy toward secreting-protein deficiency including hemophilia.

P072

Expression and activity of microRNAs during neural stem cell differentiation

Daniela Corno[1], Annalisa Lattanzi[1], Bernhard Gentner[1], Tiziano Di Tomaso[1], Luigi Naldini[1,2], Angela Gritti[1]

[1]*TIGET, San Raffaele Scientific Institute, Milan, Italy,* [2]*Vita-Salute San Raffaele University, Milan, Italy*

Several neuronal- and astroglial-specific microRNAs (miRNAs) are known but the identification of miRNAs modulating somatic neural stem (NSC) biology is still elusive. Here we looked for miRNAs enriched in NSC/early progenitor cells and their potential role in regulating self-renewal and/or lineage restriction. We used global miRNA expression profile to select miRNAs whose expression is up- or down-regulated in NSC-derived populations and miRNA-regulated bidirectional lentiviral vectors (bdLV.miRT; Brown et al, 2007) to monitor the activity of shortlisted miRNAs. Results of global miRNA profiling led us to focus on miR-93 and miR-125b, whose function in neural cells has not been fully elucidated. Both miRNAs were enriched in NSCs and progenitors. miR-93 and miR-125 expression and activity were significantly down- and up-regulated, respectively, along differentiation. Immunofluorescence confocal analysis on bdLV.miRT-transduced NSC cultures indicated increase of miR-125b activity during lineage committment and persistent acivity in 40% of GFAP+ astrocytes and in 20% of immature TUJ1+ neurons. Activity of miR-93 was high during all the differention steps in the majority of nestin+ immature glial cells and GFAP+ astrocytes, while it was low in the neuronal compartment. In the attempt to elucidate the role of these miRNAs in modulating NSC function we are currently evaluating NSC self-renewal and multipotency upon miR-125b and miR-93 perturbation obtained using a "squelching" approach based on the saturation of miRNA target sequences by LV-mediated miRT overexpression, with consequent decrease of endogenous miRNA expression/activity. This work lays the framework for studying regulation of transgene expression in specific neural compartments.

P073

Isolation, characterization and hepatocyte differentiation of human adult progenitor cells from liver and pancreas

Cedric Duret[1], Irena Iankova[1], Jeanne Ramos[2], Sabine Gerbal-Chaloin[1], Jean-Michel Fabre[3], Anne Wojtusciszyn[4], Patrick Maurel[1], Martine Daujat-Chavanieu[1]

[1]*INSERM U1040, Montpellier, France,* [2]*CHRU Guy de Chauliac, Montpellier, France,* [3]*CHRU St Eloi, Montpellier, France,* [4]*CHRU Lapeyronie, Montpellier, France*

During embryogenesis liver and pancreas are generated from a common endodermic precursor. Furthermore, adult hepatocyte clusters could be observed in pancreatic tumors. These observations raised the hypothesis that progenitors from the endoderm developmental stage could persist during adult life. In this study we established a similar strategy for isolation, proliferation and differentiation of human progenitor cells from adult liver and pancreas.

Organ fragments were dissociated with collagenase and extracted cells were seeded in medium for human adult hepatic progenitor cell proliferation. After 4–7 days, various cellular populations appeared. The proliferation medium was replaced at confluence by the hepatocyte differentiation medium. We observed an increased expression of hepatocyte genes albumin and alpha-1-antitrypsin, and a decreased expression of pancreatic genes insulin and glucagon. At day 21, clusters of positive cells for albumin were observed.

In a second set of experiments, we isolated a population negative for CD105 and CD90 antigens after 7–14 days of amplification. The populations from both liver and pancreas had an identical morphology, close membrane antigen expression profiles, and expressed endodermic progenitor cell antigens CD24, CD49f and temporary AC133, EpCAM. In the hepatocyte differentiation medium expression of albumin and CYP3A4 mRNAs increased while insulin and glucagon gene expression was not detected. At day 21 positive clusters for albumin were observed.

In conclusion, we showed the feasibility to isolate a progenitor cell population with hepatocyte differentiation capacity from human adult liver and pancreas. This progenitor population could be a model for basic research and a cell source for biotherapy.

P074

Aggressive malignant tumors are revealed during *in vivo* differentiation of partially reprogrammed human induced pluripotent stem cells

O Féraud[1], L Tosca[1,2], N Oudrhiri[1], E Gobbo[1], AG Turhan[1,3], A Bennaceur-Griscelli[1,4], F Griscelli[1,5]

[1]*INSERM UMR-S 935, ESTeam Paris-Sud, Villejuif, France,* [2]*Hôpitaux Universitaires Paris-Sud, APHP, Service of histology and embryology, Clamart, France,* [3]*Université de Poitiers et Service d'Hématologie et d'Oncologie Biologique, Poitiers, France,* [4]*Hôpitaux Universitaires Paris-Sud, APHP, Service of Hematology, Villejuif, France,* [5]*Laboratoire de Génétique, Université de Paris Descartes, Paris, France*

We have generated by lentivirus-mediated gene transfer of four (Oct4, Sox2, Klf4, c-Myc) or six (Oct4, Sox2, Klf4, c-Myc, Nanog, Lin28) transcriptional factors, two types of induced Pluripotent Stem Cells (iPSCs) from different kinds of somatic cells: fully reprogrammed iPSCs with a silencing of the all the transgenes and partially reprogrammed iPSCs that still express one or several transgenes. We have assessed their behaviour during their differentiation and proliferation using *in vivo* teratoma assays in NOD/SCID mice. We report that in contrast to fully reprogrammed iPSCs, partially reprogrammed iPSCs, generated major dysplasia and malignant tumors with Yolk Sac Tumor and Embryonal Carcinoma positives for AFP, cytokeratin AE1/AE3 and CD30 that correlated with the expression of one or several transgenes used for the reprogramming. Furthermore major genomic instability with several clusters of amplifications and deletions, were only observed in the teratomas suggesting that reprogramming by pluripotency factors can lead to a pre-malignant phenotype whose full potential can be revealed only in teratoma assays. In addition aggressive osteosarcoma, adenocarcinoma and undifferentiated carcinoma all showing hyperchromatic nuclei with prominent nucleoli and numerous mitosis were also revealed by this teratoma assay.

Therefore, it will be pertinent to explore a large series of iPSCs generated by non integrative approaches or recombinant proteins and plasmids and to develop alternative methods such as gene profiling or micro RNA patterns and perhaps epigenetic screening to improve the safety of iPSC before future potential applications in regenerative medicine.

P075

Dual differentiation of human mesenchymal stem cell driven by transient serum exposure regimes.

Louise France, Colin Scotchford, David Grant, Hassan Rashidi, Alex Popov, Virginie Sottile

Wolfson Centre for Stem Cells, Tissue Engineering and Modelling (STEM), School of Clinical Sciences, The University of Nottingham, Nottingham, UK

Human mesenchymal stem cells (MSCs), which can generate both osteoblasts and chondrocytes, represent an ideal resource for orthopaedic repair through autologous and allogeneic regenerative medicine approaches. One major difficulty for the development of osteochondral constructs using undifferentiated MSCs is that serum is typically used in culture protocols to promote differentiation of the osteogenic cell type, whereas existing chondrogenic differentiation protocols require the use of serum-free conditions. In order to define treatment conditions which could be compatible with both chondrogenic and osteogenic differentiation in a single bioreactor, we have analysed the efficiency of new biphasic differentiation regimes based on transient serum exposure followed by serum-free treatments. MSC differentiation kinetics were assessed either in serum-free medium or with a range of transient exposure to serum, and were compared to standard continuous serum-containing treatment. Although osteogenic differentation was not supported in the complete absence of serum, marker expression and extensive mineralisation analyses revealed that 5-day transient exposure was able to trigger a level of differentiation comparable to that observed when serum was present throughout. This initial phase of serum exposure was further shown to efficiently support the successful chondrogenic differentiation of MSCs, comparable to controls maintained in serum-free conditions throughout. This study reveals that a new culture model based on temporal serum exposure followed by serum-free treatment is compatible with both osteogenic and chondrogenic differentiation of MSCs. These results now underpin the development of novel regenerative medicine strategies for osteochondral repair using MSCs.

P076

Generation of transgene-free hiPSCs from peripheral blood of Pyruvate Kinase Deficiency patients for gene correction by Meganuclease mediated Homologous Recombination

Zita Garate[1,2], Oscar Quintana-Bustamante[1,2], Laura Cerrato[1,2], Roman Galetto[3], Fátima Rodriguez-Fornes[1,2], Luren Poirot[3], Tabita Maia[4], Leticia Ribeiro[4], Noemi Fusaki[5], Guillermo Guenechea[1,2], Agnes Gouble[3], Juan Bueren[1,2], Jose Carlos Segovia[1,2]

[1]*Differentiation and Cytometry Unit. Hematopoiesis and Gene Therapy Division. Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT), Madrid, Spain,* [2]*Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain,* [3]*Cellectis therapeutics, Paris, France,* [4]*3Serviço de Hematologia, Centro Hospitalar de Coimbra, Coimbra, Portugal,* [5]*DNAVEC Corporation, Tsukuba, Japan*

Patient specific induced-Pluripotent Stem Cells (iPSC) together with gene correction by Homologous Recombination (HR) is a promising alternative for regenerative medicine. However there are several safety and methodological issues, like the need of preventing the persistence of reprogramming genes in the genome of iPSCs and the development of more stable iPSC clones from accessible tissues, that should be solved before iPSC-derived cells could be used in the clinic. Here, we have selected non-integrative RNA Sendai viruses (SeVs) as reprogramming vectors and used peripheral blood mononuclear cells (PBMNC) from patients suffering from Pyruvate Kinase Deficiency (PKD), a human disease affordable to be modeled both *in vitro* and *in vivo*. Transduction of PBMNCs from two healthy donors and two PKD patients with SeVs carrying the 4 reprogramming factors (*OCT3/4, SOX2, KLF4, c-MYC*) allowed the generation of iPSC clones with higher efficacies than using lentiviral vectors. Pluripotent characteristics of PBMNC-derived hiPSCs were confirmed by immunofluorescence, flow cytometry and 96 pluripotency-associated genes expression array. Integrated SeVs were not found in PBMNC-hiPS lines after 15 passages, as deduced from RT-PCR analyses. Aiming the gene correction of these PKD-iPSCs, a meganuclease-mediated gene targeting approach has been designed. Preliminary studies in 293T cells showed that a meganuclease that specifically targets the PKLR locus generated double strand brakes in this locus with efficiency close to 15%, as determined by surveyor assay. This study shows the generation of integration-free hiPSC lines from PKD PBMNCs for PKD modelling and for the development of safer gene targeting strategies.

P077

SFHR correction of cystic fibrosis induced pluripotent stem (iPS) cells derived from airway submucosal gland epithelial cells

RG Sargent[1,2], Y Yang[2], A Lee[1,2], S Kim[2], A Bedeyat[2], L Jamali[2], P Renz[1,2], Z Qi[1], S Suzuki[1], MJ Yezzi[1,2], MO Meunch[3,1], L Ye[1], WE Finkbeiner[1], JW Yu[1], YW Kan[1], DC Gruenert[1,2]

[1]*University of California, San Francisco, San Francisco, CA, USA,* [2]*California Pacific Medical Center Research Institute, San Francisco, CA, USA,* [3]*Blood Centers research Institute, San Francisco, CA, USA,* [4]*University of Vermont, Burlington, VT, USA*

P078

Sleeping Beauty transposon-based system for cellular reprogramming and targeted gene insertion in induced pluripotent stem cells

Ivana Grabundzija[1], Jichang Wang[1], Attila Sebe[2], Zsuzsanna Erdei[2], Robert Kajdi[3], Anantharam Devaraj[1], Tobias Cantz[4], Axel Schambach[4], Christopher Baum[4], Zsuzsanna Izsvak[1], Balazs Sarkadi[2], Zoltan Ivics[3,1]

[1]*Max Delbrück Center for Molecular Medicine, Berlin, Germany,* [2]*Hungarian Academy of Sciences, Budapest, Hungary,* [3]*Paul Ehrlich Institute, Langen, Germany,* [4]*Hannover Medical School, Hannover, Germany*

P079

Generation of traceable "factor-free" induced pluripotent stem cells

J Kuehle[1], D Hoffmann[1], JD Suerth[1], S Turan[1], D Zychlinski[1], M Galla[1], T Maetzig[1], T Cantz[2], M Grez[3], C Klein[4], C Baum[1], A Schambach[1]

[1]*Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany,* [2]*JRG Stem Cell Biology, Hannover Medical School, Hannover, Germany,* [3]*Georg-Speyer-Haus, Frankfurt, Germany,* [4]*Children's Hospital, Ludwig-Maximilians University, Munich, Germany*

Technologies to generate induced pluripotent stem cells (iPSC) have progressed from integrating vectors to transient methods which avoid integration of foreign DNA into the host genome. We and others have proposed that the use of optimized integrating vectors allows robust generation of high quality iPSC and that integrated sequences can even be exploited for downstream applications like cell therapy, where detection of iPSC progeny is of great importance. To achieve this, we developed an efficient lentiviral reprogramming vector with a polycistronic codon-optimized cassette to express the reprogramming factors (OCT4, KLF4, SOX2, c-MYC) along with a fluorescent marker. Flp recombinase target sites were inserted into the promotor- and enhancer-deprived "self-inactivating" (SIN) LTR to allow conditional excision.

Targeting adult fibroblasts from wildtype and different knockout strains that model neutrophil disorders (e.g. p14cond.KO and gp91phoxKO), we reproducibly obtained murine iPSC with low vector copy number. Next, we excised the reprogramming cassette using retroviral particle-based protein transfer of Flp recombinase, and validated the maintenance of pluripotency and hematopoietic differentiation potential. Furthermore, using LM-PCR we mapped the remaining clone-specific integration site of the residual SIN LTR in our iPSC, often finding intergenic regions that represent potential safe harbors. After subcutaneous injection for teratoma formation in immunodeficient mice, we determined iPSC contribution to different tissues with high sensitivity (>1 in 10000 cells) using quantitative PCR.

In summary, optimized integrating vectors can be used to generate "factor-free" induced pluripotent stem cells with a clonotypic and traceable genetic identity.

P080

Generation of Induced tissue-specific stem cells by de-differentiation of somatic cells using non-integrative viral vectors

J Lelorier[1], N Moniaux[2], C Remus[1], E Gobbo[1], O Féraud[1], A Bennaceur-Griscelli[1,3], F Griscelli[1,4]

[1]*INSERM UMR-S 935, ESTeam Paris-Sud, Villejuif, France,* [2]*INSERM U 785, Centre HepatoBiliaire, Hôpital Paul Brousse, Villejuif, France,* [3]*Hôpitaux Universitaires Paris-Sud, APHP, Service of Hematology, Villejuif, France,* [4]*Laboratoire de Génétique, Université de Paris Descartes, Paris, France*

Adult tissue-specific progenitors represent an attractive approach for the generation of normal and pathological cells *in vitro* and to generate new tissue. We have developed a new generation of tissue-specific stem cells called" induced Tissue Stem Cells" (iTSCs) by de-differentiation of somatic cells using non-integrative vectors encoding several embryonic reprogramming factors (oct4/Sox2/Klf4/cMyc) enabling a partial reprogramming process.

We have recently produced iTSCs of endodermic origin from murine hepatocytes and produced more than 50 clones and subclones capable of self renewal. As expected in contrast to murine embryonic cells, all clones were negative for SSEA1 and Oct-4 and expressed endodermic markers such as GATA4, Sox17, Foxa1, Foxa2, Foxa3 and CXCR4. Furthermore, they expressed markers of liver (CK8, CK18, HNF4a, AFP) of biliary (CK7, CK19, HFN6, HNF1a, and HNF1b) and of pancreatic (Pdx1, Ngn3, Nkx6.1, Nkx2.2, NeuroD1) progenitor cells showing that there were able to differentiate into several different cell types. We have showed that endodermic iTSCs can be spontaneously differentiated into hepatocytes producing high levels of liver-specific markers (Alb, glycogen, lipid, TDO2, TAT, Cytochromes P450) and into pancreatic insulin-producing cells. *In vivo* their injections into the muscle of NOD/SCID mice have revealed the presence of two kinds of tissues, one with architecture of the hepatic parenchyma expressing albumin and another with bile duct structure.

ITSCs represent thus a new generation of tissue-specific progenitors that allow an easy way to produce tissue specific stem

cells and their derived lineages for drug and toxicity assays, and for further therapeutic applications.

P081

Patient-specific induced pluripotent stem cells (iPSCs) to model disease pathology in metachromatic leukodystrophy.

V Meneghini, G Frati, C Cavazzin, A Lombardo, L Naldini, A Gritti

San Raffaele Scientific Institute, Telethon Institute for Gene Therapy (TIGET), Milan, Italy

Metachromatic leukodystrophy (MLD) is caused by genetic defects in the activity of arylsufatase A (ARSA), a key enzyme in the catabolism of myelin-enriched sphingolipids. The establishment of an in vitro model recapitulating pathogenesis may help understanding the cellular and molecular events underlying disease manifestations. Here we report the derivation of a collection of induced pluripotent stem cell (iPSC) lines generated through somatic reprogramming of fibroblasts obtained from MLD patients and healthy donors (WT). We first transduced MLD fibroblasts with a lentiviral vector driving ARSA expression (LV.ARSA), achieving supraphysiological enzyme activity. Then, we used a monocistronic excisable LV to transfer key reprogramming genes (LV.OCT4.SOX2.KLF4), generating several stable WT, MLD and gene-corrected MLD iPSC clones characterized by the expression of pluripotency markers and by the ability to generate teratomas when injected in immunodeficient mice. Ultrastructural and molecular analyses revealed increased lysosomal storage and impaired lysosomal trafficking in MLD iPSCs. These pathological hallmarks were reduced in gene-corrected cells, indicating that transgenic ARSA expressed in iPSCs is functional, as also confirmed by specific enzymatic assays. By applying specific culture conditions we obtained iPSC-derived neural stem/progenitor-like cells (NPCs), a renewable source of cells that we are differentiating in oligodendrocytes and neurons, the relevant target cells in MLD. On iPSC-derived NPCs and differentiated cultures we are assessing functional features and we will define their biochemical, molecular and epigenetic signature. These cells may provide a powerful platform for modeling disease pathology, testing gene correction strategies and, ultimately, testing the safety and efficacy of gene/cell-based therapies.

P082

Restrictions in the generation of iPSCs from DNA-PKcs-deficient fibroblasts reveal the role of Non Homologous End Joining in Cell Reprogramming

F.J. Molina-Estévez[1], M.L. Lozano[1], S. Navarro[1], O. Quintana-Bustamante[1], Y. Torres[2], I. Grabundzija[3], Z. Izsvak[3], J.C. Segovia[1], E. Samper[2], Z. Ivics[4], J.A. Bueren[1], G. Guenechea[1]

[1]Hematopoiesis and Gene Therapy Division. Centro de investigaciones Energéticas Medioambientales y Tecnológicas (CIEMAT) and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain, [2]Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain, [3]Mobile DNA, Max Delbrück Center for Molecular Medicine, Berlin, Germany, [4]Division of Medical Biotechnology, Paul Ehrlich Institute (PEI), Langen, Germany

Generation of induced pluripotent stem cells (iPS) is having a significant impact in the establishment of disease models and will affect to regenerative medicine and gene therapy strategies in the years to come. Herein, we present data related to the reprogramming of primary mouse embryonic fibroblasts (pMEF) from severe combined immunodeficient (***scid)*** mice defective in DNA-PKcs: a key protein for Non-Homologous End Joining (NHEJ). Polycistronic lentiviral vectors carrying *Oct-3/4*, *Klf-4*, *Sox-2* plus *c-Myc* or *mCherry* were used to transduce early passage ***scid*** pMEFs. Reduced numbers of iPS-like colonies were generated from ***scid*** cells, being reprogramming efficiency decreased 4 to 7 fold from ***wt*** cells. Moreover, these ***scid*** iPS-like clones were prematurely lost or differentiated. Analysis of the transduction efficacy of ***wt*** and ***scid*** pMEFs discarded this factor as candidate to account for the low efficiency of ***scid*** reprogramming. In addition, analyses of SA-β-Gal and P16/INK^{4a} senescence markers were manifestly increased during the reprogramming process of ***scid*** pMEFs while remained unaltered in ***wt*** cells. In contrast to the limited efficacy of polycistronic LVs to reprogram ***scid*** pMEFs, polycistronic transposons harbouring the four reprogramming genes generated stable ***scid*** iPS clones, which preserved the genotype and phenotype characteristic of ***scid*** cells, such as its distinctive hypersensitivity to DNA damage. The ***scid*** iPS cell model would be useful to conduct research into the physiological consequences of the DNA-PKcs mutation during development and improve current cell and gene therapy strategies for the disease.

P083

Development of a personalized regenerative medecine using iPSC for treating inherited liver diseases: application to Crigler-Najjar type 1

Claire Poiron[1], Angélique Fourrier[1], Cécilia Abarrategui-Pontes[1], Kyle D. Fink[2], Laurent Lescaudron[3], Gary L. Dunbar[2], Anne Gaignerie[4], Anne Weber[5], Anne Dubart-Kupperschmitt[5], Khadidja Fékir[6], Ludovic Vallier[7], Philippe Labrune[8], Ignacio Anegon[1], Tuan Huy Nguyen[1]

[1]INSERM UMR 1064, CHU Hotel Dieu, Nantes, France, [2]Field Neuroscience Institute, Central Michigan University, Michigan, USA, [3]INSERM U791, CHU Hotel Dieu, Nantes, France, [4]Plateforme iPS, SFR François Bonamy, IRT-UN, Nantes, France, [5]INSERM U972, Hôpital Paul-Brousse, Villejuif, France, [6]Service de dermatologie, Hôpital Antoine Béclère, Clamart, France, [7]Anne McLaren Laboratory for Regenerative Medicine, University of Cambridge, Cambridge, UK, [8]Service de Pédiatrie, Hôpital Antoine Béclère, Clamart, France

Background: Crigler-Najjar type 1 disease (CN1) is characterized by absence of UGT1A1, resulting in hyperbilirubinemia. Allogeneic hepatocyte transplantation is a promising alternative to liver transplant, yet it suffers from major limitations. We aim to develop a personalized regenerative medecine based on induced pluripotent stem cells (iPSC) for treating CN1. The Gunn rat is an animal model of CN1, in which we showed that <1% of hepatocytes expressing transgenic UGT1A1 is therapeutic.

Methods: IPSC will be derived from skin biopsies of CN1 patients and Gunn rat fibroblasts by exogenous expression of Oct4/Klf4/Sox2/c-Myc. Using Zinc Finger Nucleases (ZFN), integration of a therapeutic cassette encoding UGT1A1 will be targeted in a safe harbor locus. Genetically-corrected IPSC will be differentiated into hepatocytes (iPS-Hep). Human and rat iPS-

Hep will be transplanted into an immunocompromised or immunocompetent Gunn rats, respectively.

Results: Skin biopsies from two CN1 patients have been scheduled in September/October and will be reprogrammed using Sendai Vectors. We constructed a donor plasmid expressing UGT1A1 for targeted vector integration into AAVS1 locus. This construct will be tested in a CN1-iPSC line that we generated using retroviral vectors. Gunn Rat iPSC were obtained using lentiviral vectors. We characterized ZFN specific for the mutated UGT1A1 exon in Gunn rat. This locus can be used to directly repair by ZFN-based gene editing or as a safe harbor.

Conclusion: This study will provide the proof-of-principle that: (i) genetically-corrected hiPS-Hep engraft and treat CN-1, a prototypic metabolic liver disorder and (ii) disease correction is maintained in immunocompetent Gunn rat using corrected Gunn iPS-Hep.

P084

Improving Retroviral Episome Transfer for transient delivery of transcription factors.

JW Schott, D Hoffmann, T Mätzig, D Zychlinski, C Baum, A Schambach

Hannover Medical School (MHH), Hannover, Germany

Retroviral Episome Transfer (RET) is based on the application of integrase-defective retroviral vectors. While this system has been widely used to achieve stable episomal gene transfer in non-dividing tissues, we improved this technology for transient delivery of transcription factors to achieve cell fate modification.

Targeted modification of cell fate enables the generation of specific cell types, which offers great potential to be applied in regenerative medicine and basic research. As the generation of both induced pluripotent stem cells (iPSCs) and induced neuronal stem cells (iNSCs) involves transfer of the transcription factor Oct4, it served as our model protein.

By performing kinetic analyses, optimal time points for repeated transduction of RET vectors in dividing cells were identified and enabled the generation of stable expression levels. Investigation of vector characteristics revealed a gamma-retroviral, LTR-driven backbone architecture to be most suitable. Expression from retroviral episomes could be enhanced by valproic acid by up to 3-fold. Oct4 performance was markedly improved by fusion to a minimal repeat of the transactivation domain of the herpes-simplex-virus viral protein 16 (VP16).

Providing proof-of-principle, the concerted action of these modifications enabled the generation of iPSCs based on the episomal transfer of Oct4, with the remaining transcription factors required for reprogramming (Sox2, Klf4, c-Myc) being provided by integrating vectors. Importantly, 70–75% of iPSC clones obtained were free of residual integration of the episomal vector.

These results provide evidence that RET is suitable to achieve transient but efficient transfer of Oct4 for cell fate modification.

P085

Induced pluripotent stem cells from home-made-mRNA transfection:a tool for stem-cell derived hepatocyte studies

Clara Steichen[1], Eléanor Luce[1,2], Joëlle Marie[3], Sylvie Goulinet-Mainot[1,2], Lucie Tosca[4], Gérard Tachdjian[4], Anne Weber-Benarous[1,2], Anne Dubart-Kupperschmitt[1,2]

[1]Inserm U972, Hôpital Paul Brousse, Villejuif, France, [2]Université Paris Sud 11, Hôpital Kremlin-Bicêtre, France, France, [3]Centre de Génétique Moléculaire, CNRS UPR 3404, Gif-sur-Yvette, France, [4]Service d'Histologie Embryologie et Cytogénétique, Hôpital Antoine Béclère, Clamart, France

Due to the lack of liver donors and the difficulty to amplify hepatocytes in vitro, stem cell-derived hepatocyte transplantation may represent aninteresting alternative to orthotopic liver transplantation, currently the only available treatment for metabolic genetic disorders of the liver. In this context, Induced Pluripotent Stem Cells (iPSCs) generated from human somatic cells are quite promising. However significant barriers must be overcome before this technology become relevant for clinical applications. One of them is the generation of iPSCs free of any exogenous DNA sequence.

We have developed inthe lab a method to generate iPSCs (adapted from the work of Warren et al.) based on repeated transfections of home-made messenger RNAs. We obtained plasmids encoding the transcription factors OCT3/4, KLF4, SOX2, c-MYC and LIN28 from stab cultures. PolyA-tails were added upon PCR to generate DNA templates that we used for in vitro transcription. Synthetic mRNAs contain modified nucleotides and are capped to ensure mRNA stability. These mRNAs have been daily transfected into human foreskin fibroblasts for 16 days. The first colonies emerged after 12 days of transfection. These iPSC clones have been characterized by immunostaining, q-PCR, embryoid bodies and teratoma formation assays etc. Our results confirmed that we did generate integration-free iPSCs having a normal karyotype.

We are currently differentiating those cells into hepatocyte-like cells. We are also applying this reprogramming method to fibroblasts obtained from biopsies of hemophilia B and familial hypercholesterolemia patients.

P086

Specific elimination of CD133+ tumor cells with retargeted oncolytic measles viruses

Tobias Abel*[1], Patricia Bach*[1], Christopher Hoffmann[2,3], Zoltan Gal[2,3], Gundula Braun[1], Iris M. Völker[1], Claudia R. Ball[4], Ian C.D. Johnston[5], Ulrich M. Lauer[6], Christel Herold-Mende[4], Michael D. Mühlebach[1], Hanno Glimm[2,3], Christian J. Buchholz[1]

[1]Paul-Ehrlich-Institut, Langen, Germany, [2]National Center for Tumordiseases (NCT), Heidelberg, Germany, [3]German Cancer Research Center (DKFZ), Heidelberg, Germany, [4]University of Heidelberg, Heidelberg, Germany, [5]Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany, [6]Medical University Hospital Tübingen, Tübingen, Germany

Tumor initiating cells (TICs) are in prime focus of current cancer research as they are supposed to improve our understanding of tumorigenesis and the development of more effective anti-tumoral strategies. The cell surface marker CD133 is frequently used to identify TICs of various tumor entities, including hepatocellular cancer and glioblastoma. Here we describe oncolytic measles viruses (MVs) retargeted to CD133. The viruses, termed MV-141.7 and MV-AC133, infected and lysed selectively CD133-positive tumor cells. Notably, both viruses exerted strong anti-tumoral effects on human hepatocellular carcinoma derived subcutaneously or intraperitoneally growing multifocal tumors with several animals remaining virtually tumor-free over long observation periods. Thus, the CD133-targeted viruses were

substantially more effective in prolonging survival than the parental virus MV-Nse which is closely related to oncolytic MVs currently assessed in clinical trials. Interestingly, target receptor overexpression or increased spreading kinetics through tumor cells were excluded as causative for the enhanced oncolytic activity of CD133-targeted viruses. Moreover, MV-141.7 was also effective in an orthotopic glioma tumor sphere and a primary colon cancer mouse model. The data demonstrate that CD133-targeted MVs selectively eliminate CD133-positive cells from tumor tissue. They do therefore form a key tool for future cancer research and therapy.

P087

Human tumor cells treated with exosomes derived from mature dendritic cells (DCs) are able to induce *in vitro* sensitization of naïve human T lymphocytes against Exo-treated and untreated tumor cells

GG Romagnoli, PA Toniolo, IK Migliori, BZ Barbosa, PC Bergami-Santos, JA Barbuto

University of Sao Paulo, Sao Paulo, SP, Brazil

Exosomes (Exo) are secreted nanovesicles that originate from multivesicular bodies and contain proteins and genetic material, which can be transferred between cells and contribute to their communication in the body. In the immune system, Exo originated from DCs contain many of the molecules involved in antigen presentation and are able to induce direct and indirect lymphocyte responses. In this work we isolated Exo from mature DCs and used them to treat tumor cells from the breast cancer cell line, SK-BR-3. The incorporation of the DC-originated Exo by the tumor cells was confirmed by fluorescence microscopy and by flow cytometry, which showed the transfer of antigen presentation-associated molecules to tumor cells. These Exo-modified tumor cells were used for the *in vitro* sensitization of CD3+ peripheral blood lymphocytes (from the same donors of the DCs) and their response to a challenge with tumor cells was then evaluated by their cytokine secretion pattern, measured by CBA and ELISPOT. Sensitized T cells' production of interleukin-6 was increased three times when challenged with tumor cells (treated or not with Exo originated from DCs), in comparison to the production induced by phytohemagglutinin. Likewise, the production of interferon-gamma induced by the challenge with tumor cells (treated or not with Exo) was significantly higher when T cells had been sensitized with Exo-modified tumor cells. These data indicate that tumor cells treated with Exo from mature DCs are able to induce sensitization of T cells against tumor antigens, a phenomenon that could be exploited in immunotherapeutic protocols against cancer.

P088

Dendritic-tumor cell hybrids in therapeutic vaccination against advanced neuroblastoma

PC Bergami-Santos[1], L Cristofani[2], V Odone-Filho[2], JA Barbuto[1]

[1]*Institute of Biomedical Sciences-USP, Sao Paulo, Brazil,* [2]*Faculty of Medicine-USP, Sao Paulo, Brazil*

Neuroblastoma is a common intra-abdominal tumor in children that may have an aggressive behavior and poor response to therapy, needing, therefore, new therapies. Monocyte-derived dendritic cells (Mo-DCs) can be applied in the development of immunotherapy against cancer, but in cancer patients present phenotypic and functional changes that impair their potential to induce effective responses. To circumvent this, Mo-DCs from healthy donors can be fused to patients' tumor cells and, after irradiation, used to initiate anti-tumor responses. Fused cells maintain the expression of both tumor and Mo-DC markers. Furthermore, the heterokaryons seem to survive and proliferate better than non-fused cells (both tumor and DCs) in culture. When fused cells were utilized to stimulate patients' lymphocytes, they were able to induce the production of a distinct cytokine pattern, characterized by a higher IFN-gamma and a lower IL-4 production. We report further, the preliminary results of a protocol using this vaccination strategy in patients with histological diagnosis of neuroblastoma. Tumor samples from 63 patients were obtained, processed into single cell suspensions and stored for later use. Seventeen patients already received the vaccination, nine patients after autologous bone marrow transplantation (as included in the Brazilian neuroblastoma treatment protocol NEURO-X-2008). Clinical responses ranged from complete clinical remission to none. The average number of monthly doses received is 3.3 (one dose/month) and no significant adverse side effects were noted. Thus, the further characterization of the responses induced by this treatment could establish this approach as an effective treatment modality for patients with neuroblastoma.

P089

MicroRNA regulation of oncolytic adenoviruses for selective targeting of pancreatic tumors

Xavier Bofill-De Ros, Anabel José, Cristina Fillat

Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) and Centro de Investigación Biomédica en Red (CIBERER), Barcelona, Spain

Misregulation of microRNAs is a defining hallmark of tumorogenesis. While the expression of some microRNAs is universally altered in tumors, often unique patterns reflect the lineage dependency of tumors, related to their tissues of origin. MicroRNA expression profiling comparing healthy pancreas with pancreatic pathologies such as chronic pancreatitis and pancreatic tumors has led to the identification of several deregulated microRNAs. In the present work, we have analyzed the expression of several miRNAs in a wide range of human and mouse pancreatic cell lines and in a variety of tumor samples; and validated few candidates that are expressed in the pancreas but not in the tumoral tissue. We have explored the feasibility to exploit pancreas specific microRNAs to provide oncoselectivity to replication-competent adenoviruses. Firstly, we have studied the ability of such microRNAs to regulate transgene expression in luciferase vectors incorporating several combinations of miRNA with variable number of target sites. Optimal miRNA target sites combinations have been used to engineer wild type adenovirus (Ad5) regulating E1A gene. We show that E1A expression in adenoviral-infected cells was controlled according to microRNA expression. Moreover, we provide evidences that the modified adenoviruses retained full activity in pancreatic cancer cells whereas they showed attenuated effects in non-tumor cells. Furthermore pancreatic intraductal administration of Ad-wt or Ad-miR-controlled viruses into wild type animals demonstrated pancreas de-targeting effects with Ad-miR viruses. These data highlights that oncolytic adenoviruses engineered with specific pancreas miRNAs while retaining full lytic potency can provide with enhanced safety in pancreatic cancer treatment.

P090

Sstr2A: a relevant target for the delivery of genes into human glioblastoma cells using fiber-modified adenoviral vectors

M Colin[1,2], K Lécolle[1,2], S Bégard[1,2], R Caillierez[1,2], D Demeyer[1,2], E Grellier[1,2], A Loyens[1,2], Z Csaba[3], JC Beauvillain[1,2], JC D'Halluin[1,2], M Baroncini[1,4], JP Lejeune[1,4], A Sharif[1,2], V Prévot[1,2], P Dournaud[3], L Buée[1,2]

[1]*Inserm UMR837, Lille, France,* [2]*Univ. Lille Nord de France, UDSL, Faculté de Médecine, IMPRT, JPARC, Lille, France,* [3]*Inserm UMR676, Université de Médecine Denis Diderot-Paris 7, Paris, France,* [4]*CHRU, Team of neurosurgery, Lille, France*

Glioblastomas are the most aggressive of the brain tumors occurring in adults and children. Currently available chemotherapy prolongs the median survival time of patients by only 4 months. The low efficiency of current treatments is partly due to the blood–brain barrier, which restricts the penetration of most drugs into the central nervous system. Locoregional treatment strategies thus become mandatory. In this context, viral tools are of great interest for the selective delivery of genes into tumoral cells. Gliomas express high levels of type 2 somatostatin receptors (sstr2A), pinpointing them as suitable targets for the improvement of transduction efficiency in these tumors. We designed a new adenoviral vector based on the introduction of the full-length somatostatin (SRIF) sequence into the HI loop of the HAdV fiber protein. We demonstrate that (i) HAdV-5-SRIF uptake into cells is mediated by sstr2A, (ii) our vector drives high levels of gene expression in cells expressing endogenous sstr2A, with up to 65-fold enhancement and (iii) low doses of HAdV-5-SRIF are sufficient to infect high-grade human primary glioblastoma cells. Adenoviral vectors targeting SRIF receptors might thus represent a promising therapeutic approach to brain tumors.

P091

Retroviral Replicating Vector (RRV)-Mediated Prodrug Activator Gene Therapy With Codon-Optimized Nitroreductase

SA Collins[1], CR Logg[1], A Inagaki[1], K Hiraoka[1], S Kamijima[1], K Herman[1], PF Searle[3], M Tangney[2], N Kasahara[1]

[1]*Department of Medicine, University of California Los Angeles (UCLA), Los Angeles, California, USA,* [2]*Cork Cancer Research Centre, University College Cork, Cork, Ireland,* [3]*Cancer Research UK Institute for Cancer Studies, University of Birmingham, Birmingham, UK*

Our studies to date have demonstrated significantly enhanced survival benefit when tumor-selective retroviral replicating vectors (RRV) are employed for prodrug activator ('suicide') gene therapy in a variety of cancer models. Clinical testing of RRV-mediated gene therapy using yeast cytosine deaminase (CD) is currently underway in a multi-center Phase I/II trial for patients with recurrent glioblastoma multiforme in the United States. In this study, we employed RRV to deliver modified versions of another prodrug activator gene, *E.coli* nitroreductase, which activates the prodrug CB1954, to a potent bifunctional alkylating agent. We constructed RRV encoding wild-type *E.coli* NTR genes (RRV-NfsA, RRV-NfsB) as well as NTR variants extensively modified to optimize human codon usage and vector stability (RRV-NAO, RRV-NBO). Sequence optimisation was confirmed to increase genomic stability of RRV-NAO and RRV-NBO viruses upon serial passage in U87 human glioma cells. Next, *in vitro* cytotoxicity was examined by MTS assay after CB1954 treatment of U87 cells transduced with each vector. After only 24-hr CB1954 exposure, viability was reduced by >80% in glioma cells transduced with RRV-NAO, which showed the most potent cell killing efficiency and bystander effect among the 4 vectors tested. Repeated exposure of RRV-NAO transduced cells to CB1954 resulted in further reduced cell viability in comparison with a single exposure, confirming that an intact virus reservoir remains following initial prodrug treatment. These data indicate that we have been successful in developing an improved prodrug activator gene with potential for therapeutic efficacy when delivered by RRV in experimental models of human glioma.

P092

Using oncolytic viruses for the targeted therapy of pancreatic adenocarcinoma: a pre-clinical study.

Hubert Lulka[1], Naima Hanoun[1], Hervé Berthommé[2], Alberto Epstein[3], Louis Buscail[1], Jean-Luc Béjot[4], Pierre Cordelier[1]

[1]*INSERM U1037, Cancer Research Center of Toulouse, Toulouse, France,* [2]*Bioviron, Villeurbanne, France,* [3]*CGMC - UCB Lyon I, Villeurbanne, France,* [4]*Karcinolys SAS, Rueil-Malmaison, France*

Recent technical advances have improved the tumor specificity of oncolytic viruses, leading to the development of new weapons for the war against cancer. Viruses derived from HSV-1 replicate in cancer cells, leading to their lysis, sparing healthy cells. In the present study, we examined for the first time whether these viruses may impair pancreatic adenocarcinoma (PDAC) tumor growth, a deadly disease with no cure. We used Myb34.5 virus in which the γ34.5 gene is controlled by B-myb-tumor specific promoter. We infected human PDAC-derived cell lines with Myb34.5, wild-type virus (HSV-F) and MGH-1 mutant virus not expressing γ34.5. Our results demonstrate that HSV-F, Myb34.5, and to a lesser extent MGH-1, replicate efficiently in PDAC-derived cell lines to provoke their lysis, with Myb34.5 being more active than MGH1. We then measured the antitumor effect of Myb34.5 in an orthotopic model of human PDAC developed in athymic mice. Myb34.5 injection does not cause acute toxicity or morbidity of tumor-bearing animals. The intratumoral injection of increasing doses of Myb34.5 and its repeated systemic injection,combined or not with gemcitabine chemotherapy, strongly inhibit tumor progression. In conclusion, we demonstrate Myb34.5 replicates efficiently in PDAC-derived cells and inhibits their proliferation. In vivo, Myb34.5 is very well tolerated and strongly inhibits cancer progression not only after intratumoral injection but also following systemic injection. The latter results strongly suggest that systemic Myb34.5 treatment may impair metastatic progression. This preclinical study stems for the use Myb34.5 for the treatment of patients diagnosed with advanced forms of pancreatic cancer.

P093

mRNA sonoporation as a novel tool in dendritic cell-based cancer immunotherapy

Heleen Dewitte[1], Sandra Van Lint[2], Stefaan De Smedt[1], Karine Breckpot[2], Ine Lentacker[1]

[1]*Lab for General Biochemistry and Physical Pharmacy, Faculty of Pharmacy, Ghent University, Ghent, Belgium,* [2]*Laboratory of*

Molecular and Cellular Therapy, Department Immunology-Physiology, Medical School of the Free University of Brussels (VUB), Jette, Belgium

Since the discovery of dendritic cells (DCs) and their role in immunity, DC vaccination has become a new and emerging strategy in cancer immunotherapy. By ex vivo loading a patient's DCs with antigens (as proteins, peptides or nucleic acids) and injecting them as a vaccine, specific antitumor immune responses can be induced.

In this work, we explored the use of mRNA sonoporation as a novel transfection technique for DC vaccination. Sonoporation makes use of microscopic gas bubbles that respond to pressure differences created by ultrasound waves. By adding mRNA-loaded microbubbles to DCs and exposing them to ultrasound, the microbubbles locally implode, causing a dual effect; (1) the cell membranes at the implosion site are locally damaged (sonoporated) and simultaneously (2) the mRNA is released and can be internalized through the created pores.

Our data demonstrated efficient ultrasound-triggered transfection of murine DCs in vitro [1]. Furthermore, in vivo assays showed that injection of DCs sonoporated with either mOVA alone or combined with TriMix (a mix of 3 mRNAs that modulates the DC's functionality [2]) induced strong proliferation of adoptively transferred OT-I cells. Upon injection of these sonoporated DCs into mice with pre-existing OVA-expressing tumor cells, tumor outgrowth was significantly slowed down.

These data provide proof that mRNA sonoporation can be used in DC vaccination. Importantly, this technique offers interesting perspectives towards future in vivo intranodal transfection of DCs, which could circumvent expensive and time-consuming ex vivo procedures.

1. Biomaterials, 2011. 32(34): p. 9128–9135.
2. Cancer Research, 2012. 72(7): p. 1661–1671.

P094

A safe and effective gene- and cell-based platform for the targeted delivery of IFN-α to tumors by infiltrating macrophages

G Escobar[0,2], D Moi[0,2], A Ranghetti[0,2], R Mazzieri[0,2], L Naldini[1,2]

[1]*Vita Salute San Raffaele University, Milan, Italy,* [2]*Angiogenesis and Tumor Targeting Research Unit, San Raffaele Telethon Institute for Gene Therapy, Milan, Italy*

TEMs (Tie2-expressing monocytes) are recruited to tumors, where they promote angiogenesis. We previously exploited their tumor-homing ability and selective expression of the angiopoietin TIE2 receptor to turn TEMs into IFN-α delivery vehicles by engineering hematopoietic stem cells (HSCs) to express an IFN-α transgene under the control of the *Tie2* promoter. This cell-based strategy strongly inhibited tumor growth in mice. Because TIE2 is also expressed by HSC, we developed vectors regulated by miR-126/130a, which de-target transgene expression from HSC and may thus reduce their exposure to the IFN-α transgene. The improved safety of these vectors was shown by monitoring cell cycle and long-term repopulating activity of HSC in mice. Detargeting from HSC did not affect the ability of TEM-mediated IFN-α delivery to inhibit both primary and secondary spontaneous mammary tumors. We then constructed miR-regulated vectors carrying the human TIE2 enhancer/promoter and IFN-a sequence and tested them in human cord blood $CD34^+$ cells transplanted in NSG mice. Human hematopoietic chimerism was not impaired by transduction with the TIE2-IFN vector. Moreover, when mice were orthotopically injected with human breast cancer cells, IFN-a activity was induced at the tumor site, as shown by upregulation of IFN-inducible genes. Importantly, by providing human IL-7, IL-15 and GM-CSF we improved the reconstitution of human T, NK and myeloid cells in the NSG mice, and achieved effective antitumor response upon transplantation of TIE2-IFN transduced HSC. These results illustrate the therapeutic potential of a gene- and cell-based human platform for the specific delivery of IFN-α to treat established tumors.

P095

CD80/IL-2 mediated inhibition of multiple myeloma induced suppression of NK cell activity

Giulia Giunti[1], Lucas Chan[1], David Darling[1], Sharham Kordasti[1], Linda Barber[1], David Malone[1], Farzin Farzaneh[1], Stephen Schey[2]

[1]*Department of Haematological Medicine, King's College London, London, UK,* [2]*Department of Haematology, King's College Hospital, London, UK*

Natural Killer (NK) cells provide a potentially useful strategy for the immune therapy of multiple myeloma (MM). However, NK-cell based therapies have so far provided limited clinical benefit, possibly reflecting the various escape mechanisms employed by tumour cells to avoid NK cell recognition.

Recent studies have shown that myeloma cells utilise different mechanisms to impair NK and T cell functions. Important amongst these is the reduced expression of CD80 on dendritic cells.

In the present study, we demonstrate that MM cell lines are able to impair the activity of NK cells isolated from healthy donors. This inhibition, which correlates with the downregulation of activating receptors such as NKG2D, NKp30 and DNAM-1, is the product of direct, contact-dependent interactions between MM and NK cells without the need for other pheripheral blood components.

On these basis, we have examined the ability of genetically modified MM cells, engineered to express CD80 and IL-2, to enhance and recover NK cell functions. Our results show that CD80/IL-2 expressing MM cells are able to expand NK and T cell numbers in vitro, and to induce a significant increase in the fraction of NK cells expressing activatory receptors such as NKp44, NKG2D and CD69, when compared to unmodified MM cells. More importantly for potential therapeutic applications, the stimulated NK cells show increased cytolytic activity against unmodified MM cells.

Therefore these data suggest that CD80/IL-2 expressing MM cells may provide a suitable strategy for NK cell stimulation and the induction of broad ranging immunological responses against MM cells.

P096

EGFR-specific retargeting of a dendrimer-coated adenovirus carrying the sodium iodide symporter (NIS) for systemic radiovirotherapy of liver cancer

Geoffrey Grünwald[1], Alexandra Vetter[2], Kathrin Klutz[1], Michael Willhauck[1], Reingard Senekowitsch-Schmidtke[3], Markus Schwaiger[3], Burkhard Göke[1], Ernst Wagner[2], Per Holm[4], Manfred Ogris[2], Christine Spitzweg[0]

[1]*Department of Internal Medicine II – Campus Grosshadern, University Hospital of Munich, Munich, Germany,* [2]*Pharmaceutical Biotechnology, Department of Pharmacy, Center for System-Based Drug Research, and Center for Nanoscience, Ludwig-Maximilians University, Munich, Germany,* [3]*Department of Nuclear Medicine, University Hospital Klinikum rechts der Isar, Munich, Germany,* [4]*Institute of Experimental Oncology, University Hospital Klinikum rechts der Isar, Munich, Germany*

Systemic administration of adenovirus leads to rapid clearance from the blood stream due to liver pooling of virus particles, induction of immune response, and unspecific biodistribution in non-target organs, thereby limiting its therapeutic use. To overcome these hurdles, polymer coating of adenoviruses has become a promising strategy in order to develop shielded and targeted gene delivery vectors.

To generate a shielded, targeted and armed vector that evades the unfavorable normal virus tropism, we physically coated a replication-selective adenovirus carrying the theranostic sodium iodide symporter (NIS) gene with a conjugate consisting of cationic PAMAM dendrimer linked to the peptidic, epidermal growth factor receptor (EGFR)-specific ligand GE11 for targeted radiovirotherapy of hepatocellular carcinoma.

Intravenous injection of the uncoated adenovirus led to a high level of radioiodine accumulation in the liver of mice due to hepatic pooling of the vector as shown by ^{123}I scintigraphy, which was significantly reduced after dendrimer coating. Evasion from liver pooling resulted in increased transduction efficiency in xenograft tumors. EGFR-specific targeting was confirmed by reduced transduction efficiency in the absence of the targeting ligand. A significantly enhanced oncolytic effect was observed following i.v. application of dendrimer-coated adenovirus in a liver cancer xenograft mouse model (virotherapy), that was further increased by additional treatment with a therapeutic dose of ^{131}I (radiovirotherapy) and was associated with markedly improved survival. These results demonstrate restricted virus tropism and tumor-selective retargeting after systemic application of dendrimer-coated, EGFR-targeted adenoviruses thereby representing a promising innovative strategy for improved systemic NIS gene therapy.

P097

Dual-targeting of oncolytic measles viruses to EGFR-positive tumor cells using DARPins and MMP

Jan Hanauer[0], Katrin Friedrich[0], Larissa Angebauer[0], Steffen Prüfer[0], Christian Jost[0], Andreas Plückthun[0], Klaus Cichutek[0], Michael D. Mühlebach[0]

[1]*Oncolytic Measles Viruses and Vectored Vaccines, Paul-Ehrlich-Institut, Langen, Germany,* [2]*Institute of Biochemistry, University of Zurich, Zürich, Switzerland*

Measles viruses (MV) are naturally cytolytic by induction of giant syncytia. Vaccine-derived MV exhibit pronounced cytotoxicity in tumors, as their ubiquitously expressed receptor CD46 is usually up-regulated in tumor cells. To further combine the intrinsic oncolytic potential of MV with enhanced safety, we aimed to generate recombinant MV fully retargeted to the tumor marker EGFR and simultaneously dependent on activation by tumor-associated proteases.

We generated a chimeric viral attachment protein composed of a mutated MV-H protein with ablated natural receptor binding fused to three EGFR-specific DARPins with different receptor affinities. By replacing the H in oncolytic MV by the DARPin-H we could generate fully EGFR-retargeted recombinant MV, which have been characterized for specificity, stability, and oncolytic efficacy. The αEGFR-DARPins resulted in specific and efficient function of the recombinant DARPin-H proteins, analysed using a receptor-transgenic CHO-cell panel. Conservation of the anti-tumoral potency of retargeted MV-EGFR was demonstrated *in vitro* by infection of a panel of EGFR-positive human tumor cell lines, and the most potent targeted virus eliminated xenograft tumors in SCID-mice. Thereby, the DARPin-MV with the highest EGFR-affinity revealed comparable efficacy as the parental non-targeted virus.

To further reduce off-target toxicity e.g. in naturally EGFR expressing healthy tissue, we combined entry-targeting with protease-targeting by generating MV containing DARPin-H and MMP-activatable F-proteins (Springfeld et al 2006, Mühlebach et al 2010). We were able to rescue these dual-targeted viruses, which revealed significant cytotoxicity on $EGFR^+/MMP^+$ tumor cells.

DARPins can be used for efficient retargeting and dual tumor-targeting of MV against EGFR expressing tumors.

P098

Evaluation of membrane-anchored versions of interleukin-12 as transgenes in armed oncolytic adenoviruses

Maria Bunuales[1], Jose Ignacio Quetglas[1], Eva Garcia-Aragoncillo[1], Raquel Casado[1], Sergia Bortolanza[1], Jesus Prieto[1,2], Ruben Hernandez-Alcoceba[1]

[1]*FIMA, Pamplona, Navarra, Spain,* [2]*CIBERehd, Pamplona, Navarra, Spain*

Oncolytic viruses armed with immunostimulatory transgenes are attractive therapeutic agents because a synergy between the direct cytolytic effect of the virus and the activation of the immune response against tumors is expected. We have developed oncolytic adenoviruses (OAVs) adapted as vectors for interleukin-12 (IL-12). However, high expression of IL-12 mediated by the replicative vector causes severe toxicity in permissive hosts such as Syrian hamsters and prevents the use of high viral doses.

With the aim of increasing their therapeutic index, we have performed modifications in the expression cassette to reduce systemic exposure to the cytokine. Target sequences for microRNA 122 were introduced in the 3′UTR of IL-12 to prevent expression in hepatocytes. In addition, membrane anchoring of IL-12 was attempted by incorporation of the glycosylphosphatidylinositol (GPI)-anchor signal from the folate receptor or a transmembrane (TM) domain from the CD4 protein.

In vitro transfection of these therapeutic cassettes in HEK293 cells indicates that both the GPI-anchor signal and the TM domain are able to increase the amount of IL-12 displayed in the surface of producing cells. However, only the TM domain reduced the release of IL-12 in the supernatant of cells. Incorporation of the miR122 target sequence efficiently inhibited the expression of the cytokine in hepatocyte-derived cell lines such as HuH-7.

We conclude that microRNA-mediated limitation of IL-12 expression combined with membrane anchorage in producing cells are feasible methods to reduce systemic exposure to the cytokine. In vivo experiments using OAVs encoding these modified transgenes are underway to validate this approach.

P099

Efficient oncolytic activity induced by the CD46-expressing adenoviral vector depends on the cumulative cellular conditions of survival and autophagy.

