SUOMEN GEENITERAPIASEURA
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Invited Speakers

INV020

Gene Therapy – Current Status and Future Direction

H Büning [1 2 3]

1: Institute of Experimental Hematology, Hannover Medical School, Hannover 2: German Center for Infection Research (DZIF), partner site Hannover-Braunschweig 3: Center for Molecular Medicine (CMMC), University of Cologne, Cologne

The first gene therapy commenced a quarter of a century ago, when a functional copy of adenosine deaminase (ADA) gene was introduced - employing a retroviral vector as delivery tool - into peripheral blood T lymphocytes of patients with ADA deficiency, a severe combined immune deficiency syndrome (SCID). Years of tremendous efforts in developing better vectors, in improving vector production and primary cell culture protocols, and in basic and translational research followed before clear signs of therapeutic efficacy could be obtained. Studies showing promise covered different monogenetic diseases as well as different forms of cancer and used a variety of vectors and delivery strategies. Fostered by the increasing numbers of reports on clinical benefit obtained for patients with detrimental inherited or acquired diseases, gene therapy strategies are now also explored for the treatment of chronic or progressive and of infection diseases. Here, we will highlight some of the most recent achievements and discuss recent technological improvements, but also remaining challenges.

INV021

Considerations and options for use as a vector for gene therapy

A H Baker [1]

1: University of Glasgow

Adenovirus has been used very broadly, both experimentally and in clinical studies. However, progress in clinical studies has been limited. There are many considerations for attempt to overcome some of the obstacles that have hampered the effective use of this vector. First, many studies have focused on the group c adenovirus serotype 5 vector. While this has many attributes that make it attractive, it has a number of serious limitations, including tropism for the liver, toxicity and poor tropism for many cell types. The adenovirus family, both human and non-human holds tremendous potential for the use of alternative vectors. Some of these, for example adenovirus 35, have been developed as more effective vectors. Second, adenovirus is a complex virus and there are many interactions of the virus with the host that dictate tropism and also toxicity. It has only been in recent years that these interactions have begun to be understood more fully. This talk will cover the above topics and illustrate the basic and applied biology relating to the use of adenovirus-based vectors for application to gene therapy.

INV022

Update on the use of Adenovirus for immunotherapy

V Cerullo [1]

1: Centre for Drug Research (CDR) and Division of Pharmaceutical Biosciences Faculty of Pharmacy University of Helsinki

Adenovirus is one of the most used viruses for gene therapy purposes, at the moment accounting for the 22.2% of all gene therapy clinical trials. Adenovirus is however mostly used for gene transfer (in particular its Helper-dependent version, HDAd), for vaccine development (mainly in its E1 deleted version known as First Generation Vector, FGAd) and finally for cancer treatment (in its oncolytic version, OAd).
Compared to other viruses adenovirus has a peculiar capability to interact with a plethora of Pattern Recognition Receptors (PRRs) triggering a potent and characteristic immune response. This feature renders this virus an excellent candidate for virus-based immunotherapy development.
In this session I will illustrate how historically the concept of oncolytic virus has evolved into oncolytic vaccine and how this has impacted the design of the on-going clinical trials. I will illustrate recent development of this vectors and recent studies highlighting these novel aspects of this virus and some future needs and direction to improve the design of the vector even further.

INV023

Oligonucleotide Therapies

C I E Smith [1]

1: Karolinska Institutet, Karolinska University Hospital Huddinge, Stockholm

Over the last few years, oligonucleotides (ONs) have been established as therapeutic agents with great potential. In this educational session I will briefly describe the development of ON medicines up until today. The various biological processes that have been targeted will be presented. Most ON therapies act through antisense mechanisms being directed against various RNA species, as exemplified by gapmers, steric block ONs, antagomirs, small interfering RNAs (siRNAs), micro-RNA mimics, and splice switching ONs. The beauty with these drugs is the conceptual simplicity in which they can be generated as compared to other forms of medicines. In contrast to antisense ONs, those binding to Toll-like receptors and those forming aptamers have completely different modes of action. Similar to other novel medicines, the path to success

has been lined with failures, where various therapeutic ONs did not perform as expected. However, from an educational point of view, this may be of great interest, since you may learn as much, or, often, even more, from failures than from successes. While ON drugs are being increasingly used in the clinic, many challenges remain until the expectations for this new form of medicine are fully met.

INV024

The challenges in large scale viral vector production

H P Lesch [1 2 3]

1: FinVector Vision Therapies Ltd 2: FKD Therapies 3: A.I.Virtanen Institute

A broad spectrum of gene therapy applications show potential for future development and commercialization. However, clinical scale vector manufacturing for phase III and beyond has proven to be challenging. Upscaling the process with suspension cells in stirred tanks in serum-free conditions is increasingly feasible. Modern bioreactors are disposable with perfusion capability and automated control of stirring, temperature, pH and dissolved oxygen. However, cellular shear stress is a common problem associated with large scale systems. Not all cells can be efficiently grown in suspension, and in addition, some cells in adherent conditions have shown higher specific vector productivity. Typically, lentivirus or AAV manufacturing have still relied on flask type two dimensional approaches such as Cell Factories. Upscaled production in flasks is limited by the production space required, it is impractical to handle and difficult to monitor/ control culture conditions. The option for adherent cell systems is the use of packed-bed bioreactors, which have provided three dimensional controlled, perfusable systems with low shear stress for the cells. Sampling of the cells and cost, especially if serum is used, are drawbacks with these systems. Microcarriers have also been used, dispersed in suspension but proven to be challenging to handle with homogenous growth. The limitation has been the expansion of large cell mass on the static vessels with limited scalability. Finally, the scalable down-stream processing to remove the contaminants from the harvested material while preserving the functionality of the vector and ensuring the quality are important key factors for successful large scale manufacturing.

INV025

Tracking vector safety in gene therapy

M Schmidt [1]

1: DKFZ / NCT Heidelberg

Viral vectors have shown their efficiency in clinical studies of rare diseases. However, also vector-mediated insertional side effects and malignant transformation occurred in few patients. These observations prompted new vector designs, such as self-inactivating (SIN) LTR configurations and tissue specific promoters that promise safe and efficient gene-correction in patients. Now, with the advent of mammalian whole genome sequence availability and next generation sequencing (NGS), large-scale identification of vector integration profiles and vector persistence studies over time became an essential part of pharmacokinetics studies for a gene therapeutic drug. Our current (linear amplification-mediated, LAM) PCR coupled to NGS strategy to identify vector integrations is continuously further extended. Here, the current state-of-the-art and future perspectives will be presented, which includes new technologies like capture based quantitative sequencing of viral vector sequences and integration sites as well as associated bioinformatical data mining tool suites, will be presented.

INV026

A mouse is not a pig is not a human – Choosing a relevant model to mimic human diseases

J Huusko [1]

1: A.I.Virtanen Institute

A laboratory mouse is the most widely used mammal species in biomedical research. It is usually the first choice animal model in preclinical studies for its relatively short life span, low cost and ease of genetic manipulation. Mouse studies have given and continue to give important data for wide variety of diseases but in many cases, and especially in more advanced studies approaching clinical trials, it is compulsory to use also large animal models. The choice of the species and model used has a major role in getting valid and accurate data on the research topic. Natural characteristics of the species in question have an effect on the response to the disease modelling as well as on the response to treatments. Thus, these characteristics should be taken into account when making the choice of the animal model.

INV027

Update of Gene and Cell Therapy of Chronic Diseases Cardiovascular VEGF gene therapy and therapeutic angiogeneisis

M Hedman [1 2] J E Lähteenvuo [1] J Nurro [1] J Hartikainen [1 2] S Ylä-Herttuala [1]

1: University of Eastern Finland 2: Kuopio University Hospital

Cardiovascular diseases are largely manifested as tissue ischemia in the areas where adequate blood supply is compromised due to atherosclerotic arteries. Therefore, promotion of new blood vessels in the ischemic tissues, called therapeutic angiogenesis, is an attractive approach for the treatment of cardiovascular diseases. Vascular endothelial growth factors (VEGFs) have been used among others for therapeutic applications. Therapeutic genes are introduced into tissues with help of viral vectors. Adenoviruses are the most widely used and best studied vectors for cardiovascular gene therapy. Our studies have focused on cardiovascular VEGF gene therapy in both preclinical animal models and in clinical trials. In preclinical models the safety and toxicology of adenoviral VEGF gene therapy has shown to be good both in cardiac and peripheral settings of induced ischemia. Also,

increased regional perfusion, improved exercise tolerance, improved myocardial function and protection against ischemic damage have been shown in animal models. During the last 15 years we have conducted four phase I/II trials with adenoviral VEGF-A and one phase I/II trial with adenoviral VEGF-D. The safety and feasibility of intracoronary and intramyocardial gene injection as well as intramuscular injections into peripheral muscles have shown to be excellent in these studies. Furthermore, in patients with coronary heart disease, some positive results have been achieved regarding myocardial perfusion parameters, even though no changes in cardiovascular morbidity and mortality were observed. In the light of these studies it seems that development of better preclinical animal models more accurately mimicking chronic ischemia and its diverse manifestations in humans would help to clarify the full potential of cardiovascular VEGF gene therapy in complexed diseases. Furthermore, larger patient populations with less severe manifestation of the disease and more carefully selected surrogate endpoints based more on modernimaging techniques and metabolic analyses should be used for clinical testing. Hedman M et al. Circulation 2003;107:2677-83 Lähteenvuo JE et al. Circulation 2009;119:845-56 Muona K et al. Gene Ther 2012;19:392-95

INV028

Update on gene and cell therapy of glioblastoma

H Miletic [1 2]

1: Department of Biomedicine, University of Bergen, Norway
2: Department of Pathology, Haukeland University Hospital, Bergen, Norway

Glioblastoma is the most frequent and most malignant primary brain tumor. Despite substantial advances in the understanding of the molecular mechanisms of this disease, newly developed therapeutic strategies including gene therapy have largely failed in the clinic. One of the main reasons for failure of targeted therapies is attributed to tumor heterogeneity. In contrast, gene therapy using suicide genes is not a targeted approach and reasons for the failure are most likely due to low transduction efficacies of tumor cells. This accounts in particular for replication defective retroviral vectors which were the first vectors tested in clinical trials. Since then more efficient viral vectors have been developed and have shown promising results in animal models. Adenoviral vectors and replication competent retroviruses are currently under evaluation in clinical trials. Furthermore, neural and mesenchymal stem cells are frequently used as delivery vehicles for therapeutic genes into tumors and have entered clinical trials as well. Recent developments in the field of gene and cell therapy of glioblastoma, both in the experimental and clinical phase will be presented and discussed.

INV029

Immunostimulatory Gene Therapy

A Loskog [1]

1: Dept of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University

Immunotherapy is gaining ground in the field of oncology and novel therapeutics such as checkpoint blockade antibodies are approved for melanoma and soon for various cancers. Chronic inflammation and regulatory immune cells promote cancer development and progression at the same time hampering effector cells such as cytotoxic T lymphocytes (CTLs) that specifically target and kill cancer cells. The suppressive tumor microenvironment utilizes a plethora of cells and molecules to combat the immune system. By utilizing gene therapy the tumor microenvironment can be modified to instead promote anti-tumor immune responses either by transferring immunostimulatory genes into the tumor or by transferring genes that interfere with regulatory pathways. Commonly, replication defective viruses have been used to transfer genes into the tumor. However, to increase transgene expression, immune activation and release of tumor antigens oncolytic viruses are now used as gene transfer vehicles. Such engineered oncolytic viruses are now approaching the market as novel cancer therapeutics. Most oncolytic viruses transfers GM-CSF but other molecules are in pipeline. We have focused on CD40 ligand (CD40L) gene therapy. CD40L is a multi-potent molecule that stimulates antigen-presenting cells and induces CTL expansion at the same time it reduces T regulatory cells and differentiates M2 macrophages into M1. CD40L can also activate the endothelium to enhance lymphocyte migration. In mice, dog patients and in human clinical trials we have shown the potent effects of adenovirus-mediated CD40L gene transfer. CD40L can also be combined with other stimulators to further optimize the immune responses against cancer. Immunostimulating gene therapy by utilizing oncolytic viruses is a very interesting approach to combat cancer.

INV030

Gene Therapy for Rare Diseases

A Biffi [1]

1: HSR TIGET, San Raffaele Telethon Institute for Gene Therapy

No abstract available.

INV031

Nanoscopy with focused light

S W Hell [1]

1: Max Planck Institute for Biophysical Chemistry

Throughout the 20th century it has been widely accepted that, at the end of the day, a light microscope relying on conventional lenses (far-field optics) cannot discern details that are finer than about half the wavelength of light (> 200

nm). However, in the 1990s, it was discovered that overcoming the diffraction barrier is realistic and that fluorescent samples can be resolved virtually down to molecular dimensions. Here we discuss the simple yet powerful principles that allow neutralizing the resolution-limiting role of far-field optical diffraction1,2. In a nutshell, features residing closer than the diffraction barrier are prepared in different molecular (quantum) states so that they are distinguishable for a brief detection period. As a result, the resolution-limiting role of diffraction is overcome, and the interior of transparent samples, such as living cells and tissues can now be imaged non-invasively at the nanoscale using focused light in 3D. Besides discussing basic principles, we will show recent advancements. In particular, we demonstrate massive parallelization of RESOLFT and STED recording using simple patterns of light, by more than 100,000 fold3. Likewise, we demonstrate the relevance of emerging 'far-field optical nanoscopy' to various areas, especially to the life sciences. 1. Hell, S.W. Far-Field Optical Nanoscopy. Science 316, 1153-1158 (2007). 2. Hell, S.W. Microscopy and its focal switch. Nature Methods 6, 24-32 (2009). 3. Chmyrov, A. et al. Nanoscopy with more than 100,000 'doughnuts'. Nature Methods (2013).

INV032

CAR therapy: the CD19 paradigm

M Sadelain [1]

1: Center for Cell Engineering Memorial Sloan Kettering Cancer Center New York, NY 10065

The genetic engineering of T cells provides a means to rapidly generate anti-tumor T cells for any cancer patient. This approach is predicated on gene transfer technology that enables the expression of receptors for antigen and other gene products in primary T cells. Tumor targeting may be achieved through the transfer of a physiological receptor for antigen, which is known as the T cell receptor (TCR), or synthetic fusion receptors, which we grouped under the general term of chimeric antigen receptor (CAR). CARs are recombinant receptors for antigen, which, in a single molecule, redirect T cell specificity and eventually enhance anti-tumor potency. Functional augmentation is achieved through the design of second generation CARs, which not only redirect cytotoxicity, but also reprogram T cell function and longevity through their costimulatory properties. The combined activating and costimulatory domains incorporated in second-generation CARs critically determine the function, differentiation, metabolism and persistence of engineered T cells. CD19 CARs that incorporate CD28 or 4-1BB signalling domains are the best known to date. Two decades ago, we selected CD19 as the prime target for developing our CAR technology and provided the first proof-of-principle that CD19-targeted human peripheral blood T cells could eradicate a broad range of B cell malignancies in immunodeficient mice (Brentjens RJ, Riviere I, et al, Nat Med, 2003). CD19 has since become the poster child for CAR therapies. Complete remissions have been reported from several centers in patients with non-Hodgkin lymphoma, chronic lymphocytic leukemia and, most dramatically, acute lymphoblastic leukemia. Two types of second generation CARs, utilizing either CD28 or 4-1BB as their costimulatory signaling components, have been used in ALL patients. Both have yielded dramatic outcomes, in adults as well as in children. Our data indicate that CD28-based CARs direct a brisk proliferative response and boost effector functions, while 4-1BB-based CARs direct a gradual T cell accumulation that may eventually overcome lesser functional potency. These distinct kinetic features can be exploited to further develop CAR T cell therapies for a variety of cancers. To this end, we have now modeled CD19 CAR therapy for ALL in a "stress test", wherein we purposefully lower the infused T cell doses to challenge the CAR therapy. We have compared novel CAR designs intended to recruit both CD28 and 4-1BB signaling. These quantitative analyses reveal striking disparities that hinge on subtle variations in the structural design of CARs and co-expressed costimulatory molecules. Remarkably, we find that some of the most effective engineering strategies activate and sustain the recruitment of the IFNβ pathway through the induction of IRF7, while lowering the induction of exhaustion markers relative to second generation CARs activating either CD28 or 4-1BB alone. These studies point to future CAR designs that may supersede those currently in use for B cell malignancies

INV033

Cancer therapy

Z Eshhar [1]

1: Weizmann Institute of Science, Rehobot

No abstract available.

INV034

CAR T cells for B cell lymphoma and leukemia– the Swedish experience

A Loskog [1]

1: Uppsala University

Chimeric antigen receptor (CAR) T cells recognize and kill tumor cells while simultaneously receiving activation and survival signaling via the CAR receptor. It consists of two basic domains, an antigen-recognizing domain and a downstream signaling domain. The antigen-recognizing domain is commonly a single chain antibody targeting a cell surface antigen on the tumor and the signaling domain is a fusion of the CD3 zeta chain and costimulatory molecules such as CD28, or 4-1BB. Hence, binding of CAR to the tumor will mimic the most important aspects of T cell interaction with antigen-presenting cells. CAR stimulation maintains T cell activation and promotes proliferation as well as survival, factors important to endure in the immunosuppressive milieu in cancer patients. CD19-targeting CAR T cell therapy for B cell malignancy has shown remarkable results, especially in leukemia, while lymphomas are more difficult to treat. In Sweden, we are evaluating the safety and efficacy of third generation CD19-targeting CAR T cells (CD28-41BB-zeta) in an ongoing clinical trial aiming to define obstacles to treat lymphoma and to find clues how to overcome these obstacles.

Different conditioning strategies are used to reduce tumor burden and suppressive immune cells prior CAR T cell therapy and all patients are evaluated for immune escape mechanisms and other biological fingerprints before and at different time points post treatment. The level of immunosuppression and other findings are being correlated to clinical responses and to how they respond to different types of conditioning.

INV035

Cell encapsulation technology for the treatment of malignant brain tumors

R Bjerkvig [1]

1: KG-Jebsen Brain Tumour Research Centre, Department of Biomedicine, University of Bergen, Norway NorLux Neuro-Oncology, Department of Oncology, Luxembourg Institute of Health

Malignant brain tumors (glioblastomas) have a very poor prognosis with an overall patient survival of 15 months. The tumors represent a local disease within the central nervous system (CNS), and are characterized by extensive angiogenesis and extensive tumor cell infiltration into the brain parenchyma. Although, the blood brain barrier (BBB) is disrupted in the tumor core, a large proportion of the tumor cells reside in brain areas with an intact BBB, which hampers the delivery of therapeutic compounds by systemic therapy. We pioneered the work on alginate microencapsulation of therapeutic cells that by local implantation, provides a site-specific delivery system of therapeutic compounds to the CNS. This technology provides a long-term release of therapeutic compounds at the tumor site, and reduces potential side effects associated by systemic delivery. In the presented work, different cell encapsulation options will be presented as well as proof-of principle in a preclinical therapeutic context.

INV036

Clinical development of oncolytic HSV-1 G47Δ

T Todo [1]

1: The University of Tokyo

Genetically engineered, conditionally replicating herpes simplex viruses type 1 (HSV-1) are promising therapeutic agents for cancer. We have developed a triple-mutated, third-generation oncolytic HSV-1, G47Δ, that exhibits enhanced replication capability in a variety of cancer, efficient induction of specific antitumor immunity, and high safety features. G47Δ also kills cancer stem cells very efficiently. The first-in-man clinical trial started in 2009 which was a phase I-IIa study in patients with recurrent glioblastoma. G47Δ was administered stereotactically into the tumor, twice within 14 days. The treatment was well tolerated by patients. Clinical observations in some patients hinted the efficacy of G47Δfor glioblastoma. The long-term efficacy seemed to have been caused by the induction of specific antitumor immunity rather than the direct oncolytic activity of G47Δ. This first trial taught us that conventional methods for efficacy evaluation such as RECIST and WHO criteria may not be adequate for oncolytic virus therapy. Because the extent of viral replication and the strength of immune responses vary considerably among patients, a preferred means of treatment using oncolytic HSV-1 may be repeating local injections until the cancer is cured. Because direct inoculation of G47Δ into human brains has proven safe, a phase II clinical trial in patients with recurrent or residual glioblastoma has recently started to test the efficacy. We aim that oncolytic virus therapy using G47Δ become a standard therapeutic option for brain tumors as well as for other cancer patients in the near future.

INV037

New insights into the adenovirus and interactions with the host

A H Baker [1]

1: University of Glasgow

At present, vectors based on human adenovirus 5 are the most preferred vector in ongoing gene therapy clinical trials. However, some major concerns about their use have been highlighted. Namely, a reported high vector-associated toxicity and widespread pre-existing immunity. Both aspects contribute to limit their effectiveness and hamper their clinical use. Nonetheless, adenovirus-based vectors yet pose several advantageous properties that make them promising therapeutic candidates. A better understanding of vector–host interactions is crucial in the search of strategies to prevent detrimental host interactions and create improved vectors for human gene therapy.

INV038

Angiogenic Gene Therapy

S Yla-Herttuala [1 2]

1: A.I.Virtanen Institute, University of Eastern Finland, Kuopio 2: Gene Therapy Unit and Science Service Center, Kuopio University Hospital, Kuopio

Therapeutic angiogenesis is a potentially useful therapeutic strategy for ischemic heart disease and peripheral arterial occlusive disease. It involves generation of new capillaries, collateral vessels or both in ischaemic muscles using either recombinant growth factors or their genes. Arteriogenesis is a process caused by increased shear stress at the arteriolar level resulting in the formation of large conduit vessels from preexisting small vessels. Most commonly used growth factors for therapeutic angiogenesis are members of the vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) families. Some other cytokines and growth factors can also have angiogenic effects. Improved perfusion and functional parameters can be achieved by angiogenesis and arteriogenesis in large animal chronic ischemia models and in man. Safety of clinical gene therapy of cardiovascular diseases has been excellent with long-term follow-up up to 10 yrs after the therapy. Small non-coding RNAs can also be used for angiogenic gene therapy. Most promising results have so far been obtained with direct catheter-based intramyocardial

injections of VEGF-D genes with adenovirus and AAV vectors. Results from a recent phase II clinical trial with adenovirus-based catheter-mediated intramyocardial VEGF-D gene therapy in refractory angina patents will also be discussed. References: Ylä-Herttuala, S., Rissanen, T.T., Vajanto, I., Hartikainen, J. Vascular endothelial growth factors. Biology and current status of clinical applications in cardiovascular medicine. J. Am. Coll. Cardiol. 49: 1015-1026, 2007. Muona, K., Mäkinen, K., Hedman, M., Manninen, H., Ylä-Herttuala, S. 10-year safety follow-up in patients with local VEGF gene transfer to ischemic lower limb. Gene Ther. 19; 392-395, 2012. Ylä-Herttuala, S. Cardiovascular gene therapy with vascular endothelial growth factors. Gene 525; 217-219, 2013.

INV039

Gene Therapy & Genome Editing for Heart Failure

R J Hajjar [1]

1: Cardiovascular Research Center, Icahn School of Medicine at Mount Sinai School, New York

Congestive heart failure remains a progressive disease with a desperate need for innovative therapies to reverse the course of ventricular dysfunction. A number of genetic mutations have also been associated with cardiomyopathies resulting in heart failure. Over the last ten years, we have undertaken a program of targeting important calcium cycling proteins in experimental models of heart by somatic gene transfer. This has led to the completion of a first-in-man phase 1 clinical trial of gene therapy for heart failure using adeno-associated vector (AAV) type 1 carrying the cardiac isoform of the Sarcoplasmic reticulum Ca2+ ATPase pump (SERCA2a). The safety profile of AAV gene therapy along with the positive biological signals and clinical outcomes were observed in phase 1 and phase 2a trials of AAV1.SERCA2a in NYHA class III/IV patients. A large (250 patients) phase 2b, multinational, double-blind, placebo-controlled, randomized study of adult patients with stable NYHA class II-IV ischemic or non-ischemic HF did not show any cinical improvements in patients receiving AAV1.SERCA2a at a dose of 1x1013 viral genomes. A mutation in the coding region of the phospholamban (PLN) gene (R14del) has been identified in families with hereditary heart failure. Heterozygous patients exhibit left ventricular dilation and ventricular arrhythmias. We derived induced pluripotent stem cells (iPSCs) from a patient harboring the PLN R14del mutation, and differentiated the iPSCs into cardiomyocytes (iPSC-CMs). We found that the PLN R14del mutation induced Ca2+ handling abnormalities, electrical instability, abnormal cytoplasmic distribution of PLN protein, and increased expression of molecular markers of cardiac hypertrophy in iPSC-CMs. Gene correction using transcription activator-like effector nucleases (TALENs) ameliorates the R14del-associated disease phenotypes in iPSC-CMs. In addition, we show that knocking down the endogenous PLN and simultaneously expressing a codon-optimized PLN gene reverses the disease phenotype in vitro. Our findings offer potentially novel strategies of targeting these pathogenic mutations associated with cardiomyopathies.

INV040

Genomic dark matter: the expanded RNA world and therapeutic targeting of non-coding RNAs

K V Morris [1 2]

1: Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla CA, 92037 USA 2: School of Biotechnology and Biomedical Sciences, University of New South Wales, Sydney NSW, 2052 Australia

Observations over the past decade have demonstrated that exogenously introduced non-coding RNAs can transcriptionally modulate gene expression in human cells by recruiting silent state epigenetic marks to target loci. We present evidence here suggesting that in human cells long non-coding RNAs (lncRNAs), which are antisense to particular protein-coding genes, function in human cells as effector molecules driving endogenous epigenetic silencing pathways. These lncRNAs, can emanate in trans from pseudogenes or cis, and antisense to their protein-coding counterparts, and guide epigenetic remodeling complexes consisting of Enhancer of Zeste (Ezh2) and DNA methyltransferase 3a (DNMT3a) to target loci. Notably, when these regulatory lncRNAs are repressed the result can be a concomitant activation of their protein-coding counter parts expression as a result of derepression of the lncRNA-targeted locus. We present several examples of antisense lncRNA directed regulation of gene expression from various diseases model systems ranging from Cancer to HIV to Cystic Fibrosis. We also present an example of an innovative genetic therapy for HIV that utilizes the endogenous lncRNA pathway. Collectively, the data presented here offers a distinctly different picture for gene regulation than has previously been appreciated. Notably, we find that an underappreciated RNA directed mechanism of action is operative in human cells that can be taken advantage of to either transcriptionally silence a genes expression in a long-term manner or activate a genes transcription by the targeted degradation of regulatory antisense lncRNAs.

INV041

Transcriptional Gene Silencing of HIV using Aptamer-siRNA Chimeras

M S Weinberg [1 2]

1: The Scripps Research Institute 2: University of the Witwatersrand

Gene-based therapies represent a promising therapeutic paradigm for the treatment of HIV-1, as they have the potential for sustained viral inhibition via reduced treatment interventions. Such an option may represent a long-term treatment alternative to highly active antiretroviral therapy. We have previously described a therapeutic approach, referred to as transcriptional gene silencing (TGS), where small noncoding RNAs directly inhibit the transcriptional activity of HIV-1 by targeting sites within the 5' LTR promoter - a feature characterized by concomitant silent-state epigenetic marks on histones and DNA. In order to deliver TGS-inducing RNAs, we developed chimeric molecules based on the previously reported dual function of the gp120 (A-1) aptamer

and anti-HIV siRNA. We show that such a system is capable of producing processed guide RNAs that could localize to the nucleus and transcriptionally repress HIV expression in infected cells and in vivo in HIV-infected humanized Rag2-/-yc-/- (RAG-hu) mice. This is the first known study to demonstrate robust silencing of an existing viral infection in vivo following systemic drug administration of TGS-inducing RNAs. Further studies will reveal the durability and specificity of suppression and the potential to add selective pressure on conserved promoter elements to evolve less pathogenic variants of HIV-1.

INV042

The macrophage epigenome and the control of the inflammatory gene expression program

G Natoli [1]

1: European Institute of Oncology, Milan

Induction of an inflammatory response requires the activation of a complex gene expression program in which hundreds of genes are activated or repressed in a kinetically complex fashion that reflects the specific functional role of their products. The activity of the transcription factors responsive to inflammatory stimuli, such as NF-kB, STAT and IRF family members, is critically influenced by the pre-existing chromatin organisation (epigenome) of the cells in which they are activated. This way inflammatory gene expression is qualitatively and quantitatively different depending on the cell type in which it is elicited. In turn, chromatin organisation in differentiated cells is controlled by lineage-determining transcription factors, such as the essential myeloid master regulator PU.1 and its binding partners (RUNX1, IRF8 and others). A wealth of genomic, biochemical and functional data accumulated in the last years has demonstrated that an important role of PU.1 is to make binding sites for inflammatory transcription factors accessible, thus enabling their recruitment to chromatin and the activation of a macrophage-specific inflammatory gene expression program.

INV043

Dissecting Endothelial-to-Hematopoietic Transition in Human iPS Differentiation with Single-Cell Analysis

C Guibentif R Rönn S Saxena C Böiers G Karlsson N B Woods

1: Lund University, Stem Cell Center

We set out to dissect the endothelial-to-hematopoietic transition (EHT) in the human developmental context, using single-cell analysis of in vitro differentiation cultures of iPS cells with a WAS-GFP reporter system. Using the newly defined surface phenotype CD43-CD34hiCD90hiCXCR4-CD73-, shown to enrich for hemogenic endothelium (HE) in pluripotent stem cells differentiation cultures, we identified day 10 of our protocol as an ideal time-point for EHT single cell analysis with 23±7,2% (n=4) of CD34+ cells being either HE or HSC-like cells (CD43+CD34+CD90+). We performed RT-qPCR of 398 single-cells for >90 genes previously identified as being relevant for EHT, and HSC emergence. Unsupervised hierarchical clustering and principal component analysis revealed a continuum of cells progressing from an endothelial to a hematopoietic transcriptional program. The single cells were clustered into 11 groups: 2 to 4 display endothelial transcriptional program with notably high levels of KDR, ENG and VE-cadherin; groups 6 to 8 have hematopoietic signature with expression of key HSC genes such as RUNX1, TAL1, LYL, PBX1, GATA2, GF1B, MEIS, some of which were downregulated in groups 9 to 11 that instead display upregulation of erythro-myeloid commitment genes such as GYPA, KLF1 and PRG2. Interestingly, Group 5 appeared at the interface of endothelium and hematopoietic lineage, displaying a clear endothelial transcriptional program as well as high levels of key genes associated with HSC development and HSC identity, including WAS-GFP. This data has allowed us to identify a molecular and FACS based sub population of cells undergoing EHT, and to propose strategies for the enrichment cells within the HE and HSC like populations that are undergoing or have recently undergone EHT, respectively. Using single-cell analysis of iPS-derived cells as a novel approach to interrogate EHT in the human setting, we show for the first time with high level of detail a subset of cells undergoing EHT.

INV044

Development of new technologies for the safer translation of iPSCs for regenerative medicine.

T Hiramoto [1] T Nii [1] M Tahara [2] C Sakamoto [1] H Ono [1] S Myiamoto [1 3] M Takeda [2] H Kohara [1 3] K Tani [1 3]

1: Division of Molecular and Clinical Genetics, Medical Institute of Bioregulation, Kyushu University 2: Department of Virology, National Institute of Infectious Diseases.
3: Project Division of ALA Advanced Medical Research, The Institute of Medical Science, The University of Tokyo

Recent development of induced pluripotent stem (iPS) cell technology has made a significant impact on the field of human regenerative medicine. We recently reported the generation of iPS cells from common marmoset (CM; Callithrix jacchus), one of the most useful experimental animals, by using lentivirally transduced reprogramming factors, including OCT3/4, SOX2, KLF4, and c-MYC into CM fibroblasts. The cells formed human dysgerminoma-like tumors in SCID mice, indicating that the transduction of reprogramming factors caused unexpected tumorigenesis of CM cells(Yamaguchi S et al, Cancer Sci 2014). For the safer translation of iPS cell technology to clinical medicine, we have recently succeeded in developing two new technologies. First, We have developed novel and non-integrating measles virus(MV) gene transfer vector which is non-transmissible, can transfer multiple genes simultaneously into human cells without affecting host's genomic DNA. We generated iPS cells from human fibroblasts or non-stimulated T cells using MV-ΔF-OSKL-EGFP. These human iPS cells were demonstrated to express pluripotent markers (NANOG, Tra-1-60 etc.) and demethylation. These iPS cells also differentiated into three germ line tissues in vitro and in vivo. Importantly, we also established ground state like pluripotent cell (GSL-iPS cells)

from hematopoietic stem / progenitor cells (CD34 positive cells) by using MV vector. In the presence of human leukemia inhibitory factor (LIF), GSK-3 inhibitor (CHIR99021), and MEK inhibitor (PD0325901), GSL-iPS cells could be cultured from dissociated single cells with rapid cell growth. GSL-iPS cells also expressed pluripotent markers (NANOG, Tra-1-60) and were able to be differentiated into three germ line cells. Second, we investigated gene transfection efficiency followed by gene editing efficiency in hPSCs(human ES and iPS cells) cultured in a single-cell-state to reduce cell density. hPSCs cultured in a single-cell-state were transfected using non-liposomal transfection reagents with plasmid DNA driven by the human elongation factor 1-alpha 1 (EF1α) promoter or mRNA encoding enhanced green fluorescent protein (eGFP). We found that most cells (DNA; >90%, mRNA; >99%) were transfected without the loss of undifferentiated pluripotent stem cell marker expression or pluripotency. Moreover, we demonstrated an efficient gene editing method using transcription activator-like effector nucleases (TALENs) targeting the adenomatous polyposis coli (APC) gene. Our new technologies would be helpful for the safer translation of iPS cells to regenerative medicine.

INV045

Genome Editing In Primary Human Cells and Organs: Towards the Goal of Engineering Genetic Cures

M C Holmes [1]

1: Sangamo BioSciences, Inc.

The juxtaposition of human genome sequencing and its editing in primary cells represents a fundamentally new way to approach the challenge of disease treatment. The notion of using genome editing in the clinic has been reduced to practice via the application of zinc finger nucleases to disrupt the CCR5 gene and create HIV resistant CD4 T cells in a cohort of HIV-positive human subjects. This preclinical and subsequent clinical effort has provided key components of a charted path for the application of editing in an ex vivo setting. We have established clinical-scale approaches for ex vivo editing-driven targeted knockout, correction, and addition in human hematopoietic stem/progenitor cells, thus making clinically tractable their genetic manipulation as a therapeutic strategy for infectious and monogenic diseases of the blood. Efficient disruption and addition within intact organs have also been attained in vivo using appropriately optimized nucleases and delivery modalities in rodent and nonhuman primate models. The talk will describe these data and highlight the use of genome editing in both basic science research and translational applications.

INV046

Gene transfer of E2F2 induces in situ regeneration of retinal pigment epithelium

D Kampik [1] M Basche [1] U F Luhman [1] A J Smith [1] R R Ali [1]

1: Institute of Ophthamology, University College London

The retinal pigment epithelium (RPE) interacts closely with photoreceptors and is important for maintaining visual function. In degenerative diseases like age-related macular degeneration (AMD), the leading cause of blindness in the developed world, RPE cell loss is followed by photoreceptor cell death. RPE cells can proliferate under certain conditions, suggesting an intrinsic regenerative potential, but this has so far not been utilised therapeutically. We have used gene transfer of E2F2, a potent transcriptional regulator of proliferation, to RPE cells to induce cell replication and thereby regeneration. In vitro, E2F2, delivered to growth-arrested ARPE19 cells by transient transfection, induced upregulation of Ki67 (2.3-fold) and uptake of BrdU (3.5-fold). Following subretinal injection in wild type mice of non-integrating lentiviral vector expressing E2F2 we observed a 40-fold increase in E2F2 positive RPE cells, and a 10-fold increase in BrdU positive cells in both young (12 weeks) as well as old (18 months) wildtype mice. LNT-E2F2 induced a mean increase in RPE cell density of 15% compared to control vector treated eyes. The concept was tested in a transgenic mouse model that features RPE loss through inducible activation of diphtheria toxin-A gene in RPE cells. Following administration a LNT-E2F2 vector, there was increased BrdU uptake (9-fold) and an increase in cell density (30%) in the central RPE where pathology was strongest. Such in situ regeneration might lead to a new treatment concept for progressive retinal degenerations such as AMD where there is RPE loss.

INV047

Expanding AAV cargo capacity in the retina

A Auricchio [1 2]

1: TIGEM 2: Dept. Of Translational Medicine, "Federico II" University Medical School, Naples, Italy

Retinal gene therapy with AAV vectors is safe and effective in humans. However, AAV's limited cargo capacity prevents its application to therapies of inherited retinal diseases (IRDs) due to mutations of genes over 5 kb (here referred as large genes), like Stargardt disease (STGD1) and Usher syndrome type IB (USH1B). We have developed dual hybrid AAV vectors each containing one half of a large transgene expression cassette which reconstitute a large gene upon co-infection of the same cell. We found that dual hybrid vectors transduce efficiently mouse and pig photoreceptors to levels that resulted in significant improvement of the retinal phenotype of mouse models of STGD1 and USH1B. More recently, we have included in dual hybrid AAV vectors signals that mediate the degradation of truncated proteins that are produced by single half vectors. This should improve the safety of this platform in view of future clinical applications. However, dual AAV cargo of about 9.4 kb would not be sufficient for retinal transfer of larger genes like ALMS1 or EYS mutated in Alstrom syndrome or retinitis pigmentosa, respectively. We are therefore developing triple AAV vectors with an overall cargo capacity of about 14 kb. Our preliminary data show that triple AAV vectors transduce mouse photoreceptors and should be thus further tested in animal models of IRDs. Overall, the data from our group and others support the use of multiple AAV

vectors for large gene transfer thus overcoming one of the major limitations of this vector platform.

INV048

Restoration of vision with ectopic expression of human rod opsin

J Cehajic-Kapetanovic [1] C Eleftheriou [2] A E Allen [2] N Milosavljevic [2] A Piennaar [2] R Bedford [2] K E Davis [2] P N Bishop [1] R J Lucas [2]

1: Centre for Ophthalmology & Vision Sciences, Institute of Human Development, University of Manchester and Manchester Royal Eye Hospital, CMFT, Manchester Academic Health Sciences Centre 2: Faculty of Life Sciences, University of Manchester

Many retinal dystrophies result in photoreceptor loss, but the inner retinal neurons can survive making them potentially amenable to emerging optogenetic therapies. Here, we show that ectopically expressed human rod opsin, driven by either a non-selective or ON-bipolar cell specific promoter, can function outside native photoreceptors and restore visual function in a mouse model of advanced retinal degeneration. Electrophysiological recordings from retinal explants and the visual thalamus revealed changes in firing (increases and decreases) induced by simple light pulses, luminance increases and naturalistic movies in treated mice. These responses could be elicited at light intensities within the physiological range and substantially below those required by other optogenetic strategies. Mice with rod opsin expression driven by the ON-bipolar specific promoter displayed behavioural responses to increases in luminance; flicker; coarse spatial patterns; and elements of a natural movie at levels of contrast and illuminance (≈50-100lux) typical of natural indoor environments. These data reveal that virally-mediated ectopic expression of human rod opsin can restore vision under natural viewing conditions and at moderate light intensities. Given the inherent advantages in employing a human protein, the simplicity of this intervention, and the quality of vision restored, we suggest that rod opsin merits consideration as an optogenetic actuator for treating patients with advanced retinal degeneration.

INV049

The value of clonal repertoire analysis for safe and efficient gene therapy

C von Kalle [1]

1: DKFZ and National Center of Tumor Diseases (NCT) Heidelberg

Gene therapy has reached considerable successes and is heading into clincis to treat a broad spectrum of diseases. The previous approval of the first medicinal gene therapy product, Alipogene tiparvovec, by the European Medical Agency (EMA) has impressively underlined the fact that gene therapy is already on its way to become a regular treatment strategy. However, concerns have been raised on the safety of gene therapy, mainly triggered by first generation retroviral vectors which application resulted in severe adverse events in individual patients. While current generation self-inactivating lentiviral vectors and non-active integrating AAV are considerably safer, these safety concerns could not be eradicated completely. With the advent of next generation sequencing and new gene editing tool suites, previously unreachable precision in treatment, diagnostics and consequently safety evaluation can be reached nowadays. This not only allows a sophisticated and thorough vector persistence and toxicity (insertional mutagenesis and immunity) analysis, but also guides the future route of gene therapy approaches aimed for sole or combinatorial treatment strategies.

INV050

A genome-free integration site analysis strategy allows studying cell clones with integrations in repeated sequences, readdressing the hematopoietic reconstitution in gene therapy patients.

E Montini [1]

1: San Raffaele Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milan

High-throughput retrieval and mapping of genomic lentiviral vector insertion sites in blood cells from hematopoietic stem cell (HSC) gene therapy patients is a powerful strategy that enables studying the clonal composition, diversity and abundance in different cell lineages isolated over time after transplantation. However, these analyses are normally performed only using integrations that can be precisely mapped on the reference genome, while the remaining 50% of the cell clones marked by vector integrations flanking genomic repeated sequences are simply excluded from the analyses. We implemented novel powerful noise filtering procedures and a novel graph theory-based method for genome-free sequence alignments allowing for the first time to identify and study the behavior of cell clones harboring integration sites in repeated sequences. We applied this new methodology for the molecular follow-up of seven metachromatic leukodystrophy gene therapy patients (up to 48 months after transplant). By using this approach, when compared to the genome-bound bioinformatics analyses, we doubled the number of observations available for clonal tracking across lineages and time points allowing studying in greater detail and precision the clonal abundance and repopulation dynamics during the early and late phases of hematopoietic reconstitution. Our HSC number estimations show that patients were repopulated by up to 10,000 active HSCs that contribute to lymphoid and myeloid cell lineages, especially at early time points. These refined analyses confirm the positive safety and efficacy profile of these therapies and significantly improved our capability to quantify and track tens of thousands of cell clones from different cell lineages from the early and late phases of hematopoietic reconstitution.

INV051

Gene therapy experiences

A Fischer [1]

1: Hôpital Universitaire Necker – Enfants Malades, Paris

No abstract available.

INV052

Thalassemia programme including the preclinical and clinical data

G Veres [1]

1: bluebird bio, Cambridge MA

No abstract available.

INV053

Short and long non coding RNA therapeutics for cardiac remodelling

T Thum [1]

1: Medizinische Hochschule Hannover (MHH)

Cardiac Remodelling after cardiac stress leads to genetic and structural changes of the heart finally ending in heart failure development. I here will discuss latest own findings based on RNA therapeutics to combat heart failure. The presentation will include microRNA-based and long noncoding RNA-based therapeutic approaches in small and large animals to pave the way for future development of next-generation noncoding RNA-based therapeutics for heart failure treatment.

INV054

Promoter-targeted shRNAs differentially regulate vascular endothelial growth factors.

N Laham-Karam [1] M Lalli [1] N Leinonen [1]
S Ylä-Herttuala [1 2 3]

1: Department of Biotechnology and Molecular Medicine, A.I.Virtanen Institute, Kuopio FI-70211 2: Gene Therapy Unit, Kuopio University Hospital, Kuopio FI 70029 3: Science Service Center, Kuopio University Hospital, Kuopio FI 70029 4: A.I.Virtanen Institute 5: Gene Therapy Unit, Kuopio University Hospital

The vascular endothelial growth factors (VEGF) and their receptors (VEGF-R) are central regulators of vasculogenesis, angiogenesis and lymphangiogenesis. They are key modulators of these processes in embryonic development, in normal adult physiology and in many pathologies. In particular, VEGF-A is critical for angiogenesis and vascular permeability, whereas VEGF-C is essential for lymphangiogenesis. In this study, we investigated the ability of small hairpin (sh)RNAs to regulate VEGF-A, VEGF-C and VEGF-R1. Promoter-targeted shRNAs were expressed in the context of lentiviral vectors. We identified shRNAs that can upregulate VEGF-C both at the mRNA and protein levels, and differentially regulate VEGF-A depending on the cell type. Likewise, we identified a shRNA targeting mVEGF-R1 promoter that downregulated the expression of this protein. Hence, promoter-targeted shRNAs can affect endogenous gene expression not only bimodally but also differentially in a cell-type specific manner. The level of regulation across the panel of shRNAs varied maximally from a 2.2-fold increase to a 4-fold decrease. This level of change should be useful in fine-tuning and modulating target gene expression, which for potent molecules, such as VEGF-A and VEGF-C, can be very beneficial. Furthermore, the increase or decrease in gene expression by promoter-targeted shRNAs corresponded to enrichment for activating or suppressing histone marks, respectively. These data support a role for epigenetic modifications in the mechanisms of promoter-targeted shRNAs activities. These promoter-targeted shRNAs may facilitate the design and development of targeted, context-dependent strategies for both pro- and anti-angiogenic therapies for the treatment of vascular-related pathologies.

INV055

Development of a neuronal gene therapy approach for Friedreich Ataxia

F Piguet [1 2 3 4] C de Montigny [1 2 3 4] N Vaucamps [1 2 3 4]
L Reutenauer [1 2 3 4] H Puccio [1 2 3 4]

1: Institut Génétique Biologie Moléculaire Cellulaire 2: INSERM U596, Illkirch, France 3: CNRS, UMR7104, Illkirch, France 4: Université de Strasbourg, Strasbourg, France

Friedreich's ataxia (FRDA), the most common autosomal recessive ataxia, is characterized by a sensory and spinocerebellar ataxia, hypertrophic cardiomyopathy and increase incidence of diabetes. FRDA is caused by reduced levels of frataxin (FXN), an essential mitochondrial protein involved in the biosynthesis of iron-sulfur clusters. Impaired mitochondrial oxidative phosphorylation, bioenergetics imbalance, deficit of Fe-S cluster enzymes and mitochondrial iron overload occur in individuals with FRDA. To date there are not effective treatment for FRDA, and cardiac failure is the most common cause of mortality in FRDA. We recently showed that AAVrh.10 vector expressing human FXN injected intravenously not only prevented the onset of the cardiac disease in a faithful FRDA cardiac mouse model, but also, when administered at the time of heart failure, reversed rapidly and completely cardiac disease at the functional, cellular and molecular level in treated animals. In addition, we have recently generated a novel mouse model that recapitulates faithfully the sensory ataxia associated to FRDA. As soon as 23 days of age, they exhibit an ataxic phenotype, which is progressive and worsening over time. Electrophysiological studies reveal a significant decrease of sensory wave at 4.5 weeks and almost a complete loss at 8 weeks of age. A significant loss of sensory neurons within dorsal root ganglia is observed at 17.5 weeks of age compare to age matched controls at lumbar levels. This mouse model will be essential to dissect the pathophysiological pathway associated to the disease but also to test therapeutic approaches, including gene therapy.

INV056

Hematopoietic stem cell based gene therapy for the treatment of lysosomal storage disorders

A Biffi [1]

1: HSR TIGET, San Raffaele Telethon Institute for Gene Therapy

In most Lysosomal Storage Disorders (LSD) hematopoietic stem cell (HSC) transplantation is not or poorly effective. HSC gene therapy could ameliorate the outcome of allogeneic transplant and provide an expectation of efficacious treatment for these LSD. HSC can be genetically modified to express supra-normal levels of the therapeutic enzyme, and become a quantitatively more effective source of functional enzyme than normal donor's cells. Moreover, autologous HSC are immediately available, thus saving precious time in rapidly progressing forms, and can significantly reduce transplant-related morbidity and mortality. We are thus implementing an innovative approach based on the transplantation of autologous, gene corrected HSC for the treatment of severe LSD lacking efficacious and safe therapeutic opportunities. To this goal, we exploit the features of lentiviral vectors (LV). By using LV for HSC gene correction, we proved the therapeutic potential of HSC gene therapy in the murine model of three different LSDs. In the case of metachromatic leukodystrophy (MLD), a severe dysmyelinating LSD, preclinical research led to Phase I/II clinical testing. Indeed, a clinical trial of HSC gene therapy for MLD is currently on going. Evidence of tolerability and safety of the proposed approach, as well as of therapeutic efficacy in the treated patients have been obtained. The same approach has been applied with success to the murine models of type I Mucopolysaccharidosis (MPS I), a LSD characterized by visceral organ, skeleton and nervous system involvement, and of globoid leukodystrophy (GLD), a demyelinating LSD similar to MLD. We are also experimentally addressing the critical need of enhancing brain microglia turnover with donor cells following HCT in order to anticipate the time of clinical benefit and improve the efficacy of the transplant procedure. This work thus far provided hints for designing novel and less invasive approaches for treating LSDs having a prevalent or exclusive CNS involvement.

INV057

Gene therapy for primary immunodeficiencies in Japan

M Onodera [1]

1: National Center for Child Health and Development

Primary immunodeficiencies (PID) are caused by mutations of genes encoding proteins related to immune functions. There are two clinical approaches toward the diseases, one of which is transplantation of normal hematopoietic stem cells (HSCs) from healthy individuals as a replacement for their own HSCs (allogeneic stem cell transplatation) and the other is an infusion of their own HSCs genetically corrected by the transduction of normal genes into them (gene therapy). In particular, recent many clinical trials have proven the efficacy of hematopoietic stem cell gene therapy (HSC-GT) for the diseases and established the firm position of gene therapy as an alternative therapeutic option for patients with PID, as shown in clinical trials for X-linked severe combined immunodeficiency (X-SCID), adenosine deaminase (ADA) deficiency, Wiskott-Aldrich syndrome (WAS), and chronic granulomatous disease (CGD). On the other hand, the severe adverse effect, that is, leukemogenesis by integration of vectors has arisen in the considerable number of patients who received HSC-GT using retroviral vectors. We have also performed HSC-GT for two patients with ADA-SCID and one with CGD in Japan and are now in preparation for gene therapy for WAS. In this symposium, I would like to introduce the results of our clinical trials and explain the current situation of gene therapy in Japan.

INV058

Gene therapy for beta-thalassemia: moving from the preclinical phase to the clinical trial.

G Ferrari [1]

1: San Raffaele-Telethon Institute for Gene Therapy (TIGET), San Raffaele Scientific Institute and "Vita-Salute" San Raffaele University Medical School, Milan

Beta-thalassemia is a severe congenital anemia caused by reduced or absent beta-globin chain production of the adult hemoglobin tetramer. More than 300 mutations leading to the disease have been described, affecting all the steps related to the expression of the β-globin gene. It represents the most common autosomal recessive syndrome to cause a major health problem worldwide with an estimated annual birth incidence of 40.000/year. Treatment of beta-thalassemia is essentially supportive. Patients require a lifelong transfusion regimen combined with iron chelation therapy to reduce hemosiderosis that is ultimately fatal if not continuously treated. At present, the only curative approach is represented by allogeneic hematopoietic stem cell transplantation (HSCT), which, however, is limited by HLA compatibility and toxicity due to graft versus host disease, graft rejection and immunosuppressive regimens required. Along years, medical management and HSCT improved the quality of life and survival of thalassemic patients. Nevertheless, they are both burden by complications and limitations, outlining the need for testing innovative curative approaches. Gene therapy for beta-thalassemia, as an alternative cure to allogeneic HSCT, is based on the autologous transplantation of hematopoietic stem cells (HSCs) engineered by viral vectors expressing a transcriptionally regulated human beta-globin gene. The development and production of lentiviral vectors and the optimization of gene transfer protocols in human CD34+ cells have progressed this field to the pioneering clinical trials in France and in U.S.A. The results so far available are encouraging and will help also to better understand current limitations and ways for improvement. Our contribution to this field was devoted to the clinical development of a safe gene therapy approach, relying on the high-titer globin vector GLOBE (Miccio et al, PNAS 2008), new source of HSCs and an innovative clinical protocol favoring efficient engraftment of genetically modified cells with reduced toxicity. Starting from the demonstration of proof of efficacy of gene therapy in thalassemic mutant mice and in hematopoietic cells from

thalassemic patients (Miccio et al, PNAS 2008 and PLoSOne 2011, Roselli et al, EMBO MolMed 2010; Milsom and Williams, 2010), we moved towards the clinical development by assessing the risk/benefit ratio prior to administration in humans, in comprehensive in vivo pre-clinical studies. Evaluating the biosafety of gene therapy medicinal products following EMA and ICH guidelines, in the GLPs (good laboratory practices) framework, provides results of scientific significance within regulatory standards, paving the way towards future market registration. GLP main studies of toxicology and tumorigenicity (in thalassemic mice) and biodistribution (in NSG mice) proved the safety of GLOBE vector. The clinical trial proposal was anticipated for Scientific Advice to EMA and approved by institutional ethical committee and Italian regulatory authorities (AIFA/ISS). The clinical protocol of gene therapy for transfusion dependent beta-thalassemia (TIGET BTHAL, NCT02453477) will be presented.

INV059

Gene Therapy for Inherited Muscle Diseases

F Mavilio [1]

1: Généthon

Mutations in a large number of genes affect development and function of skeletal and heart muscles, and lead to severe, often fatal genetic diseases. These include Duchenne muscular dystrophy (DMD), limb-girdle muscular dystrophy and many other severe, though less frequent disorders for which no therapy is currently available. Recent progress in the design and manufacturing of adeno-associated viral vectors (AAVs) allow for the first time the development of gene therapies for monogenic muscle disease, based on systemic administration of high doses of AAV vectors carrying therapeutic gene expression or splicing-modulating cassettes. We will present pre-clinical studies of gene therapy for DMD and X-linked myotubular myopathy.

INV060

Defining, Modifying, and Improving the Specificities of CRISPR-Cas9 Nucleases

J K Joung [1 2]

1: Massachusetts General Hospital 2: Harvard Medical School

CRISPR-Cas9 nucleases have recently emerged as important technologies for biomedical research and as potential platforms for gene-based therapeutics. Here I will present our recent work on the CRISPR-Cas9 RNA-guided nuclease platform for introducing targeted genome sequence alterations, including discussion about our most recent efforts to define, modify, and improve the cleavage specificities of these nucleases in human cells. Our work expands the range of sequences that can be targeted by CRISPR-Cas9 and provides important advances for the ultimate clinical translation of these nucleases to treatment of genetic diseases.

INV061

DNA integration in human gene therapy

F D Bushman [1] S Hacein-Bey-Abina [2 4] S Y Pai [3 8] L Caccavelli [4] E Six [2 4] E Sherman [1] N Malani [1] C Berry [6] F Male [1] D B Kohn [11] A Filipovich [10] L Notarangelo [3] A Fischer [2 4 5] D Williams [3 8] M Cavazzana [2 4] A Thrasher [9]

1: University of Pennsylvania 2: Université Paris Descartes 3: Boston Children's Hospital 4: Hôpital Necker-Enfants Malades 5: Inserm 6: University of California, San Diego 7: Boston Children's Hospital 8: Dana-Farber Cancer Institute 9: Institute of Child Health 10: Cincinnati Children's Hospital Medical Center 11: University of California Los Angeles (UCLA)

To correct inherited disorders, it is often necessary to integrate therapeutic sequences into host cell chromosomes to achieve long-term correction. Gene transfer for to treat diseases such as inherited immunodeficiencies involves harvesting of CD34+ stem cells, transduction ex vivo with retrovirus-based vectors, and reinfusion of the gene-modified cells. Transduced cell populations can then be monitored by sampling cells from blood. Retroviral vectors have now been used successfully to correct SCID-X1, WAS, beta-thalassemia and other disorders. Results from recent trials will be presented, along with improved methods for tracking and analyzing integration site distributions and the outcome of gene correction.

INV062

Evolving Gene Therapy for Primary Immunodeficiency

A J Thrasher [1]

1: University College London

Over the last 15 years, several studies have demonstrated highly effective gene therapy for the X-linked form of SCID (SCID-X1) and ADA deficiency, using retroviruses to deliver the therapeutic genes into haematopoietic stem cells ex vivo. Similar ‘proof of principle’ studies have been conducted in patients with Chronic Granulomatous Disease and Wiskott-Aldrich Syndrome. Bearing in mind the outcome and adverse effects of conventional therapy, these are remarkable results and the first clear indication that gene therapy can offer a cure for some human diseases. Despite successful immunological reconstitution, several patients treated on these trials developed mutagenic side effects manifesting as leukaemia and myelodysplasia. The understanding of mechanisms behind these events paved the way to the development of refined vector technologies including use of self-inactivating vectors in which the powerful viral enhancer sequences are deleted.
Clinical trials using self-inactivating gammaretroviral and lentiviral vectors have now been reported, and one again demonstrate the huge potential of gene therapy for haematopoietic disorders including SCID-X1, Wiskott-Aldrich Syndrome, and ADA-SCID. At the same time, the safety profile appears to have been significantly enhanced, and efforts are being made to develop these agents for medicinal licensing. New technologies including homologous recombination or gene repair to accurately correct genetic

mutations may eventually supersede gene addition once limitations of efficiency and toxicity have been addressed.

INV063

Oligonucleotides for Splice-Switching and DNA Duplex Invasion

C I E Smith [1]

1: Dept Laboratory Medicine, Karolinska Institutet, Karolinska University Hospital Huddinge, Stockholm

The inherited immunodeficiency, X-linked agammaglobulinemia (XLA), is caused by mutations in the BTK gene, and results in a B-lineage developmental block. We have recently assessed the treatment potential of splice-correcting oligonucleotides (SCOs) targeting a mutated BTK transcript, which contains a pseudo-exon. In order to study the potential of SCOs, we engineered a novel, Bacterial Artificial Chromosome (BAC)-transgenic mouse carrying a mutated human BTK gene. In this model it was possible to correct the defect both in pro-B-cells in vitro, and also in mature B-cells, as demonstrated by the injection of SCOs in vivo. For correction of exon inclusion defects we have generated reporter constructs, which have been studied in cell lines and used oligonucleotides, which simultaneously bind to the pre-mRNA and splicing factors. In preliminary experiments we have been able to promote exon inclusion. Regulation of plasma cholesterol levels is very complex and several proteins are involved. From these, the proprotein convertase subtilisin/kexin type 9 (PCSK9) has emerged as a promising pharmacologic target. We have recently been able to inactivate PCSK9 by splice-switching oligonucleotides (SSOs), converting the normal splice form to a natural, less abundant and inactive, splice variant. While targeting of RNA by oligonucleotides is rather straightforward, strand-invasion into duplex DNA is considerably more challenging. We have developed a series of bis-locked nucleic acids (bisLNAs), which bind though combined Hoogsteen and Watson-Crick hybridization. These constructs show promising results for invasion into duplex DNA.

INV064

The TargetAMD project – Using Free of Antibiotic Resistance gene (pFAR4) miniplasmids for a Sleeping Beauty (SB100X) mediated gene therapy to treat neovascular Age-Related Macular Degeneration (nAMD)

G Thumann [1] D Scherman [2] Z Ivics on behalf of the TargetAMD consortium [3]

1: Department of Ophthalmology, University Hospitals of Geneva, Rue Alcide-Jentzer 22, Geneva 14, 1211, Switzerland 2: Unité de Pharmacologie Chimique et Génétique et d'Imagerie, INSERM U1022 - CNRS UMR8151, 75270 Paris, France 3: Division of Medical Biotechnology, Paul Ehrlich Institute, Langen, Germany

To avoid possible risks associated with viral gene delivery, TargetAMD has established protocols to deliver the PEDF gene to Iris (IPE) and Retinal (RPE) pigment epithelial cells using the non-viral hyperactive Sleeping Beauty (SB100X) transposon, which integrates the gene into the host cell's genome. The SB100X transposase and the PEDF gene are encoded in "Free of Antibiotic Resistance gene" (pFAR) miniplasmids increasing safety and efficiency. In theory, PEDF-transfected cells transplanted subretinally increase PEDF levels and prevent choroidal neovascularization (CNV) in AMD patients replacing current therapy, which requires monthly intraocular injections of anti-VEGFs. Using pFAR4 miniplasmids and SB100X as few as 5,000 hIPE/hRPE cells have been efficiently transfected and express (63.2-times) and secrete (1.18±0.89 ng PEDF/h/104 cells vs. 0.07±0.03 ng PEDF/h/104 control cells) increased rPEDF. Using the modified electroporation device and the new developed buffer transfection efficiency and cell viability, expressed as Electroporation Score (EC), were comparable than obtained with commercial buffers (EC=66-77 vs. EC=64-82 in controls). In soft agar PEDF-transfected cells did not proliferate, whereas HeLa cells formed 89.4±26.4 colonies and verified randomized integration pattern by LAM-PCR/deep sequencing strategy minimizes the risk for gene integration into cancer genes. Subretinal transplantation of 10-20,000 PEDF-transfected rat IPE/RPE cells in a CNV rat model showed significant reduction in neovascular area, 15.25/27.38 µm2 vs. 58.53 µm2 in controls, after 14 days. We have shown that non-viral transfection of RPE and IPE cells with the PEDF gene does not alter cell functions and stability indicating that PEDF-transfected cells are safe for transplantation in nAMD patients.

INV065

Systemic Administration of VSV Expressing cDNA Libraries to Treat Established Tumours

T Kottke [1] K Rajani [1] J Thompson [1] L Evgin [1] J Pulido [1 2] K Shim [1] M Schuelke [1] E Ilett [3] P Selby [3] A Melcher [3] R Vile [1 2 3]

1: Departments of Molecular Medicine Mayo Clinic, Rochester, Minnesota 2: Department of Immunology, Mayo Clinic, Rochester, Minnesota 3: Leeds Institute of Cancer Studies and Pathology, University of Leeds

Expression of a cDNA library from the immunogenic platform of the Vesicular Stomatitis Virus (VSV) generated Th17 T cell responses against tumor associated self antigens (TAA), which eradicated established tumors. Three immunogenically active VSV-TAA– VSV-N-RAS, VSV-Cytochrome-C and VSV-TYRP-1 – isolated from the library treated melanomas associated with a CD4+ T cell dependent Th17 response to tumor cells, but with no detectable IFN-γ response. We show here that the immunogenically active cDNA were all truncated versions of the full length proteins, generated as a result of selection to allow better viral replication. However, VSV-mediated presentation of truncated self proteins generated CD4+ Th17 responses which were more effective, and longer lasting, than Th1 anti tumor responses raised to the normal full length proteins. Optimal anti tumor responses were raised by cumulative signaling from different types of APC, each presenting specific, truncated antigens. Full length, properly folded self TAA were processed through a Th1 type, CD8+

dependent pathway. In contrast, truncated versions of the same self TAA (likely to be poorly and incompletely folded) were presented through a Class II-dependent, Th17 pathway, showing that full length and truncated proteins are rigorously distinguished and trafficked into separate pathways of antigen presentation. Finally, the Th17, CD4+ response against truncated self TAA was both more therapeutic against tumor, and had longer lasting memory, than the Th1 response raised against the corresponding full length self TAA. These data show that the type/potency of anti tumor immune responses against self TAA can be manipulated in vivo through the integrity of the self protein (full length or truncated), inclusion of multiple TAA to recruit the optimal combination of APC and the resultant skewing of the T cell response to either a CD8+ Th1, or a CD4+ Th17phenotype. These results open new approaches for cancer immunotherapy against self TAA, and are significant in other contexts in which aberrant folding of normal cellular proteins can lead to the generation of T cell reactivity against self proteins.

INV066

Oncolytic adenoviruses: versative tools for killing tumor cells, inducing anti-tumor immunity and facilitating T-cell therapy. A summary of rodent and human data.

A Hemminki [1 2 3]

1: Cancer Gene Therapy Group, Department of Pathology and Transplantation Laboratory, Haartman Institute, University of Helsinki, Finland 2: Helsinki University Hospital Comprehensive Cancer Center, Helsinki, Finland 3: TILT Biotherapeutics Ltd., Helsinki, Finland

Oncolytic viruses have been used for treatment of cancer for more than a century. However, the finding that a major part of their anti-tumor activity may be due to activation of the immune system, is a relatively recent one. Thus, currently these agents are viewed more as immunotherapy than sterile oncolytic drugs. Adoptive cell therapy (ACT) with tumor infiltrating cells (TIL) has shown promising results in melanoma trials, with up to half of patients benefiting and a significant proportion alive and without disease even a decade later. However, in melanoma solid tumors ACT is currently not working. Based on clinical and preclinical work done at CGTG, including treatment of >300 patients with oncolytic adenoviruses, and the data emerging from clinical substrates, we proposed that oncolytic adenovirus could overcome the obstacles identified for ACT in the past decade Adenovirus per se was able to dramatically enhance efficacy of ACT in a preclinical model of melanoma but mice were not completely cured. To enhance efficacy further, cytokine arming devices were compared and the best ones were incorporated into viruses for preclinical development. A bicistronic oncolytic adenovirus coding for tumor necrosis factor alpha (for counteracting immunosuppression) and interleukin 2 (for graft stimulation) was identified as the optimal candidate. Clinical trials (sponsored by TILT Biotherapeutics Ltd) using this virus for enhancing tumor TIL and CAR therapy are planned to start in 2016.

INV067

rAAV-mediated Gene Therapy for Metabolic Liver Disease; Prospects and Challenges in Paediatric Patients

I E Alexander [1 2 3] S C Cunningham [2] S M Siew [2]

1: The Children's Hospital at Westmead 2: The Children's Medical Research Institute 3: The University of Sydney

Vectors based on Adeno-Associated Virus (rAAV) are currently showing exciting therapeutic promise in clinical trials for adults with Haemophilia B (Nathwani et al., NEJM, 2015). This evidence of efficacy, combined with ongoing development of the AAV vector toolkit, gives cause for optimism about the possibility of achieving similar successes in a broader spectrum of liver diseases. Many of the most attractive disease targets, however, require treatment in infancy or early childhood while the liver is still growing, and progressive loss of episomal AAV vector genomes can be anticipated. A further challenge is the relatively higher gene transfer efficiencies required to treat many of the most troubling metabolic liver disease phenotypes, such as urea cycle defects. Accordingly, near term success demands careful target disease selection, such that the required gene transfer efficacy and durability is within reach of existing AAV vector technology. For more demanding disease targets therapeutic success requires further advances in AAV-based technology. The most immediate prospects lie with the discovery/development of AAV capsid variants that are highly tropic for the human liver, and possess other desirable properties, such reduced recognition by neutralizing antibodies prevalent in patient populations. Additional strategies being explored include the stabilization of AAV-mediated transgene expression using hybrid AAV/transposon systems and the pharmaco-genetic expansion of gene-modified hepatocytes up to therapeutically useful thresholds. Genome editing strategies are also emerging using AAV-mediated homologous recombination (HR), alone or in combination vectorized user designed nucleases. Here liver growth may actually prove to be advantageous, making the paediatric liver particularly attractive.

INV068

AAV-based delivery of APP and PS1 in adult mouse hippocampus identifies initial steps of Alzheimer's disease

M Audrain [1 2 3] R Fol [1 2 3] P Dutar [4] B Potier [4] J M Billard [4] J Flament [3 5] S Alves [1 3] M A Burlot [1 2 3] G Dufayet-Chaffaud [1 3] A P Bemelmans [3 6] J Valette [3 6] P Hantraye [3 5 6] N Déglon [7 8] J Braudeau [1 3] N Cartier [1 3]

1: INSERM UMR1169, Université Paris-Sud, Fontenay-aux-Roses 2: Université Paris Descartes 3: CEA, DSV, FBM, MIRCen, Fontenay-aux-Roses 4: INSERM UMR894, Centre de Psychiatrie et Neurosciences, Université Paris Descartes, Sorbonne Paris Cité, Paris 5: INSERM UMS27, Fontenay-aux-Roses 6: CNRS UMR9199, Université Paris-Sud, Université Paris-Saclay, Fontenay-aux-Roses 7: Department of Clinical Neurosciences, Laboratory of Cellular and Molecular Neurotherapies, Lausanne University Hospital, Lausanne 8: Neuroscience Research Center, Laboratory of

Cellular and Molecular Neurotherapies, Lausanne University Hospital, Lausanne

Alzheimer's disease (AD) is the most frequent form of dementia in the elderly with no effective treatment. The mechanisms triggering disease onset and progression are still imperfectly dissected. We aimed to decipher the modifications occurring in vivo during the very early stages of AD, before the development of amyloid deposits, neurofibrillary tangles, neuronal death and inflammation. Classical transgenic models with constitutive and high level of APP expression and rapid progression to amyloid aggregation are not appropriate to study initial phases of disease progression. We used an AAV-based transfer of human mutated APP and presenilin 1 (PS1) genes to the hippocampus of adult mice to express stable and moderate levels of human APP and increase amyloid cleavage. The APP, βCTF and Aβ ratios were similar to those in hippocampal tissues from AD patients. High levels of GSK-3β were associated with increased phosphorylation of Tau protein. Rapid synaptic failure was observed with decreased synaptic markers and subtle electrophysiology and spectroscopy abnormalities associated with early behavioral impairments in the open-field, Y-maze and Morris water maze tasks. Altogether, we demonstrate that moderate levels of amyloid products and an Aβ42/Aβ40 ratio similar to that in humans are sufficient to rapidly trigger early steps of the amyloidogenic and Tau pathways in vivo. This AAV-designed AD mouse closely mimicking human APP processing, allows to define a sequence of early events responsible for synaptic dysfunction and cognitive defects before the classical hallmarks of AD. This model should help to evaluate neuroprotective strategies at initial step of AD progression.

INV069

Pluripotent stem cell (PSC)-derived myeloid cells as a novel source for cell and gene therapy strategies

T Moritz [1]

1: Reprogramming and Gene Therapy Group, REBIRTH Cluster of Excellence, Division of Experimental Hematology, Hannover Medical School

Hematopoietic in vitro differentiation of human PSCs not only constitutes an important model system of embryonic hematopoietic development but also holds great promise for gene and cell therapy. Whereas approaches to generate long term repopulating hematopoietic stem cells (LTR-HSC) from pluripotent sources have met limited success, more differentiated cells may represent valuable alternatives. In this context our group has established highly efficient embryoid body (EB)-based differentiation protocols to generate large numbers of functional myeloid cells such as granulocytes or macrophages from murine and human pluripotent cell sources. Furthermore, we have demonstrated genetic repair of differentiated myeloid cells derived from disease-specific iPSCs in the context of a number of genetic disease entities including pulmonary alveolar proteinosis (PAP) due to granulocyte/macrophage–colony stimulating factor (GM-CSF-alias CSF2-) receptor deficiency. Whereas current therapeutic options for this disease are limited, we recently demonstrated that a gene and cell therapy approach based on the endotracheal application of healthy, gene-corrected macrophages (Pulmonary macrophage transplantation, PMT) maybe feasible and effective. The use of iPSC-derived cells in this context will be discussed as well as the potential transfer of this technology to other disease entities.

INV070

Engineered extracellular vesicles for biomedical applications

J Z Nordin [1] O Wiklander [1] Y Lee [2] E CI Smith [1] I Mäger [2] M JA Wood [2] S EL Andaloussi [1 2]

1: Karolinska Institutet 2: University of Oxford

Extracellular vesicles (EVs) (40-100 nm in diameter) are secreted by nearly all cells in the body and play an important role in intercellular communication. They are found in all body fluids and have the ability to convey not only genetic material and bioactive lipids to recipient cells, but also various types of proteins to regulate a range of biological processes. Hence, they have gained increasing attention as new drug delivery vehicles. EVs per se have been used as therapeutic agents in various disease models, particularly in relation to immunotherapy for cancer treatment, immunosuppressive therapy for autoimmune disorders and for induction of tissue regeneration. We have recently developed a platform to generate engineered EVs by recombinantly expressing fusion proteins comprising of known EV proteins and targeting peptides in producer cells. EVs displaying such proteins have subsequently been loaded with exogenous macromolecular drugs for targeted delivery in mice. In parallel, we have exploited the same strategy for loading of protein therapeutics on the surface of EVs derived from a range of cells, including different stem cells that have inherent immunosuppressive properties, to sequester disease-associated factors in blood. To facilitate pre-clinical applications of EVs, we have developed scalable method of purifying EVs based on filtration and subsequent size exclusion chromatography, generating high yields of EVs with retained biophysical properties. Finally, we have characterised, in detail, the content of EVs derived from different cells, how their composition changes with culturing conditions and how that ultimately affect their biodistribution in mice.

INV071

G-force loading of virus vectors into vesicles for enhanced gene therapy vehicles

Z Fitzpatrick [1] A Maus [1] B György [1] D Mu [1] K Csomos [1] J E Walter [1] C A Maguire [1]

1: The Massachusetts General Hospital 2: Harvard Medical School

While adeno-associated virus (AAV) vectors are remarkable for gene therapy, pre-existing immunity and the high vector load required to achieve adequate transduction of a desired organ remain challenges to stable transgene expression upon systemic delivery. Extracellular vesicles (EVs) have innate

tendencies to deliver biomolecules which may have utility in creating better AAV vectors for human gene therapy. We have previously reported that AAV in 293T producer cell media associates with EVs (ev-AAV) during vector isolation. Excitingly, 293T-derived ev-AAV can evade neutralizing anti-AAV antibodies and enhance transduction in mice. Our new strategy, manual loading of purified AAV into EVs may have several benefits, as formulation is controlled. First, EVs with desired biological activity, including patient-derived EVs, can be used. Second, two different AAVs may be co-loaded into a single EV which may enhance the efficacy of two-component systems in vivo. Here we demonstrate a simple method for loading EVs with AAV vectors using high-speed g-forces. Purified AAV was mixed with conditioned media containing EVs from a cell line and also primary human peripheral blood mononuclear cells and then ultracentrifuged. The resuspended ev-AAV was found to outperform standard AAV in transduction of cells in culture (~36-fold) as well as resistance to neutralizing anti-AAV antibodies. Through depleting EVs before addition of AAV, we demonstrate that EVs were essential to this enhancement. These data suggest that g-force loading of AAV into EVs represents a promising method to increase vector performance. We will next investigate co-transduction enhancement, as well as gene delivery in vivo.

INV072

Latest News from the CAT – Progresses and Challenges in development of ATMPs

P Salmikangas [1]

1: Finnish Medicines Agency

With the release of Regulation 1394/2007, a new framework for gene and cell therapy medicinal products and tissue engineered products was established in the European Union. For all three product classes, called Advanced Therapy Medicinal Products (ATMP), a centralized marketing authorization became mandatory. For evaluation of the marketing authorisations (MA) of ATMPs Committee for Advanced Therapies (CAT) was established in 2009. After six years of CAT being in operation five products have been granted a MA in EU, ChondroCelect and MACI for cartilage repair, Provenge for prostate cancer, Glybera for LPL deficiency and Holoclar for limbal stem cell deficiency. However, the pipeline of new ATMPs is much bigger, as seen from the significant numbers of different products discussed by the CAT in scientific advice and classification procedures. For a new application, data and information relating to manufacturing processes and quality control of the active substance and the final product have to be submitted for evaluation together with data from non-clinical and clinical safety and efficacy studies. Technical requirements for ATMPs are defined in the legislation and guidance for different products is available through several EMA/CAT guidelines. Due to the diversity of ATMPs, a tailored approach for regulating these products is considered necessary. Thus, a risk-based approach has been introduced for ATMPs allowing flexibility for the regulatory requirements. Recently, also amended GMP requirements have been proposed by the European Commission (specific GMP guidance under consultation). The presentation will give a short introduction to EU regulatory framework for ATMPs and information concerning current situation of MA activities and products in clinical trials will be provided. Furthermore, a brief introduction to EU guidelines for ATMPs will be given and the current key questions/ problems of the ATMP field will be discussed.

INV073

Accelerating the development of gene therapy products: a global regulatory view.

A V Eggimann [1]

1: bluebird bio

This presentation will discuss the different mechanisms available today in the existing regulatory paradigms in the EU and the US to accelerate the development of innovative products with high therapeutic potential intended to treat serious and life-threatening conditions. A case study will be used to illustrate certain aspects. Some consideration on potential improvements in existing regulations will be provided.

INV074

Obstacles and Opportunities to Gene Therapy Approval and Usage for Clinical Disease

Y Fong [1]

1: City of Hope Medical Center, Duarte CA

In this session we will discuss the regulatory and clinical trials obstacles for clinical study and approval of gene therapies. Discussed will be the differences between classical medicinal development and gene therapy development, and possible paths for overcoming these obstacles. Now that the US FDA's Oncologic Drugs Advisory Committee and Cellular, Tissue and Gene Therapies Advisory Committee have voted for approval of the oncolytic immunotherapy talimogene laherparepvec (T-VEC), a new era of clinical gene therapy is about to begin. The obstacles to clinical deployment and use once this therapy is finally approved will be discussed as an example of what we will all be facing in the gene therapy field. These will include problems with administering, storing, transporting and disposing of the viruses and vectors. These and other financial, public safety, and industrial concerns will be presented in order to frame the problems that societies such as the European Society of Gene and Cell Therapy will need to lead in formulation of guidelines and policies.

INV075

Translational Insights into Vascular Growth Factors

K Alitalo [1]

1: Translational Cancer Biology Program and Wihuri Research Institute, Biomedicum Helsinki, 00014 University of Helsinki

During embryonic development, the blood vasculature develops from embryonic mesoderm via the migration and assembly of endothelial progenitor cells, a process called vasculogenesis. The embryonic blood vasculature then expands via angiogenesis, vessel remodelling and functional specialisation, giving rise to a second vascular system, the lymphatic vasculature. Our understanding of the cellular and molecular mechanisms underlying the formation and morphogenesis of the developing vasculature has progressed significantly in recent years, and this knowledge can now be used for therapeutic manipulation of the vascular system. - Anti-angiogenic drugs are used in the treatment of cancer patients, but most patients are either refractory or eventually acquire resistance to anti-angiogenic therapeutics. A combination of angiogenesis inhibitors based on solid knowledge of the major interacting angiogenesis signaling pathways could significantly advance the efficacy of the tumor therapy. - The opposite idea of pro-angiogenic therapy in cardiovascular disease is to grow new functional blood vessels and thus restore blood flow to ischemic tissue. Several attempts have been made to stimulate angiogenesis and arteriogenesis in tissue ischemia, with limited success. One of the obstacles has been the property of vascular endothelial growth factor to promote vascular permeability, which can be therapeutically targeted in a variety of disease models by using angiopoietins. - The growth of lymphatic vessels, lymphangiogenesis, regulates a number of pathological processes including tissue inflammation and tumor dissemination but is insufficient in patients suffering from lymphedema, a debilitating condition characterized by chronic tissue edema and impaired immunity. Lymphangiogenic growth factors provide possibilities to treat these diseases. – Increased understanding of vascular growth factor biology should facilitate development of therapeutics also for cardiovascular and regenerative medicine.

INV076

Cardiovascular Molecular Imaging in Health and Disease

J Knuuti [1 2]

1: University of Turku 2: Turku University Hospital

Molecular imaging means an imaging technique that can assess cell and tissue processes in vivo at molecular level. Molecular imaging is feasible using nuclear imaging techniques such as SPECT and PET, optical imaging and MRI. PET is the most versatile molecular imaging technique due to characteristics of the imaging tracers and possibility to image also targets deep in the body. Translation from small animals to humans is straightforward. Currently, quantification of perfusion, oxygen consumption, glucose utilization, fatty acid uptake and oxidation are possible using in vivo molecular imaging methods. For PET several tracers have been validated for quantification of perfusion in absolute terms ([15O]H2O, [13N]ammonia, 82Rb). [11C]acetate and [15O]O2 have been used for measuring oxygen consumption with PET. [11C]acetate has been most commonly used and in majority of the studies simple and robust washout analysis has been applied. The most commonly applied metabolic imaging has been measurement of glucose uptake using [18F]FDG. Quantification of glucose utilization with FDG is potentially limited by the differences between natural glucose and [18F]FDG. [11C]glucose has been also used in limited sites and has been shown to provide accurate quantitation of glucose uptake. Several PET tracers have been used to measure free fatty acid metabolism. [11C]palmitic acid has been traditionally used and the retention of the tracer is thought as an index of FFA uptake and the washout as an index of oxidative metabolism. Also full quantitative model for [11C]palmitic has been introduced. [18F]FTHA has been recently used to study fatty acid metabolism and its accumulation is suggested to be mainly tracing FFA -oxidation in the heart. Also other molecular imaging methods have been developed for imaging of neuronal function, sympathetic and parasympathetic receptors. Imaging of myocardial neural function and innervation has been shown to have strong prognostic value in patients with heart failure. The development of new specific tracers for inflammation, receptors, angiogenesis, apoptosis, reporter genes and other specific targets holds great promise for the future clinical use of PET. In the imaging of vulnerable plaque, non-invasive techniques such as multislice CT provide accurate localisation of plaques and also characterise morphological criteria associated with a high risk of atherosclerotic plaque rupture. In contrast, PET uses radiolabelled molecules designed to specifically target individual biological activities in atherosclerotic plaques. A variety of cellular molecular targets involved in the progression and potential rupture of vulnerable plaques have been identified, including macrophage density, calcification, apoptosis and protease activity. In heart failure the efficiency of cardiac work reflecting the imbalance between oxidative metabolism and cardiac function appears to be a sensitive marker of myocardial pathology. Therapeutic interventions that improve outcome are associated with restoration of efficiency. Hybrid imaging combines different modalities in order to obtain complementary anatomical and functional information in a single imaging study. Morphological imaging with CT and MR has benefited from the improvement of spatial and temporal resolution. PET/CT, SPECT/CT and more recently PET/MRI systems have become commercially available and increasingly used also in cardiac imaging for both small animals for research purposes and humans for clinical research and routine.

INV077

Gene therapy for heart and lung transplantation

A Nykänen [1]

1: University of Helsinki

Organ transplantation often remains the only available treatment option for end-stage organ failure. The surgical techniques and immunosuppressive medications have developed so that over 4000 heart transplantations and almost 4000 lung transplantations are performed annually worldwide with good early and long term results. Nevertheless, significant obstacles that restrict transplant patient survival remain including primary graft failure, acute and chronic rejection, and side-effects of life-long systemic immunosuppression such as infections and malignancies. The ex vivo time during organ

transplantation makes the graft an attractive target for gene therapy. Ideally transplant gene therapy would prevent pathological responses in the graft and allow minimization or discontinuation of systemic immunosuppression. Several experimental studies have successfully used non-viral or viral vectors to introduce therapeutic genes into cardiac and lung transplants that target alloimmunity or fibroproliferative processes. We have used adenovirus and AAV vectors that encode vascular growth factors such as VEGF and angiopoietins in experimental heart and lung transplantation models aiming to prevent vascular dysfunction and pathological fibroproliferation. Issues involved with vector choice, therapeutic genes and safety need to be resolved before pursuing to clinical applications.

INV078

Rebuilding the Heart via Paracrine Factor modRNA and Human Ventricular Progenitor Cell- Based Tissue Engineering

K R Chien [1]

1: Department of Cell and Molecular Biology and Medicine, Karolinska Institutet, Stockholm

Insights from studies of human cardiogenesis are forming a blueprint for new strategies to regenerate the heart and its components in specific subsets of cardiovascular disease. Both cell free and cell based therapies can now be envisioned, driven by the ability to deliver paracrine factor synthetic, chemically modified mRNA (modRNA) to drive effects on a small subset of endogenous heart progenitors that are mobilized following myocardial infarction, as well as other circulating vascular progenitors, with concomitant effects on coronary vascular regeneration (Zangi et al, NBT, 2013). Recent studies have documented the ability to express VEGF modRNA in both small and large animals, at physiological levels, without cationic lipid based carriers and with no effects on triggering innate immunity, setting the stage for first in human studies in mid-2016. Towards strategies to regenerate ventricular muscle, a new approach has been developed to build mature, functional, human ventricular muscle via "organ-on-organ" tissue engineering. Human ventricular progenitors build the chamber wall during cardiogenesis and their large scale generation, purification, in vivo maturation, and vascularization during transplantation is central to heart tissue engineering (For review, see Sahara et al, EMBO J., 2015). While rare, transient murine ISL 1+ventricular progenitors have been isolated via genetic markers in transgenic lines (Domian et al, Science, 2009), committed human ventricular progenitors have been difficult to generate in large scale and to purify by cell surface markers. Recently, we developed a two-step method for the large scale generation of fully committed ISL1+ ventricular progenitors (HVPs) from human embryonic stem cells and the identification of a single cell surface marker that allows their one step purification to complete homogeneity. In vivo transplantation of HVPs results in the generation of a wall of a pure, mature, vascularized, and functional human ventricular muscle in kidney capsule transplantation studies, thereby documenting a completely cell autonomous pathway for human ventriculogenesis from purified HVPs. Intramyocardial in vivo transplantation studies in normal murine hearts reveal the ability of the HVPs to spontaneously migrate to the outer epicardium, proliferate, mature, and assemble into a human ventricular muscle patch on the surface of the heart without an exogenous matrix. These studies show that human ventriculogenesis can proceed via a completely cell autonomous pathway from purified ISL1+ progenitors and suggests a new clinically tractable paradigm for in vivo human ventricular chamber tissue engineering. Also, a new in vivo human heart model system has been created to allow direct chemical and genetic screening in intact human ventricular muscle in the in vivo context.

INV079

Role of HMGB1 in tissue injury.

K Tamai [1] Y Kaneda [1]

1: Osaka University

High mobility group box1 (HMGB1) is a non-histone chromatin protein which binds to DNA as a chaperon molecule for modulating chromatin structure and gene expression. In injured tissue, HMGB1 is released from necrotic cells as complexes with DNA/histone chromatin molecules and activates innate immune/inflammatory responses via ligating to Toll-like receptors (TLRs), resulting in recruiting neutrophils and macrophages into the necrotic tissues and inducing inflammatory tissue remodeling. We have reported that PDGFRa-positive mesenchymal cells in bone marrow sense injury-dependent elevation of blood HMGB1 level to be activated and move into the circulation (PNAS 2011). We then demonstrated that the circulating bone marrow-derived mesenchymal cells then specifically accumulated into the injured/inflamed tissues via CXCL12/CXCR4 axis, and released anti-inflammatory molecules such as IL-10 and TSG-6 to shift the tissue remodeling reactions from inflammatory phase to regeneration phase (J Immunol 2015, Scientific Reports 2015). Systemic HMGB1 administration induced accumulation of bone marrow-derived mesenchymal cells into the injured tissue, and suppressed inflammatory reactions and induced mesenchymal and epithelial tissue regenerations in the mouse skin graft model. Depletion of the mesenchymal cells in mouse bone marrow not only impaired the action of HMGB1 administration but also worsened inflammation in the skin graft. These findings clearly demonstrate pivotal roles of extracellular HMGB1 in tissue injury, that is to evoke initial inflammatory cell activation to remove necrotic tissues and then recruit mesenchymal cells from bone marrow to coordinate tissue regeneration process.

INV080

Towards optimized large scale production of viral vectors

O W Merten [2] D Boudeffa [1] D Fenard [2] M Mormin [2] A Galy [2]

1: Université de Montréal 2: Généthon

The evolution of gene therapy using viral vectors to advanced phase clinical trials and commercialization requires

implementation of large scale vector manufacturing methods. In the past different culture systems have been assessed for vector production. In particular, fixed bed reactor systems showed very interesting production levels and we could generate 10x higher levels of GaLV-MLV vectors (→ 3.5x107 TU/ml) than in T-flasks (Merten et al., 2001). In case of lentiviral (LV) and AAV vectors, transfection of HEK293(T) cells grown in Cell Factories (CF-10) is the classical production means and vector levels of 4x1011 ig (LV) (Merten et al., 2011) and 2x1013 vg (AAV) (unpublished) can be obtained/run using 24 and 10 CF-10, respectively. These systems are sufficient for generation of vector quantities required for early phase but not for advanced clinical trials because not scalable. Only suspension culture systems are scalable and allow large scale vector production. In case of stable producer cell lines, the establishment of suspension processes is straightforward and titers in the range of 0.5-5x107 ivp/ml have been obtained for MLV (Ghani et al., 2009) and LV vectors (Broussau et al., 2008). For large scale production of LV and AAV vectors, Généthon has implemented a transfection process of HEK293T cells adapted to suspension growth and the Sf9/baculovirus system, respectively. Production levels of 3.5±2x107 ig/ml (LV, 50L scale) and 4.2±1.2x1013 vg/ml (AAV, 200L scale) are obtained. Nevertheless, further improvements are required for producing larger vectors quantities more efficiently. Some approaches for process intensification will be presented.