Sujin Kang

Institute for Cancer Research, College of Medicine, Yonsei University, Seoul, Republic of Korea

Abstract: Transduction efficiency of adenovirus serotype 5(Ad5) is poor in several cancer cell types because of low expression of Ad5 receptors. To overcome this limitation, the fifer region of Ad serotype 5, except its tail, was replaced by Ad serotype 35(Ad35). Unexpectedly, the chimeric Ad5/35 did not show any significant enhancement of transduction efficiency. Reason was that CD46, a receptor for Ad35, was expressed in relatively small amounts, too. Therefore, we investigated the factor that induces the transduction efficiency in cancer. We found that the tumor transduction efficiency of Ad5/35 was enhanced in the presence of rapamycin, an autophagy inducer. Analysis of the survival potential and cell proliferation rate of cancer cells revealed that higher survival potential with rapamycin was responsible for a greater oncolytic effect induced by Ad5/35.

P100

Prodrug activator gene therapy for brain tumors by Retroviral Replicating Vectors delivered via allogeneic cell carriers: translational update

Shuichi Kamijima, Michelle Hickey, Akihito Inagaki, Kei Hiraoka, Masamichi Takahashi, Quincy Tam, Emmanuelle Faure-Kumar, Janet Treger, Brooke Bogan, Colin Malone, Kate Erickson, Yuki Kato, Linda Liau, Carol Kruse, Noriyuki Kasahara

University of California, Los Angeles (UCLA), Los Angeles, CA, USA

We have previously demonstrated highly efficient tumor-selective replication and therapeutic efficacy of prodrug activator ('suicide') gene therapy delivered by retroviral replicating vectors (RRVs). In the United States, an improved RRV expressing the yeast cytosine deaminase (yCD) prodrug activator gene is now being evaluated in multicenter Phase I/II clinical trials for patients with recurrent glioblastoma. To further improve the effiency of intratumoral vector dissemination and extend this approach to multi-focal malignancies, we are pursuing studies to employ alloreactive cytotoxic T lymphocytes (alloCTL) and human mesenchymal stem cells (MSCs) as tumor-homing cellular carriers that will produce and deliver RRV to multiple tumor sites. AlloCTL activated against HLA expressed in brain tumors are also currently being tested in clinical trials, a strategy that takes advantage of the lack of HLA expression in normal brain parenchyma to achieve tumor-selective allorecognition and killing at high effector:target (E:T) ratios, while alloCTL engineered to be RRV producer cells show highly efficient vector transmission to tumor cells at even at very low E:T ratios. The combined effect results in highly effective tumor suppression and prolonged survival benefit in intracerebral tumor models in vivo. In parallel studies, we are also developing clinically translatable methods to engineer allogeneic human mesenchymal stem cells (MSC) to serve as RRV carrier cells, and we have observed highly efficient intratumoral vector dissemination and transduction associated with accelerated therapeutic benefit after MSC-mediated RRV delivery in preclinical glioma models. These approaches hold promise for clinical development of "off-the-shelf" allogeneic cell carriers mediating tumor-infiltrating RRV delivery.

P101

Chemovirotherapy of malignant melanoma with an armed and targeted oncolytic measles virus

JK Kaufmann[1], S Bossow[2], C Grossardt[2], S Sawall[2], J Kupsch[3], P Erbs[4], AH Enk[5], G Ungerechts[2], DM Nettelbeck[1]

[1]Helmholtz University Group Oncolytic Adenoviruses, German Cancer Research Center and Heidelberg University Hospital, Heidelberg, 69120, Germany, [2]Department of Translational Oncology, National Center for Tumor Diseases, German Cancer Research Center, Heidelberg, 69120, Germany, [3]RAFT Institute of Plastic Surgery, Mount Vernon Hospital, Northwood, HA6 2RN, UK, [4]Transgene S.A, Strasbourg, 67082, France, [5]Department of Dermatology, Heidelberg University Hospital, Heidelberg, 69115, Germany

We report the generation of a fully retargeted melanoma-specific oncolytic measles virus (MV) for the treatment of malignant melanoma. It is engineered to enter cells specifically via the High Molecular Weight Melanoma-Associated Antigen (HMWMAA), a surface molecule expressed on >90% of resected melanoma specimens. To this end, an optimized single-chain antibody against HMWMAA was C-terminally fused to an otherwise "receptor-blind" attachment protein H.

Viral spread led to syncytia formation specifically in melanoma cell cultures, and infection efficacy correlated with HMWMAA surface expression levels. Virtually no off-target infection was detected and viral replication was attenuated 1000-fold in HMWMAA-negative control cells. In an *ex vivo* approach, living tissue cultures of human melanoma metastases were susceptible to infection with the HMWMAA-retargeted reporter virus.

A strong cytotoxic effect with up to 100% cell killing was achieved after infection of melanoma cells with a retargeted MV encoding the yeast-derived prodrug-activating enzymes cytosine deaminase and uracil phosphoribosyltransferase (FCU1) and addition of 5-fluorocytosine (5-FC). Prodrug conversion was highly efficient, as the transfer of supernatant from cells infected with MV-FCU1-HMWMAA and treated with 5-FC exerted potent bystander killing of uninfected melanoma cells.

A chemovirotherapeutic treatment regimen of melanoma xenograft tumors in immunodeficient mice led to a three-fold reduction of average tumor volume. This translated into a highly significant prolongation of survival as compared to mock-treated animals.

The highly selective MV-FCU1-HMWMAA is the first armed and retargeted oncolytic virus for advanced melanoma and may become a potent building block of novel therapies in the future.

P102

A Del1 peptide improves the efficiency of FasL gene therapy with a non-viral vector in a mouse explanted tumor model

Hisataka Kitano[1], Atsushi Mamiya[2], Masamichi komiya[1], Shinichiro Kokubun[2], Chiaki hidai[2]

[1]*Division of Oral Surgery, Nihon University School of Medicine, Tokyo, Japan,* [2]*Department of Biomedical Sciences, Nihon University School of Medicine, Tokyo, Japan*

Background: The FasL gene is one of the target genes of cancer gene therapy. In this study, we present a novel method for maintaining a high concentration of the FasL protein around target cells, which employs the extracellular matrix protein, Del1. The Del1 protein is composed of five domains; E1, E2, E3, C1 and C2. The C1 domain is essential for the deposition of Del1 in the ECM. The E3 domain can increase Del1 transfection efficiency and, at high concentrations, it induces apoptosis. In the present study, mouse explanted tumors were treated with a non-viral vector that contained DNA encoding this fusion protein.

Methods: Cells of the human oral squamous cell carcinoma cell line, SCCKN, were injected into nude mice to generate explanted tumors. cDNAs encoding FasL, or a fusion of FasL and E3C1 (FasL-E3C1) were inserted into pcDNA3D and injected into the tumors every 7 days with a transfection reagent, jet-PEI.

Results: All of the mice that were treated with the negative control vector or the FasL-encoding vector died over the 49-day observation period. In contrast, 83% of the mice that were treated with the FasL-E3C1-encoding vector survived.

Conclusion: Fusion of an E3C1 fragment with FasL improved the efficiency of gene therapy of mouse explanted tumors using a non-viral vector. Repeated local injection of a DNA encoding a fusion protein of FasL and E3C1 using this vector could be a novel approach to decreasing tumor volume and improving the prognosis of cancer patients.

P103

A role of microRNAs for sensitization to cisplatin in ovarian cancer cell lines

Saki Kondo[1,2], Yumi Kanegae[1], Nic Jones[2], Izumu Saito[1]

[1]*Institute of Medical Science, University of Tokyo, Tokyo, Japan,* [2]*The Paterson Institute for Cancer Research, Manchester, UK*

Common treatment strategies for ovarian cancer include debulking surgery in combination with chemotherapy, however, the acquisition of clinical drug resistance is the major challenges in patient survival.

Several mechanisms have been suggested to participate in conferring platinum-resistant properties to a tumour cell. Recently, it has been proposed that aberrant epigenetic marks can critically contribute to the acquisition of drug resistance. Several genes are identified to be silenced after acquisition of cisplatin resistance. We are focusing on microRNAs (miRNAs) since they are also silenced in expression due to hypermethylation of host genes.

In this study, we identified a miRNA, which was silenced in several cisplatin-resistant ovarian-cancer cell lines and ectopic expression of the miRNA sensitized cells to cisplatin, the miRNA would be very important for acquisition of chemoresistance in ovarian cancer.

P104

[^{18}F]-FDG-PET versus CT for Evaluation of Oncolytic Virus Treatment in Advanced Cancer Patients

Anniina Koski[1], Anu Koskela[2], Helena Ahtiainen[3], Anne Roivainen[3], Heidi Liljenbäck[3], Minna Oksanen[1], Kaarina Partanen[2], Leena Laasonen[2], Kalevi Kairemo[2], Akseli Hemminki[1]

[1]*Cancer Gene Therapy Group, University of Helsinki, Helsinki, Finland,* [2]*International Comprehensive Cancer Center Docrates, Helsinki, Finland,* [3]*Turku PET Centre and Turku Center for Disease Modeling, University of Turku, Turku, Finland*

Evaluating the efficacy of oncolytic virus treatments is problematic as approaches developed for chemotherapeutics may be poorly applicable. Particularly, viruses armed with immunostimulatory transgenes may inflict significant inflammation, resulting in tumor swelling. [^{18}F]-FDG positron emission tomography (PET) is a promising alternative for computer tomography (CT) in response evaluation, as it measures tumor metabolism instead of size. Our aim was to compare PET and CT as predictive and prognostic markers in cancer patients treated with oncolytic viruses.

We used an immunocompetent Syrian hamster model with orthotopic pancreatic tumors to investigate PET imaging and possible false positive PET signals after intratumoral oncolytic adenovirus treatments. PET signals from hamster tumors generally corresponded well to tumor size, but highly positive lymph node signals were detected in hamsters treated with GMCSF expressing viruses.

In addition, 20 patients with advanced cancer were treated with oncolytic adenoviruses in the context of an Advanced Therapy Access Program (ATAP) (ISRCTN 10141600) and both diagnostic CT (RECIST 1.1 criteria) and FDG-PET imaging were performed prospectively, to compare response evaluation by these methods. Further, retrospective response and survival data of all imaged patients treated with oncolytic adenoviruses in the context of ATAP were analyzed ($n \approx 150$) to evaluate the power of CT and PET separately in assessing prognosis. In patient treatments, responses from the imaging methods were concordant. However, PET imaging seemed more sensitive than CT in predicting disease control and was a better surrogate of overall survival. Therefore, [^{18}F]-FDG-PET may be a valuable method for response evaluation in oncolytic adenovirus treatments.

P105

A library for microRNA target identification by drug selection

Carol A Kreader, Kevin P Forbes, Adam McKillip

Sigma-Aldrich, St. Louis, MO, 63103, USA

miRNAs regulate gene expression post-transcriptionally by inhibiting mRNA translation, and frequently, destabilizing the targeted mRNA. A single miRNA may regulate several hundred mRNAs to control a cell's response to developmental and environmental signals. Identifying and validating target mRNAs is essential in determining a miRNA's role and function in these pathways. However, target identification is not straightforward because, in animals, miRNAs and their target sites are not fully complementary. In silico predictions identify many valid miRNA targets, however, most predicted genes fail experimental validation and many actual targets are not predicted. Our approach to identification of functional targets of a miRNA via an experimental method pairs a dual selection technology with a comprehensive cDNA library to develop what we call the **Mission® Target ID Library**. Here we present preparation of the library and its use to identify targets of miR-373.

P106

Oncolytic Sindbis Virus: blood stability and microRNA control

G Kueberuwa, L W Seymour

University of Oxford, Oxford, UK

Background: Sindbis Virus (SV) displays natural tumour selectivity and anti-tumour activity. During transmission from infected mosquitoes to humans, virus particles are exposed to blood. As such, we hypothesised that SV may have significant stability in human blood, making it compatible with intravenous (IV) delivery.

Human infection with SV can cause long lasting pathological symptoms. Our study looks to prevent this by introducing binding sites for tissue-specific microRNAs (miRs) to exert RISC mediated degradation of SV genomic and subgenomic RNA in possible sites of human toxicity, whilst retaining full stability and replication competence within cancer cells.

Methods: SV has been evaluated *in vitro* for stability in blood and *in vivo* for anticancer efficacy following IV delivery. Also, miR binding sites were engineered into SV. These sites are complementary sequences to miRs 122, 133, 142 and 206; tissue specific miRs present in cell types that are possible contributors to pathological symptoms during SV infection.

Results: Whole human or mouse blood had very little effect on the ability of SV to infect cells *in vitro*. IV delivery to C57BL/6 mice achieved effective inhibition of tumour growth in the syngeneic, B16 metastatic melanoma model *in vivo*.

Inhibition of SV replication, transgene expression and cell killing was successful in liver, muscle and dendritic cells by endogenously expressed miRs or 293 cells transfected with pre-miRs.

Conclusion: SV is compatible with IV delivery and miR mediated attenuation provides a means to alleviate possible pathological side effects without affecting its potency as an oncolytic agent.

P107

Novel mucosal orthotopic pre-clinical murine model of HPV-associated head and neck cancers for the validation of anti-tumoral immunotherapy

Rodney Macedo[1], Geraldine Lescaille[1,2], Véronique Mateo[1], Claude Baillou[1], Bertrand Bellier[1], François Lemoine[1]

[1]*Université Pierre et Marie Curie–Université Unité Mixte de Recherche, Centre National de la Recherche Scientifique 7211/ INSERM 959, Paris, France,* [2]*Université Denis Diderot, Paris, France*

Papillomavirus (HPV) infection has been recently associated with oropharyngeal head and neck cancers (HNCs), particularly HPV16 serotype that express the E6 and E7 oncoproteins. The licensed vaccines are efficient for the prevention of HPV infection, but not for established tumors. Therefore innovative curative vaccines targeting HPV oncogenes are required. Previously, we have developed a DNA-based vaccine strategy using plasmo-VLP carrying a non-oncogenic mutated, but immunogenic, form of E7 from HPV16 (pVLP-E7). We showed that pVLP-E7 can elicit specific anti-E7 immune responses and cure mice from E6/E7 HPV16-positive TC1 tumors injected subscutaneously in the flank (ectopic model).

The present study aims at developing an orthotopic model of HNCs in mice as a pre-clinical model. Thus, TC1 cells were injected into the tongue or cheek, tumor growth and anti-E7 immune response were compared to the ectopic model. The cheek model was better suited for subsequently test of immunization, than intralingual or subcutaneous tumor models, due to better survival rates and slower growth kinetics. Next, different routes of vaccination (intranasal, intra-cheek or intradermal) were tested in order to induce local and systemic immune responses and anti-tumor curative effects. Intradermal vaccination with pVLP-E7 was the better route of administration to achieve a systemic anti-E7 T-cell response. Interestingly, intradermal and intra-cheek vaccinations associated with electroporation appeared to be both equally efficient to stimulate immune and curative anti-tumoral responses by using the orthotopic model. Experiments are now in progress to better characterize our orthotopic model, and to improve the anti-tumor responses using intra-cheek vaccinations.

P108

Genetically modified free cells versus cell complexes as preventive vaccines against melanoma.

A Miguel[1], L Sendra[1], MJ Herrero[1,5], R Botella[3,6], R Algás[4], M Sánchez[1], S Aliño[1,2]

[1]*Departamento de Farmacología, Facultad de Medicina, Universidad de Valencia, Valencia, Spain,* [2]*Unidad de Farmacología Clínica, Hospìtal Universitario y Politécnico La Fe, Valencia, Spain,* [3]*Servicio de Dermatología, Hospìtal Universitario y Politécnico La Fe, Valencia, Spain,* [4]*Servicio de Radioterapia, Hospital Clínico Universitario, Valencia, Spain,* [5]*Instituto de Investigación Sanitaria La Fe, Valencia, Spain,* [6]*Departamento de Medicina, Facultad de Medicina, Universidad de Valencia, Valencia, Spain*

The objective of this work is to evaluate the antitumor response of cell complexes (tumor cells + genetically modified cells) versus free cells genetically modified in a preventive vaccination for melanoma.

Methodology: B16 mouse melanoma cells or mouse fibroblasts were transfected with GM-CSF and IL-12 genes. Complexes were formed by adding PEI (0.025 or 0.05 mg/mL 800KDa) to 2×10^5 B16 cells plus 2×10^5 modified fibroblasts mixture. For vaccination C57BL6 mice were injected with three doses of vaccine at −21, −7 and 7 days with respect to tumor implantation with 10^5 B16 cells (day 0). Each dose of vaccine consisted of 2×10^5 B16 cells transfected and irradiated or complexes. Vaccination groups were cell complexes or B16 transfected with p2F_GM-CSF, p2F_GM-CSF/IL-12, pMok_GM-CSF, p2fØ. Tumor volume was measured on different days, and blood samples were taken to quantify immunoglobulin production. Survival curves were also performed.

Results: The groups vaccinated with free cells had slower tumor growth, a longer average life and a greater production of Ig G anti-TMP. Groups vaccinated with cells transfected with p2F_GM-CSF and pMoK_GM-CSF obtained a survival at 40 days of 40% and 20% respectively compared to 0% of the other groups.

Conclusions: The groups vaccinated with genetically modified cells had a greater anti-tumor response than the vaccinated with genetically modified cell complexes. GM-CSF producing groups showed a greater anti-tumor effect and an increase in survival, while the combination with IL-12 did not seem to provide any benefit in anti-tumor response. Partially supported by SAF 2011-27002.

P109

LV-assisted shRNA Interference to CD44v6 Reverts Drug Resistance and Blocks Tumor Dissemination

Benedetta Nicolis di Robilant, Monica Casucci, Laura Falcone, Barbara Camisa, Massimo Bernardi, Magda Marcatti, Bernhard Gentner, Luigi Naldini, Fabio Ciceri, Chiara Bonini, Attilio Bondanza

S. Raffaele Hospital, Milano, Italy

Introduction: The microenvironment shapes tumor-cell biology, including resistance to therapy and promotion of metastasis. The isoform variant 6 of CD44 (CD44v6) acts as an adhesion receptor for osteopontin, as well as a co-receptor for cytokines produced by the microenvironment. CD44v6 is widely expessed on tumors, including leukemia and myeloma, and correlates with a bad prognosis

Aim: To validate a platform for shRNA interference to CD44v6 for cancer therapy

Results: CD44v6 was up-regulated on human primary leukemic blasts cultured with bone marrow (BM)-derived mesenchymal stroma cells. CD44v6 up-regulation associated with acquired drug resistance, which could be blocked with a CD44v6 mAb. Acquired drug resistance was independent from adhesion to osteopontin and could not be blocked with a VEGF mAb. Aiming at interfering with all signals converging to CD44v6, we therefore generated specific shRNAs and expressed them in LVs optimized for the transduction of hematopoietic cells. LV transduction completely and specifically silenced CD44v6 expression. Despite normal rates of in vitro proliferation, CD44v6-silenced leukemic blasts failed to initiate leukemia in xenografted NSG mice in competitive assay. CD44v6 also played a role in acquired drug resistance of myeloma cells. CD44v6-silenced myeloma cells failed to engraft when infused i.v., but gave rise to big plasmocytomas when injected intra BM. Interestingly, CD44v6-silenced plasmocytomas only grew locally, without disseminating to the remaining of the body

Conclusions: CD44v6 plays a major role in acquired drug resistance and appears to mediate widespread dissemination of local tumors. Targeting CD44v6 with LV-assisted shRNA interference may be of therapeutic value in cancer

P110

Induction of anti-tumor immune responses by oncolytic vaccinia virus armed with CD40-ligand

Suvi Parviainen[1], Marko Ahonen[1], Iulia Diaconu[1], Akseli Hemminki[1], Vincenzo Cerullo[0,2]

[1]*Cancer Gene Therapy Group, Molecular Cancer Biology Program & Transplantation Laboratory & Haartman Institute & Finnish Institute for Molecular Medicine, University of Helsinki, Helsinki, Finland,* [2]*Laboratory of Immunovirotherapy, Division of Biopharmaceutics and Pharmacokinetics, Faculty of Pharmacy University of Helsinki, Helsinki, Finland*

Oncolytic vaccinia virus is an attractive platform for immunotherapy. In addition to potent lytic effect, oncolysis releases tumor antigens and provides co-stimulatory danger signals which can activate the immune system against tumor cells. Arming the virus is an efficient strategy to improve the immunotherapeutic effect. CD40 ligand (CD40L, CD154) can suppress tumor cell proliferation, induce apoptosis of tumor cells and triggers several immune mechanisms. One of these is a T-helper type 1 (Th1) response which leads to activation of cytotoxic T-cells and reduction of immune suppression. CD40L has also been shown to be a potent activator of antigen-presenting cells. Therefore, we constructed a vaccinia oncolytic virus expressing human soluble CD40L (vvdd-CD40L), which also includes the td-tomato fluorochrome for detection of the virus.

We show effective expression of functional CD40L both *in vitro* and *in vivo*. In a xenograph model of bladder carcinoma sensitive to CD40L treatment, we show that tumor growth was significantly inhibited by the oncolysis and apoptosis exerted by vv-DD-CD40L following both intravenous and intratumoral administration of the virus. In a CD40-negative tumor model the virus lost its advantage over the unarmed virus. We also show that the vv-DD-CD40L promotes more immunogenic form of cell death compared to the control virus by assessing a panel of immunogenic cell death markers such as HMGB1 and ATP release and calreticulin exposure.

In summary, oncolytic vaccinia virus coding for CD40L showed potent oncolytic anti-tumor activity and it was able to stimulate multiple immune-responses.

P111

Cooperation of chemokin with extracellular matrix fragment to inhibit tumour angiogenesis, lymphangiogenesis and lymph node metastasis.

Anne-Catherine Prats[1], Loic Van den Berghe[2], Audrey Rayssac[1], Nadéra Ainaoui[1], Florent Morfoisse[1,2], Françoise Pujol[1], Andreas Bikfalvi[3], Hervé Prats[2], Barbara Garmy-Susini[2]

[1]*TRADGENE EA 4554 Université Paul Sabatier, Toulouse, France,* [2]*Inserm U1037, Toulouse, France,* [3]*Inserm U1029, Talence, France*

Anti-angiogenic and anti-lymphangiogenic drugs slow tumour progression and dissemination. However, an important difficulty is that tumour reacts and compensates to obtain the blood supply needed for tumour growth and lymphatic vessels to escape to distant loci. Therefore, there is a growing consensus on the requirement of multiple anti-angiogenic proteins to stop cell invasion efficiently.

Here we developed IRES-based bicistronic lentivectors and adeno-associated virus derived vectors (rAAVs) to co-express anti-angiogenic molecules, targeting different angiogenesis pathways, i.e. the matrix proteolysis fragments endostatin and fibstatin, and the chemokin CXCL4L1. Anti-angiogenic factors was analysed either by local delivery following transduction of pancreatic adenocarcinoma cells with lentivectors, or by distant delivery resulting from intramuscular administration in vivo of rAAVs followed by tumour subcutaneous injection. We demonstrated that fibstatin and CXCL4L1 cooperate in inhibition of angiogenesis in vitro. Such a cooperative effect of fibstatin and CXCL4L1 was also observed in tumoural angiogenesis in vivo, either by local or by distant delivery. The different anti-angiogenic molecules significantly inhibit tumour spread, however no beneficial effect was found on tumour growth inhibition using molecules combinations compared to molecule alone. In contrast we observed that fibstatin and CXCL4L1 cooperate in the blockade of tumour invasion of draining lymph nodes. Furthermore fibstatin and CXCL4L1 strongly inhibit tumour lymphangiogenesis. These data reveal the cooperation of a chemokin with a matrix proteolysis fragment resulting in strong inhibition of tumour (lymph)angiogenesis, providing new perspectives in anti-angiogenic gene therapy of invasive cancers.

P112

Gemcitabine allows tumor selective adenovirus to preserve the oncolytic enhancing properties of i-leader truncation in immunocompetent animals

Cristina Puig-Saus, Eduardo Laborda, Alba Rodríguez-García, Rafael Moreno, Ramon Alemany

Institut d'Investigació Biomèdica de Bellvitge- Institut Català d'Oncologia, Barcelona, Spain

Adenovirus i-Leader protein is a small protein with unknown function. During a bioselection of adenoviruses with enhanced replication in cancer associated fibroblasts (CAFs) we isolated a mutant with a truncated i-leader protein. This mutation increases the release of wild type adenovirus leading to more cytotoxicity and antitumor activity especially on cells resistant to adenovirus replication such as CAFs. Now we have extended these results to a tumor-selective oncolytic adenovirus (ICOVIR-15i). *In vitro*, this i-leader truncated oncolytic adenovirus is released faster to the supernatant of infected cells and shows large-plaque phenotype and increased cytotoxicity. *In vivo*, in immunodeficient mouse tumor models this i-leader truncated oncolytic adenovirus is more efficacious compared to the parental oncolytic adenovirus. Aiming at a clinical application of this mutant in pancreatic cancer, we combined it with gemcitabine. In a Syrian hamster immunocompetent tumor model only the combination of gemcitabine with the i-leader truncated oncolytic adenovirus shows antitumor efficacy, compared to single-agent treatments or to the combination with the parental virus without the i-leader mutation. These results reveal the advantage of the i-leader mutation when chemotherapy is used. We speculate on the role of cell lysis pathways or the immune system behind the synergy of i-leader truncated oncolytic adenoviruses and chemotherapy.

P113

From total success to complete failure: The outcome of VA7 virotherapy in syngeneic CT26 murine colon carcinoma model reflects clonal origin and type I interferon sensitivity of the tumors

JJ Ruotsalainen[1], MU Kaikkonen[1], JC Bell[2], AE Hinkkanen[1], MJ Vähä-Koskela[3]

[1]*A. I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, Kuopio, Finland,* [2]*Ottawa Health Research Institute, Ottawa, Canada,* [3]*Molecular Cancer Biology Program, University of Helsinki, Helsinki, Finland*

Background: Previously, Semliki Forest virus vector VA7 completely eliminated type I interferon (IFN) unresponsive, intracranial U87 human glioma xenografts while IFN responsive GL261 mouse gliomas proved refractory. Here we report that the clonal origin and type I interferon sensitivity of CT26 murine tumors determines the success of VA7 virotherapy.

Method: CT26WT and CT26LacZ tumors were infected with VA7 in vitro and in vivo while VSV-Δ51 mutant was used as a control virus. The ability of the different tumor clones to secrete and respond to type I interferon was studied. Double-flank and rechallenge in vivo experiments where the CT26LacZ and CT26WT tumors were implanted into separate flanks of the same Balb/c mice either simultaneously, or the latter after CT26LacZ infection were performed to see if cross-immunity had been induced.

Results: The viruses killed the two CT26 clones efficiently in vitro, and only CT26WT cells were sensitive to type I interferon treatment. Both tumor clones could secrete IFN beta upon infection. The in vitro results were reflected perfectly in vivo, and only CT26LacZ tumors could be eradicated upon VA7 i.t. injections. Infection of CT26LacZ tumors induced protective immunity against delayed CT26WT rechallenge in half of the treated mice, while the eradication of CT26LacZ tumors did not influence the CT26WT outgrowth in the double-flank experiments.

Conclusion: In line with our previous results using VA7 in different orthotopic mouse glioma models, the therapeutic outcome in CT26 colon carcinoma heavily relied on the type I IFN sensitivity of the cancer cells.

P114

Targeted Gene Delivery using Novel Folate-Conjugated Cationic Liposomes

Sridevi Gorle, Mario Ariatti, Moganavelli Singh

Non-Viral Gene Delivery Laboratory, Discipline of Biochemistry, University of KwaZulu-Natal, Durban, KwaZulu-Natal, South Africa

Gene therapy is fast becoming a promising strategy for the treatment of human diseases rooted in genetic disorders such as cancer. Its success largely depends on the efficient delivery of therapeutic genes into target cells in vitro and in vivo. The development of safe, efficient and targeted gene delivery vehicles that will elicit the required response in the desired diseased tissue is crucial. Liposomes have shown the potential to be ligand-conjugated and receptor targeted. Our aim is to develop and test a lipid-based system for efficient targeted gene delivery via the folate receptors found overexpressed in many malignant tumors.

Folate conjugated liposomes were prepared using cationic cholesterol derivative *N,N*-dimethylaminopropylamidosuccinylcholesterylformyl-hydrazide (MSO9), the helper-lipid, dioleoylphosphatidylethanolamine (DOPE), and $DSPEPEG_{2000}$-folate. The physicochemical characterization of the liposomes and lipoplexes were assessed by zeta sizing and TEM. DNA-binding and protection capabilities of all liposomes were confirmed by band shift assays, dye displacement assays, and nuclease protection assays. In vitro cytoxicity was determined using the MTT assay, and gene expression using the luciferase reporter gene assay in three human cell lines, nasopharyngeal carcinoma (KB), cervical carcinoma (HeLa), both folate receptor positive cells, and the embryonic kidney cells (HEK293), a folate receptor negative cell line.

Relatively low cytoxicities were observed for all cell lines with promising gene expression levels noted for the receptor positive cells. These liposome formulations are promising candidates for future *in vivo* studies and may play a significant role in gene/drug targeting to cancer cells.

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Personalized SmartDC/tWT1 immunotherapy to boost the graft-versus-leukemia reactivity against acute myeloid leukemia

B Sundarasetty[1], VK Singh[0,2], L Macke[1], T Woelfel[0,2], A Ganser[1], R Stripecke[1]

[1]*Department of Hematology, Hemostasis, Oncology and Stem Cell Transplantation, Hannover Medical School, 30625 Hannover,*

Germany, [2]3rd Medical Department, University Medical Center of the Johannes Gutenberg University Mainz, 55101 Mainz, Germany

Clinical trials to assess the ability of immunotherapy using WT1 peptides to generate immune responses in patients with AML were compromised by the low consistency and outcomes of the immunization. Thus, we addressed the feasibility of endogenous and long-lasting WT1 antigen presentation with "SmartDCs": monocytes genetically programmed with lentiviral vectors (LVs) designed to provide immune modulation and maintain antigen-loaded DCs alive, functional and activated for three weeks. The combination of transgenes expressed in the LV consisted of GM-CSF, IL-4 and truncated WT1 (lacking the zinc finger domain). Overnight LV transduction of monocytes obtained from peripheral blood induced self-differentiation of monocytes into immunophenotypically stable "SmartDC/tWT1" that were viable in Nod-Rag$^{-/-}$-IL-2gc $^{-/-}$ (NRG) mice for four weeks. In order to validate the use of SmartDC/tWT1 in the autologous or HSCT setting, we set up a patient-donor proof-of-concept model. Remission samples from an ITD$^+$ AML patient and surplus material from the donor lymphocyte infusion (DLI) obtained from the matched related donor were used to produce SmartDC/tWT1. Both recipient and donor SmartDC/tWT1 expanded the respective CD8$^+$ T cells *in vitro* (approximatelly 35-fold). *In vitro* immune monitoring for reactivity of the CD8$^+$ T cells against WT1 using K562/ HLA*A02:01$^+$/WT1$^+$ or against the primary AML blasts was confirmed by IFN-g ELISPOT and cytotoxicity assays. NRG mice challenged with K562/ HLA*A02:01$^+$/WT1$^+$ cells, immunized with SmartDC/tWT1, and infused with *in vitro* expanded CD8$^+$ T cells showed higher frequencies of persisting human CD8$^+$ T cells and delayed tumor development in comparison to control mice.

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Lentiviral vector-induced SmartDC-TRP2 for melanoma immunotherapy: immunemonitoring validation assays and GMP development

R Stripecke[1], B Sundarasetty[1], L Macke[1], R Gutzmer[2], L Chan[3], F Farzaneh[3]

[1]Dep. Hematology, Hemostasis, Oncology and Stem Cell Transplantation, Hannover Medical School, Hannover, 30625, Germany, [2]Dep. Dermatology, Hannover Medical School, Hannover, Hannover, 30625, Germany, [3]Dep. Haematological and Molecular Medicine, King's College, SE5 9NU, London, UK

"SmartDCs" are monocytes transduced overnight with lentiviral vectors (LVs) co-expressing GM-CSF and IL-4 resulting in long-lasting (>3 weeks) and persistently activated DCs. A tricistronic LV co-expressing a melanoma self-antigen (tyrosinase-related protein 2, TRP2) was sufficient to generate long-lived SmartDC-TRP2, which potently stimulated CD8$^+$ cells to inhibit melanoma growth in mice. Human SmartDC-TRP2 injected s.c. in NOD.Rag1$^{-/-}$.IL2r$\gamma^{-/-}$ mice were viable for a month *in vivo* and did not cause any signs of pathologies. Leukapheresis samples obtained from five HLA*A02:01 melanoma patients during remission were used for production and pre-clinical immune potency testing of SmartDC-TRP2. We established an *in vitro* immunemonitoring IFN-g ELISPOT assay using K562 target cells transgenic for HLA*A02:01 and TRP2. Stimulation of antigen-specific IFN-g production and cytotoxicity by CD8$^+$ T cells was confirmed (three patients). For GMP development, CD14$^+$ monocytes (CliniMACs) obtained from two independent leukapheresis were transduced with the LV (MOI 5) in a closed bag system and the cells were cryopreserved. After thawing and *in vitro* seeding we observed 50% viability a week after with approximately 2.5 copies of integrated LV per cell. A lentiviral vector production batch using a 293T master cell bank, highly pure plasmids and GMP-compliant methods is underway at Kings College London for characterization of the LV purity and stability. The pilot LV/GMP batch will be subsequently used for further *in vitro* and *in vivo* identity and biosafety characterizations of SmartDC-TRP2. With these analyses we intend to develop the SmartDC GMP platform with broad capabilities of clinical development for cancer immunotherapy.

P117

A small G protein inhibitor, bisphosphonates, produces synergistic cytotoxicity on wild-type *p53*-bearing mesothelioma with adenoviruses up-regulating the p53 expression level

Masatoshi Tagawa[1,2], Kiyoko Kawamura[1], Shinya Okamoto[3], Yuan Yuan Jiang[1,2], Masato Shingyoji[4], Yuji Tada[5], Yuichi Takiguchi[6], Koichiro Tatsumi[5], Hideaki Shimada[7], Kenzo Hiroshima[8]

[1]Division of Pathology and Cell Therapy, Chiba Cancer Center Research Institute, Chiba, Japan, [2]Department of Molecular Biology and Oncology, Graduate School of Medicine, Chiba University, Chiba, Japan, [3]Department of Biochemistry, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan, [4]Department of Thoracic Disease, Chiba Cancer Center, Chiba, Japan, [5]Department of Respirology, Graduate School of Medicine, Chiba University, Chiba, Japan, [6]Department of Medical Oncology, Graduate School of Medicine, Chiba University, Chiba, Japan, [7]Department of Surgery, School of Medicine, Toho University, Tokyo, Japan, [8]Department of Pathology, Tokyo Women's Medical University Yachiyo Medical Center, Yachiyo, Japan

Bisphosphonates are synthetic analogues of pyrophosphate and have a strong affinity for bone matrix and are currently used as an agent for hypercalcemia and osteoporosis. The third generation of bisphosphates inhibits farnesyl pyrophosphate synthetase and subsequently decreases isoprenoid pools, which results in suppressed prenylation of small G proteins since the unprenylated proteins cannot access to cell membrane. We found that zoledronic acid (ZOL), one of the third generation agents, produced cytotoxic effects on mesothelioma with the wild-type *p53* gene by inducing cleavages of caspases and then apoptosis. Furthermore, ZOL treatments induced phosphorylation of p53 at Ser 15 and elevated expression levels of p53 target molecules, suggesting a possible activation of the p53 pathways. Transfection of p53-siRNA in mesothelioma suppressed the p53 expression levels and inhibited the phosphorylation, but did not influence ZOL-mediated cleavages of caspases or apoptosis, indicating that ZOL produced p53-independent cytotoxicity. We also tested a possible p53-dependent cell death in mesothelioma since the majority possesses the wild-type *p53* gene but are defective of the *p14* gene, which leads to loss of p53 functions. Transduction of mesothelioma with adenoviruses expressing the *p53* gene (Ad-p53) or Ad defective of E1B55kDa molecules (Ad-delE1B55) produced cytotoxicity through activating p53 pathways and apoptosis. We then tested a possible combinatory use of ZOL and Ad-p53 or Ad-delE1B55 and found that the combination produced synergistic anti-tumor effects. These data collectively suggest that Ad-mediated p53 activation and inhibited

small G proteins' functions with p53-independent cytotoxicity are targets for mesothelioma treatments.

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In vivo validation of suicide gene therapy against cancer

Walid Touati[1], Johanne Seguin[2], Thi Tran[3], Eric Tartour[3], Claude Baillou[4], François M. Lemoine[4], Philippe Beaune[1], Isabelle de Waziers[1]

[1]*INSERM Unité mixte de recherche 775, Université Paris Descartes, Sorbonne Paris Cité, Faculté de Médecine, 45 rue des saints-pères 75006 Paris, France,* [2]*Unité de pharmacologie chimique génétique et imagerie, INSERM U1022, CNRS UMRS 8151, Université Paris Descartes, Sorbonne Paris Cité, Faculté de Pharmacie, 4 avenue de l'observatoire 75006 Paris, France,* [3]*INSERM U970, PARCC, Université Paris Descartes, Sorbonne Paris Cité, AP-HP, Hôpital européen Georges Pompidou, service d'immunologie biologique, 20 rue Leblanc 75015 Paris, France,* [4]*Université Pierre et Maris Curie-Université Paris06, Unité mixte de recherche CNRS 7211 / INSERM 959, Groupe hospitalier Pitié Salpêtrière, 83 boulevard de l'hôpital 75013 Paris, France*

In a "suicide gene" strategy for cancer therapy, cyclophosphamide (CPA) was as a prodrug along with a modified cytochrome P450 2B6 (CYP2B6TM)/NADPH cytochrome P450 reductase (RED) gene as produg-activating gene, vectorized in VSV-G pseudotyped lentivirus. Given the relatively low affinity of CYP2B6 for CPA (Km ~ 5 mM), CYP2B6 was modified by site directed mutagenesis in order to increase by 10 fold its catalytic efficiency to metabolize CPA into cytotoxic metabolites (CYP2B6TM). Due to heterogenous expression of RED in tumor cells, a fusion gene (CYP2B6TM-RED) was constructed allowing the expression of both proteins at high levels in tumor cells.

In vitro validation of the strategy was demonstrated by infecting human (A549) and mouse (TC1) pulmonary cell lines, both resistant to CPA, with lentiviral particles containing the CYP2B6TM-RED fusion gene. Interestingly, both CYP2B6TM-RED transduced cell lines were sensitized to lower doses of CPA (IC_{50} ~ 0,5 mM).

Using TC1 cells in an *in vivo* tumor model, non-infected and CYP2B6TM-RED transduced TC1 cells were mixed at different ratios (0%, 25%, 100%) and sub-cutaneously injected into C57Bl/6 mice. When tumor volume reached ~ 500 mm^3, mice were treated with CPA (140 mg/Kg, ip, once a week during 4/5 weeks). After 4/5 injections, a total eradication of the tumor was observed without any recovery of the tumor more than 8 weeks after the last injection. Interestingly, mice injected with 25% or 100% of CYP2B6TM-RED transduced cells exhibited roughly the same response to CPA treatment.

Also preliminary, these results open the way for a future clinical application.

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LCMV-Pseudotyped VSV-Based Systems for Treatment of Malignant Glioma

Alexander Muik[1], Roza Zehtab Jahedi[2], Yvonne Geiß[1], Catherine Dold[3], Andreas Volk[1], Ursula Dietrich[1], Hrovje Miletic[2], John Bell[4], Dorothee von Laer[3]

[1]*Georg Speyer Haus, Frankfurt am Main, Germany,* [2]*Department of Biomedicine, University of Bergen, Bergen, Norway,* [3]*Institute for Virology, Innsbruck Medical University, Innsbruck, Austria,* [4]*Ottawa Hospital Research Institute, Centre for Innovatice Cancer Research, Ottawa, Canada*

Malignant glioma is the most frequent primary brain tumor and still has a very poor prognosis. A promising new treatment approach is the use of vesicular stomatitis virus (VSV)-based oncolytic virotherapy. But, even though effective against multiple tumor entities in preclinical animal models, VSV exhibits inherent neurovirulence, which has so far hindered clinical development.

To improve glioma specificity and to abrogate neurotropism, we pseudotyped VSV with the LCMV glycoprotein (LCMV-GP). Retargeting to glioma cells while sparing neurons was successfully shown *in vitro* and *in vivo* for replication-deficient pseudotype vectors [1]. Consistently, both systemic toxicity and neurotoxicity were strongly reduced for a replication-competent rVSV(GP) in mice compared to VSV-WT. In addition, rVSV(GP) shows a potent anti-glioma effect *in vitro* and *in vivo*. Intratumoral rVSV(GP)-treatment of subcutaneous tumors led to a 100% response rate, eventually translating into long-term tumor clearance in all treated animals. Furthermore, a multi-dose systemic treatment of orthotopic glioma-xenograft-bearing mice led to effective oncolysis of the tumor with 8/9 mice showing event-free survival up to >125 days post-transplantation (dpt) compared to a median survival of 32 dpt for the PBS-treated controls. In animal models of ovarian cancer and melanoma, rVSV(GP) was also found to be effective.

Provided that the highly beneficial toxicity and efficacy profile can be confirmed in current animal studies, a clinical implementation of LCMV-pseudotyped VSV-based systems appears warranted.

[1] Muik, A.; Kneiske, I.; Werbizki, M.; Wilflingseder, D.; Giroglou, T.; Ebert, O.; Kraft, A.; Dietrich, U.; Zimmer, G.; Momma, S.; von Laer, D., *J Virol*, **2011**, 85:5679–84.

P120

Intratumoral delivery and biodistribution of oncolytic alphaviral vectors in mouse melanoma model

Jelena Vasilevska, Dace Skrastina, Svetlana Lubina, Tatjana Kozlovska, Anna Zajakina

Biomedical Research and Study Centre, Riga, Latvia

Recombinant vectors based on alphaviral replicon are promising tools in the field of oncolytic cancer gene therapy because of their strong cytopathic effect through the induction of p53-independent apoptosis. However, broad tissue tropism and transient expression make it more difficult to develop an optimal cancer treatment strategy. In this study we have investigated the intratumoral delivery and distribution of replication deficient Semliki Forest virus (recSFV) in B16 melanoma-bearing mice as a consequence of intraperitoneal (i.p.) and itratumoral (i.t.) vector administration strategies. For this purpose a group subcutaneous B16 tumor-bearing mice were i.p. or i.t. inoculated with recSFV particles encoding firefly luciferase. The tissues homogenates (heart, bran, liver, kidney, lung) and subcutaneous tumor nodules were subjected for luciferase expression analysis at 24 h postinoculation. We observed a broad recSFV distribution in mice upon i.p. vector administration and the predominant tumor targeting of recSFV upon i.t. inoculation. In order to investigate the vector distribution within subcutaneous tumor nodule, two vectors expressing green fluorescence protein (EGFP) and red fluorescence protein (DS-Red) were injected into different points

of tumor nodule. The analysis of tumor cryosections revealed only local expression of the corresponding fluorescence protein without virus intratumoral dissemination. Our results show that the i.t. vector inoculation is biosafe approach for the development of therapeutic treatment. Nevertheless the additional strategies to enhance the vector dissemination within the tumor have to be developed in future. A detailed evaluation of vector distribution properties could have an impact on cancer therapy clinical trial safety and efficacy.

P121

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P122

Efficacy of hIL10 gene transfer to infarcted pig heart

L Sendra[1], V Bodi[2], A Miguel[1], MJ Herrero[3], G Miñana[2], MJ Forteza[2], M Sanchez[1], A Diaz[4], F Chaustre[2], SF Aliño[1,5]

[1]Departamento de Farmacologia, Universidad de Valencia, Valencia, Spain, [2]INCLIVA Fundacion Investigacion Hospital Clinico, Valencia, Spain, [3]Instituto Investigacion Sanitaria La Fe, Valencia, Spain, [4]SCSIE Universidad de Valencia, Valencia, Spain, [5]Unidad de Farmacologia Clinica Hospital La Fe, Valencia, Spain

Introduction: Efficiency of gene transfer in pig cardiac tissue after induced heart infarct is evaluated. After heart infarction it develops an inflammatory environment that is able to damage healthy cells. Heart gene transfer of anti-inflammatory human IL10 gene could be a good procedure to improve infarct evolution.

Material and Methods: A balloon catheter is placed in coronary artery to blockade circulation and provoke the infarct during 75 minutes. Then, a 7F Swan-Ganz balloon wedge catheter is located in coronary sinus for retrograde injection of 50 mL of saline solution bearing p2F-hIL10 plasmid (20 μg/mL), after balloon inflation. Transfer was performed at two different flow rates, 5 and 10 mL/s and its efficiency was evaluated as hIL10 DNA and RNA copies in heart tissue. Tissue samples were collected 3 days after gene injection from both ventricles (anterior and posterior), auricles and infarct zone and were analyzed by real-time PCR.

Results: The main observations were: (i) gene is efficiently delivered in cardiomyocytes (10^1-10^2 hIL10 DNA copies/ng of total DNA); (ii) highest RNA levels (10^4-10^5 hIL10 RNA copies/μg of total RNA) were reached at 5 mL/s flow rate; (iii) infarcted zone presents higher delivery level ($>10^4$ copies of hIL10 DNA/ng of total DNA); (iv) injection does not induce heart toxicity as indicated by monitored hemodynamic parameters. Results indicate that non-invasive retrovenous injection in heart is efficient and could be a model with potential clinical application for recovery improvement. Partially supported by SAF2011-27002 project.

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Evaluation of different parameters involved in cardiac function by mri after intramyocardial administration of treatments with ASCS in a porcine ami model

Guadalupe Gómez-Mauricio[1], Antonio Bernad[3], Verónica Crisóstomo[4], María Fernanda Martín-Cancho[2], Diego Celdrán[2], Claudia Báez[4], Jose Mijares[1], Sonia Martínez-Caballero[1], Verónica Álvarez[1], Javier G Casado[1], Francisco Miguel Sánchez-Margallo[1]

[1]Stem Cell Therapy Unit, Minimally Invasive Surgery Centre Jesús Usón, Cáceres, Spain, [2]Pharmacy Service, Minimally Invasive Surgery Centre Jesus Uson, Cáceres, Spain, [3]Translational Platform, Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Cáceres, Spain, [4]Endoluminal Therapy and Diagnosis Unit, Minimally Invasive Surgery Centre Jesus Uson, Cáceres, Spain

The improvement of heart function caused by the transplantation of Adipose-Derived Stem Cells (ASCs) has been demonstrated in mice and the pro-angiogenic capacity of this cells can be mediated by the secretion of several growth factors. This work raises the possibility of combining cell and gene therapy to evaluate different treatments with these cells in a porcine acute myocardial infarct (AMI) model. The magnetic resonance imaging (MRI) was used for in vivo monitoring. In this work we created an endoluminal AMI model in four different groups of Large White pigs. The transplantation was performed, on the day of the creation of the AMI, by thoracotomy and intramyocardial administration using different allogeneic porcine ASCs (Group I: control group using saline serum; Group II: ASCs transduced with GFP and labeled with SPIO; Group III: ASCs transduced with GFP; Group IV: ASCs that overexpress growth factors IGF1 and HGF). Animals were monitored by MRI for one month. In our model a significant infarct area was observed, that progressively was reduced during the monitorization period. The labeled cells allowed us the localization of transplanted cells *in vivo*. We observed a reduction of infarct area and improved

cardiac functionality in all groups (although not statistically significant). The labeling of ASCs with SPIO was safe in short term, allowing the location of cells *in vivo*. Finally besides of the variability observed in terms of cardiac function, the result of this study suggests the need to extend the monitoring period for further conclusions.

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Histological and immunohistochemical studies after intramyocardial administration of different treatments with ASCS in a porcine ami model.

Guadalupe Gómez-Mauricio[1], Antonio Bernad[4], Verónica Crisóstomo[3], María Fernanda Martín-Cancho[2], Diego Celdrán[2], Claudia Báez[3], José Mijares[1], Sonia Martínez-Caballero[1], Verónica Álvarez[1], Javier G Casado[1], Francisco Miguel Sánchez-Margallo[1]

[1]*Stem Cell Therapy Unit, Minimally Invasive Surgery Centre Jesús Usón, Cáceres, Spain,* [2]*Pharmacy Service, Minimally Invasive Surgery Centre Jesús Usón, Cáceres, Spain,* [3]*Endoluminal Therapy and Diagnosis Unit, Minimally Invasive Surgery Centre Jesús Usón, Cáceres, Spain,* [4]*Translational Platform, 2Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid, Spain*

Several studies suggest that the angiogenic capacity in myocardial repair may be mediated by various substances secreted by the ASCs, such as growth factors. Also, superparamagnetic nanoparticles of iron oxide (SPIO) are used to mark and trace cells in vivo by MRI. On the other hand, the anatomical and physiological similarities between human and pig are essential to conduct the comparative studies in cardiac diseases. We used different treatment groups with allogeneic ASCs in porcine endoluminal AMI models and transplanted them by thoracotomy and intramyocardial administration. (Group I: control group using saline serum; Group II: ASCs transduced with GFP and labeled with SPIO; Group III: ASCs transduced with GFP; Group IV: ASCs that overexpress growth factors IGF1 and HGF, labeled with compatible fluorophores GFP and Cherry respectively (1:1)). Different histological and immunohistochemical analyses at infarcted area were performed after euthanasia. Histological studies show an angiogenesis increase in all groups studied, having obtained the best results in the animals treated with ASCs that overexpress growth factors. Immunohistochemical studies indicated the presence of GFP+ cells in all animals treated with ASCs labeled with this protein. Finally, there was a clear expression of IGF1 around the vessels, in the samples corresponding to the animals treated with ASCs-IGF1/HGF. The viability and location of all the treatments has been demonstrated after one month of monitoring. After transplantation of porcine ASCs, it was also confirmed on-going angiogenic processes in the ischemic tissue which seems to be potentiated by the local overexpression of IGF1 and HGF.

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Intracoronary administration of porcine cardiac stem cells in a porcine model of acute myocardial infarction: A preclinical safety evaluation

Claudia Báez[1], Verónica Crisóstomo[1], Jose Luis Abad[2], Virginia Nunes[2], Itziar Palacios[2], Mónica García-Lindo[1], Manuel González[1], Fei Sun[1], Guadalupe Gómez-Mauricio[1], Javier G Casado[1], Francisco Miguel Sánchez-Margallo[1]

[1]*Minimally Invasive Surgery Centre Jesús Usón, Cáceres, Spain,* [2]*Coretherapix S.A., Madrid, Spain*

Our aim was to determine in a swine model of acute myocardial infarction (AMI) a safe dose and time for the intracoronary administration of porcine cardiac stem cells (pCSC). Through a percutaneous femoral arterial approach, a myocardial infarction was created in 18 Large-White female swine by a 90 minutes balloon occlusion of the left anterior descending coronary artery immediately distal to the origin of the first diagonal branch. Intracoronary pCSC administration was carried out after a 2h reperfusion period in 8 animals injecting 25×10^6 pCSCs (n=5) and 50×10^6 pCSCs (n=3). A further 10 animals received the same cell doses but delivered 7d after AMI creation (n=5 per dose). Troponin I (TpnI) levels were measured before and after infarction, 2 and 24h after cell administration and, in the case of immediate pCSCs infusion, also at one week after cell injection. Infarct creation was successful in all animals as demonstrated by the significant increase in TpnI from baseline to reperfusion (from $0.015\pm0.014\mu g/L$ to $23.722\pm3.754\mu g/L$). TpnI levels measured 7d after cell delivery in the post-reperfusion cell administration groups were significantly higher in the animals receiving 50×10^6 pCSC ($0.323\pm0.204\mu g/L$ *versus* $2.000\pm0.707\mu g/L$); two pigs belonging to the higher dose group died post-cells. However, Tpn I showed no statistically significant differences between 25×10^6 and 50×10^6 cells when administered 7d after AMI induction. In conclusion, the intracoronary administration of 25×10^6 and 50×10^6 pCSCs appeared to be safe when performed 7d after AMI induction. Post-reperfusion delivery was safer when the lower dose was used.

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Role of neuropilins 1 and 2 in angiogenic properties of endothelial progenitor cells.

Isabelle Galy-Fauroux[1,2], Anna Lokajczyk[1,2], Catherine Boisson-Vidal[1,2], Anne-Marie Fischer[2,3], Dominique Helley[2,1]

[1]*INSERM UMR-S 765, Faculté des Sciences Pharmaceutiques, Paris, France,* [2]*Université Paris Descartes, Sorbonne Paris Cité, Paris, France,* [3]*AP-HP, Hôpital Européen Georges Pompidou, Paris, France*

Neuropilins (NRP) are transmembrane co-receptors for members of the VEGF (vascular endothelial growth factor) family. We show for the first time that both NRP1 and NRP2 are expressed not only by mature endothelial cells but also by endothelial colony–forming cells (ECFC) from cord blood which have been proposed as a tool for cell therapy in ischaemia. The ability of ECFC to mobilize and migrate to ischaemic sites appears to be an important factor in the success of cellular therapy. We have previously shown that alpha6-integrin subunit ($\alpha6$) played a major role in the proangiogenic properties of ECFC. Moreover, Lal Goel *et al.*, showed that NRP2 regulates $\alpha6\beta1$ integrin in epithelial cells. We report here that the expression of NRP1 and NRP2 do not change during the time course of culture. To understand the roles of NRP1 and NRP2, we have invalidated NRP1 or/and NRP2 by RNA interference in ECFC. Invalidation for NRP1 as well as NRP2 inhibits the wound healing showing their implication in migration. Invalidation of NRP1 induces a decrease of 30% of the proliferation of ECFC ($p<0.01$). Interestingly, invalidation of NRP1 induces a 1.75 fold increase of NRP2 expression ($p<0.01$). Invalidation of NRP2 or $\alpha6$ induces a decrease respectively, of $\alpha6$ or NRP2 expression, showing that NRP2 and $\alpha6$ expression by ECFC are linked. Our data indicates

that neuropilins play an important role in proangiogenic properties from ECFC; a better knowledge of these proteins is necessary for using ECFC in cell therapy in ischaemia.

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A novel electrospun biograft for heart function stabilization after myocardial infarction

Marie-Noelle Giraud[1], Anne-Geraldine Gueux[2,3], Aurélien Frobert[1], Stéphane Cook[1], Guiseppino Fortunato[2], Eric Koener[2], Jérémy Valentin[1], Hendrik Tevaearai[3]

[1]Cardiology, Department of Medicine, Fribourg, Switzerland, [2]Empa, Saint Gallen, Switzerland, [3]Cardiovascular Surgery, Inselspital, Bern, Switzerland

Cardiac tissue engineering is increasingly considered as a possible curative treatment after a myocardial infarction. In the current study, a novel micro-fibrous matrices, functionalised with an oxygen functional hydrocarbon coating were designed and seeded with bone marrow derived mesenchymal stem cells (MSCs) and evaluated in a rodent model of myocardial infarction.

Methods: Microfibrous polycaprolactone matrix were produced by electrospinning and enriched with an oxygen functional hydrocarbon coating in order to enhance rat MSC adhesion. The biograft was implanted two weeks post LAD ligation. Its effect on heart function was evaluated by echocardiography 4 weeks post treatment and compared with appropriate controls.

Results: Relative to pre-treatment, MSC- seeded patches induced a stabilisation of EF after 4 weeks ($49 \pm 11\%$ and $48 \pm 8\%$ respectively, $p = 0.9$) whereas cell-free matrix did not prevent progression toward heart failure (EF of $47 \pm 10\%$ and $37 \pm 4\%$ respectively, $p = 0.006$). The beneficial outcome could explained by a stabilization of remodeling and LV volumes rather than c-kit$^{(+)}$ and sca-1$^{(+)}$ cells recruitment.

Conclusion: Our data demonstrate that the implantation of MSC-seeded, plasma functionalised electrospun matrices is safe and reverses functional alterations observed in hearts after chronic infarction. We provide evidence that the implanted biograft has a beneficial effect on heart remodeling, indicated by stabilized ejection fraction and LV-volume.

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Angiogenic therapies require collateral flow

Petra Korpisalo, Jarkko Hytönen, Jouni Taavitsainen, Anna Partanen, Laura Kiema, Johannes Laitinen, Seppo Ylä-Herttuala

A.I.Virtanen Institute, University of Eastern Finland, Kuopio, Finland

Angiogenic gene therapy has shown potential for the treatment of peripheral ischemic diseases in animal models but not in clinical trials. We hypothesised that the lack of collateral flow in patients, having long stenoses of the conductive vessels, may impair angiogenesis. Most animal models employ single occlusions that initiate effective endogenous collateralisation providing in-flow for the newly growing angiogenic vessels. But what happens to angiogenesis in the lack of collateral flow?

Long, bilateral stenosis of variable decree were created into hindlimbs of New Zealand White rabbits on cholesterol diet using endothelial denutation with 2F balloon catheter. Intramuscular gene transfers of adenoviral vascular endothelial growth factor (AdVEGF) or beta galactosidase control (10e11 viral particles) were preformed. Ischemia progression was followed using contrast enhanced ultrasound and magnetic resonance spectroscopy, and collateralisation using angiography. Ischemic damage and angiogenesis were evaluated using immunohistology.

The control animals had significantly decreased perfusion and aerobic energy metabolism in the ischemic limbs 4 weeks after the operation. Control muscles showed typical signs of chronic ischemia including fibrosis and chronic inflammation at 4 weeks. Surprisingly, the AdVEGF treated limbs did not show improvement in perfusion or aerobic energy metabolism. Instead, there were large necrotic areas within the treated muscles, showing abundant capillary growth but lacking blood flow.

Thus, we conclude that collateral flow into the newly formed angiogenic vessels is essential for the functional benefit of angiogenic gene therapy. Collateralisation differences between human patients and animal models may in great part explain why the clinical angiogenic trials have failed.

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AAV-9 gene delivery in the adult dog heart

Gilles Moulay[1], Tomohito Ohtani[1], Michael Barry[1], Atta Behfar[1], David Kaye[2], Michael O'Connor[1], Linh Pham[1], Adam Guenzel[1], Peter Psaltis[1], Matthew Swale[1], Stephen Russell[1], Margaret Redfield[1]

[1]Mayo Clinic, Rochester, MN, USA, [2]Baker Heart Research Institute, Melbourne, Australia

Introduction: Down-regulation of cardio-protective protein expression in the failing myocardium suggests that gene therapy could abrogate adverse cardiac remodeling/dysfunction by restoring cardio-protective protein expression. Adeno-associated virus serotype 9 (AAV-9) is cardiotropic in rodents but cardiotropism and immune tolerance may be species-specific. The Na/I symporter (NIS) mediates iodide (I) uptake in thyroid. Cardiac NIS expression can be serially assessed with I^{131} or Tc-99m and SPECT/CT cardiac imaging. Cardiac NIS expression after AAV-9-canineNIS via alternate delivery methods was assessed.

Methods: Adult dogs (≈ 20 Kg) were confirmed AAV-9 antibody negative using an in-vivo bio-assay (passive immunization of SCID mice prior to AAV-9-NIS or AAV-9-CEA with subsequent murine SPECT/CT imaging or CEA assay). AAV-9-canine NIS was administered via direct epi-cardial injection (thoracotomy), intra-coronary infusion, intra-coronary infusion with coronary sinus to coronary artery recirculation, and direct endocardial injection using a novel trans-arterial endocardial delivery catheter (C-cath).

Results: Serial SPECT/CT imaging after direct epicardial injection of AAV-9-canineNIS revealed a dose dependent (3 doses, 5×10^{11} to 1×10^{13} vg) cardiac NIS expression at 2, 4, 12, and 24 weeks post-injection. Direct C-cath endocardial administration enabled widespread expression with additional septum delivery. Neither intra-coronary (1×10^{13} vg) nor intra-coronary + re-circulation administration (3 doses, 5×10^{12} to 2×10^{13} vg) resulted in cardiac NIS expression at SPECT/CT at 2, 4 or 12 weeks.

Conclusion: AAV-9 does not provide sufficient cardiac expression after intra-vascular delivery at doses up to 2×10^{13} vg, but direct myocardial delivery is a clinically feasible approach for long term cardiac gene delivery.

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IRES-based vectors for a combined gene therapy of heart ischemia

Edith Renaud[1], Oksana Kunduzova[2], Françoise Pujol[1], Denis Calise[3], Jérôme Roncalli[2,4], Angelo Parini[2], Anne-Catherine Prats[1]

[1]*University Paul Sabatier TRADEGENE EA4554, Toulouse, France,* [2]*INSERM U1048 I2MC, Toulouse, France,* [3]*Unité mixte de service US006, Toulouse, France,* [4]*CIC Biothérapie CHU Rangueil, Toulouse, France*

Despite of considerable advances in the treatment of cardiovascular diseases, left ventricle dysfunction and heart failure remain an important problem of public health. In such a context, gene therapy appears as an attractive approach. Recent studies demonstrate that the combination of several therapeutic genes improves treatment efficiency. In the laboratory, we have developed an original approach of combined gene therapy, based on translational activators, IRESs (internal ribosome entry sites), to generating gene transfer vectors called "multicistronic" as they co-express several therapeutic molecules. We have validated the concept of IRES-based vector for therapeutic angiogenesis of hindlimb ischemia, using a vector co-expressing the angiogenic factors FGF2 and Cyr61. This association creates a synergistical effect of the two molecules that are more efficient at low doses.

Presently we are developing gene transfer vectors applicable to gene therapy of myocardial ischemia, based on the tools developed in skeletal muscle. The aim is to express combinations of molecules to stimulate angiogenesis and myocardium contractile function. Bicistronic lentivectors expressing two luciferase genes separated by different IRESs under the control of different promoters have been successfully assessed in mouse cardiomyocytes and cardiac fibroblasts. Luminescent vectors are also being tested in vivo by direct intracardiac injection. In addition, lentivectors have been designed to express combinations of two or three therapeutic genes including apelin, FGF2 and Cyr61. The therapeutic benefits of these vectors will be assessed in a mouse model of infarcted myocardium. The final objective is to select an optimized therapeutic vector in a clinical perspective.

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Local anti-angiogenic gene therapy reduces in-stent restenosis in a preclinical atherosclerotic triple-injury model

Jarkko Hytonen[1], Jouni Taavitsainen[1], Johannes Laitinen[1], Anna Partanen[1], Olli Leppanen[1], Petra Korpisalo[1], Kari Alitalo[2], Seppo Yla-Herttuala[1]

[1]*A.I.Virtanen- Institute, University of Eastern Finland, Kuopio, Finland,* [2]*University of Helsinki, Helsinki, Finland*

In-stent restenosis (ISR) remains a key limitation to endovascular revascularization therapies. The growth of vasa vasorum into the lesion from the adventitia has been suggested as an accelerator for the process of ISR and Vascular Endothelial Growth Factor (VEGF) is the key mediator of angiogenesis. We studied the therapeutic potential of local anti-angiogenic therapy for ISR by utilizing soluble VEGF receptors that act as decoys and reduce the amount of free VEGF in the tissue.

67 hyperlipidemic WHHL rabbit aortas were denuded of endothelium. Six weeks later a section of the aortic wall was injected with 1.5×10e10 pfu adenovirus encoding soluble VEGF Receptor 1 (sVEGFR1), sVEGFR2 or control LacZ using a needle catheter. A bare metal stent was implanted on the same segment. Control angiographies and euthanasia were performed on d6, d14, d42 and d90 followed by histological analyses.

Gene transfer efficacy was assessed at d6 with LacZ controls and found to be localized in the adventitia. At d42 sVEGFR1 group showed a 21.4% and sVEGFR2 group a 36.3% reduction in restenosis compared to control, which persisted at d90 (10.9% and 25.7%, sVEGFR1 and sVEGFR2 respectively). Proliferation was reduced in neointimas of treatment groups at d14 by 55.2% and 63.6% and by 25.0% and 73.5% at d90 (sVEGFR1 and sVEGFR2, respectively).

Local anti-angiogenic gene delivery might be a useful therapy when battling ISR without depending on strong cytotoxic stent coatings. Although the peak expression after adenoviral gene therapy occurs at d6, we show results persisting three months after treatment.

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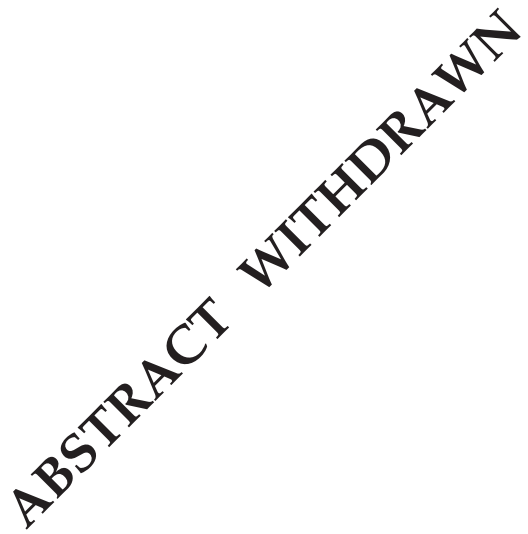
Photodynamic Therapy With Indocyanine Green Induces DNA Damage By Comet Assay *In Vitro*

Sherien El-Daly, Amira Gamal-Eldeen, Mona Abo-Zeid

National Research Center, Cairo, Egypt



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Comprehensive "integrome" analysis of lentiviral WAS gene therapy patients

Luca Biasco[1], Cristina Baricordi[1], Francesca Dionisio[1], Andrea Calabria[1], Danilo Pellin[2], Samantha Scaramuzza[1], Clelia Di Serio[2,3], Christof Von Kalle[4], Manfred Schmidt[4], Luigi Naldini[1], Eugenio Montini[1], Alessandro Aiuti[1,5]

[1]*San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Milan, Italy,* [2]*CUSSB, Università Vita-Salute San Raffaele, Milan, Italy,* [3]*Università Vita-Salute San Raffaele, Milan, Italy,* [4]*National Center for Tumor Diseases (NCT-DKFZ), Heidelberg, Germany,* [5]*University of Rome "Tor Vergata", Rome, Italy*

Hematopoietic stem cell (HSC) gene therapy (GT) is a valid therapeutic option for Wiskott-Aldrich syndrome (WAS) patients. A gammaretroviral (RV) vector clinical trial for WAS achieved significant benefit but this was associated with the development of leukemias and clonal dominance due to LMO2 and MDS/EVI1 vector insertions (IS). We developed a GT approach based on infusion of autologous HSC transduced with a SIN lentiviral (LV) vector carrying the WAS transgene under its 1.6kb promoter. To assess safety and to study the early hematopoietic reconstitution we profiled at high resolution the "integrome" of 3 WAS patients with a total of 23.156 unique insertions (8.012 from Pt1, 6.446 from Pt2, 8.698 from Pt3) allowing a deep clonal tracking of BM CD34+, PB Myeloid, T and B cells in the first phases after transplant. By variation of the Shannon Index we showed an increase in the clonal diversity up to 12 months after GT with different dynamics according to the cell population analyzed. Additionally, top contributing IS were stochastically fluctuating overtime with no sign of sustained expansions. Multipotent progenitors were identified by shared integrations between CD34+ cells/CFCs and mature blood lineages and tracked over time. We also performed a comprehensive comparison with RV GT clinical trial identifying two histone marks (H3K4me3 and H2AZ) whose combination appears to account for the differential genomic distribution of the two vectors. Overall, our LV did not generate selection for oncogenic loci or clonal dominance bringing a safer insertional profile in WAS GT patients as compared to RV.

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Increased incidence of hepatocellular carcinoma (HCC) in mice following a single intra-hepatic injection of adeno-associated virus (AAV) in the neonatal period

RJ Chandler, CP Venditti

National Human Genome Institute, National Institutes of Health, Bethesda, Maryland, USA

We have previous reported that AAV gene delivery was capable of rescuing mice with methylmalonic acidemia (MMA) from lethality (Chandler and Venditti, Mol Ther 2010 18(1): 11–16). As part of these studies, forty-eight mice received a single injection of 1 or 2×10^{11} GC of AAV-CBA-mMut at birth; greater than 70% of these animals developed HCCs between 12 and 21 months of life. Sixteen mice were treated with AAV-CBA-GFP, in the same fashion; greater than 50% of these mice developed HCCs between 14 and 25 months. Forty-one uninjected littermates were also followed for 18 to 25 months and only a single mouse in this group developed HCC, indicating that a strain effect did not explain the increased rate of tumorigenesis seen in the animals that received AAV. While numerous toxicology studies in a variety of species have demonstrated the safety of AAV, a small number of publications have documented hepatic tumorigenesis in mice treated with AAV, and one report has implicated insertional mutagenesis by an AAV vector as a factor in the development of HCC. We are currently trying to identify the sites of vector integration in the HCCs to determine any possible relationship between AAV integration and carcinogenesis. However, an AAV integration near a gene, or microRNA implicated in cancer will not definitely prove causation and other variables may play an important role in the tumorigenesis we have observed. Determining why AAV treatment of mice is infrequently associated with increased tumorigenesis should lead to improved safety in AAV gene delivery.

P135

Functional correction of an X-CGD model cell line by targeted genome engineering

Anne-Kathrin Dreyer[1], Kerstin Kaufmann[2], Christian Brendel[2], Manuel Grez[2], Toni Cathomen[3]

[1]*Experimental Hematology, Hannover Medical School, Hannover, Lower Saxony, Germany,* [2]*Georg-Speyer-Haus, Johann Wolfgang Goethe University, Frankfurt, Hesse, Germany,* [3]*Center of Chronic Immunodeficiency, University Medical Center Freiburg, Freiburg, Baden-Württemberg, Germany*

X-linked chronic granulomatous disease (X-CGD) is a rare hereditary disease characterized by impaired neutrophil function due to mutations in the gp91phox gene, leading to a non-functional NADPH oxidase complex. A new promising gene therapeutic approach is based on designer nucleases, such as the zinc-finger nuclease (ZFN) technology, that has proven useful for rational genome engineering in a variety of cell types. AAVS1-specific ZFNs can mediate the targeted integration of a therapeutic expression cassette into the AAVS1 locus in intron 1 of the PPP1R12C gene, which is considered a safe harbor for the expression of transgenes. As a proof-of-principle, the gp91phox-deficient cell line PLB-985 X-CGD was transfected with AAVS1-specific ZFNs and a donor plasmid containing the gp91phox cDNA under a granulocyte specific promoter as well as a selection marker. Flow cytometric analysis of the selected polyclonal population showed that 37% of the treated cells express gp91phox. PCR-based genotyping of the selected single clones showed 12 % of targeted integration without additional random integration. Positive clones were differentiated into granulocytes and assayed for restored NADPH oxidase function by dihydrorhodamin (DHR) assay, which verified functional restoration of the NADPH oxidase complex to wild type levels. In conclusion, functional correction of an X-CGD model cell line was achieved after targeted integration of a therapeutic gp91phox cassette into the AAVS1 safe harbor locus using the ZFN technology.

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Meganuclease mediated homologous gene targeting: impact of locus and cassette design on transgene expression

Justin Eyquem, Laurent Poirot, Roman Galetto, Andrew Scharenberg, Julianne Smith

Cellectis Therapeutics, Paris, France

Integrative gene transfer is a powerful tool to analyze gene function and is widely used for bioproduction, drug screening and therapeutic applications. However, classical gene transfer methods can lead to random and multi-copy insertions, contribute to unstable or silenced transgene expression and disturb endogenous gene expression. Homologous gene targeting using engineered Meganucleases (MNs) allows the efficient integration of a single expression cassette into a specific locus and is a potential solution to overcome these issues. MNs (homing endonucleases) are sequence specific endonucleases that recognize and cleave a large (14–40bp) target, generating a DNA double-strand break. A combinatorial approach has been used to redesign the MN DNA-binding interface and tailor its specificity, thus allowing engineered MNs to target almost any locus. In order to better understand the impact of the locus on transgene expression and stability, we have designed MNs to specifically target

three human genomic locations spanning intergenic, intronic and enhancer regions. We established the optimal meganuclease dose to mediate homologous gene targeting in HEK293 cells by assessing activity and toxicity of the MNs. Then, we performed and molecularly characterized targeted insertions of a GFP expression cassette in both orientations and under three different promoters, monitoring transgene expression as well as the transcriptional effect of insertions on flanking endogenous genes. The stability and predictability of random, multicopy and single targeted integration were also compared. Results will be presented showing that the locus, the promoter and the cassette orientation can all have a strong impact on transgene expression levels.

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Efficient gene targeting in human hematopoietic stem and progenitor cells by Zinc Finger Nucleases and Integrase-Defective Lentiviral Vectors

Pietro Genovese[1,2], Angelo Lombardo[1], Tiziano Di Tomaso[1], Giulia Escobar[1], Davide Moi[1], Claudia Firrito[1], Lucia Sergi Sergi[1], Philip D. Gregory[3], Michael C. Holmes[3], Luigi Naldini[1,2]

[1]San Raffaele Telethon Institute for Gene Therapy, Milan, Italy, [2]"Vita Salute San Raffaele" University, Milan, Italy, [3]Sangamo BioSciences, Richmond, USA

The development of ZFN technology for gene targeting enables *targeted* rather than *random* integration, and gene *correction* rather than *replacement*, as new paradigms for gene therapy. Whereas high levels of targeted gene modification has been reported in some clinically relevant primary cells, the demonstration of highly efficient targeted integration in human Hematopoietic Stem/Progenitor Cells (HSPC) remains challenging. To achieve this goal, we developed a strategy for efficient delivery of the molecular machinery required to induce homology-driven transgene insertion into a selected genomic target site in HSPC. We combined Integrase Defective Lentiviral Vectors (IDLV) to deliver a donor template for homology-driven repair and mRNA transfection to drive a short but robust spike of ZFN expression in the transduced HSPC. Optimization of this protocol facilitated targeted integration of transgene expression cassettes into the AAVS1 "safe harbor" site or of a corrective cDNA into a mutational hotspot of the IL2RG gene of HSPCs with high efficiency and reproducibility (average efficiency 6%, n = 14 cord blood donors). In colony forming assays, ZFN-treated CD34+ cells generated both erythroid and myeloid transgene positive colonies. Molecular analysis displayed site-specific transgene integration in ~95% of the transgene positive CFC analyzed (n = 79), confirming at the clonal level the high specificity of ZFN-induced targeting. Upon xeno-transplantation of the treated cells in NSG mice we were able to find GFP+ cells at 13 weeks post transplantation in both myeloid and lymphoid lineages and also in the CD34+CD38− compartment, providing the indication of targeting in long term repopulating stem cells.

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Non-toxic targeting of a human chromosomal locus with a lentiviral vector-associated meganuclease

Chenxia He[1], Agnès Gouble[2], Alix Bourdel[1], Laurent Poirot[2], Frédéric Pâques[3], Philippe Duchateau[3], Aleksander Edelman[1], Olivier Danos[1,4]

[1]INSERM U845, Université Paris Descartes, Faculté de Médecine Necker, Paris, France, [2]Cellectis therapeutics, Paris, France, [3]Cellectis SA, Paris, France, [4]Cancer Institute, University College London, London, UK

Site specific endonucleases - Zinc Finger Nucleases, TALE Nucleases or naturally occurring Meganucleases (MN) - can be engineered for custom recognition of any genetic locus and used for gene targeting. Yet, the prolonged expression of these nucleases into cells is usually toxic, due to the accumulation of DNA double strand breaks and efficient methods for transient delivery of these proteins are therefore required. Meganucleases can be produced as single chain proteins and are advantageous for developing approaches of protein transduction.

Here we explore the possibilities of associating I-*Cre*I-derived single chain MNs to lentiviral vectors. Protein fusions were constructed to drive the incorporation of a highly active but toxic MN, CLS4076, into lentiviral particles. The viral protein Vpr as well as cyclophilin A (CypA), both known to interact with HIV-1 Gag, were tested as fusion partners. The MN activity was measured in a quantitative extra-chromosomal assay based on the recombination-mediated rescue of a luciferase reporter gene. CLS4076 fused to CypA at its C-terminus was highly active. It was also able to mediate homologous recombination at the targeted chromosomal locus, as efficiently as when a lentiviral vector encoding the MN was used. Cell toxicity was measured over 7 days following transduction and found to be absent or minimal even at high multiplicities of infection. In contrast, transduction by the CLS4076 encoding lentiviral vector resulted in increased apoptosis and up to 45% of cell death. We conclude that I-*Cre*I MNs associated to lentiviral particles can mediate gene targeting in the absence of toxicity.

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Group II introns as potential tools for genomic modification: Expression and splicing in eukaryotic cells

Madeleine Zerbato[1,2], Nathalie Holic[1,2], Sophie Moniot-Frin[1,2], Dina Ingrao[1,2], Anne Galy[1,2], Javier Perea[1,2]

[1]Inserm, U 951, Molecular Immunology and Innovative Biotherapies, Genethon, Evry, F-91002, France, [2]University of Evry Val d'Essonne, UMR_S951, Genethon, Evry, F-91002, France

Group II introns are self-splicing mobile elements found in prokaryotes and eukaryotic organelles and that propagate by homing into precise genomic locations. Group II intron homing occurs following assembly of a ribonucleoprotein complex containing the intron-encoded protein (IEP) and the spliced intron RNA. Group II introns could therefore be exploited for targeted genomic modifications. Engineered group II introns are now commonly used tools for targeted genomic modifications in prokaryotes but not in eukaryotes. We speculate that the catalytic activation of currently known group II introns is limited in eukaryotic cells. The brown algae *Pylaiella littoralis* Pl.LSU/2 group II intron is uniquely capable of *in vitro* ribozyme activity at physiological levels of magnesium and therefore could be a good candidate for use in eukaryotic cells, but this intron remains poorly characterized. We purified and characterized recombinant Pl.LSU/2 IEP which displayed high levels of reverse transcriptase activity without intronic RNA. The Pl.LSU/2 intron could be engineered to splice accurately in *Saccharomyces cerevisiae* and splicing efficiency was increased by the maturase activity of the IEP. However, spliced transcripts were not expressed.

Furthermore, intron splicing was not detected in human cells. These data provide the first functional characterization of the PI.LSU/2 IEP and the first evidence that the Pl.LSU/2 group II intron splicing occurs *in vivo* in eukaryotes in an IEP-dependent manner. However, further tool development is needed, to obtain efficient gene modification to envision applications in gene therapy models.

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Safe AAV integration profile in LPLD gene therapy

C Kaeppel[1], SG Beattie[2], R Fronza[1], R van Logtenstein[2], F Salmon[2], A Nowrouzi[1], H Glimm[1], C von Kalle[1], H Petry[2], D Gaudet[3], M Schmidt[1]

[1]*National Center for Tumor Diseases, German Cancer Research Center, Heidelberg, Germany,* [2]*uniQure, Amsterdam, The Netherlands,* [3]*ECOGENE-21 Clinical Research Center, Université de Montréal, Chicoutimi, Canada*

Adeno-associated virus (AAV) vectors are promising for clinical gene therapy but their wider application is curtailed due to concerns of AAV integration-mediated tumourigenesis previously reported in mice. However, a comprehensive integration site (IS) analysis in AAV injected patients has not been performed to date. The vector system AAV1-LPL^{S447X} has been developed for the therapy of lipoprotein-lipase deficiency (LPLD), a disorder of the lipid metabolism. We present a large-scale IS analysis by LAM-PCR and pyrosequencing of muscle biopsies collected from five LPLD patients who received multiple intramuscular vector injections. This study was completed with preclinical mouse muscle and liver IS analyses derived from wild-type mice injected intramuscularly or intravenously with AAV1-LPL^{S447X}, respectively. We characterized 1968 unique AAV IS in patient muscle and 1735 or 892 IS in mouse muscle and liver, respectively. Most AAV-derived LAM-PCR amplicons represented concatemeric rearrangements with large inverted terminal repeat deletions. The integration patterns were largely random in respect to gene coding regions, CpG-islands and palindromic regions. In the analyzed patient samples, we identified two AAV IS-hotspots in the mitochondrial (mt) genome. In mouse muscle, three hotspots were detected, one on the mtDNA and two in the muscle specific nuclear genes *Myh* and *Ttn*. Interestingly, after intravenous injection, no prominent hotspot was detected. AAV integration is safe, its preference for the mtDNA differs from the elucidated AAV trafficking route from the endosome to the nucleus and may broaden AAV vector application. In future, the nuclear and the mt genome should be included in any integration study.

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Self-inactivating and wild-type MLV vectors have the same integration profile in human CD34$^+$ hematopoietic progenitor cells

Arianna Moiani[1], Annarita Miccio[2], Julia Suerth[3], Ermanno Rizzi[4], Marco Severgnini[4], Danilo Pellin[5], Axel Schambach[3], Christopher Baum[3], Gianluca De Bellis[4], Fulvio Mavilio[2,5]

[1]*Division of Genetics and Cell Biology, Istituto Scientifico H. San Raffaele, Milan, Italy,* [2]*Center for Regenerative Medicine, University of Modena and Reggio Emilia, Modena, Italy,* [3]*Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany,* [4]*Institute for Biomedical Technologies, Consiglio Nazionale delle Ricerche, Milan, Italy,* [5]*Genethon, Evry, France*

MLV-derived gamma-retroviral vectors integrate preferentially near regulatory regions in the human genome, and are associated to a significant genotoxic risk. Self-inactivating (SIN) vectors are depleted of the strong enhancer and promoter in the LTR, and have improved safety features. In this study we report at a large scale study the integration preferences shared by SIN gamma-retroviral vectors in clinically relevant primary cells. We compared 13,011 SINMLV unique integration sites to 32,574 wt MLV previously generated integration sites in Human CD34+ hematopoietic progenitor cells (HPCs). We observed that MLV vector depleted of the LTR recapitulates the same pattern of integration previously reported for wt MLV, with the characteristic distribution of the integrations around enhancer and promoter regions (marked by H3K4me3 and H3K4me1 histone modifications) as well as specialized chromatin configurations (H2A.Z) and PolII binding sites. More over SIN-MLV integrations are highly clustered with most of the clusters overlapping with MLV wt clusters, and the hot spots of MLV integrations are targeted at the same frequency by SIN-MLV. This study demonstrates that the U3 region of the LTR is not involved in the integration site selection of MLV vectors in human CD34+ HPCs. The design of SIN vectors should thus take into account that the presence of strong regulatory elements inside the expression cassette will likely increase the risk of insertional activation of nearby oncogenes at a similar extent of the deleted MLV enhancer.

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Safety and efficacy studies of a self-inactivating lentiviral vector in a mouse model of Fanconi anemia subtype A

F.J. Molina-Estevez[1], A. Nowrouzi[2], M.L. Lozano[1], S. Charrier[3], A. Galy[3], G. Guenechea[1], M Schmidt[2], J.A. Bueren[1]

[1]*Hematopoiesis and Gene Therapy Division. Centro de investigaciones Energéticas Medioambientales y Tecnológicas (CIEMAT) and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain,* [2]*Abteilung für Translationale Onkologie. Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, Germany,* [3]*Unité INSERM U951. Genethon, Evry, France*

Fanconi anemia (FA) is a rare genetic disease characterized mainly by bone marrow failure and cancer predisposition, principally acute myeloid leukemia. Aiming to develop a clinical trial for FA patients of the A subtype (the most frequent FA subtype), a PGK-*FANCA-wPRE** SIN-LV was selected. To verify the stability of expression and the safety properties of this vector, in vivo gene therapy experiments were conducted in *Fanca*$^{-/-}$ mice. Purified Lin- BM progenitors from donor male *Fanca*$^{-/-}$ mice were transduced ex vivo for periods shorter than 48h with MOIs of 20–50 TUs/cell of the FA therapeutic vector, and transplanted into irradiated *Fanca*$^{-/-}$ female recipients. Under the tested transduction conditions no significant toxicity was observed in transduced colony forming cells (CFCs). Similarly, good engraftments were observed in all transplanted recipients. Freshly transduced CFCs, and transduced CFCs from serially transplanted *Fanca*$^{-/-}$ recipients showed a significant and stable correction of the hypersensitivity to DNA cross-linking drugs that characterizes to FA cells. In no instance hematological abnormalities indicative of myelodisplasia, leukemia or aplasia was observed in any of the gene therapy treated *Fanca*$^{-/-}$ mice. The clonal repopulation analyses of recipients transplanted with the

FA-therapeutic lentiviral vector showed a polyclonal repopulation pattern, while an oligoclonal repopulation pattern was observed in *Fanca*$^{-/-}$ recipients transplanted with SFFV-*EGFP* RV-transduced cells. The insertion profile of the therapeutic FA vector in *Fanca*$^{-/-}$ HSC and clonal dynamics of corrected HSC in serial transplanted mice have been characterized by LAM-PCR and pyrosequencing, in order to further investigate the safety properties of the PGK-*FANCA-wPRE** LV.

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Development of safety improved lentiviral vectors containing ubiquitous chromatin opening elements

Uta Müller-Kuller[1], Christian Brendel[1], Simone Scholz[2], Hana Kunkel[1], Linping Chen-Wichmann[1], Manfred Schmidt[2], Manuel Grez[1]

[1]*Georg-Speyer-Haus, Frankfurt, Germany,* [2]*DKFZ, Heidelberg, Germany*

Ubiquitous chromatin opening elements (UCOEs) are methylation-free CpG islands spanning divergently transcribed promoters of housekeeping genes with chromatin opening capability. We and other have used UCOE to counteract epigenetic effects on transgene expression. In our initial studies we used a 1.5 kb DNA fragment derived from the core region of the A2UCOE from the human HNRPA2B1-CBX3 gene locus. This element includes the promoter region and the first exon plus part of the first intron of the HNRPA2B1 and CBX3 genes. Introduction of the A2UCOE in combination with the myeloid specific promoter MRP8 into a lentiviral vector resulted in high, persistent, specific and copy number dependent transgene expression *in vitro* and *in vivo*.

However the inclusion of the A2UCOE within lentiviral vectors resulted in low titers due to the antisense effect of transcripts initiated at the HNRPA2B1 promoter. Moreover, HNRPA2B1 transcripts splice into cellular transcripts leading to the overexpression of aberrant fusion transcripts. We deleted the HNRPA2B1 mioety from the A2UCOE to avoid these severe disadvantages. This 674 long CBX3-alone fragment sustained eGFP expression from the SFFV promoter in P19 cells and protected the SFFV promoter from CpG methylation although at a lower efficiency than the 1.5 full A2UCOE fragment. Likewise the A2UCOE, the CBX3-alone element provided copy dependent expression in a myelomonocytic cell line and supported myeloid specific expression when fused to a myeloid promoter. Thus the CBX3-alone fragment is a new promising element to protect transgene expression from positions affects without altering the innate properties of tissue specific promoters.

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Transcription Activator-like Effector Nuclease-based Genome Editing for Fanconi Anemia (FA)

Mark Osborn, Colby Starker, Anthony DeFeo, Daniel Voytas, John Wagner, Bruce Blazar, Jakub Tolar

University of Minnesota, Minneapolis, MN, USA

Transcription activator like effector nucleases (TALENs) are genome-editing reagents that generate a DNA break at a user-defined site in the genome and hold great promise as a tool for precision gene repair. Our goal is to apply TALENs for the Fanconi anemia (FA) group of gene disorders. Two TALEN candidates, specific for the Fanconi anemia complementation group C (FANCC) IVS4 mutation site, were constructed using the Golden Gate TALEN assembly procedure and tested for their ability to mediate gene editing by homologous recombination of a marker sequence. Rates of genomic modification were assessed in 293 cells as well as fibroblasts from a FANCC patient. In 293s we observed activity rates of 8% and 51% and in FANCC cells we saw rates of 2% and 24.5% for the same two TALEN candidates. We then employed the TALEN with the highest level of activity in conjunction with an exogenous synthetic donor DNA that contains the corrective base for the IVS4 mutation as well as sequences that serve as a platform to perform linear amplification-mediated PCR (LAM-PCR). Following introduction of the TALEN and donor into FANCC fibroblasts we were able to document permanent genotypic correction of the IVS4 mutation by direct sequencing.

Conclusions: Our results to date show the applicability of genome-editing strategies using engineered nucleases for FA. This provides proof-of-concept for patient-specific gene-editing/correction in primary human fibroblasts and serves as a platform for translational ex vivo therapies.

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A humanized model to assess the genotoxicity of viral vectors in the context of O^6- methylguanine DNA methyltransferase (MGMT) mediated *in vivo* selection

R Phaltane[1], R Haemmerle[2], M Rothe[2], U Modlich[2], T Moritz[1]

[1]*REBIRTH Research Group Reprogramming, Hannover Medical School, Hannover, Lower Saxony, Germany,* [2]*Institute of Experimental Hematology, Hannover Medical School, Hannover, Lower Saxony, Germany*

Integrating vector-induced insertional mutagenesis represents a major problem for human gene therapy. Currently most preclinical genotoxicity assessment is performed in mice. These studies in relevant human models appear highly desirable and humanized mice may represent suitable preclinical models. Detailed genotoxicity studies seem particularly important for chemotherapy-mediated *in vivo* selection strategies, as here the insertional risk may be augmented by cytotoxic agent-induced genotoxic lesions and the proliferative stress exerted on hematopoietic stem cells (HSCs). Thus, we investigated SIN lentiviral vector-mediated $MGMT^{P140K}$ *in-vivo*-selection system in a NOD.SCID.IL2Rgc$^{-/-}$xenotransplant model using human cord blood-derived $CD34^+$ cells. Two doses of combined O^6-benzylguanine (BG; 20mg/kg) plus 1,3-Bis-(2-chlorethyl)-1-nitroso-urea (BCNU;5–10mg/kg) given one week apart were applied. While at the highest BCNU doses animals succumbed to myelosuppression, intermediate doses yielded significant enrichment of transgenic human cells in the peripheral blood(PB) from 1.2 + / − 0.4% before to 21.9 + / − 9.9% after treatment (P = 0.0053, n = 9) and in the bone marrow(BM) with 2.1 + / − 1.4% in untreated animals versus 57.6 + / − 7.8% in the treated group (P = 0.0004, n = 6–9). Human hematopoiesis and gene marking was followed for 24 weeks in primary recipients by repeated PB analysis and histopathology, BM analysis at the end of the experiment. No evidence of lineage skewing or leukemic transformation was observed in any of these analyses. Likewise when cytokine-mediated *ex-vivo* expansion of human HSCs following retroviral gene transfer was investigated in our humanized model no adverse events were observed. Currently LAM-PCR and nrLAM-PCR followed by high throughput sequencing is performed to

gain better insight into the clonal repertoire of hematopoietic reconstitution in these mice.

P146

The *in vitro* immortalization assay for preclinical risk assessment of integrating retroviral vectors - Principles and limitations

M Rothe, JD Sürth, D Zychlinski, A Schambach, C Baum, U Modlich

Hannover Medical School, Hannover, Germany

The *in vitro* immortalization (IVIM) has become a standard preclinical assay to screen new vectors for their genotoxic potential regarding their ability to transform murine hematopoietic cells by insertional mutagenesis. The assay selects mutants arising with a low frequency, based on their long-term survival in myeloid differentiation conditions, and determines their incidence (number of cells required to form a mutant) and fitness (number and size of subclones obtained by replating). The assay has revealed some important principles: (1) Relocating gammaretroviral enhancer-promoter sequences to an internal position of a "self-inactivating" (SIN) vector reduces the fitness of mutants, as do mutations in transcription factor binding sites or enhancer-blocking insulators. (2) Only a subset of insulators was potent enough to attenuate the transforming potential of a strong retroviral enhancer-promoter. (3) Cellular promoters located in SIN vectors, depending on their enhancer activity, may reduce the risk of transformation below the detection limit (>3 logs compared to standard gammaretroviral vectors). (4) Compared to gammaretroviral vectors, the lentiviral integration pattern reduces the incidence of insertional mutants by a factor of ~2, and the alpharetroviral by a factor of ~6. Even though the assay has been established in several laboratories worldwide, there is a constant need for standardization of the specific steps during the complex procedure. Several important aspects and pitfalls regarding transduction, cultivation and final analysis will be highlighted and discussed.

P147

In vivo mouse models for vector genotoxicity testing and lentiviral vector-based cancer gene discovery

Daniela Cesana[1], Monica Volpin[1,2], Marco Ranzani[1], Cynthia Bartholomä[3], Stefania Merella[1], Fabrizio Benedicenti[1], Lucia Sergi Sergi[1], Francesca SanVito[4], Chiara Brombin[5], Alessandro Nonis[5], Clelia Di Serio[5], Claudio Doglioni[4], Christof VonKalle[3], Manfred Schmidt[3], Luigi Naldini[1,2], Eugenio Montini[1]

[1]San Raffaele Telethon Institute for Gene Therapy, Milan, Italy, [2]Vita-Salute San Raffaele University, Milan, Italy, [3]National Center for Tumor Diseases (NCT), Heidelberg, Germany, [4]Department of Pathology, San Raffaele Hospital, Milan, Italy, [5]OSR-CUSSB, Milan, Italy

We developed a novel *in-vivo* vector integration genotoxicity assay based on systemic vector injection into newborn tumor-prone *Cdkn2a*$^{-/-}$ and heterozygous *Cdkn2a*$^{+/-}$ mice. Treatment with a LV with self-inactivating (SIN) LTR, harboring the Spleen-Focus-Forming-Virus (SF) enhancer/promoter in internal position and driving the GFP expression, caused a significant acceleration in hematopoietic tumor onset with respect to controls in both mouse models. The treatment with a vector devoid of the Open-Reading-Frames (ORF) downstream the SF promoter induced a more dramatic acceleration of tumor onset even in a SIN LTR configuration. By analyzing >7500 tumors-derived LV integrations, we identified 72 Common Insertion Sites (CIS), indicating that tumor onset acceleration was caused by insertional mutagenesis. Interestingly, we found that the ORF-less LV induced oncogenesis mainly by the activation of *Braf* by a read-through/splicing-capture mechanism. On the other hand the LV containing the ORF, being unable to activate *Braf*, activated/disrupted other oncogenes or tumor-suppressor genes through enhancer-mediated and/or aberrant splicing mechanisms. Braf activation appears to be a highly dominant and powerful oncogene in our mouse models while the activation of other oncogenes induces a delayed tumor onset. The expression levels of the human orthologs of the CIS genes found in our study in Acute Myeloid Leukemia patients (n = 229, 2 independent cohorts) were correlated by multivariate analysis to their survival. Interestingly, the expression of 2 CIS genes significantly impacted the patients' survival. Hence, our approach can be used to study the genotoxicity of integrative vectors and to discover novel clinically relevant cancer genes.

P148

Dose effects and genomic integration profiling after Sleeping Beauty mediated transposition from adenovirus in female and male mice

Wenli Zhang[1,2], Martin Mueck-haeusl[1], Anja Ehrhardt[1,2]

[1]Max von Pettenkofer-Institut, Munich, Germany, [2]University Witten/Herdecke, Witten, Germany

Our pervious work demonstrated efficient transduction and somatic integration from adenovirus/transposase (AdV/SB) hybrid-vectors utilizing a hyperactive Sleeping Beauty transposase (HSB5). However, before pursuing clinical studies, the safety profile of these hybrid-vectors with respect to vector dose and genotoxicity in vivo remains to be investigated. Herein, we evaluated this vector system in female and male mice with different vector dose settings, and analyzed the fate of the transposon in murine liver after stabilized transgene expression levels was observed.

Our results illustrated that efficacy of the AdV/SB hybrid-vector was dose-dependent. Efficient liver transduction in mice was achieved after injection of a virus dose above 4×10^{10} TU/kg and a non-toxic dose should be below 2×10^{11} TU/kg. Viral vector copies quantified by real-time PCR from the AdV/SB hybrid-vectors treated murine hepatocyte revealed genome copy numbers of up to 0.811 copies/cell of the transposon-encoding vector and 0.106 copies/cell of the transposase-encoding vector. Quantification and analysis of duration of SB transcription levels are ongoing.

Our genome-wide integration site analysis identified 163 independent transposition events from liver of HSB5 treated mice (3 female and 2 male). 77% (105/136) were found in non-gene areas and 23% (31/136) in genes indicating a random integration pattern. In addition, 27 extra-chromosomal integration events were observed, most likely caused by transposon excision and subsequent transposition into the delivered vectors. To our surprise, the chromosomal integration site analysis displayed a strong bias towards the X-chromosome. To clarify this, a LAM-PCR method is being performed aiming at a high resolution of integration events.

P149

Efficacy of a gene therapy approach using a humanized model of Fanconi anemia.

Rocío Baños[1], Antonio Valeri[1], Lara Álvarez[1], Begoña Díez[1], Roser M. Pujol[2], Rebeca Sánchez-Domínguez[1], José C. Segovia[1], Jordi Surralles[2], José A. Casado[1], Juan A. Bueren[1], Paula Río[1]

[1]*Hematopoiesis and Gene Therapy Division. CIEMAT/CIBERER, Madrid, Spain,* [2]*Universidad Autónoma Barcelona/CIBERER, Barcelona, Spain*

Gene Therapy can be a good alternative for Fanconi anemia (FA) patients without a HLA identical donor. Although the efficacy of gene therapy to correct *in vitro* the phenotype of hematopoietic progenitors (HPCs) from FA patients has been previously shown, *in vivo* long term studies have been hampered mainly due to the reduced number and the fragility of cells available from these patients. We have recently developed a humanized model of FA based on the transplantation of *FA*-like $CD34^+$ cells obtained from healthy cord bloods using LVs carrying an shRNA against *FANCA* and *EGFP* as a marker gene (shFA-LVs). We initially demonstrated that *FANCA*-interfered $hCD34^+$ cells behaves *in vitro* as FA cells (FA-like cells) and once transplanted into inmunodeficient mice they engrafted, though with a proliferative disadvantage compared to control $hCD34^+$ cells. Now, we have tested the efficacy of gene therapy to correct *in vitro* and *in vivo* phenotype of FA-like cells. FA-like $CD34^+$ cells were transduced with a LV:PGK*FANCA*, a LV:PGK*FANCA**(carrying three silent mutations to avoid the recognition by the shFANCA) or with a control LV. Both LV:PGK*FANCA* and LV:PGK*FANCA** reverted the MMC sensitivity in these cells. FA-like cells corrected by gene therapy were transplanted into immunodeficient mice and the level of engraftment was analysed up to six months. BM analysis showed expression of *hFANCA* and reversion of the MMC sensitivity in FA-like human HPCs. These results confirm that gene therapy can correct the hematopoietic phenotype of FA-like cells *in vivo* long term after transplantation into immunodeficient mice.

P150

Efficient mobilization and transduction of WASP-deficient haematopoietic cells in a murine model

Sabine Charrier[1], Mike Blundell[2], Grégory Cédrone[1], Fawzia Louache[3], William Vainchenker[3], Adrian Thrasher[2], Anne Galy[1]

[1]*INSERM U951, GENETHON, Evry, France,* [2]*INSTITUTE OF CHILD HEALTH, UCL, London, UK,* [3]*INSERM U1009, Institut Gustave Roussy, Villejuif, France*

Transplantation of gene-corrected autologous hematopoietic stem cells (HSC) is a potential therapeutic option to treat patients with Wiskott Aldrich Syndrome (WAS). HSC can be obtained from bone marrow or from circulating blood following G-CSF mobilization. WAS is caused by lack of the WAS protein (WASp) which is a regulator of hematopoietic cell cytoskeleton and motility. It is presently not known in detail how the lack of WASp affects the mobilization process and the engraftment of HSC. This prompted us to study this question in a murine model of WAS. In the steady-state adult WASp-deficient mice (WKO) exhibit abnormal haematological parameters with B cell lymphopenia, marked neutrophilia. WKO mice also have a splenomegaly with higher levels of CXCL12 expression and higher numbers of circulating haematopoietic progenitors than normal wild-type (WT) mice. Administration of human rec. G-CSF was able to mobilize peripheral blood progenitor/stem cells (MPBC) in WKO mice as effectively and with the same kinetic as in WT mice. These WKO MPBC could be transduced efficiently and engrafted in both WT and WKO recipients providing long-term multi-lineage reconstitution. WKO MPBC provided better survival and reconstituted higher levels of peripheral blood granulocytes than WT MPBC. Surprisingly the peripheral blood recovery of the B lymphocytes was dependent of the background of the host, and was higher in WT recipient, regardless of the origin of transplanted cells. Thus, in the absence of WASp, effective mobilization can be achieved but partial correction may be caused by an abnormal haematopoietic environment.

P151

Hematopoieitic safety evaluation of Vectofusin-1, a new culture additive enhancing lentiviral vector entry into human hematopoietic progenitor cells

J Buisset[1,2], AK Seye[2], D Ingrao[1,2], A Galy[1,2], D Fenard[1,2]

[1]*Genethon, Evry, France,* [2]*INSERM U951 - UEVE, Evry, France*

Several ongoing clinical gene therapy applications are based on *ex vivo* transduction of CD34+ hematopoietic stem cells (HSCs) with HIV-1-based lentiviral vectors (LVs). *Ex vivo* transduction of HSCs usually requires the use of highly concentrated LV or repeated infection cycles, and remains limited by incomplete transduction of the target cell population. To safely potentiate lentiviral transduction, we developed a new class of viral entry enhancers called Vectofusins®. Results presented here show that the lead peptide, Vectofusin-1, enhances transduction of hCD34+ cells with various LV pseudotypes at least as efficiently as other transduction additives (*i.e.* SEVI and Retronectin®). With this peptide, about 80% of CD34+ cells can be transduced with unconcentrated RD114-TR-LV. Enhancing effects are also observed with optimal concentrations of VSV-G-LV without modifying average vector copy number per cell (VCN). Vectofusin-1 is particularly useful to reduce vector usage. Low concentrations of purified VSV-G-LV (10^E7 ig/ml) transduce only 10% of cells, but the addition of Vectofusin-1 increases transduction to 37%±11 of cells which is a 5 fold gain in vector amount since this level of transduction is normally achieved with 5×10^E7 ig/ml vector in absence of additive (32%±8). Vectofusin-1 exhibits a TC_{50} around 60μg/ml on HSCs, one log above the EC_{50} of 6μg/ml. At an optimal concentration of 12μg/ml, the hematopoietic safety of Vectofusin-1 was demonstrated *in vivo* following multi-lineage engraftment of hCD34+ cells in BALB/rag2-/-gC-/- mice. Vectofusin-1 is therefore a safe, effective and versatile additive that could significantly enhance the efficacy of hCD34+ cell-based gene therapy.

P152

Histone deacetylase 3 modulates the expansion of human hematopoietic stem cells

Jon Fernández Rueda, Carina Elizalde, Juan Manuel Salcedo, Akaitz Dorronsoro, Izaskun Ferrin, Emma Jakobsson, César Trigueros

Inbiomed, Donostia-San Sebastián, Spain

Epigenetic modulation by acetylation/deacetylation of histones plays a central role in the self-renewal and differentiation of hematopoietic stem cell (HSC) compartment. Inhibitors of histone deacetylases such as valproic acid (VA) favour the expansion of HSC in vitro. In this work, we have identified the VA target HDAC3 as a negative regulator of umbilical cord blood HSC expansion. We have demonstrated that knockdown of HDAC3 gene improves the expansion of CD34+ cell population, and concomitantly increases the potential of these cultures to generate colony forming units in functional assays. Furthermore, HDAC3 inhibition affects neither cell proliferation nor monocytic commitment and differentiation, which are the mainly altered processes upon VA treatment. Thus, our results identify HDAC3 as a promising target for HSC-based cell therapies.

P153

Itgb2 hypomorphic mice: a novel approach for ex vivo LAD gene therapy

D Leon-Rico[1], M Aldea[1], R Sanchez[1], JC Segovia[1], G Santilli[0,2], AJ Thrasher[0,2], JA Bueren[1], E Almarza[1]

[1]*CIEMAT/CIBERER, Madrid, Spain,* [2]*ICH, UCL, London, UK*

Leukocyte Adhesion Defficiency Tipe-I (LAD-I) is an autosomal recessive primary inmunodefficiency caused by mutations on the *ITGB2* gene (CD18 leukocyte integrin) and characterized by defects on leuckocyte firm adhesion to the activated endothelium.

Hypomorphic CD18 mouse model (*itgb2*^{tm2bay}) shows reduced expression of CD18, and consequently of CD11a, b and c, in both peripheral blood and bone marrow cells. *itgb2*^{tm2bay} mice bone marrow analysis revealed higher proportion of progenitor cells (LSK), reduced apoptosis levels and higher clonogenic capacity compared to WT mice. Analysis of cell cycle status of *itgb2*^{tm2bay} mice BM LSK cells showed higher proportion of G_o cells. In competitive repopulation assays where total BM or lineage negative BM cells from itgb2^{tm2bay} and WT mice were mixed at the same proportion, itgb2^{tm2bay} grafts had an improved contribution to recipient hematopoietic reconstituion, compared to WT grafts. These results suggest that CD18 deficiency results in an improved hematopoietic stem cell function.

On this model we have performed *ex vivo* gene therapy experiments using self-inactivating lentivectors where hCD18 was regulated by different physiological promoters. hCD18 was expressed on peripheral blood and bone marrow cells and, as a consequece, higher mCD11a expression levels could be observed in recipient *itgb2*^{tm2bay} PB cells. After secondary transplants, hCD18 was still expressed in all transplanted groups, indicating that all tested LVs conferred an stable expression of the therapeutic gene, and were able to transduce long-term repopulating cells. Functional assays will be required for testing whether hCD18 expression is able to revert the hematopoietic phenotype on this LAD-I model.

P154

The expression of a truncated form of Epo receptor increases the HSCs pool without inducing their proliferative expansion

MR Lidonnici[1,4], F Tiboni[1], G Mandelli[1], C Cattoglio[2], M Galletti[3], A Cavazza[3], A Grande[3], F Mavilio[3], G Ferrari[1,4]

[1]*H.San Raffaele-Telethon Institute for Gene Therapy (HSR-TIGET), Milan, Italy,* [2]*San Raffaele Scientific Institute, Gene expression Unit, Milan, Italy,* [3]*Center for Regenerative Medicine, University of Modena and Reggio Emilia, Modena, Italy,* [4]*Università Vita-Salute San Raffaele, Milan, Italy*

Gene therapy for diseases affecting the hematopoietic system requires transplantation and engraftment of a high number of autologous genetically engineered HSCs. An adequate dose and clonal diversity of engrafted cells would lower the risk of skewed HSCs expansion and stressed hematopoiesis. Therefore, new tools to increase the size of the transplanted, corrected HSC pool need to be explored. Towards this aim, we explored the signalling triggered by a truncated erythropoietin receptor (tEpoR). Our previous results suggest that expression of tEpoR might confer competitive repopulation ability to murine and human HSCs by favoring their survival and/or engraftment.

To test if the in vivo competitive engraftment is due to expansion of the incoming stem cells pool, we performed a competitive repopulation experiment in transplanted mice. We observed that LV-mediated expression of tEpoR leads to competitive advantage of transduced HSCs promoting an in vivo expansion of transduced HSCs compared to untransduced cells. Competitive repopulation unit assay demonstrates that tEpoR has a robust effect in expanding LT-HSCs but not beyond the normal HSC levels, providing a tool to select transduced cells towards the therapeutic dose.