INV081

Recombinant adeno-associated virus production in invertebrate cell suspension cultures

R Kotin [1]

1: Voyager Therapeutics, Cambridge, Ma

Conventional rAAV production approaches based on plasmid DNA co-transfection of adherent cells requires either pooling or sequential multiple batch processing to produce sufficient quantities of clinical grade rAAV vectors to complete most non-clinical and clinical studies. [The cytotoxic effects of the helper virus gene products, for example adenovirus E4orf4 or HSV-1 ICP0, preclude establishing continuous producer cell lines thereby necessitating transient gene expression.] For pre-clinical development, minimizing the number of production runs needed to generate rAAV improves the reliability of data thereby increasing the predictability of the outcomes. Additionally, fewer production batches provide economic benefits by reducing time, materials, and eliminating repetitive costs associated with in-process and release assays. As an alternative to adherent cell culture, suspension cell cultures may be expanded volumetrically thereby greatly increasing the vector production capacity vis à vis an areally expanded adherent cell culture. However, efficiently introducing the necessary AAV and helper virus genes into the host cells represents the main challenge for suspension culture production of rAAV. A pivotal discovery at the National Institutes of Health demonstrated that lepidopteran cells derived from Spodoptera frugiperda (Sf9) supported Rep-dependent replication of single-stranded rAAV vector genomes enabling large-scale rAAV manufacturing in suspension culture. Due to genetic instability resulting from the duplicated Rep ORFs, the 1st generation system was sufficient for non-GMP vector production but probably not suitable for commercial development. The 2nd generation system overcame the genetic instability and also consolidated three baculovirus expression vectors (BEV), into two BEVs. The AAV non-structural Rep proteins and structural Cap proteins ORFs were engineered for stoichiometric protein expression and a second BEV introduces the rAAV vector genome. Capsids assembly appears unaffected by the switch to the invertebrate cells that are hosts for the invertebrate Densovirinae parvovirus. In addition, with the exception of AAV2, X-ray crystallographic capsid structures were determined with capsids produced in Sf9 cells. Adapting the methodologies and materials from the National Institutes of Health, Voyager Therapeutics has developed current Good Manufacturing Practice (cGMP) compliant processes for large-scale rAAV production in single-use bioreactors. Upstream and downstream process optimization has improved yields, recovery efficiency, product purity, and batch-to-batch consistency. Together with rigorous quality control, a single large-scale production run eliminates the variability and reduces the costs associated with multiple batch production processes. Multiple runs with this scalable system will enable the cost effective supply of drug substance to larger patient populations.

INV082

Improving liver-directed gene therapy and CRISPR/Cas9-based hepatic genome engineering

T vandenDriessche [1] N Nair [1] H Evens [1] K Singh [1] M Rincon [1] S Sarcar [1] M Di Matteo [1] F Mingozzi [2] C Le Guiner [3] P De Bleser [4] A Cantore [5] N Ward [6] S Waddington [6] J Mc Vey [6] P Moullier [3] L Naldini [5] M KL Chuah [1]

1: Free University of Brussels & University of Leuven 2: INSERM U649 3: University of Nantes 4: VIB, Ghent, Belgium 5: San Rafaelle Telethon Institute for Gene Therapy, Milano 6: Institute of Child Health, University College London

The robustness and safety of liver-directed gene therapy for hemophilia could be substantially improved by enhancing factor VIII (FVIII) and factor (FIX) expression in the liver. To achieve this, we developed and validated a new computational approach of rational in silico vector design. This approach relies on a genome-wide bio-informatics strategy to identify cis-acting regulatory modules (CRMs) containing evolutionary conserved clusters of transcription factor binding site motifs that determine high tliver-specific gene expression. Incorporation of these CRMs into adeno-associated viral (AAV) and non-viral plasmid and transposon vectors enhanced gene expression in mice liver 10 to 100-fold, depending on the promoter used. In particular, these CRMs resulted in robust and sustained liver-specific FIX expression and induction of FIX-specific immune tolerance in mice. FIX peak levels of 20-35% could be attained in cynomolgus macaques after AAV-based liver-directed gene therapy. Given its small size (72 bp), the most potent CRM is particularly well suited to boost FVIII

expression using AAV vectors, allowing the production of physiologic FVIII at low, clinically relevant vector doses. This obviated the need to boost FVIII expression through modification of its coding sequence by incorporating artificial glycosylation sites. Using a sensitive hepatocellular carcinoma-prone mouse model, we found no evidence of increased genotoxic risk of these CRM elements when integrating vectors are employed. By combining codon usage optimization with the hyperactivating FIX-R338L Padua mutation we obtained a 15-fold gain in potency which was validated using different vector platforms including AAV, integration-competent and –defective lentiviral vectors and piggyBac transposons, allowing the use of lower and thus potentially safer vector doses. The use of computational vector design, synthetic (hyperactive), FVIII and FIX transgenes are promising avenues to further improve the efficacy, feasibility, and safety of hemophilia gene therapy with broad implication for liver-directed gene therapy. Finally, we built further upon this concept of computational vector design to develop a robust platform for tissue-specific gene inactivation in the liver using CRISPR/Cas9. As a proof of concept, we demonstrated that liver-specific over-expression of Cas9 in combination with FIX-specific guide RNAs resulted in the selective inactivation of the endogenous murine FIX gene consistent with the emergence of a hemophilic phenotype. This opens new perspectives for the use of CRISPR/Cas9 for in vivo somatic genome engineering in specific target organs. Funding: FWO, EU FP7, AFM, SRP VUB (‘Grower’), IOF VUB GEAR

INV083

CAR T-Cells; where do we go next?

M K Brenner [1]

1: Baylor College of Medicine

Much excitement has been generated by the success of CAR-T cells for the treatment of B cell malignancies. Achieving equivalent success for solid tumors will be more problematic. The solid tumor environment is immunosuppressive, and there are few consistently expressed antigens that are unique to the tumor and can be recognized by a CAR. Moreover, the genetic instability of tumors means that their antigenic profile evolves with time and location in the patient. These characteristics require us to think differently about how best to develop and use CAR T-cells. In this presentation, I will outline the need to develop CAR T-cells that recognize tumor patterns rather than discrete tumor targets; to employ natural selection and not just intelligent design for this purpose; and to modify the developmental model for conventional drugs to take account of these opportunities and challenges.

INV084

Gene transfer by lentiviral vectors and gene editing from bench to bedside

L Naldini [1]

1: HSR TIGET, San Raffaele Telethon Institute for Gene Therapy

Lentiviral vectors are one of the most widely used tool in biomedical research and, upon recently entering clinical testing, are providing a long-sought hope of cure for some otherwise deadly human diseases. I was lucky enough to be involved in the original development of this technology and contribute to advance it until clinical testing. Over the years, as we faced the challenge to achieve safe and effective gene transfer, we devised novel strategies to broaden the reach and improve the precision of genetic manipulation, such as microRNA mediated regulation to sharply target vector expression to the desired cell type or stage, and gene editing by artificial endonucleases and lentiviral donor template. We have exploited the improved tools to gain novel insights into relevant biological processes such as hematopoietic stem cell function, induction of immunological tolerance and tumor angiogenesis. These advances are now being translated into new therapeutic strategies for genetic disease and cancer. My presentation will account these scientific undertakings as well as my lifetime journey, which took me over several Institutions throughout the world and gave me the opportunity to work with many talented scientists who as mentors, colleagues and alumni gave an invaluable contribution to these results.

INV085

Applications of integration-deficient lentiviral vectors (IDLVs)

R J Yáñez-Muñoz [1]

1: School of Biological Sciences, Royal Holloway, University of London

Lentiviral vectors have shown great efficacy in many research applications and a number of clinical trials, but their integration in the host cell genome precludes some uses and implies a risk of insertional mutagenesis. We developed integration-deficient lentiviral vectors (IDLVs) in the previous decade to expand the range of lentiviral applications and address the mutagenesis concern. While previously IDLVs had been used as negative controls for standard integrating lentiviral vectors, our observation of their transient transduction proficiency in dividing cells in culture prompted a re-examination of in vivo utility. Initial eGFP expression experiments in quiescent cells in eye and brain showed effective and stable transduction by IDLVs. This proficiency was confirmed by rescue of two rodent models of retinal degeneration, the Rpe65 mouse and the Mertk rat. More recently we have demonstrated IDLV-mediated rescue in a rat model of Parkinson disease and effective transgene expression in the spinal cord. The usefulness of IDLVs is not limited to gene expression, as they are also very effective for delivery of cassettes designed for site-specific integration, transposition and gene editing. For the latter application, we have recently demonstrated partial rescue of T-cell deficiency by ex vivo gene editing and transplantation of haematopoietic stem/progenitor cells in the Prkdc scid mouse, a model of primary immunodeficiency, using IDLVs to deliver both the gene editing template and the designer nuclease. Finally, modification of culture conditions at the time of transduction has led to highly elevated frequencies of IDLV episome establishment in dividing cell populations, prompting further

efforts to achieve stable expression from IDLV episomes in proliferating cells.

OR079

Reprogramming, iPS cells and human artificial chromosomes for gene and cell therapy of muscular dystrophy and beyond

F S Tedesco [1]

1: Department of Cell and Developmental Biology, University College London, London, United Kingdom

Successful autologous cell therapies for muscular dystrophies are challenged by hurdles related to the target tissue, myogenic cells and gene therapy vectors. Skeletal muscle is the most abundant tissue of the human body, whose self-renewal is sustained by a pool of stem/progenitor cells that exhaust with disease progression and are difficult to expand in vitro. An archetypical example is Duchenne muscular dystrophy (DMD), the most common incurable muscle disorder of childhood caused by mutations in the largest gene known in nature: dystrophin (2.4Mb).
Here I present our portfolio of technologies developed to overcome the above-mentioned hurdles. In order to allow transferring of the entire dystrophin genetic locus to host cells, novel human artificial chromosomes (HACs) are currently being engineered and transferred to myogenic progenitors. When isolation of muscle-derived stem/progenitor cells proves challenging, host cells can be reprogrammed to pluripotency and the resulting iPS cells differentiated towards the myogenic lineage, as we reported for Limb-Girdle 2D and Duchenne muscular dystrophies. We have recently validated this approach using human embryonic stem cells. We are also refining the human iPS cell-based platform using non-integrating vectors and xeno-free media. Notably, recent data showing extension of our human iPS cell-derived, inducible myogenesis platform to muscle tissue engineering, disease modelling and drug development will also be presented. Finally, a novel strategy based upon direct reprogramming of satellite cell-derived myoblasts to muscle pericyte-like cells will be presented; this strategy provides evidence of a druggable pathway that might have a clinically relevant potential, allowing systemic delivery of myoblasts in cell therapy protocols for muscle diseases. Overall the above-described platforms are likely to advance the development of novel, successful gene and cell therapy protocols for muscular dystrophies.

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Selected Oral Presentations

OR001

Immunomodulatory progenitor cells: a novel allogeneic therapy for patients with ischaemic cardiomyopathy undergoing coronary artery bypass grafting

S Sultan[1] A Reginald[1] M J Evans[1] P Antonitsis[2] S Westaby[3] K Anastasiadis[2]

1: Cell Therapy Limited 2: AHEPA University Hospital, Thessaloniki, Greece 3: Oxford Heart Centre, The John Radcliffe Hospital, Headington, Oxford, UK.

Immuno-modulatory progenitor cells or iMP cells, are a novel and distinct mesodermal progenitor cell that do not meet the International Society for Cellular Therapy mesenchymal stromal cell (MSC) definition but do exhibit an immune-modulatory (MIC A/B, CD178, CD289, CD99 and EGF-R) and cardiac specific phenotype (CD181, CD126, CD304, CD363 and CD182). Thus allogeneic iMP cells were used in a phase II (Heartcel) clinical trial of advanced heart failure patients incompletely re-vascularized (ICR) by Coronary Artery Bypass Graft (CABG). ICR affects ~37% of CABG patients and is associated with a ~40% increase in mortality and ~49% increase in major adverse cardiac events (MACE). The Heartcel trial completed in Q4 2014. Concomitant with CABG, iMP cells were injected intra-myocardially into the areas of hypo-kinetic myocardium bypass would not re-vascularise. SPECT imaging was used pre-operatively to identify these iMP injection sites and post-operatively to monitor/measure change in viability and contractility. The study met all endpoints: 100% 1 year MACE-Free survival in all patients that persists to date (18–24 months). Clinically and statistically significant mean improvement in LVEF (30%), LV scar size (40%) and quality of life (50%). The results suggest the potential for in situ myocardial regeneration to mitigate the effect of incomplete revascularization in heart failure.

OR002

Lessons learned from first-in-man gene therapy clinical trial for pancreatic cancer: targeting tumor metabolism to tackle chemoresistance

M Gayral[1] E Saland[1] H Lulka[1] A Vignolle-Vidoni[1] N Hanoun[1] A Lemarié[1] L Ligat[1] N Saint-Laurent[1] F Lopez[1] L Buscail[2] J E Sarry[1] P Cordelier[1]

1: INSERM 1037, Toulouse, France 2: CHU Toulouse Oncopole

Pancreatic cancer (PDAC) remains a deadly disease with no cure. In this dismal context, we demonstrated that non-viral gene therapy to sensitize cancer cells to chemotherapy was safe and feasible, and offers therapeutic benefit in patients (Thergap trial). However, alternative molecular pathways must be targeted to relieve resistance to treatment for best therapeutic benefit. We identified cytidine deaminase (CDA), that catalyzes the hydrolytic deamination of cytidine and deoxycytidine to uridine and deoxyuridine, as overexpressed in cohorts of patients resisting to gemcitabine and in PDAC as compared to normal parenchyma. Targeting CDA using genetic tools sensitizes cancer cells to chemotherapy both in vitro and in vivo. In the absence of chemotherapy, loss of CDA unexpectedly alters cell proliferation and tumor progression. In treated cells, nucleotide levels are decreased and Krebs cycle is altered, strongly suggesting mitochondrial dysfunction, as we demonstrate decreased mitochondrial ATP and down expression of key proteins of the mitochondrial OXPHOS complexes and b-oxidation. Consequently, mitochondrial ROS are elevated with major changes in the redox balance in PDAC cells. Remarkably, tumor cells retaliate using compensatory mechanisms including Pasteur Effect. Taken together, this study illustrates that genetic depletion of CDA reverses the chemoresistance of PDAC cells to standard-of-care therapy, and, for the first time, that CDA is essential to the energetic metabolism of PDAC cells, with new insights in synthetic lethality pathways that may afford new targeting opportunities. This study stems for the genetic targeting of CDA, alone or in combination, for the management of patients with PDAC.

OR003

Early and self-limiting cytokine release syndrome by CD44v6 CAR-T cells in human hematochimeric NSG mice

M Norelli[1 2] M Casucci[2] B Camisa[2] L Falcone[2] A Saudemont[3] P Genovese[4] L Naldini[1 4] F Ciceri[5] C Bordignon[1] C Bonini[6] A Bondanza[1 2]

1: Vita-Salute San Raffaele University 2: Innovative Immunotherapies Unit, San Raffaele Hospital and Scientific Institute, Milano IT 3: Immunotherapy Group, Anthony Nolan Institute, London UK 4: Gene Transfer Technlogy Unit, San Raffaele Hospital and Scientific Institute, Milano IT 5: Hematology and Bone Marrow Transplantation Unit, San Raffaele Hospital and Scientific Institute, Milano IT 6: Experimental Hematology Unit, San Raffaele Hospital and Scientific Institute, Milano IT

Background: The antitumor efficacy of chimeric antigen receptor (CAR) T cells often associates with the cytokine release syndrome (CRS). Currently available xenograft mouse models are poorly predictive of the potential of CAR-T cells to cause this life-threatening complication. Severely immunocompromised NSG mice indeed lack functional monocytes, which are the main in vivo source of IL-6, the master cytokine of CRS.

Aim: To preclinically assess the risk of CRS by CAR-T cells targeted against the CD44v6 antigen.

Results: NSG mice were simultaneously humanized with cord blood-derived hematopoietic stem cells (HSCs) and with

the CD44v6 + ALL-CM leukemia semi-cell line. After full hematopoietic reconstitution and leukemia engraftment, hematochimeric NSG mice were infused with human autologous T cells genetically modified with a CD28-endocostimulated CD44v6 CAR following ex vivo activation with CD3/CD28-beads and IL-7/IL-15. In vivo leukemia clearance associated with transient malaise, high fevers and weight loss. Differently from control mice infused with CD19 CAR-T cells, this syndrome was however significantly anticipated (median 3 vs. 8 days) and self-limited, coinciding with monocyte depletion by CD44v6 CAR-T cells. Interestingly, this syndrome overlapped with a surge in human IL-6 levels, which were not observed in NSG that had not been previously humanized with HSCs. Moreover, there was a clear correlation between CRS severity and the leukemic burden prior to CAR-T cell infusion, mimicking what has been observed in clinical trials

Conclusions: These results validate an innovative xenograft mouse model predictive of clinical CRS and suggest that monocyte depletion by CD44v6 CAR-T cells may restrain the severity of this complication.

OR004

Preclinical efficacy of a chemo-virotherapy treatment for pancreatic cancer

E A Cafferata[1] H L Weber[1 2] S Werbajh[1] E Salvatierra[1] C Rotondaro[1] L Sganga[1] G Acosta Haab[3] D T Curiel[4] M Gidekel[2] O L Podhajcer[1]

1: Laboratory of Molecular and Cellular Therapy, Fundación Instituto Leloir, IIBBA-CONICET, Argentina 2: Universidad de La Frontera, Chile. 3: Laboratory of Pathology, Hospital de Oncología Marie Curie, Buenos Aires, Argentina 4: The Division of Cancer Biology, Washington University School of Medicine, St. Louis USA.

Pancreatic cancer is predicted to become the second leading cause of cancer-related death around 2020. Early stages are usually asymptomatic and patients present with advanced metastatic disease. The high resistance to conventional and targeted therapies is largely due to the dense extracellular matrix devoid of an extended blood vasculature that hampers drug penetration deeply inside the tumor mass. Novel therapeutic tools designed to target the tumor stromal cells in addition to the malignant cells might then become a valuable tool. We designed an oncolytic adenovirus (OAV) whose replication was driven by the cdc25B promoter. The corresponding gene is highly expressed in primary and metastatic pancreatic cancer both in human malignant cells and cancer associated fibroblasts. The combination of the OAV AV25CDC and gemcitabine exhibited the largest therapeutic effect on orthotopically implanted human xenografts tumors in nude mice and on syngeneic tumors in Syrian hamsters. Indeed, nude mice harboring 15-days old SW19990 orthotopic tumors, treated i.t. or systemically with AV25CDC combined with gemcitabine, exhibited 70%-80% reduction in tumor size that lasted for at least 60 days. Chemo-virotherapy treatment induced a return to normal levels of biochemical parameters of hepatic toxicity; mice also exhibited more than 90% reduction in CA19.9 serum levels. Chemo-virotherapy efficacy was confirmed in mice harboring Mia PaCa-2 tumors and in Syrian hamsters harboring syngeneic HaP-T1 tumors. Biosafety studies showed no evidence of toxicity exerted by AV25CDC. We observed that viral treatment disrupted the tumor architecture and induced an increase in MMP-9 activity that might facilitate gemcitabine penetrability.

OR005

New insights into AAV vectors integration profile: internal vector regions

I Gil-Farina[1] C Kaeppel S Wilkening M A Pañeda Rodriguez E Lopez-Franco A Fontanellas J Prieto L Spronck C von Kalle H Petry G Gonzalez-Aseguinolaza M Schmidt

1: DKFZ and National Center of Tumor Diseases (NCT) Heidelberg 2: Digna Biotech S.L. 3: CIMA 4: University Clinic of Navarra 5: uniQure

Recombinant adenoassociated viruses (rAAV) allow long-term transgene expression in post-mitotic tissues due to their ability to persist as concatemeric structures. However, although at rare frequencies, rAAV randomly integrates into the host genome requiring the study of vector integration to ensure therapy's biosafety. The inverted terminal repeats (ITR) constitute the preferred breakage points within these vectors, nonetheless recent studies suggest that breakage may occur along the whole vector sequence. Current integration analyses are mostly based on the linear amplification-mediated (LAM)-PCR technology, where genomic regions adjacent to the ITRs are amplified for integration site identification, meaning that new methods are required to identify the integration of internal vector regions. We present a novel LAM-PCR-based approach simultaneously employing five primer sets covering the whole vector sequence. Concurrently to standard LAM-PCR, multiplex LAM-PCR was performed in tissues from monkeys injected with the AAV2/5-AAT-coPBGD vector, which has been used in studies for the treatment of the acute intermittent porphyria. Subsequent 454 sequencing and data analysis of ~1million raw sequences for each tissue allowed the identification of near 800 integration sites and identified the C-region of the ITR as preferred vector breakage site. In addition, multiplex LAM-PCR showed breakage at different positions of the AAV vector genome in all the organs analyzed. Our data show the suitability of this method for the analysis of viral integration occurring at inner regions of the vector, thus providing a deeper insight into rAAV integration and contributing to increase the thoroughness of the safety studies in gene therapy clinical trials.

OR006

Enhanced AAV vector transduction through genetic and pharmacological inhibition of U2 snRNP components

C A Schreiber T Sakuma Y Izumiya R K Bressin U Basu K Koide A Asokan Y Ikeda

1: Mayo Clinic 2: University of California, Davis 3: University of Pittsburgh 4: University of North Carolina

Adeno-associated viruses (AAV) have evolved to exploit the dynamic reorganization of host cell machinery during co-infection by adenoviruses and other helper viruses. In the absence of helper viruses, host factors such as the proteasome and DNA damage response machinery have been shown to effectively inhibit AAV transduction by restricting processes ranging

from nuclear entry to second-strand DNA synthesis. To identify host factors that might affect other key steps in AAV infection, we screened an siRNA library that revealed several candidate genes including the PHD finger-like domain protein 5A (PHF5A), a U2 snRNP-associated protein. Disruption of PHF5A expression selectively enhanced AAV transduction by increasing transcript levels and appears to influence a step after second-strand synthesis in a serotype and cell type-independent manner. Notably, genetic disruption of U2 snRNP and associated proteins, such as SF3B1 and U2AF1, also increased AAV vector transduction, suggesting the critical role of U2 snRNP spliceosome complex in this host-mediated restriction. Moreover, pharmacological inhibition of U2 snRNP by meayamycin B, a potent SF3B1 inhibitor, substantially enhanced AAV vector transduction of clinically relevant cell types. Further analysis indicated that U2 snRNP proteins suppress AAV vector transgene expression through direct recognition of intact AAV capsids. In summary, we identify U2 snRNP and associated splicing factors, which are known to be affected during adenoviral infection, as novel host restriction factors that effectively limit AAV transduction. Concurrently, we postulate that pharmacological/genetic manipulation of components of the spliceosomal machinery might enable more effective gene transfer modalities with recombinant AAV vectors.

OR007

Harnessing the potential of miRNAs to enhance production of recombinant adeno-associated virus (rAAV) vectors

V V Emmerling[1] S Fischer[2] S Stiefel[2] R Handrick[2] M Hoerer[3] K Otte[2] S Kochanek[1]

1: Ulm University 2: University of Applied Sciences Biberach 3: Rentschler Biotechnologie GmbH

Recent success of vectors based on recombinant Adeno-associated virus (rAAV) used in diverse gene therapy applications raises the demand for clinical and market supply material. However, substantial process improvements are required to achieve robust and economic manufacturing of rAAV vectors at large scale. Attempts to increase the yield of these systems by using biphasic production processes or host cell engineering, like it is done for classical protein expression systems have not been implemented yet. In the present study, we investigated whether pro-productive effects of mild hypothermia can be transferred from established Chinese hamster ovary (CHO) based productions to human expression hosts used for manufacturing of viral vectors. We observed significantly increased rAAV yields when HeLa producer cells were maintained at decreased culture temperature during production. Strikingly, microRNA (miRNA) profiling studies revealed miR-483 to be one of the most dramatically up-regulated miRNAs in response to mild hypothermia in both CHO and HeLa cells. In functional validation experiments we discovered that enforced expression and inhibition of miR-483 is directly linked to rAAV vector productivity. Furthermore, target gene analysis resulted in identification of temperature and miR-483 modulated genes which are directly involved in signaling pathways regulating cellular survival and productivity. Our results suggest that miR-483 is a universal regulator during adaptation of cells to mild hypothermia and a key inductor of viral vector productivity. Thus, miRNAs represent novel molecular tools to improve challenging viral vector productions in the future.

OR008

Increasing endothelialization of coronary stents with local adenoviral VEGF-A gene therapy in naïve pig coronary arteries

J P Hytonen[1] P Halonen[1] S Tarvainen[1] J Taavitsainen[1] A Kuivanen[1] A Koistinen[1] J Laakkonen[1] J Hartikainen[1] S Yla-Herttuala[1]

1: A.I.Virtanen Institute, University of Eastern Finland

We aimed to increase stent endothelialization with local gene therapy known to promote endothelial proliferation. In addition, we evaluated OCT and angioscopy imaging as tools for detecting endothelium with comparison to ex-vivo analyses of endothelialization. 12 bare metal (BMS) and 12 drug eluting (DES) stents were implanted in porcine coronary arteries and received either AdVEGF-A or control AdLacZ gene transfers with a porous drug delivery catheter. Stents were imaged in-vivo with angiography, OCT and angioscopy immediately after, one week and two weeks after stenting. At d14 the stents were collected for histology, multi-photon microscopy and scanning electron microscopy for assessment of endothelialization. Strut coverage was analyzed from SEM images and graded 0 (no coverage) to 3 (fully covered). Angioscopy pullbacks were analyzed for thrombus in the stented artery and graded similarly 0 (no thrombus) to 3 (occluding thrombus). SEM analyses two weeks after intervention showed increased strut coverage with BMS after VEGF-A gene therapy compared to control LacZ (2.8 ± 0.4 vs. 1.8 ± 0.4, $p = 0.0031$, respectively). Gene therapy did not improve strut coverage in DES (1.6 ± 0.5 and 1.8 ± 0.4, p = NS, AdLacZ and AdVEGF respectively). Angioscopic thrombus formation was low in all groups with a trend towards less thrombus on BMS groups (0.2 ± 0.4 and 0.3 ± 0.5, AdLacZ and AdVEGF) compared to DES (0.8 ± 0.8 and 0.8 ± 0.7, AdLacZ and AdVEGF). AdVEGF treatment increased coverage of BMS but did not improve healing of DES two weeks after stenting. Most likely the strong cytotoxic drugs in DES hamper stent healing even with local supraphysiological growth factor concentrations.

OR009

GRK2 expression levels appear to determine the beneficial outcome of AAV9-mediated βARKct gene therapy in different mouse models of inherited cardiomyopathy

R Bauer[1] H Enns[1] A Jungmann[1] B Leuchs[2] C Volz[1] S Schinkel[1] W J Koch[3] P W Raake[1] P Most[1] H A Katus[1] O J Müller[1]

1: Internal Medicine III, University Hospital Heidelberg, Germany 2: Tumor Virology, German Cancer Research Center Heidelberg 3: Center for Translational Medicine, Temple University School of Medicine, Philadelphia, USA

In many patients with muscular dystrophies evolving cardiomyopathy has strong impact on mortality. However, effective strategies to treat cardiomyopathy in these patients are not clearly defined. Previously, treatment with β-blockers showed beneficial effects in a mouse model of Duchenne muscular dystrophy (mdx), but not in δ-sarcoglycan-deficient mice (Sgcd-/-), a model of limb girdle muscular dystrophy type 2F (LGMD2F). We therefore aimed to study a more specific approach to target maladaptive β-adrenergic signalling in these models of cardiomyopathy.

Lowering cardiac G protein-coupled receptor kinase-2 (GRK2) activity with βARKct expression, a peptide inhibitor of GRK2, has resulted in improvement of heart failure in several different animal models. Therefore, we investigated whether βARKct gene delivery using adeno-associated virus type 9 (AAV9), a more specific approach in preventing desentization of the G protein-coupled receptor by displacing GRK2 from the plasma membrane than beta-blockers, could ameliorate development of cardiomyopathy in mdx and Sgcd-/- mice. We found that long-term treatment with AAV9- βARKct-cDNA with a cardiac-specific promoter could significantly improve left ventricular systolic function and ameliorate myocardial hypertrophy in mdx mice, whereas beneficial effects on cardiac function in Sgcd-/- mice were mild without protection from hypertrophy. Interestingly, in contrast to mdx mice neither GRK2 nor nuclear factor-kappaB (NFκB) were upregulated in Sgcd-/- mice, indicating the existence of distinct pathogenic mechanisms in these cardiomyopathic mouse models. Taken together, effectiveness of AAV-mediated βARKct therapy may vary between different genetic causes and presumably dependent on the state of adrenergic dysregulation mediated through the upregulation of GRK2.

OR010

Therapeutic angiogenesis: novel insights in the mechanism of action of FGF-4

G M Rubanyi C Basilico

1: Angionetcs Inc. 2: New York University, School of Medicine

Generx (Ad5FGF-4) is currently in Phase 3 clinical trial as a therapeutic angiogenesis product candidate for the treatment of patients with refractory angina. Experiments were performed in cultured cells to explore whether FGF-4 is capable to interact with the adenoviral vector and endogenous VEGF. The potential interaction with the Ad5 vector was tested in cultured RCS chondrocytes and 3T3 fibroblasts. Ad5GFP (M.O.I. 10vp/cell) was added to the cells, which were treated 24 hours later by FGF-4 (10ng/ml). FGF-4 stimulated gene expression 5-10 fold (GFP fluorescence and Western blot) in both RCS (growth inhibition) and 3T3 cells (growth promotion). This novel observation suggests a positive interaction between the Ad5 vector and the transgene of Generx, potentially leading to increased extent and duration of gene expression after Ad5FGF-4 administration. The interaction between FGF-4 and endogenous VEGF was studied in a co-culture system of human dermal fibroblasts (HDF) and human umbilical vein endothelial cells (HUVEC). FGF-4 (1-8 ng/ml) stimulated new vascular network formation, including tube elongation and branching, which was significantly inhibited by both anti-VEGF (4 ug/ml) and anti-VEGFR2 (30 ug/ml) antibodies. FGF-4 stimulated the release of VEGF from HDF, but not from HUVEC. These results demonstrate that FGF-4 stimulates the release of VEGF from non-endothelial cells and FGF-4 and VEGF act synergistically on endothelial cells in the formation of new vascular structures.

OR011

Immunomodulatory properties of human MuStem cells: assessing their impact on adaptive and innate immunity

J Lorant[1 2 3] N Jaulin[3 4] I Leroux[1 2 3] C Schleder[1 2 3] C Zuber[1 2 3] M Charrier[1 2 3] B Lieubeau[5] Y Péréon[6] A Magot[6] A Hamel[7] O Adjali[3 4] K Rouger[1 2 3]

1: INRA UMR703 PAnTher, F-44307 Nantes, France 2: LUNAM Université, Oniris, École nationale vétérinaire, agro-alimentaire et de l'alimentation Nantes-Atlantique, Nantes, F-44307, France 3: Atlantic Gene Therapies, F-44000 Nantes, France 4: INSERM UMR1089, Nantes University Hospital, F-44000 Nantes, France 5: INRA, USC 707 Université, EA 4644 IECM, Nantes, F-44307, France 6: Centre de Référence des maladies neuromusculaires rares Nantes-Angers, Service des Explorations Fonctionnelles, Centre Hospitalier Universitaire Hôtel-Dieu, Nantes, F-44093, France 7: Service de Chirurgie Infantile, Centre Hospitalier Universitaire Hôtel-Dieu, Nantes, F-44093, France

Several preclinical approaches based on allogeneic stem cell delivery were shown to be attractive for the treatment of genetic muscular dystrophies. Nevertheless, a significant hurdle for their clinical translation is the immune rejection of donor cells. Immunosuppressive regimens are generally used to overcome host immunity and can allow the improvement of graft survival. Nevertheless, they are associated with a number of side effects, limiting their long term use. Recently, some tissue-specific adult stem cell populations were described to exhibit immunomodulatory properties that could increase their ability to engraft in an allogeneic recipient and improve their regenerative potential. We have previously demonstrated that allogeneic muscle-derived delayed adherent stem cells (that we called MuStem cells) are able to phenotypically and clinically correct the Duchenne dystrophic canine model (Rouger et al., 2011; Robriquet et al., 2015). Recently, we isolated human MuStem cells and assessed their immunomodulatory potential. We evaluated their ability to inhibit T cell proliferation and to modulate the complement pathway. Interestingly, our preliminary data showed that human MuStem cells were able to modulate allogeneic T cell proliferation and to express immunomodulatory molecules such as prostaglandin-E2, indoleamin-2,3-deoxygenase-1 and TGFβ2. Moreover, MuStem cells were also able to secrete Factor H molecule suggesting a potential effect on the alternate pathway of the complement system. Overall, our study is critical for the understanding of the crosstalk between MuStem cells and the immune system, as well as the design of safe and efficient allogeneic stem cell-based therapy for the treatment of muscle dystrophies.

OR012

Targeted genome editing in human long-term repopulating hematopoietic stem cells for the correction of SCID-X1

P Genovese[1] G Schiroli[1 2] A Conway[3] D Guschin[3] G Escobar[1 2] T Di Tomaso[1] V Marin[4] E Giuliani[4] C Firrito[1] P D Gregory[3] M C Holmes[3] B Gentner[1] C Bovolenta[4] G J Cost[3] A Lombardo[1 2] L Naldini[1 2]

1: San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET) 2: Vita-Salute San Raffaele University 3: Sangamo BioSciences, Inc. 4: MolMed S.p.A.

Targeted genome editing has brought gene correction within the reach of gene therapy. We recently showed that gene targeting in the most primitive hematopoietic stem/progenitor cells (HSPC) is constrained by gene transfer efficiency and a bias against the use of homology directed repair. By combining Integrase Defective Lentiviral Vectors (IDLV) for donor template delivery and mRNA transfection for ZFN expression and

tailoring culture conditions, we overcame in part these barriers and provided evidence of targeted integration (TI) in human HSPC. We achieved TI of a corrective cDNA into a mutational hotspot of IL2RG gene with high efficiency (~6%) and specificity (>95%) in long-term repopulating HSPC. The targeted cells generated polyclonal lymphoid cells that were functionally indistinguishable from wild-type, proving functionality of the edited gene. Here, in order to establish a transferable-to-the-clinic gene correction protocol we further optimized reagents and scaled-up the gene editing procedure. We developed new nucleases targeting the upstream region of the IL2RG gene to correct the majority of SCID-X1 mutations with only one ZFN/donor set. We compared the performance of IDLV and AAV6 as donor vehicles and found a similar rate of cDNA insertion into intron-1 of IL2RG gene. We found that inclusion of modified nucleotides during mRNA production and clinical grade purification of IDLV allow decreasing cellular innate response and electroporation toxicity, respectively. Currently, we are producing large-scale lots of gene-corrected cells using the Maxcyte electroporator and have successfully treated up to 40 million HSPCs and have shown repopulation capacity in NSG mice.

OR013

Innate immune sensors restrict lentiviral gene delivery to human natural killer cells: novel perspectives on the use of viral vectors

E C Sayitoglu[1 2] A D Duru[3 4] M Chrobok[3 4] C Chiang[4] A Parlar[1 2] G H Senturk[1 2] B Erman[2] E Alici[3 4] T Sutlu[1]

1: Sabanci University, Nanotechnology Research and Application Center, Istanbul, Turkey 2: Sabanci University, Faculty of Engineering and Natural Sciences, Istanbul, Turkey 3: Karolinska Institutet, Department of Medicine Huddinge, Centre for Hematology and Regenerative Medicine, Stockholm, Sweden 4: Vaccine and Gene Therapy Institute of Florida, Centre for Diseases of Aging, Port St. Lucie, Florida, USA

Natural Killer (NK) cells are members of the lymphocytic lineage that have an essential role in immunosurveillance against tumors and infected cells. The potential of using NK cells for cancer immunotherapy continues to inspire research on various perspectives ranging from ex vivo expansion to genetic modification for maximizing and directing anti-tumor activity. Genetic modification of NK cells can be carried out with lentiviral vectors, however the gene transfer efficiency is markedly lower when compared to other cells of hematopoietic origin. We have hypothesized that the innate antiviral defense systems in NK cells restrict lentiviral vector entry and the inhibition of these signals can lead to better gene delivery. Our results show that blocking the intracellular antiviral defense mechanisms by inhibiting the TBK1/IKKε kinase complex that acts downstream of RIG-I, MDA-5 and TLR3, provides a significant increase in the efficiency of lentiviral gene delivery to NK cells. RNA sequencing analysis of the differential gene expression profile during lentiviral vector entry to NK cells in the presence or absence of the inhibitor BX795 has provided a detailed map of pathways that trigger antiviral responses against gene therapy vectors. In this study, we present the analysis of the NK cell response to lentiviral gene delivery and our efforts to modulate these pathways using small molecule inhibitors in order to increase gene delivery efficiency, not only in the specific case of NK cells but also for applications in gene therapy protocols with other cell types.

OR014

Assessing the role of LRRK2 G2019S mutation and the genomic background to the development of PD-related neurodegeneration

C Calatayud[1 2] A Faella[1] S Jiménez[2 3] I Fernández[1] Y Richaud-Patin[2 3] E Tolosa[5 6] C Mussolino[7 8 9] T Cathomen[7 8 9] A Raya[2 3 10] A Consiglio[1 4]

1: Institut de Biomedicina Universitat de Barcelona 2: Institute for Bioengineering of Catalonia (IBEC) 3: CIBER-BBN 4: University of Brescia and National Institute of Neuroscience 5: CIBERNED 6: Universitat de Barcelona-IDIBAPS 7: University Medical Center Freiburg-Institute for Cell and Gene Therapy 8: University Medical Center Freiburg-Center for Chronic Immunodeficiency 9: Hannover Medical School-Institute of Experimental Hematology 10: ICREA

Despite the advances in the identification of genes and proteins involved in Parkinson's Disease (PD), there are still appreciable gaps in our understanding of the mechanisms underlying the chronic neurodegenerative process in PD. We have recently demonstrated that iPSC technology can be used to observe phenotypes relevant to neurodegeneration in PD, and also provided first proof-of-principle evidence that neurons with the genome of a sporadic PD patient exhibited similar phenotypes as seen in iPSC derived from patients with monogenic LRRK2 (G2019S) PD. Here we planned to generate a complementary set of iPSC lines from asymptomatic patients carrying pathogenic LRRK2 mutations, in order to test the relative contribution of pathogenic mutations and gene susceptibility factors to PD-related DAn neurodegeneration. We then corrected the LRRK2 mutation by using TALEN-mediated genetic engineering in the asymptomatic LRRK2-iPSC lines, as well as well as in our already established LRRK2-PD iPSC lines. Dopaminergic neurons differentiated in parallel from this subset of iPSC lines have been cultured over a long time span and monitored for the appearance of neurodegeneration phenotypes. Transcriptomic profiling is also being considered to unveil possible stress mechanisms DAns may face. The availability of a refined set of PD patient-specific iPSC lines representing symptomatic and asymptomatic cases of familial PD sharing the same pathogenic mutation in LRRK2, as well as isogenic iPSC lines in which the mutation has been edited out, will provide a unique test bed for revealing the specific genetic determinants contributing to or preventing the neurodegeneration in PD.

OR015

Insulin mutation causing neonatal diabetes corrected with CRISPR in patient-derived iPSC

D Balboa[1] J Saarimäki-Vire[1] S Eurola[1] K Wartiovaara[1] H Huopio[2] T Otonkoski[1]

1: University of Helsinki 2: University of Eastern Finland, Kuopio

Permanent neonatal diabetes is commonly caused by mutations affecting genes encoding proteins that are important for beta-cell function, such as ATP-sensitive potassium channels or insulin. Insulin gene mutations lead to the accumulation of misfolded insulin protein, causing endoplasmic reticulum (ER) stress that eventually triggers beta-cell apoptosis and subsequent diabetes. In order to establish a human disease model of ER stress-caused diabetes, we derived iPSC lines from two Finnish families carrying heterozygous mutations in the insulin gene. These mutations are dominant and the carriers develop diabetes at 3–4 months of age. The patient-specific iPSC lines were differentiated in vitro successfully to pancreatic endocrine progenitors using an optimized protocol and, further, into functionally immature beta cells. To overcome the variability in the differentiation between the control and patient-specific iPSC, we generated isogenic cell lines by correcting the insulin mutation using CRISPR technology. A combination of guide RNAs targeting next to the mutation site, together with 70 bases single stranded DNA repair template and Cas9 resulted in efficient correction of the insulin mutation by homologous recombination. After electroporation of these components to the patient iPSCs, 22.5% of isolated clones had integrated the correction repair template. Corrected cells were differentiated in parallel with mutant cells to the beta cell lineage and differentiated endocrine progenitors were transplanted under the kidney capsule of immunodeficient mice to obtain beta cell maturation in vivo. This model provides insight into the pathogenetic mechanisms of beta-cell failure not only in these families, but also in more common forms of diabetes.

OR016

CRISPR-Cas9 Assisted Cassette Exchange (CACE) of MHC alleles in antigen presenting cells enables altered immune activity

W Kelton A Waindok M Pogson C Parola S Reddy

1: ETH Zürich

Allogeneic (non-self) cell transplantations are commonly used to treat various genetic diseases and hematological malignancies. Finding suitable donors for these therapies is often challenging, as the highly polymorphic MHC/HLA gene alleles require matching to the host to prevent transplant rejection. In order to address this limitation, we have developed an ex-vivo genome editing approach-CRISPR-Cas9 Assisted Cassette Exchange (CACE)–which enables the precise exchange of MHC alleles ($\sim$5kb) at the native genomic locus, therefore offering the potential to create perfect matches between donor and recipient in cellular transplantation. For initial evaluation, the CACE was established in immortalized mouse antigen-presenting cells (RAW264.7 macrophages from a C57BL/6 mouse strain), which express high levels of surface MHCs (H2-Kd). Genomic exchange at the H2-K locus was achieved via co-transfection of cells with a plasmid containing CRISPR/Cas9 and guide RNA and the cassette exchange DNA template encoding the alternate MHC allele (H2-Kb originating from Balb/c mouse strain). Following CACE, modified cells were easily isolated by selecting for phenotypic differences using FACS (antibody based detection of H2-Kb bound with antigenic peptide). Cell lines engineered to express the H2-Kb allele in place of one of the H2-Kd alleles were able to activate the T cell hybridoma line B3Z (TCR engagement triggers β-galactosidase expression) as efficiently as a wild type H2-Kb cell line (JAWSII dendritic cells). Finally, we have also investigated several designs of repair template in order to optimize the conditions for higher efficiency CACE.

OR017

Genome editing of the BCL11A erythroid-specific enhancer in bone marrow-derived CD34+ cells for the treatment of hemoglobinopathies

K H Chang[1] S Tan[1] S Smith[1] K Chen[1] T Sullivan[1] A Reik[2] F Urnov[2] E Rebar[2] P Gregory[2] O Danos[1] H Jiang[1]

1: Biogen 2: Sangamo BioSciences

BCL11A is a critical mediator of the fetal-to-adult globin switch; its knockout reactivates fetal globin in adult erythrocytes. Here, we describe targeted knockout of a single key regulatory element in an erythroid-specific enhancer in intron 2 of the BCL11A gene using zinc finger nucleases (ZFNs). Transient expression of these ZFNs in CD34+ cells results in permanent editing of BCL11A gene locus in all differentiated lineages with erythroid-restricted loss of BCL11A expression. As elevation of fetal hemoglobin alleviates the clinical symptoms of beta-hemoglobinopathies, once fetal program is reactivated via BCL11A editing, patients' autologous hematopoietic stem and progenitor cells (HSPCs) can be infused back to provide a one-time, durable treatment for these diseases. While autologous HSPCs can be obtained from mobilized peripheral blood (MPB) or bone marrow (BM), in contrast to beta thalassemic patients, patients with sickle cell disease (SCD) cannot at present safely undergo HSPC mobilization, necessitating the use of BM-derived CD34+ cells for gene editing. We found a markedly reduced levels of gene editing at the BCL11A enhancer in BM relative to MPB-CD34+ cells. We established methods to increase gene editing efficiency in BM-derived HSPCs cells, with robust fetal globin elevation in their erythroid progeny following targeted disruption of the BCL11A enhancer. Furthermore, these edited BM-CD34+HSPCs achieved multilineage long-term engraftment in NSG mice. These data demonstrate that genome editing of erythroid-specific BCL11A enhancer in BM-CD34+ cells is compatible with preserving critical HSPC functions, and provide strong support for the feasibility of therapy of SCD using a gene editing approach.

OR018

Epigenetherapy for cardiovascular disease: the role of nuclear non-coding RNAs in the regulation of VEGF-A

M P Turunen

1: A.I.Virtanen Institute

Transcriptional gene silencing (TGS) and activation (TGA) of gene expression by double stranded RNAs involve epigenetic changes in the promoter but the exact mechanism of action has been elusive. We have reported TGS and TGA by lentivirus expressed shRNAs targeted to murine VEGF-A promoter both in vitro and in vivo. This ''Epigenetherapy'' was surprisingly effective in the treatment of hindlimb ischemia and myocardial infarction. Therefore, we postulated that these shRNAs

mimic endogenous miRNAs acting in the nucleus and that they are also secreted from cell to cell. We performed miRNA-sequencing from normoxic and hypoxic endothelial cells that were separated into nuclear and cytoplasmic fractions. We found hundreds of miRNAs that are nuclear enriched and many of them change their localization upon hypoxia. Importantly, we found several previously unknown putative miRNAs, many of which were also nuclear enriched. Interestingly, 5′ and 3′-arms of some miRNAs were enriched either in nucleus or cytoplasm. Furthermore, hypoxia-associated miRNA change occurs in general in either the cytoplasm or the nucleus. Hypoxia regulated mmu-miR-466 and mmu-miR-669c are expressed from the intron of SFMBT2, which itself is a polycomb protein regulating H3K27me3 histone mark. SFMBT2 is also induced upon hypoxia. These miRNAs seem to regulate VEGF-A expression via TGS and/or TGA by targeting non-coding transcript present at the VEGF-A promoter. Here we propose a molecular mechanism of action for both TGS and TGA in mouse VEGF-A promoter. These findings represent a novel nuclear biology for miRNAs and pave way for clinical use of Epigenetherapy.

OR019

Targeted genome editing in mouse Hematopoietic Stem/Progenitor Cells (HSPC) to model gene correction of SCID-X1

G Schiroli[1 2] P Genovese[1] V Capo[1] M C Castiello[1] P D Gregory[3] M C Holmes[3] G Sitia[4] A Villa[1] A Lombardo[1 2] L Naldini[1 2]

1: San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET) 2: University Vita-Salute San Raffaele 3: Sangamo BioSciences, Inc. 4: San Raffaele Scientific Institute

We are developing a gene editing strategy in HSPC to correct the IL2RG mutations causing SCID-X1 disease. To model SCID-X1 gene correction in preclinical studies, we developed a mouse model carrying the human IL2RG gene including a common disease-causing mutation in place of murine Il2rg. To assess the minimal level of corrected HSPC required to achieve immune reconstitution we first performed competitive transplantation with wild-type (WT) and IL2RG-/- HSPC and found that 1% of WT cells are sufficient to partially reconstitute the lymphoid compartments. We then developed a protocol to obtain gene correction in murine Lin- HSPC based on the delivery of donor template by IDLVs followed by transfection of ZFN mRNAs. This protocol yielded high on-target nuclease activity (40%) and a mean of 6% transgene integration by homology-directed repair. Upon transplant into lethally irradiated mice, only the gene corrected cells were able to generate B and T lymphoid lineages, showing clear selective advantage over uncorrected cells. These data indicate functional correction of the defective IL2RG gene in an in vivo model by our strategy. Whereas editing was nearly undetectable in the engrafted HSC, gene corrected lymphoid cells persisted in the mice up to 7 months post transplantation within all the hematopoietic organs. Furthermore, upon challenging the mice with a murine pathogen we observed viral-specific IFN-g production by CD8 + gene corrected cells, proving their in vivo functionality. These results suggest that our protocol achieves biologically relevant levels of gene correction in progenitors capable of sustaining long-term lymphopoiesis.

OR020

Humoral immune response against AAV2/2-ND4 vector (GS010) after intravitreal administration in non-human primates and patients with Leber Hereditary Optic Neuropathy (LHON)

C Bouquet A Galy S Fitoussy S Meunier S Uretsky J P Combal N Thomasson

1: GenSight Biologics

LHON is the most common inherited genetic mitochondrial disease and leads to retinal ganglion cell death and bilateral vision loss. rAAV2/2-ND4 (GS010) is proposed for the treatment of LHON-ND4 patients. As AAVs are known to trigger host immune response, circulating anti-AAV2 antibody titers (IgG and neutralizing antibodies (NAb)) were measured after single intravitreal injection of GS010 in non-human primates (NHP, GLP toxicity study) and in LHON patients (Phase I/IIa trial, EudraCT no. 2013-001405-90). At baseline, 55% of NHPs contained undetectable NAb titers and 45% had titers 1:5–1:400. For all animals, titers increased up to 1:12800 from Day 15 post-injection. Between 2 and 6 months titers remained stable. All treated NHP retinas contained high ND4 mRNA copy numbers (1E3-1E5 per μg of DNA). Preliminary data from the Phase I/IIa trial showed that, at baseline, 5 out of 9 patients had no anti-AAV2 antibodies. Two weeks after injection, 3 of these 5 patients remained negative. At two weeks 3 patients had IgG and NAbs titers variably increased by 3 to 85-fold and up to 20-fold respectively. Three patients had unchanged elevated NAb titers compared to baseline. No correlation with local inflammatory adverse events was noticed. In conclusion, a serum humoral immune response against AAV2/2 capsid was generated starting two weeks after intravitreal GS010 injection. This was not associated with loss of transgene expression or safety issues. Additional understanding of AAV2/2 immunogenicity in the aqueous humor and serum over efficacy and potential for second eye injection will be discussed.

OR021

Retinal degeneration in Cln6nclf mice, a model for vision loss in transmembrane neuronal ceroid lipofuscinoses (NCL), amenable to AAV mediated gene therapy targeting the inner and outer retina

S M kleine Holthaus[1 2] U F Luhmann[1] S A Azam[1] L Abelleira Hervas[1] Y Duran[1] R A Pearson[1] A J Smith[1] S E Mole[2] R R Ali[1]

1: UCL Institute of Ophthalmology 2: MRC Laboratory for Molecular Cell Biology

The neuronal ceroid lipofuscinoses (NCL) are inherited lysosomal storage disorders representing the most common neurodegeneration during childhood. A major obstacle to developing therapies for NCL is the delivery of agents to the brain. In the eye adeno-associated virus (AAV) mediated gene therapies have been used to restore the expression of proteins and improve retinal morphology and function. As vision loss is a key symptom in NCL, we hypothesize that an AAV mediated gene therapy could preserve eyesight and may pave the way towards widespread therapeutic treatments for NCL. The Cln6nclf mouse is an NCL model that presents with

vision loss before the onset of neurological symptoms. These mice harbour a mutant Cln6 gene resulting in a short-lived protein. To determine the time window for treatment, we investigated the retinal phenotype in Cln6nclf animals. Histological and functional alterations occur as early as 2 and 3 weeks leading to photoreceptor dysfunction and dramatic photoreceptor loss. Immunostaining on unaffected retinas reveals a weak Cln6 expression level in photoreceptors and a strong expression level in bipolar cells in the inner retina, one of the cell types least amenable to viral transduction. Our data demonstrates that AAV mediated supplementation of Cln6 in photoreceptors does not prevent the retinal degeneration in Cln6nclf mice. We investigated the transduction efficacy of a novel AAV vector and show that bipolar cells can be targeted efficiently. Currently, we are assessing whether the viral delivery of Cln6 to bipolar cells exclusively or in combination with photoreceptors is therapeutic in mutant mice.

OR022

Target enrichment sequencing for detection of vector integration sites

S Wilkening S Afzal R Fronza E Senís Herrero C Bartholomä A Deichmann C von Kalle D Grimm M Schmidt

1: DKFZ and National Center of Tumor Diseases (NCT) Heidelberg 2: Heidelberg University Hospital

To understand viral integration mechanisms and assess biosafety, the determination of the exact positions of viral integration sites in the host genome is crucial. Current detection methods are based on primer binding within the vector genome, its elongation into the host genome, ligation of a common adapter on the host part, and subsequent PCRs. Thus, these methods depend on the presence of the primer binding site in the viral genome and biases may be introduced during restriction digest (if applied), ligation, and PCR. Here, we present results from a Target Enrichment Sequencing (TES) approach in which fragments of genomic regions that contain vector sequences are captured by vector-specific Sure Select probes. This approach has major advantages over primer-based approaches:

- A more quantitative estimation of cell clonality is possible as fewer biases are introduced.
- Relative quantification of vector copies per genome is possible by the capturing a genomic reference (omitting the need for qPCR).
- Integrations of incomplete vectors can be detected.
- As the entire vector is sequenced, mutations within the vector (and possible the transgene) are be detected.

Comprehensive analysis of the data obtained either by TES or by the linear amplification mediated (LAM-PCR) revealed so far undescribed recombinations within the Inverted Terminal Repeats (ITRs) of recombined Adeno-Associated Viruses (rAAVs). In summary, we show that TES is an important complementary tool to primer based approaches like LAM-PCR for mapping of vector/viral integration sites and we provide new and so far unpublished information about rAAV integration pattern.

OR023

HIV-1 mediated insertional mutagenesis increase the persistence of infected T cells in patients under ART by triggering their differentiation into long lived T-regulatory and T-central memory cells

D Cesana[1] F R Santoni de Sio[1] L Rudilosso[1] P Gallina[1] A Calabria[1] L Passerini[1] S Nozza[2] E Vicenzi[2] G Poli[2] G Tambussi[2] E Montini[1]

1: San Raffale Telethon Institute for Gene Therapy (HSR-TIGET) 2: San Raffaele Scientific Institute

It has been suggested that HIV-1 by integrating near cancer-associated genes could promote the expansion and persistence of infected cells in patients under Anti-Retroviral Therapy (ART). However, the molecular mechanism/s of insertional mutagenesis used and the physiological impact on the cells harboring these integrations are unknown. Here, we found that in the blood of 34% of HIV-1 patients under ART (30/87) there is an enrichment of cell clones with proviral integrations driving the expression of aberrant chimeric transcripts containing viral sequences fused to the first protein coding exon of BACH2 or STAT5B and predicted to encode for unaltered full-length proteins. Forced expression of these transcription factors in naïve CD4+T-cells significantly skewed their differentiation towards functional T-regulatory cells with immunosuppressive potential. Importantly, tracking the expression of HIV-1/STAT5B transcripts in T cell subpopulations and monocytes purified from the blood of patients under ART, we found that, in all patients tested (N=6), chimeric mRNAs were present only in T-regulatory and T-central memory cells but not in CD8+T-cells, T-naïve, T-stem-cell-memory, T-effector-memory nor monocytes. Our findings provide novel evidence that HIV-1 takes advantage of insertional mutagenesis to favor its persistence in the host by activating STAT5B and possibly BACH2. However, the selective advantage conferred by these integrations does not involve T-cell transformation but rather the modification of their phenotype into functional T-regulatory and T-central memory cells which are long-lived, potentially able to diminish the immune surveillance against infected cells and thus favoring the escape from the immune system and long-term viral persistence.

OR024

Genes located in the proximity of a retroviral insertion site can work cooperatively and affect hematopoiesis

T C Ha[1] O Kustikova[1] M Stahlhut[1] M Morgan[1] A Schambach[1] C Baum[1]

1: Institute of Experimental Hematology, Hannover Medical School

Retroviral insertional mutagenesis (RIM) is a known adverse effect in gene therapy trials, but is also a powerful tool for identifying novel oncogenes and cooperative pathways. However, focusing only on the gene closest to the insertion site may lead to underestimation of vector insertion effects. We hypothesized that multiple genes within a defined region of a vector insertion site could cooperatively influence the outcome of RIM. Therefore, we examined genes surrounding Bcl-xL, a common insertion site (CIS) found in two RIM databases (IDDb

and RTCGD). As Bcl-xL and Id1 in this locus are known to influence hematopoiesis, both genes were studied individually or co-expressed in hematopoietic stem and progenitors cells (HSPC) in a murine transplantation setting. We demonstrated that, similarly to Bcl-2, Bcl-xL functions as a fitness enhancing gene in HSPC by increasing the number of HSPC in recipients. In contrast, overexpression of Id1 favored myelopoiesis but depleted the transgene-positive population in long-term. Interestingly, co-expressing Bcl-xL and Id1 induced a sustained survival advantage of myeloid cells, without overt transforming events (observation time 18 weeks). Our data showed that the expression of more than one gene within a common retroviral insertion site cooperatively affects hematopoiesis. The combined expression of Bcl-xL and Id1 may be of interest for therapeutic approaches requiring a selective advantage of myelopoiesis. More generally, multiple genes within a CIS should be examined when searching for novel effectors via RIM and the combined effects should be limited when using vectors which lack long-distance enhancer activity.

OR025

A first-in-human phase I/II trial demonstrates the safety and the immunogenicity of a lentiviral-based therapeutic HIV vaccine eliciting potent polyfunctional multispecific CD8 and CD4 T-cell responses in HIV-infected individuals

H Toussaint[1] S Agaugue[1] E Sarry[1] A Bejanariu[1] E Sabbah-Petrover[1] C Bauche[1]

1: Theravectys

We assessed the safety and efficacy of a therapeutic anti-HIV-1 prime-boost vaccine regimen based on intramuscular injection of two integrative lentiviral vectors (ClinicalTrials.govIdentifier: NCT02054286). The randomized, placebo-controlled trial enrolled 38 HIV-infected individuals on suppressive ART and aimed at comparing the safety, tolerability and immunogenicity of the therapeutic vaccine candidate at 3 incremental doses (5.106, 5.107 or 5.108 TU) versus placebo. The vaccination regimen consisted of two intramuscular injections 8 weeks apart with lentivectors encoding for immunogenic regions of the HIV GAG, POL and NEF proteins under the regulation of the ß2-microglobulin human promoter. With the lack of any serious adverse events in all 38 participants and no safety concerns related to the treatment, the clinical data confirmed safety and tolerance of the lentiviral-based therapeutic vaccine. Analysis of the immunological data demonstrated the ability of the vaccine to elicit multi-specific and poly-functional cellular immune responses in vaccinated subjects:

i) 93% of the vaccinated subjects showed vaccine specific CD4+ and CD8+T-cell responses compared to 66,6% of the placebo group;
ii) a high frequency of functional T-cells able to produce at least 2 or 3 cytokines among IFN-γ, TNF-α and IL-2 was evidenced;
iii) a dose effect was observed when comparing the 3 groups;
iv) sustainable responses were characterized up to 24 weeks. We are currently evaluating the impact of ART interruption of vaccination on CD4-T cell levels, plasma viral load and viral reservoirs of the induced immune response to optimize the design of the planned Phase II.

OR026

AAVrh10–SGSH intracerebral gene therapy in Mucopolysaccharidosis Type IIIA

M. Hocquemiller[1]

1: Lysogene, 92 200 Neuilly-sur-Seine, France

Mucopolysaccharidosis type IIIA (MPSIIIA) is a lysosomal storage disorder caused by mutations in N-sulfoglucosamine sulfohydrolase (SGSH), resulting in heparan sulfate (HS) accumulation and progressive neurodegeneration. There is currently no treatment. Our approach is intracerebral gene therapy. The efficacy of AAV serotype rh.10 carrying the human SGSH cDNA has been demonstrated in the MPSIIIA mouse model. Toxicity has been examined in rats and juvenile dogs. A Phase I/II clinical non-comparative, open-label study in 4 children with MPSIIIA has been completed with the primary objective of assessing tolerance and safety. A secondary objective was the collection of data to determine potential future efficacy endpoints. The study showed that the treatment was safe and well-tolerated after one year in the four children. All patients showed improvement in behavioral disorders, hyperactivity and sleep disorders. Furthermore a cognitive enhancement was suggested by neurocognitive evaluation in the youngest child at one year but not sustained at two years. A phase II/III multi-centric clinical study in Europe and the US is planned to start in 2016. The trial will be an open-label, single arm intracerebral administration of an AAV serotype rh.10 carrying the human SGSH cDNA. Our clinical development plan also includes a multi-center natural history study in untreated patients to function as a non-concurrent control. This talk will focus on pre-clinical studies and elaborate on current clinical development.

OR027

Lentiviral vector infection of CD34+hematopoietic cells induces cellular DNA methylation changes independently of vector genomic integration.

T Aranyi[1] D Stockholm[1] R Yao[1] C Poinsignon A Paldi[1] A Galy

1: GENETHON, Integrare research unit, UMR_S951, Inserm, UEVE, EPHE, Genethon Evry, France

Hematopoietic gene therapy currently relies on ex vivo transduction of CD34+ cells during a short period of culture. Epigenetic changes may be induced by this procedure, but remain poorly characterized. We were the first to report that lentiviral vector (LV) transduction of CD34+ cells causes genome-wide cellular DNA methylation changes as measured on 27K chips on cord blood cells (Yamagata et al PlosOne 2012). We now extend these initial observations by investigating simultaneously the methylation level of 450 000 CpGs in CD34+ cells from apheresed blood (15 donors, 5 batches of GFP LV, 4 Illumina chips). As there is a strong inter donor variability, we have also developed a new algorithm called ''double average technique'' to identify very small, but reproducible differences between two series of samples after normalization of the raw data. We confirmed the induction of cellular DNA methylation changes during transduction and exposure to LV. Levels ranged from minimal to high, were batch-dependent even though all led to similar transduction levels (40–60%). The effects were independent of genomic integration and were induced by non-integrating vectors. They required cellular entry as non-

infectious particles produced without envelope glycoprotein always had minimal effects. The CpG methylation changes were broadly distributed in the genome but the strongest DNA hyper methylation effects were clustered and concerned only 4000 CpG sites. Thus, genome-wide DNA methylation can occur during ex vivo manipulation of CD34+ cells. Mechanisms should be further investigated to assess possible biological consequences on target cells and to minimize this effect.

OR028

Nuclear miRNAs mmu-miR-466/699, SFMBT2, YY1 and HIF1α in the regulation of VEGF-A expression

P Laitinen[1] T Husso[1] I L Kolari[1] M Väänänen[1] T C Roberts[2 3] M Weinberg[4] K Morris[5] S Ylä-Herttuala[1 6] M P Turunen[1]

1: Department of Biotechnology and Molecular Medicine, A.I.Virtanen Institute, University of Eastern Finland 2: Sanford-Burnham Medical Research Institute, Development, Aging and Regeneration Program, La Jolla, CA, 92037, USA 3: Department of Physiology, Anatomy and Genetics, University of Oxford, South Parks Road, Oxford, OX1 3QX, UK 4: Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, USA 5: Biotechnology and Biomedical Sciences, The University of New South Wales, Sydney, Australia 6: Science Service Center and Gene Therapy Unit, Kuopio University Hospital, Kuopio, Finland

Small non-coding RNAs targeted to gene promoters have been observed to modulate transcriptional silencing (TGS) and transcriptional activation (TGA) of gene expression. We hypothesized that endogenous nuclear localized microRNAs (miRNAs) mediate TGS and TGA by targeting promoter associated non-coding transcripts. Using bioinformatics approaches to predict miRNA targets in mouse VEGF-A promoter, we found two miRNAs, mmu-miR-466 and mmu-miR-669 which were predicted to target both VEGF-A and HIF1α promoters and their 3'UTRs. These miRNAs were found to be expressed from the intron of Scm-like with four mbt domains 2 (SFMBT2), which itself is a polycomb protein regulating histone silencing found in TGS. Notably, SFMBT2 is induced in response to hypoxia and its promoter contains predicted targets for YY1 transcription factor and hypoxia inducible factor 1, alpha subunit (HIF1α). When we cultured endothelial cells in hypoxia (0h, 2h and 24h, 1% O2), in addition to induction of HIF1α, expression of YY1 and mmu-miR-466 and mmu-miR-669 were found to be upregulated in nuclear fractions of hypoxic endothelial cells. YY1 has been observed to be involved in both up- or downregulation of gene transcription and is known to interact with SFMBT2. Collectively, the observations presented here suggest that non-coding transcripts in nucleus may compete with miRNA binding to mRNA 3′-UTRs in cytoplasm where miRNAs regulate post transcriptional gene silencing in a novel model for VEGF-A regulation in hypoxia by miRNAs acting in the nucleus.

OR029

Pre-clinical evaluation of AAV5-miHTT gene therapy of Huntington's disease

J Miniarikova[1 2] I Zanella[1] B Blits[1] V Zimmer[3] A Spoerl[3] A Southwell[4] M Hayden[4] S V Deventer[2] N Deglon[3] H Petry[1] P Konstantinova[1]

1: uniQure biopharma B.V. 2: Leiden University Medical Center 3: Lausanne University Hospital 4: University of British Columbia

The most upstream therapeutic target in Huntington's disease (HD) is the mutated huntingtin (mtHTT) and gene silencing with artificial miRNAs (miHTTs) delivered by adeno-associated viral vector (AAV) is expected to have therapeutic benefit. We have taken two approaches for the development of RNAi-based gene therapy of HD: total HTT silencing by targeting exon 1 and allele-specific inhibition by targeting heterozygous SNPs linked to mtHTT. Anti-HTT target sequences were incorporated in different miRNA scaffolds and their knockdown efficacy, allele selectivity and pri-miHTT processing were analyzed in vitro. The best miHTT candidates were incorporated in AAV5 vector and produced using the established uniQure baculovirus-based manufacturing platform. Proof of concept studies have shown efficacy of AAV5-miHTT in the lentivirally-derived HD rat model and in the humanized Hu97/18 mouse model. In both models, AAV5-miHTT delivery resulted in a reduction of the disease-related HTT mRNA and protein which was associated with a delay of neurodegeneration and in reduction of mtHTT aggregates. Ongoing research with best miHTTs aims to assess the selectivity, long-term therapeutic benefits in improving the behavioral, cognitive and electrophysiological symptoms in the Hu97/18 model. Furthermore, the miHTT processing, safety and off-target potential are being determined by next generation sequencing (NGS) on RNA isolated from murine striata to support the selection of the therapeutic candidate for clinical development. These preclinical results suggest that AAV5-miHTT may provide important therapeutic benefit for the HD patients and will allow for long-term HTT suppression upon a single vector administration.

OR030

Chemically modified guide RNAs enhance CRISPR/Cas genome editing in human primary cells

R O Bak[1] * A Hendel[1] * J T Clark[1] A B Kennedy[2] D E Ryan[2] S Roy[3] I Steinfeld[4] B D Lunstad[3] R J Kaiser[2] A B Wilkens[1] R Bacchetta[1] A Tsalenko[2] D Dellinger[3] L Bruhn[2] M H Porteus[1]

*1: Department of Pediatrics, Stanford University, Stanford, CA 94305, USA 2: Agilent Research Laboratories, Santa Clara, CA 95051, USA 3: Agilent Research Laboratories, Boulder, CO 80301, USA 4: Agilent Research Laboratories, Tel-Aviv, Israel *R.O.B. and A.H. contributed equally to this work*

CRISPR/Cas-mediated genome editing relies on guide RNAs to direct site-specific DNA cleavage mediated by the Cas endonuclease. The guide RNAs are typically expressed intracellularly from viral or non-viral vectors or delivered directly as in vitro-transcribed RNA. In this study, we chemically synthesized 100-nucleotide single guide RNAs (sgRNAs) targeting three different genes (IL2RG, HBB, and CCR5), alongside sgRNA variants containing modified nucleotides at both ends selected for their potential to impact the stability and immunostimulatory properties of nucleic acids. We demonstrate that chemically synthesized sgRNAs can facilitate genome editing in human cell lines when co-delivered with Cas9-encoding plasmid. We further show that sgRNAs containing modified nucleotides dramatically enhance genome editing compared to unmodified sgRNAs. When co-delivering chemically modified sgRNAs and Cas9 mRNA we observe > 90% in/del frequencies in human cells lines, ~70% in human primary T

cells, and ~40% in CD34+hematopoietic stem and progenitor cells (HSPCs), despite detecting no activity for unmodified sgRNAs in the latter two cell types. We observe no apparent cellular toxicity and analyses of off-target activities indicate that chemically modified sgRNAs typically retain high specificity. We also tested the simultaneous use of two chemically modified sgRNAs that cut 205bp apart in the CCR5 gene. When co-delivered with Cas9 mRNA in human primary T cells and CD34+HSPCs we observe 93% and 43% allele modification, respectively, of which the majority of modification events are deletions between the two sgRNAs. We believe that chemically modified sgRNAs will significantly improve a wide array of CRISPR/Cas biotechnological and therapeutic applications.

OR031

Intra-cerebral administration of AAV vector containing the human alpha-N-acetylglucosaminidase cDNA in children with Sanfilippo type B (MPSIIIB) syndrome: results at 12 months of a phase I/II trial.

M Tardieu M Zerah M L Gougeon J Ausseil S De Bournonville B Husson D Zafeirou G C Parenti C Broissand C Artaud B Poirier K Deiva T Baugnon T Roujeau J M Heard

1: Université Paris sud 2: Universite Paris Descartes 3: Institut Pasteur 4: Université d'Amiens 5: University of Thessaloniki 6: University of Napoli 7: Université de Montpellier

Four children with MPSIIIB mutated in the NAGLU gene received intracerebral deposits of highly purified rAAV2/5-hNAGLU vector particles produced by Sf9 insect cells engineered with baculovirus vectors (manufactured by Uniqure, NL). At the time of treatment, the youngest patient (20 months) had normal cognitive evaluation, two patients (26 and 30 months) were slightly below normal, and the oldest patient (53 months) was in the mild delay range. None of the children had autistic behaviour. The AAV vector dose of 4x1012 viral genomes/patient in 60μl was delivered over 2 hours at 16 sites in the cerebral and cerebellum white matter. Gene therapy was combined with immunosuppression (mycophenolate mofetil 8 weeks, tacrolimus, long-term) to prevent immune rejection. Low titer vector was detected in blood ($\leq$2x103vg/μg DNA) and urine for 48hours post-deposition. Safety data collected over the one-year follow-up showed good tolerance. We observed no reactive inflammation on brain images, no adverse events related to product or procedure, no increase in number of infectious events, no sign of toxicity related to immunosuppressive drugs (except a short period of high transaminases in one child). Data on efficacy will be presented. Available preliminary results indicate release of catalytically active NAGLU in CSF, appearance of NAGLU-responsive T-lymphocytes in blood, normal brain development without atrophy at sequential MRIs, cognitive progression after one year, as measured through a large set of complementary neuropsychological testing

OR032

Patient-specific gene-corrected iPSC-derived neural stem/progenitor cells for autologous cell therapy applications in lysosomal storage diseases

V Meneghini[1 7] G Frati[1] S De Cicco[1] M Luciani[1] C Cavazzin[2] F Morena[4] M Paulis[5] M Comi[1] W Mentzen[6] S Gregori[1] F Sanvito[3] A Villa[1 5] A Bulfone[6] S Martino[4] A Gritti[1]

1: San Raffaele Scientific Institute, San Raffaele Telethon Inst. for gene Therapy (TIGET), Milan, Italy 2: MolMed S.p.A, Milan, Italy 3: San Raffaele Scientific Institute, Milan, Italy 4: University of Perugia, Italy 5: Humanitas Clinical and Research Center, Rozzano, Italy 6: Crs4, Biomedicine, Pula (CA), Italy. 7: Imagine Institut

Metachromatic Leukodystrophy (MLD) is a fatal neurodegenerative lysosomal storage disease (LSD) due to genetic defects of the arylsulfatase A (ARSA) gene. Transplantation of autologous hematopoietic stem cells genetically modified to over express the missing enzyme is in advanced clinical translation. Human neural stem/progenitor cells (hNSCs) are also under clinical evaluation for LSD and other neurodegenerative diseases. The only available source of NSCs for clinical trials is currently represented by foetal-derived allogeneic NSCs (hfNSCs), which pose obvious safety and ethical concerns. For diseases amenable to gene therapy, patient-specific gene-corrected iPSCs might be differentiated into neural cells with potential value for autologous cell therapy applications. In this perspective, the achievement of hiNSC lines displaying a consistent and reliable ''NSC signature'' is mandatory. We have recently generated a collection of iPSC clones through reprogramming of skin fibroblasts from normal donors (ND; n=3) and MLD patients (n=2). Lentiviral vector (LV)-mediated gene transfer of a functional hARSA gene in MLD iPSCs resulted in stable supraphysiological enzymatic activity. Also, we have developed a protocol to efficiently differentiate hiPSCs into bona-fide NSCs (hiNSCs) that are phenotypically and functionally similar to hfNSC, and have a comparable global expression profiling. Finally, we provided evidence of enzymatic CNS correction upon intracerebral transplantation of LV.hARSA-MLD hiNSCs in immunodeficient MLD mice. Assessment of safety and long-term efficacy of autologous gene-corrected MLD hiNSCs in relevant pre-clinical models is a mandatory step to validate them as safe, homogeneous and renewable cell sources to be considered in future clinical application.

OR033

AAV- APPsα brain delivery rescues synaptic failure in an Alzheimers disease mouse model

R Fol §[1 2] J Braudeau §[1] S Ludewig[5] T Abel[4] S W Weyer[7] J P Roederer[7] M Audrain[1 2] A P Bemelmans[3] C F Buchholz[4] M Korte[5 6] U C Müller #[7] N Cartier #[1]

1: INSERM U1169 / MIRCen CEA Fontenay aux Roses 92265 France, and Université Paris-Sud, 91400 Orsay 2: Université Paris Descartes 3: CNRS UMR9199 MIRCen CEA Fontenay aux Roses 4: Molecular Biotechnology and Gene Therapy, Paul-Ehrlich-Institut, Langen 5: Zoological Institute, Division of Cellular Neurobiology, TU Braunschweig, Braunschweig 6: HZI, AG NIND, Braunschweig 7: Department of Bioinformatics and Functional Genomics, Institute of Pharmacy and Molecular Biotechnology, Heidelberg University § indicates joint first authors # indicates joint senior authors

Alzheimer's disease (AD) is characterized by synaptic failure, dendritic and axonal atrophy, neuronal death and progressive loss of cognitive functions. It is commonly assumed that these deficits arise due to β-amyloid accumulation and plaque deposition. However, increasing evidence indicates that loss of physiological APP functions mediated predominantly by neurotrophic APPsα produced in the non-amyloidogenic α-secretase pathway may contribute to AD pathogenesis. We used a gene therapy approach

to directly overexpress APPsα in the brain using AAV-mediated gene transfer and explored its potential to rescue structural, electrophysiological and behavioral deficits in APP/PS1ΔE9 AD mouse model. Sustained APPsα overexpression in aged mice with already preexisting pathology and amyloidosis restored synaptic plasticity and partially rescued spine density deficits. Importantly, AAV-APPsα treatment also resulted in a functional rescue of spatial memory. Moreover, we demonstrate a significant reduction of both toxic soluble Aβ42 and plaque load. In addition, APPsα induced the recruitment of microglia with ramified morphology towards plaques and upregulated IDE and TREM2 expression suggesting enhanced plaque clearance. Collectively, these data indicate that APPsα may improve synaptic and cognitive deficits, despite established pathology. Increasing APPsα using AAV-based brain gene delivery may therefore be of therapeutic relevance for AD.

OR034

Lentiviral-mediated correction of mobilized CD34+ progenitors and repopulating cells from Fanconi anemia patients

P Rio[1 2] S Navarro[1 2] G Guenechea[1 2] R Sanchez-Dominguez[2 7] M L Lozano[1 2] J A Casado[1 2] R Yañez[1 2] M L Lamana[1 2] A Galy[3] J C Segovia[2 7] J Surralles[4] C Diaz de Heredia[5] J Sevilla[6] J A Bueren[1 2]

1: Division of Hematopoietic Innovative Therapies, CIEMAT/CIBERER, 28040 Madrid, Spain 2: Advanced Therapies Unit, IIS-Fundacion Jimenez Diaz (IIS-FJD, UAM), 28040 Madrid, Spain 3: Genethon, 91000 Evry, France 4: Departamento de Genética y Microbiología, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain 5: Hospital Universitario Vall d'Hebrón, 08035 Barcelona, Spain 6: Hospital Infantil Universitario Niño Jesús, 28009 Madrid, Spain 7: Differentiation and Cytometry Unit-Division of Hematopoietic Innovative Therapies, CIEMAT/CIBERER, 28040 Madrid, Spain

Fanconi anemia (FA) is an inherited disease mainly characterized by congenital abnormalities, progressive bone marrow failure, and cancer predisposition. In contrast to other hematopoietic disorders already treated by hematopoietic gene therapy, marked proliferation and differentiation defects have been observed in FA hematopoietic stem cells (HSCs), complicating the harvesting of high numbers of CD34+ cells from these patients. On the other hand, such HSC proliferation defects might facilitate the engraftment of limited numbers of corrected FA HSCs. To evaluate the safety and efficacy of a HSC mobilization procedure of FA patients with filgrastim and plerixafor, a clinical trial has been recently opened. None of the six patients that have been already included in this trial have shown severe adverse events related to the mobilization procedure. Additionally, compared to previous CD34+ collection efforts in FA patients, high numbers of CD34+ cells have been collected in those patients that had a higher hematopoietic reservoir. Aiming at the genetic correction of mobilized FA-A HSCs, a protocol based on the transduction of mobilized CD34+ cells with a clinical grade lentiviral vector that carries the FANCA gene has been validated in the GMP facility of our Institution. These transduction conditions facilitated the phenotypic correction of mobilized FA-A hematopoietic progenitors. Moreover, the transplantation of transduced FA-A progenitors into NSG mice showed for the first time the repopulating ability of gene corrected mPB FA-A CD34+ cells. An update of our preclinical and clinical studies will be presented.

OR035

A new conditional mouse model to unravel the platelet defect in Wiskott-Aldrich syndrome

L Sereni[1 2] M C Castiello[1] A Anselmo[3] D Inverso[4] E Draghici[1] R Aiolfi[4] A J Thrasher[5] L D Notarangelo[6] M Iannacone[4] M Bosticardo[1] A Villa[1 7]

1: HSR TIGET, San Raffaele Telethon Institute for Gene Therapy 2: University Vita-Salute San Raffaele 3: Humanitas Clinical and Research Center, Rozzano, Milan, Italy 4: Division of Immunology, Transplantation, and Infectious Diseases, San Raffaele Scientific Institute, Milan, Italy 5: Molecular Immunology Unit, Institute of Child Health, University College London, London, UK 6: Division of Immunology, Children's Hospital, Harvard Medical School, Boston, MA, USA 7: Milan Unit, Istituto di Ricerca Genetica e Biomedica, Consiglio Nazionale delle Ricerche, Milan, Italy

Wiskott-Aldrich Syndrome (WAS) is a X-linked primary immunodeficiency caused by mutations in the Was gene. Patients show thrombocytopenia, eczema, infections and susceptibility to autoimmunity and malignancies. The treatment of choice is bone marrow transplantation, however we and others have recently demonstrated that patients lacking HLA-matched related or unrelated donors, can be successfully treated with gene therapy (GT). Although WAS GT patients show significant improvement of the clinical signs of the disease, platelet (PLT) counts never reach normal levels. Two hypotheses can explain the platelet defect in WAS: a defect in PLT production by megakaryocytes (MKs) or an increased peripheral elimination. To better clarify the bases of thrombocytopenia, we have generated a conditional mouse model (CoWas) that lacks the expression of WASp only in the megakaryocytic lineage. The CoWas mice have PLT counts comparable to the complete Was-/- mice (WKO) and show a normal PLT production by MKs. Moreover, Was-/- MKs are able to restore PLT counts after in vivo PLT depletion. Was-/- PLTs are more activated in vivo, leading to premature peripheral elimination. Our model suggests that PLTs lacking WASp have per se an intrinsic defect that leads to their elimination even in the presence of normal immune cells, as seen in CoWas mice. Our mouse model also represents a suitable tool for GT studies aimed at clarify whether a full correction of MK compartment is necessary to normalize PLT counts.

OR036

A prospective clinical trial of autologous TARGT™ prolonged EPO secretion showed EPO-independence in EPO-dependent ESRD patients

S Blum[1] N Shapir[1] R Miari[1] A Liran[1] B Lerner[1] K Doenyas-Barak Keren[2 3] S Efrati[2 3] D Schwartz[4] G Chernin[4] G A Neil[5]

1: Medgenics Medical Israel, Ltd., Misgav, Israel 2: Research & Development unit and Nephrology unit, Assaf-Harofeh Medical Center, Zerifin, Israel 3: Sackler School of Medicine,

Tel-Aviv University, Tel Aviv, Israel 4: Nephrology and Dialysis Department, The Tel Aviv Sourasky Medical Center, Tel Aviv, Israel 5: Medgenics, Inc., Wayne, PA, USA

Recombinant human erythropoietin (rHuEPO) along with iron supplementation corrects anemia in most patients with End Stage Renal Disease (ESRD), but is associated with supra-physiological peak serum concentration (C_{max}) of EPO and may cause thromboembolic complications. The Transduced Autologous Restorative Gene Therapy system (TARGT™) is an *ex-vivo* gene therapy, providing autologous, continuous protein or peptide therapies at physiological ranges. We present here initial results from 2 ongoing open label ascending dose clinical studies of $TARGT_{EPO}$ in patients with anemia due to ESRD. Patients are enrolled into pre-defined low or mid dose cohorts. In these studies patient dermal tissue biopsies (MOs) are transduced with a Helper-Dependent Adenoviral Vector containing the EPO gene (called $TARGT_{EPO}$s) which then re-implanted for delivering the required EPO dose. Thus far 11 patients were enrolled in both protocols and follow up is ongoing with the first implanted patient being followed with stable EPO secretion and resulting stable Hb for over 11 months from a single implantation without the need for rHuEPO or blood transfusion. Results obtained suggest stabilization of serum EPO levels at the physiological range of ≤ 20 mIU/ml and the resulting Hb levels between 9-12 g/dL. Comparative analysis of serum EPO levels revealed significantly lower C_{max} with $TARGT_{EPO}$ compared to rHuEPO and an order of magnitude less overall EPO exposure as calculated by Area Under the Curve which may confer significant clinical benefit. No treatment related serious adverse events have been reported. $TARGT_{EPO}$ is a promising novel therapy for anemia and potentially for other protein deficient diseases.

OR037

Correction of CTLs cytotoxic function defect by SIN-lentiviral mediated expression of Munc13-4 in type 3 familial hemophagocytic lymphohistiocytosis

T Soheili[1] J Rivière[1] I Ricciardelli[2] E Verhoeyen[3] G de Saint Basile[1] P Amrolia[2] I André-Schmutz[1] M Cavazzana[1]

1: Institut Imagine, Inserm U1163, Paris, France 2: Institute of Child Health, London, UK 3: Centre International de Recherche en Infectilogie, Lyon, France 4: INSERM U951,Genethon, Evry, France

Familial hemophagocytic lymphohistiocytosis type 3 (FHL3) is a life-threatening disorder of the immune system due to mutations in the UNC13D gene encoding Munc13-4 protein. Munc13-4 controls docking of lytic granules before they fused with the plasma membrane in cytotoxic T and NK lymphocytes and its defect results in defective cytotoxic function of these cells. The only curative treatment of FHL3 is allogenic hematopoietic stem cell (HSC) transplantation. For those patients without compatible bone marrow donor, gene therapy could represent a therapeutic option. As Munc13-4's function is to allow proper cytotoxic activity in mature CD8 + T cells, both HSCs and CD8 + T cells may constitute targets for gene correction. We constructed a self-inactivating HIV-1 derived lentiviral vector encoding human Munc13-4 and show that it could effectively transduce T cells of FHL3 patients. The expression of Munc13-4 mediated by the two different pseudotypes of this vector, high tropism VSV-G and lymphoid specific measle-pseutopyted H/F enveloped, resulted in the correction of the defective cytotoxic function of FHL3 T cells. Adoptive transfer of these gene-corrected T cells in SCID mice bearing autologous B-LCL lymphoma led to significant tumor regression due to an efficient homing and longer persistence of corrected T cells in mice compared to non-corrected T cells. Our study shows for the first time that a lentiviral mediating gene transfer in T-cells could be proposed for a hemophagocytic lymphohistiocytosis disorder.

OR038

Next-generation muscle-directed gene therapy using skeletal-muscle specific transcriptional modules identified by genome-wide computational analysis

S Sarcar[1] M Rincon[1 5] M Keyaerts[1] E Samara-Kuko[1] J Tipanee[1] T Athanasopoulos[2] G Dickson[2] T Lahoutte[1] P De Bleser[3] T VandenDriessche[1 4] M K Chuah[1 4]

1: Vrije Universiteit Brussel 2: Royal Holloway, University of London 3: University of Gent 4: KU Leuven 5: Fundacion Cardiovascular de Colombia

There is a need to generate the next-generation vectors for gene therapy of muscle disorders in order to express higher levels of the therapeutic transgene at lower vector doses. This should minimize the risk of immune responses and toxic effects. Moreover, the availability of more potent vectors would also ease manufacturing constraints. To increase the potency of gene therapy for muscle disorders, such as Duchenne muscular dystrophy (DMD), we explored the use of novel genome-wide computational strategies to boost vector performance. This resulted in the identification of hyperactive transcriptional cis-regulatory modules (i.e. CRMs) associated with a robust 200 to 400-fold increase in skeletal muscle-specific gene expression following AAV9-mediated transduction in mice. We then confirmed by chromatin immunoprecipitation that the CRMs contained clusters of muscle-specific transcription factor binding sites that accounted for high muscle-specific expression. We further validated the performance of these muscle-specific CRMs to boost expression of micro-dystrophin and follistatin, as an effective combination gene therapy, in mdx/SCID mice. Subsequently, we injected AAV9 vectors that expressed micro-dystrophin (micro-DYS) and follistatin (FST) from the de novo designed CRMs into dystrophic mdx/SCID mice. This resulted in stable and widespread expression of the therapeutic micro-DYS and FST proteins in skeletal muscle and heart, as confirmed by immunohistochemistry and ELISA, respectively. Most importantly, the high expression levels of micro-DYS and FST led to a robust functional correction of the dystrophic phenotype of the treated mdx/SCID mice by assessing physical fitness and endurance using a treadmill assay. This opens new perspectives for muscle-directed gene therapy.

OR039

Neuroprotection in an ALS mouse model following peripheral delivery of motor neuron targeted aCAR-IGF-1 Lentiviral vector

I Eleftheriadou I Manolaras E Irvine N D Mazarakis

1: Imperial College London

We have engineered HIV-1 based lentiviral vectors (LVs) with tropism to spinal cord motor neurons (MNs) capable of

being delivered non-invasively to the CNS through retrograde transport via the Neuromuscular Junction. We have previously demonstrated that, in vivo intramuscular application of coxsackievirus and adenovirus receptor (αCAR)-EGFP-targeted vector in mouse leg muscles results in specific retrograde transduction of lumbar MNs. Here we investigated in vivo the neuroprotective effects of αCAR-targeted expressing insulin-like growth factor 1 (IGF-1) for inducing neuronal survival and ameliorating the neuropathology and behavioral phenotypes associated with the SOD1G93A ALS mouse model. Human IGF-1 was cloned into these vectors and high titer vectors were produced. Vesicular Stomatitis Virus glycoprotein (VSV-G) pseudotyped LVs expressing IGF-1 were also produced so as to compare to neuroprotection resulting from retrogradely transported muscle-produced IGF-1. Single bilateral delivery of αCAR-targeted or VSV-G-pseudotyped LVs expressing IGF-1 into key muscle groups of SOD1G93A mice was performed at day 28. Motor performance, coordination and gait analysis were assessed weekly from day 65. We observed substantial therapeutic efficacy in vivo with αCAR IGF-1 LV pretreatment with up to 23% extension of survival compared to VSV-G IGF-1 LV and non- treated controls. Efficacy was linked to improved motor performance. End-point histological analysis of lumbar and thoracic spinal cord samples revealed that αCAR IGF-1 LV increases MN survival. These data support that αCAR IGF-1 LV is a good candidate for non- invasive neuroprotective gene therapy of ALS.

OR040

Spell-checking nature: interrogating the versatility of the CRISPR/Cas9 system for the treatment of Duchenne Muscular Dystrophy

D Wojtal[1 2] D U Kemaladewi[1] Z Malam[1] S Abdullah[1] T W.Y. Wong[1] E Hyatt[1] S Pereira[1] J Stavropoulos[1] V Mouly[3 4 6] K Mamchaoui[3 4 6] F Muntoni[5] T Voit[3 4] H Gonorazky[1] J J Dowling R Mendoza-Londono4[1] E Ivakine[1] R D Cohn[1 2]

1: The Hospital for Sick Children 2: University of Toronto 3: Institut de Myologie 4: Université Sorbonne Paris Cité 5: Institute of Child Health and Great Ormond Street Hospital 6: UPMC-Paris 6

Recently, the genome-modifying technology Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) system has been harnessed as a tool for programmable, high precision gene modification in eukaryotes. However, little is known about the potentially broad therapeutic implications of this technology. Here we explore various therapeutic approaches of the CRISPR/Cas9 system in skeletal muscle cells of Duchenne Muscular Dystrophy (DMD) patients. In addition to demonstrating that targeted genome editing is able to correct a pathogenic point mutation (c.7003G > T, p.E2035X) in myoblasts, we have also explored alternative and novel CRISPR/Cas9-mediated therapeutic strategies that include duplication removal and gene modulation. We demonstrate that an adapted CRISPR/Cas9 strategy successfully removes a 139 kb duplication (exons 18-30: chrX:32,552,206-32,413,149 (hg19) duplication with an AAAT insertion in the breakpoint junction) in the DMD gene leading to restoration of full-length dystrophin and α-dystroglycan expression in patient myotubes. Moreover, as a universal, mutation-independent treatment approach, we show that a modified CRISPR/Cas9 system leads to a significant increase in compensatory utrophin expression accompanied by restored levels of β-dystroglycan, providing evidence for functional impact using this strategy. In summary, we demonstrate that CRISPR/Cas9 can have significantly broader therapeutic implications for DMD, which includes strategies to restore full-length dystrophin expression as well as mutation-independent approaches that target modulation of disease-modifying genes.

OR041

Adenoviral vector transduction of designer nucleases restores DMD reading frames in dystrophin-defective muscle cell populations

I Maggio[1] L Stefanucci[1 2] J M Janssen[1] J Liu[1] X Chen[1] K Mamchaoui[3] M AFV Gonçalves[1]

1: Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, 2333 ZC 20, the Netherlands 2: Facoltà di Scienze Matematiche Fisiche e Naturali, Universitá di Roma Tor Vergata, Rome, 00133, Italy 3: Sorbonne Universités, UPMC Univ Paris 06, INSERM UMRS974, CNRS FRE3617, Center for Research in Myology, Paris, France

Duchenne muscular dystrophy (DMD) is a fatal X-linked muscle wasting disorder caused by loss-of-function mutations in the 2.2 Mb dystrophin-encoding DMD gene. Among the broad array of DMD mutations, over 50% comprises large intragenic deletions of one or more exons that disrupt the reading frame. The integration of gene editing and gene delivery technologies based on sequence-specific designer nucleases and viral vectors, respectively, constitutes a potential therapeutic modality for permanently restoring the endogenous DMD reading frame in patient-derived myogenic cells. Here, we investigated the suitability of adenoviral vectors (AdVs) encoding CRISPR/Cas9 RNA-guided nucleases (RGNs) or transcriptional activator-like effector nucleases (TALENs) to repair the DMD reading frame through non-homologous end-joining (NHEJ). Human myoblasts carrying mutations within the major DMD mutational ''hotspot'' were used as target cells. These mutations consisted of deletion of exon 48 through 50 or exon 45 through 52. We demonstrate that AdVs encoding designer nucleases can be tailored for NHEJ-mediated DMD repair strategies involving (i) incorporation of small insertions and deletions (indels) at out-of-frame sequences for reading frame resetting, (ii) splice acceptor knockout for DNA-level exon skipping and (iii) RGN or RGN-TALEN multiplexing for excision of reading frame-disrupting exons. Importantly, the transduction procedures did not interfere with the differentiation capacity of gene-edited myoblasts ultimately resulting in selection-free detection of edited DMD alleles and corresponding transcripts in differentiated whole muscle cell populations. We conclude that AdV delivery of designer nucleases represents a robust and versatile platform for rescuing expression of in-frame transcripts from DMD alleles with intragenic deletions.

OR042

Human artificial chromosome-mediated genetic correction of human dystrophic skeletal muscle progenitors for the autologous cell therapy of Duchenne muscular dystrophy

S Benedetti[1 2] H Hoshiya[1] M Ragazzi[1] Y Kazuki[3] N Uno[3] G Ferrari[1] R Tonlorenzi[4 5] A Lombardo[6] V Mouly[7] L Naldini[6] G Messina[8] M Oshimura[3] G Cossu[9] F S Tedesco[1]

1: Department of Cell and Developmental Biology, University College London, London WC1E 6DE, United Kingdom 2: UCL Institute of Child Health, London WC1N 1EH, United Kingdom 3: Department of Biomedical Science, Institute of Regenerative Medicine and Biofunction, Tottori University, Yonago, Japan 4: Division of Regenerative Medicine, San Raffaele Scientific Institute, Milan 20132, Italy 5: Division of Neuroscience, San Raffaele Scientific Institute, Milan 20132, Italy 6: San Raffaele-Telethon Institute for Gene Therapy and Vita Salute San Raffaele University, Milan 20132, Italy 7: Universite´ Pierre et Marie Curie/Paris 6/Inserm UMR_S 974, CNRS UMR 7215, Institut de Myologie, Groupe Hospitalier Pitie´-Salpetriere, Paris, France 8: Department of BioSciences, University of Milan, Milan, Italy 9: Institute of Inflammation and Repair, University of Manchester, Manchester, United Kingdom

Dystrophin gene mutations cause Duchenne muscular dystrophy (DMD), an incurable muscle disorder resulting in disability and premature death. Gene and cell therapy for DMD is promising but complex, as dystrophin is the largest human gene (2.4Mb) and skeletal muscle the most abundant human tissue. Coupling myogenic stem/progenitor cells such as satellite cell-derived myoblasts or pericyte-derived mesoangioblasts, together with episomal large-capacity vectors such as human artificial chromosomes (HACs) can provide a solution to overcome these hurdles. We previously showed phenotype amelioration of dystrophic mice transplanted with murine mesoangioblasts corrected with a HAC containing the entire dystrophin locus (DYS-HAC). However, clinical translation of the DYS-HAC platform will require highly proliferating cells able to withstand clonal expansion to numbers relevant to treat a dystrophic patient. Here we present a strategy for the generation of DYS-HAC-corrected, transplantable DMD myogenic progenitors. Reversible immortalization using excisable human telomerase and Bmi1 cDNAs allowed bypassing of replicative senescence and DYS-HAC transfer in both mesoangioblasts and myoblasts. Immortalized clones maintained a stable karyotype, remaining myogenic, non-transformed and non-tumorigenic. These clones gave rise to dystrophin-positive fibres upon transplantation into dystrophic mice. Importantly, we are now generating a novel multifunctional DYS-HAC that will provide complete genetic correction, enhanced proliferation and dystrophin expression, controllable cell death (safety system) and inducible myogenesis in both tissue- or pluripotent cell-derived progenitors. These results provide the first evidence of translation of HAC technology to clinically-relevant patient-specific myogenic progenitors, paving the way for the establishment of an autologous HAC-based protocol for gene and cell therapy of DMD.

OR043

Gene transfer of MTMR2 prolongs survival and rescues the pathology in a murine model of myotubular myopathy

N Danièle C Moal L Julien T Jamet R Joubert S Martin A Vignaud M W Lawlor A Buj Bello

1: GENETHON 2: Medical College of Wisconsin

X-linked myotubular myopathy (XLMTM) is a severe congenital disorder leading to generalized skeletal muscle weakness in male infants, frequently associated with fatal respiratory failure. The disease is caused by loss-of-function mutations in the MTM1 gene, which encodes myotubularin, the founder member of a family of 15 homologous proteins in mammals (including MTMR1 to 14). We recently demonstrated the therapeutic efficacy of intravenous delivery of recombinant adeno-associated viral vectors (rAAV) expressing myotubularin in murine and canine animal models of the disease. As an alternative approach, we tested in the present study whether Mtm1 closest homologues, Mtmr1 and Mtmr2, could also rescue the XLMTM phenotype. Recombinant serotype-9 AAV vectors encoding either MTMR1 or MTMR2 under the control of the desmin promoter were injected into the tibialis anterior muscle of two week-old Mtm1-deficient knockout mice. Two weeks after vector delivery, a therapeutic effect was observed with Mtmr2 but not Mtmr1 overexpression. The histopathological hallmarks were normalized, and muscle hypotrophy and contractility were improved. Furthermore, systemic administration of a single dose of this rAAV9-Mtmr2 vector in mutant mice improved the motor activity and contractile force, and prolonged survival throughout a 3-month study. Altogether, our results establish the proof-of concept that skeletal muscle-targeted MTMR2 overexpression represents a novel target for therapeutic development in myotubular myopathy.

OR044

Pre-clinical workup of lentiviral mediated stem cell gene therapy for mucopolysaccharidosis type IIIA

S M Ellison A Sergijenko A Langford-Smith K Langford-Smith R Wynn F Wilkinson B Bigger

1: University of Manchester 2: Central Manchester Foundation Trust

Sanfilippo disease (MPSIIIA) is a devastating lysosomal storage disease (LSD) that causes progressive mental decline leading to death in early childhood. Absence of functional N-sulphoglucosamine sulphohydrolase (SGSH) leads to accumulation of Heparan Sulphate (HS) in the lysosomes of patients, causing cellular dysfunction, with the brain predominantly affected. There are currently no treatments. Enzyme replacement therapy is ineffective since enzyme cannot pass the blood brain barrier. Haematopoietic stem cell transplant (HSCT) can circumvent this problem via monocyte trafficking and engraftment in the brain, thereby allowing delivery of enzyme by cross correction. HSCT corrects the brain in the related HS storage disease MPSI Hurler, however this is not the case for MPSIIIA due to insufficient enzyme production. To overcome this we developed a lentiviral vector (LV) mediated stem cell gene therapy approach to significantly boost SGSH enzyme levels. Eleven percent of normal enzyme activity was achieved in the brain which corrected the behavioural phenotype in MPSIIIA mice, normalised GAG storage and neuroinflammation, corrected lysosomal compartment size and significantly improved survival. Pre-clinical toxicology studies have demonstrated safety and efficacy of GMP grade SGSH LV in human CD34+ cells. We have achieved a 9 fold increase in SGSH activity in transduced human HSCs at a vector copy number of 0.73 ± 0.1 without adverse toxicity or lineage skewing. Furthermore, we observed low transformation frequency using the in vitro immortalisation assay, comparable to other lentiviral vectors currently in the clinic. We aim to begin a phase I/II clinical trial in MPSIIIA patients by early 2016.

OR045

Efficient targeted gene addition to a safe harbor locus in long-term repopulating hematopoietic stem cells for correction of x-linked chronic granulomatous disease via genome editing

S S DeRavin A Reik L Li P Q Liu A Song J R Pearl D E Paschon M V Peshwa N Theobald U Choi J Lee S Koontz C Sweeney K A Zarember G Lee E J Rebar P D Gregory F D Urnov H L Malech

1: Sangamo BioSciences 2: National Institute of Allergy and Infectious Diseases 3: Maxcyte

Genome editing-driven targeted integration (TI) of a therapeutic transgene into a genomic safe harbor site in CD34+ hematopoietic stem cells (HSC) could provide a safer alternative to randomly integrating viral vectors for gene correction of inherited immune and hematologic disorders. Achieving efficient TI in long term engrafting human HSC in a clinically compliant setting is a key step on the path to this goal. Here, we demonstrate that optimized delivery of zinc finger nuclease (ZFN) mRNA via a clinical-grade electroporation device accompanied with adeno-associated virus (AAV) 6-delivery of a corrective donor construct achieves clinically relevant levels of TI into the well-established safe harbor locus AAVS1 in long term repopulating human HSCs. This dual-mode delivery yields up to 58% marker-positive human HSCs, with ~15% marking observed following long-term engraftment into immunodeficient mice. We applied this approach to X-linked chronic granulomatous disease (X-CGD), in which neutrophils lack the gp91phox subunit of the NADPH oxidase required to generate microbicidal oxidants. Targeted addition of a gp91phox transgene into the AAVS1 safe harbor locus in HSC from X-CGD patients resulted in 15–18% of HSC expressing normal amounts of gp91phox protein and reconstituted NADPH oxidase activity in neutrophils derived ex vivo from these HSC. In immunodeficient mice transplanted with the corrected X-CGD HSC 6% of the human cells in the mouse marrow expressed gp91phox. Our data provide a foundation for deploying genome-editing-based targeted gene addition for X-CGD in the clinic, and point to this approach as one broadly applicable to correction of other monogenic diseases.

OR046

Ex vivo liver-directed gene therapy in a pig model of hereditary tyrosinemia type 1

R D Hickey F Elgilani[1] S A Mao[1] J Glorioso[1] B Amiot[1] P Rinaldo[1] Y Ikeda[1] S J Russell[1] J B Lillegard[1] S L Nyberg[1]

1: Mayo Clinic

Hereditary tyrosinemia type I (HT1) is an autosomal-recessive inborn error of metabolism caused by deficiency in fumarylacetoacetate hydrolase (FAH), an enzyme that catalyzes the last step of tyrosine metabolism. In the current study, we tested the hypothesis that ex vivo hepatocyte gene therapy can correct the metabolic disorder in FAH-/- pigs. Recipient FAH-/- pigs (n=4) underwent partial liver resection and hepatocyte isolation by collagenase digestion. Hepatocytes were transduced with a lentiviral vector (LV) expressing the FAH cDNA under the control of the thyroxine-binding globulin promoter. Pigs received autologous transplants of hepatocytes by portal vein infusion, either after 2 hours of LV transduction or after 24 hours of LV transduction during 3-dimensional hepatocyte spheroid formation. Following transplantation, the protective drug NTBC was withheld from recipient pigs to provide a selective advantage for expansion of corrected cells. Two animals were euthanized at 2 and 4 months post-gene therapy to examine the transplanted livers. Histologically, these livers displayed robust engraftment and proliferation of FAH+ cells, with no evidence of fibrosis present. Two pigs continue to thrive independent of NTBC supplementation, indicating complete amelioration of the HT1 phenotype. Biochemically, this is characterized by normal values of AST, bilirubin, PTT and INR. Importantly, blood tyrosine and succinylacetone levels improved to within normal range off NTBC, demonstrating complete correction of tyrosine metabolism. While additional studies in large animals are needed to investigate engraftment and proliferation of transplanted hepatocytes, including safety studies, further exploration of ex vivo gene therapy for metabolic liver disease clinically appears warranted.

OR047

Guanylyl cyclase A-targeted gene therapy for polycystic kidney disease

S J Holditch A Cataliotti V Torres Y Ikeda

1: Mayo Clinic

Autosomal recessive polycystic kidney disease (ARPKD) is characterized by enlarged kidneys with dilated collecting ducts and congenital hepatic fibrosis with significant morbidity and mortality. The cardiac hormone B-type natriuretic peptide (BNP) is a guanylyl cyclase A agonist, which demonstrates potent cardiorenal protective activities. Here, we investigated the therapeutic effect of long-term BNP overexpression in the rat model of ARPKD (PCK). Littermate PCK neonates received placebo, or a single intraperitoneal injection of adeno-associated virus 9 (AAV9) vectors at 1.0E+12 genome copies/kg. Three months post treatment, kidney weight was significantly reduced in treated PCK, along with a significant reduction in renal cyst burden. Blood and urine analysis confirmed decreased disease burden with BNP treatment, with higher creatinine clearance, decreased proteinuria, and decreased renal injury markers. BNP treatment also preserved glomerular injury in PCK. Real-time RT-PCR analysis demonstrated that BNP treatment significantly reduced renal and hepatic expression of the fibrosis-associated genes. BNP-treated PCK rats also showed decreased expression of fibrosis markers, along with decreased cyst-index. Additionally, echocardiography demonstrated preserved cardiac function with treatment. Together, these observations indicate that a single intraperitoneal AAV9 injection elicits therapeutic benefit in protecting against declining hepatic and renal function, remodeling of healthy parenchyma, and increased cyst burden, while providing cardiac protection. The present study further suggests that sustained GC-A activation provides a novel opportunity for the treatment for polycystic kidney and liver diseases.

OR048

Human T effector and T regulatory cell differentiation and response are distinctly controlled by FOXP3 expression.

F R Santoni de Sio [1] L Passerini[1] S Restelli[1] M M Valente[1] G Escobar[1] L Naldini[1 2] M Porteus[3] R Bacchetta[1 3]

1: San Raffaele Telethon Insitute for Gene Therapy (HSR-TIGET) 2: San Raffaele Vita-Salute University 3: Stanford University

Forkhead-box-P3 (FOXP3) is the transcription factor mastering the function of T regulatory cells (Tregs), key controllers of immune tolerance. Mutations in FOXP3 cause Treg dysfunction resulting in the life-threatening autoimmune syndrome Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX). While the role of FOXP3 in Treg function has been clearly assessed, its role in Treg differentiation remains mainly obscure, with some observations calling for its possible function also in T effector cells (Teffs). We have exploited hu-mouse models repopulated by genetically modified hematopoietic stem/progenitor cells (HSPC) to overexpress or down-regulate the expression of FOXP3 in order to study the role of FOXP3 in the differentiation and function of Teffs and Tregs. While FOXP3 KD and KO induced significant expansion of Teffs, its overexpression dampened Teff differentiation and TCR-mediated proliferative responses. Interestingly, both FOXP3 overexpression and KD reduced TCR diversity of peripheral T cells, but they did not affect Treg differentiation, at least at steady state. Unexpectedly, FOXP3 overexpression increased the size of HSPC compartment both in vitro and in vivo. Thus, our data indicate FOXP3 as relevant player in Teff differentiation, expansion and activity, while Treg differentiation appears less affected by FOXP3 modulation. We are currently investigating the molecular mechanisms underlying this role. On the other, they call for caution in envisaging an IPEX gene therapy based on HSPC genetically modified to constitutively express FOXP3 gene. Overall, our results unveil previously unknown functions of the human FOXP3 gene and are essential to better define future gene-transfer based therapeutic approaches for IPEX syndrome.

OR049

A Phase 2b clinical trial of non-viral gene therapy in Cystic Fibrosis patients: randomized, double-blind, placebo-controlled repeated aerosol delivery to the lungs.

D R Gill[1] E W Alton[1] D K Armstrong[1] A C Boyd[1] S H Cheng[2] S Cunningham[1] G Davies[1] J C Davies[1] H Elgmati[1] A Greening[1] U Griesenbach[1] K Harman[1] T Higgins[1] S C Hyde[1] J A Innes[1] M McGovern[1] G Murray[1] D J Porteous[1] C J Saunders[1] R K Scheule[2] N J Simmonds[1] S G Sumner-Jones[1] M D Waller[1]

1: UK Cystic Fibrosis Gene Therapy Consortium 2: Genzyme Corporation

The UK Cystic Fibrosis Gene Therapy Consortium has undertaken an extensive pre-clinical programme, including repeat-dose toxicology studies in mice and sheep, together with a Phase 1/2a safety study in Cystic Fibrosis (CF) patients to assess aerosol delivery of non-viral plasmid DNA/liposomes to the lungs. The formulation is composed of GL67A liposomes complexed with plasmid pGM169, which is completely free of CpG dinucleotides and directs persistent high-level expression of codon-optimised CFTR under the control of the hCEFI promoter/enhancer (Hyde et al 2008 Nature Biotechnology 26: 549-551). The Phase 1/2a trial in CF patients identified a safe (5ml) aerosol dose delivered to the lungs via a Trudell AeroEclipse II nebuliser. In a follow-on Phase 2b study, CF patients with an FEV1 % predicted lung function of between 50 and 90% were recruited to measure potential clinical benefit following delivery of 12 aerosol doses at monthly (28 ± 5 day) intervals. The double-blind, placebo-controlled trial compared the above formulation with 0.9% saline in a 1:1 randomisation. The Per Protocol patient group (pre-defined as receiving 9 or more doses) consisted of 116 patients (52 Placebo and 64 Active). Two small ($n = 24$) patient sub-groups also underwent either additional nasal administration, or bronchoscopic measurements, to evaluate molecular surrogates (2:1 randomisation Active: Placebo). Data for safety and primary/secondary efficacy outcome measures will be presented. Supported by the NIHR EME programme and the CF Trust.

OR050

Efficient non-viral gene delivery by minicircle Sleeping Beauty transposon system into hematopoietic stem cells for gene therapy applications

M Holstein[1] C Mesa-Nuñez[2] E Almarza[2] C Miskey[1] M Schmeer[3] D Kobelt[4] W Walther[4 5] Z Izsvák[6] M Schleef[3] C Rudolph[7] J A Bueren[2] G Guenechea[2] Z Ivics[1]

1: Transposition and Genome Engineering, Division of Medical Biotechnology, Paul Ehrlich Institute, Langen, 63225, Germany 2: Division of Hematopoietic Innovative Therapies, CIEMAT/CIBERER/IIS-FJD, Madrid, 28040, Spain 3: PlasmidFactory GmbH & Co. KG, Bielefeld, 33607, Germany 4: Translational Oncology of Solid Tumors, Max Delbrück Center for Molecular Medicine, Berlin, 13125, Germany 5: Translational Oncology of Solid Tumors, Experimental and Clinical Research Center, Charité University Medicine, Berlin, 13125, Germany 6: Mobile DNA, Max Delbrück Center for Molecular Medicine, Berlin, 13125, Germany 7: Department of Pediatrics, Ludwig Maximilian University of Munich, Munich, 80337, Germany

The Sleeping Beauty (SB) transposon system is a non-viral gene delivery platform that combines simplicity, inexpensive manufacture and close-to-random profile of genomic integration. However, efficient correction of hematopoietic stem cells (HSCs) with non-viral vector systems, including SB, demands further refinement of gene delivery techniques. Our goal was to decrease excessive cell toxicity associated with electroporation of HSCs, and to increase gene delivery efficiency – both largely dependent on the amount and size of the plasmid vector to be delivered. We set out to improve SB gene transfer into hard-to-transfect HSCs by delivering SB system components in the form of minicircles. Minicircles generated from their parental plasmid DNA through a recombination step are devoid of bacterial backbone sequences and therefore significantly reduced in size. As compared to conventional plasmids, the SB transposon system is delivered into mouse and human HSCs more efficiently and with reduced electroporation toxicity in the form of minicircle DNA. The minicircle platform greatly enhances SB transposition and transgene integration resulting in higher numbers of stably modified blood precursors, which are able to engraft and reconstitute the hematopoietic system when transplanted into mouse recipients. Moreover, additional improvement by providing the SB transposase in the form of synthetic mRNA enabled us to further increase the efficacy and biosafety of HSC genome modification. Collectively, implementation of minicircle and mRNA technologies allowed us to further refine the SB transposon system in the context of HSC gene delivery to ultimately meet clinical demands of an efficient and safe non-viral gene therapy protocol.

OR051

Ultrasound-targeted delivery of chemotherapeutic drug and nucleic acids by gas-filled cationic liposomes

A Delalande[1] S Manta[2] A Peyret[1] L Horvat[1] S Ezzine[2] M Bessodes[2] P Midoux[2] P A Jaffres[3] N Mignet[2] C Pichon[1]

1: CNRS/Université d'Orléans 2: CNRS 3: Université de Brest

Ultrasound (US) is widely used in the medical imaging field for diagnostic purposes. Having first been developed as ultrasound contrast agents, nowadays, microbubbles are also proposed as tools for ultrasound-assisted gene delivery. Gas microbubbles are served as a drug reservoir that can be triggered by ultrasound. Their oscillation behaviour during US exposure cause transient membrane permeability of surrounding cells, facilitating targeted local delivery. Gas microbubbles could therefore serve as a drug reservoir that could be triggered by ultrasound. In this study, we developed gas microbubbles capable of delivering both a cytotoxic agent as paclitaxel and siRNA to promote tumor regression. Microbubbles were developed with original histidylated cationic lipids. In vitro measurements have been done to depict the behavior of these microbubbles in the presence of tumor cells after ultrasound activation and their capacity to bind siRNA. Ultrasound parameters were optimized in vitro for siRNA delivery in 4T1 cells stably expressing luciferase (luc). Cationic microbubbles complexed with anti-luc siRNA led to specific inhibition of luciferase expression under specific ultrasound parameters. A proof of principle has been validated in vivo using 4T1 orthotopic murine mammary tumor model. Our siRNA-paclitaxel formulations were injected either inside the tumor or intravenously followed by ultrasound application at tumor site. A significant inhibition of tumor growth was observed in mice after 3 weeks of weekly treatments with these formulations. In conclusion, we succeeded to produce original gas-filled liposomes made with histidylated cationic liposomes that were ultrasound-sensitive and able to co-deliver efficiently paclitaxel and siRNA for tumor treatment.

OR052

Development of oncolytic vaccine for cancer treatment

C Capasso[1] M Hirvinen[1] M Garofalo[1 3] D Romaniuk[1] L Kuryk[1] T Sarvela[1] A Vitale[4] M Antopolsky[2] A Magarkar[2] T Viitala[2] T Suutari[2] A Bunker[2] M Yliperttula[2] A Urtti[2 5] V Cerullo[1]*

1: Laboratory of Immunovirotherapy, Division of Pharmaceutical Biosciences and Centre for Drug Research, University of Helsinki, Viikinkaari 5, 00790 Helsinki, Finland 2: Division of Pharmaceutical Biosciences and Centre for Drug Research, University of Helsinki, Viikinkaari 5, 00790 Helsinki, Finland 3: Department of Molecular Medicine and Medical Biotechnology, University of Naples ''Federico II'', Via Pansini, 80131 Naples, Italy 4: Department of Movement Sciences and Wellness (DiSMEB), University of Naples Parthenope, Via Medina 40, 80133 Naples, Italy; CEINGE-Biotecnologie Avanzate, Via G. Salvatore 486, 80145 Naples, Italy 5: School of Pharmacy, University of Eastern Finland, Yliopistonranta 1, P.O. Box 1627, FI-70211 Kuopio, Finland.

Not more than ten years ago oncolytic viruses were thought to be effective mainly for their ability of replicating and directly kill cancer cells. The immune system was seen as an obstacle to be overcome in order to achieve maximum replication and efficacy. In the last few years this paradigm has been challenged and a complete new approach has been taken in the oncolytic virus field. The immune system is not seen as an obstacle anymore but rather something to activate in order to maximize the efficacy, to target uninjected lesions and to stimulate a robust and durable response; nowadays the word ''oncolytic viruses'' are often interchanged with the word ''oncolytic vaccine''.

Other and us have conducted pioneering studies demonstrating that the use of oncolytic viruses has the ability to stimulate and trigger the tumor-specific immune response. However the simple use of oncolytic viruses as immunotherapeutic agent has its own laminations due to the nature of the virus and the tumor that ultimately ends stimulating a more robust anti-viral than anti-tumor response.

To overcome this limitation we have developed a rapid and highly customizable way to increase the specificity of an oncolytic adenovirus directing the anti-viral response towards tumor antigens rather than viral-antigens. In fact we have coated an oncolytic adenovirus with MHC-I restricted tumor specific peptides. This strategy has led to an increased tumor-specific immunity. The system we have developed does not involve chemical or genetic modification of the viral capsid rendering the system very appealing for clinical translation approaches. We have characterized this complex and we have shown its efficacy in three different models of murine melanoma including humanized mice bearing human tumor and matched human immune system.

OR053

An oncolytic adenovirus (AdΔ19K) sensitizes pancreatic cancer cells to cytotoxic drugs by promoting DNA-damage and aberrant mitosis through inactivation of checkpoint mediators.

C Pantelidou[1] G Halldén[1]

1: Barts Cancer Institute, Queen Mary University of London

Replication-selective adenoviral mutants have been evaluated in numerous clinical trials targeting solid cancers with demonstrated safety but limited efficacy. Efficacy was remarkably enhanced in conjunction with cytotoxic agents. Potent synergy has been established in preclinical models in combination with DNA-damaging apoptosis-inducing cytotoxic agents. However, the underlying cellular mechanisms are mostly unknown. We identified cellular factors that are essential for synergistic/enhanced cell killing that may direct the development of novel anti-cancer therapeutics to treat pancreatic cancer; one of the most dismal prognosis of all cancers. The potent adenoviral mutant AdΔ19K, deleted in the Bcl2 functional anti-apoptotic homologue E1B19K, potently synergized with the DNA-damaging drug gemcitabine to kill pancreatic cancer cells and increased DNA damage. In the presence of high levels of gemcitabine-induced DNA-damage, checkpoint-mediated arrest via pChk1 was potently induced, which was efficiently prevented by AdΔ19K through degradation and mislocalisation of Mre11. In addition, AdΔ19K downregulated an important pChk1 adaptor-protein, thereby further attenuated pChk1-mediated signalling. Live imaging demonstrated that low doses of gemcitabine caused multiple mitotic aberrations including multipolar spindles, micro- and multi-nucleation and cytokinesis failure. AdΔ19K further enhanced cell killing, potentiated drug-induced DNA damage and the mitotic aberrations. These results reveal novel mechanisms

exploited by adenovirus to ensure completion of its life cycle in the presence of cellular DNA damage. Data will be provided to demonstrate a rationale for developing therapies to target novel cellular factors in pancreatic cancer in conjunction with conventional chemotherapies.

OR054

CD30-targeted oncolytic virotherapy of Hodgkin Lymphoma

A Muik[2] J D S Hanauer[2] T Friedel[2] I Schneider[2] B Rengstl[1] C Ayala-Breton[3] K W Peng[3] S Newrzela[1] C J Buchholz[2]

1: Goethe University Frankfurt 2: Paul Ehrlich Institute 3: Mayo Clinic

Hodgkin lymphoma (HL) is a hematopoietic malignancy with an incidence of 3-4 cases per 100.000 persons per year. HL is characterized by a unique histological pattern, as HL cells are composed of Hodgkin and Reed-Sternberg (HRS) cells representing a mononucleated or multinucleated subtype, respectively. The most important immunological marker of HL is CD30. Current treatment strategies rely on chemotherapy followed by radiotherapy. However, even though the majority of patients can be cured by the current standard of care, approximately 20% either succumb to relapse, suffer from progressive disease or develop treatment-induced secondary cancers. Here, we have generated both a CD30-specific oncolytic measles virus (MV-CD30) and vesicular stomatitis virus (VSV-CD30) for virotherapy of HL. Most importantly, this is the first report of a fully retargeted VSV. Retargeting was achieved by the fusion of a CD30-specific single-chain variable fragment to a MV hemagglutinin (H) blinded for its natural receptors. In case of VSV the VSV-G glycoprotein was replaced with the MV envelope proteins. Both viruses were selective for CD30+ cells with soluble extracellular CD30 domains blocking infection. As expected due to the inherent VSV replication machinery, VSV-CD30 replicated significantly faster and to much higher titers compared to the respective MV counterpart also leading to more efficient killing of HL cells in vitro. Moreover, VSV-CD30 was able to infect HL cells in complex HL:CD4 T cell co-cultures. First in vivo assessments of antitumoral effects in HL mouse models are currently under way.

OR055

Pulmonary macrophage transplantation employing HSC- or iPSC-derived cells as an innovative gene therapy approach in Pulmonary alveolar proteinosis

M Hetzel[1] N Lachmann[1] C Happle[1] T Suzuki[2] A Mucci[1] A Kuhn[1] A Schambach[1 3] G Hansen[1] B Trapnell[2] T Moritz[1]

1: Hannover Medical School 2: Cincinnati Children's Hospital Medical Center 3: Boston Children's Hospital

Pulmonary alveolar proteinosis (PAP) caused by mutations in the granulocyte/macrophage–colony stimulating factor (GM-CSF-alias CSF2-) receptor α chain (CSF2RA) represents a rare, life-threatening lung disease characterized by the accumulation of phospholipids and proteins in the lungs secondary to a functional insufficiency of alveolar macrophages. Whereas current therapeutic options for this disease are limited, we recently demonstrated that a gene therapy approach based on the endotracheal application of healthy, gene-corrected macrophages (Pulmonary macrophage transplantation, PMT) maybe feasible and effective. We now have evaluated the genetic correction of HSC- as well as iPSC-derived macrophages from PAP patients as a potential source for such a cell replacement therapy and employed a lentiviral construct expressing a codon-optimized human CSF2RA-cDNA from an EFS1α promoter (EFS.CSF2RA.iGFP). In PAP patient-derived CD34+HSCs transduction with the EFS.CSF2RA.iGFP vector rescued hGM-CSF dependent colony formation as well as monocyte/macrophage (M/M) differentiation, while healthy donor CD34+ cells overexpressing CSF2RA exhibited no aberrations in growth, colony formation or in vitro differentiation towards CD14 +CD11b+CD68+CD163+macrophages. The EFS.CSF2RA.iGFP construct also corrected GM-CSF dependent functionality including CD11b activation, GM-CSF uptake, phagocytosis, and CSF2R-downstream signalling in PAP-iPSC derived M/M. Thus, in summary we here demonstrate efficient and functional genetic correction of PAP-patients derived macrophages and introduce HSC- but also iPSC-derived M/Ms as a potential source for innovative, PMT-based therapy in this life-threatening disease.

OR056

Comprehensive atlas of activity and "integrome" of human hematopoietic stem/progenitor cells

S Scala[1] L Basso Ricci[1] F Dionisio[1] L Leonardelli[1] S Scaramuzza[1] M P Cicalese[1 3] F Ferrua[1 3] A Aiuti[1 2 3] L Biasco[1]

1: San Raffaele Telethon Insitute for Gene Therapy (OSR-TIGET) 2: Vita-Salute San Raffaele University 3: Pediatric Immunohematology and Bone Marrow Transplant Unit, San Raffaele Scientific Institute

Despite the wide clinical exploitation of CD34+ cells in retroviral (RV) or lentiviral (LV) gene therapy (GT) clinical trials, few information are available on the contributions of individual stem and progenitor cells (HSPC) to engineered hematopoiesis overtime. To address this issue we exploited a combination of state-of-the-art phenotypic characterization and molecular tracking by integration sites (IS) analysis to study the in vivo fate and dynamics of 7 distinct HSPC subpopulations, including hematopoietic stem cells (HSC), multipotent progenitors (MPP) and lineage committed lymphoid and myeloid progenitors. Cells were FACS-sorted from the bone marrow of 4 Wiskott-Aldrich syndrome (WAS) patients after GT with LV transduced CD34+ cells. We tracked > 700 CD34+clones, showing that individual CD34+CD38-CD90+CD45RA- HSC clones could self renew and actively generate blood cells to levels similar or higher than MPP, from 6 months up to steady-state hematopoiesis at 3 years after GT. Moreover, by preliminary phenotypic characterization we observed that WAS-treated patients showed HSPC composition similar to pediatric controls. Additionally, we are generating the first comprehensive atlas of LV and RV ''integrome'' in distinct transduced CD34+subpopulations. We FACS-sorted, at the time of theoretical re-infusion, different HSPC subpopulations and collected IS. We also transduced separately sorted HSPC to unveil preferential vector distribution within various differentiation stages. To date, we collected > 400.000 sequencing reads belonging to >6.000 unique HSPC IS. Analysis is currently ongoing. These studies will be crucial to investigate the in vivo dynamics and hierarchies of gene-corrected HSPC and to unveil vector-host interactions in the infused cell product.

OR057

The use of transgenic extracellular vesicles (EVs) expressing membrane-bound CD39/CD73 for treatment of inflammatory disease

S A Snoek[1 2] N Broekstra[1 2] J van Ittersum[1 2] J de Vrij[3] M van Geldorp[1 2] L van Baarsen[1] P P Tak[1 2] M J Vervoordeldonk[1 2] J D Finn[1 2]

1: Academic Medical Center 2: Arthrogen BV 3: Erasmus University Medical Center

Our recent data has demonstrated that the balance between pro-inflammatory extracellular ATP and anti-inflammatory adenosine is skewed in the synovial compartment of rheumatoid arthritis (RA) patients, likely contributing to ongoing inflammation. CD39 is an ATPase that converts extracellular ATP and ADP into AMP, while CD73 converts AMP into adenosine. Therefore, we aim to use CD39 and CD73 therapeutically to ameliorate the ATP:adenosine balance and reduce inflammation. CD39 and CD73 are membrane bound enzymes and previous studies have shown that removing CD39 from the membrane reduces its specific activity by >90%. Thus we are interested in using extracellular vesicles (EVs) to deliver these membrane-bound enzymes as a novel treatment for inflammatory disease. We performed a large scale purification (10L) of CD39/CD73-EVs from stably transfected HEK293 cells. We found that EV-CD39 showed >15 fold higher specific activity when compared with soluble CD39, likely due to maintaining the native structure of CD39. EVs containing CD39 and CD73 were also ~10 times more potent in reducing pro-inflammatory cytokine production in in vitro inflammation assays (IC50 in pM range). In a mouse air pouch model of inflammation, local administration of EVs expressing CD39/CD73 lowered infiltration of immune cells, CD11b expression on neutrophils, and production of CCL2 and TNF, compared to control EVs. Transgenic EVs are a promising approach for the delivery of membrane bound therapeutic enzymes and may have potential for the treatment of inflammatory disease, including rheumatoid arthritis.

OR058

Extracellular membrane vesicles from umbilical cord blood derived mesenchymal stromal cells protect against ischemic acute kidney injury, a feature that is lost after inflammatory conditioning

L Kilpinen[1] U Impola[1] L Sankkila[1] I Ritamo[1] M Aatonen[2] S Kilpinen[3] J Tuimala[1] L Valmu[1] J Levijoki[4] P Finckenberg[5] P Siljander[2] E Kankuri[5] E Mervaala[5] S Laitinen[1]

1: Finnish Red Cross Blood Service 2: Division of Biochemistry and Biotechnology, Department of Biosciences, University of Helsinki 3: MediSapiens Ltd. 4: Orion Pharma Ltd. 5: Department of Pharmacology, Institute of Biomedicine, University of Helsinki

Background: Mesenchymal stromal cells (MSC) are shown to have a great therapeutic potential in many immunological disorders. Currently the therapeutic effect of MSCs is considered to be mediated via paracrine interactions with immune cells. We investigated the production of extracellular membrane vesicles (MVs) from human umbilical cord blood derived MSCs (hUCBMSC) in the presence (MVstim) or absence (MVctrl) of inflammatory stimulus.

Methods: hUCBMSCs were cultured in serum free media with or without IFN-g and MVs were collected from conditioned media by ultracentrifugation. The protein content of MVs was analyzed by mass spectrometry. Hypoxia induced acute kidney injury rat model was used to analyze the in vivo therapeutic potential of MVs and T-cell proliferation and induction of regulatory T cells were analyzed by co-culture assays.

Results: Both MVstim and MVctrl showed similar T-cell modulation activity in vitro, but only MVctrls protected rat kidneys from reperfusion injury in vivo. The proteomic analysis revealed dramatic changes in the protein content of the MVs induced by the IFN-g stimulation. Complement factors (C3, C4A, C5) and lipid binding proteins (i.e apolipoproteins) were only found in the MVctrls, whereas the MVstim contained tetraspanins (CD9, CD63, CD81) and more complete proteasome complex accompanied with MHCI. Interestingly, two differentially produced MVs contained specific Rab proteins suggesting that same cells, depending on external signals, produce vesicles originating from different intracellular locations.

Conclusions: We demonstrate that inflammatory conditioning of MSCs influence on the protein content and functional properties of MVs revealing the complexity of the MSC paracrine regulation.

OR059

Polyplex namomicelles assembled with neprilysin mRNA augmented clearance of beta-amyloid peptide from intracerebroventricular infusion

C Y Lin[1 2] S Uchida[1] M Ikegami[1] K Itaka[1] K Kataoka[1 3]

1: The University of Tokyo 2: Japan Society for the Promotion of Science (JSPS) 3: Innovation Center of NanoMedicine (iCONM)

Gene therapy using mRNA is a promising alternative with several advantages over that of plasmid DNA (pDNA); mRNA would be simply translating proteins in cytoplasm without any risk of genome integration. In this study, we administered mRNA encoded for neprilysin (NEP) using polyplex nanomicelles in the Aβ intracerebroventricular (i.c.v.) pre-supplemented model. Since Aβ is the major component of neuritic plaques that often accumulate in the brains of Alzheimer's Disease (AD) patients and NEP play the major role for the clearance of Aβ in brain. As a proof-of-concept study to prove the feasibility of mRNA for treating AD, we constructed a mouse NEP-expressing mRNA and evaluated the capacity for degrading Aβ. The NEP mRNA exhibited superior NEP activity attributed to an apparent capability on the Aβ and human amyloid precursor protein (hAPP) degradation in primary neurons and hAPP overexpressed cells. Furthermore, we evaluated in vivo activity of NEP-expressing mRNA by i.c.v. infusion of the mRNA using polyplex nanomicelles, which allows efficient mRNA introduction in vivo into neural tissues and other various organs. ELISA evaluation revealed that the mRNA injection significantly augmented the NEP level in the mouse brain and effectively reduced the pre-supplemented Aβ in the brain. Meanwhile, a chimeric NEP mRNA engineered to express reporter green fluorescent protein (GFP) simultaneously, showed polyplex nanomicelles successfully delivered NEP mRNA into neurons in vivo. Collectively, mRNA administration is promising to be a new therapeutic approach for neurological diseases such as AD.

OR060

Exosomes from human Cardiac Progenitor Cells: development of GMP-grade manufacturing and testing methods

G Andriolo[1] V Lo Cicero[1] L Barile[1] E Cervio[1] S Soncin[1] S Bolis[1] T Torre[1] F Siclari[1] T Moccetti[1] G Vassalli[1] L Turchetto[1] M Radrizzani[1]

1: Cardiocentro Ticino

Exosomes from human Cardiac Progenitor Cells (CPC) inhibit cardiomyocyte apoptosis and improve cardiac function after myocardial infarction (Barile et al. Cardiovasc Res 2014). The development and validation of Good Manufacturing Practice (GMP)-grade methods for exosome production and testing are essential for clinical translation. To optimize expansion of CPC from atrial appendage explants, different serum-free media and adhesion surfaces were tested in comparison with the standard condition (IMDM + 20%FBS, gelatin-coated plastic), evaluating proliferation kinetics of 4 CPC batches by a colorimetric assay. For each condition, the relative proliferation (%) was calculated (= OD test condition/OD standard condition x100). At seven days the relative proliferation on non-coated plastic was 85 ± 15% with IMDM + 10% Stemulate™ (Cook General Biotechnologies); 88 ± 19% with StemMACS-MSC (Miltenyi Biotec); 57 ± 30% with Stemgro®-hMSC (Corning); on CellBIND surfaces (Corning) 49 ± 43% with IMDM + 10% Stemulate™, 77 ± 17% with StemMACS-MSC, 40 ± 10% with Stemgro®-hMSC. CPC showed the typical phenotype of mesenchymal progenitors cells (CD73/CD105 positive, CD14/CD20/CD34/CD45/HLA-DR negative), with variable expression of CD90 in different conditions; they expressed cardiac-specific transcriptional factors (GATA4/TBX5/MESP1/TBX18 mRNA). For exosome production, CPC were starved for 1 week in DMEM; exosomes were isolated by differential ultracentrifugation. Nanoparticle Tracking Analysis (NanoSight-LM10, Malvern Instruments) showed comparable profiles for exosomes harvested from CPC cultured in standard condition and in StemMACS-MSC. Western Blot Analysis showed that exosomes contain Alix and TSG101; analysis of other exosome-associated markers (CD9, CD63, CD81) is in progress, such as functional evaluation of exosomes obtained in different conditions. In conclusion, StemMACS-MSC may be suitable for GMP-grade production of human CPC-derived exosomes. Methods for exosome purification are under development.

OR061

Does the common use of national regulatory pathways undermine the general European regulation on advanced therapy medicinal products?

A Mahalatchimy A Faulkner

1: Centre for Global Health Policy, School of Global Studies, University of Sussex

In 2007, the European Union adopted a specific regulation on Advanced Therapy Medicinal Products with the main goals of promoting public health as well as the European market. It notably clarifies that all medicinal products that are covered by such regulation have to be authorised according to the centralised procedure for marketing authorisation, i.e. by the European Commission after the opinion of the European Medicines Agency and especially of the new Committee for Advanced Therapies. However, more than six years after its entry into force, only five advanced therapy medicinal products have been authorised through this regulatory pathway. Indeed, it appears that many more products are authorised and available to patients outside this general regulatory framework. Focusing on the UK and France, we will discuss the national regulatory pathways that are used for advanced therapy medicinal products. On the one hand, the hospital exemption scheme which exists in both countries as coming from European Union law is differently regulated and used. On the other hand, the UK and France also have other specific regulatory pathways. Are these national regulatory pathways a means of bypassing the European pathway or could they be used as a link to enable it? This work is supported by REGenableMED, UK ESRC Project ES/L002779/1.

OR062

Environmental risk assessment for gene and cell therapy products: Tips and tricks to avoid pitfalls with clinical trial and market authorization applications

U Jenal

1: Jenal & Partners Biosafety Consulting

The environmental risk assessment (ERA) forms a significant part of clinical trial and market authorization applications for therapies with drug substances containing or consisting of genetically modified organisms (GMO). An ERA is required regardless of the type of therapy, i.e. in vivo or ex vivo gene therapy, oncotherapy or immune therapy. This presentation addresses the most frequent and tricky questions related to an ERA, drawing from actual experiences with application processes. In order to prepare a comprehensive and compelling risk assessment document, an ERA should cover all characteristics of the GMO having a potential adverse effect in case of exposure of people other than the treated patients or the environment. Most importantly, a rational has to be given on a) how exposure is avoided due to safe application of the drug substance or b) how negative consequences from exposure are prevented due to safety features of the GMO. To this end, information will have to be derived from specific data gained from research and development, from manufacturing processes as well as from pre-clinical or clinical studies. Having these data at hand is crucial for the preparation of an ERA. Therefore, the planning of manufacturing processes, pre-clinical studies or clinical trials should ensure that these data will be generated for future clinical trial or market authorization applications.

OR063

Vammin induces a highly efficient angiogenic response through VEGFR-2/NRP-1 and bypasses the regulatory function of VEGFR-1

P I Toivanen[1] T Nieminen[1] J P Laakkonen[1] T Heikura[1] M U Kaikkonen[1] S Ylä-Herttuala

1: A.I.Virtanen Institute

Vascular Endothelial Growth Factors (VEGFs) are candidate molecules for the treatment of ischemic diseases by therapeutic angiogenesis. Properties of a novel snake venom derived VEGF

family member, Vammin, were compared to a fully soluble VEGF-A109 and ECM binding VEGF-A165 both in vitro and in vivo using adenoviral gene transfer vectors. All tested factors bound equally to VEGFR-2 and VEGF-A165 and Vammin also to NRP-1 and heparin. Vammin forms with engineered C-terminal sequences showed considerable flexibility in NRP-1 binding determinants with either a C-terminal arginine or lysine compatible for binding. Vammin induced more potently and efficiently VEGFR-2 mediated cell proliferation than either VEGF-A form. Accordingly, Vammin induced differential expression of genes associated to cell proliferation, migration and angiogenesis in HUVECs, whereas VEGF-A165 and especially VEGF-A109 induced less pronounced changes. Adenoviral gene delivery of Vammin induced a highly efficient angiogenic response in rabbit skeletal muscles. Low doses of Vammin, VEGF-A109 or VEGF-A165 induced mainly sprouting angiogenesis, whereas high doses of especially VEGF-A109 induced only enlargement of pre-existing capillaries. Binding of a ligand to VEGFR-2 is sufficient for capillary enlargement and increased perfusion, but the vector dose and binding to NRPs and HSPGs are important determinants governing the amount of sprouting angiogenesis. Vammin is a highly potent and efficient pro-angiogenic molecule and an exciting candidate for future development of pro-angiogenic therapies.

OR064

EphrinB2/EphB4 signaling controls the switch between normal and aberrant angiogenesis by VEGF

E Groppa[1 2] S Brkic[1 2] V Sacchi[1 2] M G Muraro[1 2] M Trani[1 2] S Reginato[1 2] M Heberer[1 2] R Gianni-Barrera[1 2] A Banfi[1 2]

1: Department of Biomedicine, University of Basel
2: Department of Surgery, Basel University Hospital

Vascular Endothelial Growth Factor-A (VEGF) is the master regulator of vascular growth and it induces either normal or aberrant angiogenesis depending on its dose in the microenvironment around each producing cell in vivo. However, pericyte recruitment stimulation by Platelet Derived Growth Factor-BB (PDGF-BB) prevents aberrant structures, yielding homogeneously normal angiogenesis. Here we dissected the role of specific pericyte-mediated signaling pathways in the switch between normal and aberrant angiogenesis by VEGF. Monoclonal populations of transduced myoblasts were used to homogeneously express specific VEGF doses, inducing either normal or aberrant angiogenesis, and were further transduced to secrete soluble blockers of the TGFβ-1/TGFβ-R, Tie2/Angiopoietin or EphB4/EphrinB2 pathways (LAP, sTie2Fc and sEphB4, respectively). Two weeks after implantation into mouse leg muscles, EphrinB2/EphB4 inhibition, but not TGFβ nor Angiopoietin blockade, switched normal angiogenesis by low VEGF to aberrant angioma-like structures, similar to the effects of blocking pericyte recruitment. Conversely, gain-of-function of EphB4 signaling (systemic treatment with recombinant EphrinB2-Fc) converted aberrant angiogenesis by high VEGF into normal networks of mature capillaries. We found that VEGF over-expression in muscle induces angiogenesis without sprouting, but by circumferential enlargement and longitudinal splitting (intussusception). EphB4 signaling regulated endothelial proliferation and therefore the diameter of initial enlargements, determining whether they could split successfully (normal) or not (aberrant growth), based on the achieved diameter. Mechanistically, it did not interfere with pericyte recruitment, contrary to high VEGF alone, but rather regulated VEGF-R2 downstream signaling through phospho-ERK, but not through PI3-kinase/Akt. Therefore, EphrinB2/EphB4 signaling can prevent VEGF-induced aberrant angiogenesis and expand its therapeutic window.

OR065

Tracking genetically engineered lymphocytes long-term reveals the dynamic of T-cell immunological memory

G Oliveira[1 2] E Ruggiero M T Lupo-Stanghellini N Cieri M D'Agostino[1 2] R Fronza L Biasco F Dionisio S Mastaglio S Mastaglio R Greco J Peccatori A Aiuti A Bondanza A Lambiase C Traversari L Vago C Von Kalle M Schmidt C Bordignon[5] F Ciceri[1] C Bonini[1]

1: San Raffaele Scientific Institute 2: Universita Vita-Salute San Raffaele 3: German Cancer Research Center 4: San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET) 5: MolMed S.p.A

A long-lasting immune protection to pathogens and cancer requires the generation of memory T cells able to survive long-term. To unravel the immunological requirements for long-term persistence of memory T cells in humans, we characterized and traced, over several years, T lymphocytes genetically modified to express the Thymidine Kinase (TK)-suicide gene and infused in 10 patients after haploidentical hematopoietic stem cell transplantation (HSCT). At 2–14 years after infusion and in the presence of a complete and resting immune system, we could still detect effectors/effector memory (TEM/EFF), central memory (TCM) and stem memory (TSCM) TK+ cells, circulating at low but stable levels in all patients. Longitudinal analysis of CMV and Flu-specific TK+ cells indicates that antigen recognition is dominant in driving their in vivo expansion and persistence. Characterization of TK+-cell products infused to patients showed that the amount of infused TSCM cells positively correlates with early expansion and long-term persistence. By combining sorting of memory T-cell subsets with sequencing of integration sites, TCRα and TCRβ clonal markers, we longitudinally traced T-cell clones from infused products to late follow-up time-points. We showed that although T cells retrieved long-term are enriched in clones originally shared in different memory T-cell subsets, dominant long-term clonotypes preferentially originate from infused TSCM clones. Altogether, these results indicate that long-term persistence of memory T cells after haploidentical HSCT is determined by antigen exposure and by the original phenotype of infused cells, according to hierarchical model in which TSCM are superior to TCM and TEM/EFF.

OR066

Fucoïdan promotes early step of cardiac differentiation from human embryonic stem cells and long term maintenance of beating areas

S Hamidi[1 4] D Letourneur[2 4 5] R Aid-Launais[2 4 5] A Di Stefano[1 6] W Vainchenker[1 6] F Norol[1 3] C Le Visage[2 4 5]

1: Inserm UMR 1009, Institut Gustave Roussy, Villejuif, 94805, France. 2: Inserm U698, Cardiovascular Bioengineering, X Bichat Hospital, Paris, 75019, France 3: AP-HP, Service de Biothérapie, Hospital Pitié Salpétrière, Paris, 75013, France. 4: Univ Paris Diderot, Paris, 75013, France. 5: Sorbonne Paris

Cité, Universite Paris Nord, Villetaneuse, 93430, France. 6: Université Paris-Sud, Orsay, 91400, France

Introduction: Replacing loss of cardiac cells after ischemic heart injuries using a tridimensional biocompatible scaffolds for cell delivery could be assimilated to a microenvironment mimicking culture device directly in contact with the targeted area. Our main work was to modify those scaffolds properties to improve cardiac commitment of human embryonic stem cells (hESC).

Experimental Procedures: We modified scaffolds physical and chemical properties to support human embryonic stem cells (hESCs) differentiation toward the cardiac lineage, by incorporating gelatin and Fucoidan, and analyzed hESC cardiac commitment markers expression by qPCR and histological analysis.

Results: Fucoidan scaffolds ability to locally concentrate and slowly release TGF-β and TNF-α was confirmed by Luminex technology. They support significantly higher expression of the early step of embryonic cardiac differentiation markers: NKX2.5 ($p < 0.05$), MEF2C ($p < 0.01$), and GATA4 ($p < 0.01$). We also found that Fucoidan scaffolds supported the late stage of embryonic cardiac differentiation marked by ANF expression ($p < 0.001$). Moreover, thin connecting smooth muscle cells filaments enabled maintenance of beating areas for up to 6 months.

Conclusion Perspectives: Porous scaffolds are a promising method to improve cells delivery to damaged myocardium. Absence of mechanical stress in the soft hydrogel impaired sarcomere formation, as confirmed by cardiac muscle myosin MYH6 and sarcomeric α-actinin analysis. As a consequence, further scaffolds chemical stiffening or combination with mechanical stress, could enhance sarcomere formation at terminal stages of differentiation (TEA. 2014 doi: 10.1089)

OR067

Differences in collateral-dependent muscle perfusion may explain efficacy variation in clinical angiogenic gene therapy trials

P Korpisalo[1 2] S Tarvainen[1] T Auvinen[2] K Mäkinen[2] J Hytönen[1] H Mussalo[3] T Liimatainen[3] M Hakulinen[3] J Hakumäki[3] T Laitinen[3] S Ylä-Herttuala[1]

1: A.I.Virtanen Institute, University of Eastern Finland, Kuopio 2: Heart Center, Kuopio University Hospital 3: Imaging Center, Kuopio University Hospital

Background: Angiogenic gene therapy aims to improve ischemic tissue oxygenation by opening up the capillary bed and supporting collateral growth in chronically ischemic conditions where endogenous vascular repair mechanisms are compromized. However, to date only a few patients have benefited from the clinical trials of the novel treatment.

Methods and aims: We initiated a clinical phase I-II, placebo controlled, single blind angiogenic gene therapy trial in chronic lower limb ischemia to test the safety and angiogenic potential of the mature form of vascular endothelial growth factor D in an adenoviral vector (AdVEGF-DdNdC). 30 patients undergoing peripheral bypass surgery will receive i.m. injections of 3x10e10 or 3x10e11vp of AdVEGF-DdNdC or NaCl as control. Importantly, the study utilises multimodality imaging (including MRI/S, FDG-PET contrast enhanced ultrasound and fotoacustic imaging) to objectively assess the effects of the investigational therapy and to reveal possible sources of variation in patient responses.

Results: With the lower dose group of patients now completed, the AdVEGF-DdNdC gene therapy was well tolerated and improved relative tissue perfusion with all treated patients one week after gene transfer. However, significant variation in the baseline level of collateral-dependent microvascular perfusion was observed among patients negatively affecting the potential efficacy of AdVEGF-DdNdC and increasing variation in the final results.

Conclusions: The baseline level of collateral-dependent microvascular perfusion varies considerably among patients with limb ischemia and seems to negatively affect the results of angiogenic gene therapy. Patients with initially low or low post operative microvascular perfusion may best benefit from angiogenic therapies.

OR068

Modulation of neuroinflammatory responses in the cuprizone mouse model following transplantation of mesenchymal stem cells genetically engineered to secrete IL13 coincides with the appearance of multiple alternatively activated macrophage and microglia phenotypes

D Le Blon[1 2] C Guglielmetti[3] C Hoornaert[1 2] D Dooley[4] J Daans[1 2] E Lemmens[4] N De Vocht[1 2] K Reekmans[1 2] E Santermans[5] N Hens[2 5 6] H Goossens[2] M Verhoye[3] A Van der Linden[3] Z Berneman[1 2] S Hendrix[4] P Ponsaerts[1 2]

1: Laboratory of Experimental Hematology, University of Antwerp, Antwerp, Belgium 2: Vaccine and Infectious Disease Institute, University of Antwerp, Antwerp, Belgium 3: Bio-Imaging Laboratory, University of Antwerp, Antwerp, Belgium 4: Department of Morphology, Biomedical Research Institute, Hasselt University, Diepenbeek, Belgium 5: Center for Statistics, I-Biostat, Hasselt University, Hasselt, Belgium 6: Centre for Health Economic Research and Modeling Infectious Diseases (Chermid), University of Antwerp, Antwerp, Belgium

Forced alternative activation of neuroinflammatory responses is expected to become a new therapeutic approach for a variety of central nervous system (CNS) disorders in which pro-inflammatory microglia and/or macrophage responses contribute to the neuropathology. With this multidisciplinary study, we propose a novel neuroimmune-modulating approach involving intracerebral transplantation of mesenchymal stem cells (MSC) genetically engineered to secrete interleukin 13 (IL13). In the first part of this study we investigated whether grafting of IL13-producing MSC in the corpus callosum can alter the course of cuprizone (CPZ)-induced CNS inflammation and demyelination. Using non-invasive T2-weighted magnetic resonance imaging we here demonstrate that grafting of IL13-producing MSC displays a significantly superior protection against CNS inflammation and demyelination as compared to grafting of non-engineered MSC. Further experiments in eGFP + bone marrow chimeric mice and in the CX3CR1 + /eGFPCCR2 + /RFP transgenic mouse model, both used to histologically discriminate between brain-resident microglia and CNS-infiltrating bone marrow derived macrophages, we demonstrate that grafting of IL13-producing MSC results in a significant recruitment of peripheral macrophages into the CNS parenchyma which, by the action of IL13, become forced into multiple alternatively activated phenotypes as demonstrated by differential expression patterns of MHCII and/or Arginase-1. Moreover, the appearance of these alternatively activated macrophage phenotypes following grafting of IL13-producing MSC

coincides with the observed microglial quiescence in the CPZ model. Concluding, with this study we suggest that controlled and localized introduction of alternatively activated macrophages at the onset of neuroinflammatory responses has the potential to exert a major immunomodulatory effect on pathology-associated microglial immune responses.

OR069

Extensive comparison of HEK293 transfection process and Baculovirus expression system to manufacture an AAV8-based treatment for Crigler-Najjar syndrome

L Guianvarc'h F Collaud C Le Bec F Mingozzi F Mavilio M Hebben

1: GENETHON

An AAV8 vector expressing the human UDP-glucuronosyltransferase 1 polypeptide A1 (UGT1A1) has been developed for the treatment of Crigler-Najjar syndrome. To enter phase I/II clinical phase, a robust and GMP-compliant manufacturing process is needed to produce the therapeutic vector in sufficient quantity and quality. Two production platforms have been evaluated to produce the recombinant AAV8 in stirred tank bioreactors: on one hand, the standard triple transfection process was adapted to HEK293 cells growing in suspension; on the other hand, the dual Baculovirus expression system in Sf9 cells was tested. In both cases, the AAV8 vector was produced in 2L and 10L scale bioreactors and was then purified by immunoaffinity chromatography, concentrated and formulated using tangential flow filtration. The resulting final products have been thoroughly analyzed using a wide set of analytical tools to characterize the product purity, integrity and efficacy. Both processes displayed comparable productivity and generated similar ratios of full/empty capsids. However, the results revealed that the HEK293 transfection process was the most efficient to produce AAV8-UGT1A1 vector with the foreseen target profile, particularly with regards to genome integrity, capsid proteins content and infectivity. As expected, this vector profile correlated with the highest level of transgene expression in vitro and with the best efficacy to decrease bilirubinemia in a rodent model of Crigler-Najjar syndrome. Based on these results, the triple transfection process is being scaled up in disposable bioreactors in order to produce clinical grade AAV8-UGT1A1 vector.

OR070

iCELLis® fixed-bed technology provides an efficient scalable system for viral vector production

H P Lesch[1 2 3] P Valonen[1] K M Heikkilä[1] E M Lipponen[1 2] A Müller[1] E Räsänen[1] T Tuunanen[1] J Malinen[3] K E Makkonen[3] A Valkama[3] T Heikura[3] A Martikainen[3] T Salonen[3] E M Nurminen[1] M M Hassinen[1 2] M Karhinen[1] N R Parker[1 2] R Shaw[1] S Ylä-Herttuala[3 4]

1: FinVector Vision Therapies Ltd 2: FKD Therapies 3: A.I.Virtanen Institute 4: Kuopio University Hospital

Large scale vector manufacturing has proven to be challenging. The iCELLis® 500 provides disposable fixed-bed system with perfusion capability and with automated control of stirring, temperature, pH and dissolved oxygen. The cultivation area varies between 66 and 500 m2. It is ideal to satisfy demand for phase III / commercial requirements. In iCELLis® Nano, the cultivation area 0.53-4 m2 is ideal for small batch production and process development purposes. We have used iCELLis Nano for the production of different kind of viruses, such as adenovirus, lentivirus and AAV. HEK293 and 293T cell culture parameters were optimized in adherent mode and also suspension cells in serum-free medium were tested. Adenovirus infection and harvest by chemical lysis inside the bioreactor was proven to be efficient. Lentivirus and AAV production by calcium phosphate mediated plasmid transfection was optimized. Lentivirus harvest was performed by perfusion, whereas AAV harvest was done by lysis technique. We have also tested baculovirus mediated lentivirus production in iCELLis®. The scalability of the system was demonstrated by manufacturing adenovirus using iCELLis® 500 100m2 bioreactor with the process parameters defined in a small scale. High yield productivity was achieved in a large scale system. The iCELLis® bioreactors supported cell growth, although an uneven distribution of the cells was observed, mostly in iCELLis® Nano 4m2 bioreactor. To conclude, iCELLis® equipment has shown us an efficient way to manufacture large batches of different kinds of gene therapy products suitable for large preclinical animal models and up to phase III trial and beyond.

OR071

Human cardiac stem cells for allogeneic cell therapies: integrating bioprocess development and "omics" characterization tools

P Gomes-Alves[4 5] M Serra[4 5] C Brito[4 5] M FQ Sousa[4 5] L R Borlado[7] J A López[3] J Vázquez[3] M JT Carrondo[4 6] A Bernad[8] P M Alves[4 5]

1: iBET 2: Coretherapix 3: Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain 4: iBET, Instituto de Biologia Experimental e Tecnológica, Oeiras, Portugal 5: Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal 6: Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Monte da Caparica, Portugal 7: Coretherapix, Tres-Cantos, Spain 8: Centro Nacional de Biotecnología, Madrid, Spain

Stem cells therapies stand as a promising strategy for cardiac repair as the adult heart homes a population of resident cardiac stem cells (hCSC) capable of regenerate contractile myocardium upon activation [1]. Decoding the intricate cellular pathways, growth-factors and receptors involved in this process is also of major relevance to understand cardiac repair and improve clinical intervention. With this work we aim at establishing a robust and scalable bioprocess for hCSC expansion to support allogeneic cell therapies but also to perform the proteomic profiling of hCSCs receptome and secretome to provide new insights about the molecules and pathways involved in cardiac repair events. Significantly higher expansion cell yields were obtained in stirred tank bioreactors when compared to static cultures while cells remained phenotypically and functionally similar, concerning cell viability, metabolism, GF secretion and differentiation potential. A high-throughput proteomics workflow was implemented, enabling identification of challenging proteins such as GF and receptors, which are relatively low abundant and hydrophobic membrane proteins, respectively. hCSC receptome analyses lead to the identification of more than 3000 proteins, several hundred with numerous transmembrane domains (e.g. Connexin-43), among which more

than 150 were plasma membrane receptors (e.g. IGF2R) [2]. Secretome data analysis enabled the identification of about 300 proteins (e.g. IGF-binding protein-7). These data strongly contribute to a better molecular characterization of hCSC, unveiling possible effectors of cardiac repair mechanisms.

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OR072

Automated lentiviral transduction of T cells with CARs using the CliniMACS Prodigy

L E Nickolay[1] U Mock[1] G WC Cheung[2] M Pule[2] A Thrasher[1] I Johnston[3] A Kaiser[3] W Qasim[1]

1: UCL Institute of Child Health 2: UCL Cancer Institute 3: Miltenyi Biotec GmbH

Novel cell therapies derived from human T lymphocytes are exhibiting enormous potential in early phase clinical trials in patients with haematological malignancies. Ex-vivo modification of T cells is currently limited to a small number of centres with the required infrastructure and expertise. The process requires isolation, activation, transduction, expansion and cryopreservation steps. To simplify procedures and widen applicability for clinical therapies, automation of these procedures is being developed in cooperation with Miltenyi Biotec. The CliniMACS Prodigy system has been adapted for lentiviral transduction of T cells and here we present preliminary data with the device. A lentiviral vector encoding a chimeric-antigen-receptor specific for CD19 (CAR19) was used for T-cell transductions. Using a closed single-use tubing set, freshly collected or freeze/thawed mononuclear cells from non-mobilised leukapheresis collected from healthy donors were loaded onto the CliniMACS Prodigy and activated with TransAct, a polymeric nanomatrix activation reagent incorporating CD3/CD28-specific antibodies. Cells were transduced 24–48h after activation and expanded in the CentriCult-Unit of the tubing set, allowing for stable culture conditions as well as automated feeding and media exchange. The process was continuously monitored to determine kinetics of cell expansion, transduction efficiency and the phenotype of the expanded cells. Small and large scale transductions were run in parallel to assess the efficiency of the automated T-cell modification. Finally, cells were harvested and cryopreserved to assess the functional capabilities of CAR19 T cells. Overall transduction efficiencies, cell yields and characteristics were comparable to existing manual procedures, indicating that the device has valuable therapeutic potential.

OR073

DARPins as tool for flexible vector targeting – specific manipulation of GluA4 positive interneurons in defined brain areas

J Hartmann[1] R T Freiling[1] R Münch[1] S Beyer[2] J Koch[2] M Seeger[3] K Khodosevich[4] H Monyer[4] C J Buchholz[1]

1: Paul Ehrlich Institute 2: Georg-Speyer-Haus 3: University of Zurich 4: Medical Faculty of Heidelberg University and DKFZ

Interneurons are inhibitory neurons that are responsible for establishing inhibitory circuits in the brain. Their dysfunction is associated with schizophrenia, autism and intellectual disabilities. They constitute only 20–25% of all neurons in the cortex, are strikingly diverse and therefore difficult to manipulate. To solve this problem, we generated viral vectors which are specifically retargeted to interneurons. Cell-type specificity is provided by designed ankyrin repeat proteins (DARPins) displayed on the vector particle surface which are blinded for recognition of the natural receptor. Notably, DARPins are small binding molecules (14-17 kDa), from which combinatorial DARPin libraries with more than 10 to the power of 12 variants can be generated and used to select high affinity binders for almost any target of choice. In this respect, we generated a novel combinatorial DARPin library which can be easily transferred to LV and AAV vectors. Subsequently, DARPins specific for the glutamate receptor subunit GluA4, which is a marker for a subpopulation of interneurons, were selected via ribosome display. Selected GluA4-DARPins proofed to be specific for GluA4 and showed no cross-reaction to the closely related GluA2 receptor. In addition, LV and AAV particles displaying GluA4-DARPins selectively transduced GluA4 expressing CHO cells. Furthermore, preliminary in vivo experiments indicate an efficient and specific gene transfer to interneurons upon injection of GluA4-AAV-into the motor cortex of mice. Taken together, these results indicate that a rare subpopulation of interneurons in the brain can be specifically transduced enabling more-effective therapeutic interventions in the future.

OR074

A novel RNA virus-based episomal vector system for long-term stem cell modification

A Makino R Komorizono S J Holditch B Lu T Honda Y Ikeda K Tomonaga

1: Kyoto University 2: Mayo Clinic

Genetically modified stem and progenitor cells emerged as promising regenerative platforms to treat genetic and degenerative disorders, highlighted by successful stem cell gene therapy for inherent immune deficiency. However, the biosafety concern over insertional mutagenesis, due to integrating recombinant viral vectors, has overshadowed the widespread clinical applications of genetically modified stem cells. Here we report Borna disease virus (BDV)-based vector, as a unique RNA-based episomal vector, amenable for long-term transgene expression in stem cells. Unique among animal RNA viruses, BDV non-cytopathically replicates and readily establishes a long-lasting, persistent infection in the nucleus of various cell types. BDV-based vectors allowed for long-term transgene expression in mesenchymal stem cells without affecting the morphology, cell surface CD105 expression, or the ability to differentiate into adipocytes. Similarly, replication-defective BDV vectors achieved long-term transduction of human induced pluripotent stem cells (iPSCs), while maintaining their capability of differentiating into three embryonic germ layers. Furthermore, we succeeded to reduce the induction of BDV vector in iPSC by addition of ribavirin, ribonucleic analog-type anti-viral drug. On the other hand, in this study we improved the rescue efficiency of BDV vector by identification of a host factor, which regulates BDV particle production. Knockdown of the host factor in BDV-infected cells provided 30-fold higher titer virus production, compared with control. Thus, our BDV-based vectors offer a novel

episomal RNA expression system for stem cell transduction without genomic modification.

OR075

Expression of three allogeneic MHC Class I in recipient liver significantly prolongs survival of fully-allogeneic vascularised cardiac allografts

M S Moawadh[1] M Paul[1] E C Cunningham[1] C Wang[1] S Cunningham[2] S S Tay[3] G J Logan[2] I E Alexander[2] P Bertolino[3] D G Bowen[1 3] G A Bishop[1] A F Sharland[1]

1: The University of Sydney 2: Children's Medical Research Institute 3: Centenary Institute of Cancer Medicine and Cell Biology

In previous studies, AAV-mediated gene transfer of a single mismatched donor MHC class I molecule (Kd) to C57BL/6 recipient liver induced tolerance to Kd–expressing skin grafts. However, survival of fully mismatched d-haplotype skin or heart grafts is not extended in AAV-Kd treated mice. Tolerance induction may require expression of all mismatched MHC molecules (3 class I and 2 class II). To facilitate expression of multiple MHC I in recipient liver, we created a construct in which the three d-haplotype heavy chains Dd, Ld and Kd were separated by an F2A linker (DaLeK), and then determined the effect of administration of this vector upon heart graft survival. DaLeK was packaged into a liver-specific rAAV2/8 vector. Fully-allogeneic hearts from DBA/2 (H-2d) were transplanted into C57BL/6 (H-2b) at either d7 or d14 post-inoculation. Administration of 5x1011 vector genome copies (vgc) AAV-DaLeK to C57BL/6 mice yielded strong expression of Dd, Ld and Kd on hepatocytes. Expression was enhanced by co-transduction with a vector encoding β2 microglobulin, ALT levels remained normal and no inflammatory infiltrates were detected. Survival of DBA/2 hearts transplanted into AAV-DaLeK treated mice was prolonged from a MST of 7 days to a MST of 23 days. Administration of a vector encoding third-party Kk did not alter survival. In conclusion, AAV-DaLeK permits expression of multiple MHC I from a single vector, and its administration significantly prolongs survival of fully-allogeneic heart transplants. A combination of AAV-DaLek with vectors expressing CIITA and/or allogeneic MHC class II may produce tolerance to fully-allogeneic grafts.

OR076

Crispr/Cas9-based COL7A1 editing for recessive dystrophic epidermolysis bullosa

A Izmiryan[1 2] M Bovolenta[3 4] F Mavilio[3] A Hovnanian[1 2 5]

1: INSERM U1163, Institut Imagine, Paris, France 2: University Paris Descartes Sorbonne Cité, Paris, France 3: Genethon, 1 bis, rue de l'Internationale, 91002 Evry Cedex, France 4: Department of Life Sciences and Biotechnology, University of Ferrara, 74 Fossato di Mortara, 44121 Ferrara, Italy 5: Department of Genetics, Necker Hospital, Paris, France

Recessive Dystrophic Epidermolysis Bullosa (RDEB) is a rare and severe genetic skin disease responsible for blistering of the skin and mucosa. RDEB is caused by a wide variety of mutations in COL7A1 encoding type VII collagen, the major component of anchoring fibrils which are key attachment structures for dermal-epidermal adhesion. Site-specific CRISPR/Cas9-mediated Homologous Recombination (HR) is emerging as a powerful approach for gene editing to correct disease mutations. Here we provide preliminary data for COL7A1 editing using the CRISPR/Cas9 approach without selection. We designed five guide RNAs (gRNAs) in order to correct a RDEB causative null mutation in exon 2 (c.189delG; p.Lys6Trp*40). Four of them showed efficient expression, low toxicity and up to 20% of activity in HEK293 cells. These gRNAs were cloned into a lentiviral vector and delivered as integration-deficient lentivirus (IDLVs). Among the four site-specific gRNAs tested, two showed significant activity in HEK293 cells (up to 35%), in primary keratinocytes and in fibroblasts (up to 15%) when delivered as IDLVs. To achieve gene editing in RDEB cells, primary keratinocytes and fibroblasts isolated from a RDEB patient homozygous for the c.189delG mutation were co-transduced with IDLVs encoding the site-specific CRISPR/Cas9 and the corresponding Donor. Genetic correction could be detected in bulk-transduced cells when performing allele-specific PCR and direct sequencing. In addition, type VII collagen was detected in up to 10% of these cells as assessed by immunochemistry. Next steps will aim at improving the efficiency of CRISPR/Cas9-mediated HR for the development of transplantable skin models suitable for clinical application.

OR077

Ancestral sequence reconstruction disrupts epitopes yet preserves AAV structure and function

H T Turunen[1 2 3 4] E Zinn[1 2 3 4] S Pacouret[1 2 3 4] L H Vandenberghe[1 2 3 4]

1: Schepens Eye Research Institute 2: Massachusetts Eye and Ear Infirmary 3: Harvard Medical School 4: Grousbeck Gene Therapy Center

AAV is highly prevalent in human populations resulting in long lasting immunity against capsid antigens shared between virus and vector, often referred to as pre-existing immunity (PEI). PEI has been shown to block gene transfer due to neutralizing antibodies (NAB) and implicated to negatively impact the safety and efficacy of gene therapy. It has been proposed previously to develop engineered AAVs devoid of capsid surface epitopes that govern PEI. However, due to poor understanding of the sequence–function correlation of the AAV capsid, this is particularly challenging to achieve while retaining the therapeutically relevant structural and functional properties of the vector. We hypothesized that ancestral sequence reconstruction (ASR) methodology could be used to simultaneously mutate a large percentage of capsid amino acids to disrupt NAB targeted epitopes while preserving biological functionality of the vector. By comparing sequences and phenotypes with contemporary AAVs, a more comprehensive understanding of capsid sequence–function correlation could also be obtained. Novel AAV capsid proteins with up to 95 mutated amino acids (12.8%) as compared to their contemporary evolutionary descendants were generated and used to produce functionally viable AAV vectors with potential broad applicability in gene therapy. Vector neutralization by human serum samples from different populations was studied with in vitro and in vivo assays. Also, NAB cross-reactivity between ancestral and contemporary vectors was assessed by using serum from pre-immunized non-human primates. Our results indicate that ASR methods have potential in generating novel AAV vectors with unique immunological properties.

OR078

Introducing the 'Transgene Repression in vector Production' (TRiP) system: a universal viral vector production system for enhancing therapeutic vector titre.

H E Maunder J Wright V Scripps S Iqbal S Ellis Y Lad S Tatoris N G Clarkson K B Reddy C R Vieira K A Mitrophanous D C Farley

1: Oxford BioMedica

One of the main impediments to the realisation of the potential of gene therapy is manufacturing sufficient amounts of vector at consistently high quality. For most vector platforms, the high 'benchmark' vector titres achieved with 'inert' reporter genes are seldom achieved with therapeutic vectors expressing biologically active proteins. This is often due to constitutive expression of the transgene protein during vector production, which can affect some aspect of production cell metabolism/viability and/or vector assembly/activity. Severely reduced titres can greatly impede, or even prevent clinical development. Typically, powerful promoters are used to drive the transgene transcription unit in order to achieve therapeutically relevant concentrations in vivo. Whilst the use of tissue-specific promoters can be employed to lower transgene expression in production cells, they can suffer from leakiness, and have poor/unpredictable activity in target cells of different species during pre-/clinical development. We have developed the TRiP system for the production of both RNA- and DNA-based viral vectors, achieving high titres independently of transgene or promoter element utilised. The TRiP system employs a translational block of one or more transgenes by using the bacterial 'Tryptophan RNA-binding Attenuation Protein' (TRAP) to repress transgene translation only during vector production. We have demonstrated the TRiP system in HEK293T cells, where therapeutic lentiviral vector titres can be increased by up to 100-fold (in some cases close to the benchmark). The TRiP system has the capacity of standardising manufacture within viral vector platforms, since transgene activity is minimal/absent.

Poster Presentations

P001

Development of a cellular model of pyruvate kinase deficiency in human progenitors using CRISPR/Cas9 lentiviral vectors

S Lopez-Manzaneda[1 2] R Torres[3] R Sanchez-Dominguez[1 2] J C Ramirez[4] J A Bueren[2 5] J C Segovia[1 2]

1: Differentiation and Cytometry Unit-Division of Hematopoietic Innovative Therapies, CIEMAT/CIBERER, 28040 Madrid, Spain 2: Advanced Therapies Unit, IIS-Fundacion Jimenez Diaz (IIS-FJD, UAM), 28040 Madrid, Spain 3: Unidad de Vectores Virales, Centro Nacional de Investigaciones Cardiovasculares, 28029 Madrid, Spain 4: VIVEbioTECH, 20009 Donostia-San Sebastian, Spain 5: Division of Hematopoietic Innovative Therapies, CIEMAT/CIBERER, 28040 Madrid, Spain

Pyruvate Kinase Deficiency (PKD) is an autosomal recessive disease caused by mutations in the PKLR gene producing chronic non-spherocytic hemolytic anemia, which can be fatal in some patients during early childhood. The only curative treatment for PKD is allogeneic bone marrow transplantation, making PKD a good scenario for gene therapy. We designed a successful preclinical gene therapy protocol in a PKD mouse model with a lentiviral vector (LV) carrying a codon-optimized version of the PKLR cDNA (coRPK) that conferred therapeutic levels of PKLR expression. This vector has been recently designated as an Orphan Drug for PKD treatment by the European Commission (PGK-coRPK LV, EU/3/14/1130). Due to difficulties for obtaining primary CD34+ cells from PKD patients to assess the efficacy of the LV, an alternative source of human PKD-hematopoietic progenitors is needed. For this purpose, we have generated a CRISPR/Cas9 system with guide-RNAs designed against different regions of the PKLR gene, to knock-out RPK expression. Up to six different Guide-RNAs were specifically developed to cleavage the wild type PKLR gDNA. These Guide-RNAs contain at least 3 mismatches with coRPK, to avoid the cleavage of the therapeutic transgene. 293T cells and primary CD34+ cells were electroporated with plasmids containing these constructs. Three of them showed the capability to generate double strands breaks in the PKLR gene. In order to identify and select the edited cells the Cas9-gRNAs were cloned into a lentiviral backbone construct also expressing the ZsGreen1 protein. The efficacy of these new vectors is currently being tested.

P002

Optimization of gene editing in human hematopoietic progenitors to insert a therapeutic matrix in the PKLR gene

O Quintana-Bustamante[1 2] S Fañanas-Baquero[1 2] I Orman[1 2] F Rodriguez-Fornes[2 4] R Sanchez-Dominguez[1 2] A Gouble[3] R Galetto[3] L Poirot[3] J A Bueren[2 4] J C Segovia[1 2]

1: Differentiation and Cytometry Unit- Division of Hematopoietic Innovative Therapies, CIEMAT/CIBERER, 28040 Madrid, Spain 2: Advanced Therapies Unit, IIS-Fundacion Jimenez Diaz (IIS-FJD, UAM), 28040 Madrid, Spain 3: CELLECTIS SA, 75013 Paris, France 4: Division of Hematopoietic Innovative Therapies, CIEMAT/CIBERER, 28040 Madrid, Spain

Gene editing is emerging as a promising gene therapy approach. However, its direct application to hematopoietic stem cells (HSCs) is challenging because it needs a balance between gene editing efficacy and maintenance of HSCs properties. Pyruvate Kinase Deficiency is a rare erythroid metabolic disease caused by mutations in the PKLR locus, producing an energetic imbalance in erythrocytes. We developed a gene editing approach through a knock-in strategy using a therapeutic

matrix carrying an exon 3-11 partial codon optimized version of the PKLR gene and the puromycin selection gene, inserted in the second intron of the PKLR gene assisted by TALE nucleases. We have previously used these tools in patient-specific induced pluripotent stem cells (iPSCs), and now, we have adapted them to hematopoietic progenitors (HPCs). The therapeutic matrix and a PKLR TALEN were electroporated in purified CD34+ cells that were expanded and puromycin-selected to enrich in gene edited HPCs. Up to 96% of colonies derived from selected CD34+ cells showed the specific integration of the transgene in the target site. To reduce the toxicity of the process the cell expansion and puromycin selection steps have been shortened. Finally, when PKLR TALEN were electroporated as mRNA, gene edited human HPCs could be detected after transplantation in NSG mice, showing the feasibility of performing gene editing of PKLR in human hematopoietic repopulating cells.

P003

Gene correction of IVS I-110 β-thalassaemia

P Patsali[1 2] C Mussolino[3] C Loucari[1 4] C Stephanou[1 2] M N Antoniou[2] T Cathomen[3] M Kleanthous[1 4 5] C W Lederer[1 4 5]

1: Department of Molecular Genetics Thalassaemia, The Cyprus Institute of Neurology and Genetics, 2370 Nicosia, Cyprus 2: Department of Medical and Molecular Genetics, King's College London, SE1 9RT London, United Kingdom 3: Institute for Cell and Gene Therapy, University Medical Center Freiburg, 79106 Freiburg, Germany 4: Cyprus School of Molecular Medicine, 2370 Nicosia, Cyprus 5: These authors have contributed equally to this work

Thalassaemia is amongst the commonest single-gene disorders worldwide and caused by deficient production of α- or β-globin. The disease has limited curative treatment options, but is a promising target for gene therapy. Specific β-thalassaemia mutations have already been addressed by the burgeoning field of genome editing, albeit at efficiencies that would require enrichment of corrected cells for clinical application. This study is focused on the development of efficient gene-correction tools (TALENs and CRISPR-Cas9), specific for the common and severe HBB(IVS I-110 G > A) mutation, which in most Mediterranean and many Western countries has a frequency amongst β-thalassaemia carriers of above 20% (with 80% on the island of Cyprus). This mutation introduces an abnormal splice acceptor site in intron 1 of the β-globin gene, therefore retaining an intronic in-frame premature stop codon in the mature, aberrantly spliced mRNA. Here we illustrate the high cleavage activity of novel IVS I-110-specific genome-editing tools (TALENs and CRISPR-Cas9) in HEK293T cells and in HBB(IVS I-110 G > A)-transgenic murine erythroleukaemia (MEL) cells. Multiplex RT-qPCR for the absolute quantification of correctly and aberrantly spliced HBB mRNAs shows a significant population-wide increase of functional HBB mRNA in treated cells. In line with this, immunoblots confirm significant correction of β-globin protein expression in treated HBB(IVS I-110 G>A)-transgenic MEL cells compared to mock controls. These data demonstrate that HBB(IVS I-110 G>A) is a suitable target for efficient genome editing and phenotypic correction and that it may serve as a model for the correction of many other intronic disease-causing mutations.

P004

Codon optimization improves factor IX expression in Hemophilia B mice by more than 15-fold

T Liu[2] S Tan[2] S Patarroyo-White[2] D Drager[2] K Chen[2] Q Zhou[2] A Cantore[1] L Naldini[1] O Danos[2] H Jiang[2]

1: San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET) 2: Biogen

Adeno-associated virus (AAV)-mediated human factor IX (FIX) gene therapy has demonstrated long-term FIX activity in Hemophilia B (HemB) patients with up to 6% of normal, which, however, did not fully protect patients from spontaneous bleeds. In addition, the high AAV doses were associated, in some cases, with liver toxicity resulting in loss of FIX expression. Here we sought to maximize FIX expression by employing a variety of codon optimization strategies, which could potentially increase the circulating FIX levels or reduce the vector doses. Six FIX variants were generated by using synonymous codons, which were adjusted to maintain FIX mRNA stability, and the codon usages were changed to human bias as indicated by higher human codon adaption index (CAI). In addition to the overall increase of the CAI, the distribution of high CAI was varied through the coding sequences to potentially modulate the rate of protein translation from the start to finish. Moreover, the R338L mutation was incorporated to increase the specific activity of FIX. All the variants were then cloned into expression plasmids and tested in HemB mice by hydrodynamic injection. Compared to the wild type FIX, the codon-optimized variants achieved 3 to 15-fold higher circulating FIX antigen levels in HemB mice; in combination with R338L mutation, the circulating FIX activity level of the best variant was increased 75-fold or higher. The long-term expression of these variants delivered by lentiviral vectors will be evaluated in HemB mice, which could identify an optimal FIX transgene for efficient HemB gene therapy.

P005

Correcting the bleeding phenotype in hemophilia A using lentivirally FVIII-corrected endothelial cells differentiated from hemophilic induced Pluripotent Stem Cells (iPSCs)

C Olgasi[1] M Talmon[1] S Merlin[1] D Colangelo[1] G N Berta[2] A Lombardo[3] L Naldini[3] A Raya[4] F Valeri[5] P Schinco[5] M Messina[5] M Prat[1] A Follenzi[1]

1: Universita del Piemonte Orientale 2: Azienda Osp/Univ San Luigi Gonzaga, Orbassano, Italy 3: HSR TIGET, San Raffaele Telethon Institute for Gene Therapy 4: Institute for Bioengineering of Catalonia (IBEC) 5: A.O.U. Città della Salute e della Scienza, Torino, Italy

Hemophilia A (HA) is an X-linked bleeding disorder caused by factor VIII (FVIII) deficiency. Somatic cells can be reprogrammed generating autologous, disease-free iPSCs, differentiated into targets cells relevant for gene and cell therapy. We generated FVIII-corrected patient-specific iPSCs from peripheral blood cells and differentiated into functional endothelial cells (ECs), secreting FVIII after transplantation in HA mouse model. CD34+ cells were isolated from peripheral blood of healthy and hemophilic donors and reprogrammed with a Cre-Lox LV carrying OCT4, SOX2 and KLF4. Reprogrammed CD34+ cells originated ESC-like-iPSCs+colonies for AP

staining and stem cell markers. EBs expressed germ layers specific markers and differentiated in osteogenic, chondrogenic and adipose tissues. iPSCs showed increased telomeres length and normal karyotype. FVIII-corrected HA-iPSCs expressed hBDD-FVIII by lentiviral vectors under the control of an EC-specific promoter (VEC). iPSCs differentiated into ECs, acquired endothelial-like morphology, expressed ECs markers and formed tubules when cultured in matrigel. VEC-GFP-LV transduced ECs were transplanted intraportal in NOD/SCID(NS)-γNull mice engrafted and proliferated in the livers up to 12 weeks later. Finally, healthy and FVIII-corrected EC-iPSC-derived linked to Cytodex microbeads were transplanted in NS hemophilic mice. aPTT was performed 3 and 7 days later and FVIII activity was 2% in mice transplanted with healthy cells and 5% in mice received FVIII-corrected HA cells. Immunofluorescent staining showed the presence of transplanted EC associated with beads recovered from injected mice. In conclusion, our cells engrafted, proliferated and expressed FVIII from differentiated, gene corrected and reprogrammed factor-free iPSCs confirming the suitability of this approach for HA gene-cell-therapy.