HSCs transiently expressing the tEpoR assessed that tEpoR signal improves the engraftment of transduced cells, most likely by increasing their homing capacity. Moreover, microarrays analysis identified specific sets of gene ontology categories and pathways up-regulated in CD34+ cells expressing tEpoR. The hypothesis that tEpoR promotes engraftment of transduced repopulating cells, increasing their homing capacity rather than inducing their proliferative expansion, could have important safety implications for gene therapy.

P155

Effect of three-dimensional hydrogel scaffolds on megakaryocyte differentiation

Audrey Pietrzyk[1], Sonia Poirault-Chassac[1], Didier Letourneur[0,2], Catherine Le Visage[0,2], Dominique Baruch[1]

[1]*INSERM U765, Paris, France,* [2]*INSERM U698, Paris, France,* [3]*Université Paris Descartes - Faculté de Pharmacie, Paris, France,* [4]*Université Paris Diderot - Sorbonne Paris Cité, Paris, France*

Hematopoietic stem cells (HSC) niche is a part of the bone marrow, composed of a three-dimensional (3D) structure and stromal cells. The aim of the study was to determine the effect of a 3D structure on HSC differentiation into megakaryocytes (MK) and platelet production. HSC were cultured with thrombopoietin on polysaccharide hydrogel scaffolds (3D) in comparison to liquid phase (2D). Characterization of different stages between immature cells and mature MK was performed by *in situ* microscopic observations as well as following enzymatic lysis to extract cells from scaffolds. Markers of differentiation were quantitated by flow cytometry, and platelet production following MK exposure to high shear rates in Bioflux200® was performed by videomicroscopy. Numerous MK were observed microscopically within the pores of scaffolds up to 36 days. Mature MK are characterized by CD34 disappearance and strong expression of CD41a (αIIb) and CD42b (GPIb). In 3D condition, a population of mature MK ($CD34^-/CD41^+/CD42^+$) was present at least up to 36 days. Furthermore, a second wave of MK

progenitors ($CD34^+/CD41^+/CD42^-$) appeared from D16 on, that could be involved in prolonged production of MK up to D36. Functional platelet-producing MK were observed in 3D as determined for example at D19 by MK perfusion at high shear rate on von Willebrand factor. In contrast, in 2D condition, no MK survived after 19 days. In conclusion, 3D hydrogel scaffolds provide a means to prolong the duration of immature cells ability to differentiate into fully mature and platelet-producing MK and thus may mimick the bone marrow environnement.

P156

Improved Clinically Applicable Lentiviral Vectors for the Gene Therapy of Pyruvate Kinase Deficiency

María Garcia-Gomez, Susana Navarro, Maria Garcia-Bravo, Rebeca Sanchez-Dominguez, Israel Orman, Miguel A Martin-Rey, Juan A Bueren, Jose C Segovia

Differentiation and Cytometry Unit. Hematopoiesis and Gene Therapy Division. Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT) and Centro de Investigación Biomédica en Re, Madrid, Spain

Pyruvate kinase deficiency (PKD) is an autosomal recessive disorder caused by mutations in the *PKLR* gene, resulting in chronic non-spherocytic haemolytic anaemia. The clinical signs of the disease are very variable, ranging from mild to severe anaemia. Currently, there is no specific pharmacological therapy for severe PKD patients, and the availability of an HLA-compatible donor is still a limitation for the hematopoietic transplantation of these patients. In these cases, hematopoietic stem cell gene therapy could be an effective alternative. With this in mind we developed self-inactivating lentiviral vectors carrying a codon optimized version of the *PKLR* (coRPK), driven by the PGK ubiquitous promoter. The transduction of Lin- BM cells from RPK-deficient mice followed by the infusion in irradiated RPK-mutant recipients resulted in the stable expression of the transgene in the long-term. This gene therapy protocol was able to normalize the erythroid compartment and erythroid metabolites profile, extending the RBC life span, without affecting other hematopoietic lineages. Our results point out the feasibility of using lentiviral vectors to treat the severe forms of PKD by gene therapy. With the aim to reduce potential adverse effects, new lentiviral vectors carrying the coRPK cDNA and different erythroid promoters (human PKLR and beta-globin) with different combinations of enhancers from beta-globin locus control regions (LCR) have been developed. The efficacy and specificity of these vectors are being analysed.

P157

A zinc finger nucleases-based human cell model to mimic the Wiskott Aldrich syndrome during megakaryocytic differentiation

Miguel Toscano[1], Per Anderson[1], Pilar Muñoz[1], Gema Lucena[2], Marien Cobo[1], Karim Benabkellah[1], Philip Gregory[3], Michael Holmes[3], Francisco Martin[1]

[1]*Genomics and Oncological Research Center, Granada, Spain,* [2]*Andalusian Stem Cell Bank, Granada, Spain,* [3]*Sangamo BioSciences Inc., Richmond, CA, USA*

Wiskott-Aldrich Syndrome protein (WASP) is exclusive to blood cells and plays important roles integrating extracellular signals with cytoskeletal rearrangements. Mutations in the *WAS* gene cause the Wiskott-Aldrich Syndrome (WAS) characterized by eczema, immunodeficiency and thrombocytopenia, which is manifested even in milder forms of WAS. The role of WASP in megakaryocyte and platelet physiology is poorly understood due to the difficulty to perform experiments with megakaryocytes or platelets from WAS patients. Here, we have used zinc finger nucleases targeting the *WAS* locus to knock down the *WAS* gene in K562 cells, a well known cellular model to study megakaryocytic differentiation. K562WASKO cells mimic several of the defects encountered in WAS patients. We found defective terminal MK differentiation as evidenced by cellular morphology and lower expression of CD41a, TGFb and Factor VIII. We observed signs of premature MK differentiation and/or activation such as higher CD61 levels, increased polymerized f-actin, higher attachment and enhanced phosphatidylserine exposure (apoptosis). These phenotypic alterations were restored upon expression of *WAS* by lentiviral transduction demonstrating that WASP is the main player in all the alterations observed in K562WASKO cells. The present work demonstrates the important role of WASP in MK development and proposes K562WASKO cells as a new tool to study the role of WASP in this process.

P158

Cell recruitment fluorescence imaging

Benoit Thibault[1,3], Muriel Golzio[2,3], Eliane Mery[1,3], Magali Castells[1,3], Justin Teissie[2,3], Jean Pierre Delord[1,3], Bettina Couderc[1,3]

[1]*Institut Claudius Regaud, Toulouse, France,* [2]*UMR CNRS 5089, Toulouse, France,* [3]*Université Toulouse III, Toulouse, France*

The main causes of the high mortality of ovarian adenocarcinoma are the late detection due to an absence of symptoms and the acquisition of a chemoresistant phenotype by the tumoral cells after the first line of chemotherapy treatment. The tumoral microenvironment was described as involved in the general oncogenesis, angiogenesis, the metastatic progress, the escape to the immune system and chemo-resistance. Among the different types of cells involved in chemo resistance we are interested in the MSC/Macrophages interactions and their contribution to the regulation of the tumoral angiogenenesis and the acquisition of chemo resistance. By fluorescence imaging using genetically modified cells we evidenced the sequence of recruitment of Bone Marrow MSC (physiological MSC) and macrophages to the tumour *in vivo*. These recruitments are among other able to act on the ovarian microenvironment by stimulating the synthesis of cytokines involved in angiogenesis and chemo resistance (IL-6, IL-8, VEGF). We were able to isolate tumour associated MSC (CA-MSC) and tumor associated macrophages (TAM) from the tumour on the basis of their fluorescence in order to compare their phenotypes with those of the physiological cells. Thus we showed the variations of expression of various markers of differentiation allowing characterizing the CA-MSC and TAM. The analyses of the cells recruitment were also realized at different time (progressing tumours, treated tumours and relapse) in an *in vivo* model of ovarian cancer using Fluobeam®, an open fluorescent imaging system.

Mery *Gynecol. Oncol*. 2011
Castells *Int. J. Mol. Sci* 2012
Castells *Cancer Letters* 2012

P159

In vivo magnetic resonance imaging (MRI) and spectroscopy (MRS) identifies hamsters responding to treatment with oncolytic adenovirus coding for GMCSF

Otto Hemminki[1], Riikka Immonen[2], Johanna Närvainen[2], Pasi Soininen[2], Sari Pesonen[1], Olli Gröhn[2], Akseli Hemminki[1]

[1]*University of Helsinki, Helsinki, Finland,* [2]*University of Eastern Finland, Kuopio, Finland*

At present there is a lack of surrogate endpoints for the evaluation of oncolytic and immunological therapies as conventional modalities (CT, PET and tumor markers) of evaluating cancer treatment responses are compromised. Magnetic resonance imaging (MRI) and spectroscopy (MRS) might offer a solution to this problem, but so far these have not been tested in the context of oncolytic adenovirus. Here we show evidence that in the T2 weighted MRI an appearing black core area can be seen in the treatment responders. We also show that the following metabolites: taurine, choline and unsaturated fatty acids as observed with the MRS might be used in the evaluation of treatment response. In this immunocompetent Syrian hamster model the effect of the virus produced cytokine GM-CSF might be seen in MRI and MRS already in a few days while the effect of the GM-CSF production and oncolysis might be observed best at one week. At later timepoints induction of possible anti-tumor immunity might reflect the results observed. Our experiments suggest that MRI and MRS can be used for the evaluation of oncolytic treatment efficacy.

P160

The effect of the labeling of plasmid DNA on transfection efficiency

Koen Rombouts, Katrien Remaut, Kevin Braeckmans

Ghent University, Ghent, Belgium

Scientists all over the world are looking at biotechnological pharmaceuticals and micro and nano carriers to cure or alleviate genetic diseases. One of the challenges is to bring the pharmaceuticals from the extracellular environment to their destiny: the cell. To follow this journey from extracellular environment to cytoplasm or even the nucleus, imaging techniques based on fluorescence are commonly used. Visualization of the non-fluorescent pharmaceutical cargo is generally performed by fluorescent labeling. When designing an experiment, a lot of parameters concerning the choice of cells, carriers, type of plasmids, reporter gene, etc. are considered, but which labeling method suits the needs of the experiment best, is often overlooked.

In this work, a variety of different pDNA labeling techniques ranging from covalent binding to triple helix formation were compared for their effect on the transfection efficiency, which is a good measure for conservation of biological function. Different labeling densities, needed for often used (advanced) microscopy techniques, were tested. For one labeling technique, a negative influence was detected. We tried to identify the steps which were most critical for the drop in transfection efficiency. Dissociation from the complex and transcription appear to be significant bottlenecks, while uptake in the cell is not altered. With this information, it is possible to determine untill which step the used labeling method is reliable.

In conclusion, the information that is included in this work, should help in determining if the labeling method that is used, yields biological relevant results.

P161

Design and development of a new lentiviral based anti HIV therapeutic vaccine

Ana BEJANARIU, Marie RODRIGUEZ, Emeline SARRY, Lucie CASABAN, Stacie ABDALLA, Emmanuelle SABBAH-PETROVER, Cécile BAUCHE

THERAVECTYS, Paris, France

THERAVECTYS develops a new generation of prophylactic and therapeutic vaccines using the lentiviral vectors technology. Its most advanced product, a therapeutic anti-HIV vaccine treatment, will enter clinical Phase I/II within few weeks.

Vaccine candidates are integrative and self-inactivated live-recombinant lentiviral vectors. They encode an HIV antigen, under the regulation of a patented promoter that is preferentially induced in APC (generating of a strong, specific and long lasting T-cell immune response), and showing a basal expression level in all cells (allowing their elimination by the settled immune response). These vaccine candidates are classified as "Live recombinant vectored vaccines" (EMA, 2011)

THERAVECTYS developed an innovative manufacturing process combining high production yields, impurity profiles compatible with direct injections into humans and high immunogenicity. Pilot and GMP batches have been manufactured and GLP preclinical studies (amongst which biodistribution, shedding and toxicity) performed. These studies showed the restricted diffusion of THERAVECTYS' vaccine candidates after injection and their fast disappearance in few weeks, correlated with an absence of macroscopic and microscopic toxicity.

Altogether, these data have allowed the settlement of an anti-HIV therapeutic Phase I/II clinical trial that just had received the authorization of the French regulatory agency.

This trial will be held in France and Belgium and plans the enrollment of 36 HIV-1 infected patients. THERAVECTYS' anti-HIV vaccine treatment will be assessed at three doses and safety, tolerability and immunogenicity compared to a placebo group. Results are expected by September 2014 with intermediary analyses in September 2013.

P162

Induction of anti-capsid and anti-transgene CD4+T cells responses to rAAV2/1 vectors requires sialic-acid expression on Dendritic Cells

Maxime FERRAND[1], Cyril WILMES[1], Anne GALY[1,2], Florence BOISGERAULT[1]

[1]*Genethon, Evry, France,* [2]*INSERM U951, Evry, France*

Recombinant Adeno Associated Viral Vectors (rAAV) efficiently transfer genes into target tissues through their interactions with diverse cellular receptors. Efficacy of long-term gene expression of the transgene can be limited by T cell-mediated immune responses to capsid or transgene-encoded determinants. The initiation of specific cellular immune responses usually involves dendritic cells (DC), which efficiently present antigens

and deliver stimulatory signals to specific T cells. Whether or not the primary tissue receptors described for rAAV are also involved in the priming of anti-capsid and anti-transgene immune responses has never been addressed. In this context, we focused on rAAV1, whose primary receptors are alpha2,3 and alpha2,6 N-linked sialic acids, to determine if sialic acids contribute to the DC-mediated anti-vector immunity in mice.

Staining of splenic DC with fluorescent lectins to look for bioavailable sialic acids, showed that DC express alpha2,3 and alpha2,6 linked sialic acids. Those DC have the ability to present capsid and/or transgene antigens to specific CD4+T cells in rAAV1-injected mice and furthermore, rAAV1-loaded DC trigger immune reactivity to both determinants in vivo. Removal of sialic acids on purified DC strongly reduced antigenic presentation of the capsid in vitro and also decreased significantly the priming of CD4+T cells to the capsid but also to the transgene in vivo.

In conclusion, the receptor that is used for rAAV1 to target specific tissues is also of main importance to trigger cellular immune responses to the capsid and the transgene, arguing for the interest of selecting AAV variants unable to interact with APCs.

P163

Taming the shrew: Escaping immune activation through the use of CpG-depleted adeno-associated virus vectors

Susan M. Faust[1], Peter Bell[1], Benjamin J. Cutler[2], Scott Ashley[1], Yanqing Zhu[1], Joseph E. Rabinowitz[3], James M. Wilson[1]

[1]University of Pennsylvania, Philadelphia, PA, USA, [2]Washington University, St. Louis, MO, USA, [3]Temple University, Philadelphia, PA, USA

Adeno-associated viral vectors (AAV) are leading candidates for gene therapy in skeletal muscle diseases such as Duchenne Muscular Dystrophy. However, in Phase I clinical settings, immune responses toward the delivery vehicle or trangene product have compromised safety and long-term gene replacement success. Toll-like receptor 9 (TLR9) has the potential to recognize unmethylated CpG motifs in the therapeutic expression cassettes packaged in an AAV capsid and to induce a pro-inflammatory immune response. We assessed the requirement of TLR9 in promoting immunity toward AAV associated antigens following skeletal muscle AAV gene transfer. In muscle gene transfer experiments, we compared immunological responses in WT and TLR9 deficient mice that received an immunogenic AAV vector, AAVrh32.33, which has been characterized to generate robust immunoreactivity and transgene loss in C57BL/6 (WT) mice. TLR9 was identified as a critical element in immunoreactivity toward AAV associated antigens following intramuscular gene transfer, and the absence of TLR9 signaling resulted in suppressed Th1 (IFNg) responses toward capsid and transgene antigen, minimal cellular infiltrate, and stable, enhanced transgene expression in target muscles. Our findings were subsequently translated into a CpG-depleted AAVrh32.33 vector utilized for skeletal muscle gene transfer into WT mice. We report the development of a CpG-depleted AAVrh32.33 vector that establishes long-term, enhanced transgene expression, evades inflammatory T cell responses, and minimizes infiltration of effector T cells. CpG-depleted AAV vectors hold great promise in avoiding the undesirable activation of immune responses commonly observed in clinical trials and provide a strategy to improve the outcome of AAV directed gene therapy.

P164

The critical role of activation induced cell death and passive apoptosis in the induction of in vivo immune hyporesponsiveness toward AAV8 vectored transgene product in the liver

Susan M. Faust, Peter Bell, Julio Sanmiguel, Yanqing Zhu, James M. Wilson

University of Pennsylvania, Philadelphia, PA, USA

Gene therapy provides a significant opportunity to treat a variety of inherited and acquired diseases. However, adverse immune responses toward the vector, as well as the therapeutic transgene product, may limit gene therapy success. The underlying mechanisms responsible for immunity or tolerance toward AAV associated antigens remain poorly defined. Studies in mice demonstrate that AAV2/8 gene transfer to the liver is associated with immunological hyporesponsiveness toward both the AAV vector and the antigenic transgene product. To evaluate the role of activation induced cell death (AICD) and passive apoptosis in the deletion of mature T lymphocytes that could potentially respond to AAV vector or transgene antigen, we compared immunological responses in hepatic AAV2/8 transfer in murine recipients lacking the functional death cytokine receptor, Fas, and recipients overexpressing the anti-apoptotic factor, Bcl-xL, to wild type murine counterparts. Prolonged transgene expression was dependent on both Fas signaling and Bcl-xL-regulated apoptosis in T cells, and loss of transgene expression in these mouse models was associated with a non-cytolytic mechanism of transgene extinguishment. Abrogation of passive apoptosis enhanced Th1 responses toward transgene antigen, while AICD functioned to limit neutralizing antibody production toward AAV2/8. Our findings also revealed that immune hyporesponsiveness and stable transgene expression was dependent on upregulation of FasL expression on transduced hepatocytes and a corresponding apoptosis of infiltrating Fas(+) lymphocytes. These data collectively provide evidence that both AICD and passive apoptosis of lymphocytes are essential for immune hyporesponsiveness toward hepatic AAV2/8 encoded trangene product in the setting of liver gene transfer.

P165

Multiple Myeloma Patients Display Decreased Death Ligand Expression on T Lymphocytes

FZ Hapil[1,2], S Koksoy[3], F Ersoy[4], E Kurtoglu[5], L Undar[4], S Sanlioglu[1,2]

[1]Human Gene and Cell Therapy Center of Akdeniz University Hospitals and Clinics, Antalya, Turkey, [2]Department of Medical Biology and Genetics, Akdeniz University Faculty of Medicine, Antalya, Turkey, [3]Department of Medical Microbiology, Akdeniz University Faculty of Medicine, Antalya, Turkey, [4]Department of Haematology, Akdeniz University Faculty of Medicine, Antalya, Turkey, [5]Department of Haematology, Antalya Training and Research Hospital, Antalya, Turkey

Background: Cytotoxic lymphocytes are the warriors of the immune system against transformed cells causing cancer. One of the most important mechanisms used by these cells is the death ligand-mediated target cell apoptosis. Although other defense mechanisms have been widely studied, the significance of death

ligand expression in MM patients regarding anti-tumor immunity has not been revealed yet. Since FasL and TRAIL play major roles in immune surveillance, we investigated death ligand expression profiles on lymphocytes of newly diagnosed MM patients.

Materials and Methods: We studied peripheral blood samples of 14 newly-diagnosed MM patients and 9 healthy age/sex matched controls. Blood samples were stained with CD3-FITC, CD4-APC-Cy7, CD8-PerCp, CD25-PE-Cy7, TRAIL-PE and FasL-PE fluorophore-conjugated monoclonal antibodies. Quantibrite-PE beads were used for the estimation of antibody bound per cell (ABC) values. Flow cytometry analyses were performed using BD FACScanto-II device and SPSS software was used for statistical analysis.

Results: Flow cytometry data revealed a reduction in FasL and TRAIL protein levels on CD3+, CD3+CD4+, CD3+CD8+, and CD3− lymphocytes of MM patients compared to healthy controls, although no statistically significant alteration in death ligand expression on CD4+CD25+ T cells was detected. In addition, death ligand-expressing cell proportions were not different between MM patients and healthy controls.

Conclusions: Since downregulation of death ligand expressions on lymphocytes may represent one possible immune related defect in MM patients, reconstitution of death ligand expression via gene transfer might represent a novel therapeutic strategy against MM.

P166

PTEN frameshift mutation is correlated with a higher cytotoxic T lymphocyte tumor infiltrate in microsatellite unstable colorectal cancers and could be targeted for immunotherapy strategies.

Pauline Maby[1], David Tougeron[2], Nicolas Elie[3], Emilie Fauquembergue[1], Richard Sesboüe[1], Marie Cornic[4], Thierry Frébourg[1,5], Jean-Baptiste Latouche[1,5]

[1]Inserm U1079, Rouen University Hospital, Institute for Research and Innovation in Biomedecine (IRIB), North-West Cancéropôle, Rouen, France, [2]Gastroenterology Department, Poitiers University Hospital, Poitiers, France, [3]Imaging Core Facility, CMABIO, Caen University Hospital, Caen, France, [4]Department of Pathology, Becquerel Rouen Cancer Center, Rouen, France, [5]Department of Human Genetics, Rouen University Hospital, Rouen, France

Colorectal cancers (CRCs) with microsatellite instability (MSI) represent 15 to 20% of CRCs, including Lynch syndrome, the most frequent hereditary form of CRC. These MSI+ CRCs, overall, have a more dense lymphocytic infiltration and a better prognosis than the other CRCs. A stronger immunogenicity of these CRCs could be explained, at least in part, by the accumulation of frameshift mutations, in genes containing coding repeat sequences, that could influence the tumor immune cell infiltration. In particular, such mutations could lead to the synthesis of neo-antigens targeted by a specific anti-tumor T lymphocyte (TL) response.

To determine which mutations could influence the tumor immune cell infiltration, we quantified total (CD3+), regulatory (FoxP3+) and cytotoxic (CD8+) TL infiltrate in 88 MSI+ tumors, using tissue microarrays, and we analyzed frameshift mutations in repeated coding sequences of 19 genes, using 2 multiplex PCRs.

No correlation was found between FoxP3+ cell density and frameshift mutations. Total and cytotoxic TL densities within the tumors significantly increased with the total number of mutations. Moreover TL density was higher, especially for CD8+ T cells, when PTEN gene was mutated, arguing for the presentation by the tumor cells of neo-epitopes derived from this frameshift mutation.

Altogether, this work emphasizes the potential interest of targeting neo-antigens derived from frameshift mutations in immunotherapy strategies for CRCs with MSI, especially young Lynch syndrome patients.

P167

Development of Novel Chimeric Antigen Receptors Containing Intracellular Signaling Domain of Glucocorticoid-Induced TNF-Receptor

Yasunori Amaishi[1], Sachiko Okamoto[1], Yoshikazu Kurosawa[2], Hiroshi Shiku[3], Junichi Mineno[1], Kazuto Takesako[1]

[1]Center for Cell & Gene Therapy, Takara Bio Inc., Otsu, Japan, [2]Institute for Comprehensive Medical Science, Fujita Health University, Nagoya, Japan, [3]Department of Immuno-Gene Therapy, Mie University Graduate School of Medicine, Tsu, Japan

Adoptive immunotherapy using T cells expressing Chimeric Antigen Receptor (CAR), which can recognize corresponding antigen in non-MHC-restricted manner, is a promising strategy to treat patients suffering from autoimmune diseases and malignancies. First-generation CARs consist of the intracellular domain (ICD) of CD3z, while second-generation CARs have additional ICDs from various co-stimulatory protein receptors (e.g. CD28, 41BB, ICOS) which are expected to enhance antitumor activities of T cells. Recently, third-generation CARs have been developed by incorporating multiple signaling domains to augment T cell signaling capacity.

Glucocorticoid-induced TNF-receptor (GITR), a member of the TNF receptor superfamily, is known to inhibit the suppressive activity of regulatory T cells and extend the survival of T-lymphocytes. In order not only to extend T cell survival but also to inhibit the suppressive activity of regulatory T cells, GITR ICD was incorporated into our second and third generation CARs as a co-stimulatory molecule. We found that the order of GITR ICD in the signaling domains was crucial for proper expression of CARs, as our second generation CARs having a signaling domain "CD3z-GITR" were efficiently expressed, while expression of CARs with "GITR-CD3z" was extremely low. With a comprehensive evaluation of the CAR constructs in leader sequences, trans-membrane domains, additional co-stimulatory molecules, and their order in the signaling domains, we successfully developed the promising CARs. Our novel second and third generation anti-EGFR CARs containing GITR ICD were highly expressed with antigen specific cytokine secretion in vitro. T cells expressing our novel CARs may be promising effectors for cancer therapy.

P168

Characterization of AAV T cell epitopes in splenocytes from normal human donors

Federico Mingozzi[1], Daniel Hui[1], Etiena Basner-Tschakarjan[1], Yifeng Chen[1,2], Shyrie Edmonson[1,2], Marcela Maus[1], Gregory Podsakoff[1], Gary Pien[1], Rodney Camire[1], Katherine High[1,2]

[1]Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA, USA, [2]Howard Hughes Medical Institute, Philadelphia, PA, USA

Adeno-associated viral (AAV) vectors are among the gene delivery vehicles of choice for the correction of monogenic disorders. However, the potential for host immune responses against the AAV capsid remains a concern. To characterize the prevalence of humans carrying T cell responses against the AAV capsid, we screened splenocytes derived from subjects undergoing splenectomy for non-malignant conditions. In total, 44 spleen samples were screened by ELISpot and an age-dependent increase in frequency of positive T cell responses was observed, with 16.7% subjects <5 years of age showing positive IFN-γ responses, 66.7% positive subjects aged between 5 and 21 years, and 78.57% positive subjects aged >21 years. Engagement with CD8+ T cells was shown by polyfunctional analysis of T cell activation markers, MHC Class I pentamers staining, and CTL assay in which cross-reactivity of alternate AAV capsids was evaluated. Depletion of CD45RO+ T cells prior to testing resulted in abolition of IFN-γ response, suggesting a role for memory T cells in AAV capsid-triggered immune responses. Finally, peptide binding affinities to MHC class I for candidate T cell epitopes from alternate serotypes were measured using a fluorescent peptide binding assay. In conclusion, our studies with normal human splenocytes showed 1) an age-dependent increase in detection of reactive splenocytes in healthy humans; 2) that humans carry fully functional capsid-specific memory CD8+ T cells; 4) that conserved AAV epitopes are cross recognized by capsid-specific T cells; and 5) that capsid derived epitopes bind to MHC class I with high affinity.

P169

MHC-II targeting lentiviral vector trigger functionnal Th1 and cytotoxic T cell responses following in vivo DC targeting in mice

Ciré Séverine[1,2], Da Rocha Sylvie[1], Ferrand Maxime[1], Buchholz Christian J.[4], Collins Mary[3], Galy Anne[1]

[1]Inserm U 951, Molecular Immunology and Innovative Biotherapies, Genethon, Evry, F-91002, France, [2]Université Paris Diderot, Sorbonne Paris Cité, Paris, France, [3]Infection and Immunity, University College London, London W1T 4JF, UK, [4]Molecular Biotechnology and Gene Therapy, Paul-Ehrlich-Institut, Langen, Germany

The possibility to transfer genes *in vivo* to specific cells such as antigen-presenting cells (APC),provides an opportunity to specifically immunize towards a transgene and to study the potency of different kinds of APCs in cellular immune responses. A novel immunogenic transgene was generated by fusing GFP to T cell epitopes from the murine HY male gene (GFPHY). To target APCs, we used lentiviral vectors pseudotyped with engineered measles envelope glycoproteins targeting MHC class II with a scFV (MHC-II-LV). *In vitro*, this vector specifically transduced MHC class II+ splenocytes cells (GFP expression assessed by FACS analysis). To proceed with *in vivo* experiments, vector production methods were optimized, providing concentrated MHC-II-LV stocks containing about 2000 ng RT/ml, which were sufficient for *in vivo* injections. Intravenous (IV) injection of MHC-II-LV-GFPHY in female mice (from 30 to 100 ng RT/mice) induced male-specific cellular Th1 immune responses in spleen. The 50 ng RT dose was then used to further characterize these immune responses in comparison to same amount of VSVG-LV-GFPHY. Shortly following IV injection of MHC-II-LV-GFPHY vector, CD11c+ dendritic cells and CD3-CD19- stromal cells in lymph nodes were transduced whereas VSVG-LV-GFPHY led to low levels of expression in all splenic cell subsets. A single injection IV of MHC-II-LV-GFPHY vector induced functional effector CD4 and CD8 T cell responses, in particular the induction of *in vivo* male-target-cells lysis and the establishment of transgene-specific immune memory as shown by challenge with peptides. Thus, MHC-II-LV vectors can be used for efficient and specific T cell immunization in mice.

P170

Dual-vector system for generation of high-titer antibodies against botulinum toxin and anthrax

Na Young Kim, Jai Myung Yang, Sungho Shin

Department of Life Science, Sogang University, Shinsu-Dong, Mapo, Seoul, Republic of Korea

Multivalent DNA vaccine for highly pathogenic organism offers an attractive approach for preventing deliberately introduced disease through bioterrorism acts. The botulinum neurotoxins produced *Clostridium botulinum*, most potent toxin, and *Bacillus anthracis*, the bacterial pathogens responsible anthrax, have been reported to be potential agents for bioterrorism. In this study, we investigated feasibility of dual vector, containing heavy chain domain of *Clostridium botulinum* neurotoxin type E (HCR) and domain 4 moiety of *Bacillus anthracis* protective antigen (PA-D4). To increase the immunogenicity of the dual plasmid DNA vaccine, both antigens were fused to mouse IgM signal peptide. Following transfection of 293T cells with dual plasmids resulted in production and secretion of both HCR and PA-D4 protein in vitro that was examined by Western blotting and ELISA assay. Vaccination of BALB/c mice via intramuscular route against HCR and PA-D4 resulted in high titer antibody production. In conclusion, these studies demonstrate the effectiveness of immunization with dual-function DNA vaccine against botulinum toxin and anthrax. Further experimentation with dual vector delivery system to animals is required for the refinement of DNA vaccines to effectively prime protective immune responses against two major biothreat agents.

P171

Viral like particles derived from the hepatitis B virus capsid protein for delivery of therapeutic RNA sequences to hepatocytes

A Ely, PB Arbuthnot

Antiviral Gene Therapy research Unit, Department of Molecular Medicine and Haematology, University of the Witwatersrand, Johannesburg, South Africa

Hepatitis B virus (HBV) infection is a major medical problem with the 350 million chronic carriers worldwide at risk factor for cirrhosis and hepatocellular cancer. Anti-HBV RNA interference (RNAi) activators have been shown to effect potent silencing of HBV replication. Nevertheless, the development of effective platforms for the delivery of therapeutic sequences remains the greatest hurdle facing gene therapy. Here we aimed to develop

viral like particles (VLPs) derived from the capsid of HBV. Expression of the HBV core protein is easily achieved in bacterial systems and core proteins spontaneously assemble into viral capsid-like particles. Bacterial RNAs are automatically taken up in the capsid with the predominant species being the RNA from which the core proteins were translated. The rationale of this study was to include a therapeutic sequence on the same transcript containing the core protein sequence. Expression of core proteins from such a transcript would consequently produce VLPs containing a therapeutic RNA sequence. Electron microscopy revealed that the VLPs resembled wild-type virus capsids. Characterisation of the RNA content of the VLPs indicated the presence of the core coding sequence and the therapeutic sequence. In agreement with previous reports the core/therapeutic RNA was the predominant RNA species encapsidated. The RNA sequence appears very stable and is likely to be protected from degradation within the capsid during purification. This method offers a convenient tool for large scale synthesis of therapeutic RNA sequences encapsidated in a delivery vector. Current studies are underway to optimise delivery and silencing of RNA-containing VLPs.

P172

Potent knockdown of viral replication following lentiviral vector-mediated delivery of liver-specific micro RNA mimics targeting HBV

Dejana Ivacik[1], Abdullah Ely[1], Patrick Arbuthnot[1], Nicolas Ferry[2,1]

[1]*University of the Witwatersrand, Johannesburg, South Africa,* [2]*INSERM UMR-S 948, Nantes, France*

Globally, persistent hepatitis B virus (HBV) infection is a significant cause of public health problems. Individuals who are chronically infected are at an increased risk of developing hepatocellular carcinoma and cirrhosis. Currently available HBV treatments have variable efficacy and there is a need to develop novel and improved therapies. Although RNA interference (RNAi)-based approaches have shown promise, accomplishing safe and sustained silencing as well as efficient delivery to hepatocytes has hampered clinical translation of this promising technology. Lentiviral vectors have the useful property of stable integration into host cells enabling sustained expression of RNAi-activating sequences. To advance use of recombinant lentiviruses for HBV treatment, we have engineered the vectors to include liver specific, Pol II cassettes that generate mono- or polycistronic HBV-silencing primary micro RNA mimics. The recombinant lentiviruses were capable of transducing human liver-derived Huh7, HepaRG and HepG2.2.15 cells in vitro. Northern blot analysis verified effective processing of the anti-HBV pri miRNA sequences to form RNAi-activating guide sequences. When stably transduced cells were transfected with a HBV replication-competent plasmid, potent inhibition of markers of viral replication was achieved. Sustained, hepatotropic HBV silencing was effected with limited disruption of endogenous micro RNA function.The efficacy of the anti HBV lentiviral vectors is currently being assessed in HBV transgenic mice and UPA/SCID mice that have been xenografted with lentivirus transduced human hepatocytes. Evaluation of preclinical proof of principle in these animal models will provide insights into the potential utility of ex vivo gene therapy for chronic viral infections of the liver.

P173

Expression of a fusion inhibitor and a CCR5 agonist by lentiviral vectors confers protection against HIV infection in vitro: assessment of efficacy in vivo in a new humanized mice model of HIV infection

Petit Nicolas[1], Lambert Sidonie[2], Burlion Aude[1], Dorgham Karim[3], Marcelin Anne-Genevieve[2], Gorochov Guy[3], Marodon Gilles[1]

[1]*UMR7211 INSERM U959 Biotherapy Laboratory AP-HP, Paris, France,* [2]*INSERM UMR S 943, Virology Laboratory AP-HP, Paris, France,* [3]*INSERM UMR S 945, Immunology Laboratory AP-HP, Paris, France*

This project aims to validate a gene therapy strategy against HIV by gene transfer into hematopoietic progenitors and generation of HIV-resistant CD4+ T cells. We used lentiviral vectors expressing a fusion inhibitor (T20m), and a strong CCR5 agonist modified to reduce CCR5 expression (P2i). The human lymphoblastoid cell line CEMx174 expressing the HIV coreceptor CCR5 (CEM-R5) was transduced with the vectors and infected with CCR5- or CXCR4-tropic HIV strains. The anti-HIV effect of the transgenes was confirmed in vitro by p24 ELISA, flow cytometry and semi-quantitative PCR. Our results suggest that T20m and P2i act in synergy to prevent HIV infection and replication. To test HIV protection in vivo, we developed a new model of HIV infection in NOD.SCID.gc-/- (NSG) mice, in which the CEM-R5 cell line is injected into NSG mice. CEM-R5 grafted mice survived more than 20 days, although the lymphoma infiltrates the liver, the bone marrow and the spleen, as shown by in vivo bioluminescence assay. Viral loads could be detected in the sera of HIV-infected animals, showing that the model may be well suited for studies aimed at inhibiting HIV infection. Experiments aimed at showing HIV resistance conferred by our vectors using the model are ongoing. Future studies will determine the impact of the treatment in humanized mice reconstituted with transduced human progenitors, with perspectives for a clinical trial.

P174

Membrane-anchored and secreted C Peptides as HIV entry inhibitors

Lisa Egerer[1], Janine Kimpel[1], Kerstin Schmidt[1], Frances Brauer[0,2], Andreas Volk[0,2], Dorothee von Laer[1]

[1]*Division of Virology, Medical University Innsbruck, Innsbruck/Tyrol, Austria,* [2]*Applied Virology and Gene Therapy, Georg-Speyer-Haus, Frankfurt am Main, Germany*

Background: C peptides derived from HIV gp41 had been shown to efficiently suppress HIV entry. Here, we analysed a membrane-anchored (maC46) as well as a secreted (sC46) variant of the peptide C46 for gene therapy of HIV infection.

Methods: Membrane-anchored or secreted C46 peptides were expressed from gammaretroviral or lentiviral vectors in B and T cell lines as well as in primary human T cells. Cultures were analysed for expression of the peptides and antiviral effect. Furthermore, a humanized mouse model was estabished to test these antiviral peptides in vivo.

Results: The sC46 peptides were expressed and secreted in different lymphoid cells and exerted high antiviral activity against a variety of HIV envelope glycoproteins. In mixed cell cultures peptides secreted from transduced cells produced a

bystander effect and prevented infection of non-modified cells. However, in primary cells antiviral effect was less pronounced. Therefore further optimization is needed. In the mouse model, we observed a substantial increase of maC46+ CD4+ T cells in blood as well as in spleen, apparently due to the selective pressure of ongoing HIV infection. This increase of CD4+ T cells was neither seen in uninfected control mice nor with a control vector.

Conclusions: The clear accumulation of maC46+ cells in HIV-infected humanized mice indicates that these cells are protected from HIV infection in vivo. Further studies in this model with maC46 and sC46 will allow us to analyze the conditions that determine efficacy of T cell based immuno/gene therapy for HIV-infection.

P175

Effects of lentivirus-mediated kallistatin gene transfer on influenza virus infection in mice

Ai-Li Shiau[1], Chia-Hsing Leu[2], Mei-Lin Yang[1], Chao-Liang Wu[2]

[1]*Department of Microbiology and Immunology, National Cheng Kung University Medical College, Tainan, Taiwan,* [2]*Department of Biochemistry and Molecular Biology, National Cheng Kung University Medical College, Tainan, Taiwan*

Influenza infection induces bronchitis, pneumonia, and even severe lethal pneumonia caused by increased cytokine secretion and acute inflammation at sites of viral replication. These cytokines are associated with the recruitment of neutrophils, macrophages, and lymphocytes to the infection site. Influenza virus hemagglutinin (HA) plays a dual role in the initiation of infection by binding the virus to sialic acid-containing glycans on the host cell surface and by promoting penetration of the viral genome through membrane fusion. The cleavage of HA0 into HA1/HA2 by secreted serine proteases activates virus infectivity and is important for influenza virus pathogenicity. Kallikreins are a subfamily of serine proteases, whereas kallistatin, a tissue kallikrein-binding protein, is a serine protease inhibitor with anti-inflammatory activity. In this study, we investigated the effects of human tissue kallikrein and kallistatin on influenza virus infection in mice. We found that kallikrein expression was upregulated in influenza virus-infected mice. Furthermore, human tissue kallikrein could promote HA cleavage and enhance virus replication in MDCK cells infected with influenza virus, which could be abrogated by treatment with human kallistatin. Mice treated with lentiviral vectors encoding human kallistatin (LV-HKBP) could enhance the survival of influenza virus-infected mice compared with those treated with control vectors (LV-GFP). Moreover, the serum level of pro-inflammatory cytokine IL-6 decreased and lung injury was alleviated after LV-HKBP treatment in the infected mice. Taken together, our results suggest that kallistatin has the potential to ameliorate influenza pathogenesis by reducing influenza viral infection and inflammation.

P176

Approaches to Biosafety Testing based on Detection of novel viral sequences in Raw Materials

Alison Armstrong[1], Colette Cote[0,2], Donna McMutrie[1], Audrey Chang[0,2], John Kolman[0,2], David Onions[1]

[1]*BioReliance Ltd, Glasgow, UK,* [2]*BioReliance Corp, Rockville, MD, USA*

The safety of biologics is accomplished by a multi-level approach involving safety testing steps throughout the development and manufacturing process. The safety program commences with raw material control, and includes in-process and bulk lot release tests and assessment of the manufacturing process to inactivate or remove viruses.

Recent advances in molecular technologies have led to the detection of known and novel viral gene sequences in raw materials, including intermediates from the production process, viral seeds and master and working cell banks. The discovery of the presence of new viruses in raw materials has resulted in detection of new viral targets e.g. bovine parvoviruses, hokovirus, bocavius, and cycloviruses. Up till now, raw materials testing has involved a range of assays including in vitro tests performed in compliance with 9CFR for bovine and porcine viruses. A targeted approach to testing of new raw materials should include the use of novel molecular techniques, using directed PCR methods (testing of raw materials by PCR may highlight that sequences are present but this does not necessarily indicate that infectious virus is present). Screening for targeted sequence will also allow estimation of viral load. This data can be used in conjunction with known clearance data.

This presentation will focus on the ongoing requirement for testing of raw materials, case studies on detection of novel virus targets and specific approaches to ensure continued safety of biologics.

P177

Development of a viral vectored vaccine with an integrated regulatory strategy

Estelle De BARBEYRAC[1], Cécile BAUCHE[2], Amanda GIBBONS[1], Marie RODRIGUEZ[2], Emmanuelle SABBAH-PETROVER[2]

[1]*Voisin Consulting Life Science, Boulogne, France,* [2]*THERAVECTYS, Paris, France*

The purpose of this presentation is to demonstrate the benefits of repeated contact with the regulatory authorities throughout the development of highly innovative Medicinal Products (MPs) to ensure a successful path toward a Marketing Authorisation Application. A case study on the development of live recombinant viral vectored vaccines intended for the treatment of an infectious disease will be presented.

Highly innovative MPs such as viral vectored vaccines require a custom-made development, which means that developers cannot take a "by the book" approach to regulatory guidelines. It is especially relevant in such cases that developers seek the opinion of regulatory authorities in order to validate their strategy and development programme. Due to the complexity of the Quality and Non-clinical parts of the development programme of such gene transfer-based MP and the possible risks associated with this type of product, discussions with both national regulatory authorities and the European Medicines Agency were conducted.

In our presentation the choice of a given regulatory authority in relation with the product development stage will be discussed as well as the benefits of early interactions on the assessment of a first-in-human clinical trial application. We will also discuss the impact of the classification of this type of product on its development and its assessment by the European Medicines Agency.

P178

Development of an innovative report gene assay to detect endotoxins in biological products

Francesca Rossetti[1], Sabrina Dominici[2], Giuseppina Marano[1], Linda Palma[2], Costantina Buondelmonte[2], Mauro Magnani[2], Marina Radrizzani[5], Claudio Bordignon[1,4], Luigi Naldini[3,4], Marco Manoni[1], Claudia Benati[1], Paolo Rizzardi[1], Giuliana Vallanti[1]

[1]*Molmed S.p.A., Milan, Italy,* [2]*University of Urbino "Carlo Bò", Urbino, Italy,* [3]*Vita-Salute San Raffaele University, Milan, Italy,* [4]*San Raffaele Scientific Institute, Milan, Italy,* [5]*Cardio Centro Ticino, Lugano, Switzerland*

MolMed S.p.A. is a biotech company developing innovative antitumour therapies and offering its expertise in cell and gene therapy to third parties to develop projects from preclinical to PhaseIII trials. The detection of endotoxins (LPS, lipopolysaccharides) is a crucial release assay performed on raw materials, process intermediates and final products. The presence of LPS in infusion product can cause systemic inflammation and in the worst case scenario septic shock. Evaluation of endotoxins contaminations of biological medicinal products (e.g. plasmid DNA, vector preparation, proteins) may be sometime cumbersome due to artefacts and false positive results during the routine LAL (Limulus Amebocyte Lysate) test that then need to be investigated with the rabbit test, a product and time consuming animal experiment. As suggested in EU-EP (2.6.30), alternative biological assays can be developed and validated. The new biological innovative method proposed is a luminescent report gene assay, based on a selected clone of the stable monocytic cell line MonoMac6, transduced with a third generation lentiviral vector, LVVINV (pCCLsin.PPT.LUC.Wpre_mut_Amp). LVVINV expresses the luciferase gene under the Nf-kB consensus sequence, activated by the presence of endotoxins. The clone is optimized with the presence of a transcription blocker to minimize the endogenous activation of the promoter. The luciferase construct is inserted antisense to avoid the vector production inhibition by the transcription blocker. The system is designed for a rapid, convenient and sensitive evaluation of endotoxins contaminants and following validation may be a suitable candidate to replace the LAL and the rabbit methods as release test.

P179

Preclinical safety evaluation of gene therapy medicinal products: building up an academic Good Laboratory Practice (GLP) facility for toxicology and biodistribution studies at HSR-TIGET.

Francesca Salvatori[1], Ilaria Visigalli[1], Aisha Sauer[1], Francesca Tiboni[1], Giacomo Mandelli[1], Stefania Delai[1], Raisa Jofra Hernandez[1], Nicola Carriglio[1], Alessio Palini[2], Francesca Sanvito[2], Enzo Oriani[2], Vanda Parezanovic[2], Pamela Rodegher[3], Paola Albertini[3], Patrizia Cristofori[4], Michela Gabaldo[5], Alessandra Biffi[1], Alessandro Aiuti[1], Luigi Naldini[1,6], Giuliana Ferrari[1,6]

[1]*San Raffaele-Telethon Institute for Gene Therapy (HSR-TIGET), Milan, Italy,* [2]*San Raffaele Scientific Institute, Milan, Italy,* [3]*Histolab Verona, Verona University, Verona, Italy,* [4]*Glaxo Smith Kline, R&D, Ware, UK,* [5]*Telethon Foundation, Milan, Italy,* [6]*Vita-Salute San Raffaele University, Milan, Italy*

A few pioneering gene therapy clinical trials have demonstrated the therapeutic potential of gene-modified hematopoietic cells to correct genetic disorders. However, as this therapeutic strategy is applied to more diseases and aims to advance clinical development, rigorous preclinical studies in the most appropriate models are of paramount importance to assess the risk/benefit ratio and fulfill the requirements for future market registration. Evaluating the biosafety under regulatory compliance of gene therapy medicinal products (GTMP), utilizing GLPs (good laboratory practices) will provide results of utmost scientific significance and high regulatory standards. Therefore, we have started developing a GLP Test Facility within the academic HSR-TIGET according to OECD Principles on GLP, combining the different expertise of personnel trained in research, biotech company R&D, pathology and safety assessment and quality assurance. Several laboratories dedicated to GLP activities were identified and appropriately equipped: a BSL2 cell and molecular biology lab, a flow cytometry lab, two BSL2 labs in SPF animal facilities, a pathology lab and an archive room. Documentation required for GLP Quality management, GLP procedures and analytical methods were assembled and all personnel involved was specifically trained. Instruments were qualified and most of the analytical procedures were developed and validated according to EMA, CBER/FDA and ICH guidelines. We have already started toxicity/tumorigenicity studies for Mucopolysaccharidosis Type I and beta-thalassemia gene therapy; designing the studies according to critical aspects as the choice of the animal model, size of experimental groups, selection of dose/route of administration, sampling type and time points and analysis at termination.

P180

Potent microRNA suppression by RNA Pol II-transcribed Tough Decoy inhibitors

Rasmus Bak, Anne Hollensen, Maria Primo, Jacob Mikkelsen

Dept. of Biomedicine, University of Aarhus, Aarhus, Denmark

miRNAs have emerged as important regulators of gene expression and play a role in almost any cellular process and as potential triggers of disease. Suppression of miRNA activity by synthetic inhibitors may suffer from the need for repeated administration and poor delivery in hard-to-transfect cell types. As an alternative to synthetic agents we here investigated DNA-encoded miRNA inhibitors delivered by plasmid transfection or by lentiviral transduction. In a luciferase-based assay we compared the potency of seven types of miRNA inhibitors transcribed as short RNAs from an RNA Pol III promoter. We found superior activity of two decoy-type inhibitors, a 'Bulged Sponge' with eight miRNA binding sites and a hairpin-shaped 'Tough Decoy' containing two miRNA binding sites. We demonstrate for the first time that Tough Decoy inhibitors retain their miRNA suppression capacity when embedded in the 3′ UTR of longer mRNA transcripts expressed from an RNA Pol II promoter. This approach allows easy temporal and spatial regulation of miRNA activity using inducible and tissue-specific Pol II promoters. We also demonstrate the feasibility of simultaneously inhibiting two different miRNAs using a single Tough Decoy transcript carrying two different miRNA binding sites. Extending this approach, we inhibited six different miRNAs from a single vector encoding a Pol II transcript containing three consecutive Tough Decoys, each with two different miRNA binding sites. These novel RNA Pol II-transcribed Tough Decoy inhibitors may be employed for spatio-temporal regulation of miRNA activity as well as for therapeutic targeting of one or more aberrantly expressed miRNAs.

P181

Gene targeting of *WASp* gene in human cells by using adeno-associated virus

MA Bernardi[0], P del Pozo[0], L Pérez[0], M Simarro[0], MA de la Fuente[0]

[1]*Instituto de Biología y Genética Molecular, Valladolid, Valladolid, Spain,* [2]*Universidad de Valladolid, Valladolid, Valladolid, Spain*

Introduction: Wiskott-Aldrich syndrome (WAS) is an X-linked primary immunodeficiency characterized by thrombocytopenia, recurrent infections and eczema. It is due to mutations in *WASp gene*. There are over 200 different mutations responsible for WAS, distributed in all exons.

Objectives: Adeno-associated virus (AAV2) production for correcting *WASp* mutations in human cells by gene targeting." *In vivo*" deletion of the blasticidin resistance cassette by using recombinant Cre fusion protein.

Materials and methods: Genomic DNA was amplified by PCR to obtain the homology arms. The vectors pAAV-W1/2 and pAAV-W3/4/5/6 were generated containing the respective homologous regions of *WASp* and a blasticidin resistance cassette flanked by two loxP sites. The viral particles were produced by co-transfection of the recombinant plasmids, together with pRC and pHelper in AAV293 cells. HCT116 cells were infected with both virions at an MOI of 1000. The recombinant protein 6xHis-TAT-NLS-Cre was produced to remove the blasticidin resistance cassette.

Results and Conclusion: Two rAAV virions were produced for the correction of *WASp* mutations located in exons 1–6. The frequency of gene targeting obtained was 1–2% with both viruses, in the human cell line HCT116. We are currently working on different protocols to increase that frequency. We will test these viruses in primary fibroblast lines that we have derived from patients with Wiskott-Aldrich.

P182

Comprehensive data mining approach applied to integration sites analysis

Andrea Calabria[1], Luca Biasco[1], Fabrizio Benedicenti[1], Simone Leo[3], Martina Cesani[1], Elia Stupka[2], Gianluigi Zanetti[3], Alessandra Biffi[1,6], Alessandro Aiuti[1,5], Luigi Naldini[1,4], Eugenio Montini[1]

[1]*San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Milan, Italy,* [2]*San Raffaele Center for Translational Genomics and Bioinformatics, Milan, Italy,* [3]*CRS4, Pula, Italy,* [4]*Vita-Salute San Raffaele University, Milan, Italy,* [5]*University of Rome Tor Vergata, Rome, Italy,* [6]*Pediatric Clinical Research Unit, San Raffaele Scientific Institute, Milan, Italy*

Analysis of chromosomal vector integration sites (ISs) in vector marked cells from gene therapy patients is a key point for unraveling gene therapy progresses, like safety and efficacy, and getting new biological insights into stem cell biology. By Next-Generation Sequencing (NGS) technologies we sequenced >10×10e6 proviral/host genomic junctions from cells of 6 patients from 2 ongoing hematopoietic stem cell (HSC)-based clinical trials with self-inactivating lentiviral vectors in Milan (metachromatic leukodystrophy and Wiskott–Aldrich syndrome). Overall we retrieved 3,725,644 univocally mapped reads resulting in 56,126 unique ISs distributed into cell lineages and tissues during time. Given the large amount of IS data to be analyzed and the diversification of the variables associated to it, we designed a new comprehensive data-mining framework, divided into three main blocks: (1) NGS data processing, to handle high-throughput IS mapping; (2) data quality, to filter ISs by quality parameters and new methods for collisions detection and data normalization; (3) ISs biological analysis. The third block addresses different biological problems: (a) common insertion sites identification, using a gene-centric method; (b) clonal abundance studies using z-score statistics; (c) stem cell activity during time by analyzing shared ISs in CD34+, myeloid and lymphoid lineages; (d) lineage-based clonality and diversity analyses. By applying this framework to our clinical trials we obtained a deeper data granularity of genomic ISs allowing improved bioinformatics and statistical analyses and allowed categorizing each insertion event at lineage, source and time point level to track the clonal activity and dynamics of hematopoietic reconstitution after HSC transplantation.

P183

A safety and efficacy improved LV driven by FANCA-homologous pormoter towards gene therapy in Fanconi's aneamia

Emilie Bayart[1], Caroline Duros[1], Alexandre Artus[1], Aurore Malric[1], Stephanie Lemaire[1], Odile Cohen-Haguenauer[1,2]

[1]*Ecole Normale Superieure de Cachan UMR8113, Paris-Cachan, France,* [2]*Hôpital Saint-Louis and University Paris-Diderot, Paris, France*

Fanconi anemia is a rare inherited genomic instability syndrome, with severe BM deficiency developing around age 7. The 15 known genes are involved in DNA repair, redox metabolism and differentiation. FA patients' cells exhibit hypersensitivity to DNA crosslinking agents such as Mitomycin C (MMC). We have demonstrated that BM dysfunction can be ameliorated in protecting cells from oxidative stress with both hypoxia and NAcetylCystein and achieved in vitro and in vivo long-term reconstitution with retrovirally-transduced human unfractionated BM. (Cohen-Haguenauer et al, 2006).