P006

Intravenous administration of lentiviral vectors expressing hyperfunctional factor IX converts severe into mild hemophilia B in a canine model

A Cantore[1] M Milani[1] T Liu[2] P Della Valle[3] A D'Angelo[3] T VandenDriessche[4] M Chuah[4] H Jiang[2] T Nichols[5] L Naldini[1 6]

1: San Raffaele Telethon Insitute for Gene Therapy (HSR-TIGET) 2: Biogen 3: San Raffaele Scientific Institute 4: Vrije Universiteit Brussel (VUB) 5: University of North Carolina 6: Vita-Salute San Raffaele University

Lentiviral vectors (LVs) are attractive vehicles for liver-directed gene therapy by virtue of their ability to stably integrate in the genome of target cells and the lack of pre-existing immunity against vector components in most humans. Over the past years, we have developed a LV platform that can achieve stable transgene expression in the liver and establish correction of hemophilia in mouse models upon systemic administration. This LV is designed to stringently target transgene expression to hepatocytes through transcriptional and microRNA-mediated regulation. We then investigated the efficacy and safety of portal vein administration of LVs expressing wild-type, codon-optimized (c.o.) or c.o. hyperfunctional canine factor IX (cFIX) in a canine model of hemophilia B. We observed long-term stable reconstitution of cFIX activity up to 1% of normal and significant amelioration of the clinical phenotype in 3 treated dogs (10 years cumulative follow up). In the perspective of clinical translation and to increase therapeutic efficacy, we next treated a hemophilia B dog by peripheral vein administration of LVs expressing the c.o. hyperfunctional cFIX at a 5-fold higher dose than those previously administered. At the current follow-up (6 months after gene therapy) cFIX activity is 7–8% of normal, suggesting comparable efficacy of LV by both portal and peripheral vein administration Treatment of more hemophilia B dogs is underway to extend these results. Overall our studies position LV-mediated liver gene therapy for further preclinical development and clinical translation. LVs may thus complement other available vectors for liver gene therapy of hemophilia.

P007

Characterization and transduction of hematopoietic progenitors from pyruvate kinase deficient (PKD) patients

M Garcia-Gomez[1 2] S Lopez-Manzaneda[1 2] R Sanchez-Dominguez[1 2] O Alberquilla[1 2] M M Mañu-Pereira[3] M Muriel-Ramos[4] J L Vives-Corrons[3] J Sevilla[5] J A Bueren[2 6] S Navarro[1 2] J C Segovia[1 2]

1: Differentiation and Cytometry Unit-Division of Hematopoietic Innovative Therapies, CIEMAT/CIBERER, 28040 Madrid, Spain 2: Advanced Therapies Unit, IIS-Fundacion Jimenez Diaz (IIS-FJD, UAM), 28040 Madrid, Spain 3: IDIBAPS, H Clinic, 08036 Barcelona, Spain 4: Salamanca University Hospital, 37007 Salamanca, Spain 5: Hospital Infantil Universitario Niño Jesús, 28009 Madrid, Spain 6: Division of Hematopoietic Innovative Therapies, CIEMAT/CIBERER, 28040 Madrid, Spain

Pyruvate kinase deficiency (PKD) is an autosomal recessive disorder caused by mutations in the PKLR gene. PKD is the most common erythroid inherited enzymatic defect causing chronic nonspherocytic hemolytic anemia, high reticulocytosis, acute splenomegaly and intense iron overload in the liver, being life-threatening in severe patients. Up to date allogeneic bone marrow transplant represents the only curative treatment of patients affected by the severe form of the disease. Preclinical gene therapy studies conducted in pyruvate kinase deficient mice have shown the safety and the efficacy of a new PGK-coRPK-Wpre therapeutic lentiviral vector that provided the designation of this PGK-coRPK-Wpre vector as a new orphan drug (EU/3/14/1130). To continue with the preclinical studies required to develop a clinical trial for PKD we have characterized the hematopoietic progenitor's content and the erythroid progenitors' profile in peripheral blood (PB) from PKD patients, showing an increase in both types of cells. For transduction studies, CD34+sorted cells from PKD PB were transduced with pre-GMP viral supernatants. Hematopoietic progenitors' content and final yield of cells and CFCs was evaluated to analyze the toxicity of the vector. Percentage of transduction ranged from 31% to 100%, showing the efficacy of the transduction protocol and of the PGK-coRPK therapeutic vector. Vector copy number was quantified in both, individual CFUs and CD34+ cells maintained in liquid culture for 14 days, ranging from 0.1 to 3.0 VCN/cells. More studies are being conducted to demonstrate PGK-coRPK functionality in human PKD cells.

P008

Characterization of Oversized rAAV vectors encoding Human FVIII

S Kyostio-Moore[1] P Berthelette[1] C Sookdeo[1] B Nambiar[1] S Piraino R Jackson[1] B Burnham[1] C O'Riordan[1] K Vincent[1] S H Seng[1] D Armentano[1]

1: Sanofi Genzyme R&D Center

Clinical trials have demonstrated that a single intravenous (IV) injection of rAAV vectors encoding FIX in hemophilia B patients can provide steady-state factor IX levels for >4 years and reduce the frequency of factor infusions. Similar rAAV-mediated gene transfer for hemophilia A patients is challenging due to the combined size of FVIII cDNA and transcriptional elements that exceeds the rAAV packaging limit of 4.7 kb. To

test the feasibility of this strategy for hemophilia A, several oversized FVIII expression cassettes with codon-optimized human B-domain deleted FVIII cDNA were constructed and rAAV vectors were generated by triple transfection. The vector yields were affected by vector genome (VG) size and the majority of packaged VGs were approximately 4.7 kb. However, intact expression cassettes and comparable VG copies were observed for all the vectors in liver after IV administration to mice. An oversized 5.1 kb FVIII vector showed 2-3-fold lower plasma FVIII protein and liver VG levels compared to a 4.6 kb vector. Both vectors showed similar persistence and vector molecular forms in liver. These studies confirmed the viability of oversized rAAV vectors for the treatment of severe hemophilia A. Currently, alternate vector production methods are being evaluated to increase vector yield and quality.

P010

Persistent factor IX expression following AAV8-mediated liver transduction in macaques

H Mizukami T Ohmori[1] R Uchibori T Tsukahara Y Saga M Urabe A Kume Y Sakata K Ozawa

1: Jichi Medical University

We have been evaluating the efficacy and safety of hemophilia gene therapy using macaques, and demonstrated that liver-targeted approach with AAV8-based vector has resulted in therapeutic level of factor IX expression at the practical vector doses. Another important finding is the inhibitory effect of neutralizing antibody (NAb) against AAV capsid upon AAV vector-based gene transfer approach; none of the subjects with positive NAb showed recognizable transgene expression following IV injection of the vector. At this stage, one of the most crucial questions is the duration of this therapeutic modality. As this approach utilizes hepatocyte transduction by a non-integrating vector, the length of this therapy should depend on the lifetime of the transduced cells. During the series of experiment, we observed 5 animals for long-term outcome (longer than 3 years). All of these animals showed therapeutic levels of factor IX expression following vector injection, and the expression levels lasted throughout the observation period in all of the animals. In one animal, the expression level was kept constant within therapeutic level for more than 7 years. No adverse effect was observed in any of the animals. These findings suggest long-term efficacy and safety of this therapeutic approach, supporting application to human treatment.

P011

Targeting aurora kinase A with an enhanced TCR gene-transfer vector

N P Casey[1] H Fujiwara[1] S Okamoto[2] J Mineno[2] K Kuzushima[3] H Shiku[4] M Yasukawa[1]

1: Ehime University 2: Takara Bio Inc. 3: Aichi Cancer Center 4: Mie University 5: EB House Austria

Aurora Kinase-A (AURKA) is involved in regulating mitosis. However it is over-expressed in certain cancer types, with a 'cancer-testis' expression profile, making it a suitable target for cell-based immunotherapy. Cytotoxic T lymphocytes reactive against HLA-A*0201-restricted AURKA antigen were isolated and characterised (Ochi T, et al. Blood, 2009), and transfer of the TCR genes from these cells confers specific immunoreactivity against the target peptide in a HLA-restricted manner (Nagai K, et al. Blood, 2011). One important challenge facing TCR transfer is the potential for mispairing between exogenous and endogenous TCR chains, which may result in unknown and adverse 'off-target' combinations. TCR chains must also compete for limiting CD3 molecules, reducing efficiency. While various options are available for enhancing expression and formation of transgenic TCRs, siRNA downregulation of the endogenous TCRs is most attractive. Our retroviral vector incorporates an siRNA design active against a conserved region of the TCR sequence, without affecting the codon-optimised transgenic TCR. In addition, inclusion of a 2A self-cleaving peptide results in equimolar expression of the TCR alpha and beta TCR chains. We cloned this construct into a retroviral vector, and activity of this construct was compared with a 'conventional' vector. TCRs expressed from the siTCR-vector retained the cytotoxicity of the conventional vector, with reduced off-target activity. Furthermore, comparable rates of TCR expression, and superior rates of AURKA-peptide binding, were achieved with lower vector copy numbers. The flexibility and safety advantages of this vector design give it tremendous potential in TCR-mediated cancer immune gene-therapy.

P012

Comparison of CTS™ Dynabeads® CD3/CD28, Miltenyi TransAct CD3/28 and ExpAct Beads for large-scale CAR T cell manufacturing

X Wang[1] J Stefanski[1] O Borquez-Ojeda,[1] J Qu[1] A Hack[1] Q He[1] T Wasielewska[1] F Du[1] M Sadelain[1] I Riviere[1]

1: Memorial Sloan Kettering Cancer Center

Adoptive transfer of chimeric antigen receptor (CAR) engineered T cells is one of the most promising emerging strategies to treat patients with cancer. Large-scale manufacturing of cGMP-grade CAR T cells using patient T cells selected and activated by CTS™ Dynabeads® CD3/CD28 (Dynabeads) followed by transduction with retroviral vectors has been established by our laboratory and others. Although this method is robust, it is important to explore alternative sources for critical reagents such as magnetic beads to pre-empt supply chain limitations. To this end, we compared T cell activation and transduction with either Miltenyi TransAct CD3/28 (TransAct) beads, Miltenyi ExpAct Treg (ExpAct) beads or Dynabeads. In small-scale experiments, PBMCs were directly activated with either TransAct or ExpAct beads without CD3+ cell selection. Overall, the transduction efficiency, expansion of T cells and TEM/CM phenotypes were comparable to those of cells selected and activated with Dynabeads. In addition, CD3+ cells reached $\geq$98% by day 10 in all groups. We further tested the efficacy of these reagents using selected T cells in large-scale cGMP setting. Both the transduction efficiency and expansion of selected CD3+ cells activated with TransAct beads, ExpAct beads or Dynabeads were comparable. Moreover, we found that selected T cells activated by TransAct or ExpAct beads and transduced with CD19-targeted CAR had robust antitumor activity in vivo in NSG/CD19+NALM6 tumor mouse model. In conclusion, our pre-clinical results suggest that TransAct and ExpAct beads support efficient transduction, expansion and antitumor activity of CAR T cells that are similar to Dynabeads.

P013

Co-delivery of EGFR and HSV-tk by LCMV-pseudotyped bicistronic lentiviral vectors to enhance therapeutic gene distribution for glioblastoma treatment

J A Hossain H Miletic K Riecken B Fehse

1: University of Bergen 2: Haukeland University Hospital 3: University Clinic Hamburg-eppendorf

Malignant gliomas, the largest group of primary intracerebral tumors, are one of the most-difficult-to-cure cancers. The outcome has not significantly improved in recent years, despite considerable advances in our understanding of the molecular pathogenesis and improvement of surgical techniques, radio- and chemo-therapy. For glioblastoma multiforme (GBM), the most malignant form of glioma, the median survival time is approximately 15 months after diagnosis. Although complete remission of experimental GBM on MRI has been reported by using a lentiviral vector based suicide gene therapy approach1, recurrence of tumors at distant sites is common which is mainly caused by invasive glioma cells that escape treatment. Thus, a better distribution of the suicide gene is needed in order to target and efficiently kill the infiltrative glioma cells to prolong recurrence-free time span and improve the therapeutic effect. By introducing EGFR, a gene that has been reported to promote invasion of glioma cells2 into our LCMV-pseudotyped lentiviral vector system, we want to enhance the distribution of the suicide gene HSV-TK. This might lead to a more efficient killing of glioma cells in both tumor core and invasive areas.

P014

Inhibited interaction between p53 and Mdm2 enhances p53-mediated cytotoxic activities on INK4A/ARF-defective mesothelioma

M Tagawa[1 2] T Morinaga[1 2] K Kawamura[1] S Okamoto[1 2] S Kubo[5] M Shingyoji[7] I Sekine[6] Y Tada[2] K Tatsumi[2] H Shimada[4] K Hiroshima[3]

1: Chiba Cancer Center Res Inst 2: Chiba University 3: Tokyo Women's Medical University (TWIns) 4: Toho University 5: Hyogo College of Medicine 6: Tsukuba University 7: Chiba Cancer Center

Genetic analyses with clinical specimens revealed that malignant mesothelioma often had a homologous deletion in the INK4A/ARF region containing p14ARF and p16INK4A genes, but a majority of the p53 genotype was wild-type. Loss of p14 protein leads to augmented p53 degradation through increased Mdm2 activities which induce p53 ubiquitination, and lack of p16 functions fails to inhibit CDK4/6 actions and up-regulates pRb phosphorylation. The genetic alterations thereby decrease the p53 functions and promote cell cycle progression. We demonstrated that transduction of mesothelioma with adenoviruses expressing the wild-type p53 gene (Ad-p53) induced p53 phosphorylation at Ser 15 and 46 residues, and dephosphorylated pRb at Ser 795. The transduced cells were subjected to apoptosis with caspases activations. We examined a possible strategy to augment the Ad-p53 effects with a chemical agent that blocked p53 degradation processes. Nutlin-3a inhibited interactions between Mdm2 and p53 and subsequently augmented p53 expression levels. Heat shock protein 90 (Hsp90) inhibitors, 17-AAG and 17-DMAG, suppressed Mdm4 functions that inhibited p53-mediated transcriptional activation, and accordingly increased p53 functions. A combinatory use of nutlin-3a and Hsp90 inhibitors produced synergistic cytotoxicity on p53-wild-type mesothelioma through apoptosis, and that of nutlin-3a and Ad-p53 also achieved the cytotoxic effects in a synergistic manner. Hsp90 inhibitors were however antagonistic to Ad-p53-mediated cytotoxicity, which was due to suppressed chaperon activity of Hsp90 that targeted p53 stability. These data collectively indicates that maintenance of a high leveled p53 is crucial for Ad-p53-mediated cytotoxicity, but combination of gene medicine and a molecular targeting agent needs meticulous fine-tunings.

P015

A self-amplifying immunobooster for cancer therapy: oncolytic vaccinia virus expressing DAI

M Hirvinen[1] C Capasso K Guse M Garofalo A Vitale M Ahonen L Kuryk M Vähä-Koskela A Hemminki V Fortino D Greco V Cerullo

1: University of Helsinki 2: Finnish Institute of Occupational Health

In oncolytic virotherapy the ability of the virus to activate immune system against tumor is nowadays generally understood to be the major mechanism to fully fight the cancer and to achieve long-term anti-tumor effects. Vaccinia viruses (VV) are immunosuppressive viruses encoding genes for neutralization of the host immune responses. To overcome this problem, we developed an oncolytic VV that expresses intracellular pattern recognition receptor DAI to obtain a self-immuno-boosting system to activate the immune responses in the tumor. DAI (DNA-dependent activator of IFN-regulatory factors) is a cytosolic dsDNA sensor and a potent activator of innate immune responses. We showed that infection with DAI-expressing VV increases expression of several genes related to important immunological pathways. Treatment with DAI-armed VV resulted in significant reduction in the size of melanoma tumors in mice due to increased levels of anti-tumor T-cells in the tumors. When we re-challenged the mice with the same tumor, the DAI-VV treated mice rejected the growth of the new tumor completely, which also indicates immunity established against the tumor. To even better illustrate the immunogenicity of our virus in vivo we performed studies in PBMC humanized mice – a model closer to human system. We showed enhanced control of human melanoma tumor growth and elevated levels of human T-cells in DAI-VV treated group of mice. We conclude that expression of DAI by an oncolytic VV is a promising way to self-amplify immunostimulatory potency of oncolytic vaccinia virus to trigger the innate – and eventually the long-lasting adaptive immunity against cancer.

P016

The AdUP trial: combining prodrug activation with immune stimulation in locally recurrent prostate cancer.

P F Searle[1] V Mautner[1] E Porfiri[1] L Crack[1] R Viney[1] N D James[2] P Patel[1]

1: University of Birmingham (UK) 2: University of Warwick

The AdUP phase I clinical trial builds on our previous clinical trials of the replication-defective adenovirus CTL102, which expressed the enzyme nitroreductase (collaboration with ML Laboratories). Nitroreductase activates the prodrug CB1954 to a highly cytotoxic DNA-crosslinking agent. CTL102 was injected

into the prostate under ultrasound guidance, via the transrectal route at doses $\leq 10^{12}$ virus particles (vp), then two days later, CB1954 (24 mg/m^2) was administered intravenously. Some patients showed clear reductions in circulating PSA following treatment. After 6 months, 9/19 patients had progressive disease (PSA > 10% above baseline); 4 showed stable disease (PSA ± 10% of baseline), while 6 showed >10% reduction in PSA, including 2 with > 50% reduction. The AdUP trial incorporates two important improvements over the CTL102 trials: Firstly, the replication defective adenovirus, AdNRGM, co-expresses nitroreductase and GM-CSF. As before, the prodrug CB1954 is administered intravenously two days after the virus, and its activation by nitroreductase should lead to death of tumour cells. The expressed GM-CSF is expected to recruit antigen-presenting cells to the prostate and promote the immunogenic presentation of tumour antigens from the killed tumour cells. Secondly, AdNRGM is injected via the transperineal route under general anaesthesia, using a brachytherapy template to position the needle precisely at pre-planned locations throughout the prostate, aiming to achieve widespread intraprostatic distribution of the virus. The trial will test 5 dose-levels of AdNRGM (10^{10} to 10^{12}vp); to date 3 patients have been treated. In addition to safety monitoring, we are monitoring PSA levels and collecting samples for analysis of immune responses.

P017

Syrian hamster model for adoptive T-cell transfer and oncolytic adenovirus combination therapy

M Siurala[1 2] M Vähä-Koskela[1] S Tähtinen[1] S Parviainen[1 2] A Hemminki[1 2]

1: Cancer Gene Therapy Group, Department of Pathology, Faculty of Medicine, University of Helsinki 2: TILT Biotherapeutics Ltd, Helsinki, Finland

Adoptive transfer of tumor-infiltrating lymphocytes (TIL) has developed into a promising treatment modality for metastatic cancer. However, not all patients respond and approaches for improving the efficacy of TIL therapy are thus needed. We have combined TIL transfer with oncolytic adenovirus in the fully immunocompetent and adenovirus-permissive Syrian hamster model. TIL were obtained from syngeneic hamster tumors HapT1 (pancreatic carcinoma) and RPMI 1846 (melanoma) after culture in high-dose human IL-2. These TIL exhibited highly tumor-specific cytolytic activity in effector/target assays. In addition, MHC Class I blocking abrogated the cell killing of TIL, suggesting that cytotoxic CD8 T-cells were responsible for the observed cytolytic activity. When autologous TIL were combined with the oncolytic adenovirus Ad5-D24 in vitro, tumor cell killing was further enhanced (HapT1 cell viability 78% with TIL alone, 72% with Ad5-D24 alone and 45% with combination). Antitumor efficacy of TIL and Ad5-D24 combination was studied in hamsters bearing established HapT1 tumors; the combination of TIL and Ad5-D24 was superior to both single agents and mock treatment (combination vs. TIL, $p = 0.009$; combination vs. Ad5-D24, $p = 0.007$; combination vs. mock, $p = 0.046$). To our knowledge, this is the first time tumor-infiltrating lymphocytes of the Syrian hamster have been cultured, characterized and used therapeutically. In conclusion, we have shown that oncolytic adenovirus can be used to improve the efficacy of adoptive TIL transfer without the need to include potentially toxic concomitant regimens. Our results support the further development of T-cell therapies in combination with oncolytic viruses.

P018

Oncolytic adenovirus improves anti-tumor efficacy of adoptive T-cell therapy by breaking tumor tolerance

S Tähtinen[1] S Grönberg-Vähä-Koskela[1] D Lumen[3] M Merisalo-Soikkeli[1] M Siurala[1 2] A J Airaksinen[3] M Vähä-Koskela[1] A Hemminki[1 2]

1: Cancer Gene Therapy Group, Department of Pathology, University of Helsinki, Helsinki, 00290, Finland 2: TILT Biotherapeutics Ltd, Helsinki, 00290, Finland 3: Laboratory of Radiochemistry, Department of Chemistry, University of Helsinki, Helsinki, 00014, Finland

Background: Despite the rapid progress in the development of novel adoptive T-cell therapies (ACT), the clinical benefits in established solid tumors have remained modest due to several immune evasion mechanisms, which contribute to tumor tolerance. Of note, oncolytic adenoviruses are intrinsically immunogenic due to inherent pathogen-associated molecular patterns. Here, we studied the combination of these two established forms of immunotherapy in a highly resistant and poorly immunogenic B16.OVA mouse melanoma model.

Results: Following adoptive transfer of OT-I lymphocytes, control of tumor growth was superior in Ad5/3-D24-treated mice compared to control mice, even in the absence of active oncolysis. Significant increase in infiltration of CD45+leukocytes, CD8+lymphocytes and F4/80+macrophages was seen adenovirus-treated tumors suggesting enhanced tumor immunogenicity. Pro-inflammatory tumor microenvironment mediated by adenovirus infection led to expression of co-stimulatory signals on CD11c+dendritic cells and subsequent activation of T-cells, thus breaking the tumor-induced peripheral tolerance. An increased number of endogenous CD8+T-cells specific for tumor antigens TRP-2 and gp100 was detected in combination treated mice, indicating repertoire expansion. Moreover, majority of virus/T-cell-treated mice rejected the challenge of parental B16.F10 tumors, suggesting that systemic anti-tumor immunity was induced.

Conclusions: Treatment with oncolytic adenovirus can overcome resistance of B16.OVA murine melanoma tumors to T-cell therapy by recruitment and stimulation of tumor-infiltrating immune cells, thus resulting in improved efficacy of adoptive T-cell therapy. Importantly, these two modalities are not merely an attractive combination, but could represent a way to achieve ''CD19-like'' results in the treatment of solid tumors.

P019

Microvesicle- and exosome-mediated drug delivery enhances the cytotoxicity of paclitaxel in autologous prostate cancer cells

H Saari[1] E Lázaro-Ibáñez[1] T Viitala[1] E Vuorimaa-Laukkanen[2] P Siljander[1] M Yliperttula[1]

1: University of Helsinki 2: Tampere University of Technology

Background: Extracellular vesicles (EVs) are naturally occurring membrane particles that mediate intracellular communication by delivering molecular information between cells. In this study we investigated the effectiveness of two different subtypes of EVs (microvesicles and exosomes) as carriers of Paclitaxel to autologous prostate cancer cells.

Methods: EVs were isolated from LNCaP- and PC-3 prostate cancer cell cultures using differential centrifugation. EVs were

characterized by electron microscopy, nanoparticle tracking analysis, and western blot. The uptake of microvesicles and exosomes by prostate cancer cells was assessed by flow cytometer and confocal microscopy. The EVs were loaded with Paclitaxel by incubation and the effectiveness of EV-mediated drug delivery was assessed with viability assays. The distribution of EVs and EV-delivered Paclitaxel in cells was inspected by confocal microscopy.

Results: Loading of Paclitaxel to EVs increased the cytotoxic effect of the drug regardless of the EV subtype, while EVs without the drug increased cancer cell viability. EVs were observed to deliver Paclitaxel to the recipient cells through endocytosis, leading to the release of the drug from within the cells. Conclusions Cancer cell-derived microvesicles and exosomes can be used as effective carriers of Paclitaxel to their parental cells, bringing the drug into the cells through an endocytic pathway and increasing its cytotoxicity. However, due to the increased cell viability, their use must be further investigated before any clinical applications.

P020

Utility of platelet vector containing inactivated Sendai viral particles for multi-modal cancer therapy

We have reported multiple anti-cancer activities of inactivated Sendai virus particles (HVJ-E) including enhancement of anti-cancer immunity and induction of cancer cell-selective apoptosis mainly by the signal transduction through RIG-I/MAVS pathway (Clin. Cancer Res. 2012, etc). Clinical trials to treat melanoma and prostate cancer using HVJ-E are on-going in Japan. The limitation of HVJ-E is the inability of systemic administration due to the hemagglutinating activity. Since we found a number of areas with fibrin clots and detected high thrombin activities in tumor microenvironment (TME), we developed the platelet vector incorporating HVJ-E (PH complex) and injected the vector into tail vein of melanoma-bearing mice. The PH complex dominantly accumulated in TME but not in other tissues. Consecutive systemic injection of the PH complex recruited a number of immune cells to TME, suppressed tumor growth and extended mouse survival. NK cells and CTL against melanoma were activated by the PH complex. We discovered that the secretion of RANTES was up-regulated in TME following the PH administration, which was needed for activating CTL against melanoma. However, intratumoral injection of recombinant RANTES without HVJ-E failed in tumor suppression. Therefore, the presence of HVJ-E in melanoma tissue is essential for enhancing anti-tumor immunity in vivo in addition to inducing the secretion of RANTES. PH complex also significantly reduced the number of metastatic melanoma foci in lung where micrometastatic foci had been formed. The platelet vector will be also available for incorporating various oncolytic viruses and will provide a new approach for cancer therapy.

P021

Multiplex high-throughput analysis of IDH-gene mutations

W Wang[1] A Bertoni[1] A Korshunov[1 2] J Kirchhof[3] K Beck[3] P Lichter[1 3] B Radlwimmer[1]

1: DKFZ 2: Heidelberg University Hospital 3: Heidelberg Center for Personalized Oncology (HIPO)

Systematic characterization of somatic mutations in cancer genomes has improved our understanding of the disease and provided opportunities for the development of targeted therapies. Validation of key mutations requires sensitive high-throughput techniques. The Isocitrate dehydrogenase 1 and 2 (IDH1/IDH2) genes encode NADP+-dependent isocitrate dehydrogenases playing prominent roles in cellular metabolism including lipid metabolism and glucose sensing. Somatic gain-of-function mutations of IDH have been identified in multiple cancers including diffuse astrocytic glioma, sarcoma and acute myeloid leukemia. Their potential role in disease pathogenesis and prognosis have made IDH1/IDH2 both promising biomarkers and potential therapeutic targets. We established a high-throughput screening method for IDH mutations in early- and late-stage glioma using a multiplex mass spectrometry-based approach. Based on amplicon length and similarity, the 11 most frequent IDH1/IDH2 mutations were grouped in 4 multiplex assays. 139 diffuse astrocytic glioma, including 76 glioblastoma and 63 WHO grade II and III glioma, and 20 cytogenetically normal (CN)–AML samples were analyzed. IDH1/IDH2 combined frequency was approximately 8% (6/76) in GBM, 86% (54/63) the other glioma and 45% (9/20) in AML. Mutations of IDH1-R132 and IDH2-R172 were most frequently mutated in all three tumor entities. IDH1 and IDH2 mutations largely occurred mutually exclusive, and could be detected even when present only in a subpopulation (>5%) of cells. All mutations were independently validated by pyrosequencing with 100% concordance. This study demonstrates that quantitative mutation analysis by MassARRAY is a sensitive and effective method for mutation analysis.

P022

Antitumoral effect of mesenchymal stem cells infected with oncolytic adenovirus in canine spontaneous tumors.

T Cejalvo E Laborda I del Portillo M A Rodriguez-Milla E Rincon R Moreno C Jimenez M Ramirez D Sardon F Vazquez N del Castillo R Alemany J Garcia-Castro

1: Instituto de Salud Carlos III 2: ICO - IDIBELL 3: Hospital Infantil Universitario Nno Jesus 4: Universidad Alfonso X

Our group has previously published beneficial antitumor effects of oncolytic virotherapy delivered by autologous mesenchymal stem cells (MSC) in human neuroblastoma. A phase I clinical trial is ongoing (ClinicalTrials.gov Identifier: NCT01844661) using this approach. Now we are focused on elucidating the mechanisms underlying this therapeutic effect. We are treating canine patients with spontaneous tumors within an approved compassionate use programme in a veterinary hospital. In a previous work we developed ICOCAV17, a canine oncolytic adenovirus. Canine MSC were obtained from adipose tissue from healthy dogs and they were characterized, grown and used as adenovirus cell carriers. Twenty dogs with different tumor types were treated every 15 days (for 4 treatments) intravenously with allogeneic MSC with ICOCAV17 (canine CELYVIR). A 75% of the cases showed clinical response. Complete remission was documented on 30% of the cases. Dogs were monitored weekly evaluating hematologic, renal and hepatic injury parameters. No systemic toxicity was observed in all cases. The histopathological analysis from biopsies of tumors pre- and post-CELYVIR treatment revealed intratumoral infiltrates of T cells (CD4+ and CD8+) and/or macrophages/granulocytes (MAC387+) in post-CELYVIR infusion biopsies. We have also observed changes in immune cell subsets in peripheral blood after treatment. We postulate that an immune response may be playing a principal role on this antitumoral effect.

P023

Towards targeted virotherapy in gynecological cancer

A N Candia[1 4] A L Alfano[1 4] C M Malnero[1 2] I R Bermúdez[1 2] N Cuneo[3] M A Gangemi[1] A Soderini[3] V M Lopez[1] O L Podhajcer[1]

1: Lab. of Molecular and Cellular Therapy, Leloir Institute-CONICET, Buenos Aires, Argentina 2: Universidad Argentina de la Empresa 3: Hospital de Oncología Marie Curie. Buenos Aires, Argentina 4: Authors contributed equally to this work

Worldwide more than 1 million women are diagnosed with a gynecological cancer. Non-specific symptoms (ovary cancer) and disparities in accessibility to health services (cervical cancer) explain the differences in gynecological cancer outcome globally. Most gynecological cancer types are characterized by an accompanying stroma that not only supports malignant cells growth but is also largely responsible for the high resistance of the cancer to conventional and targeted therapies. We have recently developed a stroma-targeted oncolytic adenovirus (AR-2011) whose replication is driven by a triple chimeric promoter that drives a ΔRB E1A gene and was pseudotyped with a chimeric fiber 5/3. By using E4 gene levels we observed a large increase in lytic activity of AR-2011 under hypoxia and in the presence of TNFα. We assessed the lytic capacity of AR-2011 on fresh explants of gynaecological cancers. AR-2011 replicated in 5/8 cervical cancers, 3/4 uterine cancer and 3/4 ovarian carcinomas. AR-2011 lytic activity was also assessed in combination with chemotherapy. No viral lytic effect was observed already in explants from 2 normal uterus, 2 normal cervix and 9 normal ovaries. In addition to the lytic effect we are currently assessing biomarkers such as the expression levels of the receptor for the virus fiber (CD46 and desmoglein-2) and of the SPARC gene, and markers of an inflammatory and hypoxic environment. We propose that this approach combined whenever possible with in vivo assessment of virotherapy efficacy in patient derived xenografts, might be very useful in helping guide personalized therapeutic decisions in these devastating diseases.

P024

High levels of iCasp9 expression are needed for suicide gene transfer

S Donnou-Triffault[1 2 3] C Poinsignon[1 2 3] A Galy[1 2 3] S Fisson[1 2 3]

1: GENETHON 2: INSERM UMRS951 3: Université d'Evry Val d'Essonne

Primary central nervous system lymphomas are aggressive tumors with no efficient treatment. We wanted to test the inducible caspase 9 (iCasp9)(*) strategy to evaluate the feasibility of an in vivo targeted suicide gene therapy in a murine model of primary intraocular B-cell lymphoma. Firstly, we showed that apoptosis could not be induced in the A20.IIA lymphoma tumor cells transduced in vitro with a MHC II targeted LV expressing iCasp9, in the presence of the inductor of dimerization. Clones obtained from transduced cells showed that only 1 copy of the transgene was integrated, suggesting that levels of iCasp9 were insufficient or that cells were resistant to treatment. In contrast, clones transduced with only 1 copy of a gamma retroviral LTR-driven-iCasp9 vector expressed higher levels of transgene than LV clones and 90% of the cells died 24 hours after iCasp9 activation. Clearly, a threshold of iCasp9 expression must be achieved to induce apoptosis by gene transfer. In other cells such as 3T3, Jurkat, HCT116 and Raji cells, more than 2 copies of the LV PGK-iCasp9 must be integrated to induce apoptosis since cells expressing only 1 copy survive. It is very difficult to integrate more than 2 copies of LV per B-cell in spite of repeated cycles of infection coupled with various pre-activation stimuli. Altogether, our results provide benchmark values for iCasp9 efficacy and show that highly expressing vectors must be developed to apply iCasp9 suicide gene therapy strategies in B-lymphoma but also with other target cells, to avoid escape to treatment.

P025

Suicide gene therapy using AAV/GALR for head and neck cancer cells.

Galanin and its receptors, GALR1 and GALR2, are known tumor suppressors and potential therapeutic targets in head and neck squamous cell carcinoma (HNSCC). Previously, we demonstrated that, in GALR1-expressing HNSCC cells, the addition of galanin suppressed tumor proliferation via upregulation of ERK1/2 and cyclin-dependent kinase inhibitors, whereas, in GALR2-expressing cells, the addition of galanin not only suppressed proliferation, but also induced apoptosis. In this study, we first transduced HEp-2 and KB cell lines using a recombinant adeno-associated virus (rAAV)-green fluorescent protein (GFP) vector and confirmed a high GFP expression rate (>90%) in both cell lines at the standard vector dose. Next, we demonstrated that GALR2 expression in the presence of galanin suppressed cell viability to 40–60% after 72 h in both cell lines. Additionally, the annexin V-positive rate and sub-G0/G1 phase population were significantly elevated in HEp-2 cells (mock vs GALR2: 12.3 vs 25.0% ($P<0.01$) and 9.1 vs 32.0% ($P<0.05$), respectively) after 48 h. These changes were also observed in KB cells, although to a lesser extent. Furthermore, in HEp-2 cells, GALR2-mediated apoptosis was caspase-independent, involving downregulation of ERK1/2, followed by induction of the pro-apoptotic Bcl-2 protein, Bim. These results illustrate that transient GALR2 expression in the presence of galanin induces apoptosis via diverse pathways and serves as a platform for suicide gene therapy against HNSCC.

P026

GaLV-pseudotyped semi-RRV carrying HSV-TK; single-shot, single-cycle suicide gene delivery system for eradication of experimental glioblastoma

M Kang[1] A Song[2] J Kim[1] Y -S Kim[1]

1: Chungnam National University 2: Inje University

In this study, we tested a gibbon ape leukemia virus (GaLV) envelope-pseudotyped semi-replication-competent retrovirus vector (spRRV) system in experimental glioblastoma animal model. This spRRV system is composed of two trans-complementing replication-defective retroviral vectors sRRVgp and spRRVe encoding MuLV gag-pol genes and GaLV env gene, respectively. We found that single intratumoral injection of the sRRVgp/spRRVe particles shows considerable improvement on gene transfer efficiency in both human glioblastoma cells and pre-established human glioblastomas mouse model without spread to adjacent normal brain as well as other organs compared with a

sRRV system. Furthermore, single cycle of prodrug ganciclovir (GCV) administration after the intratumoral injection of sRRVgp/spRRVe particles expressing the herpes simplex virus 1-thymidine kinase (HSV-TK) gene into pre-established U-87 MG glioblastomas resulted in, if two outlier mice harboring secondary tumors formed by early metastatic process or from small numbers of tumor cells injected accidentally at nearby primary tumor site were discarded from analysis, a 100% survival of treatment group over a follow-up period that lasts longer than 150 days, while all mice in control groups were expired between 30~45 days after tumor injection. Thus, improved delivery of suicide gene by the GaLV-pseudotyped spRRV system can achieve successful tumor eradication without repeated prodrug administration cycle, showing promise in using spRRV system as a cancer therapeutic agent.

P027

Toxicological and bio-distribution profile of a GM-CSF-expressing, double-targeted, chimeric oncolytic adenovirus ONCOS-102 – support for clinical studies on advanced cancer treatment

L Kuryk[1 2 3] L Vassilev[3] T Ranki[3] A Karioja-Kallio[3] O Levälampi[3] A Vuolanto[3] V Cerullo[1 2] S Pesonen[3]

1: University of Helsinki 2: Immuno Viro Therapy lab 3: Oncos Therapeutics

The purpose of this work was to carry out preclinical toxicity and bio-distribution studies required for regulatory approval of a clinical trial application for a Phase I clinical studies of ONCOS-102 for therapy of advanced cancers. The study was carried out with 300 hamsters divided into nine tested groups – three BIO groups and six TOX groups. Hamsters received the appropriate dose of the Test item ONCOS-102 in NaCl solution by intracardial, intraperitoneal or subcutaneously injections on designated days. Additionally, one group was administered twice a week, including the same days as those of the administration of ONCOS-102, with intraperitoneal injections of Cyclophosphamide in dose of 20 mg/kg. The control animals were administered with NaCl solution without ONCOS-102 in the same volume and the same way. No adverse effects of repeated administration of ONCOS-102 on clinical signs including body weight, food consumption, hematology and clinical chemistry parameters, histopathology and bio-accumulation were observed in the course of 6-month administration and following 3-month recovery period. All obtained findings indicate the treatment clinically safe.

P028

Automated manufacturing process for the production of genetically modified lymphocytes using the CliniMACS Prodigy® platform

S La Seta Catamancio[1] M Cota[1] R Arnaudova[1] P Mangia[1] A Arendt[2] S Peiffer[2] D Dietrich[2] P Morandi[1] M Manfredini[1] S Fracchia[1] G P Rizzardi[1] G Vallanti[1] C Traversari[1]

1: MolMed SpA 2: Miltenyi Biotec GmbH

MM-TK is a medicinal product constituted of T lymphocytes engineered ex vivo with the retroviral vector (RVV) SFCMM-3 Mut2 #48 to express a mutated form of the Herpes Simplex Virus Thymidine kinase (HSV-TK Mut2) and a truncated form of the human Low affinity Nerve Growth Factor Receptor (ΔLNGFR) genes. It was developed as adjunctive therapy in leukaemia patients undergoing stem cell transplantation, to promote immune reconstitution and to control viral infections and disease relapses. In order to optimize the cellular manufacturing process, MolMed have strengthened an alliance with Miltenyi Biotec for tailoring of CliniMACS Prodigy® platform. A closed and automated process was developed with a specific software designed to manufacture a robust, safe and consistent product. The manufacturing process consists in PBMC stimulation, RVV transduction in RetroNectin®-coated bags and immunoselection of transduced cells by CD271-LS Microbeads reagent. Selected cells are then further expanded before final formulation in freezing medium. Four development full-scale runs demonstrated that cell processing can be performed in the closed-automated-system. Good results were achieved for all in-process parameters evaluated (cell recovery and viability, total process yield), for quality attributes (product and process related impurities), and potency (ΔLNGFR expression, ganciclovir sensitivity, Vector Copy Number; immunophenotype for identity, activation and sub-populations markers) of the final product. A formal process GMP validation is ongoing. These results will support discussion with Regulatory Authorities for the introduction of this new process in the late phase of the ongoing clinical study and for the market.

P029

Retroviral vector manufacturing productions using disposable fixed-bed bioreactors

S La Seta Catamancio[1] M Cota[1] P Mangia[1] R Arnaudova[1] L Alievi[1] A Lennaertz[2] F Rossetti[1] W Soncin[1] G Frizzale[1] V Bozzola[1] S Fracchia[1] G P Rizzardi[1] G Vallanti[1] C Traversari[1]

1: MolMed SpA 2: Pall Life Sciences

Retroviral vector (RVV) are largely used in cell and gene therapy studies. For early clinical development, RVV can be manufactured at medium-scale by different open and manual systems. Moving to advanced clinical and commercial phases, one of the major bottlenecks is to find an adequate large-scale platform for vector production from adherent cells. In order to address this issue, different systems were evaluated to obtain a scalable and robust process producing RVV with low level of impurities and high transduction efficiency. This evaluation was carried out in the context of the MM-TK therapy constituted of T lymphocytes genetically modified ex vivo with the γ retroviral vector SFCMM-3 Mut2 #48. Here, three different systems were compared: traditional packed-bed bioreactor (NLF32), compact 2D Xpansion® Multiplate and iCELLis® fixed-bed, disposable bioreactors. In these systems we investigated the role of culture regulations (temperature, pH and air saturation) on cell growth and on the RVV quality attributes by evaluating key metabolites, as glucose and lactate, and infectious viral titer. A development study was performed using the Xpansion® system: results from three runs were comparable to the ones obtained with the classic NLF-32 bioreactor, for infectious viral titer, transduction efficiency on T cells and level of impurities. In particular, viral titer was 2.5x106 vs 2.0x106 TU/ml; infectivity was 4.5x103 vs 3.2x103 TU/ng p30; transduction efficiency on target T cells was 18.2% vs 19.9%. The development study using the iCELLisTM nano bioreactor is on-

going. Process development and comparability data will be presented in more details.

P030

Gene therapy with soluble VEGFR2 and sTie2 reduces the growth of tumors and formation of ascites fluid in ovarian cancer mouse model

L Tuppurainen[1 2] H Sallinen[1 2] A Karvonen[1 2] H Hakkarainen[1 2] T Liimatainen[1 2] E Hytönen[1 2] K Hämäläinen[4 6] V M Kosma[4 6] M Anttila[1 4 5] K Alitalo[8] S Ylä-Herttuala[1 2 7]

1: A.I.Virtanen Institute 2: University of Eastern Finland, Kuopio 3: University of Helsinki 4: Institute of Clinical Medicine, Gynecology, and Pathology and Forensic Medicine 5: Department of Gynecology, Kuopio University Hospital 6: Department of Pathology, Kuopio University Hospital 7: Gene Therapy Unit, Research Unit, Kuopio University Hospital 8: Molecular/Cancer Biology Laboratory Biomedicum Helsinki and Haartman Institute, University of Helsinki, Helsinki

Angiogenesis plays a crucial role in the development of ovarian cancer tumors. In the present study, we used the anti-angiogenic gene therapy and chemotherapy as a treatment for human solid ovarian cancer in a mouse model. Immunodeficient Balb/cA-nu mice (n = 49) received SKOV-3m ovarian cancer cells i.p. Gene therapy was dosed i.v. at day 11, a day after the first MRI and visible tumors. Chemotherapy with carboplatin and paclitaxel was dosed i.p. one week after the gene therapy. Afterward the growth of the tumors and effectivity of gene- and chemotherapy were followed with weekly MRI and diffusion-weighted MRI by using relaxation times T2, T1ρ, TRAFF. Study groups were AdCMV (group I), AdCMV + chemotherapy (group II) as controls and AdsVEGFR2 + AdsTie2 (group III), AdsVEGFR2 + AdsTie2 + chemotherapy (group IV). The highest survival was in groups III and IV (mean survival 35 and 39 days respectively) and lowest survival in control group I (mean survival 26 days). Tumor growth was significantly smaller in gene therapy groups III and IV as compared to control group II. Formation of ascites fluid was also significantly lower in therapy groups III and IV as compared to control group I and also in group IV when compared to group II. These results confirm, that antiangiogenic AdsVEGFR2 and AdsTie2 gene therapy with chemotherapy is effective as reducing the growth of tumors and formation of ascites fluid in ovarian cancer mouse model.

P034

Antitumor effect of GM-CSF genetically modified cells combined with CTLA4 and Foxp3 gene silencing in mice

A Miguel[1] L Sendra[1] V Noé[2] C J Ciudad[2] M J Herrero[1] S F Aliño[1]

1: Universidad de Valencia 2: Universitat de Barcelona

Treg cells, which express the inmunoregulator molecules CTLA4 and Foxp3, are involved in the failure of therapeutic vaccination. We combine therapeutic vaccination with the CTLA4 and/or Foxp3 gene silencing. Methodology: Cell entrance and gene silencing efficacy of 2-OMe-phosphorotioates ASO (antisense oligonucleotides) and PPRH (polypurine reverse Hoogsteen hairpins) were evaluated in EL4 cells and culture primary lymphocytes by qPCR and flow cytometry. C57BL6 mice were injected with B16 tumor cells. For gene silencing anti-CTLA4 and/or anti-Foxp3 ASO (500μg/mouse, days 2, 4, 7, 9) were injected. For vaccination, 2x105 irradiated B16 GM-CSF genetically modified cells were injected (days 3, 10, 17). The CTLA4 and Foxp3 expression were measured by RT-qPCR and flow cytometry from blood samples. Tumor development and survival were monitored. Results: a) to achieve the same percentage of labeled cells in vitro, concentration of FAM-PPRH needed was higher than ASO (10-200 folds); b) gene silencing with ASO inhibited mRNA expression of CTLA4 and Foxp3 up to 52% and 50%, respectively, whereas PPRH without vector reached lower than 13%; c) synergistic survival effect was observed combining gene silencing (CTLA4 and Foxp3) with therapeutic vaccine (20 and 50% ($p < 0,05$), respectively); d) early expression (day 4) of CTLA4 and Foxp3 ($p < 0,05$) mRNA is related with tumor development; e) blood CD4 + CD25 + Foxp3 + and CD4 + CTLA4 + ($p < 0,05$) cells is higher in mice that developed tumor on sacrificed day. Conclusions: Combination of gene silencing (mainly Foxp3) with antitumor therapeutic vaccination mediates synergistic effects with potential interest in cancer treatment. Partially supported by SAF 2011-27002.

P035

Next generation sequencing of nuclear and cytoplasmic microRNAs in response to hypoxia

T Husso[1] P Laitinen[1] I L Kolari[1] M Väänänen[1] T C Roberts[2 3] M P Turunen[1] S Ylä-Herttuala[1 4]

1: A.I.Virtanen Institute, University of Eastern Finland 2: Sanford-Burnham Medical Research Institute 3: Department of Physiology, Anatomy and Genetics, University of Oxford 4: Science Service Center and Gene Therapy Unit, Kuopio University Hospital

Recent studies have suggested that mature miRNAs are present in the nucleus, although their nuclear functions have remained unclear. In this study, we performed miRNA-sequencing from normoxic and hypoxic (1% O2, 2 and 24 hours) endothelial cells which were divided into nuclear and cytoplasmic fractions. Fraction purity was confirmed by western blot analysis of tubulin, lamin and histone H3 proteins. We found that 238 miRNAs are cytoplasmic at all time points, 147 miRNAs are nuclear at all time points and 260 miRNAs change localization over time. Importantly, we found several previously unknown putative miRNAs, which many of them were nuclear enriched. We also found that the 5′- and 3′-arms of some miRNAs were enriched either in nucleus or cytoplasm. Curiously, the data show that when a miRNA changes its expression in response to hypoxia, the change happens in either the nuclear or cytoplasmic fraction for a specific miRNA. These data suggests a novel, non-canonical mechanism of action for miRNAs contributing to the hypoxic response in endothelial cells.

P036

Processed small hairpin RNAs are present in nucleus and cytoplasm and secreted from transduced cells in vivo in mouse myocardial infarction model

I L Kolari[1] T Husso[1] P Laitinen[1] M Väänänen[1] P Mäkinen[1] S Ylä-Herttuala[1 2] M P Turunen[1]

1: Department of Biotechnology and Molecular Medicine, A.I.Virtanen Institute, University of Eastern Finland 2: Science Service Center and Gene Therapy Unit, Kuopio University Hospital, Kuopio, Finland

We have reported that lentiviral shRNAs targeted to murine VEGF-A promoter are able to repress or induce VEGF-A expression both in vitro and in vivo. In this study we focus on clarifying the molecular mechanism behind shRNA-451 mediated transcriptional gene activation of mouse VEGF-A. Our data demonstrates that both mature shRNA-451 sense and antisense strands are found from the nucleus and cytoplasm in endothelial cells in vitro. Also, efficient secretion of mature shRNA-451 strands was demonstrated. Still, neither C166 nor MS-1 cells were found to intake secreted shRNA-451 strands in vitro. However, when cells from transduced hearts were sorted for co-expressed GFP (positive and negative fractions), we detected mature strand of shRNA-451 also in negative population by qRT-PCR analysis, suggesting that in infarcted mouse heart shRNA-451 transfer from cell to cell occurs, proposing that transfer may be cell type or tissue dependent. Further studies are required to find out if epigenetic changes are passed on to recipient cells in vivo. Mature form of shRNA-451 is also found in serum of MI mice that have received intra-myocardial injection of lentiviral vector expressing shRNA-451. Therefore, also tissue to tissue transfer of shRNAs is possible. This study explains why promoter targeted shRNAs have surprisingly good in vivo efficiency and also point out that safety of traditional RNAi therapeutics should be reconsidered because possible off-target effects in chromatin.

P037

Angiogenesis and vasoconstriction in the treatment of limb ischemia in a disease model

G Wirth P Korpisalo S Yla-Herttuala

1: A.I.Virtanen Institute

Aims: As clinical trials of angiogenic gene therapy have failed to reproduce the promising results from preclinical animal studies, experimental models have been criticized for generating an unrealistic setting for testing of therapies in young and healthy animals. Thus, we now studied angiogenic gene therapy in hindlimb ischemia of genetically modified diseased and aged mice.

Methods: LDLR-/-ApoB100/100 mice (age 6–15 months) underwent unilateral hindlimb ischemia operation and simultaneously received either intramuscular adenoviral hVEGF-A165 or AdLacZ-control gene transfer (2x1010pfu/ml). Contrast enhanced ultrasound imaging, photoacustic imaging and histological studies were carried out to evaluate perfusion recovery, oxygen saturation and tissue responses, respectively. Additionally, L-NAME (50mg/kg) or NaCl-control were given intraperitoneally to modulate blood flow starting four days after hindlimb ischemia operation.

Results: Although inducing significant capillary enlargement, AdhVEGF-A165 gene transfer could not improve muscle recovery in the old and diseased mice one week after operation, but instead enhanced necrosis as compared to the AdLacZ controls. As the overdilation of capillaries seemed to worsen the outcome of ischemia, we tested constriction of the vessels with L-NAME after initial opening of collaterals. Surprisingly, the vasoconstriction treatment did not result in a worsened outcome and in fact especially the very old LDLR-/-ApoB100/100 animals (13–15 months of age) seemed to even benefit from the treatment.

Conclusions: Old age and disease background greatly modify the results of AdhVEGF-A165 gene therapy and overdilation of vessels may even augment cell death in ischemia. Vasoconstriction treatment may help control vascular overdilation and warrants further studies in ischemic diseases.

P038

Molecular mechanisms of chronic vascular hyperpermeability in VEGF therapy

J P Lappalainen J P Laakkonen H Pulkkinen M Vattulainen A Tirronen P I Toivanen T Nieminen H Niskanen M U Kaikkonen S Ylä-Herttuala[1]

1: A.I. Virtanen Institute, University of Eastern Finland

Therapeutic angiogenesis, utilizing VEGF induced vascular growth has been developed for treatment of coronary and peripheral artery disease. In pre-clinical trials VEGF therapy has been shown to induce blood vessel growth and skeletal muscle perfusion. At present, the main hurdle in VEGF therapy is the increased plasma protein extravasation and vascular hyperpermeability, leading to tissue edema followed by instability of newly formed vessels. In this study we define factors that are involved in chronic vascular hyperpermeability induced by VEGF-A165. To study this we stimulated angiogenesis by adenovirus mediated VEGF-A165 gene transfer via systemic delivery. Empty adenovirus without a transgene was used as a control. Angiogenic response and endothelial permeability profiles of VEGF-A165 and control virus was studied in primary human vein endothelial cells and from various vascular beds of C57BL/6 mice. Transcriptional alteration of genes known to be involved in vascular permeability and angiogenesis was monitored by next-generation RNA sequencing. VEGF-A165 was shown to regulate various genes involved in cell adhesion, migration and calcium signaling. In vivo extravasation of fluorescent microspheres, used as a detection marker for vascular permeability, was shown to increase in VEGF-A165 group but not with the control vector group. Additionally, angiogenic response and increased VEGF-A plasma concentration was detected with VEGF-A165 group. To conclude, this data can be used to find novel molecular targets to reduce edema in pro-angiogenic therapy and vascular diseases.

P039

Equivalence of allogeneic human cardiac stem/progenitor cells (CPCs) batches used in CAREMI clinical trial

J L Abad[1] V Álvarez[1] B Sánchez[1] R Rosado[1] N Dam[1] I Palacios[1] L Rodríguez-Borlado[1]

1: Coretherapix

Coretherapix is promoting cardiac regenerative therapies based on administration of allogeneic stem/progenitor cells into patients suffering Acute Myocardial Infarction. Off-the-shelf product is sent to hospital and stored frozen until it is used in acute phase of the pathology. For the development of allogeneic therapies it is essential to guarantee batch consistency among cell products from different donors. Moreover, quality of

cryopreserved cells may be impaired respect to the same cells in ''fresh'' state. Our purpose is to assess the equivalence of CPCs batches and to study the influence of freezing over CPCs phenotype. Equivalence among different CPCs batches was assessed by genome wide expression arrays. Viability and phenotype of CPCs were compared in recently thawed and in culture cells. Viable cells were counted by Trypan Blue, apoptosis was assessed by Anexin V staining, metabolic state was tested by Alamar Blue assay and phenotype was obtained by flow cytometry. Expression arrays showed a high degree of equivalence among CPCs obtained from different donors. Batch consistency within donors was also displayed. Phenotype and viability of recently thawed and in culture CPCs were comparable and no significant differences were found. Phenotype continuity at different expansion steps was also evidenced. Array studies demonstrated the cardiac origin of CPCs and clearly differentiated them from mesenchymal cells. Therefore, our manufacturing process of CPCs is highly reproducible and robust, as it was demonstrated by batch consistency and equivalence among donors. CPCs keep intact their viability and phenotype throughout the culture and cryopreservation processes.

P040

Optimizing cellular delivery of Hcn2/SkM1 to induce long-term biological pacing

L Cócera Ortega[1] A MD Végh[2] E M Lodder[1] A O Verkerk[1] M Klerk[1] E Oosterwerff[1] V M Christoffels[1] C R Bezzina[1] H L Tan[1] M JTH Goumans[2] G JJ Boink[1]

1: Academic Medical Center 2: Leiden University Medical Centre

Background: Biological pacemakers based on adenoviral overexpression of the pacemaker channel Hcn2 and the skeletal muscle sodium channel Skm1, have shown efficient in vivo function. An important next step is the optimization of a cellular delivery to induce long-term function. Human cardiomyocyte progenitor cells (CMPCs) have been shown to efficiently deliver HCN4-based biological pacing, and persist in the heart for at least 3 months after injection. In the present study we therefore optimized electroporation protocols to efficiently introduce HCN2/SkM1 into CMPCs.

Methods and Results: Human CMPCs were isolated from fetal hearts using magnetic beads coated with a Sca-1 antibody, cultured in non-differentiating conditions, and electroporated (Amaxa-Nucleofector) with constructs encoding eGFP, Hcn2 + eGFP, Skm1 + eGFP or Hcn2 + Skm1 + eGFP. To enhance expression, a Woodchuck hepatitis virus Posttranscriptional Regulatory Element (WPRE) motif was added to the CMV-driven backbones. Addition of the WPRE was highly effective in correcting the suboptimal expression levels of Hcn2 and SkM1 which resulted in an increase in the electroporation efficiency from $\sim$30% and $\sim$10% to $\sim$80% and $\sim$50%, respectively, as detected by immunocytochemistry. Functional presence of the relevant ion currents was confirmed using perforated patch clamping at 37°C. The Hcn2 current density at -120 mV was -32 ± 6 pA/pF (n = 3), which is comparable to previously reported values for mesenchymal stem cells electroporated with Hcn2.

Conclusions: Electroporation is an efficient method to introduce relatively large ion channel genes into CMPCs. Introduction of the WPRE motif in the expression backbone importantly increased expression. Hcn2/SkM1-loaded CMPCs hold significant promise for the introduction of long-term biological pacing.

P041

Characterization of a novel diabetic animal model: SUR1-E1506/IGF-II mice

T Valkama[1] A Laine[1] E Gurzeler[1] J Huusko[1] S Ylä-Herttuala[1]

1: A.I.Virtanen Institute

Diabetes Mellitus is one of the health challenges of the world affecting over 200 million people worldwide. Animal models have an important role in studying the mechanisms and potential new treatments for the complications of diabetes. Mice overexpressing insulin growth factor (IGF)-II have been reported to be hyperglycemic, hyperinsulinemic and insulin resistant. In order to strengthen the diabetic phenotype, we crossbred IGF-II overexpressing mice with mice carrying a mutation in sulfonylurea receptor 1 (SUR1) gene (SUR1-E1506 mice). This mutation in humans causes hyperinsulinemia in infancy, developing to type 2 diabetes in adulthood. The aim of the study was to characterize this novel SUR1-E1506/IGF-II mouse model due to its diabetic phenotype. SUR1-E1506 mice without the IGF-II background were used as controls. The mice were on a regular chow diet until 6 months of age and on a high fat diet (HFD) from 6 to 12 months of age. Glucose- and insulin tolerance tests (GTTs & ITTs) and insulin ELISAs were performed at 3, 6, 9 and 12 months of age. Echocardiographic assessment of the heart was done and blood samples taken for HbA1c and lipid analysis at the same time points. Weight and blood glucose were monitored monthly. At 3 months of age SUR1-E1506/IGF-II mice were significantly less glucose sensitive than the controls, but developed high glucose sensitivity by 12 months of age. Both strains also developed a notable insulin resistance. Total cholesterol and HbA1c levels were increased with age on both strains. End-point measurements to finalize the characterization are ongoing.

P042

Enhancers unfolding the complexity of transcriptional responses during atherogenesis - emerging potential of 'enhancer therapy'

H Niskanen M Liivrand S Ylä-Herttuala C K Glass M U Kaikkonen

1: A.I.Virtanen Institute 2: University of California, San Diego

Atherosclerotic cardiovascular diseases are the leading cause of death in the Western world. However, we are still far from understanding how different cell types in the developing atherosclerotic lesion contribute to the disease: what makes these cells different and how do the differences in the cellular responses contribute to the disease process? Recent studies suggest that cell type-specific enhancer selection, involving the binding of lineage-determining transcription factors, is responsible for cell-types specific responses to extracellular stimuli. This study aims to identify the gene regulatory processes that take place in endothelial cells, macrophages and smooth muscle cells during atherogenesis by following enhancer dynamics during exposure to hypoxia, oxLDL and LPS. Our analysis focuses on novel non-coding RNAs originating from enhancer regions, called enhancer

RNAs (eRNAs) and their role in the transcriptional responses. We find that targeting of eRNAs using anti-sense oligonucleotides, siRNAs and CRISPR-Cas9 system allows modulation of the expression of nearby target genes in a cell-type specific manner. We therefore propose to investigate the feasibility of 'Enhancer Therapy' by selecting cell-specific enhancers that express eRNAs and interact with cardiovascular disease-relevant genes.

P043

Characterizing chromatin architecture and hypoxia-regulated gene expression in human primary vascular endothelial cells

H Niskanen[1] M Heinäniemi[1] S Ylä-Herttuala[1] M U Kaikkonen[1]

1: University of Eastern Finland

Endothelial cells (EC) are important for the maintenance of vascular homeostasis. Their ability to sense oxygen levels in the blood and neighboring tissues is crucial for vascular development and angiogenesis. Our goal was to study the transcriptional response of ECs to hypoxia, characterize hypoxia-responsive enhancer elements and to investigate how chromatin interactions are involved in the regulation of hypoxia response and EC-specific gene expression. We also investigated how cohesin regulates transcription and chromatin interactions during the hypoxia response. We used global run-on sequencing (GRO-seq) to determine the transcriptional response of HUVECs to hypoxic conditions (1% O2, 8 h). GRO-seq revealed differential regulation of 602 genes and hundreds of eRNAs, non-coding RNAs transcribed from enhancer regions. We also investigated genome-wide chromatin interactions by using tethered conformation capture and ChIP-seq for RAD21, a component of the cohesin complex. Comparisons of chromatin interaction data available from other cell types allowed us to determine EC-specific chromatin compartments. These compartments were enriched for genes important for endothelial functions and were found to be transcriptionally active only in endothelial cells. We also show how cohesin is involved in maintaining the cell-type specific gene expression and in regulating hypoxia-responsive genes. In addition, our data revealed that eRNA expressing enhancers are more involved in chromatin interactions and their expression correlates with interacting hypoxia-regulated genes. Finally, we show that the chromatin architecture remains unchanged in hypoxic conditions. In conclusion our data provides new insights into the epigenetic landscape of ECs and their ability to respond to hypoxia.

P044

High plasma lipid levels reduce efficacy of adenovirus-mediated gene therapy

A Laine[1] A M Kivelä[1] J Huusko[1] E Gurzeler[1] M H Dijkstra[1] G Wirth[1] C B F Andersen[2] S K Moestrup[2] S Ylä-Herttuala[1]

1: A.I.Virtanen Institute 2: Aarhus University

We previously reported differences in transgene expression after systemic adenoviral VEGF-A gene transfer in different hyperlipidemic mouse models. The aim of this study was to elucidate whether high lipid levels and specific lipoproteins could decrease adenoviral transduction efficiency and relate to insufficient transgene expression after adenoviral gene transfer in clinical trials. Adenoviral transduction efficiency was studied in C57Bl/6 mice and four hyperlipidemic mouse models (ApoE-/-, LDLR-/-, LDLR-/-ApoE-/- and LDLR-/-ApoB100/100 mice) after adenoviral gene transfers of human (h) VEGF-A165 or LacZ. Mice were fed with a regular chow diet (RCD) or high fat diet (HFD) for five weeks. Transgene expression was studied four days after gene transfers. In addition, different tissue samples were collected for mRNA measurements and histology. Plasma levels of hVEGF-A on a HFD were lower in LDLR-/- and LDLR-/-ApoB100/100 mice compared to C57Bl/6 and apoE-deficient mice after systemic gene transfers. In addition, transgene expression in general was higher on a RCD compared to levels on a HFD in all LDLR-/- based models. Transduction efficiency after local intra muscular gene transfers in LDLR-/-ApoB100/100 mice on a HFD was correspondingly reduced compared to mice on a RCD. High plasma lipid levels, especially apoE-containing lipoproteins, are protective against adenoviral transduction in mice, which implies that high cholesterol levels in humans could also be protective against viral infections leading to insufficient transgene expression. This is an important aspect that should be appreciated in cardiovascular clinical adenoviral gene therapy trials as the treated patients in most cases have significantly elevated blood lipid levels.

P045

Lentivirus-mediated SERCA2a gene transfer improves left ventricular function in heart failure

M Mattila[1 2 3] J Koskenvuo[4] M Savontaus[1 4]

1: Centre for Biotechnology, University of Turku, Finland 2: Pharmacology, Drug Development and Therapeutics, University of Turku, Finland 3: Drug Research Doctoral Program, University of Turku, Turku, Finland 4: Turku University Hospital, Finland

Background: Despite significant treatment advances, severe heart failure carries a dismal prognosis, underscoring the need for novel therapeutic approaches. In the heart, during relaxation, calcium is reaccumulated in the sarcoplasmic reticulum by the sarcoplasmic reticulum Ca2 + ATPase pump (SERCA2a). It has been shown that SERCA2a expression and activity is decreased in cardiac dysfunction leading to diminished calcium uptake by sarcoplasmic reticulum, thus providing a rationale for SERCA2a-based gene therapy for heart failure.

Methods: Lentiviral vector LV-SERCA2a-GFP expressing human SERCA2a gene and GFP reporter gene was constructed. GFP expressing vector LV-GFP was used as a control. Heart failure was induced by administrating Doxorubicin to C57Bl mice. LV-SERCA2a-GFP, LV-GFP or saline was delivered by ultra-sound guided intramyocardial injection into the anterior wall of the left ventricle. Several functional parameters were measured by echocardiography at the injection day and day 28 after injection. RNA was extracted for RT-PCR, and tissue sections were prepared for immunohistochemical analysis.

Results: The in vivo method was shown to be feasible and reporter gene analysis from frozen tissue samples exhibited robust GFP expression confirming that cardiomyocytes had been transduced by the viral vector. Echocardiography analyses demonstrated a significant increase in the ejection fraction in SERCA2a group when compared to control virus group or saline-injected group. Furthermore, end-systolic and end-diastolic left ventricle volumes were significantly smaller in SERCA2a group as compared to controls.

Conclusions: SERCA2a-based gene therapy resulted in significant improvement in left ventricular function in experimental heart failure. These results encourage further clinical development of SERCA2a gene therapy.

P046

Comparison of intramyocardial gene transfer of adenovirus, adeno-associated virus and lentivirus vectors: their effects on gene transduction efficiency, inflammation, fibrosis, left ventricular function and electrocardiography of mouse heart

M Merentie[1] L Lottonen-Raikaslehto[1] V Parviainen[1] J Huusko[1] M Mendel[7] N Laham-Karam[1] V Kärjä[5] M Hedman[2 6] S Ylä-Herttuala[1 3 4]

1: A.I.Virtanen Institute 2: Diagnostic Imaging Center, Kuopio University Hospital 3: Gene Therapy Unit, Kuopio University Hospital 4: Science Service Center, Kuopio University Hospital 5: Department of Clinical Pathology, Kuopio University Hospital 6: Heart Center, Kuopio University Hospital 7: Department of Medical Biotechnology, Jagiellonian University

Gene therapy with viral vectors is a promising new treatment option for cardiac diseases. The aim of this study was to compare adenovirus (AdV) vectors to adeno-associated (AAV)- and lentivirus (LeV) vectors delivered with percutaneous intramyocardial injections in mice regarding transduction efficiency, myocardial damage, effects on the left ventricular function and electrocardiography (ECG) in order to find the most suitable and safe vector for cardiac gene therapy. AAV9 was also studied by using intravenous (iv) injections. AdV had the highest transduction efficiency in cardiomyocytes followed by AAV2 and AAV9 which were at a similar level compared to each other. The lowest efficiency was seen with LeV. The tissue damage, seen in the form of local fibrosis and inflammation in the left ventricle (LV), was proportional to transduction efficiency. AdV caused LV dilatation and systolic dysfunction. Neither of the locally injected AAV serotypes impaired the LV systolic function, but AAV9 caused diastolic dysfunction to some extent. LeV did not affect the cardiac function. Surprisingly, LV dilatation and a significant decrease in LV systolic function were also seen in the NaCl control group. After iv injection of AAV9, transgene positive cells were seen throughout the myocardium. However, also diffuse fibrosis was present leading to significantly impaired LV systolic and diastolic function and pathological ECG changes. Compared to AdV vector, AAV2, AAV9 and LeV were less effective in transducing cardiomyocytes, but also less harmful. Local administration of AAV9 was safer and more efficient compared to systemic administration.

P047

Secreted LDL receptor/transferrin chimeric protein improves dyslipidemia in LDL receptor-deficient mice

E Leggiero[2] G Labruna[4] F Salvatore F Salvatore[2] L Sacchetti L Pastore[2 3]

1: CEINGE-Biotecnologie Avanzate 2: CEINGE-Biotecnologie Avanzate, Napoli, 80145, Italia 3: Università di Napoli ''Federico II'', Napoli 80131, Italia 4: Fondazione IRCCS SDN, Napoli, Italia 5: Università di Napoli ''Federico II'', Napoli, Italia

Familial hypercholesterolemia (FH) is a well-characterized genetic hyperlipidemia due in most of the cases to mutations in the LDL receptor (LDLR) gene; FH is characterized by elevated concentration of plasma LDL cholesterol (LDL-C) with consequent deposition of LDL-C in tendons, skin and arteries. Statins can lower cholesterol levels but are not effective in all patients and prognosis is still quite poor. In the past we have developed gene-therapy strategies based on liver transduction using PEGylated helper-dependent adenoviral (HD-Ad) vectors. However, intravenous administration is often associated to a host response that can narrow the therapeutic window and reduce the clinical applicability of gene transfer. We are devising a therapeutic strategy for reducing LDL using a secreted protein. At this aim, we developed an HD-Ad vector for the expression of a soluble form of the extracellular portion of the human LDLR fused in frame with transferrin (LDL-R/Tf). We have evaluated the efficacy of this chimeric protein in cellular models as 293, COS and CHOldla7; subsequently, we have intravenously administered the HD-Ad vector expressing LDLR/TF and demonstrated the efficacy of the above-mentioned vector in reducing total cholesterol levels in LDLR-deficient mice, the mouse model of FH.. Additional experiments for the evaluation of the efficacy of the LDLR/TF chimeric proteins expressed using alternative routes of administration are ongoing in order to develop a gene transfer protocol more compatible with clinical applications.

P048

IRES-based vectors for a combined gene therapy of heart ischemia

E Renaud-Gabardos[1] B Lebas[1 3] F Hantelys[1] D Calise[1 2] P Sicard[1 2] O Kunduzova[1 2] F Pujol[1] J Roncalli[3] X Chaufour[1 3] B Garmy-Susini[1 2] A Parini[1 2] A C Prats[1]

1: Université Paul Sabatier 2: Inserm 3: CHU Rangueil

Heart failure remains an important problem of public health, to which gene therapy appears as a promising perspective. Our laboratory develops combined gene therapy of cardiovascular diseases, based on translational activators, IRESs (internal ribosome entry sites), to generate combined gene transfer vectors. We have validated this concept 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. Here, we have designed IRES-based vectors for gene therapy of myocardial ischemia. The aim is to express combinations of molecules stimulating angiogenesis, myocardium contractile function and cardiomyocyte survival. A bicistronic lentivector expressing two luciferase genes separated by the FGF1 IRES has been successfully assessed by direct intracardiac injection in a mouse model of infarcted myocardium, showing that the IRES drives increased transgene expression when heart is ischemic. Lentivectors have been designed to express combinations of therapeutic genes including Apelin, FGF2, Cyr61 or Serca2a. The triplet Apelin-FGF2-Cyr61 is by far the most angiogenic, whereas the tricistronic lentivector Apelin-FGF2-Serca2a shows the best therapeutic benefits in improvement of cardiac function, hypertrophic features, fibrosis and angiogenesis, by comparison with monocistronic and bicistronic lentivectors. These data validate the use of IRES-based lentivectors for combined gene therapy of heart ischemia and reveal the benefits of the

apelin-FGF2-Serca2a triplet. The final objective is to develop this therapeutic vector in a clinical perspective.

P049

Slit2 modulates Vascular endothelial growth factor receptor 2 – mediated angiogenic effects in rabbit hindlimb by decreasing the activity of eNOS

T Nieminen[1] P I Toivanen[1] J P Laakkonen[1] T Heikura[1] M U Kaikkonen[1] K J Airenne[1] S Ylä-Herttuala[1 2]

1: University of Eastern Finland, Kuopio 2: Kuopio University Hospital, Finland

Aims: Vascular endothelial growth factor (VEGF) - induced angiogenesis can likely be modified with Slit2, but the data concerning its effects in large animal models is missing. The effect of Slit2 on therapeutic angiogenesis induced by two VEGF receptor 2 (VEGFR-2) ligands, Vammin and VEGF-DΔNΔC, was studied in vivo in rabbit hindlimb skeletal muscles. The function of Slit2 was also studied as a single factor.

Methods: We performed adenoviral intramuscular gene transfers into New Zealand White rabbit hindlimbs. Blood vessel imaging was done with confocal and multiphoton microscopy. Signaling tests were performed to investigate mechanisms of Slit2 function, and RNA sequencing was used to find out the target genes.

Results: Slit2 reduced VEGFR-2-mediated vascular permeability as well as VEGFR-2-mediated increase in blood perfusion and capillary enlargement. However, sprouting of the capillaries was increased. Vascular functions or morphology were not changed after Slit2 gene transfer alone. Slit2 did not alter VEGFR-2 activation, but VEGF-induced eNOS phosphorylation was decreased. RNA sequencing revealed Slit2 down-regulating several angiogenesis-related genes such as Nuclear receptor subfamily 4 group A member 1 (NR4A1) and Stanniocalcin-1 (STC-1).

Conclusions: VEGFR-2-mediated angiogenic effects can be adjusted towards more physiological level when Slit2 is used in combination with VEGFs. This may enable the use of higher VEGF vector doses to accomplish wider distribution of the vector and the therapeutic protein in the target muscle. This approach would lead to a better therapeutic response and keep the level of vascular permeability low.

P050

Nuclear translocalisation of VEGF-B and VEGFR-1 during cardiac hypertrophy and development

A K Tirronen[1] J Huusko[1] M H Dijkstra[1] M U Kaikkonen[1] P Tavi[1] S Ylä-Herttuala[1]

1: A.I.Virtanen Institute 2: University of Eastern Finland

Chronic hypertension, a common pathological condition, can lead to left ventricular hypertrophy (LVH) and subsequently to heart failure. It has been shown that vascular endothelial growth factor B (VEGF-B) and it's receptor VEGFR-1 are involved in these processes. The aim of the study was to define the subcellular localisation and associated functions of VEGFR-1 and VEGF-B in cardiac development and LVH in cardiomyocytes both in vitro and in vivo. The expression pattern of VEGF-B and VEGFR-1 in cardiac development, normal myocardium and cardiac hypertrophy was studied in vitro on cultured neonatal cardiomyocytes and in vivo in C57Bl/6j mice using angiotensin II induced hypertrophy and physiological exercise. Specific localisation of the proteins was assessed using flow cytometry and western blotting of cellular protein fractions and immunostaining of cultured cardiomyocytes and paraffin embedded cardiac tissues. Chromatin immunoprecipitation sequencing of VEGFR-1 was also performed. Surprisingly, VEGF-B localisation was mainly cytosolic whereas VEGFR-1 was strongly localised in the nucleus of neonatal cardiomyocytes. ChIP sequencing results suggest nuclear VEGFR-1 may acts as a regulator of several cellular functions. The nuclear expression of VEGFR-1 decreased during cardiac development from birth to adulthood. Unexpectedly, both pathological and physiological hypertrophy induced translocalisation of VEGFR-1 and VEGF-B from the cytosol to the nucleus in adult hearts. In this study, we were able to show that VEGFR-1 and VEGF-B seem to respond to both pathological and physiological LVH with nuclear translocalisation. Additionally, VEGFR-1 might be an important regulator of cardiac development and postnatal hypertrophy.

P052

Impaired VEGFR3 signaling affects cardiac lymphatic vessel organization and alters accumulation of lipids

T Vuorio[1] J P Laakkonen[1] H Niskanen[1] H Hakkarainen[1] S Ylä-Herttuala[1]

1: A.I.Virtanen Institute

The role of lymphatic vessels in lipoprotein metabolism has emerged in the recent years. In our previous study, we showed that blocking VEGFR3 signaling in atherosclerotic mouse model increases circulating cholesterol levels and accelerates atherogenesis. Here we studied mechanisms behind the previous findings and analyzed the cardiac lymphatic vessel organization and cardiac function. Mice expressing soluble VEGFR3 (sVEGFR3) and mice with inactivating point mutation in VEGFR3 gene (Chy) were crossed with atherosclerotic LDLR-/-/ApoB100/100 mice. Mice were fed with chow diet for 6 months or 14 months followed by high-fat diet for 84 days. Cardiac lymphatic vessel organization and cardiac function were analyzed with confocal microscopy and echocardiography. The distribution of adipose tissue was analyzed with MR imaging and histological stainings. The expression of lipoprotein metabolism related genes were analyzed with RNA sequencing and verified with quantitative PCR. Compared to the control mice, cardiac lymphatic vessels were dilated in sVEGFR3 x LDLR-/-/ApoB100/100 mice and had almost completely lost their organization in Chy x LDLR-/-/ApoB100/100 mice. However, only a slight increase in left ventricle diastolic volume was observed. Body fat percentage was significantly lower in sVEGFR3 x LDLR-/-/ApoB100/100 mice (42.5% vs 33.2%, respectively). In addition, sVEGFR3 x LDLR-/-/ApoB100/100 mice had less liver steatosis and the expression of fatty acid transporters were significantly decreased. To conclude, here we show that VEGFR3 signaling is required for the correct organization of cardiac lymphatic vessels. In addition, attenuated VEGFR3 signaling increases cholesterol levels possibly by affecting lipoprotein accumulation in the tissues.

P053

Targeted integration of FANCA in the mouse AAVS1-homolog of Fanca-/- hematopoietic progenitor cells

M J Pino-Barrio[1 2] S Navarro[1 2] R Chinchon[1 2] P Rio[1 2] R Sanchez-Dominguez[1 2] C Mussolino[3 4] T Cathomen[3 4] J A Bueren[1 2]

1: Division of Hematopoietic Innovative Therapies, CIEMAT/CIBERER, 28040 Madrid, Spain 2: Advanced Therapies Unit, IIS-Fundacion Jimenez Diaz (IIS-FJD, UAM), 28040 Madrid, Spain 3: Institute for Cell and Gene Therapy, University Medical Center Freiburg, 79106 Freiburg, Germany 4: Center for Chronic Immunodeficiency, University Medical Center Freiburg, 79106 Freiburg, Germany

Fanconi anemia (FA) is an inherited syndrome mainly associated with bone marrow failure and cancer predisposition. Mutations in any of the 17 genes that participate in a DNA-repair pathway known as the FA pathway are responsible for this rare disease. However, mutations in FANCA account for more than 50% of the FA patients. In this project we aim to investigate the feasibility and efficacy of inserting the therapeutic FANCA gene in the murine Mbs85-locus, a site homologous to the human AAVS1 safe harbor, in Fanca-/- cells. To facilitate the specific targeting of FANCA in the Mbs85-locus, a TALEN pair was generated that showed targeted cleavage of 44% or 25% of mouse AAVS1 alleles in murine embryonic fibroblasts (MEFs) or hematopoietic progenitors, respectively. Additionally a donor vector harboring the PGK-FANCA sequence flanked by Mbs85 homology arms was constructed. Both TALENs and the donor were nucleofected in Lin– bone marrow cells from Fanca-/- mice and gene corrected hematopoietic colonies were subsequently selected in clonogenic cultures with 10 nM of mitomycin-C (MMC). According to PCR analyses of MMC-resistant colonies and based on CFCs survival rates after MMC exposure, the targeted integration of FANCA in these cells was in the range of 1–2%. Estimated efficacies of targeted integration in Fanca-/- MEFS were around 2–5%. Importantly, evidence of phenotypic correction has been observed in both cell types. In a next set of experiments the in vivo repopulating ability and therapeutic effect of gene edited Fanca-/- HSCs will be tested by transplantation into Fanca-/- recipient mice.

P054

Targeted gene therapy in the AAVS1 locus in CD34+ cells from healthy donors and Fanconi anemia patients

B Diez[1 2] P Genovese[3] G Schiroli[3 4] L Alvarez[1 2] F J Roman-Rodriguez[1 2] P Gregory[5] M Holmes[5] A Lombardo[3 4] L Naldini[3 4] J A Bueren[1 2] P Rio[1 2]

1: Division of Hematopoietic Innovative Therapies, CIEMAT/CIBERER, 28040 Madrid, Spain 2: Advanced Therapies Unit, IIS-Fundacion Jimenez Diaz (IIS-FJD, UAM), 28040 Madrid, Spain 3: San Raffaele Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milan, 20132, Italy 4: Vita Salute San Raffaele University, Milan, 20132, Italy 5: SANGAMO BioSciences, Richmond, 94804, CA

Gene targeting constitutes a new step in the development of safe and efficient gene therapy for patients with inherited diseases, such as Fanconi Anemia (FA). Since FA is associated with mutations in any of the 17 different FA genes so far discovered, we decided to develop a gene targeting strategy that can be applicable to all FA complementation groups, based on the integration of FA genes in a ''safe harbor'' locus. To assess the feasibility of targeting therapeutic transgenes in the AAVS1 locus of human hematopoietic stem cells we transduced cord blood CD34+ cells with a donor IDLV that carried an eGFP reporter, followed by the nucleofection with AAVS1-specific ZFNs-mRNAs. Efficiencies of 10% of gene targeting were obtained in in vitro cultured cells. After xenotransplantation, gene edited human hematopoietic cells engrafted both in primary and secondary NSG recipient mice, confirming the successful targeting of long-term repopulating cells. In subsequent experiments, the eGFP reporter gene was substituted by the hFANCA therapeutic gene. Gene targeting experiments conducted with the therapeutic vector in lymphoblastic cell lines from FA-A patients showed the reversion of MMC sensitivity and FANCD2 foci formation after DNA damage, confirming the phenotypic correction of these cells. A final set of experiments was performed with CD34+ cells from FA patients using similar conditions. In these studies a partial reversion of the MMC sensitivity was observed, suggesting that gene targeting is feasible in hematopoietic progenitors from FA patients.

P055

Targeted gene therapy in a ''Safe Harbor'' gene island in primary CD34+ cells: applications in Fanconi anemia

F Rodriguez-Fornes[1 2] O Quintana-Bustamante[1 2] M L Lozano[1 2] A Gouble[3] R Galetto[3] L Poirot[3] J C Segovia[1 2] G Guenechea[1 2] J A Bueren[1 2]

1: Division of Hematopoietic Innovative Therapies, CIEMAT/CIBERER, 28040 Madrid, Spain 2: Advanced Therapies Unit, IIS-Fundacion Jimenez Diaz (IIS-FJD, UAM), 28040 Madrid, Spain 3: CELLECTIS SA, 75013 Paris, France

Fanconi anemia (FA) is a rare monogenic disorder characterized by congenital defects, bone marrow failure and cancer susceptibility. Although the lentiviral-mediated transduction of hematopoietic stem cells constitutes an attractive approach for the treatment of FA, the specific insertion of the therapeutic FA gene in non-coding safe harbor (SH) loci would constitute a safer approach to limit the impact of gene insertion in functional endogenous sequences. In these studies we aimed the specific insertion of a transgene in a human SH locus (SH6). With this purpose a donor EGFP construct together with a SH6-meganuclease (SH-6 MN) or a SH6-TALEN were nucleofected in human cord-blood-CD34+ cells, either as DNA or mRNA. Thereafter the efficacy and toxicity of the gene targeting approach was investigated in these cells. Flow cytometry analyses of CD34+ that had been transfected with the nucleases delivered as DNA showed percentages of up to 3% of EGFP+ in primitive hematopoietic progenitors (CD34+/CD38-/CD90-). Homologous Recombination (HR) events were verified by PCR analysis both in liquid cultures and in colonies, confirming the gene editing of the hematopoietic progenitor cells. When nucleases were delivered as mRNA, flow cytometry analyses showed a 3-fold reduction of toxicity and percentages of EGFP+ cells of up to 6%. We also tested a new donor vector carrying the FANCA gene in HEK-293H cells and HR events were detected with this therapeutic vector. Current studies aim to demonstrate the phenotypic correction of FA progenitor cells by the specific insertion of FANCA in the SH6 locus of FA CD34+ cells.

P056

Aptamer delivery of a potent antitumor LNA-oligonucleotide

O Gissberg[1] E Zaghloul[1] K Lundin[1] R Zain[1] J Wengel[2] C I E Smith[1]

1: Karolinska Institute 2: Univsersity of Southern Denmark

Aptamers are oligonucleotides (ONs) containing secondary structures that allow them to specifically interact with target receptors. AS1411 is an aptamer capable of selectively binding to and enter cancer cells through nucleolin receptor-mediated uptake. The aptamer has also proven to induce apoptosis through various mechanisms due to its interaction with nucleolin. In this report, we aimed at taking advantage of the cell internalization properties of AS1411 by conjugating it to different Locked Nucleic Acid (LNA) containing ONs and deliver them into lung cancer cells. For one of the LNA ONs we demonstrate a potent cytotoxic effect and the corresponding aptamer conjugate is efficiently taken up by the cancer cells. We also show that AS1411 maintains its secondary structure (G-quadruplex) important for the selective uptake when conjugated to the LNA ONs, and the anti cell proliferative ON is still functional with the aptamer attached. Moreover, we found that uptake kinetics and efficiency is highly governed by ON cargo chemistry and composition since AS1411 loses its inherent anti cell proliferative ability during naked uptake when attached to a non-toxic LNA ON. All put together, these findings add new insights to the design and future applications in the developing field of aptamer-guided specific delivery of ON cargo to cancer cells.

P057

Generation of isogenic cell lines by gene editing based on cancer drug resistance screens

T Rath S Fröhling C Scholl P Chudasama H Glimm C von Kalle A Nowrouzi M Schmidt

1: DKFZ / NCT Heidelberg

Despite great advances in targeted, gene and immunotherapy approaches development of secondary mutations and drug resistance mechanisms are major drawbacks of cancer therapy. We want to identify novel candidate genes modulating drug response and resistance in functional genomic screens and we are further aiming for an in vitro system using gene editing to specifically introduce chosen mutations to model drug resistance in live cells and to investigate the functional impact of those mutations. To set up our model, TALENs (Transcription activator-like effector nucleases) and CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9) system RNA guided endonucleases together with corresponding donor vectors targeting the two cancer-relevant genes BRAF and SF3B1 were designed for generation of mutant alleles. Nuclease fidelity was determined by T7EI assay and deep sequencing. Insertions and deletions at the target loci were found in up to 42 per cent of sequences with striking variations dependent on the individual target sites. Successful gene targeting was detected by allele-specific PCR and western blot. We found 293T clones positive for the BRAF V600E mutation on gene, transcript and protein level using mutation-specific PCR primers and antibodies. Showing proof of principle of our approach, we have started to set up a universal cancer drug resistance screening assay based on viral vector integration and shRNA-mediated drug resistance gene tagging to identify novel candidate genes involved in therapy resistant sarcoma. Our universal drug resistance approach may substantially contribute to uncover resistance mechanisms and, in long-term, translation of these findings into the clinics.

P058

Trans-splicing gene therapy in the KRT14 gene

J S Breitenbach[1] T Lettner[1] T Kocher[1] W Muss[3] P Peking[1] S Hainzl[1] S K Kitzmüller[2] M Ablinger[1] F J Pötschke[1] J W Bauer[1] V Wally[1]

1: EB House Austria 2: University of Salzburg 3: Paracelsus Medical University Salzburg

EB simplex Dowling Meara (EBS-DM) is a genetic skin blistering disease caused by mutations in the KRT5 or KRT14 gene. Keratinocytes of affected patients show enhanced migration and invasiveness, an increased sensitivity to heat and osmotic shock, and keratin aggregates in the periphery of the cytoplasm upon minor traumatization. Our aim is to establish a gene therapy for EBS-DM using Spliceosome-mediated RNA-trans-splicing, which is well established for many genes and mutations in EB. To achieve genetic correction of a dominantly inherited KRT14 missense mutation in protein position 125 (EBDM-1 keratinocytes, K14/R125H) in an EBS-DM keratinocyte cell line, we stably transfected these cells with a correcting RNA trans-splicing molecule (RTM), expressing GFP linked to the KRT14 coding region as reporter. In the corrected patient keratinocytes, we could show restoration of the intermediate filament network and decreased migration and invasiveness. To lay the base for a potential clinical application, we constructed 3D skin equivalents from the corrected human EBS-DM keratinocytes and transplanted them onto xenograft compatible immunodeficient mice. After differentiation of the grafts, we analyzed their function, morphology, differentiation status and integrity: we subjected them to mechanical pressure and analyzed the expression of skin markers by immunofluorescence microscopy and their morphology by electron microscopy. We could demonstrate correct expression of GFP and KRT5. Our data indicate that functional correction of the KRT14 gene in EBS-DM by trans-splicing is possible and therefore a promising gene therapy approach for EBS-DM. Supported by DEBRA International.