To help reducing the risk for GT induced malignancies in this cancer-prone disease, we have designed a new generation of insulated vectors to shuttle the FANCA cDNA driven by either of three promoters: the strong viral FrMuLVU3, housekeeping hPGK or FANCA homologous promotor (FANCendo) which mediates low basal expression levels since EGFP1 fluorescence is reduced by one or two range compared to hPGK or viral promoters, respectively. Transduction of HSC72 FA cells resulted in functional correction with overcoming the G2M block on high doses MMC and restoring FancD2 mono-ubiquitination. We next genetically corrected primary mesenchymal cells from FANCA-/- patients' BM. Which could recover resistance to both oxidative stress and MMC, the most efficient being with FANCendo. CD34+ cells were efficiently transduced with the FANCendoInsLV and grown over 8 weeks in LTCIC without toxicity. Experiments are underway in order to address the feasibility of generating diseased hiPS, as a model towards the establishment of best conditions for cells regeneration.

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P184

non integrative lentiviral vectors for the gene therapy of pancreatic adenocarcinoma

Emmanuel Ravet[2], Gérard Tiraby[2], Louis Buscail[1], Pierre Cordelier[1]

[1]*INSERM U1037, Cancer Research Center of Toulouse, Toulouse, France,* [2]*Cayla-Invivogen, Toulouse, France*

Locally advanced and metastatic pancreatic adenocarcinoma (PDAC), one of the most common malignancies worldwide, can't be cured. Consequently, the 5-year survival of patient diagnosed with advanced PDAC is less than 3%. In this dismal context, we conducted pioneering studies using therapeutic gene transfer to inhibit PDAC growth and are currently performing a "first-in-man" gene therapy clinical trial for this disease. Meanwhile, we demonstrated that HIV-1 lentiviral vectors outshine other vectors in delivering therapeutic genes to impede PDAC growth. However, the inherent genotoxicity of this vectors negatively impact their use in clinical trials. Thus, we developed a novel anticancerous approach based on non-integrating lentiviral vectors (NILVs). We generate SIN NILVs using two different packaging constructs encoding for HIV-1 integrase with single, class I mutations (D64V and D116N). We produced high titer NILVs following ultracentrifugation. Using GFP and secreted Lucia luciferase as markers, we demonstrate that NILVs at MOI = 5 transduce human PDAC-derived cell lines with high efficacy (96,5% ± 1%) without selection. Gene expression persists for 12 days in highly proliferating PDAC cells but was reduced ten-fold as compared to parental vectors. Interestingly D116N NILVs persisted longer in PDAC-derived cells as compared to D64V mutant. We generated a NILVs encoding for DCK:UMPCK fusion gene which metabolizes gemcitabine chemotherapy. Transduction with this vector and gemcitabine treatment successfully killed PDAC-derived cell lines. To our knowledge, we provide herein the first characterization of using NILVs as cancer gene therapy delivery vectors. This work stems for the use of NILVs in forthcoming gene therapy trial for PDAC

P185

Defining transduction thresholds for therapeutic benefit in the treatment of urea cycle disorders using recombinant adeno-associated viral vectors

SC Cunningham[1,2], CY Kok[1], IE Alexander[1,3]

[1]*Gene Therapy Research Unit, The Children's Medical Research Institute and The Children's Hospital at Westmead, Westmead, NSW, Australia,* [2]*Sydney Medical School, University of Sydney, Sydney, NSW, Australia,* [3]*Discipline of Paediatrics and Child Health, University of Sydney, Sydney, NSW, Australia*

Recombinant adeno-associated viral vectors (rAAVs) are the most promising delivery system for liver-targeted gene therapy. We are interested in developing therapies for urea cycle disorders, which result in impaired ammonia detoxification and amino acid metabolism. Patients with severe neonatal disease have high morbidity and mortality necessitating liver transplantation. Given the shortage of donor livers and transplantation-associated morbidities, gene therapy offers an attractive alternative. A major translational challenge of early gene therapy with rAAV is the maintenance of therapeutically adequate levels of transgene expression during liver growth. AAV vectors exist largely as episomes which are rapidly lost from proliferating hepatocytes. A subset of cells, however, remain stably transduced as a consequence of genomic integration. To determine the stable transduction threshold for therapeutic benefit following neonatal treatment we used ornithine transcarbamylase (OTC) and argininosuccinate synthetase (ASS) deficient mouse models.

Neonatal OTC deficient (Spf^{ash}) mice were injected with rAAV2/rh10.LSP1-mOTC and, upon reaching adulthood, residual endogenous OTC activity was knocked down using a short-hairpin RNA. All mice became severely hyperammonaemic despite liver-wide OTC activities above 30% wild-type levels, and stable hepatocyte transduction levels of ~10%. Hence the number of hepatocytes transduced appeared more important than absolute enzyme levels achieved. This conclusion was strongly supported by studies in the neonatal lethal ASS knock-out mouse. After in utero injection, and subsequent vector re-delivery with alternative capsid serotypes, L-arginine injections and protein restriction, mice survived to adulthood. Protection against hyperammonaemia correlated with stable transduction of approximately 25% of hepatocytes, but as little as 15% liver-wide ASS activity.

P186

Human galectin 3 binding protein interacts with recombinant adeno-associated virus type 6

Jérôme Denard[1], Cyriaque Beley[2], Robert Kotin[3], René Lai-Kuen[4], Stéphane Blot[5], Hervé Leh[8], Aravind Asokan[6], Jude Samulski[6], Philippe Moullier[1,7], Thomas Voit[2], Luis Garcia[2], Fedor Svinartchouk[1]

[1]*Généthon, Evry, France,* [2]*Institut de Myologie, Paris, France,* [3]*Molecular Virology and Gene Therapy Laboratory, Bethesda, USA,* [4]*Paris Descartes University, Paris, France,* [5]*Université Paris-Est Créteil, Maisons-Alfort, France,* [6]*University of North Carolina School of Medicine, Chapel Hill, NC, USA,* [7]*Université de Nantes, Nantes, France,* [8]*ENS Cachan, Cachan, France*

Recombinant adeno-associated viruses (rAAVs) hold enormous potential for human gene therapy. Despite the well-established safety and efficacy of rAAVs for in vivo gene transfer, there is still little information concerning the fate of vectors in blood following systemic delivery.

Here, by using a multidisciplinary approach (proteomics, binding assays, electron microscopy and in vivo experimentations), we screened for serum proteins interacting with different AAV serotypes in humans, macaques, dogs and mice. We report that serotypes rAAV-1, −5, −6, but not −2, −7, −8, −9 and −10 interact in human sera with galectin 3-binding protein (hu-G3BP), a soluble scavenger receptor. Between the three serotypes, rAAV-6 has the most important binding capacities to G3BP. rAAV-6 also bound G3BP in dog sera, but not in macaque and mouse sera. Furthermore, interaction of the hu-G3BP with rAAV-6 led to the formation of aggregates and hampered transduction when co-delivered into the mouse.

Our results suggest that proteins of the innate immune system with high structural similarity might provide different host defense functions against AAV serotypes in a species-specific fashion. Interactions with these proteins need to be considered when designing pre-clinical studies in animal models for systemic gene transfer and evaluating vector biodistribution and toxicity.

P187

Development of novel targeted lentiviral vectors for gene therapy of motor neuron diseases

Ioanna Eleftheriadou, Antonio Trabalza, Stuart M Ellison, Nicholas D Mazarakis

Imperial College London, London, UK

Motor Neuron Diseases (MND) including Amyotrophic Lateral Sclerosis (ALS) and Spinal Muscular Atrophy (SMA) are neurodegenerative diseases that can cause progressive paralysis and premature death, while there are no treatments available up to date. Gene therapy using lentiviruses has been successful at alleviating symptoms and extending survival in murine models of both diseases. Lentiviral tropism is determined by their viral envelope protein, which upon identification and interaction with their receptors induce fusion of the viral envelope with the target cell membrane. Restricting infection to specific cells, known as 'targeted transduction', is critical for safe and efficient *in vivo* gene delivery. An alternative to pseudotyping could be modification of viral surface through genetic engineering.

Our aim was to generate novel lentiviral vectors with tropism to motor neurons (MNs) via the neuromuscular junction (NMJ). Targeting is based on a method that involves incorporation of an antibody and a fusogenic protein into lentiviral surface as two distinct molecules. We have cloned and engineered membrane-bound and single-chain antibodies against rat Thy1.1 and p75LNGFR presynaptic terminal receptors. The resulting high titer vectors were proven to be absolutely specific for Thy1.1 and p75 expressing cells, respectively. Their specificity has extensively been assessed *in vitro* through a series of binding and competition assay experiments. *In vivo* intramuscular delivery of these vectors to rat and mouse gastrocnemious muscles is currently being performed to assess retrograde transport to spinal cord and transduction of spinal MNs. These vectors have the potential to be innovative tools for gene transfer to MNs.

P188

Restricted transgene expression in microglia using microRNA-9 regulated lentiviral vectors

Erika Elgstrand Wettergren[1], Malin Åkerblom[0,2], Rohit Sachdeva[0,2], Luis Quintino[1], Giuseppe Manfre[1], Cecilia Lundberg[1], Johan Jakobsson[0,2]

[1]CNS Gene Therapy Unit, Dept. of Experimental Medical Science, BMC A11, Lund University, Lund, Sweden, [2]Molecular Neurogenetics, Dept. of Experimental Medical Science, BMC A11, Lund University, Lund, Sweden

Microglia has been shown to play an important part in maintaining homeostasis in the central nervous system (CNS). The cell type has also been implicated in the pathogenesis of several diseases. Microglia has however been difficult to study and the exact role of this cell type in healthy and diseased brain is therefore still unclear. Gene therapy paradigms aimed at targeting microglia has been largely unsuccessful since this cell type is difficult to target specifically. In this study, we use microRNA detargeting to restrict transgene expression to microglia in vivo. We have constructed a lentiviral vector containing the PGK promoter driving GFP coupled to four copies of the target site for microRNA-9 (LV-PGK-GFP-miR9T). Rats were injected into the striatum with either the LV-PGK-GFP-miR9T or a LV-PGK-GFP (control) vector. There was a significant increase in microglia specificity using the LV-PGK-GFP-miR9T vector compared to using a LV-PGK-GFP vector ($68.4 \pm 2.4\%$ versus $6.2 \pm 1.5\%$, $p < 0.0001$). Transgene expression could also seen in activated microglia when the virus injection was followed by an excitotoxic insult. These results indicate that miR9 regulated lentiviral vectors can be used to efficiently target microglia and monitor the activation of resident microglia following a lesion.

P189

A Highly Effective Equine Infectious Anaemia Virus-based Lentiviral Gene Therapy Platform for the Treatment of Ocular Diseases

S Ellis, J Miskin, K Binley, J de Belin, J Loader, J Ferrige, D Angell-Manning, M Carlucci, M Kelleher, S Naylor, K Mitrophanous

Oxford BioMedica (UK) Ltd, Oxford, UK

Oxford BioMedica has developed ocular gene therapies based on the recombinant Equine Infectious Anaemia Virus (EIAV). Currently we have four ocular therapies: RetinoStat®, StarGen™, UshStat® and EncorStat®, for the treatment of age-related macular degeneration, Stargardt macula dystrophy, Usher Syndrome 1B, and the prevention of corneal transplant rejection respectively. RetinoStat® and UshStat® are in Phase 1/2 under an IND, StarGen™ is in Phase 1/2 with an open IND and CTA (France).

The EIAV lentiviral vector system has been extensively characterized in GLP safety and biodistribution studies following subretinal administration for up to 6 months as part of the RetinoStat®, StarGen™ and UshStat® programmes. Subretinal administration of vector caused no long-term detrimental changes within the eye. Ocular inflammation (in the absence of prophylactic anti-inflammatory medication) following administration of all three ocular products was only mild-to-moderate and transient, completely resolving within one month. Biodistribution studies demonstrated the lentiviral vector stayed within the ocular compartment, and little or no antibody responses were observed in these studies.

This safety profile has translated to the clinic, with over 10 patients treated with three different ocular therapies with no adverse events. Additionally, clinical expression of the secreted proteins in the RetinoStat®-treated patients has shown to be sustained (out to one year so far) and follows a clear dose response.

The EIAV-based vector platform is an effective and safe system for the delivery of large transgenes into target retinal cells resulting in stable and long-term therapeutic expression.

P190

Cationic synthetic polymers enable high-efficiency transduction of human hematopoietic progenitor cells by adenovirus vectors

Sigrid Espenlaub[1], Tatjana Engler[1], Ian C.D. Johnston[2], Alexandra Vetter[3], Manfred Ogris[3], Stefan Kochanek[1], Florian Kreppel[1]

[1]Department of Gene Therapy, University of Ulm, 89081 Ulm, Germany, [2]Miltenyi Biotec GmbH, 51429 Bergisch Gladbach, Germany, [3]Center for System-Based Drug Research, Department of Pharmacy, Pharmaceutical Biotechnology, Ludwig Maximilian University, 81377 Munich, Germany

Human hematopoietic stem and progenitor cells (HPCs) - while being an attractive target for various gene transfer approaches - are largely refractory to transduction by human Ad5. Here, we describe the utility of cationic synthetic polymers that allow for high-efficiency transduction of this cell type by Ad5.

The dendrimeric, positively charged poly(amidoamine) PAMAM is able to form complexes with Ad5-based vectors upon a simple mixing procedure. This complexation of Ad5 with PAMAM allowed for transduction of up to 41% human $CD34^+$ stem and progenitor cells purified from buffy coats.

In depth analysis confirmed significantly increased uptake of PAMAM/Ad complexes by a wide variety of different cell lines of hematopoietic origin.

Further, we found that MACSductin^{TM} reagent, polycationic magnetic particles from Miltenyi Biotec (Germany) in a magnetic field allowed to concomitantly transduce and purify human $CD34^+$ stem and progenitor cells.

In summary, our data suggest that non-covalent charge-based targeting strategies are favourable compared to genetic or chemical coupling strategies for efficient gene delivery into HPCs by Ad5-based vectors.

P191

Development of a physiologically relevant in vitro model of AAV8 transduction of primary mouse hepatocytes

Susan M. Faust[1], Frank Jacobs[2], Sadik H. Kassim[3], Roberto Calcedo[1], Benjamin J. Cutler[5], Yanqing Zhu[1], Peter Bell[1], Joseph E. Rabinowitz[4], James M. Wilson[1]

[1]University of Pennsylvania, Philadelphia, PA, USA, [2]University of Leuven, Leuven, Belgium, [3]Janssen Research and Development, Philadelphia, PA, USA, [4]Temple University, Philadelphia, PA, USA, [5]Washington University, St. Louis, MO, USA

The study of AAV8 transduced hepatocytes in culture is of great importance in researching the full mechanism behind AAV8 transduction, in producing and isolating soluble factors following hepatic AAV8 gene transfer, and in assaying the function of AAV vectors prior to the initiation of in vivo experiments. Poor in vitro transduction efficiency of AAV8 in hepatoma cell lines and primary hepatocytes poses a major challenge to this line of investigation. A second barrier–dedifferentiation and rapid death of hepatocytes in response to inadequate in vitro culture environments–precludes use of such systems as relevant models. We report the development of a physiologically relevant in vitro model of AAV8 transduction of primary mouse hepatocytes. We demonstrate a cell culture system that supports normal cell structure and maintenance of differentiated hepatocytes with highly efficient AAV8 gene transfer, in which >96% of hepatocytes express the reporter gene. AAV8GFP gene transferred hepatocytes cultured in vitro demonstrated spherical, clustered morphology and robust GFP expression for up to one week. Morphological characteristics typical of healthy, differentiated hepatocytes were observed for the length of the study. In conclusion, we report an in vitro microenvironment that supports long-term maintenance of AAV8 gene transferred hepatocytes.

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MicroRNA-Regulated Cardiotropic AAV Vectors with Skeletal Muscle-Specific Suppression of Transgene Expression

Anja Geisler[1], Jens Kurreck[1], Roland Vetter[0,2], Henry Fechner[1]

[1]Institue of Biotechnology, University of Technology Berlin, Berlin, Germany, [2]Institute of Clinical Pharmacology and Toxicology, Charité - Universitätsmedizin Berlin, CCM, Berlin, Germany

AAV vectors are currently the most specific and promising vector type used for gene transfer into the heart. However, these vectors also tranduce other organs, in particular the liver and the skeletal muscle. Recently we have shown that insertion of miR-122-specific target sites (TS) in the 3′UTR of a transgene is a powerful method to prevent transgene expression in the liver. Among miRs expressed in the heart and skeletal muscle, only miR-206 is highly expressed in skeletal muscle and rarely expressed in cardiac tissue. However, we found that a transgene which contains three complete complementary copies of miR-206TS was strongly suppressed in the heart after systemic AAV9 vector application. *In vitro* analysis revealed that this resulted from binding of a member (miR-1) of the same miR family, that was strongly expressed in the heart. To prevent miR-1-mediated transgene repression, we introduced single nucleotide substitutions into the miR-206TS. Several mutated miR-206TS (m206TS) were resistant to miR-1, but remained fully sensitive to miR-206. All these variants had mismatches in the "seed" region of the miR-m206TS duplex in common. Systemic *in vivo* application of AAV9 vectors confirmed that m206TS-bearing transgene was repressed in the skeletal muscle but not in the heart. Furthermore, we found that miR-206-mediated repression of a transgene can be improved by use of imperfect complementary miR-206TS. Thus, site-directed mutagenesis of miR-TS provides a new strategy to differentiate transgene de-targeting by miRs of the same family.

P193

Restoration of transduction efficacy in the liver through a surgical approach in a model of preimmunized rats against VSVg lentiviral envelop

SCHMITT Françoise[1,2], LE BOULC'H Morwenn[2], BONI Sébastien[2], ANEGON Ignacio[1], FERRY Nicolas[2], PODEVIN Guillaume[2]

[1]INSERM U1064, Nantes, France, [2]INSERM U948, Nantes, France

Introduction: gene therapy appears as an innovative treatment in the field of inherited liver diseases. Nevertheless, *in vivo* efficacy of lentiviral vectors (LV) used in humans is limited by a non specific innate immune response against its VSVg protein envelop. The aim of this study was (1) to validate a rat model of pre-immunization against VSVg protein, and (2) to prove the ability of our "Lentiviral in Isolated Liver (LIL)" approach to restore an efficient transduction in the liver of these rats.

Material and Methods: ten rats were immunized through intravenous injection of a LV with a VSVg envelop. Three weeks later, ELISA and LV neutralization assays by rats seri confirmed the immunization. Then, a GFP-encoding LV was injected either through the portal vein or using the LIL approach, with or without liver washing. Transduction efficacy was tested 7 days later through qPCR and anti-GFP immunohistochemistry in the liver.

Results: every pre-immunized rat developed anti-VSVg antibodies. Transduction efficacy after portal vein injection significantly decreased in pre-immunized animals (0.52% versus 0.01%, $p = 0.03$) and LIL without liver washing restored the expected transduction level (0.33%) in those rats. No transduction was

found with the "LIL with liver washing" method, whatever the immune competence of the rats.

Conclusion: pre-immunization of rats against VSVg protein efficiently mimics human seric prevention in transduction efficacy of LV. The LIL approach in pre-immunized rats allows a restoration of transduction efficacy in the liver.

P194

A single baculovirus for the production of rAAV8 vectors

Lionel Galibert, Aurélien Jacob, Christophe Lecomte, Christel Rivière, Otto-Wilhelm Merten

Genethon, Evry, France

In this work, we present the realization of a single baculovirus encoding the AAV *rep2*, *cap8* genes and the recombinant AAV genome, allowing the production of recombinant AAV8 vectors in Sf9 cells. This set up was performed in a modified bacmid system deleted of the *chitinase*, *cathepsin* and *p10* genes. The *rep2/cap8* expression cassette was inserted in the *egt* locus of the bacmid genome. The recombinant AAV genome was inserted in the classical Tn7 site of the bacmid. This baculovirus, named "Monobac", was used for the production rAAV2/8 encoding the human γ-sarcoglycan gene, of clinical interest for the treatment of LGMD2C (γ-sarcoglycanopathy). A gain by a factor 5 in rAAV8 productivity in the cell culture was observed using the Monobac compared to a production using two baculoviruses. The observed productivity gain was (i) due to the expression of the AAV *rep2/cap8* expression cassette from the *egt* locus and (ii) due the use of a single baculovirus for the production allowing the simultaneous transfer of all gene functions to the infected cells. The produced rAAV8 capsids displayed a reduced degradation profile of VP1/VP2 due to the elimination of the baculovirus *cathepsin* protease gene. This optimized system allows the production of an improved quantity of rAAV vectors with improved vector quality of high importance for pre-clinical and, in particular, for clinical use.

P195

Trafficking characteristics of lentiviral vectors in motor neurons *in vitro*

TA Islam[1], JN Hislop[1], DCJ Carpentier[0,2], ND Mazarakis[1]

[1]*Imperial College London, London, UK,* [2]*University of Cambridge, Cambridge, UK*

A common strategy in gene therapy involves the use of lentivectors derived from HIV-1 and EIAV. These vectors are targeted to neurones by pseudotyping them with rabies-G glycoprotein (RVG). Lentivectors are ideal for distal targeting of neurones *in vivo* because they are retrogradedly transported from the synapse via the axon to the nucleus where they integrate and express the transgene. However, transduction efficiencies in motor neurones in non-human primates have been low, hindering clinical studies. We endeavoured to characterise the processes involved in the entry and retrograde trafficking of RVG-pseudotyped lentivectors which may allow us to increase the efficacy of gene transfer.

For the first time, we demonstrate the co-internalisation of RVG-pseudotyped lentivectors with 3 specific receptors: p75 neurotrophin receptor ($p75^{NTR}$), neural cell adhesion molecule (NCAM) and nicotinic acetylcholine receptor (nAChR). The levels of association remain relatively the same, suggesting the lentivectors traffic with their specific receptors throughout the axonal pathway. Furthermore, the lentivectors follow a sequential Rab5 and Rab7 dependent pathway similar to that observed for tetanus toxin and consistent with $p75^{NTR}$ mediated trafficking.

Additionally, RVG-pseudotyped lentivectors were engineered to contain a small tetracysteine genetic tag, producing fluorescent vectors. These were utilised in live-imaging studies in primary rat motor neuron cultures. We show that while many vectors traffic inefficiently, a number of RVG pseudotyped lentivectors undergo fast, long distance retrograde transport, when compared to VSV-G pseudotyped lentivectors. This explains why the neurotropic RVG pseudotype can transduce motor neurones even when administered in the muscle *in vivo*.

P196

Difference between secreted and cellular rAAV vectors produced by human embryonic kidney 293 cell line

Christine Jenny[1], Jérôme Denard[1], Véronique Blouin[2], Jérémy Rouillon[1], Philippe Moullier[2], Fedor Svinartchouk[1]

[1]*Généthon, EVRY, France,* [2]*Université de Nantes, Nantes, France*

Recombinant Adeno-associated viruses (rAAV) produced after transient transfection of human embryonic kidney 293 cell line are efficiently released into culture medium {Vandenberghe, 2010 #348}. In the present study we compared proteins associated with rAAV-6 and rAAV-8 released into the medium (secreted fraction of rAAVs, SF) and those purified after cell lysis (cellular fraction of rAAVs, CF) and looked for the impact of these proteins on the transduction efficacy of the vectors. We report that human embryonic 293 cell line produced galectin-3 binding protein (hu-G3BP), and this protein was associated with rAAV-6 SF and rAAV-8 SF, but not with vector CF. Interestingly, rAAV-6 CF but not rAAV-8 CF was able to interact with hu-G3BP from human serum. Interaction of the hu-G3BP with rAAV-6 led to the formation of aggregates and hampered transduction when co-delivered into the mouse {Denard, 2012 #2}. Intriguingly, rAAV-8 SF (with G3BP) or rAAV-8 CF (without G3BP) had very similar efficiency and biodistribution in mice. Nevertheless, rAAV8 purified from culture medium induced a strong anti huG3BP immune response after being injected intravenously. These results demonstrate for the first time that biological properties of rAAVs released into the medium or purified from cells can be different.

P197

Generation of LeGO-SWITCH, an inducible all-in-one lentiviral vector for stringent tet-controllable gene expression

Rüdiger Klapdor, Kristoffer Weber, Johannes Polke, Adele Mucci, Boris Fehse, Stefan Horn

Research Dept. Cell and Gene Therpy, Clinic for Stem Cell Transplantation, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

Lentiviral vectors have been widely used in basic research and gene therapy. Following the building brick principle we recently introduced the LeGO vector platform for easy design and cloning of lentiviral vectors. LeGO vectors facilitate ectopic transgene expression as well as shRNA-mediated RNA interference in conjunction with a number of different fluorescent proteins and/

or selectable markers. The aim of this study was to expand the versatility of the LeGO vector system by introducing tet-controllable KRAB-domain repressors to generate inducible all-in-one lentiviral vectors. The new vectors named LeGO-SWITCH were optimized for conditional expression by designing a variety of multi-cistronic expression cassettes. Addition of an adjustable destabilisation domain to the fluorescent marker opens the possibility for secondary regulation of the marker protein level. Thus too high marker protein levels can be avoided and regulable cells can easily be identified. LeGO-SWITCH vectors mediated tightly controlled expression with low background and up to 1600 fold induction in bulk cultures of various cell lines and primary mesenchymal stromal cells (MSCs). We demonstrated functionality of LeGO-SWITCH vectors by exploring the effect of inducible positive (p110a) and negative (SHIP1) regulators of the PI3K/AKT pathway in haematopoietic cells. Tet-dependent regulation of transduced cells resulted in strict regulation of the signalling cascade and downstream biological processes including proliferation, cell survival and transformation. In conclusion the novel LeGO-SWITCH vectors allow for controlled expression of genes of interest in conjunction with fluorescent and selectable markers and thus offer new possibilities to investigate gene functions by conditional and tunable expression.

P198

A modular system for transduction targeting of viral vectors for gene therapy

Feliks Kochan[1], John A. Dangerfield[1,2], Walter H. Günzburg[1,3], Brian Salmons[3], Christoph Metzner[1]

[1]Institute of Virology, University of Veterinary Medicine, Veterinarplatz 1, 1210 Vienna, Austria, [2]Anovasia Pte Ltd, 20 Biopolis Way, #05–518 Centros, 138668 Singapore, Singapore, [3]SG Austria Pte Ltd/ Austrianova Singapore Pte Ltd, 20 Biopolis Way, 138668 Singapore, Singapore

Gene delivery approaches based on lentiviral and retroviral vectors are among the most promising with a great potential for clinical applications. Still, the predominant obstacles remain a great challenge: poor selectivity of the vectors, genotoxicity and low efficiency of gene transfer *in vivo*. In order to address some of these issues, we have developed a method to modify viral envelopes with engineered proteins in a fast, flexible and versatile way. This system employs the unique property of purified glycosylphosphatidylinositol (GPI)-anchor proteins to associate with biological membranes in a process termed "Molecular Painting". Decoration of the viral surface can be done in solution after release from the producer cells ("Post-exit"). To facilitate higher selectivity of infection, proteins post-translationally modified with GPI anchors will provide binding capabilities to the vectors. Fusion of vectors with membranes of the target cells can be achieved through the action of a mutated Sindbis virus glycoprotein, which lacks a functional binding portion. We are currently testing lentiviral and retroviral vectors modified with GPI-anchored epidermal growth factor (EGF) and CD4, which should target EGF receptor(s) overexpressing cells and HIV-1 infected cells, respectively. While similar approaches have been tested using conventional genetic modification of the virus producing cells, we believe that this system will create a more flexible and efficient method for targeted infection of specific cell types using retro- or lentiviral vectors.

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P199

BrainCAV: a nonhuman adenovirus vector for gene transfer to the brain

P Alves-Marques[1,2], A Baker[3], A Bosch[8], M Carrondo[1,2], EJ Kremer[5], R Luquin[9], I Saggio[10], J Schwarz[4], G Schiavo[7], JM Verdier[6]

[1]Instituto de Biologia Experimental e Tecnológica,, Oeiras, Portugal, [2]Instituto de Tecnologia Química e Biológica–Universidade Nova de Lisboa, Lisbon, Portugal, [3]British Heart Foundation Glasgow Cardiovascular Research Centre, University of Glasgow, Glasgow, UK, [4]Department of Neurology, University of Leipzig, Leipzig, Germany, [5]Institut de Génétique Moléculaire de Montpellier, Montpellier, France, [6]Universities of Montpellier II, Montpellier, France, [7]Molecular NeuroPathobiology Laboratory, Cancer Research UK London Research Institute, London, UK, [8]Center of Animal Biotechnology & Gene Therapy, and Dept of Biochemistry & Mol Biology, Universitat Autònoma de Barcelona, Barcelona, Spain, [9]Laboratory of Regenerative Therapy, CIMA and Dept of Neurology, Clínica Universidad de Navarra,, Pamplona, Spain, [10]Department of Genetics and Molecular Biology, University 'La Sapienza', Rome, Italy

BrainCAV is an FP7-funded collaborative research project that focuses on the development and assessment of a gene transfer vector to target the brain. As suggested by recent clinical results, brain gene transfer offers substantial potential, yet brings unique obstacles - in particular the need to address feasibility, efficacy and safety. BrainCAV's foundation is based on the potential of canine adenovirus type 2 (CAV-2) vectors to preferentially transduce neurons, undergo efficient targeting via axonal transport and provide long-term expression. BrainCAV is a translational project that spans basic research through pre-clinical model feasibility, efficacy and safety. The BrainCAV partners developed cell lines & purification schemes, improved production protocols, assayed the transcriptional response of vector-transduced neurons in vitro and in vivo, characterized the mechanism of receptor engagement and axonal transport, and assayed biodistribution and interaction with brain fluids and cells. To provide a proof-of-principle of the effectiveness of CAV-2 vectors, we also tackled mucopolysaccharidosis type VII, a global, orphan disease commonly affecting children, and Parkinson's disease, a focal degeneration of dopaminergic neurones commonly affecting aged population. A collection of BrainCAV posters describing our latest results will be presented.

For the second consecutive year, a 1500 € "BrainCAV prize" will be awarded to a selected author of poster for gene transfer to the brain.

P200

Profiling serum miRNA in models of muscular pathologies as a tool for diseases monitoring

Nicolas Vignier[1,5], Fatima Amor[2], Fogel Paul[3], Angélique Duvallet[3], Jérôme Poupiot[3], Montus Marie[3], Isabelle Richard[3,6], Carrier Lucie[4,5], Voit Thomas[1,5], Gisèle Bonne[1,5], David Israeli[2]

[1]Université Pierre et Marie Curie- Paris 6, UM 76, CNRS, UMR 7215, Institut de Myologie, IFR14, Paris, France, [2]Genethon, Evry, France, [3]Statistical consulting, 4 Rue Le Goff, France, [4]Department of Experimental Pharmacology and Toxicology, Cardiovascular Research Center, University Medical Center, Hamburg, Germany, [5]Inserm, UMRS_974, Paris, France, [6]Inserm, UMRS_974, Evry, France

Biomarkers (BM) are critically important in diseases diagnosis and prognosis as well as in monitoring of clinical trials and therapeutic attempts. Classical monitoring methods in muscular dystrophies (MD) are based on histological and molecular analyses of muscle biopsies. Such methods are time consuming, expensive, not quantitative, and require biopsies. The serum BM mCK is not specific to particular muscle pathology and badly correlate with severity. Expression profile of miRNA molecules is specific to tissue of origin and pathophysiological state. In the context of MD, specificity of muscle-miRNA profile to muscle pathology was recently demonstrated. Moreover, it was recently discovered that intracellular miRNA molecules are released into body fluids, stably expressed, and might be used for BM discovery. The present study aimed at identification of circulating miRNA BM in five mouse MD models. To obtain high quality data we elaborated a two-steps screening strategy, employing two independent and complementary miRNA quantification technologies. We have identified a set of serum miRNA commonly deregulated in a number of MD, and other miRNA which are pathology-specific, providing together pathology-specific circulating miRNA profile for every studied model. This study demonstrated clearly the utility of circulating miRNA for diagnosis and monitoring in muscular dystrophies.

P201

Predictive markers of clinical outcome in the GRMD dog model of Duchenne muscular dystrophy: a step forward in preclinical trials

F Pinto-Mariz[1,3], I Barthélémy[2], E Yada[1], T Voit[1], SD Silva-Barbosa[4], W Savino[1,3], GS Butler-Browne[1], S Blot[2]

[1]UPMC Um76 INSERM U974 CNRS UMR 7215 Institut de Myologie, Paris, France, [2]Université Paris-Est Ecole Nationale Vétérinaire d'Alfort, Maisons-Alfort, France, [3]Oswaldo Cruz Foundation, Rio de Janeiro, Brazil, [4]National Cancer Institute, Rio de Janeiro, Brazil

Observed in Duchenne patients and in the canine counterpart model, the inter-individual heterogeneity is a drawback when evaluating therapies. Identifying predictive markers may help to solve this problem.

Predictive biomarkers of the evolution of 2 month-old GRMD dogs, either toward the severe form (SF, loss of ambulation before the age of 6 months) or toward the moderate form (MF, no loss of ambulation) were identified. The proportion of circulating T-lymphocytes expressing high levels of the α-chain of the integrin VLA-4 (progression biomarker of DMD) was assessed in 10 SF and 23 MF dogs. Gait analysis using accelerometry was performed on 15 SF and 38 MF dogs, to assess if the early gait impairment could differentiate the clinical forms.

The proportion of circulating CD4+VLA4hi T-lymphocytes was increased ($p = 0.002$) in SF dogs. The value of 12% was defined as a predictive threshold for SF, with 80% specificity and 70% sensitivity. Interestingly, a 14.43% threshold could be used to obtain 100% specificity. Additionally, the spontaneous speed of SF dogs was slower ($p < 0.0001$), mainly due to a stride frequency decrease ($p < 0.0001$). A speed (normalized by height) lower than 3.2759/s, and a stride frequency lower than 2.44s-1 were able to predict SF respectively with 86.8% and 89.5% specificity, and 86.7% and 73.3% sensitivity.

These predictive markers are simple to obtain and offer good level of reliability which could be improved by combining them. Their use should improve the design of therapeutic trials according to the predicted evolution of dogs, thus reducing the interfering clinical heterogeneity.

P202

Gene therapy strategy for Amyotrophic Lateral Sclerosis using recombinant AAV9 vectors to silence the mutant SOD1 gene

Maria Grazia Biferi, Mathilde Cohen-Tannoudji, Marianne Rode, Martine Barkats

Equipe Biothérapie des Maladies Neuromusculaires (Groupe « SNC/ Motoneurone »); UPMC-CNRS-AIM-INSERM UMRS 974, Institut de Myologie, 105, Bd de l'Hôpital - 75013 Paris, France

Amyotrophic Lateral Sclerosis (ALS) is an incurable motor neuron (MN) disease characterized by MNs death leading to muscle atrophy, paralysis and premature death.

We have recently shown that self-complementary (sc) AAV9 gene vectors can efficiently target the central nervous system in neonatal and adult mice as well as in cats by intravenous injection (Barkats-Patent PCT/EP2008/063297,05-10-2007) (Duque et al., 2009). Taking advantage of this vector property, we obtained an efficient and non-invasive gene therapy strategy for Spinal Muscular Atrophy, opening new perspectives in the field of gene therapies for MN diseases (Dominguez et al. 2011).

Since scAAV9 transduces principal histotypes involved in ALS (MN, glia, muscle cells), we now intend to employ the powerful target capabilities of scAAV9 to establish a valid gene therapy approach for the most common form of familial ALS (fALS).

The major part (20%) of fALS forms is related to toxic gain of function mutations in the SOD1 gene. Transgenic mice overexpressing human mutated SOD1 (SOD1G93A mice) recapitulate ALS clinical features. We intend to assess the therapeutic effect of the body reduction of mutated SOD1 levels in SOD1G93A mice by artificial miRNA to human SOD1. Specifically, we produced recombinant scAAV9 carrying the SOD1-miRNA or one CTL-miRNA. Each vector allowed co-cistronically expression of the miRNA and EGFP, regulated by phosphoglycerate (PGK) promoter.

In the next future, neonatal (PND1) (presymptomatic) and 90 day-old (onset of symptoms) SODG93A or control mice will be intravenously injected with these vectors to evaluate the effects of the treatment on disease onset and progression.

P203

Long term study in dystrophin deficient dogs after AAV1-U7 exon skipping reveals decline of dystrophin expression

A Vulin[1,4], I Barthélémy[2], A Goyenvalle[1], JL Thibaud[2], G Griffith[1], R Benchaouir[1], M le Hir[1], Y Unterfinger[2], S Lorain[1], P Dreyfus[1], T Voit[1], PG Carlier[3], S Blot[1,2], L Garcia[1]

[1]UPMC Um76 INSERM U974 CNRS UMR 7215 Institut de Myologie, Paris, France, [2]Université Paris-Est Ecole Nationale Vétérinaire d'Alfort UPR de Neurobiologie, Maisons-Alfort, France, [3]Laboratoire RMN AIM-CEA Institut de Myologie, Paris, France, [4]The research Institute Nationwide Children's Hospital, Columbus, USA

Duchenne Muscular Dystrophy (DMD) is an X-linked recessive disorder resulting from lesions of the gene encoding dystrophin. These usually consist of large genomic deletions, the extents of which are not correlated with the severity of the

phenotype. Out-of-frame deletions give rise to dystrophin deficiency and severe DMD phenotypes, while internal deletions that produce in-frame mRNAs encoding truncated proteins can lead to a milder myopathy known as Becker Muscular Dystrophy (BMD). Widespread restoration of dystrophin expression via AAV-mediated exon skipping has been successfully demonstrated in the mdx mouse model and in cardiac muscle after percutaneous transendocardial delivery in the Golden Retriever Muscular Dystrophy Dog (GRMD) model. Here, a set of optimized U7snRNAs carrying antisense sequences designed to rescue dystrophin were delivered into GRMD skeletal muscles by AAV1 gene transfer using intramuscular injection or forelimb perfusion. We show sustained correction of the dystrophic phenotype in extended muscle areas and partial recovery of muscle strength. Muscle architecture was improved and fibers displayed the hallmarks of mature and functional units. A five-year follow-up ruled out immune rejection drawbacks but showed a progressive decline in the number of corrected muscle fibers, likely due to the persistence of a mild dystrophic process such as occurs in BMD phenotypes. Although AAV-mediated exon skipping was shown safe and efficient to rescue a truncated dystrophin, it appears that recurrent treatments would be required to maintain therapeutic benefit ahead of the progression of the disease.

P204

The striatal molecular marker St102 (2010001M06rik) Protects Striatal Neurons against an N-terminal Fragment of Mutant Huntingtin *in vivo*.

Emmanuel Brouillet[1,2], Laurie Galvan[1,2], Marie-Claude Gaillard[1,2], Nad'a Lepejová[3], Fanny Petit[1,2], Laetitia Francelle[1,2], Carole Malgorn[1,2], Benoit Bernay[3], Martine Guillermier[1,2], Carole Escartin[1,2], Gilles Bonvento[1,2], Noelle Dufour[1,2], Jean-Marc Elalouf[3], Philippe hantraye[1,2], Michel de Chaldée[3], Nicole Déglon[1,2]

[1]CEA, DSV, I[2]BM, Molecular Imaging Research Center (MIRCen), F-92265 Fontenay-aux-Roses, France, [2]CNRS, CEA URA 2210, F-92265 Fontenay-aux-Roses, France, [3]CEA, Institut de Biologie et de Technologies de Saclay (iBiTec-S), Service de Biologie Intégrative et Génétique Moléculaire (SBIGeM), F-91191 Gif-sur-Yvette Cedex, France

The mechanisms underlying the preferential vulnerability of striatal neurons to mutant Huntingtin (mHtt) in Huntington's disease (HD) remain unknown. One hypothesis is that genes selectively expressed in the striatum may play a role. In the present study we have studied the product of the 2010001M06rik (St102) gene, previously identified based on its preferential expression in the striatum and its significantly reduced levels in the striatum of R6/2 mice (Brochier *et al*., Physiol Genomics, 2008). We tested whether modifying St102 expression could change the neurotoxic effects of an N-terminal fragment of mHtt expressed in the mouse striatum using the HD lentiviral model (LV-Htt171-82Q). We developed lentiviral vectors to overexpress St102 (LV-St102) or knock-down St102 using a selective shRNA (LV-shRNA-St102). RT-PCR analysis infection of the striatum of adult mice with LV-St102 or LV-shRNA-St102 led to a significant increase or decrease of St102 expression respectively. LV-St102 and LV-shRNA-St102 were co-injected with LV-Htt171-82Q in the striatum of adult WT mice. Six weeks after injections, LV-Htt171-82Q consistently produced striatal lesions characterized by a loss of NeuN and DARPP32. Interestingly, the co-expression of Htt171-82Q and shRNA-St102 led to a significant increase in the lesion volume. On the contrary, the co-expression of Htt171-82Q and St102 led to a significant decrease of the lesion size. These results suggest that the loss of St102 could play a role in striatal degeneration in HD.

P205

Evaluation of AAV-mediated gene transfer of anoctamin 5 in dysferlin deficient mice.

Karine Charton, Laurence Suel-Petat, François Monjaret, Nathalie Bourg, Isabelle Richard

Genethon, Evry, France

Mutations in dysferlin and anoctamin 5 have been shown to lead to Limb-girdle Muscular Dystrophy type 2B and 2L respectively and also lead both to Miyoshi Myopathy. These two proteins have been both implicated in sarcolemmal resealing. We are testing the hypothesis that ANO5 protein could compensate for dysferlin deficiency based on their similarities in phenotypes and role. We constructed an Adeno-Associated Virus (AAV) vector that encoded for human ANO5 and performed intramuscular injection in wild-type (WT) and dysferlin deficient [B6.A/J-Dysf prmd] mice. We observed that ANO5 overexpression did not lead to toxicity in WT mice one month after injection. Evaluation of muscle histology, resistance of muscle to damage following eccentric exercise and sarcolemmal repair capacity are ongoing in the injected B6.A/J-Dysf prmd. These results would determine if anoctamine 5 overexpression could compensate dysferlin-deficiency.

P206

An adeno-associated virus-based intracellular sensor of pathological Nuclear Factor-kappaB activation for disease-inducible gene therapy.

Abdelwahed Chtarto[1], Katia Vermoesen[2], Elias Gebara[3], Olivier Bockstael[1], Catherine Melas[1], Marc Levivier[4], Olivier De Witte[1], Ruth Luthi-Carter[3], Ralph Clinkers[2], Liliane Tenenbaum[0]

[1]Université Libre de Bruxelles (ULB), Brussels, Belgium, [2]Vrije Universiteit Brussel, Brussels, Brussels, Belgium, [3]Brain and Mind Institute, EPFL, Lausanne, Lausanne, Switzerland, [4]Lausanne University Hospital, Lausanne, Switzerland

Stimulation of resident cells by NF-kB activating cytokines is a central element of inflammatory and degenerative disorders of the central nervous system (CNS). This disease-mediated NF-kB activation could be used to drive transgene expression selectively in affected cells, using adeno-associated virus (AAV)-mediated gene transfer.

We have constructed a series of AAV vectors expressing GFP under the control of different promoters including NFkB- responsive elements. As an initial screen, the vectors were tested *in vitro* in HEK-293T cells treated with TNF-a. The best profile of GFP induction was obtained with a promoter containing two blocks of four NF-kB -responsive sequences from the human JCV neurotropic polyoma virus promoter, fused to a new tight minimal CMV promoter, optimally distant from each other.

A therapeutical gene, glial cell line-derived neurotrophic factor (GDNF) cDNA under the control of serotype 1-encapsidated NF-kB -responsive AAV vector (AAV-NF) was protective in senescent cultures of mouse cortical neurons.

AAV-NF was then evaluated *in vivo* in the kainic acid (KA)-induced status epilepticus rat model for temporal lobe epilepsy, a major neurological disorder with a central pathophysiological role for NF-kB activation. We demonstrate that AAV-NF, injected in the hippocampus, responded to disease induction by mediating GFP expression, preferentially in CA1 and CA3 neurons and glial cells, specifically in regions where inflammatory markers were also induced.

Altogether, these data demonstrate the feasibility to use disease-activated transcription factor-responsive elements in order to drive transgene expression specifically in affected cells in inflammatory CNS disorders using AAV-mediated gene transfer.

P207

Injection of rAAV-Orai1 in adult wt and dystrophic mice up-regulates store operated calcium entry

TR Cully[1], BW Purdue[1], WG Thomas[1], P Gregorevic[2], BS Launikonis[1]

[1]*School of Biomedical Sciences, The University of Queensland, Brisbane, Queensland, Australia,* [2]*Muscle Research and Therapeutics Development, Baker IDI Heart and Diabetes Institute, Melbourne, Victoria, Australia*

Duchenne Muscular Dystrophy (DMD) is a degenerative X-linked muscle disease that affects 1: 3500 live male births. Store operated calcium entry (SOCE) is one Ca^{2+} influx pathway that has been implicated in contributing to the DMD phenotype. Edwards *et al.*, 2010a has previously reported an upregulation of SOCE in the mdx mouse model of DMD with the mdx mice displaying a milder phenotype. Mdx mice undergo major muscle degeneration/regeneration at approximately 4 weeks of age. We aimed to investigate whether upregulation of Orai1, a protein necessary for SOCE to occur, pre and post degeneration/regeneration phase will affect the degree of SOCE influx seen and therefore determine if upregulation of SOCE is compensatory. An Orai1 expression cassette was delivered to the extensor digitorum longus muscle of C57BL10 and mdx mice at 3 weeks and 2.5 months of age using recombinant adeno-associated viral vectors. Calcium imaging was performed in single, live, mechanically skinned muscle fibres to observe SOCE. We observed an age-depedent effect of Orai1 over-expression upon SOCE in the muscles of wt and mdx mice, as SOCE was increased ($p = 0.3$ and 0.06 respectively for wt and mdx mice, two tailed t test) in muscles examined 4 weeks after adminstration of vectors to 2.5 month-old mice, but no effect on SOCE was observed in animals treated similarly at 3 weeks of age.

P208

Delivery of an adeno-associated virus 9 vector encoding MTMR2 corrects targeted muscles in a murine model of myotubular myopathy

Nathalie Danièle, Thibaud Jamet, Christelle Moal, Alban Vignaud, Anna Buj-Bello

Généthon, Evry, France

X-linked myotubular myopathy is the most severe form of centronuclear myopathy, a group of muscular diseases characterised by the presence of nuclei centrally localised within hypotrophic myofibres. Patients present at birth with profound muscle hypotonia and weakness, respiratory insufficiency, and often die prematurely. No specific treatment is available. The pathology is caused by mutations in the *MTM1* gene, which encodes myotubularin, a lipid phosphatase showing substrate specificity for PI_3P and $PI_{3,5}P_2$. Myotubularin is the founding member of the MTMR protein family (myotubularin-related-proteins), classified together in reason of sequence homology.

In order to develop a therapeutic approach for myotubular myopathy, we tested the ability of *Mtmr1* and *Mtmr2* genes, the closest *Mtm1* homologues, to rescue the histological and functional abnormalities of myotubularin-deficient mice. Pseudo-typed recombinant rAAV2/9 vectors encoding either myotubularin, MTMR1 or MTMR2 under the control of the desmin promoter were injected into the tibialis anterior muscle of two week-old *Mtm1*-KO mice. Two weeks after vector delivery, a therapeutic effect was observed with *Mtmr2* but not with *Mtmr1* overexpression. Muscle weight and myofibre mean diameter increased significantly, and centronucleation was reduced to a level comparable to the one observed after *Mtm1* replacement. DHPR1α, Ryr1 and dysferlin stainings were also partially normalised, signing a reorganisation of the triads, a structure involved in excitation/contraction coupling. Most importantly, this treatment also led to an increase in the total and specific isometric force of myotubularin-deficient muscles. Altogether, these results suggest that *Mtmr2* overexpression in skeletal muscle may represent a powerful therapeutic strategy for myotubular myopathy.

P209

Heme oxygenase-1 in regeneration of skeletal muscles: significance of regulated expression

Jozef Dulak, Agnieszka Jazwa, Magdalena Kozakowska, Maciej Ciesla, Jacek Stepniewski, Agnieszka Loboda, Alicja Jozkowicz

Department of Medical Biotechnology, Jagiellonian University, Krakow, Poland

Heme oxygenase-1 (HO-1) is a cytoprotective enzyme degrading heme, which exerts numerous effects in various physiological and pathological conditions.

Recently we showed that HO-$1^{-/-}$ mice have impaired neovascularization in ischemic limbs and increased muscle cell death compared with wild-type animals. Injection of plasmid with HO-1 driven by hypoxia response element significantly improved blood flow after femoral artery ligation while reduced apoptosis and inflammation and improved muscles regeneration. HO-1 gene therapy decreased Pax3 and Pax7 expression while enhanced the expression of transcriptional (MyoD, myogenin) and post-transcriptional (miR-206) regulators of myoblasts differentiation (*Jazwa et al, in revision*).

Cytoprotective and pro-proliferative effects of HO-1 were demonstrated in C2C12 myoblasts continuously overexpressing HO-1 by retroviral transduction. However, permanent expression of HO-1 attenuated myoblasts differentiation, strongly affecting almost 20% out of 700 microRNAs. Particularly, mir-1, mir-29, mir-31, mir-133a, mir-133b, mir-206, mir-378 which promote differentiation were downregulated, while mir-146a, an inhibitor of differentiation via Notch pathway was upregulated. Carbon monoxide, a product of HO-1, appears to be responsible for regulation of microRNAs via inhibition of C/EBPDd and influence on other pathways. Interestingly, expression of HO-1 in injected myoblasts led to tumor formation and metastases of HO-1 overexpressing cells were detected in the lungs (*Kozakowska et al, Antioxid Redox Signal,16: 113–27; 2012*).

Our results suggest the therapeutic potential of HO-1 for treatment of leg ischemia. However, prolonged expression of HO-1 may results in uncontrolled proliferation and impaired myoblasts differentiation, causing the risk for rhabdomyosarcoma development. Our studies indicate for the necessity of regulated HO-1 expression.

P210

The Murine Dystrophic Muscle Environment Reduces Adeno-Associated Virus Vector-mediated Transgene Expression

Jean-Baptiste Dupont[1], Adrien Léger[1], Magalie Penaud-Budloo[1], Emilie Bertil[2], Christophe Georger[2], Richard Snyder[3,4], Philippe Moullier[1,2]

[1]*INSERM UMR1089, Nantes, France,* [2]*Genethon, Evry, France,* [3]*Department of Molecular Genetics, College of Medicine, University of Florida, Gainesville, Florida, USA,* [4]*Center of Excellence for Regenerative Health Biotechnology, University of Florida, Alachua, Florida, USA*

Duchenne Muscular Dystrophy (DMD) is a devastating neuromuscular disease caused by mutations in the X-linked dystrophin gene. To date, there is no definitive treatment for DMD, but novel options emerged during the last decade. Among them, gene therapy using recombinant Adeno-Associated Virus (rAAV) vectors have proved efficacy in several animal models for DMD, such as the *mdx* mouse. After *in vivo* administration, rAAV genome is stably maintained as episomal DNA in the host cell nucleus, and associates with cellular histones. This raises questions about the influence of epigenetic modulation on transgene expression. Interestingly, several epigenetic disruptions were highlighted in *mdx* muscle cells, and are thought to participate in the pathological process. Regarding rAAV-mediated gene therapy for DMD, these perturbations could also impact the expression of the 'chromatinized' genome and therefore the efficiency of the treatment. To examine this point, we followed the expression of a reporter transgene in C57B/6J (B6) and *mdx* mice after rAAV intramuscular delivery, in correlation with specific histone modifications. First, we found that rAAV genome expression is reduced in the muscle of *mdx* mice, compared to their B6 counterparts. Additionally, preliminary results in B6 muscles indicate the existence of rAAV genomes carrying repressive histone marks. Whether this feature is more prominent in *mdx* muscles remains to be elucidated but it could explain the reduced transgene expression in this context. In such case, specific chromatin-modifying drugs could be combined to rAAV vectors in an attempt to optimize gene therapy protocols for DMD.

P211

Identification of decorin derived peptides with a Zn2+ dependent anti-myostatin activity

S Guiraud[2], N El Shafey[2], L van Wittenberghe[1], C Geoger[1], D Scherman[2], A Kichler[1,2]

[1]*Genethon, BP60, 91002 Evry Cedex, France,* [2]*UMR 8151-U1022 INSERM, Université Paris Descartes, Chimie Paristech, 75006 Paris, France*

Decorin is a member of the small leucine-rich proteoglycan (SLRP) family and it is a component of the extracellular matrix. Decorin was previously shown to bind different molecules, including myostatin, in a zinc-dependent manner. Here, we investigated in detail the anti-myostatin activity of decorin and fragments thereof. We show that this protein displays in vitro anti-myostatin activities with an IC50 of $2.3.10^{-8}$ M. After intramuscular injection of decorin in dystrophic mdx and g-sarcoglycan$^{-/-}$ mice, we observed a significant increase of the muscle mass and this effect was maximal 18 days after administration. Further, we show that the myostatin-binding site is located in the N-terminal domain of decorin. In fact, a peptide encompassing the 31–71 sequence retains full myostatin binding capacity and intramuscular injection of the peptide induces muscle hypertrophy. The evaluation of three additional peptides suggests a crucial role of the four cysteines within the conserved C*X3*C*X*C*X6*C motif of class I of the SLRPs. Altogether, our results show that the N-terminal domain of decorin is sufficient for the binding to myostatin and they underscore the crucial role for this interaction of zinc and the cysteine cluster. We are now exploring gene and cell therapy approaches based on the use of expression vectors encoding the decorin protein or peptide.