P060

Development of MFP-inducible system for gene therapy of chronic diseases in the liver

J M Liefhebber[1] R Martier[1] R Logtenstein[1] B Blits[1] H Petry[1] P Konstantinova[1]

1: uniQure

Gene therapy offers long term solutions for chronic diseases, whereby the transgene is continuously expressed upon single vector administration. However in some cases it would be desirable to tightly regulate or switch off transgene expression. We are investigating regulated gene expression based on the mifepristone (MFP)-inducible GeneSwitch system. The GeneSwitch protein comprises yeast Gal4 DNA-binding domain, a

human p65 activation domain and a MFP controlled domain derived from the human progesterone receptor. The classical GeneSwitch system consists of two expression cassettes on two separate vectors; one containing the GeneSwitch sequence and one containing the transgene. We compared this two-vector system to a single-vector system, where the two cassettes were put into one vector. We show in vitro and in vivo data on constructs made to regulate recombinant erythropoietin (EPO) expression in the liver using a liver-specific promoter, AAT. EPO is characterized by clear expression kinetics in plasma and raises blood hematocrit, hence provides a reliable in-life readout for gene inducibility. Mice were injected with different doses of AAV5-AAT-GeneSwitch-EPO and gene expression was induced in two separate rounds at 4 and 8 weeks p.i. EPO plasma levels increased approximately 2-logs in the single or two-vector system-injected mice, compared to un-induced groups. Moreover in the absence of MFP background expression of EPO was lower in the single-vector system and hematocrit levels were unaffected. Overall, our data indicate that transgene expression can be repeatedly regulated in the liver using the GeneSwitch system and provides us with a novel AAV5 vector for further development.

P061

Genome-wide lentiviral vector integration profiles in human and murine Hematopoietic Stem/Progenitor Cells

V Poletti[1] G Corre[1] S Charrier[1] F Pichon[1] C Cattoglio[3] A Recchia[2] F Mavilio[1]

1: GENETHON 2: Università di Modena e Reggio Emilia 3: University of California, Berkeley

In the last 25 years, patients with rare immunodeficiencies have been successfully treated by ex-vivo gene therapy approaches based on transplantation of autologous hematopoietic stem/progenitor cells (HSPCs) transduced with gamma-retroviral vectors. However, insertional proto-oncogene activation leading to myelodysplastic or leukemic outgrowth was observed in some of these trials. In this and other cases, preclinical studies conducted in mice did not recapitulate several key findings observed in the clinical trials, namely, preferred integration into the LMO2 locus and T-cell leukemia induction, highlighting the weakness of murine models in predicting the genotoxic consequences of vector integration. Lentiviral vectors are considered a safer alternative to gamma-retroviral vectors, since they lack LTR enhancers and preferentially target active transcriptional units instead of gene regulatory elements. Clinical trials are so far confirming this predictions, although there is still no preclinical assay that can ''guarantee'' the safety, and fully uncover the potential genotoxicity, of integrating viral vectors as therapeutic agents. In this study, we present genome-wide lentiviral vector integration profiles obtained from over 250,000 integration sites in human CD34+HSPCs derived from cord blood, peripheral blood and bone marrow, and murine Sca+and LIN- HPSCs. We compared these LV profiles by rigorous statistical analysis to discover shared, species- and cell-specific LV integration features, target regions and genes. We show that lentiviral vectors target at high frequency a substantially different set of genes in murine vs. human cells, underlying the need to have appropriate controls in analyzing mouse studies, particularly those addressing proto-oncogene activation, clonality and clonal dominance.

P062

CRISPR/Cas9 mutation-specific gene editing for ADA SCID

M Calero-Garcia[1] M Carmo[1] A J Thrasher[1] H B Gaspar[1]

1: UCL Institute of Child Health

Adenosine deaminase (ADA) deficiency is a rare metabolic disease that results in the accumulation of adenosine and deoxyadenosine-related metabolites and causes numerous multi-organ pathologies; severe combined immunodeficiency (SCID) being the most life threatening. The only curative option for the SCID manifestation is bone marrow hematopoietic stem cell transplant from a suitable donor. Alternatively, gene therapy protocols employing retroviral vectors have been developed over the past two decades with varying successful results. However, these integrating vectors have been shown to carry a risk for insertional mutagenesis in other clinical trials. Gene editing enables the genetic engineering of DNA in a sequence-specific manner and opens the possibility of correcting ADA SCID mutations without the risk for insertional mutagenesis, while achieving near physiological ADA expression. The CRISPR/Cas9 system is the latest gene editing platform to emerge and allows for accessible targeting via Watson-Crick base pairing. In the present work we study the CRISPR/Cas9 cutting efficiency at the locus of one mutation (Q3X) described in UK residents of Somali origin. To assess NHEJ, Jurkat cells were transfected with Cas9 and 20, 18 or 17nt gRNA plasmids and western blot and enzymatic activity assays were performed, showing efficient disruption of ADA expression. To study HDR, Jurkat cells were co-transfected with the Cas9 and 20nt gRNA plasmid together with a donor plasmid, from which a small library of the on-target site was cloned and sequenced, presenting approximately 3% HDR efficiency. We are currently optimizing the HDR results and translating the approach into Q3X CD34+ patient cells.

P063

Reactivation of latent HIV-1 expression by engineered TALE transcription factors

P Perdigão[1 2 3 4 5] T Gaj[3 4 5] C F Barbas III[3 4 5] M Santa-Marta[1 2] J Gonçalves[1 2]

1: Research Institute for Medicines (iMed ULisboa), Faculdade de Farmácia,Universidade de Lisboa, Lisboa, Portugal 2: Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal 3: The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, California, USA 4: Departments of Chemistry, The Scripps Research Institute, La Jolla, California, USA 5: Department of Cell and Molecular Biology, The Scripps Research Institute, La Jolla, California, USA

The presence of replication-competent HIV, which residues mainly in populations of resting CD4+T cells, is a major hurdle for HIV eradication. While pharmacological approaches have been useful for inducing the expression of latent HIV, to date, they have been unable to purge virus from all reservoirs. Further, many of these compounds are associated with off-target effects, underscoring the need of alternative strategies capable of reactivating latent virus. Here we show that engineered transcription factors based on customizable transcription activator-like effector (TALE) proteins can induce gene expression from the HIV-1 long terminal repeat (LTR) promoter, and that combinations of TALE transcription factors can synergistically

reactivate latent virus expression in cell line models of HIV-1 latency. In addition, we show that complementing TALE activators with vorinostat, a histone deacetylase inhibitor, further enhances HIV-1 reactivation in latency models where provirus is integrated in condensed chromatin regions inaccessible to transcription factors. Critically, TALE activators did not increase the expression of immune surface markers in primary resting CD4 + T cells or impact viability. Collectively, these findings indicate that TALE transcription factors are a potentially effective alternative to current pharmacological routes for reactivating latent virus and that combining synthetic transcriptional activators with histone deacetylase inhibitors could lead to the development of improved therapies for HIV-1.

P064

Multiple emulsion can be used as microreactors for synthesizing cationic lipid coated magnetic nanoparticles for gene targeting

H Akbaba Y Selamet A G Kantarci

1: Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Ege University, Izmir, 35100, Turkey 2: Department of Physics, Faculty of Science, Izmir Institute of Technology, Izmir, 35433, Turkey

Nanoparticles can be used as non-viral gene delivery systems because of their low-cost, low immunogenicity and ease of production. Nanoparticle systems can deliver genetic material either with passive or active transport techniques. One of the active transport techniques, showing great promise for both in vitro and in vivo transfection is magnetofection. Magnetofection is defined as the nucleic acid delivery to cells, site-specifically guided by the attractive forces of magnetic fields acting on nucleic acid vectors, which are associated with magnetic nanoparticles. For this purpose, we developed a novel method, with new physics and chemistry based techniques to obtain non-toxic, biocompatible and targetable magnetic nanoparticles with optimal size and surface properties. In this method we combined microemulsion and multiple emulsion techniques and synthesized magnetic nanopaticles (MNPs) in the core of multiple emulsion. Afterwards obtained MNPs were characterized and then complexed with green fluorescence protein (GFP)-encoding plasmid (pEGFP-C1). Characterization studies include; Particle size and zeta potential analysis, Vibrating Sample Magnetometer (VSM) studies, Transmission Electron Microscopy (TEM) and Cell Proliferation Test (XTT). Obtained MNP's are superparamagnetic (According to magnetic hysteresis - VSM), cationic (+41.6 mV), nano-sized (139.9 nm), have narrow particle size distribution (PDI level is below 0.3) and uniform magnetic core (according to TEM photos), relatively non-toxic (Over %70 cell viability for desired dose- According to XTT test). At last transfection studies has done under magnetic field. pEGFP-C1 transfection were observed with fluorescence microscope and GFP expression level has been observed comparable to commercially available products.

P065

Study of NLS-modified peptide carriers for nuclear gene delivery.

M S Bogacheva A A Slita A A Egorova V S Baranov A V Kiselev

1: D.O.Ott Research Institute of Obstetrics, Gynecology and Reproductology, St.Petersburg, Russian Federation

Gene therapy faces the problem of effective non-viral DNA delivery into cells. One of the main cellular gene delivery barriers is a nuclear envelope. DNA transport through the nuclear pores is the main route for nucleus penetration in non-dividing cells. Carrier's modification with nuclear localization signal (NLS) can promote DNA delivery into cell nucleus. SV40 large T-ag NLS has been renowned to enhance nuclear DNA-uptake while modular peptide carriers have been suggested as a promising platform for the development of ''artificial viruses''. This work aimed to study cross-linking peptide-based carriers modified with SV40 NLS as vectors for gene delivery into mammalian cells. We studied three NLS-containing arginin-rich peptides with different molar content of NLS-molecules (90, 50 and 10 mol%). Study of physicochemical properties included DNA-binding and DNA-protection tests. Transfection efficacy and toxicity properties were studied in HeLa and A172 cells with lacZ as a reporter gene. Cell cycle blockage was conducted using hydroxyurea. YOYO-labelled DNA localization in cells after transfection was studied by confocal microscopy. We showed that NLS-modified peptides are capable to condense plasmid DNA and protect it from nuclease degradation. It was revealed that the NLS-modified polyplexes can transfect cells after cell cycle blockage in G1 stage and an increase in the polyplex' NLS-content leads to augmentation of transfection efficacy up to 5-10-fold compared to the unmodified ones. Thus we revealed direct relationship between rate of NLS-content in the polyplexes and their nuclear translocation properties. This work was supported by RFBR grant 15-04-00591.

P066

pFAR4/SB100X-mediated PEDF gene delivery in primary murine pigment epithelial cells

S Diarra[1] C Marie[2] D Scherman[2] Z Izsvák[3] Z Ivics[4] P Walter[1] G Thumann[5] S Johnen[1]

1: University Hospital RWTH Aachen 2: Unité de Technologies Chimiques et Biologiques pour la Santé, INSERM U1022 – CNRS UMR8258 3: Max Delbrück Center for Molecular Medicine 4: Paul Ehrlich Institute 5: University Hospitals of Geneva

Patients suffering from neovascular age-related macular degeneration (AMD), leading to blindness within several months, are commonly treated by repeated intravitreal injections of vascular endothelial growth factor (VEGF) antibodies. The aim of TargetAMD is to develop a non-viral gene therapy, where autologous iris (IPE) and retinal (RPE) pigment epithelial cells are transfected with the pigment epithelium-derived factor (PEDF) gene, the natural antagonist of VEGF, and re-transplanted within one surgical session. Stable transgene expression is ensured by the Sleeping Beauty (SB) transposon system, carried by pFAR4-plasmids, a vector free of antibiotic resistance markers. Here, we report the establishment of efficient transfections of primary murine IPE and RPE cells, mandatory for subsequent safety studies. IPE and RPE cells of C57/BL6 mice were isolated and cultivated for up to 28 days. 5×10^3 cells were electroporated with pFAR4-plasmids encoding the SB100X transposase and an SB transposon carrying either the PEDF or the Venus reporter gene at a ratio of 1:16, using the Neon® Transfection System. Transgene expression was analyzed by fluorescence microscopy and immunoblotting. Transfection of

5×10^3 cells showed a stable transgene expression for at least 2 months. Using 1100 V/20 ms/2 pulses, efficiency in Venus-transfected cells was 6.57 times higher for IPE than for RPE cells. Compared to non-transfected cells, PEDF secretion was increased up to 5.80 ± 4.36 fold. The proof of efficient primary murine IPE and RPE cell transfection leads to the next step of safety studies, such as the subconjunctival transplantation of transfected cells and subsequent large-scale transposon integration site analysis.

P068

How to optimize TFO binding of DNA in the context of bisLNA

Y V Pabon[1] K E Lundin[1] J Wengel[2] C I Edvard Smith[1] R Zain[1 3]

1: Karolinska Institutet 2: University of Southern Denmark 3: Karolinska University Hospital

Triplex forming oligonucleotides (TFOs) are used in the anti-gene strategy to modulate gene expression. TFOs bind to the major groove of double stranded DNA (dsDNA) through Hoogsteen or reverse-Hoogsteen hydrogen bonds. To enhance specificity and binding efficiency of oligonucleotides to DNA, we previously reported an alternative construct (bisLNA) having a dual binding mode and chemically modified locked nucleic acids (LNA). The purpose of this study is to optimize the TFO portion of bisLNA by examining the effect of position and amount of LNA within the sequence. Different TFOs (13 – 15mers) containing varying proportions of LNA substitutions at the 3′-, 5′-ends or throughout the sequence were hybridized to a 45mer dsDNA target using intra-nuclear salt conditions and pH. To probe for triplex formation, TFO hybridization was carried out in the presence of a triplex-specific intercalating compound, Benzoquinoquinoxaline (BQQ), and DNA complexes were analyzed using EMSA. We found that the LNA content and position at either ends of a TFO have different impact on the rate of formation and stability of the triplex. Interestingly, the absence of LNA substitution at 6 constitutive positions at the 3′-end tremendously reduced its binding capacity. On the other hand, a TFO lacking an equivalent LNA substitution at the 5′-end was able to bind to the cognate dsDNA target and we observed triplex formation at low TFO concentrations. In all cases, the DNA complexes were highly stabilized by BQQ demonstrating for the first time an efficient binding of the compound to LNA-containing triplex structures.

P069

CRISPR-Cas9 gene editing of CD34+ cells to increase fetal hemoglobin (HbF) production

K Wartiovaara[1] D Muñoz[2] W Magis[2] D Martin[2]

1: University of Helsinki 2: Children's Hospital Oakland Research Institute

The gene defects of the hemoglobin beta gene cluster are responsible for the hemoglobinopathies of tens of millions of people globally, most often suffering from the sickle cell disease (SCD) or beta thalassemias. The functional treatment modalities of these diseases are either frequent blood transfusions or bone marrow transplantation, both of which are expensive and problematic procedures and not suitable for everybody. Since the gene involved in HbF production is usually intact in SCD and thalassemia, an alternative strategy to ameliorate the symptoms of hemoglobinopathies is to increase the production of HbF. Pharmacological interventions are also used, but with limited efficiency and unwanted side effects. Here, we aimed to increase the HbF by editing the genomic locus with CRISPR-Cas9 system. Hereditary Persistence of Fetal Hemoglobin (HPFH) is a syndrome in which the switch from HbF to adult is incomplete, and 10–30% of the Hb is of the fetal form. HPFH can be caused by several mutations in the hemoglobin gene(s). We have used the CRISPR-Cas9 genetic editing system to introduce different HPFH mutations to the Hb beta-cluster locus in CD34+ hematopoietic stem/progenitor cells. Our results show that the gene editing is functional and we can get the desired genotype of cells produced. However, the efficiency needs to be increased in order to reach clinical significancy.

P070

AAV-delivered donor template and adenoviral vector-delivered TALENs allow efficient gene edition for the correction of a recurrent mutation in the Col7A1 gene causing recessive dystrophic epidermolysis bullosa in the Spanish population

C Chamorro[1 4] A Mencia[1 2 4] H Bunning[3] B Duarte[1 4] M Del Rio[2 4] F Larcher[1 2 4] R Murillas[1 2 4]

1: Epithelial Biomedicine Division, CIEMAT, 28040 Madrid, Spain 2: Department of Biomedical Engineering, UC3M, 28911 Madrid, Spain 3: Center for Molecular Medicine Cologne (CMMC), University of Cologne, 50931 Cologne, Germany 4: Instituto de Investigación Sanitaria de la Fundación Jimenez Diaz, 28040 Madrid, Spain

Recent developments in gene editing technologies provide researchers with a wide choice of tools for the targeted correction of disease-causing mutations. We have combined AAV-mediated delivery of donor template DNA with TALEN nucleases expressed by adenoviral vectors to address the correction of the c.6527insC mutation, highly prevalent in recessive dystrophic epidermolysis bullosa (RDEB) Spanish patients. Efficient transduction of keratinocytes was achieved by using capsid engineered, optimized adeno-associated viral (AAV) vectors of the serotype 2. Clonal analysis demonstrated high frequencies of gene correction via HDR using these viral vectors in a keratinocyte cell line derived from a RDEB patient carrying the c6527insC mutation. A targeting construct based on promoterless, gene trap-mediated expression of the neo selection marker resulted in much higher targeting frequency than a constitutive, PGK-driven neo expression construct. After excision of the neo selection cassette, gene edited clones recovered the expression of Col7A1 at physiological levels, and these keratinocyte clones were used to regenerate corrected skin displaying ColVII expression in the basal lamina. In addition, treatment of patient keratinocytes with TALEN nucleases in the absence of a donor template DNA resulted in the generation of indels in the vicinity of the c.6527insC mutation in a high percentage of keratinocyte clones. A subset of these indels restored the ORF of Col7A1 and resulted in abundant, supra-physiological expression levels of quasi-ColVII.

P071

The design and analysis of double RNA trans-splicing molecules for COL7A1 repair

C Hüttner[1] S Hainzl[1] E M Murauer[1] J W Bauer[2] U Koller[1]

1: Laboratory for Epidermolysis bullosa research, EB House Austria, University Hospital of Dermatology, Paracelsus Medical University Salzburg, Austria 2: University Hospital of Dermatology, Paracelsus Medical University Salzburg, Austria

Mutations in COL7A1, encoding type VII collagen, lead to the dystrophic form of the blistering skin disease epidermolysis bullosa (DEB), in which the formation of anchoring fibrils is impaired. Using the methodology of internal exon replacement (IER) we want to substitute internal COL7A1 exons in EB patient cells to correct the underlying mutations. We have established a GFP-based screening system in order to identify highly efficient double RNA trans-splicing molecules (dRTMs) prior to their introduction in patient keratinocytes. Co-transfection experiments with the most efficient dRTM and an artificial COL7A1-mini gene (COL7A1-MG), carrying the target region for RTM binding, in HEK293 cells leads to the fusion of all GFP parts, carried by both screening molecules, restoring GFP expression in over 70% GFP of all analyzed cells. The full-length expression of GFP was further confirmed on protein level by Western blot analysis. Prior to endogenous experiments in patient cells the internal GFP part on the dRTM was replaced with the wildtype coding sequence to introduce. First experiments with the endogenous dRTM in HEK293 cells, stably expressing the screening COL7A1-MG, showed expression of trans-splicing products on RNA level. Binding domain variations for 5′ and 3′ trans-splicing induction, respectively, revealed a great impact on the general splicing characteristic of the tested dRTM. Therefore we assume that our screening system accelerates the design of double RNA trans-splicing in order to obtain a molecule capable to correct hot spot mutations in the genes involved in several genetic disorders.

P072

The combined application of antisense-based strategies for RNA repair

C Hüttner E M Murauer S Hainzl T Kocher J W Bauer U Koller

1: Laboratory for Epidermolysis bullosa research, EB House Austria, University Hospital of Dermatology, Paracelsus Medical University Salzburg 2: University Hospital of Dermatology, Paracelsus Medical University Salzburg

In recent years we have developed a fluorescence-based screening system to define and analyse factors which influence the efficiency of RNA trans-splicing-mediated RNA repair. In addition to the design of RNA trans-splicing molecules (RTM) this approach can be applied to study the impact of antisense RNAs (AS-RNA) on the trans-splicing characteristics of a given RTM. We show the possibility to increase the trans-splicing efficiency of a selected RTM in order to replace two internal exons of COL7A1 by the combined application of both antisense strategies. Defects in type collagen VII encoded by COL7A1 are associated with the dystrophic form of the skin blistering disease epidermolysis bullosa (DEB). A selected AS-RNA, which interferes with the competitive splicing elements on a stably expressed COL7A1-minigene (COL7A1-MG), was co-transfected with a highly functional double RNA trans-splicing RTM (dRTM) into HEK293 cells leading to a significant increase of the RNA trans-splicing efficiency. Thereby, accurate trans-splicing between the RTM and the COL7A1-minigene is represented by the restoration of full-length GFP, that can be measured by flow cytometric analysis. We assume that the inclusion of this second antisense-based application can improve RTM-mediated correction of genes on the pre-mRNA level significantly. Our fluorescence-based screening system is therefore a useful tool to prepare a wise RNA repair strategy prior to experiments in collagen type VII deficient patient cells.

P073

Correction of type VII collagen in a murine recessive dystrophic epidermolysis bullosa model using RNA trans-splicing

P Peking[1] U Koller[1] S Hainzl[1] T Kocher[1] E Mayr[1 2] S Kitzmüller[1] T Lener[3] L Bruckner-Tudermann[4] J W Bauer[5] E M Murauer[1]

1: Laboratory for Epidermolysis bullosa research, EB House Austria, University Hospital of Dermatology, Paracelsus Medical University Salzburg, Austria 2: Laboratory for Molecular Therapy, EB House Austria, University Hospital of Dermatology, Paracelsus Medical University Salzburg, Austria 3: University Clinic of Blood Group Serology and Transfusion Medicine, Core Facility Microscopy, SCI-TRECS, Paracelsus Medical University, Salzburg, Austria. 4: Department of Dermatology, University Medical Center Freiburg, Germany 5: University Hospital of Dermatology, Paracelsus Medical University Salzburg, Austria

We use spliceosome-mediated RNA trans-splicing (SMaRT) as an RNA therapy approach, where designed RNA trans-splicing molecules (RTM) specifically substitute mutated exons during the natural occurring splicing process in the cell. In this study we investigate the functionality of the trans-splicing technology in a murine recessive dystrophic epidermolysis bullosa (RDEB) model, expressing only 10% of normal type VII collagen due to an introduced Neo-cassette in intron 2. We designed a 5′ RTM exchanging Col7a1 exons 1 to 15 in order to restore a correctly spliced mRNA. In vitro we have shown successful trans-splicing on RNA and protein level after transfection of a minicircle 5′ RTM expression plasmid into a HEK293 cell line stably expressing a GFP-target-minigene. Correct trans-splicing could be confirmed in murine wildtype keratinocytes. For in vivo experiments we delivered the FLAG-tagged RTM expressing full-length GFP into the skin of wildtype mice using a gene gun approach. The correct localization of the RTM expression within the basement membrane zone was proven by H&E staining of skin sections showing the gunned gold particles as well as by immunofluoresence analysis of GFP expression. Using an anti-FLAG-tag antibody we detected a trans-spliced type VII collagen protein in Western blot analysis. In this study we showed that our 5′ RTM is functional concerning restoration of type VII collagen in vitro and in vivo. We are currently adapting this protocol for the hypomorphic mice.

P074

Genome editing mediated by microvesicles derived from mesenchymal stem cells expressing designer nucleases.

S Bobis-Wozowicz[1] K Sit[1] K Kmiotek[1] C Mussolino[2] M Sekula[5] M Paw[1] D Boruczkowski[4] Z Madeja[1] A Schambach[3] T Cathomen[2] E K Zuba-Surma[1]

1: Jagiellonian University 2: University Medical Center Freiburg 3: Hannover Medical School 4: Polish Bank of Stem Cells 5: Malopolska Centre of Biotechnology

Designer nucleases (DN) such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), or RNA-guided endonucleases (RGENs) constitute useful tools in genome modification and regulation. These site-specific nucleases induce DNA double strand breaks at a predefined locus and harness cellular DNA-repair machinery for the break repair, thus can be used for genome editing. Although there are variety of routes to deliver DNs to cells, some of them are associated with a considerable risk of genotoxicity, whereas others are inefficient. In this study, we investigated the utility of microvesicles (MVs), cellular membrane-derived circular fragments isolated from umbilical cord-derived mesenchymal stem cells (UC-MSC), in transferring biologically active nucleases to target cells. MVs were isolated from genetically modified UC-MSCs stably expressing ZFN, TALENs or RGENs that target the EGFP locus. Both MVs and their parental cells were verified to contain DNs in the form of mRNA and proteins using RT-PCR and Western blots. Efficacy of MV-delivered DN activity was evaluated in a transwell system and via direct DN-MVs transfer. Our results show that MVs act as natural liposomes and transfer DNs to different cells including human embryonic kidney cell line (HEK), MSCs and induced pluripotent stem cells (iPS), leading to disruption of the EGFP gene. The level of gene knockout, as measured by flow cytometry, ranged between 2% and 10%, depending on the cells. Our proof-of-concept study shows that transfer of genetic material via MSC-derived MVs can be successfully explored for genome engineering purposes.

P075

Efficient gene editing in hematopoietic stem/progenitor cells with the CRISPR-Cas9 system

J L Gori[1] J M Heath[1] C Lee[1] W Selleck[1] G G Welstead[1] C Cotta-Ramusino[1] D Bumcrot[1]

1: Editas Medicine

Transplantation of ex vivo gene-modified autologous hematopoietic stem/progenitor cells (HSPCs) is an effective gene therapy strategy for patients with hematologic genetic disease. Genome editing with the CRISPR-Cas9 system modifies gene targets in human cell lines and in preclinical animal models. Here, we evaluated CRISPR-Cas9 mediated gene editing in primary human CD34 + HSPCs. Co-delivery of Cas9 with gene-target specific guide RNAs supported editing in human CD34 + cells. Gene-edited human HSPCs maintained viability, proliferative potential, and hematopoietic colony forming potential. These findings provide further evidence of CRISPR-Cas9 gene editing in relevant human primary cell populations.

P076

pCR3::GITRL transfected MSCs induce apoptotic cell death and SIVA1 expression in SCLC-21H lung cancer cell line

C Z Kopru[1] P Korkusuz[4] I Cagnan[2 5] I Akar-Soycan[2 5] G Esendagli[3] A G Ozcan[2 5]

1: Department of Nanotechnology and Nanomedicine, Institute of Science, Hacettepe University, Ankara, 06100, Turkey 2: Department of Stem Cell Sciences, Center for Stem Cell Research and Development, Institute of Health, Hacettepe University, Ankara, 06100, Turkey 3: Department of Basic Oncology, Institute of Oncology, Hacettepe University, Ankara, 06100, Turkey 4: Department of Histology and Embryology, Faculty of Medicine, Hacettepe University, Ankara, 06100, Turkey 5: Center for Stem Cell Research and Development, Hacettepe University, Ankara, 06100, Turkey

GITR (glucocoticoid induced tumor necrosis factor receptor) and its ligand GITRL have been known to play important roles in cancer immunology. In some studies it has been reported that GITR inducing with recombinant GITRL or agonist antibodies can lead to tumor regression. When GITR induced, it can bind pro-apoptotic protein Siva and cause apoptosis. SCLC-21H and NCI-H82 are small cell lung cancer (SCLC) cells which are GITR positive and negative respectively. It has been known that mesenchymal stem cells (MSCs) have inhibitory or stimulatory effects on cancer cells. However the interaction between MSCs and SCLC are not investigated. Herein, we aimed to investigate the effect of pCR3::GITRL transfected MSCs on two different SCLC cells. First, bone marrow derived MSCs (BM-MSCs) were transfected with pCR3::GITRL by using Neon Electroporation System. Transfection efficiency was determined by flow cytometry and qPCR. Apoptotic cell death in SCLC-21H and NCI-H82 cells co-cultured with transient and stable transgene carrying MSCs, were assessed by TUNEL assay and qPCR with Siva1 Taqman probes. TUNEL assays showed significantly higher apoptotic cell index in SCLC-21H (but not in NCI-H82) co-cultured with pCR3::GITRL carrying MSCs. qPCR analysis revealed 5,65 fold increase of SIVA1 expression in SCLC-21H cultured with transient pGITRL carrying MSCs compared to untransfected MSCs. The decline of SIVA1 expression was not observed in stable transfectants in where GITRL was downregulated. In conclusion, pCR3::GITRL carrying MSCs cause apoptotic cell death in GITR positive small cell lung cancer and Siva1 signalling may be involved in this effect. (Supported by TUBITAK 112T474)

P077

Mesenchymal stem cells induce proliferation of NCI-H82 small cell lung cancer

C Z Kopru[2] I Cagnan[1 5] I Akar-Soycan[1 5] G Esendagli[3] P Korkusuz[4] A G Ozcan[1 5]

1: Department of Stem Cell Sciences, Institute of Health, Hacettepe University, Ankara, 06100, Turkey 2: Department of Nanotechnology and Nanomedicine, Institute of Science, Hacettepe University, Ankara, 06100, Turkey 3: Department of Basic Oncology, Institute of Oncology, Hacettepe University, Ankara, 06100, Turkey 4: Department of Histology and Embryology, Faculty of Medicine, Hacettepe University, Ankara, 06100, Turkey 5: Center for Stem Cell Research and Development, Hacettepe University, Ankara, 06100, Turkey

Mesenchymal stem cells (MSCs) are non-hematopoietic stem cells with the capacity to differentiate into tissues of both mesenchymal and non-mesenchymal origin. The specific homing capacity towards sites of malignant growth makes MSCs attractive cells for the treatment of autoimmune disease and solid cancers. Small cell lung cancer is a malignant and aggressive tumor cell type that is hard to treat. In several studies whether MSCs support or suppress tumor growth is unclear. In our study, we aimed to investigate the proliferative

or apoptotic effects of MSCs to two different SCLC lines (SCLC-21H and NCI-H82). Thus bone marrow derived MSCs (BM-MSCs) were expanded in vitro and characterized for their cell surface markers by flow cytometry and differentiated into adipogenic and osteogenic lineages. After characterization, we have co-cultured MSCs with CFSE stained SCLC-21H and NCI-H82 cell lines. Cell proliferation was assessed by detecting the decrease in CFSE stain of cancer cells after 48 hours. Apoptosis was determined with both Annexin-PI staining and the TUNEL assay. Results indicated that human BM-MSCs induced cell proliferation when co-cultured with NCI-H82 cells but not with SCLC-21H. There was no difference in apoptotic cell death of both SCLC lines. In conclusion, MSCs stimulate proliferation of specific cancer cells and should be considered in studies using MSCs as a vehicle for anti-tumor biological agents. (This study was supported by TUBITAK-112T474)

P078

Novel subventricular zone early progenitor cell-specific adenoviral vector for in brain stem cell bioengineering

J Reetz S Hildebrandt A Schmidt C Meier O Herchenröder A Gläser M Witt A Wree B M Pützer

1: Institute of Experimental Gene Therapy and Cancer Research, Rostock University Medical Center 2: Department of Anatomy, University of Rostock

Neural stem/progenitor cells (NSPC) have the potential to self-renew and to generate all neural lineages as well as to repopulate damaged areas in the brain. Our previous targeting strategies have indicated precursor cell heterogeneity between different brain regions that warrants the development of NSPC-specific delivery vehicles. Here, we demonstrate a target-specific adenoviral vector system for in vivo manipulation of progenitor cells in the subventricular zone of the adult mouse brain. For this purpose, we identified a series of peptide ligands via phage display. The peptide with the highest affinity was expressed in conjunction with a bispecific adaptor molecule. In order to verify the targeting potential of the specific peptide, green fluorescent protein-expressing Ad vectors were coupled with the adaptor molecule and injected into the subventricular region of adult mice by stereotaxic surgery. An efficient and selective transduction of NSPC in the SVZ was achieved, whereas hippocampal NSPC were negative. Our results offer an expeditious and simple tool to produce target-specific viral vectors for selective and direct bioengineering of stem cells in situ. This powerful technique provides an excellent opportunity to gain new insight into the fundamental mechanisms that control stem cell fate and to develop innovative strategies for a successful treatment of brain disorders. This work has been supported by the German Research Foundation (DFG).

P079

Systemic and specific sonoporation-mediated gene transfer to the kidney

D Kami R Ishida T Kusaba Y Kirita O Mazda T Adachi S Gojo

1: Kyoto Prefectural University of Medicine

Background: Sonoporation can deliver agents to target local organs despite of systemic administration with decreasing the risk of adverse effects of systemic administration. In addition, oligonucleotides are thought to become a useful class of therapeutic drugs, however, what remains to be solved to ubiquitous drugs is the methodology for them to reach the cytosol in the target organs. Herein, we demonstrated that a local sonoporation to the kidney could offer highly efficient oligonucleotides transfer, which were systemically administrated, to tubular epithelium with high specificity.

Methods and Results: C57BL/6 male mice were anesthetized and injected a mixture of Dylight547 labeled oligonucleotides (DLO) and microbubble via inferior vena cava under the laparotomy and exposed an ultrasonic wave directly to the left kidney for one minute immediately after injection. The immunofluorescent findings showed that DLO was detected only in the left kidney, while DLO was not detected in the right kidney and other organs. The microbubbles coupled siRNA electrostatically on its surface and prevented siRNA to be filtered from the kidney. The ultrasonic wave irradiation to the kidney collapsed the microbubbles and released siRNA specifically into the kidney. Moreover, the ultrasonic wave transiently disturbed the glomerular filtration barrier and increased the glomerular permeability. Oligonucleotides were passed through the barrier, and absorbed by tubular epithelium.

Conclusion: We showed that sonoporation with microbubble could deliver siRNA into the kidney specifically and effectively.

P080

Human in vitro and murine in vivo models for gene therapy studies of Retinitis Punctata Albescens

K Damodar[1] M O Pequignot[1] M Cavalier[1] L Guillou[1] A Conscience[1] L Robert[1] C P Hamel[1] V Kalatzis[1]

1: Inserm U1051, INM, Montpellier, France

Retinitis punctate albescens (PRA) is an inherited retinal disorder. It is characterized by night blindness in childhood and a progressive loss of visual acuity, due to macular degeneration, leading to legal blindness from 40 years of age. RPA is caused by mutations in the RLBP1 gene, encoding the visual cycle protein CRALBP. A CRALBP-deficiency results in retinal pigment epithelium (RPE), and subsequently photoreceptor, cell death. The existing Rlbp1-/- mouse model that partially mimics the disease phenotype could be useful for gene transfer studies. As a complement, we are generating a human cellular model of the RPA RPE. To generate such a model, we isolated fibroblasts from skin biopsies of RPA patients, which we reprogrammed into induced pluripotent stem cells (iPSc). We then differentiated the iPSc into RPE. To date, iPSc have been obtained from one of the three RPA patients, have been validated by genetic and pluripotent tests, and are in the progress of being differentiated. We have previously shown that iPSc-derived RPE is morphologically and functionally characteristic of the RPE in vivo and constitutes an excellent model for gene transfer studies. In parallel, the white Rlbp1-/- mouse model was backcrossed onto a pigmented background. The pigmented Rlbp1-/- mouse colony has a CRALBP deficiency and a delayed visual cycle as shown by HPLC studies, which can be used as readout of gene transfer efficiency. Along this line, we are generating a viral vector carrying the RLBP1 gene for in vitro and in vivo gene transfer studies.

P081

Dual-regulated lentiviral vector for gene therapy of x-linked chronic granulomatous disease

G Farinelli[1] R Jofra Hernandez[1] M Migliavacca[1] E Zonari[1] V Capo[1] M Chiriaco[2] S Scaramuzza[1] G Di Matteo[2] L Sergi Sergi[1] A Rossi[3] S Ranucci[3] A Bragonzi[3] A Kajaste-Rudnitski[1] D Trono[4] M Grez[5] P Rossi[2] A Finocchi[2] L Naldini[1 7] B Gentner[1] A Aiuti[1 7]

1: Telethon Institute of Gene Therapy (HSR-TIGET) 2: Children's hospital Bambino Ges and Tor Vergata U 3: San Raffaele Scientific Institute 4: École Polytechnique Fédérale de Lausanne 5: Georg-Speyer-Haus 6: Georg-Speyer-Haus 7: Universita Vita-Salute San Raffaele

Chronic Granulomatous Disease (CGD) is caused by defective NADPH oxidase function in phagocytes causing increased susceptibility to fungal and bacterial infections. Gene therapy with hematopoietic stem cells (HSC) may represents an alternative to conventional transplantation. We designed different lentiviral vectors (LVs) expressing gp91phox driven by either PGK or a Myeloid Specific Promoter (MSP) with miRNA126 target sequences and we tested them on human and murine HSC and in X-CGD mouse model. All vectors restored gp91phox expression and function in human X-CGD primary monocytes and differentiated myeloid cells. Regulated LVs reduced off-target expression in CD34+ cells while guaranteeing a good level of expression in differentiated cells. Combining the MSP with the miRNA126 target sequence we achieved high levels of myeloid-specific transgene expression, sparing the most primitive CD34+CD38-CD90+ HSC compartment. In vivo studies in NSG immunodeficient mice transplanted with LV transduced CD34+ cells show effective engraftment of human cells. X-CGD mice transplanted with murine Lin- transduced with different vectors engrafted and restored gp91phox expression persisting up to 10 months after gene therapy. MSP-driven vectors were superior in maintaining regulation during BM development and allowed higher expression in myeloid cells. Gene therapy-treated mice were then infected with S.aureus in order to test their ability to clear one of the most recurrent bacteria causing infections in CGD patients. All gene therapy treated mice were able to clear pulmonary infection in contrast to untreated X-CGD untreated mice. The dual-regulated LV represents a promising approach for further clinical development of gp91phox therapeutic vectors.

P082

Improving gene edition tools for Wiskott-Aldrich Syndrome gene therapy.

A Gutierrez-Guerrero[1] S Sanchez-Hernandez[1] G Galvani[1] M G Toscano[1 3] M Cobo[1] P D Gregory[2] M C Holmes[2] K Benabdellah[1] F Martin[1]

1: GENyO 2: Sangamo BioSciences, Inc. 3: Amarna Therapeutics S.L.

Specific nucleases, ZFNs, TALENs and CRISPR, are powerful tools for genome editing. Primary immunodeficiencies (PID) are a main target for gene editing strategies since a small number of corrected cells could cure patients. Our final aim is to develop gene edition tools for efficient genetic rescue of human hematopoietic stem cells (hHSCs) of WAS patients. We have designed: 1) Different WAS-specific CRISPR-based nucleases expressed in Lentiviral vectors (LV) plasmids. These plasmids will allow the comparison of different delivery systems using the same backbone (nucleofection, and Integrative-deficient LVs (IDLVs)). 2) A reporter cellular model harboring GFP coding sequences disrupted by WASP sequences (K562-GF-WASP-P). This cellular model will allow an easy readout for the efficiency and safety of different gene editing tools for the WAS locus. 3) A donor DNA, containing WT-GFP cassettes under the SFFV promoter and an expression cassette for dsRED outside the homology arms. This donor will allow us to measure the unspecific insertion of the donor outside the targeted locus (red cells). 4) A second donor DNA containing 500bp 3′ and 5′ homology arm corresponding to WASp gene and an eGFP cassette, this donor will allow the estimation of the efficiency and specificity of our tools within the endogenous WASp locus. Using these tools we showed an efficient genetic rescue of the K562-GF-WASP-P model generating up to 4% of eGFP positive cells upon nucleofection of the different components. A similar efficiency was obtained for gene addition inside the endogenous locus using the donor harboring an eGFP expression cassette.

P083

A novel computational tool for vector-genome integration sites analysis

S Afzal[1] R Fronza[1] S Wilkening[1] C Bartholoma[1] C Von Kalle[1] M Schmidt[1]

1: German Cancer Research Center (DKFZ), National Center for Tumor Diseases (NCT), Heidelberg Germany

The biosafety assessment of viral vector system is crucial to ensure safe and efficient gene therapy, as insertion of therapeutic vectors near proto-oncogenes can give rise to leukemia. Determination of exact vector integration sites, their abundance and distribution pattern in the genetically modified cells is the most feasible approach to address vector safety concerns. With the advent of next generation sequencing technologies, large amount of sequencing data are being generated for comprehensive IS analysis. Therefore, there is a constant need for automated and fast bioinformatics methods for efficient downstream analysis. Here we present a new bioinformatics tool for reliable analysis of vector-genome junctions. This tool is designed to analyze data generated from different experimental techniques (e.g., LAM-PCR or targeted sequencing technologies as Agilent SureSelect). It is a user-friendly tool implemented at Linux platform with minimal software dependencies. The generated result file contains information about sequence ID, genomic and vector integration sites, strand orientation and sequence etc. The additional result files provide clustered and annotated integration sites with their sequence count and overview of basic statistics of analysis process. It takes about 30 minutes for complete processing, starting from raw reads till annotation, of 10 million paired end reads generated by targeted sequencing. In case of LAM-PCR, 30 million reads are sorted in about 30 minutes (50 different PCR) and time required for rest of processing to obtain annotated IS is also approximately 30 minutes for 15 million reads. Our tool is highly appropriate for reliable and efficient large scale analysis.

P085

Proof of concept for AAV-mediated gene transfer in iPSc-derived retinal pigment epithelium

N Cereso[1] M O Pequignot[1] L Robert[1] F Becker[2] V De Luca[1] N Nabholz[1] V Rigau[3] J De Vos[2] C P Hamel[1] V Kalatzis[1]

1: Inserm U1051, INM, Montpellier, France 2: Inserm U1041, IRMB, Montpellier, France 3: Department of Anatomy and Pathological Cytology, CHU, Montpellier, France

Inherited retinal dystrophies (IRDs) are a large group of genetically and clinically heterogeneous diseases leading to progressive vision loss, for which a paucity of disease-mimicking animal models can render pre-clinical studies difficult. To circumvent this issue, we proposed that pertinent human cellular IRD models, such as retinal pigment epithelium (RPE) derived from induced pluripotent stem cells (iPSc), could be used for gene transfer studies. As an example, we focused on choroideremia, which represents 2% of IRDs and the mouse and zebrafish models are lethal. We reprogrammed patient-specific fibroblasts into iPSc that were differentiated into RPE. We demonstrated that the iPSc-derived RPE is a polarised monolayer with a classical morphology, expresses characteristic markers, is functional for fluid transport and phagocytosis, and mimics the biochemical phenotype of patients. We assayed a panel of AAV vector serotypes in this human RPE and identified the serotype with the highest transduction efficiency (up to 80% transduction). This high in vitro transduction efficiency is likely due to the phagocytic capacity of the iPSc-derived RPE and therefore this model mimics the scenario an AAV vector encounters in vivo in the subretinal space. Furthermore, we demonstrated that AAV-mediated gene transfer normalises the biochemical phenotype in the patient RPE. To conclude, we identify the superior AAV serotype (that is different to the one currently used in clinical trials) in functional human RPE, and, for the first time, show the potential of patient iPSc-derived RPE to provide a proof-of-concept for gene replacement in the absence of an appropriate animal model.

P086

Lentiviral-mediated transgenesis of mesenchymal stromal cells with IL-10 enhances their therapeutic potential in GVHD

E Galli[1] M Noviello[1] B Camisa[1] F Gullotta[1] G Andolfi[2] G Locafaro[2] F Ciceri[3] L Naldini[4 5] C Bonini[6] F Locatelli[7] M E Bernardo[8] S Gregori[2] A Bondanza[1 5]

1: Innovative Immunotherapies Unit, San Raffaele Hospital and Scientific Institute, Milano IT 2: Tolerogenic Dendritic Cells Unit, TIGET-San Raffaele Hospital and Scientific Institute, Milano IT 3: Hematology and Bone Marrow Transplantation Unit, San Raffaele Hospital and Scientific Institute 4: Gene Transfer Technology Unit, TIGET-San Raffaele Hospital and Scientific Institute, Milano IT 5: Vita-Salute San Raffaele University, Milano IT 6: Experimental Hematology Unit, San Raffaele Hospital and Scientific Institute 7: Ospedale Pediatrico Bambino Gesù, Rome IT 8: Pathogenesis and Therapy of Primary Immunodeficiencies Unit, TIGET-San Raffaele Hospital and Scientific Institute, Milano IT

Introduction: Graft-versus-host disease (GvHD) is a severe complication of allogeneic hematopoietic stem cell transplantation (HSCT). Due to their immunomodulatory properties, mesenchymal stromal cells (MSCs) hold great promises for successfully treating GvHD. Clinical results so far have however failed to demonstrate a definitive clinical benefit. Although recognized as a key mechanism of their immunomodulatory action, IL-10 production by MSCs is microenvironment-dependent and may become limiting at inflammatory sites.

Aim: To pre-clinically investigate whether forcing IL-10 expression by lentiviral-mediated transgenesis could enhance their potency in GvHD.

Results: In this study human bone marrow-derived MSCs were efficiently transduced with a lentiviral vector (LV) encoding for IL-10 ($47.81 \pm 6.7\%$ SD) resulting in the production of high (2.31 ± 0.59 ng/mL per 10E5 cells) and stable levels of the cytokine (up to 10 days). In co-culture experiments, IL-10-modified MSCs proved to be significantly more effective than unmodified MSCs at inhibiting the proliferation of polyclonally activated T cells ($P < 0.01$). Moreover, in a xenograft GvHD model based on the co-infusion of human T cells, IL-10-modified MSCs displayed superior therapeutic effects ($P < 0.001$). Although un-modified MSCs have been proven to be safe, concerns may arise when genetically modifying them. To clear any concern on their potential genotoxity, MSCs were transduced with a LV encoding for the inducible caspase-9 suicide gene, enabling their efficient ablation upon administering a clinical-grade chemical inducer of dimerization.

Conclusions: Our study suggests that IL-10-modified MSCs could be an innovative cellular product for advanced therapies of life-threatening immunological conditions like GvHD.

P087

Development of safer & optimized CAR-T cells using lentiviral vectors

S Agaugue[1] L Tibaldi[1] E Sarry[1] K Even-Desrumeaux[1] C Bauche[1]

1: THERAVECTYS

Chimeric antigen receptors (CAR) modified T cells have shown very promising clinical results in hematological malignancies. Despite their efficacy, CAR-T cells still show toxicity due to a cytokine storm, even if it is manageable. Improving the safety while maintaining or even improving the anti-tumor efficacy of CAR-T cells is therefore a crucial therapeutic challenge. Based on our proprietary lentiviral vector technology, we are developing a CAR-T cell platform aiming at producing innovative CAR-T cells. The development conducted by Theravectys are: - Development of proprietary lentiviral vectors coding for CAR of the second (containing the CD3ζ and the 4-1BB cosignaling domains) and third generations (containing the CD3ζ, the CD28 and the 4-1BB domains) directed against CD19 (for CD19+ leukemias and lymphomas), LMP-1 and -2 (for EBV-induced leukemias). Our lentiviral vectors will allow optimal expression of CAR therefore leading to a better efficacy of CAR-T cells. Hematological malignancies will be used as a benchmark. - Development of an inducible RUSH (Retention Using Selective Hook) system which is based on the streptavidin anchored to the membrane of the endoplasmic reticulum (ER) through a hook, and on the SBP (streptavidin binding protein) introduced into the CAR structure. The interaction between the streptavidin-hook and the SBP-CAR allows the CAR retention inside the ER. The addition of biotin displaces the equilibrium of binding of streptavidin towards biotin instead

of SBP, thus leading to the release of the CAR from the ER and its expression to the cytoplasmic membrane

P088

New oncolytic vaccine platform enhance anti-tumor immunity in humanized mice with human melanomas

C Capasso[1] M Hirvinen[1] M Garofalo[2] D Romaniuk[1] L Kuryk[1] T Sarvela[1] A Vitale[3] M Antopolsky[1] A Magarkar[1] T Viitala[1] T Suutari[1] A Bunker[1] M Yliperttula[1] A Urtti[1] V Cerullo[1]

1: University of Helsinki 2: Università di Napoli Federico II 3: Università di Napoli Parthenope

Oncolytic adenoviruses (OAds) are effective in different preclinical models, although they show modest efficacy in cancer patients for different reasons. One of these is that immune responses generated by OAds target mainly the virus and not the tumor. Therefore, we need to re-direct the immune response towards cancer. We developed a new cancer vaccine platform, exploiting the immunogenicity of oncolytic adenoviruses, used as adjuvants for MHC-I tumor epitopes. In contrast to genetic vaccines or conventional vectors, epitopes bind the viral capsid electrostatically, allowing for changin the target without genetic or chemical modifications. By using the OVA-derived MHC-I restricted epitope SIINFEKL, we showed that peptide-coated OAds (PeptiCRAds) display intact infectivity and oncolytic activity. Most importantly, the SIINFEKL-coated PeptiCRAd enhanced the antigen-specific CD8+T-cell response and increased the population of mature and epitope-specific dendritic cells, increasing significantly the anti-tumor efficacy. Then, by targeting multiple clinically relevant antigens (tyrosinase-related protein 2 and human gp100) at the same time, PeptiCRAd was able to reduce the growth of distant, untreated B16-F10 melanomas by promoting the expansion of antigen-specific T-cell populations. Finally, we studied humanized mice bearing human melanomas and a human immune system: a PeptiCRAd targeting the human melanoma-associated antigen A1 (MAGE-A1) and expressing granulocyte and macrophage colony-stimulating factor (GM-CSF) was able to increase the human MAGE-A1-specific CD8+T-cell population and eradicate established tumors. For the first time, we show that oncolytic adenoviruses act as adjuvants for exogenous MHC-I tumor epitopes and that oncolysis and immunogenicity cooperate synergistically to promote the regression of tumors

P089

CD40L and 4-1BBL immunostimulating gene therapy reduces myeloid-derived suppressor cells (MDSCs) while promoting T and NK cell activation

E Svensson[1] J Wenthe[1] I Milenova[1] R Moreno[2] R Alemany[2] A Loskog[1 3]

1: Uppsala University 2: Institut Catala d'Oncologia 3: Lokon Pharma AB

LOAd703 is an adenovirus-based gene therapy that introduces the expression of TMZ-CD40L and 4-1BBL into tumor cells. The immunostimulatory capacity of a murine LOAd703 (mLOAd703) was evaluated in experimental models of pancreatic cancer with focus on its effect on myeloid cell populations since these cells are crucial in the pathogenesis of pancreatic cancer. In vitro culture of murine Panc02 with splenocytes demonstrated that survival of MDSCs was promoted. However, TMZ-CD40L and 4-1BBL-expressing Panc02 cells reduced MDSCs in the co-cultures, especially the suppressive monocytic MDSCs. Panc02 tumor cells promoted CD206- macrophages independently of transduction while CD206+macrophages were reduced. Panc02 tumor cells reduced the presence of activated T and NK cells in splenocytes co-culture but if the Panc02 cells expressed TMZ-CD40L and 4-1BBL, activated T and NK cells could still be detected. The supernatants of the co-cultures with transduced tumor cells showed significantly increased levels of Th1 cytokines. Co-culture of transduced Panc02 with a murine DC cell line (D1) increased activation markers and cytokine production. The transduced tumor cells were then used as a cellular vaccine (3x) in syngeneic immunocompetent mice. The cells activated anti-tumor immunity and rejected the transduced cells. Vaccinated mice showed tumor control after challenge with wild type tumor. In conclusion, mLOAd703 gene therapy suppresses development of monocyte MDSCs. Instead, they promote T and NK cell activation as well as Th1 cytokine production. Further, transduced tumor cell vaccines promoted generation of anti-tumor immune responses resulting in a substantial control of tumor growth.

P090

Durable leukemia remissions without myeloablation in a long-term xenotolerant mouse model of CD44v6 CAR-T cell immunotherapy

M Norelli[1 2] M Casucci[2] B Camisa[2] L Falcone[2] A Saudemont[3] P Genovese[4] L Naldini[1 4] F Ciceri[5] C Bordignon[1] C Bonini[6] A Bondanza[1 2]

1: Vita-Salute San Raffaele University 2: Innovative Immunotherapies Unit, San Raffaele Hospital and Scientific Institute, Milano IT 3: Immunotherapy Group, Anthony Nolan Institute, London UK 4: Gene Transfer Technlogy Unit, San Raffaele Hospital and Scientific Institute, Milano IT 5: Hematology and Bone Marrow Transplantation Unit, San Raffaele Hospital and Scientific Institute, Milano IT 6: Experimental Hematology Unit, San Raffaele Hospital and Scientific Institute, Milano IT

Background: Despite the remarkable clinical results of chimeric antigen receptor (CAR) T cells in B-cell tumors, their long-term efficacy is limited by the emergence of CD19-loss tumor escape variants. Unfortunately, currently available xenograft mouse models are not suited for studying the antitumor activity of CAR-T cells beyond 3-4 weeks, because of the occurrence of xenograft-versus-host disease (X-GVHD).

Aim: To investigate the long-term antitumor activity of CAR-T cells targeted against the CD44v6 antigen in an innovative xenotolerant mouse model.

Results: NSG mice triple transgenic for human IL-3, GM-CSF and stem-cell factor (NSG-3GS) were sub-lethally irradiated and injected intra-liver with human HSCs soon after birth, enabling an accelerated lympho-hematopoietic reconstitution compared with NSG mice. Reconstituting human T cells were single CD4/CD8 positive, indicating successful thymic maturation. After ex vivo isolation, NSG-3GS T cells were transduced with a CD28-endocostimulated CD44v6 CAR following ex vivo activation with CD3/CD28-beads and IL-7/IL-15, and infused in secondary NSG recipients previously engrafted with the CD44v6+ALL-CM leukemia semi-cell line. CD44v6 CAR-

T cells persisted in vivo for at least 20 weeks and mediated durable tumor remissions ($P < 0.001$ vs controls) in the absence of X-GVHD. Importantly, when infused in human hematochimeric NSG mice, long-term persisting CD44v6 CAR-T cells did not cause significant myeloablation, indicating that effective tumor targeting could be achieved without damaging the hematopoietic stem cell pool.

Conclusions: These preclinical results demonstrate the potential of CD44v6 CAR-T cell immunotherapy to induce durable leukemia remissions in the absence of irreversible hematopoietic toxicities.

P091

Development of immunotherapy with chimeric antigen receptor targeting intracellular WT1 gene product presented on HLA-A*24:02 molecule

Y Akahori H Ikeda M Yoneyama[1] Y Orito Y Miyahara Y Amaishi[2] S Okamoto K Takesako J Mineno H Fujiwara M Yasukawa H Shiku[1]

1: Mie University Graduate School of medicine 2: Takara Bio Inc. 3: Ehime University Graduate School of Medicine

Adoptive cell therapy with lymphocytes transduced with Chimeric antigen receptor (CAR) is a promising strategy to treat cancer patients. However, the target of CAR is limited to the cellular surface molecules, making difficult to expand CAR therapy for broad range of cancer patients. Inspired by the physiological recognition of epitope peptide and MHC molecule (pMHC) by T cells, we generated a series of antibodies that recognize the pMHC with peptides derived from intracellular tumor antigens. We isolated an scFv antibody clone WT#213 that recognizes WT1 p235-243 peptide (CMTWNQMNL) complexed with HLA-A*24:02 by the screening of a human scFv phage display library. We constructed a retrovirus that encodes the CAR consists of WT#213 and intracellular signal transduction domains of CD3ζ and GITR (WT#213 CAR), confirmed the specific recognition of WT1-expressing cells by the CAR-T cells, and evaluated the effectiveness and safety of WT#213 CAR in vivo by the use of NOG mouse model. To predict the potential property of WT#213 CAR in cross-reacting to normal human tissues, we conducted an alanine scan analysis of WT1p235-243 peptide to define the amino acids that were critical in the recognition. After BLAST search, we synthesized the peptides with potential risk, and tested the recognition of these peptides by WT#213 CAR. None of these peptides showed the stimulation of WT#213 CAR. The results here suggest that the immunotherapy with WT#213 CAR will be effective for the treatment of the leukemia patients without the predicted risk at least in the condition we performed.

P092

Lentiviral vector gene therapy for Omenn Syndrome

V Capo[1] M C Castiello[1] E Draghici[1] V Maina[1 5] P L Poliani[2] E Fontana[2] P Carrera[3] N P van Til[4] G Wagemaker[4] M Bosticardo[1] A Villa[1 5]

1: HSR TIGET, San Raffaele Telethon Institute for Gene Therapy 2: Department of Pathology, University of Brescia, Brescia, Italy 3: Genomics for the diagnosis of human pathologies, San Raffaele Scientific Institute, Milan, Italy 4: Erasmus University Medical Center 5: Milan Unit, Istituto di Ricerca Genetica e Biomedica, CNR, Milan, Italy

Omenn syndrome (OS) is a rare severe combined immunodeficiency, caused by defects of the lymphoid-specific V(D)J recombination. Most patients carry hypomorphic mutations in recombination activating genes (RAG) 1 or 2, leading to immunodeficiency and autoimmunity. The standard treatment is the hematopoietic stem cell (HSC) transplantation, however HSC gene therapy (GT) may represent a valid alternative, especially for patients lacking matched donor. Our goal was to verify the efficacy of lentiviral vector (LV) mediated GT in the murine model of OS (Rag2R229Q/R229Q). OS Lin-cells, transduced with a LV encoding for human RAG2 under the control of the ubiquitous chromatin opening element (UCOE), were injected into previously irradiated OS recipients. Control mice were transplanted with WT (BMT-WT) or untransduced (BMT-OS) Lin-BM cells. Peripheral blood analysis showed the appearance of B cells and the increase of T lymphocytes, as early as 6 weeks post GT. Concomitantly, constant decrease of myeloid cells indicated partial redistribution of immune subsets. Serum IgE levels declined to levels comparable to BMT-WT controls. At sacrifice, B cell progenitors were detectable in the BM, although counts were lower than BMT-WT controls. Accordingly, marginal zone and follicular B cells arose in the spleen of GT mice. Moreover, partial restoration of thymic architecture sustained the development of thymocytes. In the spleen, we retrieved naïve, memory and effector T cells, which showed in vitro proliferative capacity and polyclonal TCR repertoire. Concluding, preliminary results demonstrated the feasibility of GT for OS, although further experiments to evaluate long-term efficacy and safety are required.

P093

TCR modification by highly specific TALEN and CRISPR

F Knipping[1] K Petri[1] E Ruggiero[1] M J Osborn[2] J Tolar[2] C von Kalle[1] M Schmidt[1] R Gabriel[1]

1: DKFZ / NCT Heidelberg 2: University of Minnesota

Adoptive transfer of T-cells with transgenic high avidity T-cell receptors (TCR) is a promising therapeutic approach, however it comprises certain challenges. Endogenous and transferred TCR chains compete for surface expression and may pair inappropriately, potentially leading to autoimmunity. This can be prevented by designer nucleases such as transcription activator-like effector nucleases (TALEN) and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) system. In order to disrupt endogenous TCR expression, we assembled TALEN and CRISPR guide RNA (gRNA) targeting the constant regions of the TCR α-chain (TRAC) and TCR β-chain (TRBC1/TRBC2) respectively. Here we show the specific induction of DNA double strand breaks (DSB) by TALEN and CRISPR-Cas nuclease, examined in K562 and T-cells using Cel1-assay and deep sequencing. Using TRAC-TALEN or TRBC-TALEN mRNA, surface CD3 expression was successfully eliminated in about 75% of T-cells. The nuclease specificity was examined by identifying integrase-deficient lentiviral vector (IDLV)-marked DSB using LAM-PCR and deep sequencing.

Although about 1000 IDLV integration sites where analyzed for each TALEN and gRNA, clustered integration sites were only detected at their respective target sites, indicating high specificity. Additionally, K562 cells were nucleofected with TALEN-expressing plasmids and a donor template containing a GFP expression cassette flanked by 800bp TRAC- or TRBC1-homologous sequences. Thereby we were able to verify targeted gene addition through homology directed repair (HDR) of TALEN-mediated DSB in 10% of treated cells. TALEN and CRISPR gRNA that show high efficiency and specificity to their target sequence will be used for generating T-cells with high avidity TCR.

P094

A novel tracking system of transgene using droplet-based single cell PCR in hematopoietic stem cell gene therapy

Y Igarashi[1] T Uchiyama[1] N Watanabe[1] Y Nakazawa[1] M Otsu[2] T Ariga[3] M Onodera[1]

1: National Center for Child Health and Development 2: University of Tokyo 3: Hokkaido University Graduate School of Medicine

Tracking of the transgene in each lineage downstream of hematopoietic stem cell is extremely important in evaluating the efficacy of gene therapy clinical trials for primary immunodeficiencies. In this study, we have established the single cell-based gene tracking system using droplet digital PCR (ddPCR). The encapsulation of a single cell into each emulsion and the following emulsion PCR with TaqMan probes, enable the accurate calculation of the ratio of gene-transduced cells in the target population as follows. (Number of vector positive droplets) / (Number of reference gene positive droplets) In preliminary experiments using K562 cell samples containing EGFP positive cells at various ratios, we could calculate correctly the ratios of target gene (EGFP) positive cells at the lowest level of 0.3%. We next analyzed 2 ADA-SCID patients who received gene therapy 10 years ago without preconditioning chemotherapy. Single cell ddPCR for detecting vector sequence, revealed the skewed engraftment of the gene-transduced cells. While almost all of T cells contained vector-derived ADA gene, B and NK cells showed 10-40% of vector integrations in peripheral blood. Remarkably, CD34+ cells contained only 2-6% of gene-transduced cells in bone marrow. Further analysis showed 3-4 copies/cell integration in vector-containing B and NK cells, while almost 1 in T cells. These results imply that lower copy vector integration could provide selective advantage to T cells, while other lineages required higher copy integration for their engraftment. We conclude that single cell ddPCR is a powerful tool for tracking the transgene in gene therapy clinical trials.

P095

GvHD kinetics after haploidentical TK-cells: in-vivo HSV-TK suicide machinery is effective in GvHD control and provides a long-term immune-suppressive treatment-free survival

M T Lupo-Stanghellini[1] C Bonini[1 3] G Oliveira[1 3] A Bondanza[3] L Vago[1] R Greco[1] J Peccatori[1] S Colombi[2] A Lambiase[2] F Ciceri[1 3] C Bordignon[2 3]

1: San Raffaele Scientific Institute 2: MolMed S.p.A 3: University Vita-Salute San Raffaele

Haploidentical SCT (haplo-SCT) is limited by high rate of transplant mortality and relapse due to delayed immune-reconstitution (IR) induced to prevent graft-versus-host-disease (GvHD). We investigated the HSV-TK suicide gene strategy for the selective elimination of gene-modified donor T cells during GvHD, and preservation of graft-versus-leukemia and graft-versus-infection effects. We report the outcome for acute (a-) and chronic (c-) GvHD in haplo-SCT after TK-cell infusions. We analysed 57 adult patients (pts) who underwent haplo-SCT according to TK-trial (Ciceri, Bonini et al, Lancet Oncol 2009; Phase III TK008, NCT0091462), in 2002–2014 at our Center. Donor TK cells were infused in 34/57 pts (median 2 infusions/pt), 25/34 achieved IR (median time from SCT 84 days; from last TK-cells infusion 27 days). Twelve of 25 immune-reconstituted pts developed aGvHD (grade I–IV; median time of onset 84 days post SCT; 19 days post last TK-infusion) and one cGVHD. Direct association of TK-cells and GvHD was confirmed by immunostaining. Eleven pts required GvHD treatment: 4 pts received ganciclovir iv (GCV 5 mg/Kg/12h/14 days), 7 pts valganciclovir per os (VGCV 900 mg/12h/14 days). GvHD was controlled in all cases in a median of 14 days concomitanly with a significant reduction in numbers of circulating TK-cells, and no changes in numbers and function of circulating CD3+ TK negative lymphocytes. No cases of quiescent or progressive cGvHD were observed after a median follow-up of 679 days. Our 12-years experience confirms that infusion of TK-cells accelerates IR and controls GvHD, providing a long-term immunosuppressive therapy-free and GvHD-free survival.

P096

In vivo gene transfer into MHCII+lymph node stromal cells leads to CD8+ T cell anergy

S Ciré[1] A Galy[1]

1: Généthon

Stromal cells in lymphoid organs play important roles in the development of immune responses. In mice, several types of MHC class II-positive (MHCII+) stromal cells exist and contribute to self-tolerance. To target these cells and to assess their specific immuno-modulatory properties, we used an MHC-II targeting lentiviral vector to express a neoantigen (GFP-HY) adding miR142.3p target sequences to prevent transgene expression in cells of hematopoietic origin. Intravenous administration of the vector targeted MHCII+ cells in peripheral lymphoid organs and the transgene was expressed in lymph node CD45- MHCII+ stromal cells which were also positive for CD86 and partly for CTLA4 or B7-H4. The transgene was not detected in lymph node- or in spleen- CD11c+MHCII+CD45+ dendritic cells. In the vector-injected mice, transgene-specific effector CD4 and CD8 T cell responses did not develop although IL-6, IL-2 and IL-10 were induced. When subsequently challenged by the antigen in the form of a peptide with adjuvant, mice responded but develop lower CD8 T cell cytokine responses and were unable to kill antigen-positive target cells in vivo suggesting CD8+ T cell anergy. In contrast, control mice injected with the vector lacking miR target sequences expressed the transgene in CD45+ CD11c+ dendritic cells, developed robust memory effector Th1 and cytotoxic responses. Thus, gene transfer into lymph node

stromal cells revealed that these cells can control the development of peripheral effector CD8 T cell responses post-natally. Gene modification strategies targeting such stromal cells may contribute to the induction of stable peripheral tolerance.

P097

Co-delivery of a Gag encoding plasmid enhances the Th1 immune response generated by DNA vaccine

L Lambricht[1 2] K Vanvarenberg[1 2] V Préat[1 2] G Vandermeulen[1 2]

1: Université catholique de Louvain 2: Université catholique de Louvain

DNA vaccines are promising for cancer immunotherapy, as they trigger a broad immune response. Nevertheless, they require additional strategies to induce effective immunity against poorly immunogenic tumor antigens. Human immunodeficiency virus Gag polyprotein are able to self-assemble, leading to virus like-particles formation. The aim of this study was to assess the potential of a plasmid encoding Gag (pGag) to enhance Th1 immune response. DNA vaccine encoding ovalbumin (pOVA) was delivered to mice by intramuscular electroporation with or without co-delivery of pGag. After prophylactic vaccination, total antigen-specific IgG were decreased when pOVA was combined to pGag. This lower humoral response was explained by a shift towards Th1 as demonstrated by the 3200 fold increase of the IgG2a/IgG1 ratio. Consistent with these results, protective immunity against challenge with B16F10-ova cells was significantly improved by pGag. Tumor growth was significantly delayed from 23 and 6 days following prophylactic and therapeutic immunization respectively. To confirm these results, DNA vaccines coding for Melan-A and gp100 were constructed. The increased protective efficacy due to pGag was also observed in the case of prophylactic immunization with GP100, where tumor growth was delayed by one week. Our results demonstrated that pGag enhances the Th1 immune response generated by DNA vaccines and appears therefore as a promising genetic adjuvant for cancer DNA vaccines.

P098

Can expression of allogeneic MHC class II in recipient liver induce regulatory transplantation tolerance?

M S Moawadh[1] M Paul[1] E C Cunningham[1] C Wang[1] S S Tay[3] S Cunningham[2] M Hu S I Alexander[1] G J Logan[2] I E Alexander[2] P J Bertolino[3] D G Bowen[1 3] N Dudek[4] A Purcell[4] G A Bishop[1] A F Sharland[1]

1: The University of Sydney 2: Children's Medical Research Institute 3: Centenary Institute of Cancer Medicine and Cell Biology 4: Monash University

Regulatory tolerance to allogeneic cardiac grafts after donor MHC II gene transfer to recipient bone marrow is reported. Allogeneic MHC II is strongly expressed in recipient liver following AAV-mediated gene transfer, accompanied by a dose-dependent increase in liver T regs. However, survival of allogeneic heart grafts in transduced recipients is unchanged. Hepatocytes are not professional APC, lacking significant expression of co-stimulatory molecules, and chaperones required for antigen processing and presentation. To determine whether augmenting expression of molecular chaperones and/or co-stimulatory molecules by hepatocytes would facilitate induction of allograft tolerance, we treated C57BL/6 mice with 1×10^{11}vgc AAV2/8 encoding Class II transactivator (CIITA) and 5×10^{11}vgc IAd or IAd alone. MHC II, co-stimulatory molecules, chaperones and inflammatory infiltrate were assessed. IAd-binding peptides eluted from livers expressing IAd alone or IAd/CIITA, were identified by mass spectrometry. DBA/2 hearts were transplanted at d7 or d30 post-inoculation. CIITA transduction upregulated expression of native IAb. Expression of H-2M α and β and of Invariant chain were increased 70 to 500-fold by CIITA, attaining levels comparable to those in spleen. Peptides eluted in the presence of CIITA conformed to the IAd-binding motif. Expression of co-stimulatory molecules on hepatocytes was not increased by CIITA. Survival of DBA/2 grafts was not altered by the addition of CIITA to IAd. In conclusion, expression of CIITA and a single mismatched MHC II in hepatocytes was not sufficient to confer tolerance to fully-allogeneic heart grafts. Ongoing experiments are evaluating the combination of two mismatched MHC II and/or CD86 with CIITA.

P099

Disruption of CD8-coreceptor binding abrogates tolerance induction via liver- directed expression of donor MHC class I

M Paul[1] E C Cunningham[1] D LJ Bunker[1] Z Wang[1] C Wang[1] S S Tay[2] C McGuffog[2] G J Logan[3] I E Alexander[3] P Bertolino[2] G A Bishop[1] D G Bowen[1 2] A F Sharland[1]

1: The University of Sydney 2: Centenary Institute of Cancer Medicine and Cell Biology 3: Children's Medical Research Institute

We previously used an AAV vector to express donor MHC (H-2Kb) in recipient livers and induce donor-specific tolerance in a mouse skin transplant model. Kb expression in B10.BR mice led to impaired production of IFN-γ in response to Kb, despite persistence of Kb-specific CD8 T cells, suggesting that tolerance was associated with functional silencing. To determine the role of direct recognition of class I by CD8-dependent T cells in tolerance induction, we generated rAAV-D227K-Kb where a point mutation abrogates CD8 binding. Expression of Kb, D227K-Kb and PD-L1 on hepatocytes was assessed by FACS and IHC. Kb-bearing 178.3 skin was grafted onto uninjected recipients and mice injected with rAAV-D227K-Kb or rAAV-Kb $\pm$ PD-L1 blockade. Uninjected B10.BR mice rejected 178.3 skin (MST = 16 d, n = 6), while grafts onto rAAV-Kb-injected mice survived long term (MST > 250, n = 5). rAAV-D227K-Kb only slightly prolonged graft survival (MST = 27, n = 6), suggesting that CD8 coreceptor engagement is needed for tolerance induction. Kb but not D227K- Kb expression in B10.BR livers or Kb expression in C57BL/6 livers increased hepatocyte surface PD-L1, suggesting that alloreactive CD8 T cell activation results in PD-L1 upregulation on liver parenchyma. Blockade of PD-L1 plus rAAV-Kb caused hepatitis, with elevated ALT levels (721 ± 73) compared to rAAV-Kb (33 ± 4) or anti-PD-L1 (35 ± 2) alone. In conclusion, disrupting CD8 coreceptor engagement abolishes tolerance induction via liver-directed expression of donor MHC class I, indicating that direct recognition by CD8-dependent T cells is essential for the process. PD-L1 upregulation might play a role in the functional silencing of CD8 T cells in this model.

P100

Analyzing therapeutic efficacy of melanoma-specific T cell receptor gene therapy in an HLA-A2-transgenic syngeneic cancer model

W Uckert[1 3] M Leisegang[2] T Kammertoens[2] T Blankenstein[1 2]

1: Max Delbrück Center for Molecular Medicine 2: Charité University Medicine 3: Humboldt University Berlin

Antigen recognition by T cells is determined by two interactions: (i) binding of antigenic peptide to major histocompatibility complex molecules (pMHC) and (ii) binding of the T cell receptor (TCR) to pMHC. When using TCR-engineered T cells for immunotherapy of cancer, the strength of each interaction contributes to therapeutic efficacy. In vitro analyses and xenograft models are often not reliable in predicting in vivo efficacy of TCR:pMHC combinations. We established an HLA-A2-transgenic syngeneic cancer model for testing the efficiency with which human tumor antigens can be recognized by a given transgenic TCR and analyzed rejection of established tumors. We compared two target peptides derived from human melanoma that differ in binding affinity to HLA-A2 (MART-1: low, tyrosinase: high). While MART-1-specific TCR gene therapy did not affect tumor progression, transfer of tyrosinase-specific TCR-engineered T cells induced tumor destruction. In regressing tumors we detected infiltration of tyrosinase-specific T cells, destruction of the tumor vasculature and high local concentrations of IFN-γ. We next compared two tyrosinase-specific TCRs that recognize the same target peptide with different affinity. Only the high affinity TCR achieved long-term tumor regression while the low affinity TCR selected for relapse. The difference in therapeutic outcome was related to improved expansion of T cells and increased IFN-γ concentrations in tumor and blood. Because differences in therapeutic efficacy of both TCRs were not detected in standard in vitro experiments, we conclude that human TCR:pMHC combinations capable to reject large established tumors can only be predicted experimentally by using appropriate in vivo models.

P101

Impact of T cell expansion conditions and starting material on CAR T cell product cell number and composition

T Kaartinen[1] A Luostarinen[1] H Belt[2] J Koponen[2] P Mäkinen[2] S Ylä-Herttuala[2] S Mustjoki[3] K Porkka[3] M Korhonen[1]

1: Finnish Red Cross Blood Service 2: A.I.Virtanen Institute 3: University of Helsinki

Generation of effective T cell based therapeutics requires a delicate balance between final cell dose and quality of cells. Since T cell proliferation is tightly coupled to cell activation it may lead to cell exhaustion yielding less potent CAR T cells. Lymphocytes were transduced lentivirally with either CD19/CD28/4-1BB/z CAR or GFP genes and expanded using CD3/CD28 Dynabeads and varying concentrations of IL-2. Expansion kinetics, transduction efficiency and T cell phenotype were analyzed from the starting material and at days 10 and 20 during expansion. Killing efficiency was also evaluated. T cell expansion with low IL-2 yielded both CD4 and CD8 with 50 % Tcm (CD45RO+CD27+) and Tmsc (CD45RO-CD27+CD95+), 30 % Tem (CD45RA-CD27-) and 20 % terminal effectors (CD45RA+CD27-). Higher IL-2 concentrations (100–300 IU/ml) and the length of the expansion favored CD4 over CD8 T cells and effectors over earlier memory cell types. Successful T cell expansion requires the cells to be seeded at low cell concentration and nourished with fresh medium prior to the initial proliferation peak at day 3. Our static cell culture protocol reliably resulted in a 70-fold T cell expansion. Expansion was as effective for both healthy volunteers and leukemia patients, however, transduction efficiency was lower for patients (38 vs 79%). In conclusion, we show that cell culture parameters impact critically on T cell phenotype and final cell number and will likely have a major influence on therapeutic effectiveness.

P102

Immunotherapy of experimental glioblastoma with 70-kDa heat shock protein magnetic nanoparticles

M Shevtsov[1] B Nikolaev[2] L Yakovleva[2] A Dobrodumov[3] Y Marchenko[2] B Margulis[1] I Guzhova[1]

1: Institute of Cytology of the Russian Academy of Sciences 2: Research Institute of Highly Pure Biopreparations 3: Institute of Macromolecular Compounds of the Russian Academy of Sciences

Application of the nanoparticles for delivery of vaccine antigens is appealing because their size allows efficient uptake by dendritic cells (DC) and their biological propeties can be tailored to the desired function. We modified the surface of the nanoparticles with Hsp70 (Hsp70-MNPs) known to bind tumor-derived peptides and deliver them to DC. Developed Hsp70-nanoconjugates effectively chaperoned the synthesized hydrophobic substrate – peptide BP3 as shown by microcalorimetry and 31P-NMR methods. Hsp70-MNPs with binded tumor peptides were efficiently phagocytized by DC that induced increased expression of costimulatory molecules CD45, CD80, CD86, and MHC class II. In the orthotopic C6 glioma model in rats on the 14th day following tumor inoculation the animals recieved series of injections of Hsp70-conjugates with tumor lysate. Administration of the nanoparticles resulted in the delay of tumor volume growth as was hown by high-field MRI (11T) and increased survival. Injection of the nanoparticles increased the activity of NK-cells (Granzyme B production) and CD8+ T-lymphocytes (IFNγ ELISPOT assay). Immunotherapeutic efficacy was associated with infiltration of the glioma by NK-cells (Ly-6c+), CD45RA+ cells and T-cells (CD3, CD4, CD8). This study provides evidence of the potential of Hsp70-MNPs for the development of new anti-cancer vaccines. The study was supported by a grant from the Russian Science Foundation (No 14-50-00068) and with financial support from the Federal Agency of Scientific Organizations.

P103

An in silico tool designed to improve the immunogenicity of MHC-I peptides for cancer immunotherapy using oncolytic adenoviruses.

V C Carrascon[1] C Capasso[1] A Magarkar[1] M Garofalo[1] M Hirvinen[1] L Kuryk[1] T Sarvela[1] A Bunker[1] A Urtti[1] V Cerullo[1]

1: University of Helsinki

Oncolytic viruses have been successfully used for immunotherapy as vectors for genetic vaccines. In this context, the choice of the antigen or the epitopes is not trivial. In silico screening of antigen determinants is a valid tool to select

candidates. Most databases efficiently predict several aspects of the antigen presentation process such as peptide-MHC affinity or their ability to stimulate T-cell receptors (TCR). However, the integration of different parameters would increase the reliability of such predictions. Therefore we developed an Epitope Discovery and Improvement in Silico (EDIS) platform that integrates results from MCH-I affinity and TCR binding prediction servers. Most importantly, we designed the EDIS platform to improve the initial sequence and find more immunogenic peptides that could eventually break the tolerance to the tumor antigens. To validate our tool we used the B16-OVA tumor related peptide (SIINFEKL). We studied the candidates by ex vivo T-cell activation assays and we compared their efficacy in a cancer vaccine approach using B16-OVA murine xenografts and oncolytic adenoviruses as adjuvants.

P104

Development of a validated QPCR Assay for ADA-SCID using human CD45+ cells in NSG mice to support ADA-SCID pre-clinical biodistribution studies

R M Lowe[1] A V Sauer[2] N Carriglio[2 3] H Staton[4] J Towler[4] J Klapwijk[1] J Richards[1] P Cristofori[1] A Aiuti[2 3]

1: GlaxoSmithKline, Platform Technology & Science, Ware, UK 2: San Raffaele Telethon Institute for Gene Therapy, HSR-TIGET, Milano, Italy 3: University degli Studi di Roma Tor Vergata, Rome, Italy 4: Covance Laboratories Limited, Otley Road, Harrogate, North Yorkshire, UK

In October 2010 GSK initiated a research and development alliance with the Telethon Institute of Gene Therapy and the San Raffaele Foundation. The alliance launched a GSK funded gene therapy development program using first generation retroviral vector for the treatment of Adenosine Deaminase Severe Combined Immunodeficiency (ADA-SCID).

Here we describe the experimental approach taken for the validation of an assay system to support biodistribution studies in NSG mice transplanted with human hematopoietic stem cells (HSCs), transduced with a retroviral GIADAI vector containing the human adenosine deaminase (hADA) gene sequence. The assay used Quantitative Polymerase Chain Reaction (QPCR) to detect DNA sequences specific to the 3000 bp region of the retroviral GIADAI vector containing the Human Adenosine Deaminase (hADA) gene sequence and also to detect the presence of sequences specific to Human GAPDH and Murine Beta Actin housekeeping genes. The assay was validated in accordance with the ICH harmonised tripartite guidelines. The following parameters were addressed in the validation; Linearity, Range, Precision, Quantification Limit, Limit of Detection with 95% confidence, Specificity, Accuracy and Robustness. We will then share data from the pre-clinical biodistribution study where this assay was used.

P105

Treatment of experimental autoimmune encephalomyelitis by replicative herpes simplex virus vectors

M Nygårdas[1] H Paavilainen[1] M Röyttä[1] B Sodeik[2] V Hukkanen[1]

1: University of Turku, Finland 2: Medizinische Hochschule Hannover (MHH)

Herpes simplex virus type 1 (HSV-1) can be exploited for the development of gene therapy vectors. HSV is a neurotropic virus with a non-integrating large DNA genome. The HSV neurovirulence gene γ134.5 can be replaced by therapeutic transgenes. Experimental autoimmune encephalomyelitis (EAE) is an inducible, T-cell mediated autoimmune disease of the central nervous system (CNS) and is known as the disease model for multiple sclerosis. We and others have earlier developed gene therapy for EAE using HSV vectors expressing Th2 type cytokines such as interleukin-4 and -5. We constructed an HSV-1(17+)-BAC-based replicative vector deleted of the neurovirulence gene and expressing leukemia inhibitory factor (LIF) as a transgene [HSV-1(17+)Lox-Luc-$\Delta\gamma$134.5-LIF, here: HSV-LIF]. LIF is a neuroprotective cytokine that has potential to limit demyelination and oligodendrocyte loss in the CNS. SJL/J mice, induced for EAE, were treated with the HSV-LIF vector intracranially and the subsequent changes in disease parameters and in the immune responses were studied. Replicating HSV-LIF and its DNA were detected in the CNS during the acute infection, and the vector did spread to the spinal cord but was non-virulent. The use of HSV-LIF significantly ameliorated the EAE and improved the number of oligodendrocytes in the brains when compared to untreated EAE mice. The HSV-LIF therapy also induced favorable changes in the expression of immunoregulatory cytokines and in T-cell population markers in the CNS. These data suggest that BAC-derived HSV vectors are suitable for treatment of CNS autoimmune disease and that LIF has therapeutic potential in EAE.

P106

CD40L/4-1BBL-expressing oncolytic adenovirus (LOAd703) stimulates human dendritic cell production of Th1 cytokines and antigen-specific T cells

E Svensson[1] I Milenova[1] R Moreno[2] R Alemany[2] A Loskog[1 3]

1: Uppsala University 2: Institut Catala d'Oncologia 3: Lokon Pharma AB

We hypothesized that an immunostimulatory oncolytic adenovirus serotype 5/35 (LOAd703) is a potent inducer of tumor immunity and tested its biological functions in models of pancreatic cancer. LOAd703 introduces the expression of a trimerized membrane-bound CD40L and 4-1BBL in the tumor aiming to induce potent Th1-mediated immunity. Virus replication is restricted to tumor cells by E1Adelta24 deletion while a separate promoter drives transgene expression. CD40L is a potent stimulator of myeloid cells that in turn induce robust T cell responses. 4-1BBL is known to provide expansion of preactivated T and NK cells. Tumor cell oncolysis by LOAd703 was as efficient as a similar oncolytic virus without transgenes demonstrating that the double transgene expression did not interfere with viral replication. Repeated peritumoral injections of LOAd703 in a xenograft Nu/Nu/Panc01 model showed growth control and complete responses. The effect could be further enhanced by gemcitabine in both xenograft mice and in a syngeneic C57BL/6/Panc02 model. Transduction of DCs did not result in lysis of the cells but they matured as shown by high CD83 and IL12 expression. The addition of 4-1BBL significantly enhanced DC maturation compared to a virus containing only TMZ-CD40L by expressing higher levels of CD70, IL12, TNFa, IFNg and IL21. Further, LOAd703-transduced DCs pulsed with pp65-CMV peptides potently expanded antigen-

specific T cells as well as NK cells. In conclusion, LOAd703 is a novel, double-armed immunostimulatory oncolytic gene therapy that initiates Th1 immunity, and eradicates pancreatic cancer in experimental models. A clinical trial using LOAd703 for pancreatic cancer is underway.

P107

Mesenchymal stem cells suppress the proliferation and differentiation of keratinocyte in both in vitro and in vivo models

S K Sah S W Lee T Y Kim

1: The Catholic University of Korea 2: Dankook University

Skin homeostasis and defenses are maintained by cross-talks among keratinocytes and various immune cells either residing or recruited in the skin, through production of various cytokines, chemokines and growth factors. The influence of mesenchymal stem cells (MSCs) on keratinocyte function in altered microenvironments is poorly understood. Here, we co-cultured umbilical cord blood-derived MSCs to evaluate the paracrine effect on normal human epidermal keratinocytes in the presence of cytokines (IL-22, TNF-α, and IL-17) or high extracellular calcium level. We found that MSCs are able to suppress the production of proinflammatory cytokines in keratinocytes and influence their proliferation and differentiation fate in a high calcium environment. Furthermore, we determined that MSCs regulate the RhoA/ROCK-II and PI3K/AKT pathways, and inhibit TLR-7 and downstream NFκB activation in differentiated keratinocytes. In addition, MSCs inhibited epidermal hyperproliferation and abnormal differentiation in the mouse model of imiquimod-induced psoriasis-like skin inflammation. Our findings reveal basic regulatory network between stem cells and keratinocytes, which could be promising for the development of therapeutic approaches to treat or prevent diseases caused by keratinocyte dysfunction.

P108

Transplantation of human SCID stem cells in NSG mice gives new insights into human T-cell development

K Pike-Overzet A S Wiekmeijer M H Brugman
I L Wolvers-Tettero A C Lankester R G Bredius
J J van Dongen[2] W E FibbeA W Langerak[2] M van der Burg
F J Staal

1: Leiden University Medical Center 2: Erasmus University Medical Center

Severe combined immunodeficiency (SCID) is characterized by a deficiency of T-cells. Until recently, it was not possible to study the effect of the genetic defects on the T-cell development, because thymus biopsies are difficult to obtain from SCID patients. Additionally, the clonal contribution of stem cells to the T cell lineage is currently unknown. We made use of the NSG xenograft model and lentiviral cellular barcoding to study hematopoiesis and T cell development at a clonal level. Barcoded HSCs showed and reproducible myeloid and lymphoid engraftment, with T cells arising 12 weeks after transplantation. A very limited number of HSC clones (<10) repopulated the thymus, with clear restriction of the number of clones at the double negative stages. Yet, T-cell receptor rearrangements were polyclonal and showed a diverse repertoire, demonstrating that a multitude of T-lymphocyte clones can develop from a single HSC clone. Next, we characterized the blocks in T-cell development for different types of SCID, (ADA, Artemis, IL7RA and IL2RG).We observed blocks in T-cell development for IL7RA- and IL2RG-SCID at the CD4-CD8- DN stage. TCRB rearrangements are initiated much earlier than previously thought, as determined from the developmental block and the extent of rearrangements observed in mice transplanted with Artemis-SCID HSC. These data provide previously unattainable insight into human T-cell development using SCID as human loss-of-function models.

P109

Lentiviral vector platform To develop anti-cancer and anti-infectious diseases immunotherapies

D Revaud[1] S Agaugue[1] M Jamiluddin[1] E Sarry[1] A Bejanariu[1]
C Bauche[1]

1: Theravectys

THERAVECTYS' vaccine candidates based on a proprietary lentiviral vector technology enable a direct vaccination mechanism in patients by inducing a broad, intense and long-lasting T cell mediated response. Our lentivectors encompasses up to 15kb DNA, the resulting polypeptide targeting a combination of different antigens which mobilizes different actors of the anti-tumor immune system. Lentivectors not only provide sufficient amounts of antigens to DCs but also ensure an efficient processing and prolonged presentation of the antigens, an efficient maturation of DCs that possess all the stimulating abilities to induce an efficient activation of T cells Non replicative and non-persistent lentivectors-antigen expressing cells and transduced cells are eliminated by the immune response elicited after injection. Different pseudotypes of lentivectors are developed to avoid specific immune reaction against the vector during boost injection(s). The phase I/II clinical trial with our first vaccine candidate has been completed in HIV-infected patients. The vaccine is safe and highly immunogenic as a multi-specific and poly-functional CD4 + and CD8 + cellular immune responses is induced in vaccinated patients. These results support the potential of the lentiviral vector platform developed by THERAVECTYS for the future development of therapeutic vaccines and immunotherapies in oncology and infectious diseases. As a second indication, THERAVECTYS is developing an anti-HTLV-1 vaccine candidate to treat patients who have developed a virally-induced adult T-cell-leukemia. This vaccine candidate should enter into the clinics in mid-2015. Other indications in the pipeline are TB, HBV, urogenital cancers, triple-negative breast cancer, EBV-induced nasopharyngeal cancers, HPV-induced cancers and multiple myeloma.

P110

Disease-regulated expression of anti-inflammatory Interleukin-10 for the treatment of Rheumatoid Arthritis.

M GA Broeren M B Bennink[1] O J Arntz E A Vermeij
W B Van den Berg F AJ Van de Loo

1: Radboud University Medical Center

Introduction: We have previously shown the feasibility of local disease-inducible gene therapy in a mouse model of arthritis

(Vermeij, Broeren et al, ARD, 2014). Our goal is to translate this approach to the human rheumatoid arthritis (RA) patient.