P212

Macaque immune responses following AAV-based gene transfer to the skeletal muscle: addressing the method of vector delivery

Gwladys Gernoux[1], Marie Devaux[1], Laurence Dubreil[2], Nicolas Jaulin[1], Mickaël Guilbaud[1], Jack-Yves Deschamps[2], Thibaut Larcher[2], Lydie Guigand[2], Maëva Dutilleul[2], Yan Cherel[2], Philippe Moullier[1,3], Oumeya Adjali[1]

[1]*INSERM UMR1089, Institut de recherche thérapeutique, Nantes, France,* [2]*INRA UMR703, ONIRIS, Nantes, France,* [3]*Department of Molecular Genetics and Microbiology department, University of Florida, Gainesville, Florida, USA*

Recombinant AAV (rAAV) vectors are vehicles of choice for gene transfer to the skeletal muscle. However, translation of studies from rodents to large animals and more recently humans indicate that the risk of transgene and/or capsid-specific immune responses is high, particularly following intramuscular (IM) rAAV delivery. As an alternative, locoregional intravenous delivery (LR) is a promising method, although still poorly addressed in large animals. In addition to a better diffusion of the vector in the muscles, LR route seems to be less immunogenic towards the transgene product than IM delivery. To confirm this hypothesis and using the macaque model, we injected an AAV8 vector encoding GFP either via IM or LR routes, in the absence of any immunosuppression. To characterize the cross-talk between the AAV vector and the host immune system, vector biodistribution was analyzed in muscles and lymphoid organs. Immune responses against transgene and viral capsid were also monitored. Our preliminary results suggest that vector copy number in the draining lymph node is significantly higher after IM delivery than after LR administration. Even if we detected anti-transgene antibodies as well as IFNγ secreting T cells in both conditions, GFP expression was surprisingly maintained at least until day 90 after LR vector injection whereas transduced cells were already eliminated at this time after IM delivery. These results further confirm immune differences between IM and LR routes in a large animal model. The characterization of the underlying immune signals appears then critical for future translation of LR-based protocols to Humans.

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Limb Girdle Muscular Dystrophy 2I: Generation of a new mouse model and test of therapeutic approaches by rAAV2/9 gene transfer

Evelyne Gicquel, Karine Charton, Nathalie Danièle, Isabelle Richard

Genethon, Evry, France

Limb Girdle Muscular Dystrophies (LGMD) constitute a group of myopathies affecting muscles of shoulder and pelvic girdles. Among them, LGMD2I, a recessive autosomal muscular dystrophy, is caused by mutations in the Fukutin Related Protein (FKRP) gene. FKRP, whose function remains unclear, is supposed to participate in alpha-dystroglycan glycosylation, which is important to ensure the cell/matrix anchor of muscle fibers.

We generated a knock-in mouse model of LGMD2I, carrying the most frequent mutation (L276I) encountered in LGMD2I patients. Molecular characterization of this mouse model showed that the introduction of the mutation did not alter the expression of FKRP, neither at transcriptional or translational level. However, the protein appears to have altered function since abnormal glycosylation of alpha-dystroglycan was observed. Histologically, the muscles of this model show a moderate dystrophic pattern starting from 6 months of age, consisting both in the presence of central nuclei and in fiber size variability. The functional muscle impairment is also moderate.

To evaluate gene transfer therapeutic approach, we cloned the FKRP cDNA in an expression cassette of a rAAV2/9 vector under the transcriptional control of the muscle-specific desmin promoter. The rAAV vector was injected intramuscularly or intravenously in the mouse model. A strong expression of the FKRP transgene was obtained, both at RNA and protein levels. In systemic conditions, a histological rescue was observed. The functional efficacy of this vector is under evaluation after systemic injection in KI-FKRP mice.

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Lentiviral shRNA knock-down of Macrophage inflammatory protein-1γ ameliorates experimentally induced osteoarthritis in mice

Jeng-Long Hsieh[1], Po-Chuan Shen[2], Ai-Li Shiau[3], Chao-Liang Wu[4]

[1]*Department of NursingChung Hwa University of Medical Technology, Tainan city, Taiwan,* [2]*Department of Orthopedic Surgery, Tainan Hospital, Department of Health, Executive Yuan, Tainan citiy, Taiwan,* [3]*Department of Microbiology and Immunology, National Cheng Kung University College of Medicine, Tainan city, Taiwan,* [4]*Department of Biochemistry and Molecular Biology, National Cheng Kung University College of Medicine, Tainan city, Taiwan*

Objective: Immune cells are involved in the pathogenesis of osteoarthritis (OA). $CD4^+$ T cells were activated during the onset of OA and induced macrophage inflammatory protein (MIP-1γ) expression and subsequent osteoclast formation. In this study, we evaluated the effects of local knock-down of MIP-1γ in a mice model of OA induced by anterior cruciate ligament-transection (ACLT).

Design: The mouse macrophage cell line and osteoclast-like cells generated from immature haematopoietic monocyte/macrophage progenitors of murine bone marrow were co-cultured with $CD4^+$ T cells. The osteoclastogenesis was evaluated by tartrate-resistant acid phosphatase (TRAP) staining. Using ACLT, we induced OA in one hind-leg knee joint of B6 mice. Lentiviral vector encoding MIP-1γ small hairpin RNA (shRNA) and control vectors were individually injected intraarticularly into the knee joints of mice. The expression of MIP-1γ and the inflammatory responses was examined using immunohistochemistry (IHC) and ELISA. The knee joints were histologically assessed for manifestations of OA.

Results: $CD4^+$ T cells induced the expression of MIP-1γ and osteoclast formation. Neutralization of MIP-1$\gamma$with specific antibody can both abolish receptor activator of NFκB ligand (RANKL)-stimulated and $CD4^+$ T cells-stimulated osteoclast formation. Significant increase of MIP-1γ levels was seen 90 days after surgery. Histopathological examinations revealed that mice treated with MIP-1γ shRNA had less severe osteoarthritis than control mice had. The effect of treatment was accompanied by decreased macrophage infiltration and osteoclast formation.

Conclusions: Local inhibition of MIP-1γ expression may ameliorate disease progression and provide a potential treatment for OA.

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MicroRNAs as seric biomarkers of Duchenne Muscular Dystrophy in the GRMD dog model

Laurence Jeanson-Leh, Soraya Krimi, Julien Buisset, David Israeli

Genethon, Evry, France

Duchenne Muscular Dystrophy (DMD) is an X-linked neuromuscular disease that affects 1 child in 3500. This recessive lethal disorder is the most frequent and severe form of muscular dystrophy. The best clinically relevant animal model for DMD is the GRMD dog (Golden Retriever Muscular Dystrophy).

Creatine kinase is a well known seric biomarker of muscular pathologies but it lacks specificity and shows important variability and bad correlation to disease course. MicroRNAs (miRNAs) are small size RNA molecules involved in post-transcriptional control of gene expression. While these molecules are normally present and function inside cells, it has recently been discovered that they are also secreted and can be detected in every body fluid. Moreover, miRNAs represent great potential as specific and reliable biomarkers as their abundance varies according to many pathophysiological states.

In order to identify new and robust biomarkers for DMD, we quantified different miRNA species and realized a miRNA expression profile in the GRMD dog serum by Q-RT-PCR. We show that several seric miRNAs are highly dysregulated in GRMD dogs and are presenting correlation to disease course, giving a relevant view of the pathological state at a precise time.

Considering the strong sequence conservation of miRNAs between dog and human, the identified GRMD miRNAs represent powerful biomarkers which could be potentially transposable to DMD patients follow-up and to the evaluation of upcoming DMD gene therapy clinical trials.

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Intramuscular delivery of an AAV vector carrying a mutated myostatin propeptide ameliorates muscle hypotrophy and specific force in myotubularin deficient mice

Romain Joubert[1], Thibaud Jamet[1], Karine Poulard[1], Nadia Messaddeq[2], Karima Relizani[2], Alban Vignaud[1], Philippe Moullier[1], Anna Buj-Bello[1]

[1]*Genethon, Evry, France,* [2]*IGBMC, Illkirch, France*

Myotubular myopathy (XLMTM) is a severe congenital disease that affects skeletal musculature, which is characterized by the presence of small myofibres with altered distribution of organelles and weakness. The disease is due to mutations in the *MTM1* gene, encoding a phosphoinositide phosphatase named myotubularin. The mechanisms leading to XLMTM-related muscle hypotrophy, which correlates with prognosis severity in patients, are currently unknown. We thus investigated whether the expression level of genes involved in the ubiquitine-proteasome and autophagy pathways are deregulated during disease progression in *Mtm1* deficient muscle. In addition, we tested the capacity of a recombinant AAV vector that expresses a dominant negative mutated myostatin propeptide (AAV1-CMV-Prop-D76A) in ameliorating the XLMTM phenotype. The vector was injected into the tibialis anterior and EDL muscles of 2 week-old *Mtm1* deficient mice and muscles were analysed 2 later at the histological and functional level. This short-term treatment led to an increase in myofibre size and improved the contractile force function of myotubularin deficient muscles. Thus, inhibition of myostatin signaling is a potential therapeutic target for XLMTM.

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Toward a rescue of huntingtin transcripts by using a trans-splicing strategy

Maëva LE HIR[1], Stéphanie LORAIN[1], Nicole DEGLON[2], Deniz KIRIK[3], Gabrielle CALLANDER[3], Thomas VOIT[1], Luis GARCIA[1]

[1]*Groupe « Biothérapies des maladies du système neuromusculaire » Um76 - UPMC / U974 - Inserm / UMR7215 – CNRS / Institut de Myologie, Paris, France,* [2]*Laboratoire « Neurothérapies Cellulaires et Moléculaires » (LNCM) Département des Neurosciences Cliniques (DNC) Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland,* [3]*Groupe « Brain Repair and Imaging in Neural Systems » (BRAINS) BMC D11 Lund University, Lund, Sweden*

Huntington's disease (HD) is the most common inherited neurodegenerative disorder (5–10 cases per 100,000 worldwide). This disease leads to cognitive decline and dementia. The single genetic cause is a dominant CAG expansion of over 40 repeats in the first exon of the HTT gene encoding huntingtin. The resulting longer polyglutamine tract causes huntingtin inclusion formation, leading to cell death by a mechanism that remains unclear. The loss of striatal neurons is a major cause of cognitive and physical symptoms.

We are currently developing a trans-splicing (TS) strategy to repair HTT transcripts. TS consists of an artificial splicing reaction between a mutated HTT pre-mRNA and a TS RNA, which contains the wild-type (wt) version of the exon(s) to be corrected, splicing sequences, a linker and an antisense sequence to anneal the targeted pre-mRNA. The TS strategy offers many advantages: (1) the repair is done on the native pre-mRNA, thereby retaining the endogenous gene expression context; (2) limited vector packaging size is no longer problematic; and (3), for dominant diseases like HD, TS can reduce the level of the mutated transcript while simultaneously generating wild-type transcripts.

As HD is always due to the same mutation in exon 1, we created a series of TS molecules to repair the 5′ region of the HTT transcript.

We detected trans-spliced transcripts in the human cell line HEK 293T and are now evaluating the efficiency of our candidate molecule in cellular and animal models of HD to validate its therapeutic relevance.

P218

A microrna regulated vector prevents cardiac toxicity of CALPAIN 3 gene transfer

Florence LE ROY[1,2], Carinne ROUDAUT[1], Laurence SUEL-PETAT[1], Isabelle RICHARD[1]

[1]*Genethon, CNRS UMR8587 LAMBE, Evry, France,* [2]*Université Val d'Essonne, Evry, France*

Genetic defects in calpain 3 protease leads to Limb-Girdle Muscular Dystrophy type 2A (LGMD2A), a disease of the skeletal muscle that affects predominantly the proximal limb muscles. There is no treatment for this disease to date. We previously demonstrated the efficient recovery of calpain 3 (*CAPN3*) proteolytic activity and muscle pathology following intramuscular injection of recombinant adeno-associated virus (rAAV) vectors expressing *CAPN3* under the control of the desmin promoter in a murine model for LGMD2A (C3KO mouse). However, systemic administration of the same vector led to cardiac toxicity that we related to an unregulated proteolytic activity of calpain 3 in the heart. Following these results, we set up a strategy based on microRNA regulated vector to prevent *CAPN3* transgene expression in the heart. We cloned the target sequence of the cardiac specific microRNA-208a downstream of *CAPN3* cDNA and demonstrated *in cellulo* its capacity to down-regulate the expression of the carrier messenger in presence of miR-208a. New muscle specific promoters were also cloned and validated *in cellulo*. Several rAAV vectors carrying *CAPN3* transgene and these regulatory elements were designed and validated *in vivo*. Our results indicate that the presence of the miR-208a target sequence in combination with the different promoters tested permit *CAPN3* transgene expression in skeletal muscles without showing any cardiac toxicity in C3KO mice.

P219

***Trans*-splicing approach for muscular dystrophies.**

François Monjaret, Nathalie Bourg-Alibert, Pierre Klein, Delphine Pion, Carinne Roudau, Laurence Suel-Petat, Isabelle Richard, Karine Charton

Généthon, Evry, France

Several muscular dystrophies, including recessive **Limb Girdle Muscular Dystrophy** (LGMD2), are caused by mutations in genes encoding large proteins. The size of these genes does not fit with the encapsidation capacity of the currently used vector for gene transfer in muscle. We choose to develop RNA reprogramming by **spliceosome mediated RNA *trans*-splicing (SMaRT)** for two LGMD2, LGMD2J and LGMD2B, due to mutations in the **titin** and **dysferlin** genes, respectively.

Conceptually, *trans*-splicing is achieved using a vector encoding a **pre-mRNA *trans*-splicing molecule (RTM)**, able to target the mutated endogenous pre-mRNA and diverting the splicing machinery towards itself to replace the mutated exon with a wild type template. We constructed RTM targeting the 3′ end of both titin and dysferlin pre-mRNA. We also constructed human titin and dysferlin minigenes to be used as *trans*-splicing targets in our initial experiments. In both cases, we demonstrated at **RNA and protein** level using RT-PCR and Western Blot that *trans*-splicing has occurred. Subsequent experiments using the endogenous expressed target pmRNA as a target reveal a weaker efficiency of the RTM. Experiments are on-going to improve this efficiency and understand the shift observed between the two conditions.

P220

ProSavin® a gene therapy approach for the treatment of Parkinson's Disease: a Phase I Clinical Trial Update

Stephane Palfi[3,1], Collin Watts[4], Gurruchaga Jean Marc[3], Buttery Peter[4], Ralph Scott[2], Deeley Sarah[2], Lepetit Helene[3], Miskin James[2], Iwamuro Hiro[3], Lavisse Sonia[1], Kas Aurelie[1], Ramelli Anne Laure[3], Tani Naoki[3], Fenelon Gilles[3], Brugière Pierre[3], Naylor Stuart[2], Hantraye Philippe[1], Remy Philippe[1,3], Barker Roger[4], Mitrophanous Kyriacos[2]

[1]CEA/I2BM MirCen, Fontenay aux Roses, France, [2]Oxford Biomedica Ltd, Oxford, UK, [3]Henri Mondor Hospital APHP, Creteil, France, [4]Cambridge centre for Brain repair, Cambridge, UK

ABSTRACT MOVED TO ORAL SECTION

P221

Use of miRNA-regulated AAV vector to reduce humoral immune responses against alpha-sarcoglycan

Jerome Poupiot[1], Maxime Ferrand[2], Florence Boisgerault[2], Anne Galy[2], Isabelle Richard[1]

[1]Genethon, Evry, France, [2]Inserm U951, Genethon, Evry, France

Alpha-Sarcoglycanopathy (Limb-Girdle Muscular Dystrophy type 2D, LGMD2D) is a recessive muscular disorder caused by deficiency in α-sarcoglycan, a transmembrane protein part of the dystrophin-associated complex. We previously reported efficient AAV-mediated transfer of α-sarcoglycan in α-sarcoglycan deficient mice (*Sgca*-null mice) resulting in correction at the biochemical, histological and functional levels. However, specific humoral immune response directed against the transgene were induced after intramuscular injection, leading to disappearance of transgene expression in muscle fibers. The use of a muscle-specific promoter like desmin improved transgene expression but continued to elicit a weak humoral response. Since expression of a neotransgene in antigen-presenting cells (APC) can effectively prime immune responses, we decided to switch off the expression of the transgene in APC by using microRNA (miRNAs) regulating properties. Indeed, miRNAs are small noncoding RNAs that have very specific expression profiles and regulate gene expression by repressing translation of target cellular transcripts. We incorporated a target sequence of miRNA142-3p (miR142-T) in the AAV cassette containing the α-sarcoglycan transgene under the transcriptional control of the ubiquitous promoter CMV. After viral preparations in serotype 1, we injected the constructs with and without miR142-T intramuscularly in the *Tibialis Anterior* of *Sgca*-null mice. Analyses of muscle biopsies at day 84 and blood sera showed that the use of miRNA-regulated vector led to increased survival of muscle fibers associated with long-term expression of α-sarcoglycan and reduced humoral immune responses against the transgene. This promising strategy could facilitate sustained therapeutic effects of rAAV gene transfer in muscle.

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AAV-mediated genes transfer to restore the functional dysferlin.

Marina Pryadkina[3], Nathalie Bourg[3], William Lostal[3], Joseph Roche[2], Robert Bloch[2], Martin Krahn[1], Marc Bartoli[1], Nicolas Levy[1], Isabelle Richard[0]

[1]Aix-Marseille Univ, UMR 910, Faculté de Médecine Timone, Marseille, France, [2]University of Maryland, Department of Physiology, Baltimore, USA, [3]Genethon, CNRS UMR8587 Lambe, Evry, France

Deficiencies in dysferlin are the cause of limb-girdle muscular dystrophy type 2B and Miyoshi myopathy. Dysferlin is a transmembrane protein shown to play a role in the repair of the plasma membrane by vesicle fusion, providing a possible hypothesis for the pathophysiology of these diseases. No treatment is available for dysferlinopathies and gene therapy is complicated by the fact that the dysferlin cDNA is large.

To correct dysferlin-deficiency, several therapeutic strategies were developed in dysferlin-deficient mice (B6.A/J-Dysfprmd): transfer of minidysferlin and a strategy relying on the concatemerization property of the Adeno-Associated Viral (AAV) vector to transfer the full-length dysferlin gene by means of two AAV vectors. Only the concatemerization strategy improved muscle histology. In confirm this difference; we studied muscles from animals subjected to our experimental therapeutic strategies for their ability to recover from eccentric exercise using the large-strain injury (LSI). LSI consists in 15 repetitive lengthening contractions and generates an injury from which normal muscle recovers without the need of neomyogenesis. This test showed that the minidysferlin do not prevent myofiber degeneration in dysferlin-deficient muscle and the concatemerization strategy is efficient to protect the muscle from physical stress.

P223

Regulated GDNF expression leads to functional neuroprotection in the 6-OHDA model of Parkinson's Disease

Luis Quintino, Giuseppe Manfré, Erika Elgstrand-Wettergren, Angrit Namislo, Christina Isaksson, Cecilia Lundberg

Lund University, Lund, Sweden

Using a novel system based on destabilizing domains (DD) that regulates gene expression by targeting specific proteins for destruction we have been able to effectively regulate glial cell-derived neurootrphic factor (GDNF) expression in vivo.

The next step was to determine if regulated GDNF expression could protect nigral neurons. Lentiviral vectors expressing GDNF-DD, wildtype GDNF or regulated yellow fluorescence protein (YFP-DD) were injected to the striatum of of rats. In one group of GDNF-DD was turned on (DD-ON).In another group of GDNF-DD animals the expression was turned off (DD-OFF).The GDNF and DD-ON were able to protect up to 60% the striatal projection. Furthermore, GDNF and DD-ON were able to protect 80 and 60% of substantia nigra neurons, respectively.

The neuroprotection given by the GDNF-DD resulted in behavior improvements. The DD-ON group showed significant improvements in forelimb akinesia, forelimb assymetry and decrease of drug induced rotations.

P224

Mass Spectrometry approach for Biomarkers research in Duchenne Muscular Dystrophy

Jérémy Rouillon[1], Thibaut Léger[2], Robin Pinilla[1], Jean-Michel Camadro[2], Tomas Voit[3], Fedor Svinartchouk[1]

[1]Généthon, EVRY, France, [2]Plateforme Protéomique/Spectrométrie de masse de l'Institut Jacques-Monod, Paris, France, [3]Plateforme Protéomique/Spectrométrie de masse de l'Institut Jacques-Monod, Paris, France

Duchenne muscular dystrophy (DMD) is a rare disease affecting one in 3500 boys. Due to the extremely large size of the gene coding for the dystrophin protein, a substantial proportion (~30%) of newborn DMD boys appears a consequence of spontaneous mutations in families without genetic predisposition. This particularity of the pathology creates a need for reliable biomarkers for pre-screening of all newborns in order to select candidates for further genetic analysis.

In present study, we used sera and urines as a source of biomarkers for DMD diagnostic. In order to improve outcome of protein identification by mass spectrometry, we compared different methods of sample preparations: direct digestion, prefractionation by SDS-PAGE, low abundance proteins enrichment and high abundance proteins depletion. Such pretreated samples were further analyzed by LC-MS/MS on the LTQ-Orbitrap Velos mass spectrometer. The number of identified proteins after direct digestion of serum samples was about 200. Interestingly, there was no substantial gain in the number of total identified proteins under all tested conditions. Nevertheless, depletion of high abundance proteins gave us the best results in terms of detection of low abundance proteins and effortlessness of the approach. Application of this method to a restricted number of patients allowed us to confirm some already described biomarkers as creatine kinase and to find some new proteins differentially expressed between healthy and DMD patients. The use of these proteins as biomarkers for diagnostic of neuromuscular disorders and for following up of future gene therapy trials will be discussed.

P225

Restoring dystrophin expression with antisense PPMOs in cardiac muscles in dystrophic dogs

Toshifumi Yokota[1,3], Yusuke Echigoya[1], Akinori Nakamura[4,2], Tetsuya Nagata[2], Nobuyuki Urasawa[5,2], Masanori Kobayashi[6,2], Takashi Saito[2], Yoshitsugu Aoki[2], Ryszard Kole[7], Peter Sazani[7], Terence Partridge[9,8], Eric Hoffman[8,9], Shin'ichi Takeda[2], Dharminder Panesar[1], Merryl Rodriguez[1], Ashkan Nozohourmehrabad[1]

[1]University of Alberta, Edmonton, Canada, [2]National Center of Neurology and Psychiatry, Kodaira, Japan, [3]The Friends of Garrett Cumming Research & Muscular Dystrophy Canada HM Toupin Neurological Science Research Chair, Edmonton, Canada, [4]Shinshu University School of Medicine, Matsumoto, Japan, [5]Nagano Red Cross Hospital, Nagano, Japan, [6]Nippon Veterinary and Life Science University, Tokyo, Japan, [7]Sarepta Therapeutics, Inc. (formerly AVI Biopharma Inc), Bothell, USA, [8]Children's National Medical Center, Washington, USA, [9]George Washington University School of Medicine, Washington, USA

Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy characterized by rapid progression of muscle degeneration. The exon skipping therapy leads to the production of internally deleted, but functional in-frame mRNA transcripts that are translated into short dystrophin protein. Exon skipping employs antisense DNA-like molecules against pre-mRNA to skip over (splice out) the mutated part of the gene. The truncated "quasi-dystrophin" has missing amino acids, but retains some functions like Becker muscular dystrophy. One of the major hurdles of exon skipping therapy is that antisense oligos such as unmodified morpholinos are not efficiently delivered into the heart. Canine X-linked muscular dystrophy (CXMD), a dystrophic dog model of DMD exhibits vacuolar degeneration in cardiac Purkinje fibers, thus an idel model to test the efficacy in the heart. Here, we sought to rescue the expression of dystrophin in cardiac muscles in dystrophic dogs by using morpholino conjugates with cell-penetrating peptides, called peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs). We demonstrated that the delivery moieties significantly improved dystrophin production in the heart. Intravenous injections of PPMOs led to extensive expression of dystrophin in cardiac Purkinje fibers and ameliorated their vacuole degeneration without any detectable toxicity. This is the first demonstration of rescue of cardiac muscles with systemic antisense-mediated exon-skipping in the dog model. Our results show the potential of PMO conjugates as therapeutic agents for DMD.

P226

TetR-regulated all-in-one Lentiviral vector system for conditional expression in human stem cells.

Karim Benabdellah, Pilar Muñoz, Angélica García-Perez, Miguel G. Toscano, Francisco Martin

Centre for Genomic and Oncology Research (GENYO), Granada, Spain

Among the existing inducible transcriptional gene regulatory systems, those, based on the Tet transactivator (tTA), a TetR-VP16 chimera, are the most widely used. However, citotoxicity and transactivation of undesired genes due to the expression of TetR-VP16 proteins are limiting factors of this potent conditional system. To avoid this secondary effect, a doxycycline-regulated system based on the original TetR repressor was developed as an alternative for conditional expression. In the present study, we have constructed several All-In-One TetR-based lentiviral vectors with the aim of achieving conditional gene expression in human stem cells. All the new constructs are based in a previously published CEST All-In-One LV expressing eGFP through the regulatable CMV-TetO promoter and the original TetR through the spleen focus forming virus (SFFV) promoter. The CEST LV efficiently generated primary cells responsive to doxycycline but required an average of 3 vectors per cell and performed very poorly in HSCs and hESCs. Two modifications were performed in the CEST in order to improve transgene regulation in HSC and hESCs; 1- TetR modification to include two different nuclear localization signals (TetRnls1 and TeRnls2) and 2- Use of the EF1alpha promoter to increase TetR expression levels in HSC and hESCs. Our final construct, named CEETnls2, was able to obtain conditional expression in MSCs harbouring only one integrated vector. Most importantly the CEETnls2 LV efficiently generates doxycycline-responsive hHSCs and hESCs after one single transduction. This conditional expression was maintained in CEETnls2-transduced-hESC derived cells such as hemogenic progenitors (CD31 + CD45 −) and hematopoietic cells (CD34 + CD45 + and CD34- CD45 +).

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Potent DNA transposition by protein transduction of the *piggyBac* transposase from lentiviral Gag precursors

Yujia Cai, Rasmus O. Bak, Nicklas H. Staunstrup, Anne Kruse Hollensen, Helle Christiansen, Maria N. Primo, Thomas Corydon, Jacob Giehm Mikkelsen

Department of Biomedicine, Aarhus University, Aarhus, Denmark

DNA transposon-based vectors have emerged as gene vehicles with therapeutic potential. So far, function of such vectors has relied on the delivery of the gene encoding the transposase, raising concerns related to persistent expression of the transposase and increased cytotoxicity. We have explored, therefore, new strategies for delivery of the transposase protein. Here, we demonstrate high-efficiency transposition of *piggyBac*-derived transposon vectors facilitated by transposase protein packaged in VSV-G-pseudotyped lentiviral particles as part of the Gag protein. By inserting the transposase sequence upstream of Gag and downstream of an N-terminal, heterologous myristoylation signal, we obtain robust packaging of the hyperactive *piggyBac* transposase, hyPBase, in virus particles and demonstrate that the transposase is released from the Gag-Pol polypeptide upon maturation of the lentiviral particles. Upon hyPBase protein transduction, potent VSV-G dependent DNA transposition is observed in target cells transfected with plasmid DNA harboring the *piggyBac* transposon, whereas a mutated version of the transposase is inactive. Notably, we observe that protein transduction of hyPBase results in higher levels of DNA transposition than obtained in a conventional setting with hyPBase expression from transfected plasmid DNA. Finally, we show that hybrid, integrase-defective lentiviral vectors carrying (i) the hyPBase transposase protein and (ii) the transposon sequence facilitate potent transposition in a variety of cell types. Our data reveal new means of combining DNA transposition with lentiviral gene transfer and establish lentivirus-derived nanoparticles as a new tool for transposase protein delivery. We believe that these findings will lead to improved and safer DNA transposon vector technologies.

P228

Elucidation of the sonoporation mechanism in ultrasound mediated gene delivery by confocal microscopy

Ine De Cock, Ine Lentacker, Stefaan De Smedt

Ghent University, Ghent, Belgium

Ultrasound is commonly known as a diagnostic imaging technique. However, ultrasound in combination with microbubbles recently gained interest as a very promising drug delivery method. Microbubbles consist of a gas core stabilized by a lipid shell. In an ultrasonic field, they alternately contract and expand. Depending on the ultrasound intensities used, two major types of bubble oscillations are observed: stable cavitation and inertial cavitation, which leads to bubble implosion. Those ultrasound induced microbubble oscillations can cause permeabilization of cell membranes. This phenomenon is called sonoporation and is a very exciting feature for drug and especially gene delivery, since sonoporation enables direct cytoplasmic entry of nucleic acids.

The behavior of microbubbles in an ultrasonic field has been studied extensively. Still, very little is known about the exact biophysical processes involved in the permeabilization of cell membranes. Until now, this has only been studied by indirect methods. That is why we recently implemented an ultrasound and confocal microscopy setup which enables us to study the sonoporation process real-time *during* ultrasound radiation.

The cell membrane and the microbubbles were fluorescently labeled. Propidium iodide, a cell impermeable dye, was added as a permeabilization marker. At low ultrasound intensities the microbubbles cluster and deform the cell membrane without causing permeabilization. At higher ultrasound intensities the microbubbles were shot through the cell membrane leading to sonoporation, which was shown by the influx of propidium iodide. These observations are a first step in unraveling the sonoporation mechanism and allow optimization of ultrasound mediated drug and gene delivery.

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New insights of microbubbles-cell interactions and cellular effects during sonoporation

Anthony Delalande[1], Lucie Pigeon[1], Spiros Kotopoulis[2,3], Michiel Postema[3], Patrick Midoux[1], Chantal Pichon[1]

[1]Centre de Biophysique Moléculaire, Orléans, France, [2]Haukeland hospital, Bergen, Norway, [3]Department of Physics and Technology, Bergen, Norway

ABSTRACT WITHDRAWN

P230

Utilizing protein-protein interactions for retroviral protein transfer

Tobias Maetzig, Johannes Kuehle, Melanie Galla, Christopher Baum, Axel Schambach

Experimental Hematology, Hannover Medical School, Hannover, Germany

Retroviral particles have been successfully used for the stable genetic modification of various cells types with regenerative potential. In addition, the particulate structure of the virus can also be employed to transiently transfer proteins of interest (POI). In a proof-of-principle study (Voelkel et al., PNAS 2010), we successfully transduced Flp recombinase from the gammaretroviral Gag precursor, and particles optionally maintained infectivity when produced in the presence of retroviral RNA and wild-type Gag/Pol.

In a next step, we developed approaches to synchronize Flp-mediated recombination and integration of the retroviral vector genome. To this end, we artificially separated structures responsible for both functions to independent expression constructs. Particles produced by a super-split Gag/Pol packaging design consisting of Gag, Gag-Flp, and a myristoylation deficient G2A-Gag/Pol showed almost evenly distributed recombination and integration rates. Since packaging of cytoplasmic G2A-Gag/Pol by membrane bound Gag is mediated by protein-protein interactions, we opted to explore this mechanism for protein transfer. For this purpose we made use of HIV p6 and its interaction with Vpr. Due to their simple genome structure and perspectively production from stable packaging cell lines, we equipped MLV Gag with chimeric p6. Titers were in the range of the wild-type vector, but only p6 bearing vectors transmitted GFP-Vpr into target cells. These findings indicate that we can successfully coordinate transfer of a POI and transgene integration in target cells.

Altogether, this work will help to expand the understanding and applicability of retroviral particle-mediated protein transfer for the control of cell fate or function.

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Synthesis, characterization, and evaluation of hydrophilic cationic star homopolymers based on their ability to transfect genetic material in mammalian cells.

Nikolaos Mastroyiannopoulos[1], Kyriaki Pafiti[2], Costas Patrickios[2], Leonidas Phylactou[1]

[1]*The Cyprus Institute of Neurology and Genetics, Department of Molecular Genetics, Function and Therapy, Nicosia, Cyprus,* [2]*University of Cyprus, Department of Chemistry, Nicosia, Cyprus*

DNA and RNA transfer to mammalian cells emerges as a powerful therapeutic and diagnostic tool in medicine and molecular biology. In a non-viral way to deliver genetic material four cationic hydrophilic star homopolymers based on the novel hydrophilic, positively ionizable cross-linker bis (methacryloyloxyethyl)methylamine (BMEMA) were synthesized using sequential group transfer polymerization (GTP) and were, subsequently, evaluated for their ability to deliver siRNA to mouse myoblast cells. The nominal degrees of polymerization (DP) of the arms were varied from 10 to 50. For the polymerizations, 2-(dimethylamino) ethyl methacrylate (DMAEMA) was employed as the hydrophilic, positively ionizable monomer. Subsequently, all-star and linear homopolymers were evaluated for their ability to deliver siRNA to the C2C12 mouse myoblast cell line, expressing the reporter enhanced green fluorescent protein (EGFP). All-star homopolymers and the largest linear homopolymer presented significant EGFP suppression, whereas the smaller linear homopolymers were much less efficient. Furthermore, loading high concentrations of these homopolymers into the cells showed higher EGFP suppression but also higher cell toxicity. The siRNA-specific EGFP suppression, calculated by subtracting the effect of cell toxicity on EGFP suppression, slightly increased with star polymer loading for the two smaller stars, whereas it presented a shallow maximum and a decrease for the other two stars. Moreover, the siRNA-specific EGFP suppression also increased slightly with the DP of the arms of the DMAEMA star homopolymers. Overall, the EGFP suppression efficiencies with the present star homopolymers were at levels comparable to that of the commercially available transfection reagent Lipofectamine.

P232

Bio-inspired surfactant-modified dextran nanogels mediate enhanced intracellular siRNA delivery

Lynn De Backer, Jo Demeester, Stefaan De Smedt, Koen Raemdonck

Ghent Research Group on Nanomedicines, Laboratory of General Biochemistry and Physical Pharmacy, Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

Topical administration of small interfering RNA (siRNA) nanocarriers is a promising approach in the treatment of pulmonary disorders. However, an efficient siRNA delivery vehicle should overcome the various extra- and intracellular barriers existing in the lung. Alveolar surfactant will be one of the first interfaces that siRNA nanocarriers encounter upon inhalation. Therefore, it is imperative to evaluate the impact of the surfactant on their gene silencing potential. We demonstrated that biodegradable cationic dextran nanogels (DEX-NGs) are promising carriers for siRNA. DEX-NGs loaded with siRNA (siDEX-NGs) could induce a

marked RNAi effect in various pulmonary cell lines. However, the interaction with alveolar surfactant preparations (Curosurf®) reversed the net surface charge of the particles, resulting in significantly hampered cellular uptake. Remarkably, the RNAi effect of siDEX-NGs following interaction with Curosurf® was maintained, indicating an improved intracellular siRNA distribution mediated by the surfactant. In addition, Curosurf® was able to recover the RNAi effect of inactive anionic siDEX-NGs. The impact of Curosurf® was compared with anionic synthetic liposomes (ASLs) that consist of the most abundant phospholipids in pulmonary surfactant. Both markedly impede siDEX-NG cellular internalization but in contrast to Curosurf®, ASLs almost completely abrogate gene silencing induced by siDEX-NGs. An improved RNAi effect by virtue of Curosurf® coating was also demonstrated in hepatoma cells, indicating that the positive influence of alveolar surfactant on the intracellular siRNA biodistribution is not limited to cell lines of pulmonary origin. Hybrid pulmonary surfactant coated polymeric nanoparticles will be further explored as a biomimetic siRNA delivery platform.

P233

Efficacy of gene transfer to "in vivo" whole isolated pig liver

L Sendra[1], O Carreño[2], E Montalva[2], MJ Herrero[1,3], F Orbis[2], I Noguera[4], M Sanchez[1], R Lopez-Andujar[2], SF Aliño[1,2]

[1]Departamento de Farmacologia, Universidad de Valencia, Valencia, Spain, [2]Hospital Universitario y Politecnico La Fe, Valencia, Spain, [3]Instituto de Investigacion Sanitaria La Fe, Valencia, Spain, [4]SCSIE-Universidad de Valencia, Valencia, Spain

Introduction: The optimal porta vs cava vein hydrodynamic injection conditions for gene transfer, in surgically watertight pig liver, were studied.

Material and Methods: Pig liver was isolated by vascular clamping (cava, portal veins and hepatic artery); then, the eGFP gene transfer was performed under different conditions by implantation of a 12F catheter in anesthetized pigs. Animals were injected with a 200 mL of saline solution bearing p3c-eGFP plasmid (20 μg/mL), which contains the eGFP gene via cava or portal veins at different rates. The gene transfer efficiency of anterograde vs retrograde venous injection was tested. Effect of flow rate, 10 and 20 mL/s, was also studied. Toxicity of the procedure was evaluated in every combination.

Results: The results showed: (i) the highest gene delivery (1–10 eGFP DNA copies/100pg total DNA) occurred in portal vein (anterograde) injection; (ii) cava vein (retrograde) gene transfer mediated a higher rate of transcription (10^5–10^6 eGFP RNA copies/100ng total RNA) despite a lower plasmid amount; (iii) the fastest flow rate, 20 mL/s, reached better transcription efficiency than 10 mL/s (10^4–10^5 vs 10^5–10^6 eGFP RNA copies/100 ng total RNA) in cava vein injection; (iv) no relevant hepatic toxicity was observed (AST < 151) and surgery recovery was fast; (v) gene was present in every liver lobe but was mainly located in medial lobes.

Conclusion: These data suggest that retrovenous injection of 200 mL at a 20 mL/s flow rate is a mild and safe model of hepatic gene transfer with potential clinical applications. Partially supported by AP-151-11 and SAF2011-27002.

P234

Membrane permeabilization by the electric field and/or the pluronic L64. Relationship with transfection in vitro and in vivo.

Michel Francis Bureau[1], Luc Wasungu[3], Lauriane Jugé[2], Daniel Scherman[1], Marie Pierre Rols[3], Nathalie Mignet[1]

[1]UPCGI, U1022 INSERM, UMR8151 CNRS, Faculté de Pharmacie,Université Paris Descartes, Paris 75006, France, [2]Service de RMN Chimie-Paris Tech, Paris 75005, France, [3]CNRS, IPBS (Institut de Pharmacologie et de Biologie Structurale), Toulouse, France

Electrotransfer can be obtained by the successive delivery of a high voltage short duration pulse (HV) inducing membrane permeabilization then a low voltage long duration pulse (LV) allowing DNA electrophoresis (HVLV mode). The copolymer amphiphile, pluronic L64 (L64) is able to act on cell machinery leading to gene expression and was also shown to permeabilize cell membrane. We wondered whether L64 could have an adjuvant effect on transfection by electrotransfer and whether the sequence L64 injection then application of a LV pulse could induce transfection comparable to that with HVLV. *In vitro*, we used fluorescence-activated cell sorting to evaluate the CHO cell transfection by a plasmid coding GFP, and permeabilization to propidium iodide. *In vivo*, the transfection of mice tibial cranial muscle was evaluated by optical imaging using a plasmid DNA encoding luciferase and permeabilization to a T1 contrast agent was evaluated by magnetic resonance imaging. Using the HVLV mode, transfection was low *in vitro* on CHO cells but high for muscles *in vivo*. Pre-treatment by L64 increased transfection efficiency of electrotransfer for CHO cells but not for the muscle. In mice muscles, L64 amplified the expression of DNA. Nevertheless, neither transgene expression nor permeability indices were further amplified by subsequent delivery of one LV pulse. A major finding is that the nature of the membrane modification induced by electric pulses is not comparable to the one mediated by L64. In addition we and other showed that copolymers amphiphiles with or without permeabilizing properties increased DNA expression in muscle.

P235

pEPito plasmids: an efficient vector for gene expression in human RPE cell.

SM Calado[1,2], AV Oliveira[1,2], R Haase[3], GA Silva[1,2]

[1]Centre for Molecular and Structural Biomedicine (CBME)/Institute for Biotechnology and Bioengineering (IBB/LA), University of Algarve, Faro, Portugal, [2]Department of Biomedical Sciences and Medicine, University of Algarve, Faro, Portugal, [3]Department of Pharmacy, University of Munich, Munich, Germany

Vector loss and silencing are major hindrances to the success of gene therapy. Haase et al. have developed a vector, named pEPito, containing a scaffold/matrix attachment region (S/MARs) sequence in the backbone and reduced methylated CpGs to allow replication in mitotically active liver cells and prolong gene expression, respectively. Using retinal pigmented epithelium (RPE) cells and mouse retinas, we evaluated their mitotic stability and gene expression profile.

RPE cells were transfected with pEPito, containing CMV, hCMV or hCMV/RPE65 promoters and GFP-positive cells were quantified by fluorescence microscopy and flow cytometry. Mitotic stability in dividing RPEs was evaluated by a colony-forming assay. Blasticidin-selected CFUs were counted at 32 days of culture and further selected for two additional months. The vectors were injected intravitreously in the mouse eye and GFP expression monitored up to 32 days post-injection (dpi).

GFP expression and CFU formation are markedly influenced by promoter and cell line. The magnitude of GFP expression was:

hCMV > CMV > hCMV/RPE65 in RPE cells, detectable up to 3 months of culture. In vivo GFP expression was consistent with the in vitro results and observable for at least 32 dpi.

pEPito-based vectors containing S/MARs and reduced CpG content are active expression systems in RPE cells for up to 100 cell divisions. In vivo, gene expression is detectable for at least 32 days. Based on its efficiency, the vector with hCMV promoter has been selected for further administration of a therapeutic molecule in a mouse model of retinal neovascularization.

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P236

An homologous model of gene therapy by *in vivo* administration of a plasmid containing the mouse growth hormone gene in immunocompetent dwarf mice

CR Cecchi[1,2], F Dagnaes-Hansen[2], L Aagaard[2], M Jakobsen[2], H Gissel[2], A Gothelf[3], BH Hansen[2], J Frystyk[4], E Higuti[1], NAJ Oliveira[1], ER Lima[1], P Bartolini[1], TG Jensen[2], CN Peroni[1]

[1]*Biotechnology Center, IPEN-CNEN, University of São Paulo, São Paulo, SP, Brazil,* [2]*Department of Biomedicine, Aarhus University, Aarhus, Denmark,* [3]*Center for Experimental Drug and Gene Electrotransfer, Department of Oncology, Copenhagen University Hospital Herlev, Herlev, Denmark,* [4]*The Medical Research Laboratories, Clinical Institute and Medical Department M (Diabetes and Endocrinology), Aarhus University Hospital, Aarhus, Denmark*

An alternative method for growth hormone deficiency (GHD) treatment has been developed using gene therapy associated to electroporation. Sustained circulatory levels of hGH and a highly significant weight increase were obtained in immunodeficient dwarf (lit/scid) mice. This new study aimed at setting up an homologous model of gene therapy based on electrotransfer of genomic mouse growth hormone (gmGH) in the muscle of immunocompetent dwarf (lit/lit) mice. The plasmid pUC-UBI-gmGH was first transfected into HEK 293 cells and an *in vitro* expression up to 74.0 ng mGH/10^6 cells/day, compared to 6.0 ng mGH/10^6 cells/day for the negative control, was obtained. As a positive control for *in vivo* expression, this plasmid was administered as naked DNA via hydrodynamic injection into C57BL6 normal mice, and the weight increase of treated mice after 21 days was 60.10% ± 11.02% compared to −2.05% ± 2.32% for the control group (injected with Ringer's solution). The plasmid was then administered in a protocol of gene-electrotransfer, applying eight 150 V/cm pulses of 20 ms, for a body weight gain assay into lit/lit mice. The weight increase after 15 days was 13.48% ± 3.53% for the gmGH-treated group versus 5.66% ± 2.51% for the control group (injected with saline) and 13.10% ± 7.54% for the group injected with the same plasmid encoding the ghGH gene, both groups also followed by electroporation. This previous results can be considered very promising as a pre-clinical study in a gene therapy protocol for patients suffering for GHD.

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P237

Effects of antibiotic pretreatment and bacteria-mediated reprogramming on inflammatory bowel disease in mice.

P Celec, R Gardlík

Institute of Molecular Biomedicine, Comenius University, Bratislava, Slovakia

Since the original study of Takahashi and Yamanaka in 2006 the field of induced pluripotent stemcells has made a great progress. However, all of the experiments published so far are based on ex vivo gene delivery and subsequent reimplantation of the cells. Compared to that, in vivo reprogramming might benefit from the direct administration of DNA encoding the reprogramming factors into the target tissue. In our previous experiment we proved some beneficial effects of bacteria-mediated delivery of genes encoding reprogramming factors Sox2, Oct3/4 and Klf4 on the course of colitis in mice. Preventive oral administration of the modified strain Salmonella Typhimurium SL7207 resulted in improvements in weight loss, colon length and stool consistency. Recently it has been shown that antibiotic pretreatment can alleviate the course of chemically induced colitis in mice. In the current study, we tested whether the antibiotic pretreatment of mice could result in better colonization of administered bacterial strain of colon, more effective gene delivery, cell reprogramming and, thus, also stronger therapeutic outcome. Mice C57BL/6was given streptomycin and metronidazole for 4 days before multiple oral administrations of therapeutic bacteria every other day. After two applications, mice were given dextran sulfate sodium in drinking water to induce colitis. Disease activity parameters, such as stool consistency, weight loss and bleeding were monitored throughout the experiment. Our results indicate that antibiotic pretreatment might alter the bacterial gene delivery into the colon. Moreover, in vivo reprogramming of colon cells seems to have an effect on the course of colitis.

P238

Reduced in vitro and in vivo toxicity of siRNA-lipoplexes with addition of Polyglutamate

Virginie Escriou[1], Anne Schlegel[1,2], Pascal Bigey[1], Daniel Scherman[0]

[1]*UPCGI CNRS 8151 Inserm 1022 ParisDescartes ENSCP Chimie ParisTech, Paris, France,* [2]*DNA Therapeutics, Evry, France*

We previously designed a new siRNA vector that efficiently silences genes in vitro and in vivo. The vector originality is based on the fact that, in addition to the siRNA molecule, it contains two components: 1) a cationic liposome which auto-associates to the siRNA to form particles called "lipoplexes", 2) an anionic polymer which enhances the lipoplex's efficiency. This anionic polymer can be a nucleic acid, a polypeptide or a polysaccharide. We show here how the nature of the added anionic polymer into our siRNA delivery system impact the toxic effects induced by siRNA lipoplexes. We first observed that: (i) siRNA lipoplexes-induced toxicity was cell line dependent, tumoral cell lines being the more sensitive, (ii) pDNA-containing lipoplexes were more toxic than polyglutamate-containing ones or cationic liposomes. We next determined that the toxicity induced by plasmid-containing lipoplexes is a long-lasting effect that decreased survival capacity of cells for several generations. We also found that treated cells underwent death following apoptosis pathway. We then examined the siRNA lipoplexes induced toxicity following systemic injection to mice and observed that (i) injection of siRNA-lipoplexes, rather than of liposome, triggered a production of several cytokines in mice and (ii) replacement of plasmid by polyglutamate reduced the elevation of all the assayed cytokines.

Overall, we showed that in order to enhance siRNA lipoplexes efficiency, it is better adding an anionic polymer rather than a plasmid DNA as far as toxicity is concerned. Indeed, siRNA lipoplexes containing polyglutamate exhibited only a mild toxicity.

P239

Modulation of angiogenesis using bacteria-mediated RNA interference and DNA vaccination in inflammatory bowel disease

Roman Gardlik, Peter Celec

Comenius University, Faculty of Medicine, Institute of Molecular Biomedicine, Bratislava, Slovakia

Angiogenesis as a process of blood vessel formation plays a crucial role in several diseases, including cancer and cardiovascular disorders. Recently, dysbalanced angiogenesis has been found to be a part of pathogenesis of inflammatory bowel disease (IBD). Therefore, inhibition of angiogenesis represents a novel therapeutic strategy to alleviate the inflammation and clinical outcomes of IBD. Bacteria can act as vectors for delivery of therapeutic sequences, which is especially suitable in IBD, because of natural occurrence of bacteria in gut microflora.

The aim of the current experiment is the application of bacterial gene therapy for modulation of angiogenesis in IBD. As a molecule to be targeted we used the main proangiogenic factor - vascular endothelial growth factor (VEGF). Bacterial strain Salmonella Typhimurium SL7207 was used as a gene delivery vector for oral application. We examined two strategies - DNA vaccination and RNA interference - and compared their efficiency in improving the course of the disease in an animal model of colitis. Surprisingly, both approaches showed similar beneficial results in evaluation of disease activity parameters (stool consistency, weight loss and colon length) and expression of VEGF and proinflammatory cytokine TNF alpha. Moreover, both therapies showed an improvement in all of the above mentioned parameters compared with the control groups not treated by therapeutic bacterial strains. Although the experiment is of a preliminary nature, it provides interesting and partially surprising data. Our results are consistent with the data from literature and provide a base for further studies focused on modulation of angiogenesis in IBD.

P240

Synergy of chromosomal elements S/MAR and Initiation of Replication leads to most efficient episomal gene transfer into hematopoietic progenitor cells

AP Giannakopoulos[1], EF Stravrou[1], VM Lazaris[1], A Spyridonidis[2], A Athanassiadou[1]

[1]*Department of General Biology, School of Medicine, University of Patras, Patras, Greece,* [2]*Hematology Unit, School of Medicine, University of Patras, Patras, Greece*

The chromosomal element Scaffold/Matrix Attachment Region (S/MAR) renders the prototype episomal vector pEPI-eGFP capable for "long term" nuclear retention and transgene eGFP expression in K562 cells but not in human progenitor CD34+ cells. We produced plasmids (a) pEPI-EF1/HTLV containing the transcription cassette EF1/HTLV-eGFP-S/MAR, (b) pEPI-SFFV and (c) pEP-IR, containing the transcription cassette SFFV-eGFP-S/MAR, while in plasmid (c) a second, chromosomal element, the replication initiation region (IR) from the β-globin gene locus was inserted. All three plasmids show excellent episomatic properties when transferred into K562 cells. No effect of IR insertion on the DNA sequence of plasmid (c) was documented by Stress Induced Duplex Destabilization analysis. Plasmids were transferred into CD43+ cells from peripheral blood, from adult, mobilised, healthy donors, with transfection efficiency from 24% to 30%. FACS sorted eGFP+ cells were placed in semisolid cultures to generate differentiated colony-cells within 14 days. Fluorescence was detected in 50% of such colonies containing plasmids (a) or (b) and –most efficiently- in 99,8% of colonies containing plasmid (c). Real time PCR analysis showed that the relative, average plasmid copy number of plasmid (c) is at a higher level (by 1,2 to 5,2) than of plasmids (a) and (b) in differentiated cells. Similar analysis of eGFP mRNA revealed proportional increase in transgene expression in plasmid (c) containing cells compared to plasmids (a) and (b) containing cells from eGFP+ colonies only. These data show that synergy of chromosomal elements S/MAR and IR leads to most efficient episomal gene transfer into hematopoietic progenitor cells.

P241

Multicellular spheroids as a relevant model to study and optimize the electrotransfer of (macro)molecules

Laure Gibot, Luc Wasungu, Louise Chopinet, Marie-Pierre Rols

IPBS CNRS UMR5089, Toulouse, France

Cell membrane can be transiently permeabilized under the application of electric pulses. This process is actually clinically used in Europe to potentiate anticancer drugs, while clinical trials are performed for gene transfer in tissues.

Electrochemotherapy (ECT) is an efficient technique for treatment of solid tumors that combines electric pulses with anticancer drugs. In order to increase ECT efficiency and to in vitro test new antitumor drug candidates, we use a 3D tissue model, the spheroid, more relevant to the in vivo situation than cells cultured in Petri dishes. This model is based on aggregation properties of cells when cultured in non-adhesive environment. Spheroids display nutrient, oxygen and signal gradients, cell-cell contacts as well as cell-endogenous extracellular matrix interactions. We showed that the combination of electric pulses and classical antitumor drugs, bleomycin and cisplatin, led to changes in spheroid morphology, to growth arrest and finally to its complete apoptosis-mediated dislocation, mimicking previously observed in vivo situations during ECT. Thus, spheroid appears to be a relevant in vitro 3D model to study electrical based processes.

Although up to 30% of cultured cells can be in vitro transfected, in vivo electrogene transfer efficiency is less than 5%. In order to understand mechanisms involved during DNA transfer in 3D structures and to optimize its efficiency, we used spheroid model. GFP expression was observed by confocal microscopy after modulating electric field intensity and pulses duration. Electrogene transfer was more efficient for ms pulses versus μs, and for an electric field intensity maintaining cell viability.

P242

Trans-endothelial passage of chemical vectors in a skeletal muscle endothelium model: Effect of inflammatory cytokines.