Results: Microarray analysis on joint tissue of 20 RA patients and 7 patients without joint disease showed a 10-fold upregulation of CXCL10, an interferon inducible gene known to be expressed by many cell types. The human CXCL10 promoter was cloned into a SIN-lentiviral luciferase reporter vector. Transduced primary cells obtained from patient synovium were stimulated with pro-inflammatory lipopolysaccharide (LPS) or with TNFα, a disease driving cytokine in RA. This resulted in a 3.3- and 2.3-fold upregulation of the luciferase signal respectively. The CXCL10 promoter also responded to serum from RA patients, and could significantly distinguish between RA serum and healthy donor serum ($P = 0.017$). For therapeutic testing, the luciferase reporter gene was replaced by the anti-inflammatory Interleukin-10 gene. RA synovial cells from multiple patients transduced with the CXCL10-IL10 lentivirus produced significantly less pro-inflammatory cytokines (TNFα, IL-1β, IL-8 and MCP-1) after LPS stimulation compared to control virus. These results show the functionality of the CXCL10-IL10 lentivirus in the desired synovial target cells.

Conclusion: The selected proximal promoter from the CXCL10 gene responds to inflammatory mediators (e.g. TNF-alpha) present in serum of RA patients and the lentiviral CXCL10 promoter-dependent IL10 expression vector reduces inflammatory cytokine production by RA synovial cells. CXCL10-regulated IL10 overexpression can thus provide inflammation-inducible local gene therapy suitable for RA.

P111

Pre-clinical development of lentiviral gene therapy for SCID-X1

S Charrier[1] V Poletti[1] S Martin[1] A Vignaud M Hebben F Zhang[2] K Buckland[2] M Rothe[3] A Schambach[3] B Gaspar[2] A Thrasher[2] F Mavilio[1]

1: GENETHON 2: University College London 3: Hannover Medical School

X-linked severe combined immunodeficiency (SCID-X1) is due to mutations in the gene encoding the interleukin-2 receptor γ chain (IL2RG), and is characterized by a profound immunological defects caused by a partial or complete absence of T and NK cells and the presence of non-functional B cells. To overcome the side effects caused by MLV-based retroviral vectors in previous gene therapy clinical trials, we designed a SIN lentiviral vector (LV) carrying a codon-optimized human IL2RG cDNA under the control of the human EF1α-S promoter and a mutated WPRE. The vector (EF-IL2RG) was packaged in 293T cells transfected and grown in suspension in serum-free conditions in a bioreactor, purified by ion-exchange chromatography and concentrated by tangential-flow filtration. The vector restored normal level of IL2RG in the IL2RG-deficient ED7R human T-cell line at an average VCN of 1-3, and transduced at high efficiency human CD34+ hematopoietic stem/progenitor cells with no impact on viability or clonogenic capacity. Potential genotoxicity was evaluated in vitro by an IVIM assay and analysis of abnormal splicing of genes targeted by proviral integration, and in vivo by transplantation of transduced lin-stem/progenitor cells in IL2RG-deficent mice. Engraftment of transduced cells correlated with immune T and B cell restoration with evidence of lymphoid reconstitution in spleen and thymus. Integration site analysis is ongoing to analyze the clonal repertoire of transduced BM and peripheral blood cells. These studies will enable a multicenter phase-I/II clinical trial aimed at establishing the safety and clinical efficacy of lentiviral vector-mediated gene therapy for SCID-X1.

P112

Adenoviruses armed with TNFa and IL-2 induce systemic effect on tumor growth

R Havunen[1] M Siurala[1 2] S Parviainen[1 2] M Behr[1] D Nettelbeck[3] A Ehrhardt[4] A Hemminki[1 2]

1: University of Helsinki 2: TILT Biotherapeutics Ltd. 3: DKFZ 4: University of Witten/Herdecke

We aim to boost adoptive T cell therapy and viral infection-mediated immune responses against tumor with adenoviruses carrying immune system-stimulating factors. Studies show that the most promising cytokines to enhance T cell therapy are interleukin (IL) -2 and Tumor Necrosis Factor alpha (TNFa). Nevertheless, systemic administration of these cytokines may lead to severe side effects. High local and low systemic levels can, however, be achieved when introducing cytokines from armed viruses. We now wanted to study if our murine cytokine-armed non-replicative viruses (Ad5-CMV-mTNFa/mIL2) could induce systemic effect on tumor growth, the abscopal effect. Two B16-OVA melanoma tumors were implanted to flanks of C57BL/6 mice, which were then treated with systemic adoptive T cell transfer and virus injections into one of the two tumors. Tumor size, activation of immune cells and the viral transduction of non-injected tumors were studied. To take a step forward towards human application, we also developed replicative oncolytic adenoviruses expressing human IL-2 and TNFa (Ad5/3-E2F-D24-hTNFa-IRES-hIL2). These viruses were functional in various cancer cell lines and they produced active cytokines in vitro. With murine viruses we saw the abscopal effect: also the non-injected tumors reduced in size when viruses carried TNFa and IL2. The cytokine-armed viruses also recruited natural killer cells to both tumors and reduced the appearance of immunosuppressive M2 macrophages, although the virus itself could not be detected from the non-injected tumors. Our results give important implications for human trials, as systemic improvement in efficacy of immunotherapy could be achieved with injection of only one tumor.

P113

scAAV9 does not activate immune responses in Kupffer cells, though their phenotype and function depends on heme oxygenase-1

M Tomczyk[1] I Kraszewska[1] A Jozkowicz[1] J Dulak[1] A Jazwa[1]

1: Jagiellonian University

Upon viral infection, including some gene therapy viral vectors, Kupffer cells (KCs) in the liver were shown to exert immunosuppressive functions. Heme oxygenase-1 (HO-1) is

a crucial enzyme in heme catabolism that confers the proper function of liver macrophages scavenging free hemoglobin from dying erythrocytes. Moreover, this enzyme exerts many cytoprotective effects including anti-inflammatory actions. In the present study, we wanted to characterize KCs following self-complementary adeno-associated viral vector serotype 9 (scAAV9) transduction in vitro and in vivo depending on the presence or absence of HO-1. First of all, regardless of the treatment, we observed lower number of F4/80(hi)CD11b(lo) KCs in the liver of HO-1 knockout (KO) mice when compared to their wild-type (WT) littermates. Conversely, another population of cells defined as F4/80(hi)CD11b(hi) was significantly higher in HO-1 KO than in WT. More GFP-positive cells (following scAAV9-GFP systemic injection) were detected both in parenchymal and non-parenchymal fraction of WT than HO-1 KO mice. Apparently, when WT KCs were isolated and exposed in culture to scAAV9-GFP, no GFP-positive cells could be detected despite the fact that scAAV9 was proven to be incorporated by these cells. Moreover, there was no release of inflammatory cytokines (IL-1β, TNF-α) by KCs, neither by untreated cells nor following scAAV9 infection. The expression of IFN-α/β was also unchanged. scAAV9 transduces both parenchymal and non-parenchymal cells in the liver. KCs themselves do not aggravate inflammation upon scAAV9 transduction. Absence of HO-1 may change the distribution of different populations of liver macrophages shifting the phenotype from immunosuppressive to immunostimulatory.

P114

Regulation of anti-transgene cellular immune responses following systemic gene transfer by Anterior Chamber Associated Immune Deviation (ACAID).

Q Khebizi[1 2] S Donnou-Triffault[3] J Vendomèle[1 2] M Mormin[1] C Poinsignon[1 2] S Ciré A Galy[1] S Fisson[1 2]

1: Inserm/Généthon 2: Université d'evry 3: Genosafe

Anterior Chamber-Associated Immune Deviation (ACAID), is an antigen specific peripheral immunomodulatory mechanism described when an antigen is introduced into the anterior segment of a mammalian eye. It is not known if the ACAID mechanism could impact on cellular immune responses induced by gene therapy. Our study was conducted in a murine model to evaluate if ACAID induction would trigger immune tolerance to the product of a transgene injected by systemic route (i.v.) and if this could impact on the destruction of the transduced cells. The model constits in ACAID induction by injecting intracameraly soluble peptides (DBY and UTY) of the HY male antigen into wild type female C57Bl/6 mice (H-2b background), followed by an intravenous injection of a recombinant AAV2/8 vector (rAAV2/8-GFP-HY) containing a transgene coding for a fusion GFP-HY molecule. Our results show that an ocular injection of HY peptides 7 days before i.v. infusion of vector is able to reduce the number of IFNγ producing spleen cells up to 80%. In addition, cytokine secretion assays showed an impairment of the Th1 profile in ACAID condition with a reduction in the secretion of IFNγ and TNFα (8 and 2 times lower, respectively). Moreover, a model of in vivo cytotoxic assay suggests that almost all HY-expressing cells can survive in ACAID-induced mice in contrast to the control mice. In conclusion, ACAID seems to be a powerful inhibitory mechanism in the context of systemic gene transfer, limiting cellular immune responses against the transgene and promoting transgene expressing cells survival.

P115

TARGT™ exhibits sustained and high level secretion of the therapeutic antibody adalimumab which retains functional activity in SCID mice

I Zafir-Lavie[1] R Miari[1] N Shapir[1] T Shatil[1] G A Neil[2]

1: Medgenics Medical Israel Ltd., Misgav, 20179, Israel
2: Medgenics, Inc., Wayne, PA, 19087, USA

Monoclonal antibodies (mAbs) are a mainstay of contemporary therapeutics with numerous applications. Despite the availability of high affinity human mAbs some issues remain including development of neutralizing antibodies and high production cost. To address these issues, we have developed an innovative *ex-vivo* gene therapy approach, Transduced Autologous Restorative Gene Therapy (TARGT™), to produce and secrete adalimumab (Humira™), a widely used humanized anti-TNF-α mAb shown to benefit patients with autoimmune diseases including RA, IBD and psoriasis. As previously reported by our group, the TARGT system is capable of producing autologous, continuous protein/peptides at physiological levels, including human erythropoietin, interferon-α, and GLP-II. The system allows dose flexibility and TARGTs may be added or removed according to *in-vivo* secretion levels. Pre-clinical studies using this novel technology demonstrate *in-vitro* secretion of active adalimumab antibody at levels of tens of μg per day. Preliminary *in-vivo* experiments exhibited sustained TARGT secretion of adalimumab in SCID mice. Importantly, the secreted adalimumab retains its activity in mice sera as proven by its ability to bind huTNF-α. The results presented herein provide proof-of-concept that the TARGT platform is capable secreting mAb at therapeutic levels. Moreover, as previously reported, the platform has also promise for the treatment of other endogenous protein/peptide deficiencies.

P116

Lentiviral SIN-vector for p47phox-deficient Chronic Granulomatous Disease Gene Therapy

R Valencia D Wrona C Brendel W Hänseler G Santilli A Thrasher M Grez J Reichenbach U Siler

1: University Childrens Hospital Zürich 2: Georg-Speyer-Haus 3: University College London

Chronic granulomatous diseases (CGD) are defects of phagocyte NADPH oxidase subunits leading to recurrent severe infections with bacteria and fungi as well as hyperinflammation. We developed a lentiviral SIN gene therapy (GT) vector for the treatment of the NADPH oxidase p47phox-subunit deficient autosomal-recessive form of CGD. We compared the highly myelospecific miR223 promoter to a chimeric cathepsinG/c-fes promoter, used in a current clinical phase I/II GT trial for X-linked gp91phox-subunit deficient CGD. In an ex vivo model based on CGD patient derived iPSCs, both promoters equally reconstituted NADPH oxidase function upon transduced iPSC

differentiation to monocytes and macrophages. Transgene expression was significantly upregulated upon phagocytic differentiation. However, the two promoters differed slightly with regard to background expression in undifferentiated transduced iPSCs and HSCs. In iPSCs and in HSCs derived thereof the vector encoded miR223 promoter was completely and the chimeric promoter was only partially methylated. Upon differentiation of iPSCs to monocytes the miR223 promoter was actively demethylated and activated resulting in reconstitution of NADPH oxdidase function. Ongoing animal experiments will reveal the functional consequences of promoter methylation.

P117

T cell gene therapy for X-linked lymphoproliferative disease (XLP) using a novel transduction protocol

N Panchal[1] B Houghton[1] H B Gaspar[1 2] C Booth[1 2]

1: Institute of Child Health 2: Great Ormond Street Hospital

X-linked lymphoproliferative disease is a fatal primary immune deficiency arising from mutations in the SH2D1A gene encoding SAP, a key regulator of immune function that is expressed in T cell lineages. In absence of SAP, T follicular helper (TFH) CD4 + T cells are unable to form immune synapses with B cells resulting in impaired B cell maturation and IgG class switching leading to humoral defects as seen in XLP patients. Our work so far has shown restoration of humoral immunity in a murine model using infusion of SAP wild type T cells. Based on this we have developed a human T cell transduction protocol focusing on restoring SAP function in TFH cells. We have shown that using a novel cytokine cocktail of IL-6, IL-7 and IL-21, we can differentiate CD4 + cells from PBMCs and maintain a TFH cell profile which can then be transduced using a 3rd generation lentiviral vector containing eGFP driven by a constitutive EFS promoter. So far we have observed comparable transduction efficiencies with the standard anti-CD3/CD28 + IL-2 condition along with preferential GFP expression in the CD4 + TFH compartment. Gene expression analysis also demonstrated a significant fold increase in transcription factors that regulate TFH differentiation using the cytokine cocktail compared to the control. Therefore, we have shown by using a combination of IL-6 and IL-21, both known positive regulators of STAT3-mediated TFH differentiation, that we are able maintain this population in vitro and achieve effective transduction.

P118

Prevalence of anti-AAV antibodies in healthy humans and in subjects undergoing immunosuppression

C Leborgne[1] E Masat[2] F Jouen[3] O Boyer[3] F Mingozzi[1 2]

1: Genethon, Evry 91000, France 2: INSERM U974, University Pierre et Marie Curie, Paris 6, France 3: INSERM, U905, Rouen University Hospital, Rouen 76000, France

Adeno-associated virus (AAV) vectors are among the most efficient tools for in vivo gene transfer. However, due to the exposure to the wild-type virus, from which AAV vectors are derived, humans develop antibodies directed against the capsid protein. Pre-existing immunity to AAV in humans is highly prevalent and constitutes a major limitation to in vivo gene transfer with AAV vectors, as even low neutralizing antibody (NAb) titers can completely block transduction after systemic vector infusion. Additionally, following vector administration anti-AAV NAbs develop at high titers and persist for several years, thus preventing vector readministration. Here we explored the effect of immunosuppression on anti-AAV NAb titers in a large cohort of humans receiving various immunosuppressive (IS) regiments to manage various autoimmune diseases. Serum samples from 100 subjects receiving IS and 100 healthy donors were analysed for binding and neutralizing antibodies directed against AAV-2 and AAV-8. Additionally, as comparison, a cohort of children aged 1 year was analyzed. Our data indicate that IS results in a decrease of anti-AAV titers; this drop is more evident for some drug combinations. Next we developed a novel sensitive assay to detect anti-capsid binding antibodies and we compared amounts of adults vs. children, highlighting key differences in the two populations in terms of prevalence of binding antibodies, and reflecting timing of exposure to the wild-type virus.

P119

Lentivirus-mediated intra-articular expression of microRNA-140-3p and -5p ameliorates experimental autoimmune arthritis

A L Shiau[1] J S Peng[2] S Y Chen[3] C R Wang[3] C L Wu[4]

1: Department of Microbiology and Immunology, National Cheng Kung University Medical College, Tainan, Taiwan 2: Institute of Basic Medical Sciences, National Cheng Kung University Medical College, Tainan, Taiwan 3: Department of Internal Medicine, National Cheng Kung University Medical College, Tainan, Taiwan 4: Department of Biochemistry and Molecular Biology, National Cheng Kung University Medical College, Tainan, Taiwan

Synovial fibroblasts (SF) with aberrant expression of microRNAs (miRNA) are critical pathogenic regulators in rheumatoid arthritis (RA). Previous studies have shown that miR140 is involved in the pathogenesis of osteoarthritis (OA). In this study, we studied whether dysregulation of miR-140 contributes to the pathogenesis of RA. We found that expression of miR-140-3p and −5p was significantly lower in the synovium of RA patients and in the ankle joints of mice with collagen-induced arthritis (CIA) compared with OA patients and normal mice. The expression levels of sirtuin 1 (SIRT1) and stromal cell-derived factor-1 (SDF-1), known targets of miR-140-3p and miR-140-5p, respectively, were upregulated in mice after the onset of arthritis, which accorded with the kinetic expression pattern of miR-140-3p and −5p, respectively. Lentivirus-mediated intra-articular delivery of pre-miR-140 precursor molecules significantly ameliorated arthritis symptoms by reducing arthritis, histological, and radiological scores, as well as alleviating bone erosion. Moreover, expression of cadherin-11, a specific marker for SF, was lower in the joints injected with lentiviral vectors expressing pre-miR-140 (LVmiR-140) than in those injected with control vectors (LVmiR-scramble), suggesting that intra-articular delivery of miR-140 can target SF. Downregulation of SIRT1 and SDF-1 was also detected in the synovial tissue of LVmiR-140-treated mice. In conclusion, our results demonstrate that targeting SF by intra-articular delivery of miR-140-3p and −5p can ameliorate autoimmune arthritis. These findings support a new role for miR-140 in the pathogenesis of RA and open the possibility of developing miRNA-based therapeutics for patients with RA.

P121

Partial rescue of T-cell deficiency by targeted gene editing in HSPCs followed by transplantation in an animal model of severe combined immune deficiency

H H Abdul-Razak[1 7] C J Rocca[1 7] S J Howe[2] M E Alonso-Ferrero[2] J Wang[3] R Gabriel[4] C C Bartholomae[4] C H V Gan[2] M I Garín[5] A Roberts[6] M Blundell[2] V Prakash[1] F J Molina Estévez[1 5] J Pantoglou[1] G Güenechea[5] M C Holmes[3] P D Gregory[3] C Kinnon[2] C von Kalle[4] M Schmidt[4] J A Bueren[5] A J Thrasher[2] R J Yáñez-Muñoz[1 4]

1: School of Biological Sciences, Royal Holloway, University of London, Egham, TW20 0EX, UK 2: Institute of Child Health, University College London, London, UK 3: Sangamo BioSciences, Inc., Richmond, California, USA 4: Department of Translational Oncology, National Center for Tumor Diseases and German Cancer Research Center, Heidelberg, Germany 5: Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas, Madrid, Spain 6: Department of Medical and Molecular Genetics, King's College London, London, UK 7: These authors contributed equally to this work

Gene editing is the introduction of specific modifications in the cellular genome. It can repair defective genes, inactivate target genes and direct transgenes to safe harbours. The targeted correction of mutations relies on homologous recombination (HR). The use of designer nucleases, which induce a double-strand break at the target locus and stimulate HR, has boosted the efficiency of gene editing to therapeutic levels. Ex vivo gene editing of haematopoietic stem/progenitor cells (HSPC) for correction of inherited blood diseases is an attractive proposition. Here we show the rescue of a mouse model of primary immunodeficiency, Prkdc severe combined immunodeficiency (scid), by an autologous transplant of HSPCs edited ex vivo using designed zinc finger nucleases. Gene-edited HSPC carried the expected genomic signature of ZFN-driven gene correction, and drove the restoration of double-positive CD4/CD8 T-cells in thymus and single-positive T-cells in blood after primary and secondary transplants. The molecular signature of gene correction was detected in the blood, thymus, spleen and T-cells of transplanted animals. Purified spleen CD3 T-cells showed proliferative responses after stimulation, demonstrating functional recovery. Our results provide proof-of-principle for the rescue of scid disease by ex vivo gene editing using ZFNs. This supports the feasibility of gene editing-based therapies to correct inherited diseases of the immune system.

P122

Adeno-associated viral mediated gene therapy for Mucopolysaccharidosis IIIC

C O'Leary[1] A S L Antunes[2] H Parker[1] E Henckaerts[2] F Wilkinson[1] A Pshezhetsky[3] B Bigger[1]

1: University of Manchester 2: King's College London 3: University of Montreal

MPS IIIC is caused by mutations in the gene encoding for the enzyme, heparan sulfate acetyl-CoA: α-glucosaminide N-acetyltransferase (HGSNAT). HGSNAT deficiency affects lysosomal catabolism of heparan sulfate (HS), resulting in widespread CNS pathology in children, leading to behavioural problems, cognitive decline, dementia and death before adulthood. Currently no treatments for MPS IIIC exist as the deficient enzyme cannot cross the blood brain barrier. The adeno-associated viral (AAV) vector system has been used in a number of studies for neurological correction, therefore, intracranial injection of AAV has the potential to restore brain enzyme levels, reduce HS accumulation and correct neurological input. We have developed an AAV based gene therapy for MPSIIIC and demonstrated efficacy in the mouse model. Intracerebral injection of AAV-HGSNAT, using 2 serotypes, Rh10 and AAV9, achieved high levels of enzyme activity throughout the brain exceeding WT levels. The highest levels of HGSNAT enzyme activity were localised around the injection site; sections R2 and R3. One week post-injection, AAV9 vector related HGSNAT activity was significantly higher than WT in sections R2 and R3, 355.9% and 365.8% respectively. In addition, Rh10 HGSNAT activity was significantly higher than WT levels in sections R2 and R3; 201.3% and 215.9%. Three weeks post-injection, Rh10 activity was 203.6% and AAV9 increased to 620.8% (R2). Additionally, HS levels were reduced by 50% in AAV9 treated mice. AAV-HGSNAT is effective in producing enzyme activity levels exceeding normal levels in the brain, providing evidence that this is a potential treatment for the CNS involvement in MPSIIIC.

P123

Beta-cell targeted gene delivery for enhanced beta-cell proliferation

B Lu J M Tonne S Leber K Kurmi M Munoz-Gomez E J Jacobus Y C Kudva Y Ikeda

1: Mayo Clinic

Aging and obesity are linked to an increased risk of type 2 diabetes (T2D), a chronic metabolic disorder resulting from relative insulin insufficiency. Increased beta-cell proliferation and function would benefit patients with T2D. Glucokinase (GCK) plays a role as a beta-cell glucose sensor, and mediates glucose-responsive insulin secretion and beta-cell proliferation. Previously, we have demonstrated that systemic delivery of AAV8 vectors with a mouse insulin 2 internal promoter resulted in beta-cell-specific transgene expression. Here, we employed this vector system for induced beta-cell proliferation in vivo. When SV40 large T antigen was over-expressed, a 9-fold increase in beta-cell proliferation was observed in aged mice (control, 1.7%; SV40T, 16.0%). We then assessed the induced beta-cell proliferation upon GCK over-expression in beta-cells. When young (6 weeks old) and aged (8 months old) C57BL/6J mice maintained on normal diet or high fat diet (HFD) were given an intraperitoneal injection of GCK-expressing AAV8 vectors, beta-cell proliferation was increased in all treated groups compared to control (young mice, 2-fold; aged mice, 6-fold; aged HFD mice, 3-fold), and mice maintained on HFD showed improvements in glucose tolerance tests ($p < 0.02$ by 60 minutes). In contrast, over-expression of a recently identified beta-cell trophic factor, betatrophin, resulted in a marginal increase in beta-cell proliferation. Our data support the use of beta-cell-targeted gene delivery system for enhanced beta-cell proliferation and function.

P124

Efficacy of intrathecal AAV9 delivery in canine MPS I and impact of neonatal tolerance induction to the transgene product

C Hinderer[1] P Bell[1] J-P Louboutin[2] Y Zhu[1] H Yu[1] G Lin[1] R Choa[1] B L Gurda[1] J Bagel[1] P O'Donnell[1] T Sikora[1] T Ruane[1] P Wang[1] A F Tarantal[3] M L Casal[1] M E Haskins[1] J M Wilson[1]

1: University of Pennsylvania 2: University of West Indies 3: University of California Davis

Mucopolysaccharidosis type I (MPS I) is a lysosomal storage disease resulting from genetic deficiency of α-l-iduronidase (IDUA), an enzyme essential for catabolism of ubiquitous polysaccharides. Lysosomal accumulation of these IDUA substrates causes widespread organ pathology. Central nervous system involvement is common in MPS I, and patients with the most severe form of the disease exhibit profound neurocognitive decline in early childhood. Hematopoietic stem cell transplantation can improve cognitive outcomes in children with MPS I due to migration of donor-derived cells across the blood brain barrier, where they serve as a depot of secreted IDUA in the CNS. However, transplant is associated with considerable morbidity and mortality, and the slow rate of donor cell engraftment in the CNS results in continued disease progression after transplant, leaving most patients with significant cognitive deficits. Using a naturally occurring dog model of MPS I, we found that delivery of an AAV serotype 9 vector into the cerebrospinal fluid can achieve rapid and widespread expression of IDUA in the CNS with global correction of brain storage lesions. Antibody responses were elicited against IDUA and coincided with decreased CSF enzyme activity. Antibody responses to IDUA could be circumvented by exposing newborn MPS I dogs to the enzyme, which induced persistent immunological tolerance to the protein and allowed for more effective CNS-directed gene transfer later in life. If such a neonatal tolerance induction strategy can be translated to humans it could play an important role in gene and protein replacement therapies for recessive disease.

P125

Genomic integration site analysis of 7 metachromatic leukodystrophy patients up to 48 months follow-up after lentiviral hematopoietic stem cell gene therapy

A Calabria G Spinozzi[1 2] S Brasca F Benedicenti E Tenderini L Naldini[1 3] A Biffi E Montini

1: San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET) 2: University of Milano-Bicocca 3: Universita Vita-Salute San Raffaele

The molecular analysis of the genomic distribution of viral vector genomic integration sites (IS) is a key step in hematopoietic stem cell-based (HSC) gene therapy applications, allowing one to assess the safety and the efficacy of the treatment and to study the basic aspects of hematopoiesis and stem cell biology by monitoring the clonal diversity and dynamics of multiple hematopoietic lineages after HSC transplantation. In our ongoing clinical trial for metachromatic leukodystrophy (MLD) with a self-inactivating lentiviral vector (LV), we retrieved and sequenced > 300 million proviral/host genomic junctions from cells of 7 patients with a 48 month follow up after treatment. We have observed diverse IS profiles in every patient in this study, indicating polyclonal reconstitution. The genome wide distribution of LV IS among patients was consistent with previous preclinical and early clinical data showing the characteristic bias to target expressed genes without preferences towards regulatory regions. Clonal abundance analyses showed no sustained clonal dominance in any patient. Statistical analysis of frequently targeted genomic regions indicated that integrations in these regions were the product of an intrinsic LV integration bias rather than of insertional mutagenesis. We estimated an average clonal HSC population of ~10000 active stem cells from 9 months post treatment and observed only minor fluctuations in clonal population dynamics of the hematopoietic system reconstitution after this time point. IS analysis of 7 MLD patients shows a polyclonal repertoire and common molecular dynamics up to 48 months following gene therapy without any sign of genotoxicity.

P126

Evaluating adeno-associated viral gene delivery as a treatment for Niemann-Pick disease, type C1

R J Chandler[1] I M Williams[1] A A Incao[1] F D Porter[1] W J Pavan[1] C P Venditti[1]

1: National Institutes of Health

Niemann-Pick disease, type C1 disease (NPC1) is a heritable lysosomal storage disease characterized by a progressive neurological degeneration that causes disability and premature death. NPC1 commonly manifests in childhood, and there are no approved treatments to delay, stop, or reverse the fatal neurodegeneration that is the hallmark of this disorder. New therapies for patients with NPC1 need to be developed. A murine model of NPC1, arising from a spontaneous frame-shift mutation in the Npc1 gene has been described. The Npc1-/- mice display a rapidly progressing disease, which is characterized by weight loss, ataxia, and lethality by 9 weeks of age. To test the potential efficacy of gene therapy with the goal of developing a new treatment for NPC patients, we constructed an adeno-associated virus (AAV) serotype 9 vector to deliver the human NPC1 gene under the transcriptional control of the neuronal-specific promoter, mouse calcium/calmodulin-dependent protein kinase II (CaMKII). Npc1-/- mice received 1x1012 GC of AAV9-CaMKII-NPC1 or an equivalent reporter control, AAV9-CaMKII-GFP, between 20 and 25 days of life delivered by retro-orbital injection. Relative to the untreated or AAV9-CaMKII-GFP treated Npc1-/- mice ($n = 15$, mean survival 66 days), the Npc1-/- mice that received AAV9-CaMKII-NPC1 exhibited an increased life span ($n = 9$, mean survival 105 days, $P < 0.02$). Preliminary immunohistochemistry of the treated Npc1-/- showed widespread CNS NPC1 expression and prolonged survival of Purkinje cells. Although the AAV9-CaMKII-NPC1 treated Npc1-/- mice did not achieve a normal life expectancy, our results demonstrate, the potential efficacy of systemic AAV gene therapy as a therapeutic option for NPC1.

P127

Development of a gene therapy approach for mucolipidosis Type 2

J Knickrehm[1] S Markmann[1] K Kollmann[1] B Fehse[1] T Braulke[1] K Cornils[1]

1: University Medical Center Hamburg-Eppendorf 2: Technische Universität Dresden

Lysosomal storage diseases (LSDs) describe a group of approx. 50 inherited, monogenic disorders. Mucolipidosis Type II (MLII) is an LSD resulting from deficiency in the GlcNAc-1-phosphotransferase (GNPTAB). This protein facilitates the transport of enzymes to the lysosome by adding a Mannose-6-phosphate-(M6P-) residue. ML2 is characterised by mental and motoric retardation, skeletal deformations and an early death. Today there is no curative treatment available. On the basis of the promising results of gene-therapy studies for other LSDs, we aim at developing a gene-therapy approach for ML2. The cDNA of the human GNPTAB was cloned into a lentiviral backbone and used initially for transduction of primary murine fibroblasts from a ML2-diseased mouse. Biochemical and immunological assays demonstrated that the GNPTAB-protein was expressed and functional. Phenotypic correction was observed in transduced and in neighbouring, untransduced cells that were obviously cross-corrected. Correction of the whole population required > 20% transduction efficiency. We also performed HSCT with GFP-transduced cells in ML2 mice to determine marked donor-derived microglia cells in the brain. Immunohistochemistry of sagittal brain sections revealed GFP-positive microglia cells in the cerebellum. In conclusion, we were able to demonstrate that gene transfer of GNPTAB leads to a correction of the cellular ML2 phenotype in vitro. We could also show that GFP-marked cells derived from the blood can enter the brain in the ML2-mouse model after HSCT. Improved vector design and transduction protocols need to be tested to establish an efficient preclinical protocol for gene therapy of ML2.

P128

Long-term efficacy and safety in mice and dogs following delivery of AAV9-sulfamidase vectors to the CSF for the treatment of MPSIIIA

V Haurigot[1 2] S Marcó[1 2] A Ribera[1 2] M García[1 2] S Motas[1 2] M Molas[1 2] C Roca[1 2] X León[1 2] L Maggioni[1 2] S Añor[3] A Andaluz[3] J Ruberte[1 4] F Bosch[1 2]

1: Center for Animal Biotechnology and Gene Therapy, UAB 2: Department of Biochemistry and Molecular Biology, School of Veterinary Medicine, UAB 3: Department of Animal Medicine and Surgery, School of Veterinary Medicine, UAB 4: Department of Animal Health and Anatomy, School of Veterinary Medicine, UAB

Mucopolysaccharidosis Type IIIA (MPSIIIA) or Sanfilippo A syndrome is a rare autosomic recessive Lysosomal Storage Disease (LSD) caused by deficiency in sulfamidase, a sulfatase involved in lysosomal degradation of the glycosaminoglycan (GAG) heparan sulfate (HS). Accumulation of undegraded HS leads to lysosomal pathology, which in turn results in severe progressive neurological deterioration with relatively mild somatic pathology. Patients usually die within the first 2 decades of life. We previously demonstrated in MPSIIIA mice that 4 months after intra-cerebrospinal fluid (intra-CSF) administration of AAV9 vectors encoding sulfamidase there was whole-body disease correction through transgene expression throughout the CNS and liver. The aim of the present study was to evaluate the long-term therapeutic efficacy and safety of this gene therapy approach in mice and dogs. We provide evidence for long-term (>10 months follow-up) biochemical, histopathological and functional correction of disease phenotype following CSF delivery of AAV9-Sulfamidase to adult MPSIIIA mice. Moreover, a single intra-CSF administration of AAV9 vectors encoding canine sulfamidase to healthy Beagle dogs at the therapeutic dose resulted in a long-term stable increase in sulfamidase activity in the CSF, in the absence of any safety concerns. Altogether, these results support the clinical translation of this approach for the treatment of MPSIIIA and other LSD with CNS involvement.

P129

Hematopoietic stem cell gene therapy for the treatment of Globoid Cell Leukodystrophy

S Ungari[1] A Montepeloso[2] F Morena[3] F Cocchiarella[4] A Recchia[4] S Martino[3] B Gentner[1] L Naldini[1 2] A Biffi[1 2]

1: San Raffaele Telethon Insitute for Gene Therapy (HSR-TIGET) 2: University Vita-Salute San Raffaele 3: Department of Experimental Medicine and Biochemical Science, Perugia University, Italy 4: Center for Regenerative Medicine ''Stefano Ferrari'', Department of life Sciences, University of Modena and Reggio Emilia, Modena, Italy 5: San Raffaele Scientific Institute

Globoid Cell Leukodystrophy (GLD) is a rare and severe demyelinating lysosomal storage disorder due to the deficiency of galactocerebrosidase (GALC). Our goal is to develop a safe and effective gene therapy based on lentiviral vector (LV) mediated gene transfer into autologous hematopoietic stem cells (HSCs). We demonstrated that HSC gene therapy required a regulation of enzyme expression due to a toxic effect of GALC over-expression in the murine HSC compartment and not in more differentiated cells. We thus employed a GALC-encoding LV regulated by miRNA-126 that is expressed only in HSCs. We proved the efficacy of this LV construct in vitro and in vivo in the disease mouse model improving its survival and phenotype. Then we worked at identifying the best LV design for a proper clinical translation. We cloned a codon optimized human GALC (hGALC) cDNA into a LV backbone containing miRNA regulatory elements and we proved that 1 tag of miR-126 was sufficient to provide good repression of hGALC activity in human HSCs and to guarantee a sustained enzyme expression upon cell differentiation. Importantly, no significant hGALC-overexpression related toxicity was observed in terms of viability, differentiation, clonogenic potential as compared to control cells. Moreover regulated hGALC over-expression did not cause perturbation of pro-apoptotic sphingolipids or genes that could impair HSCs physiological functions. Safety of this LV was also demonstrated in vivo upon transplantation of immunodeficient mice with transduced bone marrow-derived human HSCs while efficacy was proved upon transplantation of corrected murine HSCs in a severe GLD mouse model.

P130

Correction of hyperglycemia through adenoviral-mediated compartmentalized liver transduction in a type 1 diabetes mellitus rodent model

G Cabrera B Segura A Esparza D Velazquez A Catzín-Kuhlmann

1: Global BioTherapeutics, Mexico City, Mexico 2: National Institute of Medical Sciences and Nutrition, Mexico City, Mexico

Type 1 diabetes mellitus (T1DM) is a complex metabolic disease characterized by elevated blood glucose levels caused by the autoimmune destruction of insulin producing pancreatic β-cells. Lifelong daily injection of exogenous insulin is the cornerstone of T1DM management. Control over glycemic fluctuations and prevention of its life-threatening complications remains a daily daunting challenge for patients. Gene therapy strategies aim at ameliorating glycemic fluctuations by achieving the secretion of physiologic levels of insulin from non pancreatic-β-cells. Using streptozotocin to induce T1DM on Wistar rats, our group assessed the glycemic effect exerted by an insulin encoding expression cassette driven by a glucose response element (GRE) carried by first generation E1-E3 deleted adenovirus delivered through compartmentalized liver transduction (CLT). Implementing CLT as the mode of vector delivery -achieved by the intra-parenchymal injection of the vector into a blood flow isolated lobe, after which perfusion is reestablished once viral endocytosis has been completed (30 min)- no viremia was observed and transgene presence and insulin synthesis were confined to the lobe of vector administration. At a dose of 2.2x10^10 IFUs/rat (8.8x10^10 IFUs/kg), correction of postprandial hyperglycemia was achieved for the duration of the study (2 months) without the need of exogenous insulin. Glucose tolerance tests revealed a return to normoglycemia within 120 minutes. Our results further demonstrate that CLT is a safe and effective mode of vector delivery. Additionally, our results demonstrate that glycemic fluctuations are manageable through gene therapy strategies and should be further explored for the treatment of T1DM.

P131

In vivo AAV-mediated highly-specific transduction of Adipose Tissue in Adult Mice using microRNA Target Sequences

E Casana[1 2 3] V Jimenez[1 2 3] S Muñoz[1 2 3] C Jambrina[1 2 3] C Mallol[1 2 3] V Sacristan[1 2 3] J Rodo[1 2 3] X Leon[1 2 3] M Garcia[1 2 3] A Ribera[1 2 3] F Bosch[1 2 3]

1: CBATEG-UAB 2: Department of Biochemistry and Molecular Biology, School of Veterinary Medicine, Universitat Autònoma de Barcelona 3: Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM)

Obesity and type 2 diabetes (T2D) are strongly associated and a major health problem because of their alarmingly growing prevalence worldwide. Thus, novel approaches are needed to prevent and combat these disorders. Deregulation of metabolic and endocrine functions of adipose tissue are considered among the main contributors to obesity and T2D in experimental animal models and in humans. Genetic engineering of adipose cells offers great potential as a tool to study the molecular mechanisms underlying these pathogenic processes as well as for the development of new therapies for these disorders. In this regard, our laboratory has recently reported that systemic or intra-depot administration of AAV vectors mediates efficient transduction of fat pads in mice. In this study, as an alternative to the use of tissue-specific promoters to restrict AAV-mediated genetic engineering to adipose tissue, we evaluated de-targeting of transgene expression in off-target tissues, including in our constructs target sites of the liver-specific miR122a and the heart-specific miR1 (mirT sequences). Upon systemic and intra¬-depot administration of AAV vectors bearing an ubiquitous promoter in conjunction with the aforementioned mirT sequences efficient transduction of adipocytes was observed while ectopic transgene expression and production in the liver and heart was efficiently inhibited. Altogether, these results indicate that it is possible to achieve AAV-mediated highly-specific and robust transduction of adipose tissue in adult mice by means of mirT sequences. Moreover, AAV-mediated genetic engineering of adipose tissue represents a technological advance that may prove useful for the development of new therapeutic approaches for obesity and T2D.

P132

HSC gene therapy for the treatment of Mucopolysaccharidosis type I: towards clinical testing

S Delai[1 4] I Visigalli[1 4] F Ferro[1] F Cecere[1] F Sanvito[2] M Vezzoli[1] P Cristofori[3] L Naldini[1] A Biffi[1]

1: San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), via Olgettina 58, 20132, Milano, Italy 2: San Raffaele Scientific Institute, Department of Pathology, Milano, Italy 3: GlaxoSmithKline, R&D, Ware, SG12 0DP, UK 4: equal contribution

Type I Mucopolysaccharidosis (MPS-I) is caused by the deficiency of α-L-iduronidase (IDUA) and characterized by accumulation of mucopolysaccharides within the lysosomes, which leads to multisystemic impairment. We developed a gene therapy (GT) strategy based on the use of lentiviral vectors (LV) for transducing autologous HSCs and inducing supra-physiological IDUA enzyme production in transduced cells and their tissue progeny. This approach, when tested in MPS-I mice in comparison with HSCT from wild type donors showed a greater efficacy in preventing and correcting disease manifestations (Visigalli, Delai et al, 2010). Based on these results, a clinical development plan for moving this approach to clinical testing has started. The toxic and tumorigenic potential of the IDUA.LV transduced murine HSCs was evaluated upon transplantation in MPS-I mutant mice, demonstrating the absence of adverse events in GT treated mice and a comparable incidence of proliferative changes with respect to control mice. Concomitantly, the protocol currently employed for patients' HSC transduction in the HSC-GT clinical trials ongoing at TIGET for other diseases, have been tested in vivo and in vitro on human HSCs with a clinical-grade IDUA.LV. Results showed that the efficiently transduced IDUA.LV human CD34+ cells engraft, differentiate into mature progeny and distribute to lymphohematopoietic murine organs similarly to control un-transduced CD34+ cells. The demonstration of the efficacy and the safety of our approach strongly support a Phase I/II clinical trial of HSC GT in MPS-I patients with the goal of augmenting the benefit and reducing the risks associated to allogeneic HSCT.

P133

Combination of low-dose gene therapy and monthly enzyme replacement therapy improves the phenotype of a mouse model of lysosomal storage disease

R Ferla[1 2] M Alliegro[1 2] E Nusco[1] P Claudiani[1 2] A Auricchio[1 2]

1: Telethon Institute of Genetics and Medicine 2: Medical Genetics, Department of Translational Medicine, ''Federico II'' University, Naples, Italy

Enzyme replacement therapy (ERT) is the standard of care for several lysosomal storage diseases (LSDs). However, ERT requires multiple and costly administrations and has limited efficacy on several LSDs features. We have recently shown that a single administration of $2\times10e^{12}$ gc/kg of adeno-associated viral vector serotype 8 (AAV2/8) is at least as effective as weekly ERT in MPS VI mice. However, the administration of high doses of AAV2/8, which require a challenging and costly production process, might result in cell-mediated immune responses to AAV8. Here we evaluated whether the combination of low doses of AAV2/8 ($<2\times10e^{12}$ gc/kg) with a rarified ERT schedule (monthly) may be as effective as the single treatments at high doses or frequent regimen. While normalization of glycosaminoglycans (GAG) levels was observed in visceral organs regardless of treatment, greater reduction of urinary GAGs was observed in mice receiving the combined therapy. In particular, urinary GAG reduction was comparable to that obtained following either high doses of AAV2/8 or weekly ERT in mice receiving both $6\times10e^{11}$ gc/kg of AAV2/8 and ERT. Similarly, reduction of lysosomal storage in both heart valves and myocardium was more consistent in mice receiving $6\times10e^{11}$ gc/kg of vector, particularly in combination with ERT. These data support a similar efficacy between low-dose gene therapy combined with rarified ERT and the corresponding single treatments at high doses or frequent regimen. This should increase the safety and reduce the risks and costs associated with both therapeutic approaches.

P134

Pancreatic overexpression of IGF1 protects NOD mice from autoimmune diabetes

C Mallol[1 2 3] E Casana[1 2 3] A Casellas[1 2 3] V Jimenez[1 2 3] M Morró[1 2 3] C Jambrina[1 2 3] V Sacristan[1 2 3] L Vilà[1 2] X Leon[1 2 3] J Agudo[1 2 3] F Bosch[1 2 3]

1: CBATEG - UAB 2: Department of Biochemistry and Molecular Biology, School of Veterinary Medicine, Universitat Autònoma de Barcelona 3: Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM)

The insulin-like growth factor 1 (IGF1) signaling pathway has been associated with the regulation of β-cell growth and survival. We have previously shown that overexpression of IGF1 in β-cells of transgenic mice counteracts cytotoxicity and insulitis and regenerates the endocrine pancreas in streptozocin (STZ) induced diabetic models. In this work, we generated type 1 diabetes-prone Non-Obese Diabetic (NOD) mice overexpressing IGF1 specifically in β-cells (NOD-IGF1) to further investigate IGF1 therapeutic effects against spontaneous autoimmune diabetes. Our results showed that β-cell overexpression of IGF1 was sufficient to prevent lymphocytic infiltration of the islets, inflammatory cytokine expression, and progressive β-cell failure and death, suggesting a blockage of the autoimmune attack against β-cells that resulted in resistance to diabetes development. Similarly, a marked reduction in diabetes incidence was also observed in young adult NOD mice upon intraductal administration of adeno-associated viral vectors of serotype 8 (AAV8) encoding IGF1 under the control of a ubiquitous promoter in combination with liver- and heart-specific microRNA target sequences. This approach enabled robust, long-term IGF1 expression in the pancreas and prevented IGF1 production in off-target tissues. AAV8-mediated overexpression of IGF1 also protected islets from insulitis development, thus preserving β-cell mass and reproducing those observations made in transgenic NOD-IGF1 mice. Collectively, these results indicate that β-cell overexpression of IGF1 prevents islet infiltration and β-cell death in mice with increased susceptibility to type 1 diabetes. Thus, IGF1 may represent a key factor to prevent autoimmune destruction of β-cells and a candidate gene to develop AAV-mediated gene therapy strategies against diabetes.

P135

Correction of murine diabetic hyperglycaemia with a single systemic administration of an AAV8 vector containing a novel codon optimized human insulin gene.

M Notaridou[1] S U Gan[2] Z Y F u[2] K O Lee[2] A C Nathwani[1] M Della Peruta[1] R Y Calne[2]

1: University College London 2: National University of Singapore

Permanent cure for Type 1 Diabetes Mellitus (T1DM) has long been sought after and insulin gene therapy has been a popular approach but with no clinical application to date. The main prerequisites for successful insulin gene therapy include stable and long term production of biologically active insulin, identification of a suitable site for expression and a delivery vector with targeted cell tropism, long term efficacy as well as minimal toxicity and immunogenicity. In this study we have addressed these challenges and report correction of hyperglyceamia in STZ induced diabetic mice after one intravenous systemic administration of a single stranded adeno-associated virus serotype 8 (ssAAV8) vector encoding a furin-cleavable human proinsulin gene under a constitutive liver specific promoter (AAV8_HLP_hINSF). Initial transduction led to severe hypoglyceamia of the injected mice. Following dose titration experiments, we achieved maintenance of euglyceamia or a mild diabetic condition for more than 8 months and still ongoing, with no events of hypoglycaemia, accompanied by significant C-peptide secretion and weight gain similar to the healthy control mice. Novel DNA codon optimization of the insulin gene to create a liver specific codon template (AAV8_HLP_hINScdF) resulted in a 10-20 fold reduction of the number of vector particles required to achieve the same extent of decrease in blood glucose levels as with the non-codon optimized vector. We suggest that AAV8 mediated constitutive secretion of insulin achieved by a single administration of the AAV8_HLP_hINScdF vector at the appropriate dosage could potentially be of therapeutic value for diabetic patients.

P137

Obese and diabetic sprague dawley rats exhibited reduced GLP-1 expression in pancreatic islets of Langerhans

H B Olgun H M Tasyurek H A Altunbas S Sanlioglu

1: Akdeniz University Center for Gene and Cell Therapy 2: Department of Internal Medicine, Division of Endocrinology and Metabolism, Akdeniz University Faculty of Medicine

Glucagon-like-peptide (GLP)-1 is an incretin hormone with antidiabetic properties, which increases glucose induced insulin secretion, in addition to survival and proliferation of pancreatic beta cells. (1) Patients with Type 2 diabetes (T2DM) have insufficient incretin secretion following meals besides other problems relevant to glucose metabolism. Thus, we aimed to investigate potential variations in GLP-1 expression, a novel

therapeutic agent used in diabetes treatment, in pancreatic beta cells of obese and diabetic versus lean non-diabetic control rats. For this purpose, SD rats were fed with normal or high fat diet for two months prior to induction of diabetes by intraperitoneal (IP) STZ administration. Diabetic rats also exhibited insulin resistance and glucose intolerance. After sacrification, immunohistochemistry analysis were performed to determine the tissue distribution of GLP-1 ligand and its receptor (GLP-1R) expression on pancreatic sections. 96% loss of beta cell mass associated with a drastic decrease in GLP-1 and GLP-1R expression were detected using ImageJ software of NIH. In conclusion, GLP-1 gene replacement therapy might be beneficial to compensate beta cell loss and incretin deficiency observed in obese and diabetic rats. Grant support: TUBITAK-112S114

P138

Industrialization of a cell based autologous therapy targeting Diabetes: Industrialization of human liver cells amplification process from Culture dish to Xpansion™ multi-plate bioreactor

A Reniers[1] V Aviv[2] S Ferber[2] T Bovy[1] S Hassin[2] J C Drugmand[1] F Moncaubeig[1]

1: Pall Life Sciences 2: Orgenesis Inc.

Current therapies against diabetes rely on several injections a day of recombinant insulin and don't prevent diabetes related complications. While pancreatic islet cell implantation provides a better treatment for diabetic patients, it is restricted due to severe shortage of tissues for transplantation. Orgenesis developed a technology for activating a pancreatic lineage and the β cell function in liver cells. This approach, based on an autologous strategy, overcomes both the shortage in tissues availability and the immune suppression. These developmentally shifted human liver cells produce insulin, process the hormone and secrete it in a glucose regulated manner. The cells ameliorate diabetes when implanted in immune-deficient SCID-NOD mice for long periods indicating that this therapy should allow substantial improvement of patients' quality of life. In this context, Orgenesis and Pall combined their respective expertise to optimize, scale-up and industrialize the process used in preclinical studies. Starting from the bioprocess developed in Cell culture dishes by Orgenesis, the cell amplification process was successfully scaled up in the Xpansion™ platform technology while a fully closed system was designed. Results showed that Orgenesis' liver cells had an equivalent, if not shorter, Population Doubling Time when grown in Xpansion™ bioreactor VS Multi-layer systems while maintaining cell morphology. At the end of the process, around 1.8 Billion cells have been recovered from an Xpansion™ 200, starting from 1-2 g of human liver and representing more than the targeted dose requirement of 1Billion cells per patient.

P139

An Antibiotic-free strategy for miniplasmid production

R Selvamani[2] M Telaar[1] K Friehs[1] M Schleef[2] E Flaschel[1]

1: Fermentation Engineering, Bielefeld University, 33615 Bielefeld, Germany 2: Plasmid Factory GmbH & Co. KG, Meisenstraße 96, 33607 Bielefeld, Germany

The segregational stability of plasmids in a recombinant bioprocess is of extreme significance. Although this is commonly achieved by the selection pressure from antibiotics, their application for the production of therapeutic DNA for gene therapy or for DNA vaccines would be undesirable. The presence of antibiotic-resistance genes in the final product would have to be avoided. In addition to the minicircle approach presented by Schmeer et al., (this conference), a miniplasmid is able to fulfil the regulatory requirements. The gene tpiA is responsible for the connection of the glycerol metabolic pathway with the glycolytic pathway in Escherichia coli. The knockout of genomic tpiA rendered cells completely auxotrophic in minimal medium with glycerol as sole carbon source while allowing growth at a reduced rate with glucose or in complex medium. This was advantageous for optimizing antibiotic-free cloning and selection of recombinant plasmid. Complementation of the auxotrophy by plasmid-borne tpiA led to high segregational and structural plasmid stability, resulting in stable production of a model recombinant enzyme under antibiotic-free conditions. Thus, the complementation of tpiA represents a significant alternative to antibiotics as a selection principle and is therefore of potential interest for the recombinant production of biotherapeutics in the form of miniplasmids.

P140

Next generation sequencing: impact on the quality control of gene therapy medicinal products

M H Wisher C Cote

1: BioReliance, SAFC

Next Generation Sequencing (NGS) represents a very powerful technique to be used in the identity and characterisation of vectors used for gene and cell therapy and to screen cell banks, virus seed stocks and starting materials for the absence of adventitious contaminants. This presentation will give examples of the current use of NGS in biological product quality control and review the current discussions and acceptance of this technique by global regulatory agencies. Progress in the optimization and validation of the different steps used in this technique will be presented including discussion of sample preparation and extraction, nucleic acid library preparation, bioinformatics and database analysis.

P141

Accurate titration of infectious AAV particles requires measurement of biologically active vector genomes and suitable controls

A François[1 3] M Bouzelha[1 3] E Lecomte[1 3] F Broucque[1 3] M Penaud-Budloo[1 3] P Moullier[1 2 3] V Blouin[1 3] E Ayuso[1 3]

1: Atlantic Gene Therapies, Nantes, France 2: University of Florida 3: Inserm U1089, Nantes, France

Although the clinical use of recombinant AAV (rAAV) vectors is constantly increasing, development of suitable quality control methods is still needed for accurate vector characterization. Among the quality criteria, the titration of infectious particles is critical to determine vector efficacy. Different methods have been developed for measurement of rAAV infectivity in vitro, based on either detection of vector

genome replication in trans-complementing cells infected with adenovirus, detection of transgene expression in permissive cells, or simply detection of intracellular vector genomes following infection of indicator cells. In the present study, we have compared these titration methods for their ability to discriminate infectious and non-infectious rAAV serotype 8 vector preparations. To challenge these methods, we have produced and characterized a VP1-defective AAV8-GFP vector. Since VP1 is required to enter the cell nucleus, the lack of VP1 should drastically reduce the infectivity of rAAV particles. The AAV8 Reference Standard Material was used as a positive control. Our results demonstrated that methods based on measurement of rAAV biological activity (i.e. vector genome replication or transgene expression) were able to accurately discriminate infectious vs non-infectious particles, whereas methods simply measuring intracellular vector genomes were not. Several cell fractionation protocols were tested in an attempt to specifically measure vector genomes that had reached the nucleus, but genomes from wild-type and VP1-defective AAV8 particles were equally detected in the nuclear fraction by quantitative PCR. These data highlight the importance of using suitable controls, including a negative control, for the development of biological assays such as infectious titrations.

P142

Development of a successful lyophilization process for lentiviral vector clinical batches

F Peral[1] N Delacroix[1] J Deroyer[1] G Deplaine[1] A Bejanariu[1] A Hadri[1] C Bauche[1]

1: Theravectys

Since lentiviral vectors are increasingly used in various applications and given that so far the best way to keep these vectors is a −80°C storage, more adapted ways of formulation and storage must be developed. We developed a new method to freeze-dry our clinical grade batches of lentivectors. This method allows us to store the batches at −20°C and +4°C while maintaining the characteristics of the particles after reconstitution and the ability to use them as intended (direct injection, T-cell modification …). Our method allows the lyophilization of lentiviral vectors either produced in adherent (with FBS) or in suspension cells cultures (without FBS and with synthetic culture medium). Quality controls have been performed to evaluate the impurity profiles of the batches before the freeze-drying process and after the reconstitution of the particles (titer, total proteins, total DNA, HEK 293T proteins), demonstrating that the batches were still fitting the international pharmacopea recommendations. We also made some immunogenicity evaluation of our lentivectors before and after the lyophilization process, showing no impact of the process on the breadth and intensity of the cellular immune response elicited after a direct injection of the reconstituted particles. THERAVECTYS develops lentiviral vectors used for human vaccination and immunotherapy applications (CAR-T cells and TCR). An in house cGMP facility is now up and running, allowing us to produce and charaterize our own lentiviral vector clinical batches. A qualified freeze-dryer has been installed in the plant, to systematically encompass this formulation and repartition step in our bioproduction processes

P143

A new cGMP facility to produce clinical grade batches of lentiviral vectors and CAR-T cell in Europe

A Zemmar[1] G Deplaine[1] J Deroyer[1] C Bauche[1] A Hadri[1]

1: Theravectys

THERAVECTYS is a Paris-based, privately-owned, fully integrated discovery & clinical development biotech company. We are developing lentiviral vectors for direct vaccination and immunotherapy applications. To control costs, respect timelines and maintain the high quality and diversity of its clinical development programs, THERAVECTYS has decided to internalize the bio-production of its lentiviral vectors and the associated quality controls. A GMP facility was built in compliance with the requirements of regulatory agencies (EMA and FDA). The 340m2 plant is equipped with state-of-the-art bioreactors (up to 1000L), semi-automated downstream skids and a fill-and-finish system to conform to our fully integrated single use process. This process implies a 16 days-USP (expansion and transfection of HEK293SF cells) and a 2 days-DSP (3 purification steps). This GMP facility will allow us to produce batches from 300 up to 5 000 vials complying with up to phase III clinical trial requirements. Additionally, our GMP facility which is composed of defined distinct independent rooms will allow lentiviral vector productions and CAR-T cells engineering, without any cross-contamination. Our Quality Control unit was designed to house a vast array of analytical equipment. With more than 30 different analytical methods including environmental monitoring, contaminants evaluation and RCL testing, THERAVECTYS has gained full independence by keeping all analytical and microbiology testing in house. This cutting-edge GMP facility had been built in 9 months, and is now operational (the certification process is currently underway). It will allow THERAVECTYS to protect its unique and advanced know-how in the manufacturing of lentiviral vectors and CAR-T cells.

P144

Full characterization of the RD3-MolPack packaging cells for the stable production of lentiviral vectors

V Marin C Piovan S Corna S Bossi C Scavullo E Giuliani C Bordignon G P Rizzardi C Bovolenta

1: MolMed S.p.A.

Gene therapy with lentiviral vectors (LV), produced transiently, has been successful in several examples of rare genetic diseases. However, transient LV production is unfeasible for large scale manufacturing. In this contest, LV stable packaging cells dramatically reduce manufacturing cost and increase the safety and reproducibility of the clinical lots, which are the major limitation for the application of transiently produced LV to large number patient clinical trials. To overcome this limitation, MolMed has developed the proprietary RD-MolPack technology for the stable production of LV, which is based on the RD114-TR envelope that, in contrast to the VSV-G envelope, permits the generation of a constitutive and 'clean' -devoid of any inducers - LV packaging system. Here, we present the characterization of the prototype RD3-MolPack-SIN-GFP producer cells as a proof-of-concept of the feasibility and safety of our technology before its exploitation at the clinical level. We determined the number of copies and integrity of the packaging

and rd114-tr genes and the integrity of the transfer vector (TV) both in the packaging and target cells. Furthermore, we demonstrated the lack of mobilization of the envelope and the lack of LV auto-transduction. Finally, we show that RD3-MolPack-SIN-GFP LV (not-purified and not-concentrated supernatant titer = 1-5 × 106 TU/ml) transduce human hematopoietic stem cells, primary T lymphocytes and hepatocytes at levels comparable to that of VSV-G pseudotyped SIN-GFP LV. Overall, our data support the conclusion that RD-MolPack technology represents a straightforward tool to simplify and standardize LV-production that is ready for clinical application.

P145

Enabling tools for gene therapy viral vector development

A F Rodrigues[1 2] A S Formas-Oliveira[1] M R Guerreiro[1 2] H A Tomás[1 2] P M Alves[1 2] M T Carrondo[1 3] A S Coroadinha[1 2]

1: iBET - Instituto de Biologia Experimental e Tecnológica, Oeiras, Portugal 2: Instituto de Tecnologia Química e Biológica-Universidade Nova de Lisboa, Oeiras, Portugal 3: Faculdade de Ciências e Tecnologia/Universidade Nova de Lisboa, Monte da Caparica, Portugal

Background: Stable cell line development for viral vector production is an intensive and time consuming work requiring transfection, clonal selection and screening. In addition virus titration methods are also laborious and often lengthy. Herein, we report the development of two novel enabling technologies for the fast cell line and viral vector development – single-step cloning screening method (SSCS) - and virus titration - Visensors. Experimental approach: The SSCS merges cloning and screening by using split-GFP, a green fluorescent protein separated into 2 fragments which fluoresce only upon transcomplementation. A cell population producing retroviral or lentiviral vectors with a S11 transgene is cloned and co-cultured with a target cell line harboring the S10 fragment. The viruses produced by the clone infect the target cells and reconstitute the GFP signal. Only high-titer clones yielding high fluorescence signal are isolated.

Results and Conclusion: The method was first validated by establishing a retrovirus producer cell line allowing high-throughput screening; clones producing up to 1×10^8 IP/mL and improved ratios of IP/TP of 1/10 were isolated. The transgene can be exchanged by RMCE. Current undergoing is the validation of SSCS for the development of a lentiviral vector stable cell line. To further expand this approach to other virus we are developing Visensors; a cell based biosensor for label-free virus. Upon activation by a viral protein the cells emit fluorescence. A Visensor for adenovirus detection was successfully validated. These enabling tools will contribute for the progress in viral vector and cell line development.

Reference

Rodrigues et al. (2015)GeneTher doi:10.1038/gt.2015.44

P146

Impact of the integrity of the ITRs on the production of rAAV vectors in the baculovirus / Sf9 cells system

Y Dickx[1] L Nauwynck[1] A Savy[1] D Bonnin[1] F Mavilio[1] O W Merten[1] L Galibert[1]

1: Genethon, 91002 Evry, France

The Adeno-Associated Virus's (AAV) Inverted Terminal Repeats (ITRs) are key features of the AAV. Those GC rich structures are implied in the replication, encapsidation of the AAV genome, along with its integration in the host genome and its excision. These sequences are the only AAV DNA sequences conserved in the recombinant AAV as they allow its replication, encapsidation and long-term maintenance in the host non-dividing target cells. During its replication, the AAV is able to reconstitute incomplete or imperfect ITR sequences. Thus it is often found that rAAV genomes cloned into the backbones of the DNA plasmids used for production, have imperfect ITRs sequences which are shortened or contain mutations. In this work, we study the impact of those imperfect ITRs and their impact on the levels of encapsidated rAAV genomes and cellular/baculovirus contaminating DNA sequences, in the baculovirus/Sf9 cells production system. We show that mutations and deletions impact the level of contaminating DNA sequences.

P147

In vitro and in vivo comparability assessment of an AAV vector manufactured by triple transfection in HEK293 cells or in the baculovirus expression system

M Steel K Woodburn T Kniffin K Gebretsadik J Chan S Vijay H Chen T W Chalberg M Gasmi

1: Avalanche Biotechnologies 2: Virovek, Inc

AVA-101 is a recombinant, replication-defective adeno-associated serotype 2 viral vector encoding the soluble form of the VEGF receptor type 1 that is currently being evaluated in clinical studies for the treatment for neovascular age-related macular degeneration. For a Ph1/2a study, AVA-101 was manufactured by triple transfection in HEK293 cells and subsequently purified by ion exchange chromatography and iodixanol ultracentrifugation. To improve scalability and cGMP suitability for late stage development, the upstream process was switched to the baculovirus expression system and the downstream purification to chromatography-based system compatible with biopharamaceutical industry manufacturing technologies. In addition, the formulation was modified to improve biocompatibility with the container closure system and infusion device. Vectors manufactured in both processes were compared using qualified analytical assays for titer (qPCR), infectivity (TCID50), transgene expression (transduction/ELISA), identity (Western blot) and purity (SDS/PAGE-Silverstain®). The ratio of empty-to-full vectors by TEM was also evaluated. Vectors biodistribution and potential toxicity effects upon subretinal administration in nonhuman primates were also assessed. Analytical results show that vectors produced by both manufacturing processes exhibit similar characteristics. When observed, differences were within assay variability. Both vectors were well tolerated with no differences observed in safety and biodistribution in animals. These results support the use of the new AVA-101 manufacturing process for further clinical development

P148

Development and upscale of HEK293 triple transfection process in single-use bioreactors for industrial manufacture of AAV vectors

L Guianvarc'h F Boussicault C Le Bec F Mingozzi F Mavilio M Hebben

1: GENETHON

The triple transfection of adherent HEK293 cells is a standard procedure to produce AAV vectors. However, as this method is poorly scalable, it is usually not suited for the large scale manufacturing of AAV-based therapeutics. To allow scalability of the triple transfection process, we adapted the adherent HEK293 cells to grow in suspension culture in chemically defined culture medium. The culture parameters were defined in disposable shake flasks and in stirred tank bioreactors. The transfection of suspension cells was made possible using Poly Ethylene Imine (PEI) as transfection agent. The process was first evaluated in disposable shake flasks, then in 2L and 10L glass bioreactors and was finally scaled up to 50L in single-use bioreactors. A number of experiments was performed to produce AAV8 and scAAV9 vectors. The results showed that the suspension transfection process leads to robust vector titers with consistent characteristics especially in terms of full/empty capsid ratio. The productivity of the cell line was monitored along with cell ageing during passages to define the optimal range of passages allowing robust production of AAV. To fulfill the regulatory requirements, a Master Cell Bank of suspension HEK293 cells was manufactured following the good manufacturing practices. Based on these results, the triple transfection process is currently being scaled up to 200L single-use bioreactors in order to produce clinical grade AAV vectors.

P149

Development of innovative scalable protocols for the purification of lentiviral vectors pseudotyped with GaLV-TR or mutated measles virus glycoproteins

D Boudeffa[2] D Fenard[1] M Mormin[1] A Galy[1] O W Merten[1]

1: Généthon 2: Université de Montréal

Lentiviral(LV) vectors are powerful tools for gene transfer in dividing and non-dividing cells. Their clinical application requires complex and scalable processes of production which has only been developed for VSV-g pseudotypes. However, pseudotyping LV by different glycoproteins (gp) allows targeting of different tissues/cell types. Modified Gibbon Ape Leukaemia Virus (GaLV-TR) gp pseudotyped LVs are interesting to transduce hematopoietic stem cells (HSC). Mutated measles virus gp engineered with single chain Fv can be redirected to specific cell surface receptors such as MHC Class II antigens (MV-CMH-II) enabling the transduction of dendritic cells. However, efficient purification schemes for these pseudotypes are not yet available. Here we report novel protocols allowing high yield production of infectious LV-GaLV-TR and LV-MV-CMH-II particles. We identified critical conditions in chromatographic and tangential flow filtration (TFF) steps for preserving infectivity/functionality of LV vectors during purification. This was done by identifying for each step, the critical parameters affecting LV infectivity, including pH, salinity, and presence of stabilizers. A three-step process (1 TFF, 2 chromatographic steps (IEC, SEC)) was optimized for LV-GALV-TR purification permitting recoveries of >50% (ip – infectious particles). Purified GaLV-TR pseudotyped LV vectors enabled the transduction of 70% human CD34+ cells in the presence of Vectofusin, whereas non-purified vector transduced only 9% of the cells. A simplified purification protocol (TFF, SEC) developed for LV-MV-CMH-II vectors led to yields of 60% (ip). Our protocols will allow for the first time the purification of various biologically-active LV pseudotypes with sufficient recovery in perspective of preclinical studies and clinical applications.

P150

Novel flowthrough platform for viral vectors purification

C Peixoto[1] R Silva[1] P Nestola[1] L Villain[3] J PB Mota[4] M JT Carrondo[1 4]

1: IBET-Instituto de Biologia Experimental e Tecnológica 2: iBET/ITQB 3: Sartorius Stedim Biotech 4: FCT/UNL

Purification of viral vectors is gaining importance to fulfill the requirements of regulatory authorities for reliable manufacturing processes. Currently, ion-exchange chromatography is typically operated in positive (bind-elute) mode: most of the impurities are collected in the flow-through pool, while the virus particles and some of the impurities are retained in the matrix. Due to the differences in charge of the different components, this mode of operating does not fit the concept of platform purification, since slight changes of the charge and size of the target virus will reflect into a different elution pattern. Furthermore enveloped virus may be damage during the binding and elution steps. In this work we developed a novel flow through purification platform for adenovirus (Ad5), by combing in series STIC membrane chromatography and Captocore matrix. The optimization was performed, first by analyzing the two ligands namely polyallylamine and octylamine, with a Biacore technology. Binding isotherms for the DNA were determined; the selected conditions were confirmed by 96 well format and then scale up was performed. The optimized full process yielded a 20% higher recovery yield compared to the traditional bind and eluted process. Also, DNA clearance over 4 log was obtained. Moreover, high flow rate can be achieved, and scale up is easy to implement. Overall this process has greater performances compared to the standard bind-elute approach, and it fits into platform concepts that can be applied for other viral vectors for gene therapy.

P151

Development of large-scale downstream purification process for recombinant adenoviruses

O Rautsi[1] E M Nurminen[1] A Muller[1] V Huusko[1] S Vepsäläinen[1] E Lipponen[1 2] H P Lesch[1 2] M Hassinen[1 2] M Pollen[1] M Jääskelainen[1] O Närvänen[1] M Karhinen[1] S Ylä-Herttuala[3] N Parker[1 2] R Shaw[1 2]

1: FinVector Vision Therapies Ltd 2: FKD Therapies 3: A.I.Virtanen Institute

The aim of this work was to develop a scalable, large-scale downstream purification process for recombinant adenovirus based gene therapy products in order to overcome limitations regarding the use of centrifugation based protocols of large scale-manufacturing. The process was established to contain detergent based cell lysis followed by clarification of the lysed harvest with depth filters. The clarified harvest material was buffer exchanged using tangential flow filtration (TFF) for chromatographic purification. The successful purification of the product was achieved with two consecutive anion exchange chromatography steps, with disposable membrane technology and traditional resin column. Finally, the product was formulated in to final formulation using TFF technique. The purification process has demonstrated to provide high quality gene products, which meet regulatory requirements to supply up to 2E+15 viral particles per batch. In terms of product purity and functionality, the scalable process has been comparable to the

''golden standard'', the CsCl ultracentrifugation based process. The developed process is suitable for suspension as well as adherent cell based upstream processes. In conclusion, we have established a scalable, large-scale downstream purification process to supply high quality recombinant adenovirus based gene products in our fully-licensed GMP manufacturing facility for pre-clinical and clinical trials. Further validation of the DS process for commercial scale is currently ongoing.

P152

Production of safe and efficient AAV vectors using minicircle DNA

M Schmeer[1] M Schleef[1] M Schnoedt[2] A Gruenert[3] R Selvamani[1] T Fuchsluger[3] H Buening[2]

1: PlasmidFactory GmbH & Co. KG 2: CMMC Cologne 3: Ophthalmology, University of Erlangen

Especially in gene therapy applications, certain sequence motifs contained in plasmid DNA have to be avoided wherever possible. Such sequences are e.g. the bacterial ori or selection markers, only used for controlling the bacterial replication of the plasmid or to select for the plasmid during cloning or during production. Such sequence motifs are redundant in the intended therapeutic application and are completely removed in minicircles, i.e. circular and ccc-supercoiled expression cassettes. Since Adeno-Associated viral (AAV) vectors are produced by co-transfection of HEK293 producer cells, such bacterial sequence motifs may be an issue of an AAV vector-mediated gene transfer as well. Here, as a result of so-called reverse packaging events, an AAV vector-mediated transfer of not only the therapeutic gene but also of the antibiotic resistance gene into the target cells has been reported. Hence, this appears to be a potential risk of plasmid derived AAV vectors which can be overcome by using minicircle DNA for AAV production. Here we present first results showing that both constructs, the Helper & Packaging plasmid as well as the Transfer plasmid can be produced as minicircles although certain sequence motifs such as the ITRs have been identified to be an issue in minicircle production which has been overcome. These minicircles have been used for efficient AAV vector productions. However, only by replacing both, the Helper & Packaging plasmid as well as the transfer plasmid, encapsidation of the antibiotics resistance gene can be avoided.

P153

Scaling up adenovirus production in suspension and adherent processes, from bench top to clinics

P Valonen[1] H P Lesch[1 2 3] K M Heikkilä[1] E M Lipponen[1 2] E Räsänen[1] E M Nurminen[1] M Hassinen[1 2] M Karhinen[1] N R Parker[1 2] R Shaw[1 2] S Ylä-Herttuala[3 4]

1: FinVector Vision Therapies Ltd 2: FKD Therapies 3: A.I.Virtanen Institute 4: Science Service Center and Gene Therapy Unit, Kuopio University Hospital, Kuopio, Finland

It is challenging to scale up viral vector manufacturing into a large scale. In bench top studies the small volumes are easy to handle, but when scaling up, volumes increase remarkably in parallel with the costs. Furthermore, when working with big volumes, nutrients, pH, and gas exchange become issues of which controlling becomes more demanding. We have successfully been working both with adherent and suspension adenovirus production processes. Importantly, all our viral materials, used in toxicological and clinical trials, are manufactured according to GMP (good manufacturing practices). We have developed a suspension process, where we utilize Biostat® CultiBag RM (Sartorius Stedim Biotech S.A.) bioreactors with cultivation volumes up to 10 L with cell densities as high as 20X106 cells/ml. Because CultiBag RM has faced some challenges in larger volumes with perfusion, stirred tank systems have been evaluated for providing us scalable production system. With adherent process, we have routinely produced adenoviruses utilizing 500 cm2 flasks. Now we are up-scaling the process with iCELLis® (PALL) single use bioreactors. Process development was made with iCELLis® Nano, where the cultivation area varies between 0.53-4 m2. Then, we moved to ICELLis® 500, where the cultivation volume can be upgraded to 500 m2 (66 - 500 m2). During the process development for a low producer like adenovirus, we have gone from flasks up to 100 m2 bioreactor, increasing the productivity from 4.0-109 vp/cm2 (flasks) to 7.9-109 vp/cm2 (100 m2). As presented, we can offer alternative GMP level manufacturing processes for variety of adenoviral products.

P154

Analytical ultracentrifugation as a method for characterization of AAV vector quality

S F Povlich[1] W C Zolla[1] G M Wilmes[1] C J Morrison[1] S C Wadsworth[1] K R Clark[1]

1: Dimension Therapeutics, Inc.

Developing complex biological molecules such as recombinant AAV vectors into commercially viable pharmaceuticals requires rigorous analytical methods to evaluate product quality. An important quality attribute for rAAV vectors is the percentage of full capsids containing complete vector genomes, as opposed to empty capsids and capsids containing partial genomes or encapsidated host cell DNA. We show that analytical ultracentrifugation (AUC) methods can be used to separate and quantify empty, full and intermediate species. We use sedimentation velocity as a direct measurement of sample heterogeneity, as measured by both UV (A260 and A280) and refractive index (RI) detectors, and compared to orthogonal methods for determination of empty and full capsids. Isopycnic sedimentation equilibrium analysis using a density gradient medium, such as cesium chloride, at the appropriate buoyant densities allows for even greater separation and detection of any potential microheterogeneity. These methods allow for comparison of the quality and homogeneity of vectors with small genomes, oversized genomes and self-complementary genomes, as well as vectors produced by different purification schemes. Our results demonstrate that analytical ultracentrifugation is an important tool in the complete characterization of rAAV vectors.

P155

Optimization of lentiviral vector production in a fixed-bed bioreactor

A J Valkama[1] H P Lesch[1 2 3] A Martikainen[1] J Malinen[1] T Salonen[1] M Mähönen[1] T Heikura[1] S Ylä-Herttuala[1 4]

1: A.I.Virtanen Institute 2: FinVector Vision Therapies Ltd 3: FKD Therapies 4: Science Service Center and Gene Therapy Unit, Kuopio University Hospital

Lentiviral vectors (LVs) are promising tools for gene therapy due to their versatility. However, large-scale production of lentiviral vectors is still a major challenge. In this study, we tested LV production using calcium phosphate transfection with four plasmids in iCELLis™ Nano bioreactor with a 4 m2 fixed-bed. Our aim was to optimize the process for scale-up. 293T cells were grown adherently in the bioreactor, and transfected in the exponential phase of growth. Dissolved oxygen, pH, temperature, stirring and perfusion were monitored and controlled during the process. Lactate and glucose concentrations were recorded as well to follow the cell growth. The process was optimized by changing the perfusion rate, stirring rate, other process controls and transfection protocol along the process. The virus was produced into the culture medium, and the product was harvested at different times to also optimize the collection time. Increasing transfection efficiencies and LV titers were achieved after optimization. The production in iCELLis™ bioreactor was easy to control, and efficient compared to control production in triple flasks. However, uneven distribution of the cells in the fixed-bed caused difficulties in monitoring the cell growth. In this study, we demonstrate that the iCELLisTM technology is efficient for controlled lentivirus production in adherent systems. The small-scale optimization provided information for larger-scale production and further process development in iCELLis 500.

P156

Concentration and washing strategies for the downstream processing of human mesenchymal stem cells

B Cunha[1] T Aguiar R Silva C Peixoto[1] M JT Carrondo M Serra[1] P M Alves

1: iBET

Currently human mesenchymal stem cells (hMSC) are expanded using planar technologies or microcarrier-based stirred culture systems up to hundreds of liters of culture volume, in order to guarantee the required cell numbers to be delivered to the clinic. Tangential flow filtration (TFF) is a well established technology that, due to its several advantages, arises as an attractive solution for the downstream processing (DSP) of such large culture volumes, without compromising the cells' characteristics. The aim of this work was to develop a scalable integrated strategy for the concentration and washing of hMSC using TFF technology. For this purpose, we have evaluated the impact that i) several TFF's process parameters (e.g. membrane material and pore size, shear rate and permeate flux) and ii) operation modes (continuous and discontinuous) have on cells' quality (i.e. cell morphology, viability, identity and potency) and recovery yield. Results show that by combining polysulfone membranes of pore sizes higher than 0.45 μm with lower residence and processing times (implying higher shear rates and permeate fluxes, respectively), hMSC could be successfully concentrated up to a factor of 20. Furthermore, a continuous diafiltration process allowed higher clearance of protein impurities (98%) and higher recovery yields (80%) of viable cells, than discontinuous diafiltration (93% and 60%, respectively). Overall, the integration of the optimized unit operations of concentration and washing allowed to decrease bioprocess time by 40%, allowing the recovery of 80% of hMSC with high viability (<95%), while maintaining cells' morphology, immunophenotype, proliferation and multipotency differentiation potential.

P157

Exploration of muscle from GRMD dogs transplanted with MuStem cells using « omics » approaches

A Lardenois[1 2 3] F Robriquet[1 2 3 4] C Babarit[1 2 3] S Jagot[1 2 3 4] T Larcher[1 2 3] M Ledevin[1 2 3] L Dubreil[1 2 3] K Rouger[1 2 3 5] L Guevel[1 2 3 4 5]

1: INRA UMR703 PAnTher, F-44307 Nantes, France 2: LUNAM Université, Oniris, École nationale vétérinaire, agro-alimentaire et de l'alimentation Nantes-Atlantique, Nantes, F-44307, France 3: Atlantic Gene Therapies, F-44000 Nantes, France 4: Université de Nantes, Nantes, France 5: Equal contributions

Duchenne Muscular Dystrophy (DMD), the most common form of inherited neuromuscular disorder, is caused by mutations in the dystrophin gene leading to the protein lack. Membrane disorganization and subsequent alterations in signaling pathways and energy metabolism play important roles in muscle fibre necrosis. Systemic delivery of MuStem cells, skeletal muscle-resident stem cells isolated in healthy dog, generate a remodeling of muscle tissue and gives rise to striking clinical benefits in Golden Retriever Muscular Dystrophy (GRMD) dog. To pursue investigation of the consequences on the skeletal muscle tissue 6 months after cell transplantation with undedicated approach, we used here a combined analysis of transcriptomics and quantitative proteomics. At molecular level, we determined that MuStem cell administration enhances muscle regeneration, promotes ubiquitin-mediated protein degradation in parallel with a decrease expression of genes associated with lipid homeostasis and energy metabolism. Furthermore, the proteomic approach confirmed a main impact of MuStem cell delivery on muscle regeneration, metabolism as well as homeostasis pathways. In addition, we establish that the analysis of a limited set of miRNAs in skeletal muscle clearly discriminates between immunosuppression context and MuStem cell therapy-related effects on GRMD dogs. Overall, the combination of transcriptomics, proteomics and miRNA approaches allowed to pave the way to the understanding of MuStem cell action modalities as stimulation of muscle fibre formation. This strategy has a great potential to considerably contribute to the identification of therapeutic biomarkers of MuStem cell transplantation and thus represents an interesting tool to monitor therapeutic effects during DMD-dedicated preclinical studies.

P158

Systemic AAV8-MTM1 gene therapy for X-linked myotubular myopathy

D L Mack K Poulard M Goddard J M Snyder R W Grange J Doering J L Strande V Latournerie P Veron F Amor J Denard H Meng L Yang F Liu L Buscara C Le Bec S Martin M O'Callaghan F Mingozzi A H Beggs M W Lawlor F Mavilio F Mavilio M K Childers A Buj Bello

1: GENETHON 2: University of Washington 3: Wake Forest School of Medicine 4: Virginia Polytechnic and State

University 5: Medical College of Wisconsin 6: University of Florida 7: Audentes Therapeutics 8: UPMC Paris 6/Genethon 9: Harvard Medical School

Mutations in the myotubularin gene (MTM1) result in X-linked myotubular myopathy (XLMTM), a fatal pediatric disease of skeletal muscle characterized by small centrally nucleated myofibers containing abnormal mitochondrial accumulations. Patients typically present with severe hypotonia and respiratory failure. Previous studies in Mtm1 mutant mice demonstrated efficacy of gene therapy to treat the disease. We also previously reported that administration of a recombinant adeno-associated virus serotype 8 (AAV8) vector expressing myotubularin under the muscle-specific desmin promoter via high pressure regional hind limb perfusion prolonged life and restored muscle function in myotubularin deficient dogs. More recently, we performed a complete dose escalation study testing the efficacy of a simple AAV8-MTM1 injection into the cephalic vein of XLMTM dogs. AAV8-MTM1 was administered at 10 weeks of age and dogs were followed for 9 months. A comprehensive readout panel including limb strength, gait, respiratory function, neurological assessment, histology, vector biodistribution, transgene expression and immune response over the nine month study was performed, defining the minimally effective dose. Results from these studies are important for the design of a clinical trial in XLMTM patients.

P159

MicroRNAs regulated vectors

K Charton[1 2] J Poupiot[1 2] C Roudaut[1 2] I Richard[1 3]

1: Généthon, R&D Department, INTEGRARE Research Unit, Evry, F_91002, France 2: INSERM, U951, Evry, F_91002, France 3: CNRS UMR 8115, Evry, F_91002, France

MicroRNAs (miRNAs) are small non-coding RNAs that are involved in downregulating gene expression by recognizing in a sequence-specific manner target mRNAs. We took advantage of endogenously expressed microRNAs for regulating new AAV vectors for gene therapy for Limb Girdle Muscular Dystrophy 2A and 2D, two recessive muscular disorders caused by deficiency in calpain 3 or α-sarcoglycan, respectively. We previously reported efficient AAV-mediated transfer of α-sarcoglycan in α-sarcoglycan deficient mice, resulting in correction at the biochemical, histological and functional levels. However, a specific immune response directed against the transgene was induced after intramuscular injection, leading to disappearance of expression in muscle. In an attempt to reduce the immune response, we incorporated a target sequence of a microRNA specifically expressed in antigen-presenting cells (miRNA142-3p) in the AAV cassette. The use of this miRNA-regulated vector reduced humoral immune responses against the transgene, leading to long-term expression of α-sarcoglycan. Similarly, we previously reported efficient AAV-mediated transfer of calpain 3 intramuscularly in calpain 3 deficient mice. However, use of this AAV systemically showed adverse events in the heart due to the expression of calpain 3. We investigated the possibility to prevent cardiotoxicity of this vector by introducing a target sequence of a specific cardiac miRNA (miR208) in the AAV cassette. After systemic injection, we showed specific regulation of the transgene in the heart and prevention of cardiac adverse events. Both these examples illustrate the potency of microRNA-regulated vectors to facilitate sustained and safe therapeutic effects of rAAV gene transfer in muscle.

P160

High expression and therapeutic efficiency of systemic delivery of a dual AAV strategy in a murine model for dysferlin deficiencies

M Pryadkina[1 4] W Lostal[1 4] N Bourg[1] K Charton[1] C Roudaut[1] M L Hirsch[2 3] I Richard[1]

1: Genethon, Integrare Research Unit, UMR_S951, Evry F91002, France 2: Gene Therapy Center, University of North Carolina, Chapel Hill, North Carolina, USA 3: Department of Ophthalmology, University of North Carolina, Chapel Hill, NC, 27599 4: Equal Contribution

Recombinant AAV (rAAV) is currently the best vector for gene delivery into the skeletal muscle. However, the 5-kb packaging capacity of this virus is a major obstacle for large gene transfer. This past decade, many different strategies were developed to circumvent this issue (concatemerization-splicing, overlapping vectors, hybrid dual or fragmented AAV). In this study, we compared large gene transfer techniques to deliver the DYSF gene into the skeletal muscle. Loss of function mutations in the DYSF gene whose coding sequence is 6.2kb lead to progressive muscular dystrophies (LGMD2B, MM; DMAT). After rAAV8 intramuscular injection into dysferlin deficient mice of vectors corresponding to all existing strategies, we showed that the overlap strategy was the most effective approach to reconstitute a full-length messenger. After systemic administration, the level of dysferlin obtained on different muscles corresponded to 0.5 to 2 fold compared to the normal level. We further demonstrated that the overlapping vector set was efficient to correct the histopathology, resistance to eccentric contractions and whole body force in the dysferlin deficient mice up to one year after injection. Altogether, these data indicate that using overlapping vectors could be a promising approach for a potential clinical treatment of dysferlinopathies.

P161

AAV-mediated gene transfer of FKRP improves muscle function in a new mouse model of Limb Girdle Muscular Dystrophy 2I

E Gicquel[1] K Charton[1] I Richard[1]

1: Genethon, Integrare Research Unit, UMR_S951, Evry F91002, France

Dystroglycanopathies constitute a group of genetic diseases caused by defective glycosylation of alpha-dystroglycan (aDG), a membrane glycoprotein involved in the cell/matrix anchoring of muscle fibers. The aDG glycosylation, a very complex process, requires many proteins whose functions are not fully elucidated. In particular, mutations in the FKRP gene encoding Fukutin related protein, lead to hypoglycosylation of aDG, resulting in different forms of dystroglycanopathies, among which Limb Girdle Muscular Dystrophy type 2I (LGMD2I). 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 abnormal glycosylation of

aDG and reduction of laminin binding. Functional muscle impairment can be observed as early as 2 months of age by a decrease of the muscle resistance to eccentric mechanical stress. To evaluate gene transfer as a therapeutic approach, we cloned the FKRP cDNA in an AAV vector under the transcriptional control of the desmin promoter. The recombinant AAV2/9 vector was injected intramuscularly or intravenously in the mouse model. Expression of the FKRP transgene restored glycosylation of aDG as well as laminin binding. The AAV vector decreased centro-nucleation and improved muscle function, conferring a better resistance to eccentric stress to the injected muscles. Experiments are now in progress to evaluate the minimum dose of vector to be administrated for reversion of the phenotype.

P162

Hepatic AAV-mediated gene transfer to reduce immune responses against alpha-sarcoglycan

J Poupiot[1] I Richard[1]

1: Genethon, Integrare Research Unit, UMR_S951, Evry F91002, France, Evry, France

Alpha-Sarcoglycanopathy (Limb-Girdle Muscular Dystrophy type 2D, LGMD2D) is a recessive muscular disorder caused by deficiency in α-sarcoglycan (SGCA), a transmembrane protein part of the dystrophin-associated complex. We previously reported efficient systemic AAV-mediated transfer of SGCA in Sgca deficient mice (Sgca-null mice), resulting in correction at the biochemical, histological and functional levels. Whereas delivery of the AAV vector by the systemic route was efficient, a specific humoral immune response directed against α-sarcoglycan was observed after intramuscular injection, leading to disappearance of transgene expression in muscle fibers, production of antibodies against the transgene and presence of CD8 T lymphocytes around the transduced fibers. We hypothesized that transduction of the liver could play a crucial role in the absence of rejection of the transgene product after systemic injection. To validate our hypothesis, Sgca-null mice injected intramuscularly with an AAV6-SGCA vector inducing an immune response were concomitantly injected in liver with an AAV9-SGCA. We observed that the liver transduction reduced significantly the immunogenic features induced by the intramuscular injection. Indeed, an improvement of the stability of the transduced fibers, a decrease of antibodies directed against the SGCA protein and a reduction of CD8 T lymphocyte infiltrates around the transduced fibers were detected. In parallel, the liver injection did not change the humoral immune response against the capsid. In conclusion, we propose that AAV liver transduction plays an essential role in the tolerance of the SGCA transgene product after systemic administration.

P164

Serum and urine proteomic profiling reveals biomarkers suitable for monitoring the outcome of therapeutic interventions in muscular dystrophies

J Rouillon[1] J Poupiot[1] A Zocevic[1] T Leger[3] C Garcia[3] J M Camadro[3] B Wong[4] L Servais[2] T Voit[2] I Richard[1] F Svinartchouk[1]

1: Généthon 2: Institut de Myologie 3: Institute Jacques-Monod 4: Cincinnati Children's Hospital Medical Center

Therapy-responsive biomarkers are an important and unmet need in the muscular dystrophy field where new treatments are currently in clinical trials. By using a comprehensive high-resolution mass spectrometry approach, and antibody arrays and western blot validation, we found that several proteins and protein fragments are abnormally present in sera and urines of Duchenne muscular dystrophy (DMD) patients, limb-girdle muscular dystrophy type 2D (LGMD2D) and their respective animal models. Levels of one the found biomarker, fragments of the myofibrillar structural protein myomesin-3 (MYOM3) were assayed in therapeutic model systems where stable restoration of α-sarcoglycan expression in KO-SGCA mice was achieved by systemic injection of a viral vector. Following administration of the therapeutic agents MYOM3 was restored toward wild-type levels in a dose-dependent manner. MYOM3 fragments showed lower inter-individual variability compared with the commonly used creatine kinase assay, and correlated better with the restoration of the dystrophin-associated protein complex and muscle force. These data suggest that the MYOM3 fragments hold promise for minimally invasive assessment of experimental therapies for DMD and other neuromuscular disorders. Molecular mechanisms of serum and urine biomarkers manifestation in DMD patients will be discussed.