Jean-Pierre GOMEZ[1], Paul-Alain JAFFRES[2], Hervé CHERADAME[4], Philippe GUEGAN[4], Bruno PITARD[5], Pierre LEHN[3], Chantal PICHON[1], Patrick MIDOUX[1]

[1]*Centre de Biophysique Moléculaire, CNRS UPR4301 and Université d'Orléans, Orléans, France,* [2]*CEMCA, CNRS UMR 6521, IFR148 ScInBioS, Université Européenne de Bretagne, Université de Brest, Brest, France,* [3]*INSERM U613, IFR148 ScInBioS, Université de Bretagne Occidentale, Hôpital Morvan - CHU Brest, Brest, France,* [4]*LAMBE, CNRS UMR8587 Université d'Evry Val d'Essonne, Evry, France,* [5]*INSERM, U915, Université, Faculté de Médecine, Institut du Thorax, Nantes, France*

Upon systemic administration, DNA complexed with cationic polymers or lipids to treat Duchenne muscular dystrophy (DMD) by gene therapy face to their trans-endothelial passage (TEP) to transfect underlying skeletal muscle cells. Moreover, DMD generates an inflammatory situation with the release of cytokines which contribute to amplify the deleterious effects of the pathology and could modify the endothelium integrity. We have evaluated TEP of various DNA complexes in a model comprising an endothelium of mouse cardiac endothelial cells (MCEC) seeded on a cell insert and mouse skeletal myoblast (C2C12) cells seeded on the well bottom. Transfection efficiency of C2C12 cells was used to measure TEP of DNA complexes. Among several synthetic vectors, DNA complexed with histidinylated lPEI (HislPEI) exhibited good capacity (24%) to cross endothelium compared to that (<2%) of other vectors. One ng/ml of either IL-1β, TNF-α or both did not induce major changes whereas 10 ng/ml increased or decreased TEP depending on the vectors but HislPEI polyplexes kept high capacity to pass through MCEC endothelium. It was noticeable that cytotoxicity of C2C12 cells was greatly increased when MCEC cells were stimulated with these cytokines whereas no cytotoxicity was observed when C2C12 cells were transfected with the cytokines and without MCEC cells. This suggests that endothelial cells produced pro-inflammatory diffusible factors with deleterious effect on C2C12 cells These results indicate that the microenvironment of DMD skeletal muscles is an important parameter for the design of chemical vectors for the transfection of myoblasts. This work was supported by AFM.

P243

Non-viral approach using *Sleeping Beauty* transposons for the correction of Fanconi Anemia Cells

Esther Grueso[1], Paula Rio[2], Zsuzsanna Izsvak[3], Juan A. Bueren[2], Zoltan Ivics[1]

[1]*Transposition and Genome Engineering Group. Paul Ehrlich Institute, Langen, Germany,* [2]*Hematopoiesis and Gene Therapy Division. CIEMAT/CIBERER, Madrid, Spain,* [3]*Mobile DNA Group. Max Delbrück Center for Molecular Medicine, Berlin, Germany*

Sleeping Beauty (SB) technology, particularly with the hyperactive variant SB100x transposase, constitutes a relevant alternative to viral vector-mediated gene therapy, due to the simplicity of production under GMP conditions, and the virtually random integration profile. For Fanconi Anemia (FA), a rare genomic instability disorder mainly characterized by severe and progressive bone marrow failure early in life, gene therapy could be an alternative to allogeneic marrow transplantation, particularly in patients without HLA-identical donors. Fifteen genes necessary for DNA inter-strand cross-link resolution have been described, which mutations give rise to FA cell phenotype; i.e. increased generation of chromosomal aberrations and hypersensitivity to DNA crosslinking agents like mitomycin C (MMC). Our project aims at developing the SB system as a non-viral approach for gene therapy of FA. SB transposons with either *FANCA* or *FAND1* (*BRCA2*) genes, together with a GFP marker cassette were cloned in a "sandwich" vector, a conformation with superior ability to transpose large transgenes. Transfections in Hela cells and in patient-derived fibroblasts with a mutation in the *FANCA* gene showed stable and long term expression of GFP when using the *FANCA* or control GFP transposon. Functional analysis showed FANCD2 nuclear *foci* restoration by immunofluorescence in GFP^+ FA-A fibroblasts and increased resistance to MMC treatment in comparison to uncorrected FA-A patient cells, demonstrating reversion of the FA phenotype. Our study shows that SB transposons could be efficient and flexible tools for genetic modification of FA patient cells *in vitro*, thereby offering a non-viral alternative for a future treatment of the disease.

P244

Nucleic acid delivery to fibroblasts using nonviral vectors associated with magnetic nanoparticles

BARTOSZ Grzeskowiak[1,2], OLGA Mykhaylyk[3], CHRISTIAN Plank[3], RYSZARD Slomski[2]

[1]*The NanoBioMedical Centre, Faculty of Physics, Adam Mickiewicz University, Poznan, Poland,* [2]*Department of Biochemistry and Biotechnology, University of Life Sciences, Poznan, Poland,* [3]*Klinikum rechts der Isar, Institute of Experimental Oncology and Therapy Research, TUM, Munich, Germany*

Nucleic acid delivery to somatic cells are of interest for cloning of animals or cell reprogramming. This study aims to explore the potential of the plasmid DNA delivery to the primary fetal pig and primary mouse fibroblasts using magnetic delivery vectors and gradient magnetic fields, an approach known as magnetofection. PEI-Mag2 iron oxide nanoparticles with a magnetic core of 9 nm and a coating from fluorinated surfactant Zonyl FSA and 25 kD branched polyethylene imine and different liposomal transfection reagents were used to formulate magnetic pDNA lipoplexes. The complexes were characterized in regards to their size, magnetic moment and electrokinetic potential. Lipofection of the pig and mouse fibroblasts resulted in similar pDNA internalization and reporter gene expression patterns. Magnetofection of the pig fibroblasts with optimal magnetic lipoplexes at applied pDNA dose of 1-to-20 pg plasmid per cell resulted in about two-fold improvement of the tranfection efficiency in terms of the luciferase reporter gene expression accompanied by the increase in the plasmid internalization as compared to the lipofection. In contrast, mouse fibroblasts showed similar internalization but only moderate (30–40%) improvement of the reporter gene expression efficiency and 1.5-fold increase in the percent of the transfected cells after magnetofection. Respiration activity of the pig and mouse fibroblasts measured after 48h incubation with optimal magnetic transfcetion complexes was not inhibited up to applied plasmid dose of 8 pg per cell.

P245

Galactosylated liposomes with proton sponge capacity: a novel hepatocyte-specific gene transfer system

Saffiya Habib, Mario Ariatti, Moganavelli Singh

Non-viral Gene Delivery Laboratory, Discipline of Biochemistry, Westville Campus, University of KwaZulu-Natal, Durban, South Africa

Hepatocyte-directed liposomal gene delivery has received much attention due to the lack of suitable treatment for several liver-associated disorders. While targeting of liposomes to the asialoglycoprotein receptor (ASGP-R), nearly-exclusive to hepatocytes, is a well-documented means of achieving cell-specificity, endo/lysosomal degradation of the internalised DNA is one of several factors which hinder successful gene transfer. This study has attempted to address this concern by modifying hepatotropic liposomes with an endosomal escape-inducing proton sponge moiety.

Novel galactosylated (SH02) and imidazolylated (SH04) cholesterol derivatives were successfully synthesised with the aim of conferring the respective functions of ASGP-R-specificity and proton sponge capability upon cationic liposome formulations. These formed unilamellar vesicles with the cytofectin, 3β[*N*-(*N*′,*N*′-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) and co-lipid, dioleoylphosphatidylethanolamine (DOPE), when incorporated at 10 mol%. Liposomes effectively bound pCMV-*luc* plasmid DNA, provided protection against serum nucleases; and were well tolerated by both hepatocytes and kidney cells in culture. Competitive inhibition assays showed that liposomes containing SH02 were internalised predominantly via the ASGP-R. Acid titration experiments highlighted the endosomal pH-buffering capacity of SH04. SH04 improved the transfection activity of the Chol-T/DOPE system, but not that of its targeted counterpart, in kidney cells only. Both SH02 and SH04 individually exhibited transfection-enhancing properties and the transgene expression levels using both novel lipids were promising. With further optimisation of the proton sponge and targeting abilities, the liposomes may achieve desired transgene expression levels for use *in vivo*.

P246

Evaluation of skin angiogenesis stimulated by ointment preparations containing angiogenic genes

Karolina Hajdukiewicz[1,2], Anna Stachurska[1,2], Agnieszka Zajkowska[1], Maciej Malecki[1,2]

[1]*Medical University of Warsaw, Warsaw, Poland,* [2]*Centre of Oncology, Warsaw, Poland*

From a point of view of classic pharmacotherapy genes should be treated as active substances that condition the biological activity of a medicinal product that is used. In the case of angiogenic genes, a gene therapy product exerts angiogenic properties - and after having been introduced into appropriate cells it stimulates processes leading to the formation of new blood vessels. In this work we performed a series of experiments aimed to select a group of vehicles, ointment ingredients that could be useful in the systems that could introduce genes into the skin of laboratory animals. Experiments were conducted on plasmids encoding VEGF, FGF, SDF proteins. Appropriate ointment formulas were prepared for experiments, and they were applied on the skin of laboratory mice; after pre-determined time mice were sacrificed, transfected skin specimens were collected and the presence of a pDNA sequence in samples was analysed with qPCR. The analysis of angiogenesis stimulation was also performed. The sequences of apllied pDNAs were found in the mouse skin. Selected vehicles make it possible to introduce pDNA into skin cells; however, the *in vivo* transfection capacity is not high. Based on estimations 10–30% of pDNA molecules applied in ointment pass into the animal skin cells. Experiments also indicate that plasmid pVEGF, pSHH, pSDF stimulate angiogenesis in animal skin and proangiogenic properties depend on a plasmid dose which is used. This work was supported by a grant from Polish Ministry of Science and Higher Education (N N 405 456039).

P247

Muscle spontaneous regeneration in dwarf mice treated with a bicistronic vector followed by electrotransfer

E Higuti[1], NAJ Oliveira[1], CR Cecchi[1], ER Lima[1], P Martins[2], M Vainzof[2], CA Thomas[3], AR Muotri[3], P Bartolini[1], CN Peroni[1]

[1]*Biotechnology Department, IPEN-CNEN, São Paulo, Brazil,* [2]*Human Genome Research Center, IB-USP, São Paulo, Brazil,* [3]*Dept. Cellular & Molecular Medicine, University of California, San Diego, USA*

Gene therapy combines the correction of defective or missing gene with low risk to the patient. Our group has developed an *in vivo* gene therapy model for the treatment of growth hormone (GH) deficiency based on injection of naked DNA followed by electrotransfer. This strategy provided the presence of human growth hormone (hGH) for at least 60 days in the circulation of immunodeficient/dwarf (lit/scid) mice, that presented a weight gain of up to 33%. The aim of the present work is to verify the safety of our method, evaluating the presence of inflammatory infiltrate and the pattern of muscle regeneration at the electroporation site. A bicistronic vector containing the murine GH (mGH) and the GFP genes under the control of the CMV promoter was utilized. Lit/lit mice were treated with 50 μg of DNA or saline (control group), injected into the quadriceps muscle, followed by electrotransfer using eight 50-V pulses of 20 ms at a 0.5s interval. Histological analysis was performed on day 0, 1, 3, 6 and 12. Muscle damage was verified on the initial days after treatment, but appeared regenerated on the 12th day. GFP maximum expression was observed on the third day. Since increased circulating mGH levels were not observed, GH mediator, i.e. mouse insulin-like growth factor-I (mIGF-I), will be determined to evaluate electroporation efficiency. The results indicate that muscle spontaneously regenerates after this treatment.

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P248

Novel ultrasound-responsive gene carrier with ternary structure.

Tomoaki Kurosaki[1,2], Shigeru Kawakami[1], Ryo Suzuki[3], Kazuo Maruyama[3], Hitoshi Sasaki[4], Mitsuru Hashida[1,5]

[1]*Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29*

Yoshidashimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan, [2]The Japan Society for the Promotion of Science (JSPS), Chiyoda-ku, Tokyo 102-8471, Japan, [3]Department of Biopharmaceutics, School of Pharmaceutical Sciences, Teikyo University, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan, [4]Department of Hospital Pharmacy, Nagasaki University Hospital, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan, [5]Institute of Integrated Cell-Material Sciences (iCeMS), Kyoto University, Yoshida-Ushinomiya-cho, Sakyo-ku, Kyoto 606-8302, Japan

Backgrounds: Recently, ternary complex constructed with pDNA, cationic compounds, and anionic compounds were reported to show high gene expressions and low toxicities *in vivo*. In this experiment, we newly constructed novel ultrasound-responsive gene carrier with ternary structure for effective and secure transfection.

Methods: pDNA was mixed with some cationic polymers and cationic complexes were formed. The cationic complexes and the anionic liposomes were mixed for formations of ternary complexes. Then, perfluoropropane gas was entrapped into the ternary complex and ultrasound-responsive gene carriers were constructed.

Results: The stabilities of the gene delivery vectors were determined by gel electrophoresis and the stable complex formations were clarified. Furthermore, physicochemical properties of the gene delivery vectors were determined. Before entrapment of perfluoropropane gas, the gene carrier showed approximately 150 to 250 nm particle size and -20 to -40 mV ζ-potential. Entrapment of perfluoropropane gas increased particle size and approximately 550 to 600 nm particles were formed. Intravenous administration of the ultrasound-responsive gene carrier with ultrasound exposure from abdominal area significantly improved gene expressions in the mouse liver, kidney, and spleen.

Conclusion: This biocompatible ultrasound-responsive gene carrier with ternary structure would be novel formulation for effective and secure gene delivery.

P249

Combining MAR elements and transposon systems for improved gene expression and integration

D Ley[1], S Puttini[1], Y Bigot[2], N Mermod[1]

[1]University of Lausanne, Lausanne, Switzerland, [2]INRA Centre de Tours, Nouzilly, France

Safety, integration and long-term expression of a transgene constitute a major challenge in gene therapy applications. In this study, we combined the efficiency of transgene integration of the transposon system and the anti-silencing properties of a genetic element called matrix attachment region (MAR). We observed that the addition of the MAR 1–68 in the *PiggyBac* transposon does not interfere with transposition, by maintaining high frequency of transgene integration in CHO cells. Moreover, it seems that this association leads to higher transgene expression from few transposon integration events. This property would be particularly interesting to be tested in muscle progenitor mesoangioblast cells. These cells are important candidates for future stem cell therapy for myopathic patients and known to be difficult to transfect. Encouragingly, our first experiments show that *PiggyBac* and *Sleeping Beauty 100X* systems are greatly efficient in these hard-to-transfect cells. Since *in vivo* electroporation is a possible strategy for the local treatment of muscle disorders, we are currently testing the combination of transposon and MAR using this method in mice muscle to see if transposon systems may promote sustained gene expression over time and/or increase transgene integration. Assessing efficiency and the advantages of this new association may lead to the discovery of a novel system possessing interesting properties for gene or cell-based therapy application.

P250

Novel carotenoid lipid vectors for ocular gene therapy

Susana Machado[1], Sofia Calado[1,2], Ana Vanessa Oliveira[1,2], Susana Jorge[1], Christer L. Øpstad[3], Hans-Richard Sliwka[3], Vassilia Partali[3], Michael Pungente[4], Gabriela Silva[1,5]

[1]Gene therapy lab, Institute for Biotechnology and Bioengineering (IBB/CBME), University of Algarve, Faro, 8005-139, Portugal, [2]PhD program in Biomedical Sciences, University of Algarve, Faro, 8005-139, Portugal, [3]Department of Chemistry, Norwegian University of Science and Technology, 7491 Trondheim, Norway, [4]Premedical Unit, Weill Cornell Medical College in Qatar, Doha, P.O. Box 24144, Qatar, [5]Department of Biomedical Sciences and Medicine, University of Algarve, Faro, 8005-139, Portugal

The eye has several advantages for gene therapy: small size, low immune and inflammatory responses and minimal diffusion of drug to the systemic circulation. Cationic lipids, one of the most studied non-viral vectors, possess either rigid or non-rigid hydrophobic chains, leaving a gap in chain rigidity to be investigated. Our objective is to evaluate the efficiency of DNA delivery to human Retinal Pigmented epithelium (RPE) cells by novel cationic lipid vectors. These novel vectors, designated as C30-20 and C20-20, both possess a highly unsaturated, conjugated, rigid polyene chain, one of C30:9 and the other C20:5, respectively, plus a non-rigid saturated alkyl C20:0 chain.

Lipoplexes, formulated by solvent evaporation of ethanolic mixtures of the new polyene compound with a co-lipid, such as DOPE or cholesterol, and incubated with DNA, were characterized by gel retardation assays, and biocompatibility and transfection assays using RPE cells.

The different lipid formulations encapsulated DNA, were biocompatible with RPE cells, with better results for those with DOPE. The C20-20/DOPE formulation had transfection efficiencies above a commercial transfection agent (GeneJuice). These results show this new polyene vectors to be promising for ocular gene therapy.

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P251

Mitochondrial Gene Targeting in Mammalian Systems using Novel 'Mitochondriotropic' Liposomes

N Narainpersad, M Singh, M Ariatti

University of Kwazulu-Natal, Durban, South Africa

ABSTRACT WITHDRAWN

P252

Comparison of genomic and complementary hGH gene sequences utilized for electrotransfer in dwarf mice

NAJ Oliveira[1], E Higuti[1], CR Cecchi[1], ER Lima[1], P Martins[2], M Vainzof[2], P Bartolini[1], CN Peroni[1]

[1]*National Nuclear Energy Commission IPEN-CNEN, São Paulo, São Paulo, Brazil,* [2]*Human Genome Research Center, IB-USP, São Paulo, São Paulo, Brazil*

Poor gene expression is the main limitation for the use of naked DNA in vivo gene therapy approaches. However, this obstacle has been overcome by employing techniques as electroporation to improve the DNA delivery. The choice of the best gene sequence to be used is also essential for its success. A gene sequence used in an assay may be genomic, complementary or optimized. In this work a comparison between a plasmid containing the CMV promoter and encoding the genomic DNA (gDNA) or the complementary DNA (cDNA) sequence of the human growth hormone (hGH) was carried out. Lit/scid or lit/lit mice were injected with 50 μg of each plasmid into quadriceps muscle, followed by electrotransfer, using eight 50-V pulses of 20 ms at a 0.5-s interval. The lit/scid group that received the cDNA-hGH presented higher levels of hGH in the circulation throughout a 45 day experiment, with a peak of $\sim$20 ng hGH/ml on day six. In the immunocompetent mice (lit/lit), hGH serum level were also higher by using the cDNA-hGH vector. As expected, the use of a viral promoter in immunocompetent animals resulted in lower levels of circulating hGH, at least 2–3 fold in our case, when compared with immunodeficient mice. The bioactivity of hGH expressed by each vector and the mIGF-I levels will be also determined. These results demonstrate the importance of selecting the elements to be used in gene therapy vectors, such as the most efficient gene sequence.

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P253

Analytical tools in minicircle production.

Anja Rischmüller[1,2], Martina Viefhues[1], Marco Schmeer[2], Ruth Baier[2], Dario Anselmetti[1], Martin Schleef[2]

[1]*University of Bielefeld, Applied Nanoscience, Bielefeld, Germany,* [2]*PlasmidFactory GmbH & Co. KG, Bielefeld, Germany*

Here, we give an overview on our analytical tools in the production of minicircle DNA, a safe and efficient vector system for gene and cell therapy and genetic vaccination approaches.

This minicircle technology is removing needless sequences like marker genes, the bacterial origin of replication etc., only used for stable maintenance and amplification of plasmids in bacteria. The resulting minicircle DNA consists almost only of the gene of interest, leading to significant size reduction and improved performance. Furthermore, our minicircle is a homogenous monomer and not a cocktail of various multimeric derivatives as in other minicircle systems published so far.

Throughout the DNA manufacturing process, certain analytical samples are taken in order to ensure the quality. For minicircle production it is especially important that the recombination does not start before this is required. A technique first time used for the analysis of DNA from a minicircle system is shown here in detail: the atomic force microscopy (AFM) is a versatile tool that shows and measures surface structures with unprecedented resolution and accuracy at the nm-scale. Additionally, we show results obtained by capillary gel electrophoresis (CGE) - a tool to identify and quantify minicircle topologies. Finally, the reliability of the non-existence of recombination product during cultivation is shown by use of a novel microfluidic channel. The continuous separation of biomolecules in microfluidic channels enables the implementation of measurements of small analytic volumes along the minicircle production. As a tool for product quality control we can detect the existence of unwanted multimers.

P254

Generation of neutralizing antibodies against botulinum neurotoxin B serotype by DNA electroporation

Alice Rochard[0], Pascal Bigey[0], Virginie Escriou[0], Daniel Scherman[0]

[1]*Unité de Pharmacologie Chimique et Génétique et d'Imagerie, CNRS, UMR 8151, Paris, France,* [2]*INSERM, U1022, Paris, France,* [3]*ENSCP, Chimie ParisTech, Paris, France,* [4]*Direction Générale de l'Armement, DGA, Paris, France*

Botulinum neurotoxin B (BoNTB) is a subtype of a family of seven (A-G) distinct proteins produced by *Clostridium botulinum*, a gram-positive, spore forming anaerobic bacillus. BoNTs inhibit the release of acetylcholine at the synapse of motor neurons, leading to flaccid paralysis. They are among the most potent poisons known, and might present a potential threat as a biological weapon. BoNTs type A, B and E are commonly linked to human disease.

Current therapies for botulism consist mainly of supportive care, prophylactic vaccine and passive antibody administration. Trivalent equine-based antitoxins (A, B and E) antibodies are available and used clinically to neutralize and clear BoNT from the circulatory system.

Production of high titer antisera against BoNTA by genetic immunization mediated by intramuscular DNA electroprapration has previously been reported. However, this technique failed to produce such neutralizing antibodies against BoNTB.

In this work we investigated the reasons of the low immunogenicity of the nontoxic C-terminal half of the heavy chain

(Fragment Fc*) of BoNTB after genetic immunization. Both antigen Fc*BoNTA and Fc*BoNTB were tagged with a c-myc epitope to improve their detection and compare their expression. ELISA, Western Blot and immunofluorescence results indicated that Fc*BoNTB antigenic fragment is poorly secreted and is blocked inside the cells. New constructs aiming at improving the secretion of Fc*BoNTB have been made by addition of N-glycosylation sites, or by modifying the size of the fragment.

P255

HMGB-1 box A domain with R3V6 peptide as an efficient carrier for gene delivery

Jae Hwan RYU, Minhyung Lee

Department of Bioengineering, College of Engineering, Hanyang University, Seoul, Republic of Korea

R3V6 is a gene carrier, which is composed with hydrophilic arginines and hydrophobic valines. R3V6 formed micelles in aqueous solution and behaves larger molecular weight peptides for gene delivery. In this study, high mobility group box 1 box A domain (HMGB1A box) was used as a nuclear localization signal (NLS) peptide in combination with R3V6 for efficient plasmid DNA (pDNA) delivery. Heparin competition assay showed that the combined complex of HMGB1A box and R3V6(HMA-RV) formed more stable complex with pDNA than poly-L-lysine (PLL), HMGB1A box and R3V6 only. Measurement of the particle size showed that the HMGB1A box contributed to the formation of compact structure of HMA-RV complex. In transfection assays, HMA-RV had higher transfection efficiency than PLL, R3V6 and HMGB1A. Furthermore, MTT assay suggests that HMA-RV was not toxic to cells. Therefore, HMA-RV may be useful as a gene delivery carrier.

P256

New analytical tools in *minicircle* production

Anja Rischmueller[1,2], Martina Viefhues[2], Marco Schmeer[1], Ruth Baier[1], Dario Anselmetti[2], Martin Schleef[1]

[1]PlasmidFactory GmbH & Co. KG, Bielefeld, Germany, [2]Experimental Biophysics and Applied Nanoscience, Bielefeld University, Bielefeld, Germany

Here, we give an overview on our analytical tools in the production of *minicircle* DNA, a safe and efficient vector system for gene and cell therapy and genetic vaccination approaches.

Our *minicircle* technology is removing needless sequences like marker genes, the bacterial origin of replication etc., only used for stable maintenance and amplification of plasmids in bacteria. The resulting *minicircle* DNA consists almost only of the gene of interest, leading to significant size reduction and improved performance. Furthermore, our *minicircle* is a homogenous monomer and not a cocktail of various multimeric derivatives as in all other *minicircle* systems published so far.

Throughout the DNA manufacturing process, certain analytical samples are taken in order to ensure the quality. For *minicircle* production it is especially important that the recombination does not start before this is required. A technique first time used for the analysis of DNA from a *minicircle* system is shown here in detail: the atomic force microscopy (AFM) is a versatile tool that shows and measures surface structures with unprecedented resolution and accuracy at the nm-scale. Additionally, we show results obtained by capillary gel electrophoresis (CGE) - a tool to identify and quantify *minicircle* topologies. Finally, the reliability of the non-existence of recombination product during cultivation is shown by use of a novel microfluidic channel. The continuous separation of biomolecules in microfluidic channels enables the implementation of in-process measurements of small analytic volumes along the *minicircle* production.

P257

Epidermal growth factor targeted novel cationic lipoplexes enhance transgene expression in HepG2 cell line *in vitro*

A Sewbalas, M Singh, M Ariatti

University of KwaZulu-Natal, Durban, South Africa

P258

On small minicircles: effect on coiling and function when approaching the sterical limits

Sofia Stenler[1], Oscar Wiklander[1], Maria Badal Tejedor[2], David Hallengärd[3], Britta Wahren[3], Mark W. Rutland[4], C. I. Edvard Smith[1], Karin E. Lundin[1], Pontus Blomberg[1,5]

[1]Clinical Research Center, Department of Laboratory Medicine, Karolinska Institutet, Stockholm, 141 86, Sweden, [2]Institute for Surface Chemistry, Stockholm, 114 86, Sweden, [3]Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, 171 82, Sweden, [4]Department of Chemistry, Surface Chemistry, Royal Institute of Tehcnology, Stockholm, 100 44, Sweden, [5]Vecura, Karolinska University Hospital, Stockholm, 141 86, Sweden

In the study presented, we investigated the properties of plasmid based vectors approaching the minimal size for vector

production. We used a minicircle producing system encoding a splice correcting U7 small nuclear RNA. This resulted in a plasmid based vector of 650 base pairs (bp), to be compared with a normal plasmid carrying the same expression cassette encompassing 3.9 kbp. We also constructed minicircles of varying lengths carrying different number of cassettes. This allowed us to evaluate the implication of size on stability and efficiency of the gene transfer vector.

The coiling morphology of these constructs was characterized by AFM. The resistance to shearing forces caused by gene transfer using a needle free pneumatic system was assayed with the Biojector. We connected these findings to the functional properties of the minicircle by studying the expression and splice correction *in vitro* using lipofection and electroporation, as well as *in vivo* in mice using hydrodynamic delivery to the liver.

This study reveals that small minicircles are more robust when exposed to shearing forces than are the corresponding plasmid. Also as splice correcting vectors, they exhibited a more long-term expression as compared to plasmids *in vivo*. Despite the small size of the minicircle, we saw a distinct coiling pattern. However, the *in vitro* data showed that there seems to be negative correlation between size and expression efficiency. These are important factors that have implication for designing the optimal gene transfer vector, for example when used as a splice correcting agent or DNA vaccine.

P259

Non-viral gene therapy of collagen induced arthritis by electrotransfer of IL-35 gene

Allan Thiolat[1], Marie-Christophe Boissier[1,2], Mylene Petit[1], Delphine Lemeiter[1], Didier Lutomki[3], Anne Denys[1], Natacha Bessis[1]

[1]*Li2p EA 4222, Sorbonne-Paris Cité, University Paris 13, Bobigny, France,* [2]*Rheumatology Dpt, Avicenne Hospital APHP,, Bobigny, France,* [3]*UMR 7244, Sorbonne-Paris Cité, University Paris 13, Bobigny, France*

Background and objectives: Interleukin (IL)-35 was described as an immunosuppressive cytokine specifically produced by CD4 + FoxP3 + regulatory T (Tregs). Since Tregs play a major role in autoimmunity maintenance and protects from inflammation, we aimed at evaluating the role of IL-35 in the collagen-induced arthritis (CIA) mouse model of Rheumatoid Arthritis using a non-viral gene transfer strategy.

Methods: Electrotransfer was used to deliver plasmids encoding IL-35. Two plasmids were used and compared: pIGneo-mouse (m)IL-35-GFP (gift from Dario Vignali) and pORF-mIL-35, where IL-35 is expressed under CMV or HTLV promoter, respectively. pIGneo-mouse (m)IL-35-GFP contains a "native" form of IL-35 made of Ebi3 and p35 linked subunits, while pORF-mIL-35contains a "single chain" construct. The clinical effect of IL-35 was assessed in collagen-induced arthritis in mice receiving two intra-muscular electrotransfer (i.m.ET) of 60μg pIGneo-mIL-35-GFP or pORF-IL-35 after CIA induction. Tregs and Th17 frequencies and phenotypes were performed by flow cytometry in the spleen and lymph nodes.

Results: Surprisingly, whatever the plasmid used, IL-35 gene transfer resulted in a statistically significant increase in clinical scores of collagen-induced arthritis as compared to empty plasmid. The underlying cellular mechanisms of this effect were shown to be related to an increased Th17/Tregs ratio in the spleen of pORF-mIL-35 treated mice.

Conclusion: We show an unexpected but clear exacerbating effect of IL-35 gene transfer in an autoimmune and inflammatory model of RA. Further studies should be performed to exclude that this pro-inflammatory action isn't the result of a synergistic effect between IL-35 and the electrotransfer.

P260

Non-viral ApoA1 gene delivery promotes regression of atherogenesis in rabbit model ofcarbohydrate and lipid metabolism disorders

Olena Toporova, Tamara Gulko, Pavlo Morgunov, Dmytro Irodov, Vitalii Kordium

Institute of Molecular Biology and Genetics of National Academy of Sciences of Ukraine, Kyiv, Ukraine

Objective: To evaluate the antiatherogenic action of human ApoA1 gene expression following direct intrahepatic injection in rabbit model of diabetes complicated by dyslipidemia.

Methods: 3 month old male C*hinchilla* rabbits were used in present studies. *I*nsulin deficiency and hyperglycemia *were* induced by triple intravenous low dose streptozotocin injection. Experimental hypercholesterolemia in intact animal (model of alimentary cholesterol atherosclerosis) and in mild diabetic rabbits (hyperglycemia - up to 12 mmol/l glucose) was generated by supplementary daily feeding of pure cholesterol (2%) in sunflower seed oil (15%) for 5 weeks. Gene therapeutic vector was the plasmid containing expression cassette for full-size human apolipoprotein A1 (apoA1) gene controlled with CMV promoter and flanked by inverted terminal repeats of AAV. We have modified branched polyethylenimine (PEI) to obtain galactose-bearing PEI for enhance efficiency of DNA internalization by target liver cells.

Results: Human apoA1 gene intraliver delivery resulted in 20 mg/dl human ApoAI in blood at least for 60 days, that was accompanied by increased HDL cholesterol and decreased LDL cholesterol levels, atherogenicity coefficient decreased to normal level and accelerated regression of hypercholesterolemia. Surprisingly, glucose level in diabetic rabbits has decreased by 40 % for 3 weeks after single DNA injection. In addition, no specific morphological changes in tissues, special for experimental atherosclerosis, have been detected unlike control animals. Liver DNA of experimental rabbits were positive in transgene PCR for 2 month after gene delivery. Thus, human apoA1 gene intraliver delivery prevents further development of hyperglycemia and atherogenesis in model animals.

P261

Gene therapy for liver diseases using non-viral minicircle-DNA vector

HM Viecelli[1], RP Harbottle[2], M Chuah[3], T VandenDriessche[3], CO Harding[4], B Thöny[1]

[1]*Department of Paediatrics, University of Zurich, Zurich, Switzerland,* [2]*National Heart and Lung Institute, Imperial College London, London, UK,* [3]*Free University of Brussels, Division of Gene Therapy & Regenerative Medicine, Brussels, Belgium,* [4]*Departments of Molecular and Medical Genetics and Paediatrics, Oregon Health & Science University, Portland, Oregon, USA*

We have reported long-term correction of hyperphenylalaninemia and hypopigmentation of the PKU mouse model, C57Bl/

6-Pahenu2, after liver-directed gene transfer with recombinant adeno-associated viral vectors. However, questions of expression stability, treatment toxicity, potential for insertional mutagenesis, and safety required for targeting newborn and paediatric patients for potential life-long treatment remain a risk for viral vector-dependent approaches. To overcome these hurdles, we are developing non-viral gene transfer methods for liver targeting. Here we report the successful use of minicircle (MC) technology to treat murine PKU. MC-DNA vectors containing a *de novo* designed liver-specific hybrid promoter-enhancer fragment, the murine phenylalanine hydroxylase gene (*mPah*), and the bovine growth hormone polyA signal were constructed and delivered to the liver by a single hydrodynamic tail vein injection. While the parental plasmid did not result in any phenylalanine clearance, the corresponding MC-DNA vector normalized blood phenylalanine concomitant with reversion of hypopigmentation in a dose dependent manner up to several months (ongoing experiment). Upon sacrificing treated mice at different point points, we found MC vector persistence concomitant with sustained transgene expression and hepatic PAH enzyme activity. In conclusion, MC technology offers a better safety profile and has the potential for gene-therapeutic treatment of liver diseases.

P262

Nonviral gene delivery to melanoma combining selective cellular uptake and tumour-specific transcription

Joana Viola[1], Phil Grayeski[1], Saskia Graf[2], Florian Kopp[1], Ernst Wagner[1], Robert Besch[2], Manfred Ogris[1]

[1]*Ludwig-Maximilian_universität München, Dep of Pharmacy, München, Germany,* [2]*Ludwig-Maximilian_universität München, Dep of Dermatology and Allergology, München, Germany*

Despite extensive research, metastatic melanomas are still associated with dismal prognosis and patients face median survival times of eight months. Thus, there is a demand for alternative therapies. Clinical trials with suicide genes, like Diphtheria Toxin A (DTA), have shown to be a promising therapeutic approach. However, highly specific targeting is required to ensure tumour-selective gene expression. Therefore, we aimed at developing a nonviral delivery vector that specifically targets melanoma cancer cells by combining perceptive cellular uptake and cell-selective transcription. After screening several melanoma cell lines for different receptors, we have determined $\alpha v\beta 3$ integrin as the most suitable target. Cyclic RGD (arginine-glycine-aspartate, cRGD) was thereafter conjugated to the carrier polyethylene glycol-linear polyethyleneimine to originate the vector that supports perceptive cellular uptake. In vitro transfection studies (with luciferase as reporter gene) have revealed this vector to be most suitable. To provide for cell-selective transcription, we have designed melanoma-specific promoters. Additionally, after evaluating the levels of microRNA 143 in melanoma cell lines, we included binding sites in our pDNA to induce silencing of the DTA gene in healthy tissue. To evaluate this system in vivo, we have developed a disseminated melanoma mouse model. Luciferase-GFP stably transduced 1205 Lu cells (melanoma stage III) were administrated intravenously into nude mice inducing multiple lung tumours. Tumour growth was followed throughout 41 days by bioluminescence and confirmed by lung histology studies thereafter. In vivo assessment of the delivery efficiency of cRGD-carrier is ongoing.

P263

Administration of free extracellular DNA during pregnancy in mice

B Vlkova, H Fabryova, K Meltenburg, J Hodosy, P Celec

Institute of Molecular Biomedicine, Comenius University, Bratislava, Slovakia

Non-specific effects of DNA on the innate immune system might affect the application of non-viral vectors in gene therapy. We have previously hypothesized that the same effects of free extracellular fetal DNA might be responsible for preeclampsia or preeclampsia-like symptoms during pregnancy. Fetal DNA was applied daily during the first, second or third week of pregnancy in mice. Saline and lipopolysaccharide were applied as negative and positive controls respectively. Blood pressure and proteinuria were measured as markers of preeclampsia. In addition, fetal number and weight were evaluated. Preliminary results show that fetal DNA decrease fetal survival and weight. On the other hand, blood pressure and proteinuria was not increased significantly in this preliminary experiment. Other details will be presented. Cell-free fetal DNA similarly to non-viral vectors such as plasmids activate the innate immune system. This might have implications for the understanding of preeclampsia as well as of the side effects of non-viral gene therapy in pregnancy.

P264

Combined delivery of BCNU and VEGF small interfering RNA into the rat glioblastoma model

Na Yi, HyunAh Kim, Minhyung Lee

Hanyang University, Seoul, Republic of Korea

As the most fatal malignancy in brain, glioblastoma cannot be effectively treated with the conventional chemotherapy. The R3V6 peptides, composed of 3-arginines and 6-valines, formed self-assembled micelles in aqueous solution. Co-delivery of bis-chloroethylnitrosourea (BCNU) and VEGF small interfering RNA (VEGF-siRNA) into glioblastoma using R3V6 might be an effective therapy. The aim of this study is to evaluate the tumor suppressive effect of the complex of bis-chloroethylnitrosourea (BCNU) loaded R3V6 (R3V6-BCNU) and VEGF-siRNA in the rat glioblastoma model. The cytotoxicity of VEGF-siRNA/R3V6-BCNU complex against C6 glioblastoma cells *in vitro* suggested that VEGF-siRNA/R3V6-BCNU complexes had higher anticancer effect than BCNU alone. *In vitro* transfection assay, VEGF-siRNA/R3V6-BCNU complexes had the highest transfection efficiency at a 1:20 weight ratio (VEGF-siRNA:R3V6-BCNU). In an *in vitro* silencing assay in C6 cells, VEGF-siRNA/R3V6-BCNU complexes had higher transfection efficiency than lipofectamine. The fluorescent microscopy and FACS were used to measure the siRNA cellular uptake efficiency in C6 cells. The FITC-siRNA/R3V6-BCNU complexes had higher uptake efficiency than lipofectamine. To evaluate the anti-tumor effects of VEGF-siRNA/R3V6-BCNU complexes *in vivo*, VEGF-siRNA/R3V6-BCNU complexes were prepared in a 20 to 1 ratio and injected intracranially into the orthotopic rat glioblastoma model. In the treatment with VEGF-siRNA/R3V6-BCNU complexes reduced tumor volume compared with the untreated tumor control group. Therefore, VEGF-siRNA/R3V6-BCNU complexes may be useful for the treatment of glioblastoma.

P265

Lentiviral-directed transgenesis in swine is an efficient tool to study human dominant genetic diseases.

Corinne Kostic[1], Nicolas Grandchamp[3], Tim King[2], Sylvain Crippa[1], Stéphanie Philippe[3], Simon Lillico[2], Chamsy Sarkis[3], Jacques Mallet[4], Yvan Arsenijevic[1], Bruce Whitelaw[2]

[1]*Unit of Gene Therapy and Stem Cell Biology, Jules-Gonin Eye Hospital, Univ Lausanne, Lausanne, Switzerland,* [2]*Division of Developmental Biology, The Roslin Institute, University of Edinburgh, Scotland, UK,* [3]*NewVectys, Paris, France,* [4]*Team of Biotherapy and Biotechnology, CRICM, Paris, France*

Studies on large animal models are an important step to test new therapeutic strategies previous to human application. Considering the importance of the cone photoreceptor function in human vision and the paucity of large animal models for cone dystrophies having an enriched cone region, we propose to develop a swine model for cone degeneration.

Lentiviral vectors encoding a human double mutant GUCY2D protein under the control of the promoter of a swine cone gene (Arrestin 3) was produced and injected in pig zygotes. Genotyping of piglets identified almost 60% of transgenics, with 1 to 6 transgene copies per genome. The transgenic swine all clearly present a reduced photopic ERG response from 3 months of age on, and various degrees of general mobility alteration were observed at 1 year. RT-PCR demonstrated expression of the transgene in the retina at 1.5 years in all 8 transgenic animals tested. Histological analysis revealed no severe alteration of the retina morphology in most of the transgenic pigs except a noticeable displacement of several cone nuclei in the outer segment layer of all transgenic animals. Phenotyping of the transgenic swine showed a wide variety in the severity of the retinal alteration as is observed in human patients.

Lentiviral-directed transgenesis allowed thus to engineer a heterogeneous cohort of transgenic animals which could mimic the heterogeneity found in humans. Moreover, common degeneration features among the transgenic animals probably reflect the general effect of the dominant mutant protein studied.

P266

Improved Doxycycline (Dox)-inducible lentiviral vectors in human hematopoiesis regulating Cytidine Deaminase Expression

Nico Lachmann[1], Roman Hillje[1], Julia Dahlmann[2], Niels Heinz[3], Ina Gruh[2], Christopher Baum[3], Thomas Moritz[1]

[1]*Rebirth, Cluster of Excellence, RG Reprogramming, Hannover, Germany,* [2]*Rebirth, Cluster of Excellence, Leibniz Research Laboratories for Biotechnology and Artificial Organs, Hannover, Germany,* [3]*Rebirth, Cluster of Excellence, Institute of Experimental Hematology, Hannover, Germany*

Hematopoietic gene therapy has proven effective in a variety of congenital diseases affecting the lymphohematopoietic system, however, constitutive overexpression of the desired transgene can be toxic to specific cell types. Thus we here introduce a novel Dox-inducible lentiviral vector harbouring an expression cassette for the reverse tet-transactivator and a T11-Dox-inducible promoter with an improved signal-to-noise ratio (Heinz et al, HGT 2011). This system is evaluated in the context of myeloprotective cytidine deaminase (CDD) gene transfer, an approach currently hampered by the lymphotoxicity observed upon constitutive CDD-overexpression. CDD-mediated drug resistance was evaluated in K562 and primary human CD34+ cells. Both, transduced K562 and CD34+ cells showed robust transgene induction within 24–48h of Dox-application at doses of $>0.2\mu g/ml$. In K562 cells this resulted in protection from apoptosis and cell-cycle arrest to Ara-C doses of 2000nM and 600nM, respectively, with control cells affected at 10-fold lower doses. Furthermore, primary human CD34+ cells were protected from Ara-C doses of up to 600nM versus 25 nM for control cells and no adverse effects of CDD-overexpression were noted. Importantly, no significant background transgene expression was noted in K562 or CD34+ cells. Thus these data demonstrate Dox-inducible transgene expression as an elegant tool for robust and rapidly controlled transgene expression in human hematopoietic cells which effectively avoids background expression. The improved "all-in-one" lentiviral vector system appears particularly suited for gene therapy approaches requiring only transient transgene expression such as myeloprotective strategies in the context of anti-cancer chemotherapy and this currently is further evaluated in a murine xenotransplant-model.

P267

Lentiviral vectors displaying modified measles virus gp overcome pre-existing immunity in in vivo-like transduction of human T and B cells

Camille Lévy[1], Fouzia Amirache[1], Caroline Costa[1], Cecilia Frecha[1], Claude P. Müller[3], Hasan Kweder[2], Robin Buckland[2], François-Loïc Cosset[1], Els Verhoeyen[1]

[1]*INSERM, U758, Human Virology laboratory, EVIR team, Lyon, France,* [2]*INSERM, U758, Human Virology laboratory, Molecular Basis of Paramyxovirus Entry, Lyon, France,* [3]*Department of Immunology and WHO Collaborating Center for Reference and Research on Measles Infections, Centre de Recherche Public de la Santé; Laboratoire National de Santé, Luxembourg, Luxembourg*

T and B cell gene therapy and immunotherapy without changing phenotype is essential. Previously, we generated lentiviral vectors (LVs) pseudotyped with Edmonston measles virus (MV) hemagglutinin (H) and fusion glycoproteins (H/F-LVs), which allowed efficient transduction of quiescent human T- and B-cells without affecting their naïve and memory phenotype. However, a major obstacle in the use of H/F-LVs i*n vivo* for gene therapy is that most of the human population is vaccinated against measles. As the MV humoral immune response is exclusively directed against the H protein of MV, we mutated the two dominant epitopes in H, Noose and NE. LVs pseudotyped with these mutant H-glycoproteins were still neutralised by human serum. Consequently, we took advantage of emerged MV-D genotypes that were less sensitive to MV vaccination due to a different glycosylation pattern. The mutation responsible was introduced additionally into the H/F-LVs. We found that these mutant H/F-LVs could efficiently transduce quiescent lymphocytes in the presence of high concentration of MV antibody-positive human serum. Mimicking the in vivo situation by incubation with total blood, the mutant H/F-LVs escaped MV antibody neutralization, where the original H/F-LVs failed. Thus, these novel H/F-LVs offer perspectives for *in vivo* lymphocyte-based gene therapy and immuno-therapy.

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Attenuation of Semliki Forest Virus Neurovirulence in Mice by Neuronal Micro-RNA Targeting

Miika Martikainen[1], Erkko Ylösmäki[2], Ari Hinkkanen[1], Kalle Saksela[2]

[1]*A. I. Virtanen Institute for Molecular Sciences, Department of Biotechnology and Molecular Medicine, University of Eastern Finland, Kuopio, Finland,* [2]*Department of Virology, Haartman Institute, University of Helsinki, Helsinki, Finland*

Insertion of target sequences for tissue-specific miRNAs has recently been utilized to modify tissue tropism of RNA and DNA viruses. This miRNA-targeting allows generation of safer oncolytic viruses for cancer virotherapy by blocking replication in unwanted tissues. Semliki forest virus (SFV) is an enveloped positive-strand RNA virus of the family *Togaviridae.* Several strains of SFV exist. Strain SFV4 is highly neurovirulent in mice of all ages causing fatal encephalitis while avirulent strain A7(74) shows attenuated spread in the central nervous system (CNS) of adult mice. Replication-competent SFV vector based on strain A7(74) have been considered as promising tools for cancer virotherapy. Since naturally neurotropic, it is of particular interest in virotherapy of brain tumours. To further increase the safety of SFV virotherapy, additional measures to restrict SFV replication in CNS are needed.

Here, we have generated novel miRNA-targeted SFV vectors by inserting target elements for neuron-specific miR124 or target elements into the SFV genome between viral nsp3 and nsp4 genes (SFV4-miRT124). Following intraperitoneal injection into adult Balb/c mice SFV4-miRT124 displayed attenuated spread into CNS. Peripheral replication was not affected and infection elicted protective immunity against lethal SFV infection. When administered intracranially, SFV4-miRT124 infected dominantly oligodendrocytes of *corpus callosum*, characteristic to avirulent SFV strain. However, infected neuronal cells of the spinal cord were observed. Taken together, by inserting miR124 target sequences, we were able to modulate the SFV neurovirulence in adult mice. This approach allows generation of safer oncolytic alphavirus virotherapy agents.

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PTG1.L, a new powerful reagent for the production of recombinant lentiviral vectors.

Fabian Gross[4], Philippe Guégan[2], Hervé Cheradame[3,2], Patrick Midoux[1]

[1]*Centre de Biophysique Moléculaire, CNRS UPR4301, Orléans, France,* [2]*LAMBE, CNRS UMR8587 Université d'Evry Val d'Essonne, Evry, France,* [3]*Polytheragene, Genopole, Evry, France,* [4]*Plateforme de vectorologie de Toulouse, laboratoire de biothérapie UMR 1037/CRCT, CHU Rangueil, Toulouse, France*

Recombinant lentiviral (r-lentiviral) vectors are very powerful tools for stable gene transfer to both dividing and non-dividing cells *in vitro* and *in vivo*. Virus particles are commonly produced upon transfection of the human HEK293T packaging cells with at least three plasmids, the first shuttle vector carrying the DNA for the gene of interest, the second encoding for *gag*, *pol* and *rev* genes and the third for the VSV-G envelope. The production of r-lentiviral vectors requires an efficient protocol easy to implement, with good reproducibility for a maximum production of viral particles in a short time with low cytotoxicity and high titre. Here we report the good production of r-lentiviral vectors upon Tri-transfection with pTRIP-GFP, 8.91 and VSV-G plasmids of HEK293 packaging cells by using PTG1.L, a new powerful reagent from Polytheragene (http://www.polytheragene.fr/). PTG1.L method was compared to calcium phosphate, polyethyleneimine (PEI25k) or LipofectAmine methods. The results indicate that the production of viral particles is several folds higher with PTG1.L than with bPEI. Although PTG1.L enabled the production of r-lentiviral vectors with the viral titre comparable to that obtained by the conventional calcium phosphate method. The advantages of the PTG1.L reagent are first easy compared to other reagents tested for production of lentiviral vectors in three days and finally its low cytotoxicity allowing the maintenance of virus particles titre in the second day of harvest. PTG1.L could become for all these reasons the best partner for the production of r-lentiviral vectors for research academic or corporate laboratories.

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Large scale efficacy and safety study of two clinical grade LentiGlobin vectors in ß-thalassemia (Hbb th1/th1) mice transplanted with transduced syngeneic Lin-depleted marrow cells

Olivier NEGRE[1,3], Cynthia BARTHOLOMÄ[7], Robert KUTNER[8], Béatrix GILLET-LEGRAND[1,3], Céline COURNE[1,3], Anaïs PAULARD[1,3], Byoung RYU[8], Christophe JOUBERT[6], Anne-Laure BAUCHET[6], Véronique NEUVILLE[5], Charlotte COLOMB[1], Yves BEUZARD[3], Christof von KALLE[7], Mitchell FINER[8], Manfred SCHMIDT[7], Philippe LEBOULCH[2,4], Gabor VERES[8], Emmanuel PAYEN[1,2]

[1]*CEA, Institute of Emerging Diseases and Innovative Therapies (iMETI), Fontenay aux Roses, France,* [2]*CEA, Inserm U962 and University Paris 11, CEA-iMETI, Fontenay aux Roses, France,* [3]*Bluebird bio France, CEA-iMETI, Fontenay aux Roses, France,* [4]*Harvard Medical School and Genetics Division, Department of Medicine, Brigham & Women's Hospital, Boston, USA,* [5]*CEA, Institut of Cellular and Molecular Radiation Biology (IRCM), Fontenay aux Roses, France,* [6]*CEA, Institut of Biomedical Imaging (I2BM), Fontenay aux Roses, France,* [7]*DKFZ, National Center for Tumor Diseases (NCT), Heildelberg, Germany,* [8]*Bluebird bio, Cambridge, USA*

Gene therapy of ß-thalassemia and sickle cell disease would change the fate of thousands of patients without geno-identical sibling donor if proven safe and effective in relevant animal models and human clinical trials. In 2010, we reported the first in man proof-of-concept of *ex vivo* gene therapy in ß-thalassemia major (severe β^E/β^0) with a lentiviral vector encoding a marked ß-globin chain. This patient was transplanted more than 5 years ago and has remained transfusion independent for more than 4 years. In order to facilitate the rate of patient enrollment, we recently modified the vector to yield higher titers and allow for effective large-scale cGMP production. The aim of the present study is to assess and compare vector efficacies and toxicities before inclusion of the new clinical grade LentiGlobin vector in a human trial. Lin-depleted bone marrow cells from β-thalassemia ($Hbb^{th1/th1}$) mice were transduced with the two LentiGlobin vectors and transplanted into syngeneic myeloablated recipients. Quantification of the relative therapeutic efficacies, toxicology (histopathology, hematology) and insertion site analyses were performed in 54 primary transplants. Secondary transplants were also conducted (total of 108 mice) in order to address

further the risk of insertional mutagenesis. Data collected for the entire study, including in-depth toxicology performed by an independent clinical research organization, will be presented.

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Primer Binding Site (PBS) deficient lentiviral vectors allow safe transient gene expression

Stéphanie Philippe[1], Dorothée Altémir[1], Nicolas Grandchamp[1], Suzanna Ursulet[1], Héloïse Pilet[1], Jacques Mallet[2], Marie-José Lecomte[1], Chamsy Sarkis[1,2]

[1]*NewVectys, Paris, France,* [2]*CRICM, Paris, France*

Several breakthroughs paved the last 15 years of lentiviral vector developments, including Self-Inactivating vectors, reintroduction of the cPPT-CTS and integrase deficient vectors. With the aim of further improving safety of lentiviral vector based gene transfer, we developed a new generation of fully transient lentiviral vectors impaired for reverse transcription due to PBS deletion.

Vectors without PBS were produced with various reporter transgenes such as GFP, Luciferase and NEO, with or without internal promoter. Vectors deprived of PBS can be efficiently produced and they genuinely encapsidate RNA genomes that do not undergo reverse transcription due to the PBS deletion. The kinetic of transduction, the efficiency of the transgene expression and the residual integration activity were assessed using various reporter vectors.

Results show that this new generation of lentiviral vectors allows transgene expression directly from the RNA vector genome and in absence of promoter. Expression is transient for about 8–9 days with a maximum level reached at around 48–96 hours. Residual integration events were further characterized, showing that they are not due to recovery of WT PBS through recombination between plasmids during the production, suggesting that the deletion of the PBS that was used is not sufficient to fully abolish reverse transcription. In addition, quantitative evaluation of the residual integration activity shows that these vectors are at least as safe as integration deficient lentiviral vectors.

We show here that lentiviral vectors deprived of PBS are promising tools for safe and transient gene transfer, including delivery of genome editors such as ZFNs or TALENs.

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Reduction of hepatocyte transduction by fiber-modified adenoviruses following their strong uptake by Kupffer cells

Najat RADDI[1,2], Frédéric VIGANT[1,2], Dragomira MAJHEN[1,2], Joao Paulo PORTELA CATANI[1,2], Gaétan CORNILLEAU[1,2], Karim BENIHOUD[2,1]

[1]*CNRS UMR 8203, Villejuif, France,* [2]*University Paris-Sud, Orsay, France*

Binding of blood factor X (FX) to Adenovirus (Ad) hexon protein was shown to be responsible for liver transduction. Unexpectedly we reported previously that an Ad5 pseudotyped with the shaft and fiber from serotype 3 (Ad5S3K3) was also impaired in its ability to drive hepatocyte transduction, thus questioning a potential role of fiber protein in Ad5 liver tropism. To better understand the role of fiber potein in Ad liver tropism, we compared Ad5S3K3 and an Ad pseudotyped with Ad3 fiber shaft (Ad5S3K5) to an Ad with unmodified capsid (Adwt). All three vectors were shown to bind to immobilized FX with similar efficacy but Ad5S3K3 and Ad5S3K5 were impaired in FX-mediated transduction of CHO cells compared to Adwt. Following intravenous administration, a dose-dependent transgene expression was observed at day 2 post-infection (p.i.) for all three viruses. Interestingly, Ad5S3K3 but not Ad5S3K5 led to a reduced transgene expression at day 2 in liver compared to Adwt. In contrast, Q-PCR analyses performed at the same time point p.i. demonstrated a dramatic decrease in viral DNA content in liver for both Ad5S3K3 and Ad5S3K5. Remarkably, after Kupffer cell (KC) depletion either by pre-administration of Ad or by clodronate-liposomes, Ad5S3K3- and Ad5S3K5-injected mice displayed a liver transgene expression comparable to KC-depleted Adwt-injected mice. Finally, experiments are ongoing to confirm the role of FX in liver transduction by Ad5S3K3 and Ad5S3K5 in KC-depleted mice. Altogether, our results emphasize a role of fiber protein in controlling Ad distribution in the liver.