P165

Functionalization of Gold Nanoparticles Enhance Gene Expression in vitro

G G Lazarus[1] M Singh[1]

1: Non-Viral Gene Delivery Laboratory, Discipline of Biochemistry, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa

Gold nanoparticles have attracted strong biomedical interest for drug/gene delivery due to their low toxic nature, surface plasmon resonance and capability of increasing the stability of the payload. The present study focuses on the synthesis of nanoparticles consisting of a gold core coated with polyethyleneimine and chitosan. All functionalized gold nanoparticles (FAuNPs) were characterized by UV spectroscopy, EM and zeta sizing. Spherical particles in the size range 11.9-195 nm with narrow particle distributions and low PDI (<1.2) were observed. FAuNPand pCMV luc-DNA complexation was efficiently demonstrated in the band shift and ethidium bromide intercalation assays respectively, with serum nuclease digestion revealing some protection of the complexed DNA. MTT cytotoxicity experiments indicated that the FAuNPs elicited a dose dependent cytotoxic effect in four mammalian cell lines (HepG2, HEK293, HeLa and Caco2). Cell viability for Au-PEI/DNA was $>80\%$ across all cell lines, and approximately 126% for Au-Chit/DNA in HepG2 cells. Transfection studies were accomplished using the luciferase reporter gene assay. Results showed that the FAuNPs produced greater transgene activity than the cationic polymer/DNA complexes on their own. This was evident for the Au-PEI/DNA complex which produced a 12 fold increase in the HEK293 cells and a 9 fold increase in the HepG2 cells, compared to the PEI/DNA complexes. Results suggest that FAuNP's low cytotoxicity and good gene expression, coupled with the ability to parametrically control particle size and surface properties, necessitates their further optimization for future in vivo and possible theranostic applications.

P166

Tailored trimethyl chitosan nanoparticles for specific gene delivery to the nervous system

C P Gomes[1 2 3] A Varela-Moreira[1 2 4] M Gomez-Lazaro[1 2] C D F Lopes[1 2 4] V Leiro[1 2] P M D Moreno[1 2] A P Pêgo[1 2 3 5]

1: INEB - Instituto de Engenharia Biomédica, Universidade do Porto, Portugal 2: I3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal 3: Faculdade de Engenharia da Universidade do Porto, Portugal 4: Faculdade de Medicina da Universidade do Porto, Portugal 5: ICBAS - Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal

Herein we propose trimethyl-chitosan (TMC) for the development of a gene vector targeted to the peripheral nervous system. One of the most relevant features of non-viral vectors for gene therapy concerns their ability to be degraded and the fate of the resulting by-products of degradation. So, we first investigated the impact of polymer degree of acetylation (DA) on enzymatic degradation and biological performance of TMC-based nanoparticles, exploring top-notch bioimaging techniques as imaging flow cytometry (ImageStreamX). Our results confirm that the biodegradation rate of nanoparticles affects their intracellular trafficking and pDNA protection ability and, consequently, their transfection efficiency. Nanoparticles based on TMC with 11% DA (TMC11) were the most efficient to transfect neuronal cells among the tested formulations (DAs: 2, 11 and 17%). Subsequently, the non-toxic fragment of the tetanus toxin (HC) was grafted to TMC11-based nanoparticles via a bi-functional PEG linker. Besides being specific to neurons, HC is also able to undergo retrograde transport, which is of critical importance to allow a peripheral administration of the nanoparticles. To evaluate the targeting potential of the nanoparticles, molecular recognition force spectroscopy (MRFS) was used to characterize the interaction between the nanoparticles and HC cell receptors. The results confirm that targeted nanoparticles specifically interact with neuronal cells, with higher binding probability and force. Also, a significantly decrease in the uptake of these nanoparticles in non-neuronal cells was observed, corroborating the MRFS results. Taken together, our results suggest that the developed neuron-targeting nanoparticles are a promising tool for peripherical neuronal regeneration.

P167

Enhancement of chitosan-based transfection efficiency with nuclear localization signal peptides

J Morais[1 2] A V Oliveira[1 3] S M Calado[1 3] G A Silva[1 4]

1: Centre for Biomedical Research (CBMR), University of Algarve, Faro, 8005-139, Portugal 2: Department of Biomedical Sciences and Medicine, University of Algarve, Faro, 8005-139, Portugal 3: Doctoral Program in Biomedical Sciences, Department of Biomedical Sciences and Medicine, University of Algarve, Faro, 8005-139, Portugal 4: CEDOC - Chronic Diseases Research Center, NOVA Medical School / Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Campo dos Mártires da Pátria 130, 1169-056 Lisboa

The successful use of non-viral gene carriers has been hampered by their modest transfection efficiency results. Nuclear import is considered the major limiting step in the development of effective non-viral gene delivery systems. Recent efforts to improve non-viral gene delivery include the use of nuclear localization signals (NLS) that can mediate nuclear intake, therefore improving transfection. Most studies have used peptides derived from viruses (e.g. SV40) that can lead to increased immunological reactions by the host tissue. In this work, an endogenous NLS assisted method, based on Insulin-like Growth Factor Binding Proteins (IGFBPs), namely IGFBP-3 and IGFBP-5, is evaluated in order to ameliorate nuclear translocation without compromising the fairly low immunological profile of non-viral vectors. Several strategies were tested in order to determine their effect in chitosan polyplex-mediated transfection efficiency in HEK293T cells: co-administration, co-encapsulation and covalent ligation to chitosan polyplexes. Our results show that transfection efficiency is concentration dependent and varies with the delivery method employed. Moreover, co-encapsulation and covalent ligation yielded a 2-fold increase in transfection efficiency associated with the use of IGFBP-3. On going work will expand the range of tested concentrations as well as the type cell lines tested. Also, in order to allow for intracellular tracking of the delivered DNA it will be labeled with a fluorescent marker. The authors acknowledge the financial support of Fundação para a Ciência e Tecnologia (PTDC/SAU-BEB/098475/2008, SFRH/BD/70318/2010, SFRH/BD/76873/2011) and to IBB/LA under the project PEst-OE/EQB/LA0023/2013 and the Marie Curie Reintegration Grant (PIRG-GA-2009-249314) under the FP7 program.

P168

Improving non-viral modification of endothelial cells – magnetically driven approach.

N Voronina R David G Steinhoff

1: Referenz- und Translationszentrum für kardiale Stammzelltherapie

Aim: Controlled genetic manipulation of angiogenesis bears huge potential for treatment of various diseases. The success in the development of clinically suitable tools, nevertheless, has not been achieved yet. Targeted delivery of nucleic acids with nanoparticle-based nonviral carriers is among the most promising approaches in the field.

Methods: During preliminary studies we optimized in vitro the delivery of microRNA and plasmid DNA into endothelial cells (HUVECs) with polyethyleneimine and magnetic nanoparticle-based vectors (PEI-MNP). Flow cytometry was used for the evaluation of transfection results either with fluorescently labeled microRNA or with eGFP plasmid. Further, two types of targeting experiments in vitro were carried at optimal range of transfection conditions. First, a possibility of localized nucleic acid delivery was studied, using laser scanning confocal microscopy for results evaluation. Second, magnetic responsiveness of transfected cells was assessed. In addition, magnetic content of cells, sufficient for their responsiveness to magnetic field, was quantified using magnetorelaxometry.

Results: High cell viability (85–90%) and highly efficient transfection of HUVECs were observed under the optimized parameters. Moreover, local delivery of nucleic acids into HUVECs was demonstrated under application of magnetic field. Furthermore, the majority of transfected cells were attracted to magnet.

Conclusions: Our findings show that magnetically driven delivery of microRNA and DNA with nanoparticle-based vectors allows efficient and controlled uptake of the introduced molecules by endothelial cells. Furthermore, responsiveness of modified cells to magnetic field indicates the potential for their magnetic guidance after transplantation in vivo. This may become an important prerequisite for therapeutic modification of angiogenesis.

P169

Efficacy of human interleukin-10 gene transfer to human liver segments "ex vivo" by hydrodynamic injection

L Sendra[1] A Miguel[1] M J Herrero[2] L Sabater[3] E Montalvá[4] R Lópe-Andújar[4] S F Aliño[1]

1: Universitat de València 2: Instituto de Investigacion sanitaria La Fe 3: Hospital Clínico Universitario de Valencia 4: Hospital La Fe

Introduction: The expression of human interleukin-10 after retrovenous hydrodynamic hIL10 gene transfer, in watertight human liver segments, was studied. Material and Methods. Watertight human liver segments proceeding from surgical resection were transfected with human interleukin-10 gene by placing a 9F catheter in a suprahepatic vein, previously referenced by the surgeon. Liver segments were injected with a volume equivalent to 1/5 segment weight of saline solution bearing p2F-hIL10 plasmid (20 μg/mL), containing the hIL10 gene, via cava at 10 and 20 ml/s. The hIL10 gene transfer and expression efficiency were studied in 3 different liver zones according to injection influence (A: direct, B: intermediate, C: remote). Results were compared with control liver transfected with eGFP. Results. The results showed: (i) 10 ml/s flow rate mediated a slightly higher gene delivery in target zone-A whereas the most homogeneous gene delivery throughout the liver occurred with 20 ml/s; (ii) 10 and 20 ml/s injection rates mediated similar indexes of transcription (20–70 hIL10 RNA copies/cell, corresponding to proteins class of low-intermediate expression); (iii) slightly higher hIL10 expression (20-200 copies/cell, corresponding to low abundance proteins class) was detected with 20 ml/s. Conclusion. Data suggest that hydrodynamic retrovenous transfer of human IL10 gene to human liver segments mediates the expression of the protein. Although mild conditions (10 ml/s) mediate the expression of the protein in all liver segment zones, the use of more demanding conditions (20 ml/s) could improve the plasmid distribution to remote areas in large segments. Supported by SAF2011-27002.

P170

Micro-minicircle vectors for splice-switching in mouse model of Duchenne Muscular Dystrophy

S Stenler[1 2] S EL Andaloussi[1 3] S Hammond[3] A Goyenvalle[4] M JA Wood[3] D J Wells[5] K E Lundin[1] C IE Smith[1] P Blomberg[1 2]

1: Karolinska Institute 2: Karolinska University Hospital 3: The University of Oxford 4: Université de Versailles St-Quentin 5: Royal Veterinary College, London

Duchenne muscular dystrophy (DMD) is a fatal X-linked muscle wasting disease for which there is no effective therapy to date. Although progress is being made in developing genetic therapies to address the primary deficit (absence of functional dystrophin) the methods used are associated with diverse problems. Replacement therapy is hampered by the large size of the dystrophin coding sequence, and although rescue of protein expression by splice-switching using synthetic antisense oligonucleotides has been shown to ameliorate the disease, the effect is transient. Transgenic expression of the splice-switching antisense molecules is an appealing alternative and viral vectors encoding splice-switching RNAs have been shown to restore function in various animal models. However, the need for repeat treatment makes the viral delivery rout unsuitable due to host immunity towards the vector. The minicircle is an improved plasmid based non-viral vector, which has a higher and more stable expression than conventional plasmids. In this study, we assay the minicircle as vector for splice-switching in DMD, in vitro and in vivo in a mouse model using different methods for delivery to the skeletal muscle. We show that it is possible to use the minicircle to express small regulatory RNAs for splice-switching, and that dystrophin levels are increased. However, development of suitable delivery methods is required to realize the full potential of minicircles in vivo.

P171

Non-viral shRNA delivery system; Gene silencing of 5α-Reductase 2 for the treatment of androgenic alopecia

G Erel H Akbaba[1] A G Kantarci[1]

1: Department of Pharamaceutical Biotechnology, Faculty of Pharmacy, Ege University, Izmir, 35100, Turkey

Adrogenic alopecia (AA) is the most common form of hair loss. Causal factors of AA are genetic susceptibility and androgens. The responsible androgen of AA is dihydrotestosteron (DHT). The conversion of testosterone to DHT is catalysed by 5α-reductase-2 (5-αR2). Then, DHT binds the androgen receptors with an avidity five-fold higher than testosterone and leads to alopecia development. Therapeutics based on short hairpin RNAs (shRNAs), which act by inhibiting the expression of target transcripts, represent a new class of highly specific treatments. Therefore, we aimed to develop a non-viral gene delivery system for topical delivery of 5-αR2 shRNA plasmid in order to treat AA. Nanoparticles were produced by ionic gelation between Chitosan (CS) and Sodium Tripolyphosphate (TPP). Different ratios of CS and TPP were examined for appropriate particle size and zeta potential. Selected nanoparticles according to their characterization results were evaluated for complexation with shRNA encoding plasmid DNA using agarose gel retardation assay. Glycerol was added as a plasticizer and Tween 80 was added as permeation enhancer to the formulation. The selected formulation was evaluated for in vitro DNA release and DNase I protection ability. Cytotoxicity of complexes was determined on Human Follicle Dermal Papilla Cells (HFDPCs) using XTT cell proliferation assay kit. According to the results, developed formulation loaded with 5-αR2 shRNA-encoding plasmid has promising potential as RNAi therapeutic for treatment of androgenic alopecia.

P173

Messenger RNA-based therapy for an apoptosis-related disease in the liver

S Uchida A Matsui K Kataoka K Itaka[1]

1: The University of Tokyo

Excessive apoptosis is critical in the pathogenesis of several intractable diseases. For treating such diseases, gene therapy allows for sustainable introduction of anti-apoptotic factors. However, DNA delivery has the risk of random genomic integration, leading to overexpression of the delivered gene. Overexpression of anti-apoptotic factors would induce cancer development. Messenger RNA (mRNA) does not have such risks. In this study, we examined the feasibility of mRNA-based therapy for preventing apoptosis in diseases by using a mouse model of fulminant hepatitis. For introducing mRNA into the liver with hydrodynamic intravenous injection, mRNA was loaded into polyplex nanomicelles, which have a core-shell structure of a poly(ethylene glycol) shell and a core containing mRNA. In a reporter analysis using GFP, mRNA delivery induced efficient GFP expression in almost 100% of liver cells, whereas at most 20% of liver cells became GFP positive after plasmid DNA (pDNA) delivery. After injection of Cy5-labeled pDNA, only weak Cy-5 signal was detected in nuclei of liver cells, demonstrating that nuclear transport is a major obstacle for pDNA delivery. In contrast, Cy5-labeled mRNA was observed in the cytoplasm of almost 100% of liver cells, which would lead to homogeneous protein expression profile. Consistent with these observations, injection of Bcl-2-encoded mRNA remarkably reduced the number of apoptotic cells in the liver of mice with fulminant hepatitis compared to that in saline-treated control mice, whereas that of Bcl-2-encoded pDNA showed almost no therapeutic effect. Therefore, mRNA-based therapy using polyplex nanomicelles is promising for treatment of apoptosis-associated diseases.

P174

PDMAEMA as gene therapy vector: an intracellular insight

D B Bitoque[1 3 4] A R Costa[2 3] G A Silva[4]

1: ProRegeM PhD Program, Department of Biomedical Sciences and Medicine, University of Algarve, 8005 Faro, Portugal 2: Department of Chemistry and Pharmacy, University of Algarve, Faro, Portugal 3: Algarve Chemistry Research Centre (CIQA), University of Algarve, Faro, Portugal 4: CEDOC, NOVA Medical School / Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Campo Mártires da Pátria 130, 1169-056 Lisboa, Portugal

Ocular diseases are among the most debilitating conditions affecting all segments of the population. Gene therapy is an alternative to traditional treatments, and adequate carriers for delivery are critical. It is known that an efficient gene delivery system must overcome several steps to be successful: enter the cell by crossing the cell membrane; escape the endo-lysosomal degradation pathway; release the genetic material; traffic the intracellular milliey and nuclear entry; and lastly, express the gene of interest. We and others have demonstrated the potential of poly(2-(N,N'-dimethylamino)ethylmethacrylate) (PDMAEMA) as a gene therapy vehicle. Studies of the intracellular pathway of PDMAEMA are scarce and therefore the goal of this study is to determine the cellular entry and intracellular trafficking mechanisms of our PDMAEMA vectors. We have used rhodamine-labeled PDMAEMA vectors to transfect retinal cells and several markers of the endocytic pathways: clathrin- and caveolin- dependent pathways to assess the mechanism of cellular entry. To study intracellular trafficking we used markers for intracellular organelles that can be involved in the trafficking: early endosomes, lysosomes, endoplasmic reticulum and Golgi apparatus. Our studies so far show that PDMAEMA polyplexes have phsysicochemical properties compatible with gene transfer: the polyplexes efficiently and quickly enter the cell within minutes and seem to escape endo-lysosomal degradation and accumulate in the perinuclear area. Further studies will focus on determining the efficiency of nuclear penetration. Acknowledgements: Fundação para a Ciência e Tecnologia, Portugal (PEst-OE/EQB/LA0023/2013; PEst-OE_QUI_UI4023_2011; PD/BD/52424/2013) and EC Marie Curie Reintegration Grant (PIRG-GA-2009-249314).

P175

Chitosan and hyaluronic acid polyplexes achieve efficient retinal gene delivery

A V Oliveira[1 2] S M Calado[1 2] G A Silva[1 3]

1: Centre for Biomedical Research (CBMR), University of Algarve, Faro, 8005-139, Portugal 2: Doctoral Program in Biomedical Sciences, Department of Biomedical Sciences and Medicine, University of Algarve, Faro, 8005-139, Portugal 3: CEDOC - Chronic Diseases Research Center, NOVA Medical School / Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Campo dos Mártires da Pátria 130, 1169-056 Lisboa

Chitosan is one of the most promising candidates with several reports supporting its role as a drug and gene delivery vehicle. Its applicability is however hindered by its low transfection efficiency. Our previous work pointed to an improved performance with the incorporation of hyaluronic acid into chitosan-based vectors; to prove this hypothesis, we performed a pair-wise comparison between chitosan and chitosan-hyaluronic acid polyplexes. Their in vitro transfection efficiency was assessed in human embryonic kidney cells (HEK293T) and human retinal pigmented epithelium cells (ARPE-19), as well as in vivo in the retina of C57Bl6 mice. Our results show that chitosan-hyaluronic acid polyplexes showed a marked improvement in the in vitro and in vivo gene transfer efficiency over chitosan polyplexes. The authors acknowledge the financial support of Fundação para a Ciência e Tecnologia (PTDC/SAU-BEB/ 098475/2008, SFRH/BD/70318/2010, SFRH/BD/76873/2011) and to IBB/LA under the project PEst-OE/EQB/LA0023/2013 and the Marie Curie Reintegration Grant (PIRG-GA-2009-249314) under the FP7 program.

P176

Development of CXCR4-targeted peptide carriers for anti-VEGF siRNA delivery into endothelium for anti-angiogenic gene therapy

A V Kiselev[1] A A Egorova[1] A N Shubina[2] M S Bogacheva[1] V S Baranov[1 2]

1: D.O.Ott Research Institute of Obstetrics and Gynecology 2: Saint-Petersburg State University

Inhibition of angiogenesis by means of small interfering RNA (siRNA) for treatment of solid tumors is a promising approach to anti-angiogenic cancer therapy. For this purpose targeted non-viral vectors can be developed. Previously, we developed DNA carriers targeting CXCR4, a receptor expressed on a variety of solid tumors and on the endothelial cells of angiogenic vessels. Aim of this study is a development of CXCR4-targeted peptide carriers for down-regulation of vascular endothelial growth factor (VEGF) expression via

anti-VEGF siRNA delivery into endothelium. Group of modular peptides conjugated with CXCR4 ligand and their complexes with siRNA were studied for physicochemical and toxic properties. Transfection efficacy of the polyplexes was studied on CXCR4(+) cells, such as endothelial hybridoma E.A.Hy926 and glioblastoma A172. We showed that the peptides efficiently bind siRNA and protect it from enzymatic degradation and the polyplexes are not toxic for cells. siRNA delivery by means of ligand-conjugated carriers resulted in 2.5-3-fold decrease of VEGF expression in glioblastoma cells and in 1.5-2-fold decrease in endothelial cells in comparison with delivery of mock siRNA. Also delivery of targeted polyplexes resulted in 2-6-fold decrease in VEGF protein production by endothelial cells and resulted in significant inhibition of endothelial cells migration in vitro. The study demonstrates that down regulation of VEGF expression in endothelium can be achieved by siRNA delivered with CXCR4-targeted peptide-based carriers. The latter can be suggested as promising agents for anti-angiogenic therapy. The work was supported by RFBR grant (15-04-00591-a) and by the President of Russia personal fellowship (SP-2162.2015.4).

P177

S/MAR exerts a positive effect on pFAR plasmid function upon transfer in hepatic cells

A Giannakopoulos[1] C Marie[2] E F. Stavrou[1] M Verras[1] M Quiviger[2] D Scherman[2] A Athanassiadou[1]

1: University of Patras Medical School 2: CNRS-Inserm-Univ Paris Descartes-Chimie ParisTech

Scaffold/Matrix Attachment Region (S/MAR) are AT rich chromosomal elements that play a role in chromatin boundary formation. Their inclusion in plasmid vectors enhances episomes' retention in the nucleus, thus facilitating their long term persistence. We used S/MAR to enhance the performance of the pFAR4 vector. pFAR derivatives are plasmids Free of Antibiotic Resistance markers. Their propagation in bacteria relies on the suppression of a chromosomal amber mutation by a plasmid-borne function. They are efficiently delivered into muscle, tumours, skin and liver, leading to superior and/or prolonged transgene expression. We produced three pFAR derivatives carrying the eGFP gene driven by the composite HCRHPi liver-specific promoter and either lacking (control plasmid) or containing a S/MAR element inserted upstream or downstream from the SV40 polyadenylation signal (S/MAR-IN or S/MAR-OUT constructs). All three vectors were successfully transferred into the Huh7 human hepatic cell line by Lipofection. The S/MAR-IN plasmid showed higher performance than the control or S/MAR-OUT plasmids, in terms of transfection efficiency (11%, 5%, and 3% respectively) and mean fluorescent intensity (2000, 800, and 1600 units respectively). The establishment of cell colonies showed that only colonies of cells transfected with the S/MAR-IN construct were fluorescent after 2 months of cell culture. Thus, in comparison with the control or S/MAR-OUT construct, the introduction of the S/MAR element upstream from the SV40 polyadenylation signal appears to enhance pFAR maintenance. Future work will focus on the study involved in sustained GFP expression, including investigation of possible transgene integration into cell genomes.

P178

Solid lipid nanoparticles for gene silencing in chemoresistant cancer cells

M Kotmakçi[1] V B Çetintas[2] G Ertan[3] A G Kantarci[1]

1: Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Ege University, Izmir, Turkey 2: Department of Medical Biology, Faculty of Medicine, Ege University, Izmir, Turkey 3: Department of Pharmaceutical Technology, Faculty of Pharmacy, Ege University, Izmir, Turkey

Cancer therapy is often impaired by chemoresistance. Signal transducer and activator of transcription-3 (STAT3) is recognized as a molecule responsible for development of resistance in cancer cells. Its high expression is correlated with high survival, proliferation, neovascularization and metastasis. We aimed to prepare nonviral gene delivery systems, loaded with STAT3 shRNA-encoding plasmid, and to test their effect on cisplatin resistant (CR-Calu1) non-small cell lung cancer cells. For this purpose we prepared cationic solid lipid nanoparticles (cSLNs) by the hot microemulsion method and characterized these formulations in terms of particle size, zeta potential and morphology. cSLNs having size well below 100 nm and zetapotential above + 30mV were tested for complexing ability with the plasmid. cSLN:DNA complexes were further characterized in terms of complexation, in vitro DNaseI and serum protection ability. Toxicity of cSLN:DNA complexes was determined on normal lung fibroblast cells. Low-toxic doses were used to treat CR-Calu1 cells. Suppression of STAT3 was evaluated at mRNA level by qRT-PCR. Selected cSLNs were able to complex the plasmid and cSLN:DNA complexes with particle size below 100 nm and zetapotential about + 10 mV were obtained. These complexes maintained plasmid's integrity against DNaseI and serum treatment. Selected formulations reduced STAT3 expression in resistant Calu1 cells by 75%. The susceptibility of resistant Calu1 cells to cisplatin was enhanced about four times by silencing of STAT3 with the developed formulation. In conclusion, developed formulations provide efficient STAT3 silencing and improve the activity of cisplatin in resistant cancer cells.

P180

Building up artificial virus for gene delivery.

L Pigeon C Gonçalves C Pichon P Midoux

1: CNRS 2: Centre de Biophysique Moléculaire

Problems associated to toxicity, immunogenicity and production of viral vectors for human gene therapy continue to make non-viral vectors a pertinent alternative. Non-viral vector technology has developed plenty of strategies to formulate plasmid DNA (pDNA) with cationic lipids and/or cationic polymers. Electrostatic complexes between a plasmid DNA (pDNA) and positively charged liposomes or polymers form spontaneously supramolecular assemblies called lipoplexes and polyplexes, respectively. Nevertheless, the relatively low transfection capacity reached with those synthetic vectors still limits their clinical applications. Several advances are presented for the building of an artificial virus. pDNA must escape from endosome after endocytosis, migrate toward the nuclear envelope and pass through the nuclear pores of non-dividing cells. DNA escape from endosomes is improved by using polymers and liposomes containing histidine moieties

(1). The nuclear delivery of DNA is favoured by the insertion in the DNA sequence of nucleic acids sequences specifically recognized by the transcription factor NFkB (2). The transfection efficacy is improved when DNA is linked to a microtubule-specific peptide that allows DNA migration toward nuclear envelop (3). We show that the supra-molecular assembly of pDNA with those devices favours its penetration, step by step, into the cell nucleus.

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P181

Development of a strategy to assess Adeno-Associated Virus-based optogene transfer in a canine model of inherited retinal dystrophy

B Ameline[1] K T Tshilenge[1] M Weber[2] G Le Meur[2] J Y Deschamps[3] A Mendes-Madeira[1] N Provost[1] M Biget[1] S Nedellec[4] V Pichard[1] P Moullier[1 5] F Rolling[1]

1: Atlantic Gene Therapies, INSERM UMR1089, Université de Nantes, CHU 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: Cellular and Tissular Imaging Core Facility of Nantes University, Nantes, France 5: Department of Molecular Genetics and Microbiology, College of Medicine, University of Florida, Gainesville, Florida, USA

Purpose: Recombinant Adeno-Associated Virus (AAVr)-mediated optogene (coding for light-sensitive proteins) transfer into retinal ganglion cells (RGCs) has emerged as a promising strategy to restore photosensitivity in the context of inherited retinal dystrophies (IRD). Optogene transfer in rodent models of IRD demonstrated restoration of visual functions. Assessment of Opn4 optogene transfer in large animal models of IRD is a key step prior evaluation in clinical trials. In this study, we have validated the Opn4 optogene functionality and optimized the RGC transduction prior future injections of rAAV-Opn4 in canine models of IRD.

Methods: We produced an AAV2-plasmid vector containing the Opn4 sequence under the control of the CMV promoter. Opn4 expression (melanopsin protein) and functionality were validated by immunocytochemistry and calcium sensor analysis. Moreover, we generated rAAV2/2 vectors carrying the eGFP cDNA under control of the CMV promoter. We performed intravitreal injection of the vector in healthy dogs. Native eGFP expression was monitored by funduscopic imaging and confocal microscopy.

Results: The plasmid vector containing the opn4 transgene was able to drive the expression of a functional melanopsin protein, resulting in an increase of intracellular calcium after light stimulation in HEK293 cells. We have also demonstrated that intravitreal injection of a rAAV2/2-eGFP vector results in efficient transduction of the canine retina.

Conclusion: We have settled the tools to perform a relevant evaluation of rAAV-mediated opn4 optogene transfer in a large animal model of IRD. The next step will be the evaluation of the efficacy of this strategy in canine models of IRD.

P182

Stable genomic integration of PEDF in primary pigment epithelial cells transfected with the Sleeping Beauty transposon system to treat age-related macular degeneration (AMD)

C Prat-Souteyrand[1] S Tobalem[1] N Harmening[1] M Kropp[1] S Johnen[2] D Scherman[3] C Marie[3] Z Izsvák[4] G Thumann[1]

1: Department of Ophthalmology, University Hospitals of Geneva, Rue Alcide-Jentzer 22, Geneva 14, 1211, Switzerland 2: Department of Ophthalmology, University Hospital RWTH Aachen, Aachen, 52074, Germany 3: Unité de Technologies Chimiques et Biologiques pour la Santé, INSERM U1022 – CNRS UMR8258, Paris, 75270, France 4: Max Delbrück Center for Molecular Medicine, Berlin, 13092, Germany

Exudative AMD is a progressive pathology and the major cause of blindness in the elderly. It is characterized by a vascular endothelial growth factor (VEGF)-induced choroidal neovascularization (CNV), retinal pigment epithelial (RPE) cells and photoreceptors degeneration. Currently 30–40% of patients can be successfully treated by monthly intravitreal injections of anti-VEGFs. To develop a one-time treatment, we have defined a protocol to transplant autologous pigment epithelial cells, transfected with the anti-angiogenic pigment epithelium-derived factor (PEDF) to inhibit CNV. 10'000 primary human (h)RPE cells were electroporated with antibiotic-resistance free plasmids (pFAR4) encoding the hyperactive Sleeping Beauty (SB100X) transposase, the PEDF-gene and the fluorescent Venus protein. PEDF gene copy number and PEDF expression were quantified by qRT-PCR; recombinant (r)PEDF secretion was analyzed by Western Blot and quantified by ELISA. Efficiency of transfection was $24.67 \pm 14.92\%$ for Venus-transfected hRPE cells and showed stable secretion for up to one year. After 21 days of culture, transfected human RPE cells revealed an expression and a secretion of rPEDF at a level 16-fold higher than non-transfected cells. Medium relative number of integrated PEDF-gene copies exclude potential harmful PEDF overexpression and retain RPE cell gene expression pattern. The present work demonstrates the high efficacy of the SB100X transposon system combined with pFAR4 miniplasmids to integrate the anti-angiogenic PEDF gene into the genome of primary pigment epithelial cells. The transfected cells secreted PEDF stably and continuously; for up to one year the cells have been cultured and presented high expression and secretion levels as soon as after 21 days.

P183

Therapeutic strategies for Huntington's disease based on antisense oligonucleotide

M Imbert[1] G Griffith[1] R Benchaouir[1] C Beley[1] B Dugovic[2] C Leumann[3] L Garcia[1] A Goyenvalle[1]

1: Université de Versailles St-Quentin, INSERM U1179, UFR des Sciences de la Santé, France 2: SYNTHENA, University of Bern, Switzerland 3: Departments of Chemistry & Biochemistry, University of Bern, Switzerland

Huntington's disease (HD) is a neurodegenerative disorder caused by a CAG repeat expansion in the first exon of huntingtin gene (htt). This disease is characterized by motor, psychiatric and cognitive impairments. Currently, there is no treatment. However, reducing the expression of mutant huntingtin protein

using antisense oligonucleotide (AON) has been shown as a promising therapeutic strategy. In this project, I explore and compare the therapeutic potential of tricyclo-DNA (TcDNA)-AONs and vectorized approaches using U7snRNA-based constructs. First, we developed TcDNA-AONs, targeting either the CAG repeats (allele-specific silencing) or the exon 36 (non allele-specific silencing) of htt gene. Our preliminary data show that TcDNA-CAG reduces the RNA level of mutant htt in patient-derived fibroblast cell lines and TcDNA-ex36 silences both mutant and wild-type htt mRNA. In parallel, in order to achieve a stable expression of antisense sequences, we are developing a U7snRNA based system to deliver the therapeutic sequences. The U7snRNA gene can be engineered into a shuttle for antisense sequences and has demonstrated efficient and long term antisense effect in various disease models. We have therefore developed modified U7snRNA constructs targeting the htt mRNA and produced lentiviral vectors for in vitro evaluation in HD patient-derived fibroblast cell lines. The most promising constructs will be transferred into AAV vectors and evaluated in vivo in a HD mice model in order to check the efficiency, toxicity and biodistribution of the modified U7snRNA. This project will thus compare AON-based and vectorized antisense strategies and contribute to the development of therapeutic tools for HD patients.

P184

Over-expression of PEDF by PEDF-transfected primary pigment epithelial cells does not induce tumorigenicity

M Kropp[1] S Tian[1] N Harmening[1] S Johnen[2] D Scherman[3] C Marie[3] Z Izsvak[4] G Thumann[1]

1: Department of Ophthalmology, University Hospitals of Geneva, Rue Alcide-Jentzer 22, Geneva, 1205, Switzerland 2: Department of Ophthalmology, University Hospital RWTH Aachen, Aachen, 52074, Germany 3: Unité de Technologies Chimiques et Biologiques pour la Santé, INSERM U1022 – CNRS UMR8258, Paris, 75270, France 4: Max Delbrück Center for Molecular Medicine, Berlin, 13092, Germany

Under the auspices of FP7 HEALTH 2012 the TargetAMD consortium is developing a treatment for neovascular AMD (nAMD) based on transplantation of autologous pigment epithelial cells genetically modified to overexpress pigment epithelium-derived factor (PEDF), a potent antagonist of vascular endothelial cell growth factor (VEGF). Increased levels of PEDF would antagonize retinal VEGF, which is responsible for subretinal neovascularization, retinal pigment epithelial (RPE) cell and photoreceptor degeneration in nAMD. Bovine and human RPE and iris pigment epithelial (IPE) cells were transfected with the PEDF gene using pFAR4-plasmids together with the hyperactive Sleeping Beauty (SB100X) transposon system, which stably integrates the transgene into the host cell genome to evaluate the tumorigenic potential of the vector system. PEDF-transfected cells were analyzed using the soft agar assay. 100,000 PEDF-transfected RPE and IPE cells were seeded in 2-layer soft agar, inspected every second day microscopically for colony formation followed by crystal violet staining at day 14. ELISA and Western Blot analyses showed that as few as 5,000 transfected RPE and IPE cells stably secrete recombinant PEDF for more than one year. PEDF secretion level is 7.95 ± 2.78 ng/h/104 cells, which is $\sim$30.6-fold higher than in non-transfected cells. Importantly, in the soft agar assay no colonies were observed at any time points in the treated RPE and IPE cells. This report demonstrates that stable integration of the PEDF gene using the pFAR4/SB100X system into primary bovine and human RPE and IPE cells results in a significant increase in PEDF expression and secretion, without evolving detectable tumorigenicity.

P185

The Brain and Spine Institute's core facilities network: a state of the art integrated services for academic and industrial partners.

A Sobczyk[1]

1: Institut du Cerveau et de la Moelle épiniere

The Brain and Spine Institute (ICM) is a private, not-for-profit, Translational Neuroscience Institute based in Paris, France, and rooted in Pitie-Salpetriere, the largest French Neurology Hospital. Main research fields include Parkinson's disease, Alzheimer, Huntington, ALS, epilepsy, Multiple Sclerosis and Neurooncology, with a total of more than 80 ongoing clinical trials. The institute aggregates more than 650 researchers, a clinical research facility, a start-up incubator and a network of 24 advanced core facilities within the same building. These platforms can support early validation of targets or proof of concepts as well as translational researches: 1) Molecular approaches: High throughput genotypes, tools and services for genome analysis, bioproduction of viral and non-viral gene transfer vectors. 2) Cellular investigation: Full-service cell culture core facility with robotized culture, IPS, screening and electrophysiology activities and a histology platform. 3) Cellular imaging: Complete offer with confocal, two-photon and electronic microscopes as well as optogenetic activity 4) Preclinical studies: 3000 sqm for the development of novel animal models, rodents, zebrafish and non-human primates. State of the art behavioral tests for preclinical studies and phenotyping, small animal MRI platform (11,7T). 5) Clinical studies: neuro-imaging (CENIR, 3T MRI), brain activity measure (MEG/EEG, oculomotricity), non-invasive cerebral stimulation, gait and equilibrium studies and a virtual reality platform. These platforms are not only precious research tools for ICM's researchers but also propose integrated services for academic and industrial partners.

P186

Development of a novel anti-angiogenic tool: Tough decoy mediated inhibition of miR-10 for treatment of age-related macular degeneration (AMD)

A Holmgaard[1] A L Askou[1] T Rothmann[1] T Bek[2] A K Hollensen[1] L Aagaard[1] J G Mikkelsen[1] T Corydon[1]

1: Department of Biomedicine, Aarhus University, 8000 Aarhus C, Denmark 2: Department of Ophthalmology, Aarhus University Hospital, 8000 Aarhus C, Denmark

VEGF binding to the KDR receptor is essential in ocular angiogenic diseases including AMD, the primary cause of vision loss in the western world. VEGF either binds to KDR receptors, where binding leads to angiogenesis, or to FLT1 receptors. Binding to FLT1 does not induce angiogenesis but FLT1 functions as a decoy receptor, regulating KDR binding. Expression of FLT1 is reduced by miR-10. We designed a tough decoy (TuD) simultaneously targeting the two isoforms of miR-10 (TuD/miR-10) and cloned it (pTuD/miR-10). A psiCHECK-based vector harboring the miR-10a target sequence fused to the Renilla luciferase gene was designed and used to assess miR-10a availability. Exogenous expression of miR-10a, delivered by the pmiR-10a vector, confirmed psiCHECK

responsiveness to miR-10a. The amount of miR-10a in human embryonic kidney (HEK293) and retinal pigment epithelium (ARPE-19) cells was determined, showing significantly higher expression of miR-10a in HEK293 cells compared to ARPE-19. Next, targeting of endo- and exogenously expressed miR-10a was performed in triple transfected (pTuD/miR-10, psiCHECK, pmiR-10a) ARPE-19 and in double transfected (pTuD/miR-10, psiCHECK) HEK293 cells, respectively. The TuD/miR-10 efficacy was evaluated, showing a dosage responsive inhibition of miR-10a in both cell types. Finally, we have produced lentiviral (LV) vectors expressing LV-TuD/miR-10, -miR-10a and luciferase reporter genes and we are now aiming at assessing the in vivo efficacy of LV-TuD/miR-10 by subretinal injections in a CNV mouse model. We believe that TuDs delivered by LVs may be a novel supplement for dual-therapy together with other anti-angiogenic effectors for treatment of vascular eye diseases, including AMD.

P187

Differentiation and transplantation of embryonic stem cell-derived cone photoreceptor precursors

K Kruczek[1] A Gonzalez-Cordero[1] E L West[1] A Naeem[1] S J Blackford[1] Y Duran[1] R A Pearson[1] R R Ali[1]

1: UCL Institute of Ophthalmology

High acuity colour vision in mammals depends on cone photoreceptors. Their death leads to vision loss, for which a promising treatment strategy would be replacement of lost cones by cell therapy. Recently, it has been demonstrated that rod photoreceptor precursors differentiated from mouse embryonic stem cells (ESC) using a 3D protocol recapitulating in vivo eye development can integrate into adult recipient retinas after transplantation. Given the importance of cone-mediated vision, an analysis of cone differentiation from mouse ESC using various methods has been performed. Increasing numbers of early cone precursors expressing the gene for nuclear receptor Trβ2 appear from day 12 to 18 of differentiation, at which stage they constitute approximately 18% of cells in ESC-derived retinas. These precursors persist in high numbers until later stages of differentiation (day 26) with some beginning to show immunoreactivity for later markers of cone differentiation such as blue opsin and cone arrestin (at days 23-29). Furthermore, cone precursors label with an adeno-associated viral vector encoding a GFP reporter under the control of cone opsin promoter. When isolated using flow sorting and transplanted into adult mouse retinas by subretinal injection, these form cell masses in the subretinal space expressing cone markers. A fraction of the transplanted cells integrates into the host photoreceptor layer and expresses mature cone markers. In conclusion, we developed a method of obtaining large numbers of cone precursors, which develop in a fashion mimicking the in vivo developmental process, and could be used for establishing cell therapies for daytime vision.

P188

OXB-102: An enhanced gene therapy for Parkinson's disease

Y Lad[1] R A Badin[2] K Binley[1] N Van Camp[2] C Jan[2] J Gourlay[2] H Stewart[1] S Ralph[1] M Kelleher[1] J Loader[1] K Hosomi[2 3] P Jones[1] S Palfi[2 3] P Hantraye[2] K Mitrophanous[1]

1: Oxford BioMedica 2: MIRCen, CEA 3: Neurosurgery, INSERM U955 T.14, Henri Mondor Hospital, APHP/UPEC, Créteil, France

The standard of care for Parkinson's disease (PD) is oral dopaminergic treatments. Although initially highly efficacious, over time they lead to debilitating long term side effects. OXB-102 is a lentiviral-based vector that delivers the genes for three key enzymes in the dopamine biosynthetic pathway, tyrosine hydroxylase, aromatic L-amino acid decarboxylase (AADC) and GTP-cyclohydrolase, to non-dopaminergic striatal neurons of the sensorimotor putamen, thus providing these cells with the ability to synthesise and release their own dopamine. The effectiveness of this strategy has been demonstrated in Parkinson's patients with a precursor vector; ProSavin®. OXB-102 is an improved vector with increased dopamine production per genetically modified cell. In non-clinical efficacy studies the improved potency of OXB-102 was demonstrated relative to ProSavin®. PET imaging using 18F-FMT, a presynaptic substrate of AADC, demonstrated a significant increase in FMT signal from OXB-102 compared to ProSavin®. A 6-month toxicology and biodistribution study indicates that the OXB-102 vector is safe and well tolerated following bilateral stereotactic administration into the putamen and the vector does not significantly spread beyond the site of administration. GMP manufacture of OXB-102 for a planned clinical trial in PD patients is currently in progress.

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Safety and biodistribution evaluation of AVA-101 following subretinal administration in nonhuman primates

S Vijay J Miller T M Nork T W Chalberg K W Woodburn

1: Avalanche Biotechnologies 2: Covance Laboratories 3: OSOD

AVA-101 is a recombinant, replication-deficient adeno-associated virus vector (serotype 2) encoding the soluble form of the naturally occurring anti-VEGF protein sVEGFR-1, and is being evaluated clinically as a potential treatment for neovascular age-related macular degeneration (AMD). To support clinical development, a comprehensive preclinical development program was undertaken, including a confirmatory 3-month GLP study in nonhuman primates to evaluate the toxicity and biodistribution of two lots (one manufactured using a triple-plasmid transfection system in HEK293 cells, and the other in a baculovirus expression system in Sf9 insect cells) of AVA-101. Animals received 1x1011 vg of AVA-101 (100 μL) by subretinal administration and were observed for up to 13 weeks with a subgroup of animals evaluated at 2 weeks. Subretinal administration of AVA-101 resulted in no adverse effects. The two AVA-101 lots yielded similar results, including histopathology, biodistribution, shedding profile, and vitreous sVEGFR-1 levels. There were no effects on clinical observations, body weights, organ weights, male reproductive, or clinical pathology parameters. Histological assessment indicated no test article-related effects, and minimal impact related to the subretinal dosing procedure. AVA-101 remained largely confined in the dosed area of injected eyes with limited shedding. These results support the continued clinical development of AVA-101 in adult subjects with neovascular AMD.

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The role of GLUT1 in the pathogenesis of Diabetic Retinopathy

S M Calado[1 2] L S Alves[2 3] S Simão[2] D B Bitoque[4 5] G A Silva[2 5]

1: PhD Program in Biomedical Sciences, Department of Biomedical Sciences and Medicine, University of Algarve, 8005 Faro, Portugal 2: Centre for Biomedical Research (CBMR), University of Algarve, Campus Gambelas, 8005 Faro, Portugal 3: Masters in Biomedical Sciences, Department of Biomedical Sciences and Medicine, University of Algarve, 8005 Faro, Portugal 4: ProRegeM PhD Program, Department of Biomedical Sciences and Medicine, University of Algarve, 8005 Faro, Portugal 5: CEDOC, NOVA Medical School / Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Campo Mártires da Pátria 130, 1169-056 Lisboa, Portugal

Diabetic Retinopathy (DR) is a blinding disease defined by retinal microvascular changes caused by chronic exposure to hyperglycemia leading to low tissue oxygenation and neovascularization. An increase in glucose transporters is hypothesized as responsible for increased glucose levels in retinal cells. Retinal glucose intake occurs exclusively through GLUT1, whose expression is controlled by HIF-1, a key player in neovascularization. To understand the role of GLUT1 in DR, we have characterized its expression both in vitro and in vivo models of DR. Retinal epithelium cells were subjected to hypoxic and normoxic conditions and challenged with different concentrations of glucose. In vivo retinal samples of diabetic Ins2Akita mice were compared with non-diabetic mice for GLUT-1 expression by western blot and immunofluorescence. Our in vitro results show increased GLUT1 expression under hypoxia conditions; we observed this increase to be associated to the translocation and stabilization of GLUT1 to the cell membrane. In vivo, GLUT1 was overexpressed in the retina of Ins2Akita mice. Immunofluorescent analysis shows membrane accumulation of GLUT1 in the innermost layers of diabetic retinas, which are known to be under hypoxic conditions. In this study the expression of GLUT1 was analyzed in vitro and in vivo models of diabetic retinopathy. Our results show overexpression of GLUT1 induced by hyperglycemia and low oxygen supply. This overexpression was associated to an increase of GLUT1 in the cell membrane, which points to a significant contribution of GLUT1 to the progression of DR. Acknowledgements: Fundação Ciência Tecnologia: SFRH/BD/76873/2011, SFRH/BPD/78404/2011, EXPL-BIM-MEC-1433-2013, PEst-OE/EQB/LA0023/2013. PIRG05-GA-2009-249314–EyeSee

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Towards improved predictability in pre-clinical research: Human 3D neural in vitro model for assessment of gene therapy vectors

D Simão[1 2] C Pinto[1 2] P Fernandes[1 2] S Piersanti[3 4] I Saggio[3 4] L M Collinson[5] G Schiavo[5] E J Kremer[6] M J T Carrondo[1 2] P M Alves[1 2] C Brito[1 2]

1: IBET-Instituto de Biologia Experimental e Tecnológica 2: ITQB-UNL – Instituto de Tecnologia Química e Biológica 3: Biomedical Science Park San Raffaele 4: Sapienza University of Rome 5: Cancer Research UK 6: Institut de Génétique Moléculaire de Montpellier

Gene therapy is a promising approach with enormous potential for treatment of neurodegenerative disorders. Viral vectors derived from canine adenovirus type 2 (CAV-2) have demonstrated in rodent models attractive features for gene delivery strategies in the human brain. These include increased neuronal tropism, efficient axonal transport to afferent brain structures, 30-kb as cloning capacity and present low innate and induced immunogenicity in humans. For clinical translation, in-depth pre-clinical evaluation of efficacy and safety in a human setting is primordial. Stem cell-derived human neural cells have a great potential as complementary tools by bridging the gap between animal models, which often diverge considerably from human phenotype, and clinical trials. Herein, we explore helper-dependent CAV-2 (hd-CAV-2) efficacy and safety for gene delivery in a human stem cell-derived 3D neural in vitro model. Human neural progenitor cells (hNPC) were differentiated as neurospheres, resulting in a stable culture of cells from neuronal, astrocytic and oligodendrocytic lineages. Assessment of hd-CAV-2 transduction was performed at different multiplicities of infection, by evaluating transgene expression and impact on cell viability, ultrastructural cellular organization and neuronal gene expression. Under optimized conditions, hd-CAV-2 transduction led to stable long-term transgene expression with minimal toxicity. The evaluation of vector tropism showed that hd-CAV-2 preferentially transduces neurons, in contrast to human adenovirus type 5 (HAdV5) that showed increased tropism towards glial cells. This work demonstrates, in a physiologically relevant 3D model, that hd-CAV-2 vectors are efficient vehicle for gene delivery to human neurons, with stable long-term transgene expression and minimal cytotoxicity.

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Retinal alterations in Cre-activated transgenic mice

E Kokki[1] T Karttunen[3] V Olsson[1] J Kujala[1] K Kinnunen[3] S Ylä-Herttuala[1 3]

1: University of Eastern Finland, Kuopio 2: Kadmon Research Institute 3: Kuopio University Hospital

Objective: The aim of the study was to investigate the effects of subretinal adenoviral gene transfer of Cre-gene (AdCre) in the mouse retina.

Methods: Study was performed using a transgenic mouse model where an inactivated human VEGF-A165 expression cassette can be activated by a single adenoviral Cre gene transfer. Gene transfer was done subretinally leaving the other eye intact. The effects of AdCre gene transfer in the mouse retina were studied by optical coherence tomography and fluorescein angiography before and after gene transfer at different time points. To study retina morphology and vascularization histopathological studies were executed.

Results: Effects of AdCre were seen in mouse retinas even 3 months after gene transfer. Retinal pathology showed structural changes such as the loss of cells in outer retinal layers which was also seen in optical coherence tomography. Vascular abnormalities were seen in immunohistological stainings and fluorescein angiography.

Conclusions: Retinal alterations in Cre-activated transgenic mouse model have similarity with an established Akimba mouse model and can hence offer a new animal model for diabetic retinopathy.

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Novel self-regulated vector for optimization of osteogenic potential of mesenchymal stem cells

S Gambera V Blanca M Arroyo J García-Castro A Alfranca

1: Instituto de Salud Carlos III

Current therapies for pathologies with extensive defects of bone mass are frequently poorly effective and often have a high economic impact. There is therefore a great interest in the development of therapeutic approaches which overcome these problems. Given their ease of isolation and their ability to undergo osteogenic differentiation, mesenchymal stem cells (MSCs) have been previously employed for this purpose. We have designed a novel tool to optimize osteogenic potential of MSCs, which consists of a lentiviral system which drives self-limited overexpression of osteogenic promoting factor Dlx5. This system comprises a unique bicistronic lentiviral vector, which codes for both Dlx5 and the recombinase Cre, flanked by two LoxP sites upon integration into DNA of target cell. The expression of Dlx5 induces osteogenic factor Osterix, which triggers osteogenic differentiation and simultaneously activates the expression of Cre, thus removing provirus from host DNA. MSCs transduced with this lentiviral vector undergo changes in cell morphology and actin cytoskeleton consistent with osteogenic differentiation, and present calcium deposits in vitro. In order to evaluate osteogenic potential of transduced MSCs in vivo, we have included them in ceramic scaffolds placed subcutaneously into mice. MSCs transduced with this vector spontaneously and efficiently form bone tissue, as evidenced by microCT and histological techniques. In summary, we have developed a lentiviral vector with increased biosafety due to its self-regulation design, which efficiently promotes osteogenic differentiation of MSCs in vitro and in vivo. These characteristics make this vector highly suitable for its application in processes which involve bone mass defects.

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The study of the effect of polylactide scaffold and bone marrow stromal cells on reparative bone formation of the defect in the rabbit's mandible.

Y A Nashchekina T V Veselova P O Nikonov M I Blinova

1: Institute of Cytology Russian Academy of Science 2: Pavlov State Medical University of St Petersburg

In a modern clinical medicine are widely used methods of tissue engineering and cell biology. The choice of the optimal scaffolds for cell culture directly affects on cell proliferation and outcome of regenerative osteogenesis. The most promising is the use of biodegradable polymeric materials based on polylactide. Reproduction and growth of cells in the polylactide scaffolds having sufficient mechanical strength and a certain spatial architecture can promote the formation of differentiated tissues structural basis. This work is devoted to the study of the effect of a polylactide scaffolds and the bone marrow stromal cells (BMSC) on reparative osteogenesis in the rabbit mandible defect in the early stages of healing. Polymer scaffolds were prepared from poly (L, L-lactide) by salt leaching technique. The pore size was evaluated by SEM as 150 microns. Polylactide scaffolds were modified by collagen or fibrin solutions with a concentration of 0.1 mg/ml. It was found that BMSCs cultured in 3D polylactide scaffold modified by fibrin form colonies, while BMSCs cultured in 3D polylactide scaffold modified by collagen type I distribute inside scaffold as single cells. Rabbits aged 2–3 months, 2-2.5 kg were used for in vivo experiment. On the third day after transplantation scaffolds it was noted the increased formation of fibrous structures, that associated with fibroblasts chemotaxis increasing. These differences persist till the 7th day, indicating a more intensive process of bone healing wounds in the prototype. This work was supported by Russian Science Foundation (project №14-50-00068) and the Federal Agency of Scientific Organizations (Russia).

P196

Transgene-free reprogramming and enzymatic dissociation facilitate tumor-free islet regeneration from diabetes-specific induced pluripotent stem cells

M El-Khatib[1] S Ohmine[1] E J Jacobus[1] J M Tonne[1] A Terzic[1] Y C Kudva[1] Y Ikeda[1]

1: Mayo Clinic

Derivation of type 1 diabetes-specific induced pluripotent stem cells (iPSCs) and their differentiation into functional β-cells can provide the foundation for new diagnostic and therapeutic applications. A major concern regarding the use of iPSC-derived insulin-producing cells is the risk of teratoma formation upon transplantation. The primary source of teratomas has been considered residual undifferentiated pluripotent cells. The use of integrating reprogramming vectors can also increase the risk of tumorigenicity of iPSC progeny, due to insertional mutagenesis or sustained expression of the reprogramming factors. Here, we assessed the safety of transplanting patient-derived iPSC-generated pancreatic endoderm/progenitor cells. Transplantation of progenitors from iPSCs reprogrammed by lentiviral vectors (LV-iPSCs) led to the formation of invasive teratocarcinoma-like tumors in over 90% of immunodeficient mice. Moreover, removal of primary tumors from LV-iPSC progeny-transplanted hosts generated secondary and metastatic tumors. Combined transgene-free (TGF) reprogramming and elimination of residual pluripotent cells by enzymatic dissociation ensured tumor-free transplantation, ultimately enabling regeneration of type 1 diabetes-specific human islets in vivo. The incidence of tumor formation in TGF-iPSCs was titratable depending on the oncogenic load, with reintegration of the cMYC expressing vector abolishing tumor-free transplantation. Thus, transgene-free cMYC-independent reprogramming and elimination of residual pluripotent cells are mandatory steps in achieving transplantation of iPSC progeny for customized and safe islet regeneration in vivo. Islet regeneration from patients with type 1 diabetes would allow in vitro modeling of patient-specific immune responses against autologous islets.

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Porous titanium pylon seeded with autologous fibroblasts or mesenchymal stem cells (MSCs) for direct skeletal attachment of limb prostheses

M Shevtsov[1 2] O Galibin[2] N Yudintceva[1] A Ivanova[3] I Potokin[4] M Blinova[1] M Pitkin[5 6]

1: Institute of Cytology of the Russian Academy of Sciences, Russia, St.Petersburg 2: I.P. Pavlov State Medical University, Russia, St.Petersburg 3: Russian Research Center of Radiology and Surgical Technology, Russia, St.Petersburg 4: Research Institute of Highly Pure Biopreparations, Russia, St.Petersburg 5: Tufts University School of Medicine, United States, MA, Boston 6: Poly-Orth International, United States, MA, Sharon

Attachment of limb prostheses to a connecting device implanted to the residuum bone and having its component outside

of the residuum constitutes a technology of Direct Skeletal Attachment. We proposed original device called Skin and Bone Integrated Pylon (SBIP) and characterized by application of the totally porous titanium. Another distinguishing feature of our method is the seeding of the SBIP with cells (fibroblasts or mesenchymal stem cells) prior to implantation in vivo. In the model of the above-knee amputees in rabbits we observed enhanced osseointegrative properties of the intramedullary porous component seeded with fibroblasts induced into osteoblast differentiation, as compared to the untreated porous titanium pylon. The three-phase scintigraphy and subsequent histological analysis showed that the level of osteogenesis was 1.5-fold higher than in the control group. Treatment of the transcutaneous component with autologous fibroblasts was associated with nearly a 2-fold decrease in the period required for the ingrowth of dermal and subdermal soft tissues into the implant surface, as compared to the untreated porous titanium component. Alternative application of the MSCs also proved to be beneficial in increase of integrative properties of SBIP with soft tissues. Application of porous titanium material seeded with dermal fibroblasts or MSCs facilitates the biointegration of a pylon with surrounding tissues and could improve the effectiveness of prosthetics rehabilitation for people with limb amputations. The study was supported by a grant from the Russian Science Foundation (No 14-50-00068) and with financial support from the Federal Agency of Scientific Organizations.

P198

Tendoncel Phase II Placebo controlled Randomized Clinical Trial results of a novel allogeneic regenerative topical medicine in treatment of Chronic Tendon injury

A Reginald[1] S Sultan[1] A Mousiadou[1] M J Evans[1] P Givissis[2]

1: Cell Therapy Limited 2: Orthopaedic Clinic of Aristotle, University of Thessaloniki, General Hospital of Papanikolaou, Greece

Cell Therapy Ltd has developed a novel topical gel for the treatment of lateral epicondylitis- Tendoncel. Tendoncel is a patented regenerative allogeneic gel that incorporates a unique combination of platelet growth factors in a cellulose-derived gel, which consequently, can control growth factor release, improving their bioavailability for maximum therapeutic benefit. Preclinical in-vitro studies have shown Tendoncel to significantly enhance the proliferation of fibroblasts compared to positive controls. Tendoncel contains a number of growth factors including; PDGF-BB, VEGF, PDGF-AA, thrombospondin and angiopoietin and can be stored for up to 3 months. Growth factors derived from platelet lysate have been shown to facilitate healing in cutaneous and soft tissue injuries but with variable efficacy. Thus we investigated Tendoncel in a double blind placebo controlled Phase II clinical trial in lateral epicondylitis tendon injury. Patients were randomized to receive either treatment- Tendoncel or placebo gel for once daily application for 21 days with follow up over a period of 3 months. The Tendoncel treatment cohort showed a statistically and clinically significant improvement in DASH (65%) and PTREE (58%, functional questionnaires to assess efficacy) with no serious adverse events or change in IgE levels (assess any systemic effects). In conclusion, Tendoncel, a novel topical platelet lysate based therapy, is both efficacious and safe in the treatment of lateral epicondylitis.

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Application of allogeneic cells of different origin for bladder reconstruction.

N V Orlova T I Vinogradova A N Muravjev N M Blum N Y Semenova N M Judintceva J A Nashchekina M I Blinova M A Shevtsov M L Vitovskaja N V Zabolotnich M G Shejhov

1: Saint-Petersburg Scientific-Research Institute of Phthisiopulmonology, Russian Ministry of Health Care, Saint-Petersburg, 191036, Russia 2: Institute of Cytology, Russian Academy of Science, Saint-Petersburg, 194064, Russia 3: Science Research Institute of Children's Infections, FMBA of Russia, Saint-Petersburg, 197022, Russia

Tissue engineering is an important modern scientific trend. But urological aspects take small moiety in the total structure of topical publications nowadays. The purpose of this study is to explore the possibility of bladder wall reconstruction using multi-component graft with allogeneic bone-marrow mesenchymal stromal cells (BMSCs). Poly-L,L-lactide/silk fibroin scaffolds were filled with collagen gel, seeded with cells and transplanted to 6 rabbits after bladder resection: group1 (control, n = 2) – scaffold with allogenic smooth muscle and urothelium cells; group 2 (control, n = 2) – matrix without cells; group 3 (n = 2) – scaffold filled with allogenic BMSCs. The results were evaluated 2 months post-surgery. Transplantation of allogenic smooth muscle and urothelium cells in group 1 led to severe inflammation with reduction of bladder capacity and graft necrosis. In group 2 rabbits we also noticed graft rejection, fibrous changes at the implantation site and severe inflammation. In group 3 animals bladder capacity was similar to pre-operative values, the labeled cells were determined at the implantation site by MRI 2 month post-surgery. Histological examination revealed initial stages of tissue reparation. BMSCs ability to modulate immune response is of great scientific interest now. Foreign scientists proved BMSCs potential to differentiate in vitro into bladder smooth muscle-like cells. Application of the multi-component graft with allogeneic cells may improve treatment results in conditions when healthy autologous material is absent. The work was supported by a grant from Russian Science Foundation (project №14-50-00068) and with financial support from Federal Agency of Scientific Organizations (Russia) and RFBR №13-04-12027 ofi_m.

P200

Large-scale myeloid differentiation of human induced pluripotent stem cells (hiPSC) for cell and gene therapy strategies

M Ackermann[1] N Lachmann[1] D Lüttge[1] E Frenzel[1] C Happle[1] S Liebhaber[1] O Klimenkova[1] S Brennig[1] S Janciauskiene[1] J Skokowa[1] G Hansen[1] T Moritz[1]

1: Hannover Medical School

Hematopoietic in vitro differentiation of hPSCs holds great promise for gene and cell therapy, and represents a valuable model system of embryonic hematopoietic development. Thus, we have established an embryoid body (EB)-based differentiation protocol employing IL-3 in combination with G-CSF or M-SCF. This ''minimal cytokine'' differentiation approach continuously (up to 5 months) produce large numbers (2-10x

106/week/6 well plate) of >95% pure granulocytes (iPSC-gra) or monocyte/macrophages (iPSC-MΦ) via an intermediate ''myeloid cell forming complex (MCFC)''. IPSC-gra and iPSC-MΦ revealed typical morphology, surface phenotype, and functionality. Thus iPSC-gra migrated towards an IL8 or fMLP gradient, formed neutrophil extracellular traps, and produced ROS. IPSC-MΦ phagocytosed latex beads and secreted cytokines upon LPS stimulation, similar to their in vivo-derived counterparts. In our model production of myeloid cells was driven by a MCFC-resident, CD34+, clonogenic progenitor population. Furthermore, early embryonic events such as endothelial versus hematopoietic specification were recapitulated. In this line, analysis of MCFCs revealed expression of MIXL1, SOX17, KDR1, GATA2, and RUNX1, as well as early CD34+/CD45- cells undergoing transition to a CD34+/CD45+ and thereafter CD34-/CD45+ phenotype. The hypothesis of a primitive hematopoietic cell arising from a population with dual (hematopoietic and vascular epithelial) potential was supported by co-staining with VE-cadherin (CD144) and colony formation primarily by CD34+/CD45+/CD144- cells. Thus, our in vitro PSC differentiation model allows for large-scale production of myeloid cells for cell and gene therapy purposes. Moreover, the model faithfully recapitulates key events in embryonic hematopoiesis and thus appears highly suited to study early hematopoietic development.

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European Union support to gene and cell therapy research in Horizon 2020 (2014–2020)

D Gancberg

1: European Commission

The new European Union (EU) programme for research and innovation, Horizon 2020 (2014–2020), supports the gene and cell therapy field by publishing calls for proposals on (clinical) collaborative research on rare diseases, in regenerative medicine or for new technological developments, amongst others. These calls, derived from the ''Societal Challenges I'' of Horizon 2020, support collaborative projects from consortia composed of a minimum of three legal entities from three different EU Member States or associated Member States. Small and medium-size enterprises (SMEs) can participate as well or apply, even as single partner, via a dedicated SME instrument. Other funding opportunities from the ''Excellence in Science'' pillar from Horizon 2020 (Marie Sklodowska-Curie training actions, European Research Council frontier research grants) are also available on regular basis. The first Horizon 2020 collaborative projects dealing with gene and/or cell therapy will be presented as well as the trends for the future Health programme (2016–2017). The EU gene transfer- and gene therapy-related collaborative projects supported during the 7th framework programme have been published in 2014 and 2015 in the journal Human Gene Therapy Clinical Development. References: D. Gancberg et al., Hum Gene Ther 2014, 25: 1. D. Gancberg et al., Hum Gene Ther Clin Dev 2014, 25: 51-71. D. Gancberg et al., Hum Gene Ther Clin Dev 2015, in press. Participant portal: http://ec.europa.eu/research/participants/portal/desktop/en/opportunities/h2020 David Gancberg Directorate Health Directorate-General for Research and Innovation European Commission CDMA 00/174, B-1049 Brussels, Belgium E-mail:david.gancberg@ec.europa.eu

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Application of allogenic bone marrow stromal cells on the urology

N M Yudintceva Y A Nashchekina M I Blinova M A Shevtsov N V Orlova T I Vinogradova A N Muravjev

1: Cell Culture Department, Institute of Cytology of the Russian Academy of Sciences (RAS), St. Petersburg 194064, Russian Federation 2: Saint-Petersburg Scientific-Research Institute of Phthisiopulmonology, Russian Ministry of Health Care, St. Petersburg 190000, Russian Federation

Urinary bladder pathology is actual problem nowadays. Bowel reconstruction of non-functioning bladder remains the gold standard, despite various complications. Recently were published successful attempts of bladder replacement with in vitro generated neo-organ containing autologous cells. But that method is unsuitable for patients with total fibrous transformation of the bladder. So it is promising to use allogenic cells for bladder reconstruction. The purpose of this study is creation of grafts containing allogenic cells to restore damaged bladder tissue. The poly-L,L-lactide/silk fibroin scaffold appeared to be mechanically strong, elastic and non-toxic. In first group scaffolds were filled with collagen gel and bone marrow stromal cells; in second – with dermal fibroblasts. Grafts were transplanted in vivo after partial rabbit bladder resection. 2 months post-surgery we evaluated safety of implants and character of the implantation-site tissue organization. The graft containing bone marrow cells integrated well with surrounding tissues, caused mild inflammation without manifested bladder capacity decrease. Initial stages of tissue reparation occurred at the implantation site. Opposite transplantation of allogenic fibroblasts-containing graft led to active inflammatory response with a pronounced bladder capacity reduction and implant rejection. Although recent studies showed native-like tissue formation in neo-bladder, its functionality is not fully investigated. Many issues must be resolved before widespread use of these technologies: contractility, innervation and vascularization of tissue-engineered grafts, and its subsequent physiological functioning. This work was supported by a grant from the Russian Science Foundation (№14-50-00068) and with financial support from the Federal Agency of Scientific Organizations (Russia) and RFBR №13-04-12027 ofi-m.

P204

Genome-wide non-coding RNA expression profile indicates upregulation of hsa-miR-761 in the bone marrow mesenchymal stromal cells of Fanconi Anemia patients

I Cagnan[1 2] D Uckan[1 2] A G Ozcan[1 2]

1: Department of Stem Cell Sciences, Institute of Health, Hacettepe University, Ankara, 06100, Turkey 2: Center for Stem Cell Research and Development, Hacettepe University, Ankara, 06100, Turkey

Fanconi anemia (FA) is a rare genetic disorder characterized by genomic instability, predisposition to cancer and bone marrow (BM) failure. The current knowledge on FA cells is insufficient to explain pathogenesis of the disease. miRNAs are among the important regulators of stem cell fate and survival and dysregulation of miRNA expression may have a role in FA BM failure. We compared the whole genome non-coding RNA expression profile of bone marrow derived mesenchymal stem cells from FA patients (n=11) and donors (n=13) using

GeneChip miRNA 2.0 Array (Affymetrix, CA). Partek Genomics Suite software package was used to identify differentially expressed miRNAs and their predicted target genes. miRNA profiling revealed that hsa-miR-761 expression was significantly upregulated in FA patients compared with donor MSCs. hsa-miR-761 expression was inhibited in FA cells using specific miRIDIAN microRNA hairpin inhibitor for 72 hours. Following the treatment, hsa-miR-761 expression was 0.48 fold inhibited compared to untransfected FA MSCs. Bioinformatic analysis predicted 678 target genes for miR-761 and NOTCH2 was among these. qPCR analysis showed 10.04 fold increase in NOTCH2 expression of FA cells treated with hsa-miR-761 miRIDIAN inhibitor. Herein, we showed the increase of hsa-miR-761 expression in FA patients' BM stroma and confirmed NOTCH2 as the target of hsa-miR-761. Accordingly, higher hsa-miR-761 expression may downregulate NOTCH2 levels in FA patients' BM stroma. In view of previous studies on NOTCH2 promoting HSC self-renewal, our results indicate a role of hsa-miR-761 in the progression of hematopoietic failure in FA. (This study was supported by TUBITAK112S021)

P205

Generation of pig induced pluripotent stem cells (piPSC)-derived cardiac cells for transplantation in a pig model of myocardial infarction

C Steichen[1] R Dariolli[1] D G Biagi[1] G Mostoslavsky[2] A C Pereira[1] J E Krieger[1]

1: Heart Institute (InCor), University of São Paulo Medical School, São Paulo, Brazil 2: Center for Regenerative Medicine (CReM), Boston University School of Medicine, Boston, Massachusetts,

IPSCs hold great promise for cell therapy including for replacing damaged cardiomyocytes after myocardial infarction. However, preclinical studies are required to evaluate the safety and efficiency of this approach. Our aim is to test the hypothesis that transplantation of pig iPSC-derived cardiac cells prevents deterioration of the cardiac function post myocardial infarction in pigs. First, we generated pig iPSCs showing the typical features of pluripotent stem cells including a normal karyotype. However, in our laboratory and others, the piPSC differentiation into cardiomyocytes remain an issue, although we already obtained beating piPSC-cardiac cells in a single experiment. To overcome this problem, we test the hypothesis that differentiation capacity of ''naïve-like'' piPSCs could be higher (compared to primed piPSCs). We generated naïve-like piPSCs using specific conditions for reprogramming and used fibroblasts from atrium, expecting this will help to improve cardiac differentiation by reducing epigenetic barriers. We are adapting a human differentiation protocol, efficient in our laboratory, to achieve satisfying efficiency with piPSCs and are testing the effect of a 3D culture method. We already generated piPSC-derived cardiac specified mesodermal cells expressing specific transcription factors such as Mesp1, Isl1 and Mef2c, and are working on maturing these cells. One alternative would be to use these cardiac mesodermal cells, taking the advantage of their relative immature but committed state for transplantation. Our final aim is to transplant the cells back into the donor pig and the laboratory developed an original and efficient model of myocardial infarction in pigs using closed- artery catheter-based method.

P206

Modelling of neonatal diabetes associated with an activating STAT3 mutation with patient-specific iPS cells

J Saarimäki-Vire[1] D Balboa Alonso[1] S Eurola[1] J Ustinov[1] T Otonkoski[1]

1: Timo Otonkoski's group, Research Program Unit, Molecular Neurology and Biomedicum Stem Cell Centre, University of Helsinki

We described a patient with neonatal diabetes associated with hypoplastic pancreas and high beta-cell autoantibody levels already at birth. Screening of the patient's DNA revealed the missense mutation K392R in the STAT3 gene. Functional characterization of this mutation showed that it is highly activating. We hypothesize that the mutation may cause pancreatic developmental failure and autoimmunity through distinct mechanisms. In this study, we tested the hypothesis using patient-derived iPS cells. Patient iPSC were differentiated towards pancreas through 17-day stepwise protocol leading to efficient specification of endocrine progenitors. Expression levels of pancreatic progenitor markers, such as PDX1 and NKX6.1, did not differ between Stat3 cells and healthy controls. Thus, overactive Stat3 did not cause a developmental block or inhibit endocrine differentiation. Instead, the NGN3 and INSULIN levels were significantly higher in Stat3 mutated cells already from day 13 of differentiation. A modified protocol was developed that maintained wild-type cells as pancreatic progenitors without endocrine differentiation. In contrast, the STAT3K392R cells upregulated NGN3 and NKX2.2 4-5 fold ($p < 0{,}005$), INSULIN 10-fold ($p < 0.005$) and GLUCAGON 5-fold ($p < 0{,}005$). Interestingly, we corrected mutation with CRISPR-CAS9 technology, and these corrected, isogenic cells were similar to control cells and did not show marks of premature differentiation. Our results show that overactive STAT3 leads to NGN3 activation and consequent premature endocrine differentiation. The correction of Stat3 mutation with CRISPR-CAS9 technology rescues the differentiation phenotype. Premature differentiation result in reduction of the pancreatic progenitor pool, leading to pancreatic hypoplasia. Patient-specific iPSC are a valuable tool for recapitulating pancreatic developmental defects.

P207

Simian iPSCs differentiated into hepatocytes: towards the proof of concept for autologous cell therapy of liver diseases

E Luce[1 2 3] C Steichen[1 2 3] S Abed[4] A Weber[1 2 3] P Leboulch[4] L Maouche-Chrétien[4] A Dubart-Kupperschmitt[1 2 3]

1: INSERM U1193 2: Universite Paris Sud UMR_S 1193 3: DHU Hepatinov 4: INSERM U962/CEA/IMETI/STI

Pluripotent stem cell-derived hepatocyte transplantation is an alternative to orthotopic liver transplantation for the treatment of severe metabolic diseases. However, before their use in clinical application, the safety and efficacy of this therapeutic approach has to be established in large animal models, including non-human primates. The aim of this project is to demonstrate that simian iPSCs (siPSCs) differentiated into hepatocytes can efficiently engraft into the liver parenchyma after autologous transplantation. We therefore

have generated and characterized siPSCs. Based on a protocol we previously described for human iPSCs, we have set up chemically defined conditions to differentiate these cells into hepatocyte-like cells (HLCs). After 20 days of differentiation, we obtain HLCs expressing FOXA2, HNF4α, CK19, AFP, APOA2 and ALB. As it is reported for human iPSC-HLCs, siPSC-HLCs closer resemble fetal rather than adult hepatocytes. Before the autologous transplantation experiment, we want to assess the ability of the siPSC-HLCs to engraft and to further mature in the liver of a smaller animal. We are currently performing transplantations of these siPSC-HLCs into the liver of uPA-SCID mice. After 8 weeks, the presence of simian hepatocytes in mouse livers will be assessed and simian albumin will be quantified in the mouse plasma. In parallel, because of the large number of cells required for the transplantation into a primate, we have been defining conditions to freeze/thaw those cells for banking. The final step of the project will be the transplantation of autologous siPSC-HLCs into the macaque liver and the evaluation of the repopulation efficiency.

P208

Generation of caudalized neural stem cells from episomal hiPSCs under GMP conditions

M Martin-Lopez[1] B Fernandez-Muñoz[1 2] I Lomas-Romero[1 2] E González-Muñoz[1] B Arribas[1 2] M A Montiel[1 2] L Laricchia-Robbio[2] S Canovas[1] J B Cibelli[1 3] P J Horner[4]

1: Laboratorio Andaluz de Reprogramacion Celular (LARCEL), Spain 2: Andalusian Initiative for Advanced Therapies (IATA) Sevilla, Spain 3: Departments of Animal Science and Physiology Cellular Reprogramming Laboratory, Michigan State University (USA) 4: Center for Neuro Regenerative Medicine Houston Methodist Research Institute, TX (USA)

The implantation of autologous Neural Stem Cells (NSCs) may be a plausible treatment for spinal cord injuries (SCI) due to their ability to integrate and differentiate into different neural lineages after grafting. Once human iPSCs are obtained from the patient, they can be neuralized in order to obtain NSCs. These procedures will have to be performed under Good Manufacturing Practices (GMP) in order to be translated into clinical stage. In this study we have adapted our established neuralization protocol to GMP conditions with the aim of creating a cell resource for both clinical and preclinical experiments. hiPSCs were produced with episomal vectors and subsequently differentiated to caudalized NSCs. The cells obtained were successfully cultured both, as Neurospheres (NEs) and adhered to substrate. For the latter, plates were coated with poly-L-ornithine and human laminin. Accutase was used as a detaching solution in both culturing techniques. These NSCs derived from episomal hiPSCs were compared by immunostaining with NSCs derived from virus generated non-GMP hiPSCs for the expression of Sox 9, Hox B4, Pax 6 and Nestin. We did not detect significant differences between cell lines in the expression of these neural markers. These results were confirmed by RT-PCR. Quality Control tests and a GMP documentary system were generated to complete GMP product release with the aim of using these cells in preclinical and clinical studies. In conclusion, we demonstrated that iPSC derived NSCs can be successfully generated under GMP conditions, enabling the use of these cells in pre-clinical and clinical studies.

P209

Human oligodendrocytes generated by direct reprogramming from adult donor adipose tissue

C L Paíno[1] L Vellosillo[1 2] M P Muñoz[1] L C Barrio[1]

1: Hospital Ramon y Cajal 2: Center for Biomedical Technology

Obtaining oligodendroglial cells from dispensable tissues would be of great interest for autologous or immunocompatible cell replacement in demyelinating diseases as well as for studying myelin pathologies. Recently, two laboratories have simultaneously reported that mouse fibroblasts could be converted into oligodendroglial cells by direct reprogramming with transcription factors involved in oligodendrocyte development (Najm et al., Nat. Biotechnol. 31:426, 2013; Yang et al., Nat. Biotechnol. 31:434, 2013). In the present study, human mesenchymal stem cells (MSC), grown in culture from inguinal adipose tissue of a 69 year old donor, were lentivirally transduced with combinations of tetracycline-inducible Sox10 (S), Olig2 (O), Zfp536 (Z) and/or Nkx6.1 (N) transgenes. Induced oligodendrocyte precursor cells (iOPCs), demonstrated by monoclonal antibody O4, were observed after at least 4 months of growth in cultures transduced with S+O+N. Transdifferentiation was also achieved by S+O+Z combination at longer culture times. GFAP+ astrocytes, both of filamentous and protoplasmic morphologies, were also generated. MSC cells transduced with S+O+N+Z produced larger numbers of morphologically similar cells but did not stain with O4. We were not able to generate iOPCs from adipose tissue using combinations lacking Sox10, neither using this transcription factor alone. iOPCs proliferated, forming small colonies, and acquired galactocerebroside (O1 antibody) expression and more complex morphologies. Efforts are ongoing to shorten iOPC generation times. The present study provides evidence that oligodendrocytes can be generated from human adipose tissue even from aged donors and after long culturing periods.

P210

Mesenchymal stromal cells enhance the hematopoietic engraftment and reduce graft failures in clinically relevant models of autologous transplantation

M Fernández-García[1 2] R Yañez[1 2] R Sánchez-Domínguez[1 2] M Peces-Barba[1 2] M Hernando[1 2] G Herrera[3] J E O'Connor[4] J C Segovia[1 2] J A Bueren[1 2] M Lamana[1 2]

1: Division of Hematopoietic Innovative Therapies, CIEMAT/CIBERER, 28040 Madrid, Spain 2: Advanced Therapies Unit, IIS-Fundacion Jimenez Diaz (IIS-FJD, UAM), 28040 Madrid, Spain 3: Cytometry Service, UCIM, INCLIVA-Universidad de Valencia, 46010 Valencia, Spain 4: Laboratory of Cytomics, Universidad de Valencia, 46010 Valencia, Spain

Co-transplantation of human mesenchymal stromal cells (MSC) with hematopoietic stem cells (HSC) has been reported to reduce the risk of graft failure in determined allogeneic HSC transplants. It is unknown whether the engraftment facilitating role of MSCs is also maintained in an autologous transplantation setting, such the one considered in HSC gene therapy. Using a congenic mouse transplantation model (CD45.1/CD45.2) we have observed that the co-infusion of MSC with

low numbers of purified LSK cells significantly improved the short- and long-term hematopoietic engraftment in sublethally irradiated recipients. This improvement was MSC dose-dependent and due to an increased homing of the LSK cells in the recipient's bone marrow. We have also observed that LSK cells rapidly interact with MSCs prior to transplantation, suggesting that the reported engraftment effects of MSCs require the direct contact of these cells with the HSCs. With the aim of approaching to a more clinically relevant model, we have conducted similar experiments transplanting WT LSK cells into Fanconi anemia A (Fanca-/-) recipient mice. In these experiments, the infusion of low numbers of WT LSK cells on FA recipients resulted in 30% of graft failure. However, when the same number of WT LSK cells was co-infused with 6.105 mAd-MSCs all the transplanted animals showed significant hematopoietic engraftments. Taken together, our results demonstrate the hematopoietic facilitating engraftment potential of Ad-MSCs, not only in an allogeneic context, but also in clinically relevant models of autologous transplantation.

P211

Isolation of immunomodulatory progenitor cells of mesodermal lineage

S Sultan N Piouka A Reginald M J Evans

1: Cell Therapy Limited, Cardiff, UK

Immunomodulatory progenitor cells (iMPs) are a novel and distinct mesodermal progenitor cell discovered and isolated by Cell Therapy Limited (CTL) with cardiac-specificity for cellular therapy in cardiac regeneration. These cells have been successfully isolated in media using clinical grade and GMP-compliant supplements and a xeno-free serum substitute. Although mesenchymal stromal cell (MSC)-like and plastic adherent, iMPs are not MSCs as defined by the International Society for Cellular Therapy consensus definition of MSCs. iMP cells were found, by FACS analysis, to be a distinct population of cells different to MSCs. Comparison of the cell surface marker expression utilizing flow cytometry found 10 markers are significantly up-regulated (>15 fold increase) in expression vs. MSCs. In particular, the iMP cell surface marker expression profile consists of MIC A/B, CD304 (Neuropilin 1), CD178 (FAS ligand), CD289 (Toll-like receptor 9), CD363 (Sphingosine-1-phosphate receptor 1), CD99, CD181 (C-X-C chemokine receptor type 1; CXCR1), epidermal growth factor receptor (EGF-R), CXCR2 and CD126. iMPs do not express CD14, CD34 and CD45. Up-regulation of these markers has been reported to show greater functional properties such as immune-modulation and proliferation.

P212

Comparison of different serum-free media for in vitro expansion of Hematopoietic Stem Cells

P Sharafi[1] G Aydin[1] D Uckan-Cetinkaya[1 2] G Wagemaker[1] F Aerts-Kaya[1]

1: Hacettepe University, Institute of Health Sciences, Department of Stem Cell Sciences, Center for Stem Cell Research and Development 2: Hacettepe University, Medical Faculty, Division of Pediatric Hematology, Bone Marrow Transplantation Unit

Hematopoietic stem cells (HSCs) can be obtained from bone marrow, umbilical cord blood (UCB) and G-CSF mobilized peripheral blood collections. However, when pediatric stem cell donors or cord blood collections are used, the number of HSCs that can be collected is limited and may be insufficient for transplantation purposes. A range of different protocols to expand HSCs in vitro are being used by many laboratories. Here, we compared the effects of two different commercially available serum-free expansion media, StemSPAN (Stem Cell Technologies) and StemMACS (Miltenyi) and different combinations of growth factors on in vitro expansion of HSCs. CD34+UCB cells were cultured for 7 days. Colony forming units (CFU) and FACS analyses for CD34/CD38 expression were performed before and after culture. The growth factor cocktail SCF, TPO, aFGF, IGF2 and ANGTPL3 (STIFA3) in presence of StemSPAN and StemMACS media, resulted in 3.0 and 3.9 fold expansion of HSCs after 7 days, respectively, with relative lower frequencies of CFUs after culture in StemMACS. Similarly, the combination of SCF, TPO, FLt3-L, IGF-BP2 and ANGPTL5 (STIFA5) in StemSPAN and StemMACS resulted in 3.2 and 4.2 fold expansion, respectively, with relative fewer CFUs after culture in StemMACs. HSCs cultured in presence of the classical combination SCF, TPO and Flt3-L in presence of either StemMACs and StemSPAN performed considerably less than cultures supplemented with IGF2/IGF-BP2 and Angiopoietin-like growth factors. In conclusion, culture in StemMACS appears to perform slightly better than StemSPAN in terms of HSC expansion, whereas CFU formation is higher after culture in StemSPAN.

P213

Prospectively isolated CD271+Mesenchymal Stem Cells support expansion of Hematopoietic Stem Cells with lymphohematopoietic differentiation potential

G Aydin[1] D Uckan-Cetinkaya[1 2] F Aerts-Kaya[1]

1: Hacettepe University Institute of Health Sciences, Department of Stem Cells Sciences, Center for Stem Cell Research and Development 2: Hacettepe University, Ihsan Dogramaci Children's hospital, Division of Pediatric Hematology, Bone Marrow Transplantation Unit

Mesenchymal Stem Cells (MSCs) are multipotent stromal cells that can be isolated from many tissues, including the bone marrow. Isolation of MSCs based on plastic adherence (PA) results in contamination of cultures with multiple types of adherent cells. Here, we used the CD271 antigen to prospectively isolate MSCs from healthy human bone marrow and compared their in vitro support of hematopoietic stem cell (HSC) expansion and lymphohematopoietic differentiation with PA-MSCs. Prospectively isolated and PA-MSCs were co-cultured with umbilical cord blood CD34+ cells for 7 days in serum-free medium (StemSPAN, Stem Cell Technologies) with SCF, TPO, Flt3-L, ANGPTL5 and IGF-BP2 (STIFA5). Colony assays were enumerated and lymphohematopoietic differentiation potential was assessed by co-culturing expanded CD34+ cells on Op9 and Op9-DL1 cell lines (provided by Dr Zuniga-Pflucker). Culture of CD34+ cells in StemSPAN/STIFA5, MSCs and CD271 cells resulted in 2.6, 3.0 and 3.4 fold expansion, respectively. After 7 days, all cultures contained approximately 60% CD34+ cells. Absolute numbers of CD34+/CD38- cells and total numbers of colony forming units (CFU-GM+CFU-GEMM+BFU-E) were highest in cultures expanded on CD271 feeder layers. All culture

conditions supported expansion of HSCs with lymphohematopoietic potential. CD34+/CD1a+T-cell progenitors were observed as early as 10 days after co-cultures with Op9-DL1 cells and were followed by the appearance of CD4+/CD8+ double positive T-cell precursors at day 15. In conclusion, prospective isolation of MSCs using CD271 is effective and can be used to expand early HSCs with lymphohematopoietic potential.

P214

Unmodified Mesenchymal Stem Cells do not support in vitro T- and B-Cell differentiation of Hematopoietic Stem Cells

P Sharafi[1] I Cevher[1] A Gunel-Ozcan[1] D Uckan-Cetinkaya[1 2] F Aerts-Kaya[1]

1: Hacettepe University, Institute of Health Sciences, Department of Stem Cell Sciences, Center for Stem Cell Research and Development 2: Hacettepe University Medical Faculty, Division of Pediatric Hematology, Bone Marrow Transplantation Unit

Mesenchymal Stem Cells (MSCs) are multipotent stromal cells that are found in the bone marrow (BM) and locally interact with Hematopoietic Stem Cells (HSCs). In vitro, MSCs support expansion of HSCs. NOTCH1 signaling occurs through its ligands Delta-Like 1 (DL1) or DL4 and is important for T cell differentiation. Here, we assessed whether human BM-MSCs can support lymphopoietic differentiation of HSCs and measured gene expression levels of DLL1 and DLL4 on MSCs. Gene expression of DLL1 and DLL4 of healthy human BM-MSCs was assessed using real-time qPCR (Roche). Umbilical cord blood CD34+ cells were cultured on feeder layers of BM-MSCs, M-CSF deficient Op9 cells or Op9-DLI cells and differentiated in presence of 5 ng/mL FL and 1 ng/mL IL-7 (Immunotools). Feeder layers were refreshed every 5 days and co-cultures were maintained up to three weeks. DLL1 and DLL4 expression was low or absent. Consequently, unmodified BM-MSCs were not able to support T or B-cell differentiation. Secretion of M-CSF by BM-MSCs caused differentiation of most HSCs into monocytes (>80%). Addition of M-CSF receptor inhibitors, c-fms inhibitor III (1 uM) and c-fms inhibitor IV (80 nM), resulted in a decrease of monocytes to 30% and 60%, respectively. Dose-finding studies using the WST assay (Roche) suggest that higher doses of these inhibitors may completely block monocytic differentiation. In conclusion, unmodified BM-MSCs do not intrinsically support in vitro differentiation of HSCs towards lymphoid lineages, and constitutive overexpression of DLL1 and DLL4 or knock down/inhibition of M-CSF expression may be required.

P215

Optimal xeno-free conditions for isolation of microvesicles from umbilical cord-derived mesenchymal stem cells for the purpose of heart regeneration

S Bobis-Wozowicz[1] K Kmiotek[1] K Sit[1] E Kamycka[1] D Boruczkowski[2] Z Madeja[1] E K Zuba-Surma[1]

1: Jagiellonian University 2: Polish Bank of Stem Cells

Microvesicles (MVs) derived from stem cells are currently intensively investigated due to their high potential in regenerative medicine. They can carry proteins and nucleic acids and transfer them to acceptor cells influencing their proliferation and differentiation. One of the most promising sources of MVs are mesenchymal stem cells, however they are usually cultured in the presence of fetal bovine serum (FBS). Proteins/microvesicles of animal origin may preclude the use of MVs in application in humans. The aim of this study was to compare and select the most optimal serum-free and xeno-free medium for MSC-MVs isolation for the purpose of heart regeneration. Umbilical cord-derived MSC (UC-MSC) were cultured in 7 media - 5 experimental and 2 controls. Cell proliferation, metabolic activity (ATP concentration), phenotype (flow cytometry), differentiation potential (osteogenesis, adipogenesis and chondrogenesis), as well as senescence rate were measured. mRNA levels for genes related to cardiomiogenesis, angiogenesis, apoptosis and immunomodulation were analyzed (real-time PCR). Additionally, concentration of secreted pro- and anti-inflammatory cytokines were checked. MVs were isolated from conditioned media by ultracentrifugation at 100000g. Level of cardiomiogenic and angiogenic transcripts (real-time PCR) and phenotype (ApoGee) was evaluated. MVs were then transferred into cardiac mesenchymal stromal cells (cMSCs), which were subjected to differentiation into cardiomiocytes. Our results indicate differences in the maintenance of mesenchymal phenotype, proliferation rate, metabolic activity and gene expression levels in various xeno-free media. We observed that MVs derived from UC-MSCs were enriched in transcripts crucial for cardiomiogenesis, which had a positive impact on cardiomyogenesis of cMSCs.