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No longitudinal transmission of AAV5-PBGD vector DNA in mice

Ana Gloria Gil Royo[1], Gloria González-Aseguinolaza[2], Ismael Aizpun[1], Astrid Pañeda Rodríguez[3], María Eugenia Cornet[3], María del Mar Municio[3], Tracy Freeman[4], Harald Petry[5], Marc Sonnemans[5], Florence Salmon[5]

[1]*CIFA, Center for Applied Pharmacological Research, University of Navarra, Pamplona, Spain,* [2]*CIMA, Center for Applied Medical Research, University of Navarra, Pamplona, Spain,* [3]*DIGNA Biotech S.L., Madrid, Spain,* [4]*Covance Laboratories Ltd, Harrogate, UK,* [5]*uniQure B.V., Non-Clinical Development, Amsterdam, The Netherlands*

Prior to gene therapy administration to patients, it is crucial to assess the risk of inadvertent transmission of vector sequences into the germline of treated individuals. AAV5-PBGD is a gene therapy vector for the treatment of acute intermittent porphyria (AIP). After intravenous injection into mice and cynomolgus macaques, vector DNA was detected by QPCR in the gonads and transiently in semen, thus triggering the performance of breeding studies.

C57Bl/6 mice of either gender were injected intravenously with AAV5-PBGD at 5×10^{14} gc/kg and subsequently mated for four days with non-treated animals of the opposite gender in order to investigate if any vector DNA sequences were passed on to the offspring via the maternal or the paternal line. Females were individualized after successful mating and killed on gestation day 16.

Upon necropsy, foetuses, reproductive organs and liver samples were collected for QPCR.

No mortality or any effect on clinical signs, body weight gain, mating performance or necropsy findings were observed. No differences were observed in any developmental parameters among the groups. Vector DNA was detected in reproductive organs of treated animals but not in non-treated animals. No vector DNA was found in placentas of treated and non-treated females. No vector DNA was detected in the foetuses.

It can be concluded that there is no risk of germ line transmission via the maternal or via the paternal line to the offspring in mice.

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Non-clinical safety evaluation of AAV5-PBGD in mice and cynomolgus macaques

Gloria González-Aseguinolaza[1], Astrid Pañeda Rodriguez[2], Esperanza López-Franco[1], Cristina Olagüe[1], Antonio Fontanellas[1], Carmen Unzu[1], Ana Sampedro[1], María Eugenia Cornet[2], María del Mar Municio[2], Paul Heal[3], Harald Petry[4], Marc Sonnemans[4], Bart Nijmeier[4], Florence Salmon[4]

[1]CIMA, Center for Applied Medical Research, University of Navarra, Pamplona, Spain, [2]DIGNA Biotech S.L., Madrid, Spain, [3]Covance Laboratories Ltd, Harrogate, UK, [4]uniQure, Non-Clinical Development, Amsterdam, The Netherlands

AAV5-PBGD, a gene therapy vector for the treatment of acute intermittent porphyria (AIP), showed sustained efficacy in PBGD-deficient AIP mice and prevented acute attacks upon Phenobarbital challenge.

Vector safety was studied, prior to clinical use, in C57Bl/6 mice and Cynomolgus macaques. Male and female mice were dosed intravenously 5×10^{12}, 5×10^{13} or $5\times10^{14}\times$gc/kg and animals from each dose group were sacrificed at Day 7, 90 and 180, respectively. Male and female macaques were injected intravenously with either 1×10^{13} or $5\times10^{13}\times$gc/kg and all animals were sacrificed at Day 30.

Transduction efficacy was assessed by measuring PBGD activity in the liver and biodistribution of vector DNA was determined by Q-PCR. Shedding of vector DNA was determined by Q-PCR in excreta.

In both species, no effect was seen on clinical signs, food consumption and body weight. Haematology, clinical chemistry and histopathology were analysed in all animals at all time points. Transient subtle effects, related to the injection of the vector, were seen in both species on the haematology and chemistry parameters shortly after dosing. No evidence of toxicity was detected at later time-points. Vector DNA was evenly distributed at day 7 but was detected mainly in liver and spleen thereafter in mice and in liver, spleen and adrenal glands in macaques. PBGD expression was confirmed in the liver of all treated animals at levels corresponding to the injected dose.

Therefore, in the absence of any adverse toxicologically significant findings, the NOAEL for AAV5-PBGD is the highest injected dose ($5\times10^{14}\times$gc/kg).

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Potential Therapeutic Role of Vasoactive Intestinal Peptide in Diabetes

Ahter Dilsad Sanlioglu[1,2], Bahri Karacay[4], Mustafa Kemal Balci[1,3], Thomas S Griffith[5], Salih Sanlioglu[1]

[1]Human Gene and Cell Therapy Center of Akdeniz University, Antalya, Turkey, [2]Department of Medical Biology and Genetics, Akdeniz University Faculty of Medicine, Antalya, Turkey, [3]Department of Internal Medicine, Division of Endocrinology and Metabolism, Akdeniz University Faculty of Medicine, Antalya, Turkey, [4]Department of Pediatrics, Division of Child Neurology, University of Iowa, Iowa City, IA, USA, [5]Department of Urology, University of Minnesota, Minneapolis, MN, USA

Type 2 diabetes (T2D) is characterized by chronic insulin resistance and a progressive decline in beta cell function. Although rigorous glucose control can reduce morbidity and mortality associated with diabetes, achieving optimal long-term glycemic control remains to be accomplished in many diabetic patients. Since beta cell mass and function inevitably decline in T2D, exogenous insulin administration is almost unavoidable as a final outcome despite the use of oral anti-hyperglycemic agents in many diabetic patients. Pancreatic islet cell death, but not the defect in new islet formation or beta cell replication has been accounted for the decrease in beta cell mass observed in T2D patients. Thus, therapeutic approaches designed to protect islet cells from apoptosis could be a significant improvement in the management of T2D, because of its potential to reverse diabetes not just ameliorate glycemia. An ideal therapeutic agent should preserve beta cells, protect them from apoptosis, stimulate postprandial insulin secretion and increase beta cell replication and/or islet neogenesis. An islet endocrine neuro-peptide known as Vasoactive Intestinal Peptide (VIP) strongly stimulates postprandial insulin secretion. Because of its broad spectrum of biologic functions such as acting as a potent anti-inflammatory factor through suppression of Th1 immune response, and induction of immune tolerance via regulatory T cells, VIP has emerged as a promising therapeutic agent for the treatment of many autoimmune diseases including diabetes.

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Primer Binding Site (PBS) - deficient lentiviral vectors for transient gene expression of DNA editor enzymes

Chamsy Sarkis[1], Dorothée Altémir[1], Héloïse Pilet[1], Suzanna Ursulet[1], Sébastien Dussaud[2], Philippe Ravassard[2], Marie-José Lecomte[1], Stéphanie Philippe[1], Chamsy Sarkis[1,2]

[1]NewVectys, Paris, France, [2]CRICM, Paris, France

Development of DNA editor enzymes, such as evolved recombinases, ZFNs or TALENs has recently opened a wide range of opportunities for genetic engineering, including transgenesis in large species. The major drawback to the widespread use of these emerging technologies lies in the delivery of the proteins which has to be efficient and fully transient for persistent editor expression is deleterious. With the aim of providing a tool fulfilling these criteria, we developed transient lentiviral vectors impaired for reverse transcription by PBS deletion and evaluated their ability to deliver genome editors for transgenesis.

Lentiviral vectors lacking the PBS and an internal promoter were produced with genome editors as transgenes, such as the PhiC31 and the Cre recombinases. Transgene expression was assessed and recombinase activity was evaluated. Results show that this new generation of lentiviral vector is efficient to mediate transgene expression in target cells. For example, Cre recombinase expression by PBS-deleted lentiviral vectors allows to efficiently recombine two Lox sites in reporter cells containing a LacZ inducible expression cassette. These vectors were also successfully used for transgenesis and allowed to generate recombined embryos from ROSA26-LacZ zygotes without any persistence of the vector genome in the developed animals.

We show here that lentiviral vectors deprived of PBS are promising tools for safe transient gene transfer, including delivery of DNA editor enzymes such as ZFNs or TALENs. This new generation of lentiviral vectors will allow the efficient use of DNA editor technologies for transgenesis application, including in large species such as pig.

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Towards a clinical trial of gene therapy for malignant glioma using highly concentrated retrovirus solution

Keiji Shimizu, Masahiro Kitahara, Yu Kawanishi, Yasunori Fujimoto, Toshio Yawata

Kochi Medical School, Nankoku, Kochi, Japan

Malignant gliomas are the most common primary tumors in the central nervous system. Despite many efforts to develop effective therapies, the outcome of patients with malignant gliomas still remains poor. Clinical trials for this disease using virus producing cell producing HSV*tk* harboring retroviruses failed to demonstrate efficacy, which may be due to low tumor cell transduction rate. However, retrovirus vector has still some attractive features, including the targeted transduction of tumor cells in brain and high stability of gene expression. We established a packaging cell line that produces the high-titer recombinant retrovirus. In gene therapy against mouse glioma model by direct inoculation of concentrated high-titer recombinant retrovirus, eighty percent of treated mice were cured completely. Preclinical safety testing for replication competent retrovirus, toxicity and tumorigenicity revealed no adverse effects or abnormality. Large-scale production of the retrovirus vectors for clinical use is cumbersome and costly because of the adherent nature of packaging cells. Microcarrier suspension culture was employed to produce high-titer retrovirus instead of a conventional static culture. A higher virus titer was obtained in suspension culture compared to static culture, but density of virus producing cells was not different between both cultures. Therapeutic efficacy was also obtained in gene therapy against mouse glioma model using the high titer recombinant retrovirus produced in the suspension culture. This culture system facilitates easy scale-up of culture volume and may achieve high purity of retroviral supernatant. These promising results prompted us to plan a clinical trial to investigate this strategy.

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Prevention of silencing by UCOE deletion constructs

Wather Hänseler[1], Christian Brendel[2], Georgina Santili[3], William James[4], Sally Cowley[4], Adrian Thrasher[3], Manuel Grez[2], Janine Reichenbach[1], Ulrich Siler[1]

[1]*Children's Resaerch Center (CRC), University Children's Hospital, Div of Immunology/BMT, Zürich, Switzerland,* [2]*Georg Speyer Resarch Institute, Frankfurt a.M., Germany,* [3]*Molecular Immunology Unit, Institute of Child Health, London, UK,* [4]*James Martin Stem Cell Facility, Sir William Dunn School of Pathology, University of Oxford, Oxford, UK*

Recent clinical Chronic Granulomatous Disease (CGD) gene therapy (GT) trial brought proof-of-concept but was accompanied by transactivation and transgene silencing. The ubiquitous chromatin opening element (UCOE) comprises the sequences of two divergent oriented promoters and is known to have anti-silencing properties. We generated deletion constructs of the UCOE element. A screen of these constructs for anti-silencing properties in highly silencing P19 cells provided us with two constructs protecting the adjacent SFFV promoter from silencing. To develop a silencing resistant GT vector for the p47phox-deficient form of CGD we combined one UCOE element with a myelospecific (miR223 or cathepsinG/c-fes chimeric) or with the SFFV promoter in a lentiviral SIN vector to drive p47phox expression. In P19 cells, the SFFV promoter was protected from silencing by a UCOE deletion construct. Upon transduction of p47phox-/- iPSCs, silencing of SFFV was prevented by the adjacent UCOE deletion construct. Both myeloid promoters showed minimal activity in undifferentiated cells. Both UCOE-protected myeloid promoters continously gained activity upon differentiation to monocytes and macrophages. Furthermore all constructs restored ROS production in monocytes and macrophages.

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Enhancement of lentiviral transduction efficiency by inhibition of intracellular anti-viral defense mechanisms

Tolga Sutlu[1], Sanna Nyström[0,2], Mari Gilljam[1], Birgitta Stellan[1], Steven E. Applequist[0,2], Evren Alici[1]

[1]*Center for Hematology and Regenerative Medicine (HERM), Department of Medicine, Karolinska University Hospital Huddinge, Karolinska Institutet, Stockholm, Sweden,* [2]*Center for Infectious Medicine (CIM), Department of Medicine, Karolinska University Hospital Huddinge, Karolinska Institutet, Stockholm, Sweden*

Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) play a major role in detection of viral infections and induction of an anti-viral state. Many wild-type viruses have developed elaborate schemes for avoiding detection by these receptors in order to increase their virulence. In the case of viral vectors, the removal of various viral genes that counteract host responses but are dispensable for vector production is often preferred due to safety and practicality considerations. Inevitably, this would render viral vectors more prone to inducing strong innate responses upon target cell infection. In this study, we have observed that inhibition of innate immune receptor signaling greatly enhances lentiviral transduction efficiency in primary human natural killer (NK) cells.

Although adoptive immunotherapy with genetically modified NK cells is a promising approach for cancer treatment, optimization of highly efficient gene transfer protocols for NK cells still presents a challenge. We were able to boost the efficiency of lentiviral genetic modification on average 3.8-fold using BX795, an inhibitor of the TBK1/IKKe complex acting downstream of RIG-I, MDA-5 and TLR3. These results support the hypothesis that during transduction, intracellular anti-viral defense mechanisms including one or more of the receptors RIG-I, MDA-5 and TLR3 contribute significantly to the resistance against viral vectors. We have also observed that the use of BX795 enhances lentiviral transduction efficiency in a number of human and mouse cell lines, indicating a broadly applicable, practical and safe approach that has the potential of being instrumental for various gene therapy protocols.

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Binding of rAAV-6 to C-reactive protein improves gene transfer in mice

Jérôme Denard[1], Béatrice Marolleau[1], Christine Jenny[1], Odile Weber[2], Hans Jörg Fehling[2], Fedor Svinartchouk[1]

[1]*Généthon, EVRY, France,* [2]*Institute of Immunology, University Clinics Ulm, Ulm, Germany*

Clinical relevance of gene therapy using the recombinant adeno-associated vectors (rAAV) often requires widespread distribution of the vector and in this case systemic delivery is the optimal route of administration. In order to improve vector efficacy it is obviously important to characterize rAAV's molecular interactions in the bloodstream.

Recently we have shown that rAAV-6 interacts specifically with G3BP (galectin 3-binding protein) in humans and dogs. Furthermore, interaction of the hu-G3BP with rAAV-6 led to the formation of aggregates and hampered transduction when co-delivered into the mouse. In mouse serum rAAV-6 binds CRP (C-reactive protein). This protein belongs to the short pentraxins whose main functions are thought to include recognition of a variety of pathogenic agents. In order to evaluate a role of rAAV-6/CRP interactions we compared rAAV-6 efficiency and biodistribution in CRP knockout mice and wild-type controls, both on inbred C57Bl/6 background. After intravenous infections of rAAV-6 coding for luciferase, live imaging showed 5 to 10 times less efficient transduction of muscles and liver in CRP knockout mice compared to C57Bl/6 controls. Better gene transfer in muscle, heart and liver of mice expressing CRP was confirmed by in vitro measurements of luciferase activity as well as qPCR analysis. Interestingly, rAAV-8 which does not interact with CRP was equally efficient in both mouse models. We hypothesize that rAAV-6 binding to CRP facilitates vector penetration through the endothelium and that similar mechanisms could play.

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The essential role of the translational research coordinator in gene therapy trial

Michiko Tanaka[1,4], Megumi Kataoka[2], Yoshikazu Yonemitsu[3]

[1]Department of Health Sciences, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, [2]Department of Cardiovascular Research, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, [3]R&D Laboratory for Innovative Biotherapeutics, Graduate School of Phermaceutical Sciences, Kyushu University, Fukuoka, Japan, [4]Center for Clinical and Translational Research, Kyushu University Hospital, Fukuoka, Japan

Background: The gene therapy in Japan is categorized in translational research. As the field of gene therapy continues to evolve, translational research coordinators (TRC) and physicians form a vital partnership, ensuring effective and safe gene therapy.

Aim: The important role of the TRC, as a member of the multidisciplinary team caring for patients who receive gene therapy as part of a clinical trial, is introduced and further issues are discussed.

Method: A case report of the TRC roles in the phase I/IIa clinical study to treat chronic critical limb ischemia using non-transmissible recombinant Sendai virus vector carrying fibroblast growth factor-2 gene (SeV/dF-hFGF2).

Findings: Gene therapy trials are exception, and are likely to be more complex than most, owing to their unique technological requirements and ethical considerations. Specific supports along with gene therapy trial by the TRC were found that ensuring gene therapy related committees and regulations, educating multidisciplinary team regarding gene therapy, ensuring virus shedding before the isolation release, and involving long-term follow-up for the subjects. The TRC were acquired knowledge and skills of supporting gene therapy by the self-learning and on the job training from physicians. It was disclosed that collaboration with physicians is critical to succeed the trial, and the supports from the TRC were relied on physicians. Also, a lack of education system of gene therapy for co-medical stuffs was revealed.

Conclusion: The gene therapy in Japan is still experimental. It is important to enrich the supports from the TRC.

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Hybrid AAV/transposase vectors for somatic integration in human cells based on the hyperactive Sleeping Beauty transposase SB100X

Wenli Zhang[1,2], Christina Rauschhuber[1], Nadine Müther[1], Nicola Wolf[1], Lajos Mates[3], Zsuzsanna Izsvak[3], Zoltan Ivics Ivics[3], Anja Ehrhardt[1,2]

[1]Max von Pettenkofer-Institut, Munich, Germany, [2]University Witten/Herdecke, Witten, Germany, [3]Max Delbrück Center for Molecular Medicine, Berlin, Germany

Recombinant adeno-associated viral (rAAV) vectors have been shown to be one of the most promising tools for therapeutic gene delivery because they can induce efficient and long-term transduction in non-dividing cells. However, as AAV vectors mostly remain episomal, vector genomes and transgene expression are lost over time in rapidly dividing cells. Therefore, to stably transduce tissues and cells with a high proliferate potential, we developed a novel AAV/transposase hybrid-vector for stable integration. To facilitate SB-mediated transposition from the rAAV genome, we established a system in which one AAV vector contains the transposon and the second AAV vector the transposase encoding sequence for delivery of the hyperactive Sleeping Beauty (SB) transposase variants HSB5 and SB100X.

This AAV/transposase hybrid-vector system was first tested in human cells by performing colony forming assays (CFA). Hela cells were transfected by SB100, HSB5 and mSB expressing plasmids followed by infection of an AAV vector containing the neomycin resistance gene (AAV-Neo). In contrast to inactive mSB control group, the SB100 and HSB5 groups showed 10-fold and 2-fold increased numbers of colonies, respectively. Next, we co-infected Hela cells with vectors AAV-Neo and AAV-SB100 at increasing dosages (MOI 100, 1000, 10000 and 50000) and highest integration efficiencies were achieved at MOI 10000 (354 ± 29 colony forming units). Based on a plasmid rescue strategy we analyzed the SB100-mediated integration profile after transposition from AAV vector and of the 40 integration events identified, half of them located in genes. A large number of integration sites is currently been analyzed by deep sequencing.

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A novel pipeline for generation of adenoviral vectors with optimized features for applications in vitro and in vivo

Martin Mueck-Haeusl[1], Wenli Zhang[1,2], Manish Solanki[2], Richard Voigtlander[1], Zsolt Ruzsics[1], Anja Ehrhardt[1,2]

[1]Max von Pettenkofer-Institut, Munich, Germany, [2]University Witten/Herdecke, Witten, Germany

High-capacity adenoviral vectors (HCA) are powerful tools for a broad range of applications but modification of the capsid provided by the helper-virus and engineering of complex cargo sequences remain challenging. We established platforms based on a traceless bacterial artificial chromosome (BAC) cloning

strategy to design a panel of capsid-modified helper-viruses allowing repeated administrations and altered tropisms. Herein, we substituted the hexon hypervariable regions (HVRs) either by precise exchanges or by switching the entire hexon surface domain (SD) from respective serotypes. By this approach we successfully rescued 4 hexon-modified helper-viruses from serotype 4 and 12 with SD-exchanges, and 12 and 48 with precise HVR-exchanges. Next, we evaluated them in C57Bl/6 mice for in vivo distribution by bioluminescence measurements. Mice infected with HV-HVRs12 demonstrated a substantial increase of luciferase activity in liver in comparison to the unmodified helper-virus, indicating an improved liver tropism for hepatocyte-targeting. Using this hexon-modified helper-virus HV-HVRs12, we produced a HCA vector expressing canine coagulation factor IX (cFIX). Efficient transgene expression from this HCA was confirmed in vitro and we are currently analyzing efficacy in vivo.

Moreover, we invented a BAC-based recombination pipeline based on iterative recombination steps utilizing alternating bacterial selection cassettes. This enables generation of complex HCA genomes such as HCA-2indsys containing four transgene expression cassettes, which include two independent systems for cell type-specific and inducible expression of reporter proteins. Liver-specific mifepristone-dependent expression of renilla luciferase was demonstrated in hepatocytes, whereas doxycycline-dependent expression of firefly luciferase in induced pluripotent stem (iPS) cells is currently being evaluated.

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An adenoviral hybrid-vector system for extrachromosomal maintenance of therapeutic DNA

Richard Voigtlander[1], Rudolf Haase[4], Martin Mück-Häusl[1], Wenli Zhang[1,2], Eric Schulz[2], Armin Baiker[3], Anja Ehrhardt[1,2]

[1]Max von Pettenkofer-Institute, Munich, Germany, [2]University Witten/Herdecke, Witten, Germany, [3]Bavarian Health and Food Safety Authority, Oberschleissheim, Germany, [4]Pharmacy, Ludwig-Maximilians-University Munich, Munich, Germany

In dividing cells the two aims a gene therapeutic approach should accomplish are defined as the nuclear delivery and retention of therapeutic DNA. For stable transgene expression therapeutic DNA can either be maintained by somatic integration or episomal persistence. Extrachromosomal maintenance of therapeutic DNA would diminish the risk of insertional mutagenesis and since most monosystems fail to fulfil both tasks with equal efficiency, hybrid-vector systems represent promising tools in facing these challenges.

Our hybrid-vector system HCAdV-pEPito synergizes high-capacity adenoviral vectors (HCAdV) for efficient delivery and the S/MAR- (Scaffold/Matrix Attachment Region-) based pEPito plasmid replicon for episomal persistence. After proving that this plasmid replicon can be excised from the adenoviral genome in vitro, colony forming assays were performed in the glioblastoma derived cell line U-87. We found that an up to 7-fold increased number of colonies formed in cells which received the functional plasmid replicon proving that the hybrid-vector system is functional. Moreover, transgene expression could be maintained for 6 weeks in vitro and the extrachromosomal plasmid replicon could be rescued 6 weeks post-infection. To show efficacy in vivo, the adenoviral hybrid-vector system was injected into C57Bl/6 mice. Analysis of murine liver revealed that the plasmid replicon can be released from adenoviral DNA in murine liver cells resulting in long-term transgene expression.

In conclusion, we demonstrate efficacy of our novel HCAdV-pEPito hybrid-vector system in vitro and in vivo. This technique may provide a promising tool for efficient delivery and stable maintenance of transgenes without genotoxicity.

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Codon optimization leads to functional impairment of the RD114-TR envelope glycoprotein

Eleonora Zucchelli, Anna Stornaiuolo, Sergio Bossi, Stefano Corna, Claudio Bordignon, GianPaolo Rizzardi, Chiara Bovolenta

MolMed S.p.A., Milano, Italy

We have studied the RD114-TR envelope within a long-term project aimed at developing RD-MolPack packaging cells for stable production of lentiviral vector (LV). In this context, we decided to integrate RD114-TR via self-inactivating (SIN)-LV into RD-MolPack. Surprisingly, RD114-TR could not be expressed by SIN-LV, likely for the presence of instability sequences within its ORF. To solve this problem we tried two strategies. The first consisted in the insertion, between the CMV promoter and the RD114-TR cDNA, of the β-globin intron (β-GI) containing an RRE element (SIN-LV-β-GI-RRE) to prevent splicing arising from the splice donor (SD) of the canonical intron of the vector and the splice acceptor (SA) of the additional intron. The second consisted in codon optimizing the RD114-TR cDNA, RD114-TRco. Remarkably, in contrast to the first fully successful strategy, we found that the second one was a failure. RD114-TRco precursor (PRco) was indeed translated at high level, but weakly processed in the functional surface (SUco) and trans-membrane (TMco) subunits and highly accumulated on the RD114-TRco LV pseudovirions, which consequently showed a negative titer. We believe that silent mutation(s) derived from codon optimization may have caused either abnormal speed of the transcription/translation processes and/or folding of the protein. Therefore, the large amount of the PRco on the pseudovirions prevents the binding of the SUco to the receptor. In alternative or in addition, the SUco is in a wrong conformational state that averts receptor recognition. In conclusion, our studies suggest that RD114-TR is not suitable for recoding to improve its performance.

Additional Abstracts

Speaker Abstracts

INV007

Retroviral and lentiviral vectors have been successfully used for the correction of inherited immune diseases in clinical trials. However, with increasing efficiency and success in clinical retroviral gene therapy, the occurrence of vector-induced severe side effects has dramatically increased. The identification of viral integration sites can deliver insight into the biological consequences of viral gene therapy. High-resolution integration site profiling enables fast and sensitive identification and tracking of individual stem cell-derived clones and has become a prerequisite in oncoretroviral based clinical gene. However, the challenge that remained was the analysis of this vast amount of sequence information. Therefore, we have developed automated bioinformatics analysis tools which rapidly process and annotate vector integration sites. We were able to investigate the clonal situation in multiple preclinical trials and four clinical gene therapy trials as well as in one gene marking study and detected different possible clonal repertoires ranging from subtle and not clinically overt effects to clonal dominance and leukemogenesis.

One decade after a first draft of the human genome by the Human Genome Project the combination of highly sensitive PCR-based techniques with new sophisticated sequencing technologies and an optimized bioinformatics high-throughput integration site analysis pipeline enable us to intensively study in depth the clonal inventory and pharmacokinetics in clinical gene therapy trials.

Braincav Posters

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BRAINCAV: a nonhuman adenovirus vector for gene transfer to the brain

P Alves-Marques[1,2], A Baker[3], A Bosch[8], M Carrondo[1,2], EJ Kremer[5], R Luquin[9], I Saggio[10], J Schwarz[4], G Schiavo[7], JM Verdier[6]

[1]Instituto de Biologia Experimental e Tecnológica, Oeiras, Portugal [2]Instituto de Tecnologia Química e Biológica–Universidade Nova de Lisboa, Portugal [3]British Heart Foundation Glasgow Cardiovascular Research Centre, University of Glasgow, UK [4]Department of Neurology, University of Leipzig, Germany [5]Institut de Génétique Moléculaire de Montpellier, France [6]Universities of Montpellier II, France [7]Molecular NeuroPathobiology Laboratory, Cancer Research UK London Research Institute, UK [8]Center of Animal Biotechnology & Gene Therapy, and Dept of Biochemistry & Mol Biology, Universitat Autònoma de Barcelona, Spain [9]Laboratory of Regenerative Therapy, CIMA and Dept of Neurology, Clínica Universidad de Navarra, Pamplona, Spain [10]Department of Genetics and Molecular Biology, University 'La Sapienza', Rome, Italy

BRAINCAV is an FP7-funded collaborative research project that focuses on the development and assessment of a gene transfer vector to target the brain. As suggested by recent clinical results, brain gene transfer offers substantial potential, yet brings unique obstacles - in particular the need to address feasibility, efficacy and safety. BRAINCAV's foundation is based on the potential of canine adenovirus type 2 (CAV-2) vectors to preferentially transduce neurons, undergo efficient targeting via axonal transport and provide long-term expression. BRAINCAV is a translational project that spans basic research through preclinical model feasibility, efficacy and safety. The BRAINCAV partners developed cell lines & purification schemes, improved production protocols, assayed the transcriptional response of vector-transduced neurons in vitro and in vivo, characterized the mechanism of receptor engagement and axonal transport, and assayed biodistribution and interaction with brain fluids and cells. To provide a proof-of-principle of the effectiveness of CAV-2 vectors, we also tackled mucopolysaccharidosis type VII, a global, orphan disease commonly affecting children, and Parkinson's disease, a focal degeneration of dopaminergic neurones commonly affecting aged population. A collection of BRAINCAV posters describing our latest results will be presented.

For the second consecutive year, a 1500 BRAINCAV prize will be awarded to a selected author of poster for gene transfer to the brain.

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Generation and genetic modification of 3D cultures of human dopaminergic neurons derived from neural progenitor cells

Catarina Brito[1,2], Daniel Simãoa[2], Inês Costa[1,2], Rita Malpiquea[2], Cristina Pereiraa[2], Paulo Fernandes[1,2], Margarida Serraa[2], SC. Schwarz[3], J Schwarz[3], EJ Kremer[4] & Paula M. Alves[1,2]

[1]iBET, Instituto de Biologia Experimental e Tecnológica, Apartado 12, Oeiras, Portugal [2]Instituto de Tecnologia Química e Biológica–Universidade Nova de Lisboa (ITQB–UNL), Avenida da República, Estação Agronómica Nacional, Oeiras, Portugal [3]Department of Neurology, University of Leipzig, Liebigstr. 20, Leipzig, Germany [4]Institut de Génétique Moléculaire de Montpellier, Universities of Montpellier I and II, France

Central nervous system (CNS) disorders remain a formidable challenge for the development of efficient therapies. Cell and gene therapy approaches are promising alternatives that can have a tremendous impact by treating the causes of the disease rather than the symptoms, providing specific targeting and prolonged duration of action. Hampering translation of gene-based therapeutic treatments of neurodegenerative diseases from experimental to clinical gene therapy is the lack of valid and reliable pre-clinical models that can contribute to evaluate feasibility and safety. Herein we describe a robust and reproducible

methodology for the generation of 3D in vitro models of the human CNS following a systematic technological approach based on stirred culture systems. We took advantage of human midbrain-derived neural progenitor cells (hmNPCs) capability to differentiate into the various neural phenotypes and of their commitment to the dopaminergic lineage to generate differentiated neurospheres enriched in dopaminergic neurons. Furthermore, we describe a protocol for efficient gene transfer into differentiated neurospheres using CAV-2 viral vectors and stable expression of the transgene for at least 10 days. CAV-2 vectors, derived from canine adenovirus type 2, are promising tools to understand and treat neurodegenerative diseases, in particular Parkinson s disease. CAV-2 vectors preferentially transduce neurons and have an impressive level of axonal retrograde transport in vivo. Our model provides a practical and versatile in vitro approach to study the CNS in a 3D cellular context. With the successful differentiation and subsequent genetic modification of neurospheres we are increasing the collection of tools available for neuroscience research and contributing for the implementation and widespread utilization of 3D cellular CNS models. These can be applied to study neurodegenerative diseases such as Parkinson s disease; to study the interaction of viral vectors of therapeutic potential within human neural cell populations, thus enabling the introduction of specific therapeutic genes for treatment of CNS pathologies; to study the fate and effect of delivered therapeutic genes; to study toxicological effects. Furthermore these methodologies may be extended to other sources of human neural stem cells, such as human pluripotent stem cells, including patient-derived induced pluripotent stem cells.

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Helper-dependent CAV-2 vector corrects neuronal pathology in a mouse model of mucopolysaccharidosis type VII

Lorena Ariza[1], Aurelie Cubizolle[3], Miguel Chillón[1,2], Nicolas Serratrice[3], EJ Kremer[3], Assumpció Bosch[1]

[1]Centre de Biotecnologia Animal i Teràpia Gènica (CBATEG), Dept. Bioquímica i Biologia Molecular, Universitat Autònoma de Barcelona, [2]Institució Catalana de Recerca i d Estudis Avançats (ICREA), Barcelona, Spain, [3]Institut de Génétique Moléculaire de Montpellier, Université de Montpellier 1 & 2 France

Lysosomal storage disorders (LSD), a group of inherited metabolic diseases, are the most common cause of neurodegeneration in children. Among LSD, Mucopolysaccharidosis (MPS) VII or Sly syndrome, is caused by β-glucuronidase deficiency. This primary defect triggers glycosaminoglycan accumulation into enlarged vesicles in peripheral and CNS tissues resulting in peripheral and neuronal dysfunction and death. No effective treatment is currently available for patients with MPS VII. Gene transfer with viral vectors conferring stable, long-term correction could provide sustained therapy if a sufficient level of enzyme was secreted. Canine adenovirus type 2 vectors (CAV-2) appear particularly promising for CNS gene therapy due to their neuronal tropism and a very efficient long-distance targeting, via axonal transport. In addition, no neutralizing antibodies against CAV-2 vectors seem to be present in human population. To test the therapeutic capacity of CAV-2 vectors, we injected eight-week old MPS VII mice in the striatum with helper-dependent CAV-2 vectors (HD CAV-2) expressing β-glucuronidase. Enzyme activity and vector DNA copies were detected at 1 week but not at 6 weeks post-injection. Immunohistochemistry for Iba1 showed strong microglia activation in the transduced tissue at 1 week, suggesting that β-glucuronidase-expressing cells may be eliminated early after transduction. For that reason, we transiently immunosupressed the animals with cyclophosphamide for 3 days before and 10 days after vector administration. In these animals, β-glucuronidase activity was observed six weeks after injection of HDCAV-2 in neurons and endothelial cells in striatum, cortex and subtantia nigra spanning through 1.8-2.0 mm in the cerebrum from dorsal to caudal. Semi-thin sections showed complete correction of lysosomal accumulation in neurons and endothelial cells and significant reduction in glial cells, both in striatum and cortex. Characterization of more distal CNS areas and long-term expression in MPSVII mice after 4-month injection will be discussed.

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Bioprocess development for canine adenovirus type 2 vectors

Fernandes P[1,2], Peixoto C[1,2], Santiago VM[1,2], Kremer EJ[3], Coroadinha AS[1,2] & Alves PM[1,2]

[1]Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa (ITQB-UNL), Oeiras, Portugal [2]Instituto de Biologia Experimental e Tecnológica (IBET), Oeiras, Portugal [3]Institut de Génétique Moléculaire de Montpellier, Universities of Montpellier I and II, France

Canine adenovirus type 2 (CAV-2) vectors overcome many of the clinical immunogenic concerns related to vectors derived from human adenoviruses (AdVs). In addition, CAV-2 vectors preferentially transduce neurons with an efficient traffic via axons to afferent regions when injected into the brain. To meet the need for preclinical and possibly clinical uses, scalable and robust production processes are required. CAV-2 vectors are currently produced in E1-transcomplementing dog kidney (DK) cells, which might raise obstacles in regulatory approval for clinical grade material production. In this study, a GMP-compliant bioprocess was developed. An MDCK-E1 cell line, developed by our group, was grown in scalable stirred tank bioreactors, using serum-free medium, and used to produce CAV-2 vectors that were afterwards purified using column chromatographic steps. Vectors produced in MDCK-E1 cells were identical to those produced in DK cells as assessed by SDS-PAGE and dynamic light scattering measurements (diameter and Zeta potential). Productivities of ~109 infectious particles (IP) ml-1 and 2×103 IP per cell were possible. A downstream process using technologies transferable to process scales was developed, yielding 63% global recovery. The total particles to IP ratio in the purified product (>20:1) was within the limits specified by the regulatory authorities for AdV vectors. These results constitute a step toward a scalable process for CAV-2 vector production compliant with clinical material specifications.

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Canine adenovirus type-2 (CAV-2) transduction induces two different responses at 24 h and 28 days in the brain of the nonhuman primate Microcebus murinus

Gina Devau[1], Élodie Chazot[1], Nadine Mestre-Francés[1], EJ Kremer[2] & Jean-Michel Verdier[1]

[1]University of Montpellier 2, Montpellier, France, [2]Institut de Génétique Moléculaire de Montpellier, Montpellier, France

Canine adenovirus type-2 (CAV-2) vectors preferentially transduced neurons and undergo efficient axonal transport. We previously showed that CAV-2 survived at least 6 months in the brain of a lemur primate, Microcebus murinus, without inducing notable perturbations in neuron activity and death, suggesting that they could be used to prevent and/or treat numerous neurodegenerative diseases. A hallmark of Parkinson's disease is the loss of dopaminergic neurons, which induces a dysregulation in the striatum and in the frontal cortex producing motor disorders. How brain cells deal with the virion-induced signal transduction during attachment and internalisation is unknown. We therefore assayed changes in gene expression induced by helper-dependent (HD) CAV-2 in the striatum of 4 primate females following injection of HD-CAV-2 (109 pp) into the right caudate nucleus. We compared the gene expression profiles at 24h and 28 days post-infection using transcriptomic approach with human Affymetrix microarrays.

We detected 11,200 transcripts, and analyzed them with statistical tools. About 20 genes were highly discriminating to detect the presence of HD-CAV2 in the striatum. Among them, the most significant changes were found in genes belonging to transcriptional regulation function, to synaptic transmission, and to the immune response. In addition, clustering analysis showed different and specific profiles at 24h and at 28 days. We observed a rather non-specific innate immune response at 24h, which is modified at 28 days towards a more tolerant response, in accordance with our histological data.

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Analysis of human serum interactions with Ad5 and CAV2: a proteomic approach

C Edwards, AC Bradshaw, R Burchmore & AH Baker

[1]*BHF Cardiovascular Research Centre, University of Glasgow, UK* [2]*Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, University of Glasgow, UK*

Understanding the interactions between serum proteins and viral vectors is key to improving the in vivo safety and efficacy profiles of gene therapy vectors. The widely-used human adenovirus type 5 vector has been shown to interact with several serum proteins; however data on the interactions of novel gene therapy vectors with serum proteins remains limited. Moreover, previous studies have used recombinant virus proteins as bait rather than intact virions. This study aimed to elucidate the serum protein interactions of the novel canine vector CAV2, using whole, biotinylated virus to co-precipitate interacting serum proteins in albumin- and IgG-depleted human samples, prior to separation by 1D gel electrophoresis and identification via tandem mass spectroscopy.

Using Ad5 as a positive control, we were able to confidently identify coprecipitated proteins from human serum samples that have previously been reported in the literature, including several complement proteins and transthyretin, a serum hormone-binding protein. Complement C3, complement C4-A and transthyretin also coprecipitated with CAV2. Interestingly, more proteins were confidently identified from CAV2 coprecipitates compared to Ad5, including the heme binding proteins hemopexin and haptoglobin and the serpin protease anti-thrombin III. This may partly be due to the significant structural differences between Ad5 and CAV2 capsids. In summary, we can confirm that the use of biotinylated whole viruses to co-precipitate serum proteins is a viable approach for the identification of virus protein interactions.

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A real-time PCR assay for quantification of canine adenoviral vectors

María Mercedes[1] Seguraa, Mercé Monfar[2], Meritxell Puig[1,2], Franck Mennechet[3], Sandy Ibanes[3] & Miguel Chillón[1,2,4],

[1]*Department of Biochemistry and Molecular Biology, Center of Animal Biotechnology and Gene Therapy (CBATEG), Universitat AutŒnoma de Barcelona, Spain* [2]*Vector Production Unit (UPV), CBATEG, Universitat AutŒnoma de Barcelona, Spain* [3]*Institut de Génétique Moléculaire de Montpellier, CNRS, Universities of Montpellier I & II, France* [4]*Institut Catalá de Recerca i Estudis Avançats (ICREA), Barcelona, Spain*

The pre-existing humoral and cellular immunity found in the great majority of the population raises concerns about the clinical efficacy and safety of vectors derived from ubiquitous human adenovirus serotypes. To alleviate these concerns, canine adenovirus type 2 vectors (CAV-2) were developed. Owing to their extraordinary neuronal tropism and efficient axonal retrograde transport, CAV-2 vectors hold great promise for the treatment of neurodegenerative diseases. The development and validation of a SYBR Green qPCR assay for determination of CAV-2 titers is reported in the present study. This method uses specific primers designed to amplify a small genomic fragment of CAV-2 structural genes (pVI-hexon). The method was accurate and reproducible as determined by the low intra- and inter-assay variability (< 15% R.S.E.). It is sensitive and useful over a 5-log range (1 æ 103 to 1 æ 107 genome copies/reaction). The assay can be used to quantify purified vector samples as well as crude viral lysates. The titers obtained by qPCR correlated well with both, those obtained by OD260 and TCID50 as indicated by the high coefficients of determination obtained by regression analysis ($r2 > 0.83$). The development of this simple and rapid CAV-2 quantitation method should be helpful for process development and monitoring.

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CAR-associated vesicular transport of an adenovirus in motor neuron axons.

Sara Salinas[1,*], Lynsey G. Bilsland[1], Daniel Henaff[2,3], Anne E. Weston[4], Anne Keriel2,3, Giampietro Schiavo1* & EJ Kremer[2,3]

[1]*Molecular NeuroPathobiology Laboratory, Cancer Research UK (CRUK) London Research Institute, UK* [2]*Institut de Génétique Moléculaire de Montpellier, CNRS UMR 5535, Montpellier, France, 3 Universités de Montpellier I & II, Montpellier, France,* [4]*Electron Microscopy Laboratory, CRUK London Research Institute, London, UK*

Axonal transport is responsible for the movement of signals and cargo between nerve termini and cell bodies. Pathogens also exploit this pathway to enter and exit the central nervous system. In this study, we characterised the binding, endocytosis and

axonal transport of an adenovirus (CAV-2) that preferentially infects neurons. Using biochemical, cell biology, genetic, ultrastructural and live-cell imaging approaches, we show that interaction with the neuronal membrane correlates with coxsackievirus and adenovirus receptor (CAR) surface expression, followed by endocytosis involving clathrin. In axons, long-range CAV-2 motility was bidirectional with a bias for retrograde transport in nonacidic Rab7-positive organelles. Unexpectedly, we found that CAR was associated with CAV-2 vesicles that also transported cargo as functionally distinct as tetanus toxin, neurotrophins, and their receptors. These results suggest that a single axonal transport carrier is capable of transporting functionally distinct cargoes that target different membrane compartments in the soma. We propose that CAV-2 transport is dictated by an innate trafficking of CAR, suggesting an unsuspected function for this adhesion protein during neuronal homeostasis.

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Canine adenovirus (CAV-2) vectors induce an innate immune response and the modulation of cell cycle genes in dopaminergic differentiated human midbrain neuronal precursors

Stefania Piersanti[1], Letizia Astrologo[2], Valerio Licursi[1,3], Rodolfo Negri[1,3], Enrica Roncaglia[4], Enrico Tagliafico[4], EJ Kremer[5] & Isabella Saggio[1,3,6]

[1]Department of Biology and Biotechnology "C. Darwin", Sapienza University of Rome, Italy [2]Department of Molecular Medicine, Sapienza University of Rome, Italy [3]Pasteur Institute, Cenci Bolognetti Foundation, Rome, Italy [4]Department of Biomedical Sciences, University of Modena and Reggio Emilia, Modena, Italy [5]Institut de Génétique Moléculaire de Montpellier, UMR 5535, Montpellier, France 6 Institute of Molecular Biology and Pathology, CNR, Rome, Italy

CAV-2 vectors circumvent the ubiquitous human anti-human Adenovirus (hAd) memory immune response, are capable of long term neuron-specific expression (>1year in rat brains), do not induce the maturation of dendritic cells and have been proposed for the treatment of neurodegenerative diseases. In the prospect of clinical applications to brain diseases in humans, we investigated the toxicogenomic profile of helper-dependent (HD) CAV-2 vectors in human midbrain precursors differentiated into dopaminergic neurons. We transduced the cultures with HD CAV-2 and, for comparison, with third generation SIN lentiviral vectors (LV) and HD hAd. We evaluated gene modulation by Affymetrix gene chip at 2h and 5 days post transduction. Our analyses of the chip-contained 47,000 transcripts showed that, at the moi of 1000 genomes per cell, HD CAV-2 exhibited a specific modulation profile. It induced genes belonging to the cell cycle, DNA recombination and repair pathways, including p53, RAD51, BIRC5, FANCD2 and MAD2L1. It also up regulated genes involved in the immune response and in inflammation, including TL3 and 4, HAS3 and CD44, and genes involved in neuron projection morphogenesis. HD hAd was less efficient in transduction than HD CAV-2 and its effect on the trascriptome was milder. However, at 2h, it did have an impact on neuron remodeling and trafficking genes. LV transduced very efficiently, had a strong effect on the trascriptome, which overlapped with that of HD CAV-2 for TLR activation, and diverged in specific aspects of the immune and DNA repair gene groups. Single gene and pathway modulation data emerged from our analysis constitute useful information for toxicity prediction, vector comparison and evolution and virus-host interaction studies.

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Widespread and long-term expression in the primate CNS using helper-dependent canine adenovirus vectors

Nicolas Serratrice[1,2,3], Nadine Mestre-Francés[3,4,5], Sara Salinas[1,2,3], Neus Bayo-Puxan[1,2,3], Thierry Bru[1,2,3], Charleine Zussy[1,2,3], Franck Mennechet[1,2,3], Sandy Ibanes[1,2,3], Aurelie Cubizolle[1,2,3], Matthieu Perreau[1,2,3], Vasiliki Kalatzis[1,2,3], Jean-Michel Verdier[3,4,5] & EJ Kremer[1,2,3]

[1]Institut de Génétique Moléculaire de Montpellier, France, [2]Université de Montpellier [1], Montpellier, France [3]Université Montpellier [2], France, [4]Inserm U710, Montpellier, France, [5]École Pratique des Hautes Études, Paris, France

In the primate central nervous system (CNS), efficient, specific, widespread and long-term transduction of neurons with gene transfer vectors is challenging. In this study, we combined helper-dependent canine adenovirus type 2 (CAV-2) vectors and a novel nonhuman primate, Microcebus murinus, to address efficacy of gene transfer to the CNS. We found that injections of helper-dependent CAV-2 vectors into the striatum resulted in neuron-specific expression at the injection site and in numerous regions and nuclei that harbour neurons that project into the striatum. Transgene expression was stable for at least 6 months, the longest duration assayed, even in animals that harboured memory immunity to adenoviruses. Transgene expression correlated with the quantification of vector genomes in each region, which is consistent with retrograde transport of vectors into afferent structures. Moreover, injection of CAV-2 vectors into the hindleg muscles also led to preferential, efficient (up to 75%) and long-lasting (at least 6 months) transduction of the innervating motor neurons in the anterior horn of the spinal cord. To address the molecular basis of CAV-2 tropism, we show that retrograde transport and preferential neuronal transduction is consistent with coxsackie and adenovirus receptor expression at synapses in the CNS and neuromuscular junctions. Our model and results have notable fundamental and clinical implications for safety and efficacy of genetic modification of the CNS, in particular in the context of modelling, preventing and treating neurodegenerative diseases in long-lived primates.

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Helper-dependent CAV-2 vector injection into the CNS of mucopolysaccharidosis type VII dogs

Aurelie Cubizolle[1], Nicolas Serratrice[1], Aurelie Gennetier[1], Sandy Ibanes[1], Mark E Haskins[2], EJ Kremer[1]

[1]Institut de Génétique Moléculaire de Montpellier, Université de Montpellier 1 & 2, France [2]Department of Pathobiology, Department of Clinical Studies, School of Veterinary Medicine, University of Pennsylvania, Pennsylvania, USA

Our goals are to develop and test the clinical relevance of helper-dependent (HD) CAV-2 vectors to treat neural degeneration caused by mucopolysaccharidosis type VII (MPS VII) in a dog model. MPS VII is caused by deficiency Ô-D-glucuronidase

activity, which is responsible for the physiological turnover of macromolecules. It hydrolysis Ô-D-glucuronic acid residues present on glycosaminoglycans (GAGs) (heparan sulfate, dermatan sulfate and chondroÿtin). Ô-D-glucuronidase deficiency causes accumulation of undegraded GAGs inside vesicles, thought to be lysosomes, leading to cell death via an unknown mechanism. The neuronal degeneration caused by the majority of LSD makes them candidates for gene therapy. Because CAV-2 preferentially transduces neurons and traffics via axons, the distribution of the transgene throughout the CNS will allow widespread delivery of the missing lysosomal enzyme with a minimum number of injections. We tested a HD-CAV-2 vector in the canine model of MPS VII for their safety, efficacy and duration of expression. We produce a HD-CAV-2 vector expressing human GUSB sequence via an RSV promoter followed by an IRES-EGFP expression cassette at a titre of 1 æ 1012 pp/ml. Injections of HD- HD-CAV-2 vector expressing GFP or GUSB-GFP were in the CNS of 12 healthy or MPS VII dogs. HD-CAV-2 vector expressing EGFP in MPS VII and healthy dogs showed a minimal induction of the immune response, an efficient transduction of the neurons and an efficient biodistribution of transduced cells. Injections of HD-CAV-2-GUSB vector showed after 1 month or 4 months post injections an increase in general level of GUSB activity, especially in the sites of injections, in areas closed to the sites of injections and in the contralateral striatum and frontal and parietal cortex. Analysis of GFP level by qPCR, also permit to observe a widespread biodistribution of the vector. The healthy and MPS VII dogs did not present humoral immune response at the day of injection or at sacrifice. Together or data demaonstrate the potential of HD-CAV-2 in the CNS of large animals.

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Production of helper-dependent canine adenovirus type 2 (CAV-2) vectors for gene therapy: Impact of transcomplementing gene products on producer cell-line

P. Fernandes[1,2], E.J. Kremer[3], A.S. Coroadinha[1,2] & P.M. Alves[1,2]

[1]Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa (ITQB-UNL), Oeiras, Portugal [2]Instituto de Biologia Experimental e Tecnológica (IBET), Oeiras, Portugal [3]Institut de Génétique Moléculaire de Montpellier, Universities of Montpellier I and II, France

The manipulation of adenovirus vectors (AdV) has been extensively studied. However, modest attention is paid to the producer cell lines and the expression of their transcomplementing gene products. All AdV require for their manufacturing and replication a cell-line that expresses the adenoviral E1 functions in trans. Furthermore, helper-dependent AdV also require a recombinase expression, such as Cre-recombinase, by the transcomplementing cell-line. However, Cre is reported to induce growth inhibition and apoptosis due to DNA damage. Previously, we had show that expression of E1 had a crucial role on CAV-2 vectors production, namely on adenoviral replication and cell viability by the influence of E1A and E1B, respectively.

In this work, the impact of Cre on MDCK-E1-Cre cells and production of helper-dependent CAV-2 vectors was evaluated, by analysing several MDCK-E1-Cre cell-clones with different Cre levels. MDCK cells were selected since this is the only canine cell-line approved by regulatory agencies to manufacture biopharmaceuticals. Cell-culture (cell growth, metabolism and physiological state under oxidative stress conditions) and viral production (CAV-2 productivity and excision of helper vectors) features were evaluated in different MDCK-E1-Cre cell-clones.

Although Cre impaired cell growth, increasing susceptibility to oxidative stress injury, the typical productivity of CAV-2 vectors was maintained in MDCK-E1-Cre cells. Moreover, these cells efficiently prevented helper vector replication, with productivities of helper-dependent CAV-2 vectors similar to those obtained with human AdV.

These results demonstrate that MDCK-E1-Cre is a robust cell-line for the production of helper-dependent CAV-2. Future work aims at scaling-up the production process using stirred tank bioreactors to debottleneck HDV availability for preclinical and clinical assays.

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Chromatography purification of canine adenoviral vectors

Segura MM, Puig M, Monfar M & Chillón M

Center of Animal Biotechnology and Gene Therapy (CBATEG), Universitat Autónoma de Barcelona, Spain

Abstract Canine adenovirus vectors (CAV2) are currently being evaluated for gene therapy, oncolytic virotherapy, and as vectors for recombinant vaccines. Despite the need for increasing volumes of purified CAV2 preparations for preclinical and clinical testing, their purification still relies on the use of conventional, scale-limited CsCl ultracentrifugation techniques. A complete downstream processing strategy for CAV2 vectors based on membrane filtration and chromatography is reported here. Microfiltration and ultra/diafiltration are selected for clarification and concentration of crude viral stocks containing both intracellular and extracellular CAV2 particles. A DNase digestion step is introduced between ultrafiltration and diafiltration operations. At these early stages, concentration of vector stocks with good recovery of viral particles (above 80%) and removal of a substantial amount of protein and nucleic acid contaminants is achieved. The ability of various chromatography techniques to isolate CAV2 particles was evaluated. Hydrophobic interaction chromatography using a Fractogel propyl tentacle resin was selected as a first chromatography step, because it allows removal of the bulk of contaminating proteins with high CAV2 yields (88%). An anion-exchange chromatography step using monolithic supports is further introduced to remove the remaining contaminants with good recovery of CAV2 particles (58-69%). The main CAV2 viral structural components are visualized in purified preparations by electrophoresis analyses. Purified vector stocks contained intact icosahedral viral particles, low contamination with empty viral capsids (10%), and an acceptable total-to-infectious particle ratio (below 30). The downstream processing strategy that was developed allows preparation of large volumes of high-quality CAV2 stocks.

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