P216

Derivation and maintaince of new human embryonic stem cell subline SC6-FF in allogenic feeder-free culture conditions.

A M Koltsova T K Yakovleva G G Poljanskaya

1: Institute of Cytology Russian Academy of Science

Human embryonic stem cell (hESC) lines represent a cell population that has unlimited replicative capacity and can differentiate into all cell types. These properties makes hESC lines an excellent tool for human cell and tissue replacement therapy. Traditionally, feeder cells are used to support the hESC growth by producing components of extracellular matrix and growth factors. The use of such culture conditions could lead to contamination of hESC by feeder cells or animal pathogenes and immunogens (if xenogenic feeder cells are used). Moreover, such conditions are difficult to reproduce because of variations between different batches of feeders, so it is an unsuitable for the production of clinical grade cells. In our work a novel hESC subline SC6-FF was derived in allogenic feeder-free culture conditions from hESC line SC6, which was derived with using of human feeder cells. The used culture system consists of extracellular matrix proteins and conditioned medium, synthesized by feeder cells – mesenchymal stem cell line SC5-MSC, which was previously derived from initial hESC line SC5. The subline SC6-FF passed through more than 150 cell population doublins, retain normal diploid karyotype and ability of in vitro differentiation in the derivates of three germ layers. SC6-FF express the markers of undifferentiated hESC. The comparative analysis of some typical features does not reveal essential differences between initial SC6 line and subline SC6-FF. The study was supported by a grant from the Russian Science Foundation (№14-50-00068) and with financial support from the Federal Agency of Scientific Organizations (Russia).

P217

Safety management of iPSCs in hematopoietic regenerative medicine

A Bedel F Beliveau L Dubuisson I Moranvillier I Lamrissi-Garcia B Rousseau H de Verneuil F Moreau-Gaudry[2] S Dabernat[2]

1: Université Bordeaux Segalen 2: INSERM U1035

Several limitations prevent the use of iPSCs in hematology. A population of differentiated cells is always contaminated by residual undifferentiated iPSCs. Persistence of iPSCs in a ready-to graft cell population is a critical hurdle because they can form teratomas. Our goal was to develop potent tools to prevent iPSC-derived teratomas. iPSCs-specific expression of a suicide-gene under the control of an embryonic-promoter is supposed to specifically kill iPSCs when the appropriate pro-drug is added. We constructed lentivectors containing icaspase-9 gene and tdTomato under the control of an embryonic-pmiR302/367 promoter and transduced iPSCs. After cell-sorting of tdTomato + iPSCs, the promoter was specifically active in iPSCs. We established in vitro toxicity of the icaspase9 inducer AP20187 in iPSCs. We observed that td-Tomato + cells and TRA1-60 + cells decreases in a dose-dependent manner. Unfortunately, AP20187 treatment does not lead to complete eradication. Moreover, as suicide-gene approach will be used to eliminate iPSCs from hematopoietic cell population, we looked at AP20187 toxicity on cord-blood cells and observed an unexpected toxic effect. YM155, a surviving inhibitor, has been reported to be effective to eliminate iPSCs. We compared it to our gene-suicide approach and found to be more efficient without toxicity on cord-blood cells. We are currently testing its capability to prevent teratoma in vivo using iPSC-expressing luciferase. This study will be useful to improve the safety-management for iPSCs-based medicine.

P219

Interleukin-13 secretion by allogeneic mesenchymal stem cells reduces allograft-specific CD8 + T cell activation, induces M2a macrophage polarization, and promotes allogeneic cell graft survival in mice

C Hoornaert[1 2] E Luyckx[1 2] K Reekmans[1 2] M Dhainaut[3] D Dooley[4] D Le Blon[1 2] E Fransen[5] J Daans[1 2] L Verbeeck[1 2] N De Vocht[1 2] E Lemmens[4] H Goossens[2] A Van der Linden[6] V Roobrouck[7 8] C Verfaillie[7 8] S Hendrix[4] M Moser[3] Z Berneman[1 2] P Ponsaerts[1 2]

1: Laboratory of Experimental Hematology, University of Antwerp 2: Vaccine and Infectious Disease Institute, University of Antwerp 3: Laboratory of Immunobiology, Department of Molecular Biology, Université Libre de Bruxelles 4: Department of Morphology, Biomedical Research Institute, Hasselt University 5: StatUa Centre for Statistics, University of Antwerp 6: Bio-Imaging Laboratory, University of Antwerp 7: Stem Cell Institute, KU Leuven 8: Stem Cell Biology and Embryology Unit, KU Leuven

Transplantation of genetically engineered mesenchymal stem cells (MSCs) for in situ therapeutic protein delivery is an attractive alternative to (non-)viral gene delivery approaches for the modulation of the clinical course of several diseases and traumata. From an emergency point-of-view, the use of pre-engineered allogeneic MSCs as off-the-shelf cell preparations has numerous advantages over the use of patient-specific autologous MSCs. Although in vitro co-culture confirmed the immunomodulating properties of allogeneic MSCs on dendritic cell (DC) function, allogeneic MSC-primed DCs lost their immunomodulatory phenotype upon in vivo introduction. Similarly, although allogeneic MSCs administered intramuscularly or intracerebrally, but not intravenously, can survive 1 week following in vivo administration, they are irrevocably rejected by the host's immune system. In an attempt to modulate MSC allograft rejection, we transduced MSCs with an interleukin-13 (IL13)-expressing lentiviral vector. Upon intramuscular transplantation, IL13-expressing MSCs induced fewer alloantigen-reactive IFNγ- and/or IL2-producing CD8 + T cells than non-modified allografts. Histological analyses of intramuscular and intracerebral allografts further revealed that the robust infiltration of Iba1- and MHCII-expressing inflammatory cells was a common feature of all examined MSC allografts, whereas expression of the M2a-polarization markers Arg1, Ym1 and FIZZ1 was restricted to macrophages infiltrating IL13-producing implants. Finally, using in vivo bioluminescence imaging, we established that IL13-producing MSC allografts survive significantly longer than their wild-type counterparts upon both intracerebral and intramuscular implantation. In summary, this study demonstrates that both innate macrophage and adaptive CD8 + T cell immune responses are effectively modulated in vivo by locally secreted IL13, ultimately resulting in prolonged MSC allograft survival.

P220

Empty capsids and macrophage inhibition/depletion increase rAAV-transgene expression in joints of both healthy and arthritic mice

C J Aalbers[1 2] L Bevaart[1 2] N Broekstra[1 2] M A van Geldorp[1 2] E Kramer[1 2] P P Tak[1 2] M J Vervoordeldonk[1 2] J D Finn[1 2]

1: Arthrogen BV 2: Academic Medical Center

Objective: rAAV5 is effective in delivering genes to the joint, however, the presence of macrophages in the inflamed joint might hamper gene delivery. We determined whether administration of agents that influence macrophage activity/number and/or addition of empty decoy capsids had an effect on rAAV5-transgene expression.

Material and Methods: Healthy or arthritic mice were injected with rAAV5.CMV.Fluc in both knee joints and monitored for luciferase expression (from 3 days - 6 months) by IVIS. Where indicated, empty capsids were co-administered with full particles. Macrophages were depleted or inhibited by systemic administration of clodronate liposomes or triamcinolone 48 hours prior to vector administration.

Results: Administration of rAAV5.CMV.Fluc in arthritic mice after the onset of inflammation resulted in lower expression of luciferase compared to vector administration before the onset of inflammation. Both macrophage depletion/inhibition or empty decoy capsid improved expression over a period of 4 weeks. The combination of macrophage inhibition and empty capsid resulted in a synergistic increase in gene expression (5.85 fold, $p = 0.001$), that was sustained for 6 months. We compared the efficacy of i.a versus i.m. triamciolone administration and found that enhancement of gene expression was independent of administration route. This effect was also observed in healthy animals.

Conclusions: We provide evidence that intra-articular macrophages are a barrier to efficient gene transfer, and that combination of triamcinolone and empty decoy capsids results in

improved gene expression. These data have implications for future applications of gene therapy to the joint, or to other tissues with an abundance of macrophages.

P221

Gemcitabine and the oncolytic adenovirus AduPARE1A display synergistic antitumor effects in pancreatic cancer by NF-κB mediated uPAR activation

A Mato-Berciano[1 2] M V Maliandi[1 2] L Sobrevals[1 2] A José[1 2] C Fillat[1 2] A Mato-Berciano[3 4] M V Maliandi[3 4] L Sobrevals[3 4] A Jose[3 4] C Fillat[3 4]

1: Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) 2: Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Barcelona 3: Institut D'Investigacions Biomèdiques August Pi i Sunyer 4: Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER)

Combined treatment of oncolytic adenoviruses with chemotherapeutic agents is foreseen as a therapeutic option for cancer. Pancreatic cancer is one of the most lethal cancers. Currently, there are limited treatment options and gemcitabine continuous to be the standard of care treatment despite it is minimally effective. Here we have investigated the potential to use gemcitabine in combination with the oncolytic adenovirus AduPARE1A to treat pancreatic cancer and evaluate the underlying mechanism. BxPC-3 and PANC-1 cells were treated with AduPARE1A and gemcitabine individually or in combination and we observed a synergistic cell killing from the combination. Such treatment also produced therapeutic benefits over either individual modality in two mouse models bearing orthotopic tumors, showing reduced tumor progression and significant prolonged mouse survival. Mechanistic studies showed that the synergistic cell death was not due to an increase in viral replication but occurred through an enhancement of apoptotic cell death. Gemcitabine stimulation increased the transcription of uPAR-controlled transgenes through the induction of NF-κB acting on the uPAR promoter. Interestingly, NF-κB gemcitabine-mediated induction of AduPAR adenoviruses interfered with the activation of NF-κB regulated genes. Consequently, AduPARE1A infection sensitized cells to gemcitabine-induced apoptosis in the combined treatment. These data highlights the potential of the combination as a treatment modality for pancreatic cancer patients.

P222

Tumor-specific oncolytic adenovirus expressing trastuzumab antibody for treatment of HER2-positive cancer

I Liikanen[1] K Guse[1] T Gutmann[1] P Savola[1] S Tähtinen[1] S K Pesonen[1] M Oksanen[1] A Kanerva[1 2] A Hemminki[1 3 4]

1: Cancer Gene Therapy Group, University of Helsinki 2: Department of Obstetrics and Gynecology, Helsinki University Central Hospital 3: Department of Oncology, Helsinki University Central Hospital 4: TILT Biotherapeutics, Ltd., Helsinki, Finland

Purpose: Trastuzumab antibody therapy against HER2 has significantly improved the survival of patients with HER2-overexpressing tumors. However, systemic antibody therapy is expensive and carries the risk of severe side-effects such as cardiomyopathy. Oncolytic adenoviruses can selectively kill cancer cells and promote anti-tumor immune responses, thus appearing ideal vectors for in situ antibody production. We hypothesized we could arm an oncolytic adenovirus with trastuzumab to achieve higher local versus systemic antibody levels in combination with immunogenic cell killing.

Experimental design: We constructed a tumor-targeted chimeric serotype 5 oncolytic adenovirus Ad5/3-Δ24-tras coding for human trastuzumab antibody heavy and light chain genes, connected by an internal ribosome entry site. Antibody production, anti-tumor efficacy and mechanism of action were evaluated in several HER2-positive and negative cancer cell lines preclinically.

Results: We observed assembly and release of the immunologically active antibody from the infected cancer cells, as confirmed by Western blot, ELISA, and antibody-dependent cell-mediated cytotoxicity (ADCC) assays. Ad5/3-Δ24-tras showed potent cytotoxicity, and enhanced anti-tumor efficacy over both oncolytic control virus Ad5/3-Δ24 and commercial trastuzumab in vivo (both $P < 0.05$). Furthermore, Ad5/3-Δ24-tras resulted in markedly improved tumor-to-systemic trastuzumab concentrations. Immunological analyses indicated dendritic cell activation and natural killer cell redistribution towards draining lymph nodes following Ad5/3-Δ24-tras treatment.

Conclusions: Ad5/3-Δ24-tras is an attractive gene therapy approach combining oncolytic and immunotherapeutic potency with local trastuzumab production, resulting in improved efficacy and immune cell activation in HER2-positive cancer. Moreover, the finding that tumor cells can produce functional antibody mediated by oncolytic virus could lead to many valuable anti-tumor approaches.

P223

Development of potency assay for recombinant adenoviral gene therapy product (rAd-IFN) to treat intravesical non-muscle invasive bladder cancer

J Mykkänen[1 2] H P Lesch[1 2 3] S Paulo R Shaw M Hassinen[1 2] S Ylä-Herttuala[3 4]

1: FKD Therapies 2: FinVector Vision Therapies Ltd 3: A.I.Virtanen Institute 4: Science Service Center and Gene Therapy Unit, Kuopio University Hospital

Potency assays are one of the key assays in defining the quality of gene therapy products. Potency assay measures the therapeutic activity of drug in a biological system and was required for this product aiming to phase III clinical trials. rAd-IFN is recombinant adenoviral gene therapy vector encoding IFNα2b gene for the treatment of refractory non-muscle invasive bladder cancer. Adenovirus transduce bladder wall cells where IFNα2b gene is expressed leading to death of cancer cells. We have developed a relative potency assay measuring the killing efficacy of rAd-IFN. Assay development was started by selection of interferon sensitive human bladder cancer cell lines, preparation of cell bank(s) and proofing the concept of the assay. Cells are transduced using multiple dilutions of reference standard and test samples leading to expression of IFNα2b and subsequent cell death. Cell killing efficiency is determined using colorimetric method measuring dehydrogenase activity of the living cells. Relative potency of test sample is determined against reference standard response curve after testing parallelism by equivalence test. We have optimized the assay for cell number, sample dilutions, curve fit, post-infection time and incubation

time for colorimetric method. Equivalence test was set up to measure parallelism of test sample and reference standard response curves. Assay qualification and robustness studies are ongoing aiming to qualify the assay for relative accuracy, precision, specificity, linearity and range according to ICH Q2 (R1). Next goal is to set the assay system suitability criteria based on qualification runs and perform the assay validation.

P224

Incorporation of avß6 integrin-targeting peptide into chimaeric Ad5/fibre knob 48 vector results in efficient tumour-targeting and evasion of neutralisation in clinical ascites

H K Uusi-Kerttula[1] J A Davies[1] L Coughlan[2] J D Chester[1 3] A L Parker[1] A L Parker

1: Institute of Cancer & Genetics, Cardiff University, Cardiff CF14 4XN, UK 2: The Jenner Institute, University of Oxford, Oxford OX3 7DQ, UK 3: Velindre Cancer Centre, Cardiff CF14 2TL, UK

Adenovirus 5 (Ad5) is commonly utilised for clinical cancer virotherapy applications, although its efficacy is hampered by poor tumour cell-specificity, high seroprevalence rates and significant ''off-target'' interactions that dictate tropism and toxicity, thus limiting bioavailability for tumour-targeting following systemic delivery. Pseudotyping Ad5 with capsid proteins from less seroprevalent Ad species may facilitate evasion of pre-existing immunity and reveal novel tropisms. We generated a chimaeric Ad5 containing fibre knob protein from the species D virus Ad48 (Ad5.knob48) via AdZ recombineering. $\alpha v\beta 6$ integrin is over-expressed during carcinogenesis and has been identified as a prognostic indicator. To enhance tumour-selectivity, a 20-aa $\alpha v\beta 6$ integrin-targeting peptide (NAVPN LRGDLQVLAQKVART, A20) from Foot and Mouth Disease virus was genetically engineered into knob DG loop (Ad5.-knob48.DG.A20). Knob pseudotyping increased transduction 3-fold compared to Ad5.luc on hCARlow/$\alpha v\beta 6$high BT-20 cells, whilst A20 peptide incorporation enhanced transduction by 98- and 53-fold for Ad5.A20 and Ad5.knob48.DG.A20, respectively. On hCARlow/$\alpha v\beta 6$high primary epithelial ovarian cancer cells, a 1.5-, 96- and 88-fold increase was observed, while transduction remained unchanged on control hCARhigh/$\alpha v\beta 6$low A549 cells. Ad5.knob48.DG.A20 was shown to utilise both CAR and $\alpha v\beta 6$ for cell entry, as confirmed by competitive inhibition assays. Furthermore, the combination of knob pseudotyping and A20 peptide incorporation resulted in efficient evasion of neutralising antibodies in the presence of ovarian ascites fluids – 100% transduction was achieved at up to 32-fold lower titre (1:40) for Ad5.knob48.DG.A20 compared to Ad5.luc (1:1280) in the presence of the most neutralising fluid. Consequently, Ad5.knob48.DG.A20 may represent a promising platform for future clinical applications in ovarian cancer.

P225

Oncolytic adenovirus loaded with active drugs as a drug delivery system for the treatment of cancer

M Garofalo B Iovine L Kuryk C Capasso M Hirvinen A Vitale M Bevilacqua V Cerullo[1]

1: ImmunoViroTherapy Lab, Centre for Drug Research (CDR) and Division of Pharmaceutical Bioscience, Faculty of Pharmacy, University of Helsinki 2: Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, Naples, Italy

Many clinical studies have shown that the oncolytic approach alone could not efficiently destroy the large tumor mass, thus by limiting an efficacy virotherapy. Combination of oncolytic adenoviruses and chemotherapeutic drugs has shown promising therapeutic results due to the synergistic action of virus and drug and is considered as a potential approach for cancer therapy. In the present study, we have optimized a model to use oncolytic adenovirus as a scaffold to deliver active drugs. First, we have developed a strategy to conjugate peptides on viral capsid, based on electrostatic interactions. We have used carnosine which is a naturally occurring histidine dipeptide, with a number of biological functions, including a significant antiproliferative activity both in vitro and in vivo. Carnosine positively charged, consisting of a tail of six lysines (Carnosine6K), has been combined with the surface of oncolytic adenovirus that is negatively charged, to allow the formation of a complex (Ad5D24CpG-Carnosine6K). The oncolytic activity of the complex was tested in a lung and colon cancer xenograft model. We found that the intratumoral injection of the complex has efficiently reduced tumor growth and most of the tumors were fully eradicated within 18 days after the treatment. In conclusion, our results encourage the use of Ad5D24CpG-Carnosine6K for new therapeutic protocols for the treatment of cancer and in particular human colon cancer and lung cancer. Moreover the use of oncolytic adenoviruses loaded with active drugs could be used as a novel drug delivery system for cancer therapy.

P226

Repeated cell transduction with non-integrating retroviral vectors for cell fate modification

J W Schott[1] D Hoffmann[1] N M Jaeschke[1] T Maetzig[1] M Ballmaier[1] T Godinho[1] F J Müller[4] T Cathomen[3] A Schambach[1 2]

1: Hannover Medical School 2: Harvard Medical School 3: University Medical Center Freiburg 4: Christian-Albrechts-University Kiel

Non-integrating retroviral vectors (NiRV) express transgenes from extrachromosomal DNA episomes. Beneficial for application, NiRV bypass genotoxicity risks associated with integrating vectors. In proliferating cells, episomal transgene expression is transient as a consequence of dilution through cell division. Temporally controlled stimuli generating integration-free cell products are especially valuable in the context of cell fate modification. As the natural expression duration after a single NiRV transduction might not suffice in certain settings, we developed a repeated transduction strategy to prolong expression duration and in detail characterized the kinetics after episomal gene transfer. As validated by transfer of fluorescent proteins, repeated NiRV transduction was stochastic, suitable to generate expression windows of choice, and applicable to various cell types. Cell proliferation rate, transgene half-life and transduction intervals were identified as main parameters determining expression kinetics, and can thus serve to adjust desired expression courses. NiRV retransduction also enabled prolonged coexpression of multiple transgenes upon their repeated coadministration, or alternatively allowed the recapitulation of defined temporal expression

sequences. Two independent settings gave prospect for application in cell fate conversion. For potential modification of human induced pluripotent stem cells (hiPSCs), high transduction efficiencies exceeding 85% were reached exemplarily with GFP-expressing NiRV. Underlining the biological significance of retransduction, repeated transfer of Oct4-expressing NiRV could saturate required Oct4-levels for successful reprogramming of human fibroblasts, which stably expressed the remaining reprogramming factors (Klf4, Sox2, Myc), into hiPSCs. This study provides both general findings on NiRV expression characteristics and evidence that repeated NiRV transfer is suitable for cell fate modification.

P227

Targeted genome editing of cell lines for improved and scalable production of lentiviral vectors for human gene therapy

A Cantore[1] M Milani[1] J Lengler[2] S Bartolaccini[1] T Di Tomaso[1] P D Gregory[3] F Scheiflinger[2] A Lombardo[1 4] L Naldini[1 4]

1: San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET) 2: Baxter Innovations GmbH 3: Sangamo BioSciences, Inc. 4: Vita-Salute San Raffaele University

Lentiviral vectors (LVs) represent efficient and versatile vehicles for gene therapy. The manufacturing of clinical-grade LVs relies on transient transfection of vector components. This method is labor and cost intensive and difficult to scale-up. The development of stable LV producer cell lines may greatly facilitate overcoming these hurdles. We have generated an inducible packaging cell line, carrying the genes encoding for third-generation LV components stably integrated in the genome under the control of tetracycline-regulated promoters. In order to minimize the immunogenicity of LVs, we set out to remove the polymorphic and antigenic class-I major histocompatibility complex (MHC-I) expressed in LV packaging cells and subsequently incorporated on the LV envelope. We performed genetic disruption of the β-2 microglobulin (B2M) gene, required for the exposure of the MHC-I on the plasma membrane in LV producer cells, by CRISPR/Cas9. We generated B2M-negative cells, producing MHC-free LVs. To insert a LV genome of interest in the cell line, we performed site-specific integration in a locus of the genome of these cells, chosen for robust expression. We obtained several producer cell lines for LV expressing marker genes or a therapeutic gene, i.e. coagulation factor IX. We show that LVs produced by these cells have reproducible titer and infectivity and transduce relevant target cells, such as hematopoietic stem/progenitor cells and T lymphocytes ex vivo and the mouse liver in vivo. Overall, we provide evidence that rationally designed targeted genome engineering can be used to improve the quality, safety and sustainability of LV production for clinical use.

P228

In vivo distribution of HIV based 3rd generation of lentiviral vectors used in gene therapy in DOI animal model of type 2 diabetes

Y E Eksi[1] H M Tasyurek[1] M K Balci[2] S Sanlioglu[1]

1: Akdeniz University Center for Gene and Cell Therapy 2: Department of Internal Medicine, Division of Endocrinology and Metabolism, Akdeniz University Faculty of Medicine, Antalya, Turkey, 07058

Considering safety, duration and the level of transgene expression, 3rd generation of HIV based lentiviral vectors appeared to be superior to other virus based gene therapy vectors for the treatment of genetic diseases one of which is the Type 2 diabetes (T2DM).[1] In order to investigate the tissue tropism of lentiviral vectors in an animal model of T2DM, Diet Induced Obesity (DIO) animal model was generated by feeding C57BL6/J mice with high fat diet (where 60% of the calories come from fat) for two months. Diabetes was induced by intraperitoneal injection (IP) of 150 mg/kg streptozotocin (STZ) in animals exhibiting insulin resistance and glucose intolerance. 5 ug of lentivirus (based on p24 titer) encoding red fluorescein protein (LentiRFP) was IP injected into the obese and diabetic C57BL6/J mice. IP route of gene delivery was chosen to avoid trapping of most of the lentiviral vectors in liver and to increase gene delivery to other abdominal tissues. Animals were sacrificed 2 and 4 months after the vector injection. Quantitative PCR analyses were performed to determine genome integrated copy numbers of LentiRFP on genomic DNAs of liver, heart, pancreas, spleen and kidneys. Based on our analysis, liver (52%), spleen (28%) and pancreas (18%) appeared to be the tissues with highest integrated lentivirus vector copy number among the tissues examined following IP gene delivery. Grant support: TUBITAK-111S157

Reference

1. Sanlioglu, A., et al., Therapeutic potential of VIP vs PACAP in diabetes. J Mol Endocrinol, 2012. 49(3): p. R157–67.

P229

Targeted homologous recombination of lentivirus vectors into ribosomal DNA

A Nousiainen[1] D Schenkwein[1 2] N Laham Karam[1] S Wojciechowski[3] P Mäkinen[1] S Ylä-Herttuala[1 4 5]

1: Department of Biotechnology and Molecular Medicine, A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, Kuopio 702110, Finland 2: Section Molecular and Gene Therapy, Department of Translational Oncology, National Center for Tumor Diseases and German Cancer Research Center, Heidelberg, 69120, Germany 3: Department of Neurobiology, A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, Kuopio, 70211, Finland 4: Science Service Center, Kuopio University Hospital, Kuopio, Finland 5: Gene Therapy Unit, Kuopio University Hospital, Kuopio, Finland

Targeting vector-mediated integration to sites that allow for the stable expression of a transgene without interfering with endogenous gene expression would be a great improvement to the safety of gene therapy. Our aim was to find out whether a combination of I-PpoI endonuclease-induced double-strand breaks, a donor molecule with target site homology, and cell cycle synchronization would increase targeted integration of lentivirus vectors. Two I-PpoI cleavage sites were chosen for this study: one in an intron of the DAB1 gene on chromosome 1, and one in the 28S ribosomal RNA gene. Donor constructs bearing homology to both sites (Chr1 HR and 28S HR) were constructed and packaged into three different vector types: integrase-deficient lentivirus (IDLV), integration competent lentivirus (ICLV), and endonuclease-carrying lentivirus (ECLV). The nontoxic cell cycle modulator mimosine was found to effectively halt the cell cycle of MRC-5 lung fibroblasts into

the G1 phase and an optimal amount of cells had entered the S-phase at 7h after release. Cell cycle synchronization was found to enhance transduction efficiency when the vector contained a homologous sequence, as analyzed by flow cytometry. A significant difference in the transduction efficiencies of IDLVs and ECLVs was noticed between different target sites: transduction was more efficient with 28S HR vectors than with Chr1 HR vectors. Efforts to determine integration or homologous recombination at the intended sites are currently ongoing. Future tests will include the determination of targeting efficiency, vector copy number and transgene expression strength from the target locus in correctly modified cells.

P230

Improvement of integrative deficient lentiviral vectors (IDLV) design

S Sánchez-Hernández A Gutierrez-Guerrero J Fuster M Cobo K Benabdellah F Martín

1: Fundación Progreso y Salud/GENyO

Integrative deficient lentiviral vectors (IDLVs) have been described as an interesting alternative for stable expression of transgenes in non-dividing cells and for transient expression in highly dividing cells. However, both the titer and expression levels of IDLV are significantly reduced compared to integrative competent lentiviral vectors (LVs) precluding a broader use of IDLVs. This observation is, in part, attributed to epigenetic transcripitional silencing. In this regard we have previously shown that the inclusion of chimeric chromatin insulators based on the chicken β-globin locus control region hypersensitive site 4 (HS4), and scaffold/matrix attachment regions (SARs/MARs) in the LV backbone avoid silencing and enhance expression. In the presents study we tested the effect of these elements in combination with WPRE, in term of gene expression level and titer in IDLV. We compared the effect of the different combinations in K562 and 293T cells as well as in human hematopoietic stem cells (hHSCs). We found that the inclusion of a chimeric insulator, IS2 (combining the SAR2 and the HS4) in combination with WPRE within the IDLV, allows higher titers and expression levels in all cells analyzed. We therefore proposed IS2-IDLV as a new platform for transient transgene expression and for stable expression in non-dividing cells.

P231

Serum proteins and rAAV efficacy

J Denard[1] C Jenny[1] T Leger[2] C Garcia[2] J M Camadro[2] F Svinartchouk[1]

1: Généthon 2: Institute Jacques-Monod

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. The success of future clinical trials depends much on the adequacy of the results obtained in animal models. We have previously demonstrated that blood proteins, galectin 3 binding protein (G3BP) and C-reactive protein (CRP) interact with different AAV serotypes in a species specific manner (Denard et al., J Virol. 2012: 6620-31) crucially changing vector's efficacy. While human and dog G3BP were able to interact with rAAV-6 and diminished its transduction efficiency, the mouse CRP protein increased more than 10 times the transduction efficiency of rAAV-1 and rAAV-6 under systemic delivery (Denard et al., J Virol. 2013: 10784-91). Taking into account the importance of the blood proteins for the efficiency and biodistribution of rAAV vectors, we carried out systematic studies of proteins interacting with different AAV serotypes in sera from mice, dog, cow, macaque and human. We demonstrate for the first time that the blood of each tested species contain specific set of proteins to interact with a given serotype. The role of these particular proteins in the transduction efficiency of rAAV vectors will be discussed.

P232

Adenoviral mediated compartmentalized liver transduction resolves viremia, biodistribution, toxicity and yields long terms transgene expression in a porcine model

G Cabrera[1] B Segura[1] M Granados[1] A Esparza[1] V M Oviedo[1] A Catzin-Kuhlmann[2]

1: Global BioTherapeutics, Mexico City, Mexico 2: National Inst. of Medical Sciences and Nutrition, Mexico City, Mexico

Compartmentalized Liver Transduction (CLT) is achieved by the intra-parenchymal injection of a vector dose into a blood flow isolated portion of the liver; perfusion is reestablished after viral endocytosis has been completed (30 min). Given the encouraging results obtained in proof of principle studies done in rats, our group assessed CLT in a porcine model using 1st generation E1-E3 deleted adenovirus. With this aim, laparoscopic surgical devices were conceptualized and prototyped to achieve CLT in large species. Implementing CLT in Vietnamese pigs, no viremia was detected during CLT, biodistribution assessment revealed no vector genome presence in ten distal tissues/organs. Transgene expression and presence were confined to the site of adenovirus administration. Toxicological evaluation revealed no liver damage as determined by histology, apoptosis and liver damage enzyme levels. Using porcine alpha-fetoprotein as a reporter gene, serum levels were documented above endogenous levels with a vector dose of $1.6 \times 10\text{^}5$ and prolonged reporter gene expression was achieved (10 months). The results obtained in the present study further support that CLT has profound paradoxical effects on the toxicological interactions between the liver and 1st generation adenovirus. We hypothesize that CLT induces the local synthesis of tolerance inducing cytokines that thwart the acute, intermediate and late adenoviral induced immune responses and are critical in generating an immune privileged liver compartment capable of long-term protein synthesis and secretion. Our results further demonstrate that compartmentalized liver transduction is a safe and effective mode of vector delivery with a promising future in gene therapy.

P233

The oncolytic features of and innate immune responses to clinical herpes simplex virus isolates

J Lehtinen[1] H Paavilainen[1] V Hukkanen[1]

1: Department of Virology, University of Turku

Herpes simplex virus (HSV) based oncolytic viruses (oHSV) developed so far have proven to be safe, but the modest efficacy has left room for improvement. We suggest that it could be

beneficial to develop oHSV based on clinical HSV strain as many of the currently existing oHSVs are derived from attenuated laboratory strains. We explored the oncolytic features of several clinical HSV-1 and HSV-2 strains in cell lines representing the natural host tissues of HSV and potential cancer tissues as well. We included laboratory strains of HSV-1 and 2 in our studies to be reference for clinical strains. Among the clinical strains we selected three HSV strains for immunological studies. The selection was based on plaque morphology, cell tropism and growth properties. The selected ones were all HSV-2 strains, even though the replication of HSV-1 strains was more efficient in the studied cell lines. Selected HSV-2 strains showed favorable cell tropism in terms of oncolytic virotherapy and in general HSV-2 strains caused more lytic infections than HSV-1 strains. One of the studied HSV-2 strains caused severe infection and high replication in glioma cells compared to other HSV-2 strains. This HSV-2 strain also induced strong delayed IFN-alpha and IFN-lambda responses at 48 hours post infection. Another HSV-2 strain replicated especially in ocular RPE cells and caused mild interferon responses in all cell types, which could be a sign of advanced immune evasion mechanisms. These HSV strains show potential to be further developed into oncolytic HSV vectors.

P234

Assessing HAdV-5 immune neutralization and tropism in vitro and in vivo in immunocompetent and immunocompromised mice

E Lopez-Gordo[1] L Denby[1] A Bradshaw[1] S A Nicklin[1] A H Baker[1]

1: Institute of Cardiovascular and Medical Science, University of Glasgow

Following intravascular delivery, coagulation factor X (FX) interacts with human adenovirus serotype 5 (HAdV-5) hexon mediating liver transduction and protection from attack by the classical complement pathway (IgM-mediated). FX is not required for liver transduction in mice lacking IgM. Here we investigated HAdV-5 neutralization and the role of FX, $\alpha v\beta 3/\alpha v\beta 5$ integrins and CAR in liver transduction in mice. Intravascular administration of a non-FX binding HAdV-5 (AdT*) to C57 BL/6 mice produced 6800-fold lower liver transduction compared to HAdV-5 ($P < 0.05$) but was not different in NSG mice, which lack innate and adaptive immunity. Transduction was, however, 23-fold lower in Rag 2-/- mice liver, only lacking adaptive immunity. Administration of $\alpha v\beta 3/\alpha v\beta 5$ integrin-binding ablated HAdV-5RGE or CAR-binding ablated HAdV-5KO1 to C57 BL/6 mice resulted in 12- and 4-fold lower liver transduction, respectively. Next, in vitro transduction was assessed in the presence of Rag2-/- serum. While serum enhanced transduction in high and low CAR-expressing cells, the presence of X-BP to block hexon:FX interaction only inhibited this enhancement in low CAR-expressing cells. Wild type but not non-CAR-binding fiber knobs or HAdV-5KO1 inhibited serum enhanced transduction in high CAR-expressing cells. This data suggest innate immunity alone may be sufficient to partially neutralize HAdV-5 in vivo. Ablating FX, $\alpha v\beta 3/\alpha v\beta 5$ integrins, or CAR-binding reduced but failed to achieve total HAdV-5 liver detargeting indicating other factors are required. In vitro studies suggest the existence of an alternative transduction pathway in presence of mouse serum involving a host factor bridging HAdV-5 to CAR, which may contribute to efficient liver transduction.

P235

Assay Conditions Significantly Alter AAV Neutralizing Antibody Determination Outcome

H T Turunen[1 2 3 4] E Zinn[1 2 3 4] L H Vandenberghe[1 2 3 4]

1: Schepens Eye Research Institute 2: Massachusetts Eye and Ear Infirmary 3: Harvard Medical School 4: Grousbeck Gene Therapy Center

A major limitation of vectors based on current AAVs is the wide prevalence of memory B and T cell responses in human populations arisen following natural AAV infection. The Neutralizing Antibody (NAB) assay guides experimentation and determines inclusion or exclusion of subjects in clinical studies. It is used directly to predict in vivo neutralization of gene delivery to the target tissue, and indirectly to indicate the presence of memory T-cells reactive to the AAVs tested. We set out to improve the sensitivity, robustness, and reproducibility of the NAB assay and aimed at validating it with other measures of pre-existing immunity (PEI). We sought to evaluate the compliance of standard AAV NAB assays with the Percentage Law, formulated by Andrewes and Elford in 1933, a standard for virological NAB assays. Protocol conditions and parameters were independently tested in how they influence assay readout and outcome. Results indicate these variables quantitatively and qualitatively alter the interpretation of PEI status. Our studies led to propose a modified NAB assay with enhanced 10-1000-fold improved sensitivity and high reproducibility, currently under validation using nonhuman primate gene transfer study samples and data. Our studies highlight the importance of PEI in AAV gene therapy and limitations of the current methodologies to measure it in a robust and predictive manner. Data indicates the potential for false positive and negative readout for assays currently in clinical use. An optimized protocol was developed for further validation and evaluation. These studies may impact translational and clinical AAV gene therapy studies.

P236

Improving the therapeutic effect of adoptive T-cell therapy by oncolytic adenovirus encoding CD40-ligand

S Parviainen[1 2] S Zafar[1] S Bramante[1] O Hemminki[1] M Siurala[1 2] R Havunen[1] I Diaconu[1] A Hemminki[1 2]

1: Cancer Gene Therapy Group, Department of Pathology, University of Helsinki, Helsinki, Finland 2: TILT Biotherapeutics Ltd, Helsinki, Finland

Adoptive cell transfer is currently considered as a highly promising therapeutic option for treatment of incurable malignant cancers. However, despite these impressive development, in advanced, solid tumors benefits has been limited. The tumor micro-environment may direct immune evasion and result in insufficient numbers and function of T-cells, eventually leading to immunosuppression and tumor tolerance. Interestingly, oncolytic adenoviruses have been shown to cause biological effects which could be utilized to overcome the immunosuppression by trigger danger signals at the tumor site and enhancing the release of tumor-specific antigens. To achieve optimal activation of the transferred T-cells, we armed viruses with CD40L, a surface receptor best known for its capacity to initiate multifaceted signals in dendritic cells, leading to activation of cytotoxic T-cells. In addition, CD40L can cause apoptosis in CD40 + tumor

cells. Therefore, we constructed selectively oncolytic serotype 3 adenovirus (Ad3) featuring human telomerase (hTERT) promoter and human CD40L (Ad3-hTERT-CMV-hCD40L). One major obstacle with oncolytic adenoviruses is suboptimal systemic delivery, which can be circumvented by using a fully Ad3 platform. Previous human data has shown the ability of Ad3 to successfully reach tumors through the intravenous route. To deeply dissect if CD40L-encoding adenovirus can modulate the tumor microenvironment, we generated a murine version of the virus (Ad5/3-CMV-mCD40L). With these two viruses we are able to study the effects of the CD40L on tumor-infiltrating lymphocytes, the T-cell graft and the reduction of immunosuppression in the tumor stroma. These findings support development of clinical trials where T-cell therapy is enhanced with oncolytic adenovirus.

P237

Targeted enrichment of wtAAV to establish comprehensive wtAAV IS profiles

K Petri[1] R Gabriel[1] S Wilkening[1] R Fronza[1] S Afzal[1] C von Kalle[1] R M Linden[2] E Henckaerts[2] M Schmidt[1]

1: DKFZ and National Center of Tumor Diseases (NCT) Heidelberg 2: King's College London School of Medicine at Guy's Hospital

Wildtype adeno-associated virus (wtAAV) is able to establish latency by integrating its DNA into preferred loci in the human genome. The most frequently targeted locus is a specific region on chromosome 19, named AAVS1. To evaluate the potential of wtAAV integration as a tool for targeted transgene integration, detailed knowledge about the hallmarks of wtAAV integration will be required. Most currently available methods, which analyze viral integration site (IS) profiles, amplify vector-genome junctions with primers binding near the viral ends. Therefore, knowledge about the structure of integrated wtAAV genomes remains limited. To address this, we investigated 2,829,267 wtAAV concatemeric structures to analyze the stability of the wtAAV genome. In addition to the ITRs and the p5 promoter region, a number of other preferred breakpoints exist throughout the wtAAV genome. A novel approach involving targeted enrichment of AAV and AAVS1 sequences enabled us to consider viral breakpoints at any position of the AAV genome. Targeted HiSeq sequencing of enriched sequences revealed hundreds of viral breakpoints and confirmed that a substantial fraction of viral junctions are observed outside of AAV ITRs and the p5 region. Those cryptic integration events, not picked up by the majority of methods currently available, are highly interesting for AAV gene therapy. Our findings suggest that comprehensive wtAAV IS profiles should be established by including primer-independent methods for IS analysis.

P238

Alignment free high resolution topological mapping of recombinant adeno-associated virus (rAAV)

R Fronza I G Farina C von Kalle M Schmidt

1: DKFZ and National Center of Tumor Diseases (NCT) Heidelberg

The rare integration events and strong secondary structures present in the adeno-associated virus (AAV) have severely disadvantaged the detection of recombinant proviruses in clinical and preclinical samples. Thus, genome-wide mapping of rAAV integration sites have been partially limited due to the inadequacy of the bioinformatics efforts put in trying to understand the viral rearrangement. Vector concatemerization in reads are analyzed using a two fragments model where 5′ ends align to the megaprimer region and the remaining 3′ nucleotides align to different parts of the proviral sequence. When both ends of a sequence aligned to the vector, concatemers are assumed. We present and discuss a framework that expands the possibility of the concatemerization analysis. Using an alignment free technique we are able to detect with great precision the origin of all the vector fragments in any single reads and allowing to

1) sort reads on the basis of the complexity of the AAV genome rearrangements for better integration sites identification;
2) detect rearrangements hotspots on the viral genome;
3) evaluate rearrangement patterns, inferred by extending the current interpretation based on the multiplicity of tail/head rearrangements observed;
4) test the order randomness of the vector fragments in the reads;

To conclude, the aim of this work is to frame the characterization of AAV recombination as a statistical and computational problem. Moreover we think that using robust and reproducible criteria, future studies will help to improve our understanding on the mechanistic foundation and the functional consequences of vector rearrangements.

P239

The integration preferences of HIV-1 in viremic patients: similarities and differences to long-term cART-treated patients and lentivirus vectors in vivo

D Schenkwein[1 8] R Fronza[1] N Kootstra[2] B Link[1] F A Giordano[3] C C Bartholomae[1] C Weber[1] H G Kräusslich[4] C Stephan[5] O T Keppler[6] W Kern[7] S Usadel[7] C von Kalle[1] M Schmidt[1 9] S Laufs[1 9]

1: Section Molecular and Gene Therapy, Department of Translational Oncology, National Center for Tumor Diseases and German Cancer Research Center, Heidelberg, 69120, Germany 2: Department of Experimental Immunology, and Center for Infectious Diseases and Immunity Amsterdam (CINIMA), Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands 3: Department of Radiation Oncology; Universitätsmedizin Mannheim, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany 4: Department of Infectious Diseases, Virology, University Hospital Heidelberg, Heidelberg, Germany 5: Medical Department no. 2, Infectious Diseases Unit, University Hospital Frankfurt, Frankfurt, Germany 6: Institute of Medical Virology, National Reference Center for Retroviruses, University of Frankfurt, Frankfurt, Germany 7: Center for Infectious Diseases and Travel Medicine, University Medical Center, Freiburg, Germany 8: Molecular Medicine, Department of Biotechnology and Molecular Medicine, A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, Kuopio, 70211, Finland 9: Share Senior Authorship

Information regarding the integration sites (IS) of the Human Immunodeficiency Virus 1 (HIV-1) during long-term combination

antiretroviral treatment (cART) has increased significantly during the past year, confirming that certain infected cells undergo clonal proliferation during prolonged cART. IS of integrated viruses (proviruses) in such cells often reside in the cellular genes BACH2 and MKL2. One possible mechanism behind clonal expansion is a growth advantage gained by infected cells from proviral sequences. Analyzing IS prior to clonal expansion could yield a more unbiased view of preferred integration by wild-type (wt) HIV-1 in vivo and shed light on the factors that promote clonality. In this study, 2244 HIV-1 IS were analyzed with linear amplification mediated PCR from viremic and short-term cART –treated patients. The integration pattern and common integration sites (CIS) were compared to previously published data of long-term cART patients. This analysis revealed that IS in BACH2 and MKL2 were significantly less favored in viremic and short-term cART patients than after long-term cART. However, clonally proliferated cells harboring proviruses in these and other cellular genes were observed also in the absence of cART. Integration hotspots of wt HIV-1 in viremic and short-term cART patients partly resembled those of lentivirus vectors in gene therapy patient data, but certain CIS were more similarly favored in the two compared wt HIV-1 data sets. This study confirms that prolonged cART exposes clonally expanded cells that harbor IS in specific cellular genes, and suggests that wt virus-intrinsic sequences may have a role in promoting clonal expansion.

P240

Kallistatin ameliorates influenza virus pathogenesis by inhibiting kallikrein-related peptidase 1-mediated cleavage of viral hemagglutinin

C L Wu[1] C H Leu[1] M L Yang[2] J Chao[3] L Chao[3] A L Shiau[2]

1: Department of Biochemistry and Molecular Biology, National Cheng Kung University Medical College, Tainan, Taiwan 2: Department of Microbiology and Immunology, National Cheng Kung University Medical College, Tainan, Taiwan 3: Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, USA

Proteolytic cleavage of the hemagglutinin (HA) of influenza virus by host trypsin-like proteases is required for viral infectivity. Some serine proteases are capable of cleaving influenza virus HA, whereas some serine protease inhibitors (serpins) inhibit the HA cleavage in various cell types. Kallikrein-related peptidase 1 (KLK1, also known as tissue kallikrein) is a widely distributed serine protease. Kallistatin, a serpin synthesized mainly in the liver and rapidly secreted into the circulation, forms complexes with KLK1 and thereby inhibits its activity. In this study, we investigated the roles of KLK1 and kallistatin in influenza virus infection. We show that KLK1 expression was increased, whereas kallistatin expression was reduced in the lung of mice during influenza infection. KLK1 cleaved H1, H2, and H3 HA molecules and consequently enhanced viral production. By contrast, kallistatin inhibited KLK1-mediated HA cleavage and reduced viral production. Furthermore, lentivirus-mediated gene delivery of kallistatin protected mice against lethal influenza virus challenge by reducing viral load, inflammation, and injury in the lung. Taken together, we identify that KLK1 and kallistatin contribute to the pathogenesis of influenza virus by affecting the cleavage of the HA peptide and inflammatory responses. In conclusion, this study provides a proof of principle for the potential therapeutic application of kallistatin or other KLK1 inhibitors for influenza. Since proteolytic activation also enhances the infectivity of various viruses, kallistatin and other kallikrein inhibitors may be explored as antiviral agents to target these viruses.

P241

Therapeutic outcomes of an HIV-based lentivirus mediated Glucagon-like peptide-1 gene delivery for diabetes treatment

H M Tasyurek[1] H A Altunbas[2] H Canatan[3] S Sanlioglu[1]

1: Akdeniz University Center for Gene and Cell Therapy 2: Department of Internal Medicine, Division of Endocrinology and Metabolism, Akdeniz University Faculty of Medicine, Antalya 3: Department of Medical Biology, Faculty of Medicine, Erciyes University, Kayseri

Glucagon-like peptide-1 (GLP-1) is a metabolic hormone with insulinotropic properties manifested by the enhancement of glucose induced insulin secretion from the islets of Langerhans after eating. Additionally, reduced gastric emptying and food intake result in weight loss in the long run. Inhibition of glucagon release from the pancreatic alpha cells is another beneficial effect of GLP-1 relevant to diabetes therapy. Unfortunately, the incretin response to glucose is reduced in type 2 diabetes which is accompanied by a moderate degree of GLP-1 hypo-secretion. To compensate the reduced incretin effect, a third generation of an HIV based lentiviral vector was generated to deliver human GLP-1 encoding DNA (LentiGLP-1) [1] and its anti-diabetic efficacy was tested in a high fat diet/low dose STZ induced experimental rat model of Type 2 Diabetes (T2DM). Based on our results, intraperitoneal (IP) delivery of LentiGLP-1 into diabetic Sprague Dawley rats drastically reduced blood glucose levels and concurrently improved insulin sensitivity and glucose tolerance. Plasma triglyceride levels were also normalized in LentiGLP-1 injected diabetics without altering blood cholesterol. Based on these findings, it is safe to claim that LentiGLP-1 vector should be assessed as a novel gene therapy modality for the treatment of patients with T2DM. Grant support: TUBITAK-112S114

Reference

1. Tasyurek, M., et al., GLP-1-mediated gene therapy approaches for diabetes treatment. Expert Rev Mol Med, 2014. 16: p. e7.

P242

GLP Principles in gene therapy: experience and challenges

I Visigalli[1] M Vezzoli[1] N Carriglio[1] F Sanvito[2] S Scaramuzza[1] A Sauer[1] F Ferro[1] S Delai[1] R J Hernandez[1] G Mandelli[1] F Tiboni[1] C Rossi[1] C Villa[2] P Albertini[1] E Stradella[2] V Parezanovic[2] A Aiuti[1 4] A Biffi[1] G Ferrari[1 4] L Naldini[1 4] P Cristofori[1 3]

1: San Raffaele-Telethon Institute for Gene Therapy (HSR-TIGET) 2: San Raffaele Scientific Institute, Milan, Italy 3: GlaxoSmithKline, R&D, Ware, UK 4: Vita-Salute San Raffaele University, Milan, Italy

Gene therapy is now emerging as a medical reality with clinical efficacy demonstrated in a number of gene therapy trials and an increasing number of products entering phase ll and lll trials each year. For the promise and potential of a gene therapy medicinal

product (GTMP) to be fully realised it is important to address regulatory expectations. Guidelines for GTMP progression in clinical trials and marketing authorizations are available to facilitate a harmonized approach in the EU and US. Non-clinical studies have the primary objective of providing sufficient information for a proper risk assessment for the product's use in human subjects. The paradigm described in ICH M3 for safety evaluation of conventional pharmaceuticals is recognised as not always appropriate or relevant to GTMPs. Non-clinical studies should be designed on a case by case basis, understanding the relevant aspects of the science underpinning that product and need for specific expertise beyond the traditional pharmaceutical field. The combination of expertise of personnel trained in research, experimental pathology, safety assessment and quality assurance has enabled setting up GLP Test Facilities in an academic environment. The objective is to maximise the early collection of proof-of-concept data that address regulatory expectations, with assurance of scientific integrity, validity and reliability. Challenges in designing non-standard study protocols to investigate in vivo fate of genetically modified cells (biodistribution) and toxicological/tumorigenicity potential will be discussed focusing on GTMP characterisation, test system (animal model), sample traceability, duration of treatment and endpoints.

P244

Cell-based biosensors for detection and quantification of gene therapy label-free viral vectors

M R Guerreiro[1 2] A F Rodrigues[1 2] P M Alves[1 2] A S Coroadinha[1 2]

1: IBET-Instituto de Biologia Experimental e Tecnológica 2: Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa

The past decade witnessed the rise of interest and investment in biotherapies using viral vectors for gene therapy purposes. Clinical application of these particles depends on accurate and reliable quantification of their transduction potential. However, current methods fail to provide it, meeting at least one of the following pitfalls: extremely time-consuming, lack high-throughput potential, based on indirect measurements that over-estimate the real titer, or require the use of reporter genes (label-containing virus), unacceptable in a clinical context. A novel method capable of overcoming these drawbacks would be of great value for research, diagnostics and industry. Herein, we report the development of novel mammalian cell-based fluorescent biosensors for accurate and robust quantification of label-free viruses: VISENSORS. For that, we are implementing conditional-fluorescent biosensors, triggerable by viral enzyme activity thus, a label-free system that are stably expressed into suited mammalian cell lines. Its applicability for the detection of two gene therapy vectors, based on Human Adenovirus type 5 and HIV1 lentivirus, in a HEK 293 sensor cell line was confirmed. High-throughput potential when coupled to a fluorometer plate reader is undergoing. A signal to noise of 2 was obtained in line with previously reported mammalian cell-based fluorescent biosensors and leaves room for further improvements by means of molecular biology and computational methods. VISENSORS will deliver fast and accurate quantification methods for clinically relevant label-free viral vectors, currently missing in the gene therapy field, establishing a new platform not only for diagnostic and clinical applications but also for basic virology research.

P245

Flow cytometry analysis for a recombinant adenoviral gene therapy vector

S Paulo[1] J Mykkänen[1 2] M Hassinen[1 2] R Shaw[1]

1: FinVector Vision Therapies Ltd 2: FKD Therapies

Infection capacity of a gene therapy vector is a key aspect in demonstrating biological activity of the product. Biological activity of the vector is the most important factor in regards to patient dose as well as overall efficacy and consistency of the therapy. We have developed a flow cytometric assay to determine the infectivity a recombinant adenoviral vector. This assay will be used in conjunction with potency and expression assays for final product release in Phase III clinical trial to demonstrate biological function of the vector. In our assay, cells that support adenovirus replication are infected with different concentrations of adenovirus. After infection, percentage of infected cells is determined with a flow cytometer utilizing a fluorescently conjugated antibody against an adenoviral structural protein. Samples are analysed in parallel with a reference standard and infectivity is given as relative Infectious Units/ml. The assay has been optimized for cell density upon infection, virus contact time as well as post infection time. Especially cell density was determined to be a significant factor in this analysis. The staining procedure for flow cytometry has also been optimized and suitable virus concentrations have been selected so that a linear response is observed. Relative infectivity result is calculated using Slope Ratio method as outlined in Ph. Eur. 5.3 and USP <1034>. This assay will be qualified and validated for ICH Q2 parameters; specificity, linearity, range, accuracy and precision.

P246

iVector: a Brain and Spine Institute's facility for bioproduction of viral and non-viral gene transfer vectors.

A Sobczyk[1] B Bonnamy[1] C Ripoll[1] P Ravassard[1]

1: Institut du Cerveau et de la Moelle épiniere

The Vectorology core facility (iVector) produces batches of highly concentrated viral gene transfer vectors of controlled quality at small and medium scale. The laboratory staff has developed an important collection of lentiviral expression backbones ready to accept any gene of interest in order to provide the best solution for every in vitro or in vivo application encompassing fundamental research, cell engineering, gene therapy, cellular therapy and vaccine. Its BSL2 and BSL3 biocontained suites allow to satisfy every demand of viral production. The close collaboration between iVector and in house research teams (ie: biotechnology & biotherapy) ensures the constant evolution of gene transfer technologies made available to researchers. Main activities:

- Design and construction of viral and non-viral vectors for any application.
- Maxi and Giga-preparations of vectors (endotoxin free conditions).
- Production of Lv (lentiviral average titer 10^9 TU/mL) and Rv (retroviral) recombinant viral vectors (Lv ''ΔU3 or SIN'' vectors).
- Collection of control lenti-vectors (GFP, miR neg, …).
- Development of viral transduced cell lines.

- CAV-2 and rAAV vectors with specific serotypes needed in neurobiology are under development and will be available by the end of 2015.
- USP and DSP optimization for production and purification of viral particles.
- Scientific, technical and regulatory consulting for viral vectors design and production. iVector is part of the Brain and Spine Institute's core facility network which facilitates translational research projects. ISO9001 certification of iVector core facility is scheduled in June 2015.

P247

A new approach for stable lentiviral vector producer cell line development

H A Tomás[1 2] A F Rodrigues[1 2] A S Formas-Oliveira[1 2] P M Alves[1 2] M J T Carrondo[1 3] A S Coroadinha[1 2]

1: IBET-Instituto de Biologia Experimental e Tecnológica 2: Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa 3: Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa

Lentiviral vectors (LV) are efficient tools to mediate gene transfer. In the last 10 years, their use in gene therapy clinical trials has grown due to their specific ability to transduce both dividing and non-dividing cells and safer integration pattern, the major obstacle to clinical use being their low titer. Despite recent improvements in transient LV productions at higher scales, those are still limited to a short production period, are expensive and raise safety issues. A continuous production system where a stable producer cell line constitutively produces LV is desirable. Its development being very laborious and time-consuming; the first limiting step is the screening of a clone with a high Gag-Pro-Pol polyprotein expression. The few LV packaging cell lines available are derived from a 2nd generation LV packaging system and were generated by viral transduction of at least one LV component, raising safety concerns. To overcome those problems we have transfected and selected a 293T cell population for the expression of a 3rd generation LV packaging system (containing a less toxic protease) and the non toxic 4070A envelope glycoproteins. Together with a specific transgene we have established a stable cell population that constitutively produces LV. The best producer clones will be identified and isolated trough the single step-cloning method (Rodrigues, et al 2015). This work shows a new approach for stable LV producer cell line generation, without using transduction and avoiding inducible systems, increasing the safety standards of LV production.

P249

Evaluation of AAV variants for improved transgene expression after intravitreous injection in rodents and non-human primates

A Keravala[1] M Ni[1] G Todorov[1] S Vijay[1] T Kniffin[1] M Gasmi[1] K Woodburn[1] T W Chalberg[1]

1: Avalanche Biotechnologies

AAV-mediated gene therapy has shown promise for ocular diseases when injected subretinally. Intravitreous (IVT) AAV administration, while less invasive, is far less efficient due to the inner limiting membrane, which acts as a significant barrier in primates. For applications where subretinal injection is not a preferred route, it would be advantageous to develop AAV vectors that are capable of efficient transduction in target cells following IVT administration. First, we compared IVT transduction of AAV2.7m8 (AAV2 variant with a 10 amino acid (aa) peptide insertion in the surface-exposed loop), ShH10 (AAV6 variant), and 2.5T (chimera between AAV2 and AAV5) in non-human primates. These vectors expressed varying levels of GFP and had distinct gene expression patterns in the retina. Next, we created hybrid vectors by inserting the 10-aa peptide from AAV2.7m8 at seven positions in the receptor-binding region of 2.5T and investigated the tropism of these variants. A majority of the 2.5T/7m8 vectors were successfully packaged and resulted in varying levels of GFP expression in vitro. Improved GFP expression was observed with one of the variants (2.5T/7m8-12) compared to the parental 2.5T capsid, following IVT injection in rodents. Finally, because neutralizing antibodies (nAb) can limit efficient AAV transduction in some therapeutic contexts, possibly including IVT administration, we sought to understand whether antibodies in human serum could block transduction of 2.5T/7m8 hybrid capsids. All hybrid vectors demonstrated favorable transduction in the presence of high nAb titers in vitro, suggesting that 2.5T/7m8 variants may not be impeded by pre-existing nAbs in a therapeutic setting.

P250

Improved dual AAV hybrid vectors for efficient and safe retinal gene transfer

I Trapani[1] E Toriello[1] S de Simone[1] P Colella[1] C Iodice[1] A Somella[1] S Rossi[2] F Simonelli[2] M Giunti[3] M L Bacci[3] A Auricchio[1 4]

1: Telethon Institute of Genetics and Medicine 2: Eye Clinic, Multidisciplinary Department of Medical, Surgical and Dental Sciences, Second University of Naples, Naples, Italy 3: Department of Veterinary Medical Sciences, University of Bologna, Bologna, Italy 4: Medical Genetics, Department of Translational Medicine, Federico II University, Naples, Italy

Dual AAV vectors effectively expand AAV cargo capacity in the retina. However, they express both lower levels of transgene compared to a single AAV and truncated proteins from either the 5′- or 3′-half vectors. To increase productive dual AAV tail-to-head concatemerization, which would overcome these limitations, we have explored the use of either various regions of homology or heterologous inverted terminal repeats (ITR). In addition, we tested the ability of various degradation signals to decrease the expression of truncated proteins. We found the highest levels of transgene expression using regions of homology based on either alkaline phosphatase or the F1 phage (AK), with AK resulting in more consistent transduction of mouse photoreceptors. The inclusion of heterologous ITR does not decrease the levels of truncated proteins relative to full-length and impairs AAV vector production. Notably, we have identified a degradation signal which mediates the degradation of proteins shorter than expected from dual AAV vectors. Our results support the use of dual AAV vectors that include ITR2, the alkaline phosphatase-derived or AK regions of homology and degradation signals for further clinical translation.

P251

Simple downstream process based on detergent treatment improves yield and in vivo transduction efficacy of adeno-associated virus vectors

G Dias-Florencio[1] G Precigout[1] C Beley[1] P Buclez[1] L Garcia[1] R Benchaouir[1]

1: UVSQ

Recombinant adeno-associated viruses (rAAV) are promising candidates for gene therapy approaches. Rapid technological evolution in the last two decades led to advances in processes applied in the production and purification of rAAV resulting in better yields and higher levels of vector purity. Recently, some reports showed that rAAV produced by transient tri-transfection method can be harvested directly from supernatant, leading to easier and faster purification compared to classical virus extraction from cell pellets. We compare these approaches with new vector recovery method using small quantity of detergent at the initial clarification step to treat the whole transfected cell culture. Coupled with tangential flow filtration and iodixanol-based isopycnic density gradient, this new method significantly increases rAAV yields and conserves high vector purity. Moreover, this approach leads to the reduction of the total process cost and duration. Finally, the vectors maintain their functionality, showing unexpected higher in vitro and in vivo transduction efficacies. This new development in rAAV downstream process once more demonstrates the great capacity of these vectors to easily accommodate to large panel of methods, able to furthermore ameliorate their safety, functionality and scalability. [Work accepted for publication in Molecular Therapy – Methods & Clinical Development, 2015 may 26].

P252

Considering the wild type adeno-associated virus of serotype 2 genome as a recombinant AAV in the baculovirus/Sf9 cells system

A Savy[1] L Galibert[1] M U Kaikkonen[2] O W Merten[1]

1: Applied Vectorology, Genethon, Evry, France, 91000 2: Biotechnology and Molecular Medicine, University of Eastern Finland, Kuopio, Finland

In 2010, a study on the infectivity of wild type Adeno-Associated Virus (WT AAV2) compared to recombinant AAV (rAAV) vectors have been published. The wild type AAV2 had an up to hundred fold higher infectivity compared to rAAVs. With the aim to decipher if this higher infectivity is due to the WT AAV2 production system, or only to the overall nature of the DNA encapsidated in the AAV capsid we have used the baculovirus/Sf9 system to produce rAAV, with the recombinant genome being the WT AAV2 genome. However, expressing WT AAV2 genome in the baculovirus system has shown an early activity of the p5 promoter leading to the expression of Rep78. The expression of this protein resulted in an excision of the WT AAV2 genome during the first steps of Baculovirus stock formation. The absence of detection of the Rep68 and Rep40 proteins tended point to reduced or impaired splicing of the rep transcripts in the baculovirus/insect cells system. To put this into evidence, we have characterized the activity of the p5 promoter in the baculovirus/Sf9 context and studied the kinetics of expression of the Rep78 protein.

P253

Comparative study of liver gene therapy with AAV vectors based on endogenous and engineered AAV capsids

L Wang[1] P Bell[1] S Somanathan[1] Q Wang[1] Z He[1] H Yu[1] D McMenamin[1] T Goode[1] R Calcedo[1] J M Wilson[1]

1: University of Pennsylvania

Vectors based on the Clade E family member adeno-associated virus (AAV) serotype 8 have shown promise in patients with hemophilia B and have emerged as best in class for human liver gene therapies. We conducted a thorough evaluation of liver directed gene therapy using vectors based on several natural and engineered capsids including the Clade E AAVrh10 and the largely uncharacterized and phylogenically distinct AAV3B. Included in this study was a putatively superior hepatotropic capsid AAVLK03 [(Nature 506:382 (2014)] which is very similar to AAV3B. Vectors based on these capsids were benchmarked against AAV8 and AAV2 in a number of in vitro and in vivo model systems including C57BL/6 mice, immune deficient FRG mice that are partially repopulated with human hepatocytes, and non-human primates. Our studies demonstrated virtual equivalence of the Clade E derived vectors and equally high transduction with vectors based on AAV3B in nonhuman primate liver and in human hepatocytes. In contrast to the previous report, AAVLK03 vectors are not superior to either AAV3B or AAV8. Vectors based on AAV3B may have a role in situations where re-administration is necessary.

P254

The construction of adenovirus type 6 vector with replication control under the human telomerase reverse transcriptase promoter

M V Tarasova[1 2] E V Demidova[1] P M Chumakov[3] G V Kochneva[2] S V Netesov[1 2]

1: Novosibirsk State University 2: FSRI State Research Center of Virology and Biotechnology Vector 3: Institute of Molecular Biology named after V.A. Engelgardt

Oncolytic viral therapy is one of the new approaches for the cancer treatment. Human adenovirus serotype 6 (Ad6) is started to be used for oncolytic therapy because it has a good therapeutical potential among different adenoviral serotypes. Besides it has low seroprevalence and was shown to have less liver toxicity and escape Kupffer cells absorption after systemic administration. The purpose of this work was to construct a novel conditionally replicating Ad6 (pAd6-hTERT) in which E1A expression, and therefore viral replication, is under control of the human telomerase reverse transcriptase (hTERT) promoter. Because of high telomerase activity in nearly all immortal cell lines and in $\sim$90% of human tumors the purpose of using hTERT promoter was to restrict adenoviral replication to tumors and thus achieve tumor-selective tumor lysis. The pAd6-hTERT vector was obtained by two steps of homologous recombination in E.coli BJ5183 cells. The first was done to get the plasmid pAd6, containing entire adenovirus 6 type genome,

and the second was performed to construct the plasmid pAd6-hTERT using shuttle vector with hTERT promoter and Kozak sequence to insert them before E1A region in final vector.

P255

A novel RNA virus vector system for small RNA deliery based on Borna disease virus

T Honda Y Yamamoto Y Matsumoto A Makino K Tomonaga

1: Kyoto University

RNA interference (RNAi) mediated by small RNAs represents a promising approach for gene therapies. However, stable and long-term expression of small RNA molecules, such as microRNA (miRNA), is a major concern for application of RNAi therapies in vivo. Borna disease virus (BDV) belongs to the Bornaviridae family within the non-segmented, negative strand RNA viruses. Unique among animal RNA viruses, BDV transcribes and replicates in the cell nucleus. In addition, BDV readily establishes a long-lasting persistent infection without overt cytopathic effect in various cell types. All these features of this virus reveal that BDV could be an ideal candidate to generate a novel RNA virus vector system enabling persistent expression of RNAi molecules in vivo. In this study, we introduce the establishment of a novel BDV vector carrying a pri-miRNA-cassette sequence in an intercistronic non-coding region of the viral genome. The recombinant BDV (rBDV) having the pri-miR-155 sequence, rBDV-miR-155, stably expressed miR-155 for a long period of time in cultured cells and efficiently silenced reporter gene containing miR-155 target sequence in the 3′-untranslated region. Downregulation of Dicer, a critical enzyme for microRNA biogenesis, abrogated the target silencing by rBDV-miR-155. We also demonstrate that the pri-miRNA-cassette sequence based on miR-155 is replaceable to any miRNA sequences of interest and that such BDV vectors can efficiently suppress the expression of both endogenous and exogenous target genes. Our results show that BDV vector can be applicable to delivery and long-term expression of small RNA molecules and would provide a novel tool for RNAi therapies.

P256

Adeno-associated viral vectors expressing primary-microRNA-31 mimics from a liver specific promoter inhibit HBV

M B Maepa A Ely P Arbuthnot

1: University of Witwatersrand

Current therapies against chronic hepatitis B virus (HBV) infection are largely ineffective. Both primary microRNA (pri-miR) and precursor microRNA (short-hairpin RNAs, shRNAs) mimics have been used as RNA interference (RNAi) activators against HBV. However, expression of shRNAs from Pol III promoter is toxic in mice. Unlike shRNAs, pri-miR mimics can be efficiently and safely expressed from pol II promoters. Pri-miR-31 mimics targeting HBx gene were previously delivered using helper dependent adenoviral vectors (HDAds). As a result of high immune stimulation by HDAds, HBV gene silencing of insufficient duration was observed. For RNAi effectors to progress into clinical application, safe and efficient hepatic delivery is essential. Adeno-associated viral vectors (AAVs) have an advantage of low toxicity and immunogenicity. This study aimed to generate and characterise self-complementary AAVs expressing anti-HBV pri-miR-31 mimics from a liver specific Pol II promoter. Expression cassettes carrying mono- and trimeric anti-HBV pri-miR sequences were incorporated in to recombinant AAV genome bearing plasmid. The AAV genomes were then packaged in both AAV2 and AAV8 capsids for in vitro and in vivo studies respectively. Following vector propagation and purification, pri-miR expression and HBV replication inhibition was assessed in cultured cells. The AAVs resulted in significant expression and inhibition of HBV replication in vitro. Data on the expression of artificial pri-miRs, HBV gene silencing and safety of anti-HBV AAVs in HBV transgenic mice will be presented. AAVs may allow efficient delivery and long-term anti-HBV effect, making these vectors suitable for development of gene therapy against chronic HBV infection.

P257

Efficient in vivo gene transfer to xenotransplanted human skin by lentivirus-mediated, but not by AAV-directed, gene delivery

M Jacobsen A L Askou K Stenderup C Rosada F Dagnaes-Hansen T G Jensen T J Corydon J G Mikkelsen L Aagaard

1: Aarhus University

Skin is an easily accessible organ, and therapeutic gene transfer to skin remains an attractive alternative for treatment of skin diseases. Although we have previously documented potent lentiviral gene delivery to human skin, vectors based on adeno-associated virus (AAV) rank among the most promising gene delivery tools for in vivo purposes. Thus, we compared the potential usefulness of various serotypes of recombinant AAV vectors and lentiviral vectors for gene transfer to human skin in a xenotransplanted mouse model. Vector constructs encoding firefly luciferase were packaged in AAV capsids of serotype 1, 2, 5, 6, 8, and 9 and separately administered by intradermal injection in human skin transplants. For all serotypes, live bioimaging demonstrated low levels of transgene expression in the human skin graft, and firefly luciferase expression was observed primarily in neighboring tissue beneath or surrounding the graft. In contrast, gene delivery by intradermally injected lentiviral vectors was efficient and led to extensive and persistent firefly luciferase expression within the human skin graft only. The study demonstrates limited capacity of single-stranded AAV vectors of six commonly used serotypes for gene delivery to human skin in vivo.

P258

Frequency and characterization of recombination events in a gene therapy COL7A1 retroviral vector during the reverse transcription in human cells

M Titeux[1 2] S Charbonnier[1 2] R Löw[3] K Kuehlcke[3] A Hovnanian[1 2 4]

1: INSERM U1163, Imagine Institute, 75015 Paris, France 2: University Paris Descartes-Sorbonne Paris Cité, 75006 Paris, France 3: EUFETS GmbH, Idar-Oberstein, 55743, Germany 4: Department of Genetics, Necker Hospital, 75015 Paris, France

Retroviruses, including MLV and HIV-derived viral vectors, package two copies of genomic RNA into viral particles

which are subsequently reverse transcribed in a minus-strand DNA in the infected cells. During that step, template switching activity and low processivity of the reverse transcriptases lead to recombination events. We have developed an ex vivo gene therapy approach using a SIN retroviral vector encoding the 8.9 kb COL7A1 cDNA and Southern blot experiments demonstrated that COL7A1 recombination sometimes occur in the transduced cells (Titeux et al. 2010). To better understand this phenomenon and to quantify the recombination frequency, we have isolated and analyzed a large number of proviruses recombination events at the clonal level. A human keratinocyte cell line was transduced with the SIN gamma-retroviral vector expressing COL7A1 under conditions that favor single provirus integration, and the cells were subsequently cloned and expanded. Three panels of overlapping PCR primers were used to amplify the integrated proviruses sequences, in order to identify and sequence the recombination events. 26% of the proviruses presented with deletions ranging from 0.1kb up to 5.4kb, involving stretches of direct repeats occurring in the highly repeated collagenous domain of COL7A1. No mutation hotspot could be identified, which could have been edited to lower the rearrangement frequency. These results have relevant consequences for highly efficient retroviral or lentiviral-based gene transfer, particularly when the expressed cDNA is large and/or contains highly repeated sequences such as TALENs cDNA expressed in Integration-Deficient Lentiviral Vectors (IDLV).

P260

Detailed comparison of retroviral vector systems and promoter configurations for stable and high transgene expression in induced pluripotent stem cells

D Hoffmann[1] F Geis[1] L Lange[1] J Kuehle[1] J W Schott[1] M Grez[2] T Moritz[1] A Schambach[1 3]

1: Hannover Medical School 2: Georg-Speyer-Haus 3: Harvard Medical School

Gene replacement in induced pluripotent stem cells (iPSC) through retroviral vectors offers new treatment perspectives for monogenetic disorders. Based on their potential to grow indefinitely in culture, gene-modified iPSC clones can be individually characterized and screened for safe integration sites. Suitable clones can be differentiated into transplantable cells of interest. However, as a current bottleneck, transduction rates in iPSC are low and stable transgene expression is strongly hampered by epigenetic vector silencing. In order to identify the most suitable retrovirus-based expression system in iPSC, we systematically compared vectors derived from three different retroviral genera with regard to their transduction efficiency and transgene expression stability. Furthermore, we performed side-by-side analysis of different promoters combined with full length or minimal ubiquitous chromatin opening elements (UCOE). Among tested configurations, lentiviral vectors containing a minimal UCOE combined with an elongation factor 1α (EFS) promoter were most potent. We generated stable puromycin-resistant iPSC and observed that ''shut-down'' of the vector is prevented by minimal UCOE independently of the promoter. Interestingly, expression strength is markedly reduced from spleen focus forming virus promoter and partially altered from EFS promoter leading to high and low expressing populations. This profile was not caused by copy number differences, rather reflecting an epigenetic phenomenon of gradually and non-silenced retroviral vectors. Importantly, only high expressing populations maintained transgene expression after iPSC differentiation into endothelial cells. In summary, we systematically validated promising vector designs for long-term genetic modification of iPSC and excluded configurations strongly interfering with transgene expression in iPSC and their progeny.

P261

Insight into how lentiviral transduction modulates the human hematopoietic stem cell transcriptome

F Piras[1 2 3] M Riba[2] D Lazarevic[2] I Cuccovillo[2 3] S Bartolaccini[3] E Stupka[2] D Cittaro[2] L Naldini[1 2 3] A Kajaste-Rudnitski[2 3]

1: Universita Vita-Salute San Raffaele 2: San Raffaele Scientific Institute 3: San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET)

Recent studies suggest that innate sensing is active in hematopoietic stem and progenitor cells (HSPC). Exposure to lentiviral vectors (LV) upon gene transfer may thus trigger acute host responses that could potentially impact on their biological properties. We have performed a high throughput RNA-Seq analysis on human cord-blood-derived CD34+HSPC exposed to research- or clinical-grade VSV-g pseudotyped (SIN) LV. As controls, cells were exposed to non-transducing Env-less, genome-less or heat inactivated vectors or kept in culture untreated. RNA was extracted at different times early after transduction, processed, ran in Illumina HiSeq2000 and analyzed for Differential Expression in Time Course and key pathways. Transduction with both research- and clinical-grade LV significantly triggered DNA damage and apoptosis-related responses. In particular, p53 signaling was among the most significantly altered pathways ($p < 3.47x10\text{-}14$) and induction of several key players, including a 8-fold increase in p21 mRNA, was further confirmed by Taqman. This signaling occurred in all CD34+subpopulations, including in the most primitive CD38-CD133+fraction, required reverse transcription but was integration-independent. LV exposure lead to a slight but significant increase in the percentage of apoptotic HSPC in culture ($p < 0.001$) and experiments to further investigate the potential consequences of this signaling on their biological properties in vitro and in vivo are on-going. Overall, our results suggest for the first time that LV transduction triggers transcriptional changes in HSPC involving pathways pivotal for their biology. Better understanding of the potential functional consequences this may have will be important for the development of improved gene therapy protocols.

P262

Development of new lentiviral vectors with a reduced splicing interference potential and a safer in vivo genotoxic profile

D Cesana[1] A Calabria[1] P Gallina[1] L Rudilosso[1] G Spinozzi[1 2] E Montini[1]

1: San Raffale Telethon Institute for Gene Therapy (HSR-TIGET) 2: University of Milano-Bicocca

Systemic vector injection into newborn tumor-prone Cdkn2a-/- and Cdkn2a+/- mice demonstrated that SINLVs harboring strong or moderate enhancer/promoters in internal position caused acceleration in hematopoietic tumor onset compared to control mice. Integration sites analyses of vector-

induced tumor showed that oncogene activations or tumor suppressor inactivation occur by combining mechanisms of induction of aberrant splicing and/or enhancer-mediated overexpression of cellular transcription units. To reduce genotoxic splicing-capture events, we designed LVs harboring sequences complementary to microRNAs (mirT sequence), which are active in hematopoietic cells (mir223 and mir142-3p) within the SIN LTR (mirsT-LTR.LV) or in the vector backbone and outside the gene expression cassette (mirT-LV) that allow selective degradation of vector-mediated aberrantly spliced transcripts. We then assessed the genotoxicity of the SIN LVs harboring mirT sequences by taking advantage of our in vivo genotoxicity models. Remarkably, injection of mirsT-LTR.LV (N = 34) and mirT-LV (N = 39) in Cdkn2a-/- mice did not caused any significant acceleration in hematopoietic tumor onset compared to control un-injected mice (N = 37). Similar results have been obtained after injection in Cdkn2a+/- mice (N = 29 for mirsT-LTR.LV, N = 25 for mirT-LV and N = 40 un-injected mice). Integration site analyses in Cdkn2a-/- treated mice confirm that both the mirT LVs are effective in preventing oncogene activation by vector-induced aberrant transcript formation. Overall, these studies show that this new advanced design lentiviral vectors completely abrogated residual vector genotoxicity in highly sensitive mouse models and could represent the vector design of choice in future gene therapy applications.

P263

Safer, more randomized lentiviral vector integration using artificial LEDGF chimeras

L Vranckx[1] J Demeulemeester[1] Z Debyser[1] R Gijsbers[1]

1: KU Leuven 2: University of Leuven

The capacity to integrate transgenes into the host cell genome makes retroviral vectors interesting tools for gene therapy. Although stable insertion resulted in successful correction of several monogenic disorders, it also accounts for insertional mutagenesis, a major setback in otherwise successful clinical gene therapy trails due to leukemia development in subset of treated patients. Despite improvements in vector design, their use is still not risk-free. Retroviral integration is not random. Lentiviral vector (LV) integration is directed into active transcription units by LEDGF/p75, a cellular protein co-opted by the viral integrase acting as a molecular tether: together they determine LV integration site choice. We developed LEDGF/p75-based tethers that result in a more random integration pattern, reducing the risk of insertional mutagenesis. We generated a truncated version of LEDGF/p75, lacking the N-terminal chromatin-reading PWWP-domain that orchestrates binding to H3K36me3, an epigenetic mark enriched in active transcription units. In addition, we replaced this domain with alternative, viral pan-chromatin recognition peptides, described to bind cellular chromatin without sequence specificity. Following the generation of stable cells lines, these LEDGF-chimeras efficiently rescued LV transduction in LEDGF-depleted cells. We demonstrate that LV integrations are distributed more random throughout the host-cell genome using our artificial LEDGF-hybrids. Under these circumstances the vectors permit high-level reporter gene expression. In addition, for our LEDGF-chimeras, 18–22% of integrations meet safe harbor criteria (Papapetrou 2011) compared to only 4.2% for wild-type cells carrying LEDGF/p75. This approach should be broadly applicable to introducing therapeutic or suicide genes for cell therapy, such as patient-specific iPS.

P264

rAAV-mediated gene transfer of a truncated CFTR rescues the cystic fibrosis phenotype in human intestinal organoids and CF mice

D Vidovic[1] M S Carlon[1] M F da Cunha[2] J D Dekkers[3 4] M I Hollenhorst[2] M Bijvelds[5] A S Ramalho[6] C Van den Haute[7 8] V Baekelandt[7] K De Boeck[9] I Sermet-Gaudelus[2] H de Jonge[5] J Beekman[3 4] A Edelman[2] R Gijsbers[1 8] Z Debyser[1]

1: Molecular Virology and Gene Therapy, KU Leuven, Belgium 2: INSERM U1151, University Paris Descartes, Faculté de Médecine Necker Enfants-Malades, Paris, France 3: Department of Pediatric Pulmonology, Wilhelmina Children's Hospital, University Medical Center, Utrecht, The Netherlands 4: Laboratory of Translational Immunology, University Medical Center, Utrecht, The Netherlands 5: Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands 6: Stem Cell Biology and Embryology, KU Leuven, Belgium 7: Neurobiology and Gene Therapy, KU Leuven, Belgium 8: Leuven Viral Vector Core, KU Leuven, Belgium 9: University Hospital Leuven, Belgium

Introduction: Cystic Fibrosis (CF) is the most common monogenic life-threatening disease in the Caucasian population, caused by mutations in CFTR, a chloride channel primarily affecting airways. To date only CF-related symptoms are treated. Gene therapy offers the potential to cure the disorder. Here, we evaluate a rAAV-based gene therapeutic approach in two preclinical models: intestinal organoids derived from CF patients and in CF mice.

Results: In contrast to previous rAAV-based clinical trials, here we used an airway-tropic serotype rAAV2/5 combined with an optimally designed expression cassette carrying a truncated CFTR (CFTRΔR) that retains ion channel activity and allows incorporation of an external promoter for optimal transgene expression. The therapeutic potential of rAAV-CFTRΔR was assessed in CFTR-deficient organoids. Even though the episomal nature of rAAV does not allow stable transduction of rapidly proliferating organoids, rAAV-CFTRΔR treatment resulted in forskolin induced swelling. In parallel, we set out to correct the CF phenotype in ΔF508 mice by nasal instillation of rAAV2/5-CFTRΔR. 2–4 weeks later, by measuring nasal potential differences, we demonstrated a response to low-chloride and forskolin perfusion in 6 out of 8 mice, indicating a restoration of chloride transport.

Conclusions: This study provides a robust proof-of-principle that rAAV-mediated gene transfer of a truncated CFTR leads to functional rescue of the CF phenotype in patient-derived intestinal organoids, and in vivo across the nasal mucosa of CF mice. Taken together, our results underscore the therapeutic potential of rAAV-CFTRΔR as gene therapy vector for CF, opening new avenues towards a generic cure for all patients.

P265

ACE2 gene therapy using adeno-associated viral vector inhibits biliary fibrosis in Mdr2-knockout mice

I G Rajapaksha[1] K Y Mak[1] S C Cunningham[2] J Torresi[3] A F Sharland[4] I E Alexander[2] P W Angus[5] C B Herath[1]

1: Hepatology Research Group, Department of Medicine, University of Melbourne, Austin Health, Heidelberg, Victoria,

3084, Australia. 2: Gene Therapy Research Unit, Children's Medical Research Institute, Westmead, New South Wales, 2145 Australia. 3: Department of Infectious Diseases, Austin Health, Heidelberg, Victoria, 3084, Australia. 4: Transplantation Research Group, Bosch Institute, The University of Sydney, Sydney, New South Wales, 2006, Australia. 5: Department of Gastroenterology and Hepatology, Austin Health, Heidelberg, Victoria, 3084, Australia.

There is a major need for effective therapies for chronic biliary diseases such as primary sclerosing cholangitis. We recently showed that a liver-specific adeno-associated viral (AAV) vector carrying angiotensin converting enzyme 2 (ACE2) markedly reduced biliary fibrosis one week after ACE2 therapy in bile duct ligated mice. In the present study we investigated long-term effects of ACE2 therapy in Mdr2-KO mice which develop progressive biliary fibrosis over 6 months. Three-months-old Mdr2-KO mice received a single i.p. injection of either AAV-ACE2 or a control vector carrying human serum albumin (AAV-HSA). ACE2 gene expression and activity, and hepatic angiotensin peptide levels were measured 3 months post-treatment. To elucidate the possible therapeutic mechanisms, angiotensin-(1-7) (Ang-(1-7)), the major anti-fibrotic peptide produced by ACE2-induced cleavage of Ang II, was infused into Mdr2-KO mice for 1 month and the same end points measured. ACE2 therapy increased ACE2 gene expression (60-fold) and protein activity (>2-fold) in Mdr2-KO mice compared with HSA-treated mice and this lead to a major decrease in hepatic levels of the potent profibrotic peptide Ang II with a concomitant increase in Ang-(1-7) levels. This was associated with an approximately 50% reduction in fibrosis ($p<0.01$). Ang-(1-7) infusion also reduced liver fibrosis in this model, suggesting production of this peptide contributes to the effects of ACE2. ACE2 gene therapy improves biliary fibrosis in a long-term model of chronic biliary disease by changing the relative hepatic levels of Ang II and Ang-(1-7). We conclude that ACE2 gene therapy has potential as a therapy for patients with biliary fibrosis.

P266

Total replacement of WPRE sequence to reduce potential oncogenicity risk without compromising vector titre or transgene expression level

O Kan D Moss N Law G Binder-Scholl H Tayton-Martin B Jakobsen D Williams

1: Adaptimmune Ltd

Adaptimmune is a clinical stage biopharmaceutical company focused on novel cancer immunotherapy products based on its T-cell receptor platform. Engineered increased affinity TCRs are delivered to a patient's own T cells by a lentiviral vector that is produced using transient transfection with a transgene containing plasmid and 3 packaging plasmids. Previously, we used a transgene containing plasmid that incorporated a woodchuck hepatitis post-transcriptional regulatory element (WPRE). The presence of the full length WPRE in lentiviral vectors has been shown to increase cytoplasmic RNA accumulation and significantly enhance expression of reporter genes in a variety of cells that also results in vector production titre increases. However, this element is potentially oncogenic due to the presence of a truncated X-protein fragment with its own promoter inside the WPRE. Therefore, in order to reduce this oncogenicity risk, we replaced the whole WPRE region with a new sequence excluding the truncated X-protein fragment and we did not observe an associated reduction in vector titre or transgene expression. Details regarding this modification and its effects on vector production and gene expression will be reported.

P267

Mesenchymal stem cells that overexpress extracellular superoxide dismutase markedly ameliorate imiquimod-induced psoriasis-like skin inflammation in mice through regulating multiple anti-inflammatory and immunosuppressive pathways.

S K Sah J H Lee C O Yun K S Kang T Y Kim

1: The Catholic University of Korea 2: Hanyang University 3: Seoul National University

The immunomodulatory and anti-inflammatory properties of mesenchymal stem cells (MSCs) have been proposed in several autoimmune diseases and successfully tested in animal models, but their contribution to psoriasis and underlying pathways remain elusive. Likewise, increased or prolonged presence of reactive oxygen species (ROS) and aberrant antioxidant systems in skin are known to contribute the development of psoriasis, and therefore, effective antioxidant therapy is highly required. Here, we explored the feasibility of using extracellular superoxide dismutase (SOD3)-transduced allogeneic MSCs as a therapy in the mouse model of imiquimod-induced psoriasis-like inflammation, because SOD3 has been reported to be expressed at low levels in psoriatic lesions, and investigated poorly understood underlying mechanisms. The mouse received a booster dose of imiquimod to evaluate the chronicity and late phase response of inflammation during continued activation of antigen receptors. Subcutaneous injection of allogeneic SOD3-transduced MSCs significantly prevented psoriasis development through suppression of proliferation and infiltration of the various inflammatory cells such as T cells, neutrophils, and dendritic cells, and down-regulated the pro-inflammatory cytokine expression, inhibited multiple signaling pathways such as toll-like receptor-7 (TLR-7), nuclear factor-κB (NF-κB), p38 mitogen-activated kinase (MAPK), Janus kinase signal transducer and activators of transcription (JAK-STATs), adenosine receptor activation. Our data suggest that enhancing the immunosuppressive and antioxidant activity of MSCs, as well as modulating immune cell-mediated immunity using overproduced SOD3 with high activity, may be a powerful therapeutic approach in treatment of chronic inflammatory skin diseases such as psoriasis.

P268

Dual-vector prodrug activator gene therapy using two different retroviral replicating vectors

S Kubo M Takagi-Kimura C R Logg K Hermann N Kasahara[2]

1: Hyogo College of Medicine 2: University of California Los Angeles (UCLA) 3: University of Miami

Retroviral replicating vectors (RRVs) have been shown to achieve efficient tumor transduction and enhanced therapeutic benefit in a wide variety of cancer models. Here we evaluated two different RRVs derived from amphotropic murine leukemia virus (AMLV) and gibbon ape leukemia virus (GALV), encoding

two different prodrug activator genes, the yeast cytosine deaminase (CD) and HSV thymidine kinase (TK) in Hep3B human hepatocellular carcinoma cells. Both RRVs expressing GFP gene (AMLV-GFP and GALV-GFP) efficiently replicated in Hep3B cells and spread in culture. Additionally, AMLV-GFP can spread in GALV-mCherry pretransduced Hep3B cells but not in AMLV-mCherry pretransduced cells. Similarly, GALV-GFP can spread in AMLV-mCherry pretransduced cells but not in GALV-mCherry pretransduced cells. Notably, however, replication and spread of either RRV in culture was not affected by pre-transduction with the counterpart RRV encoated with the other envelope. In order to investigate the effect of combined prodrug-dependent cell killing vs. multiple vector copy transduction in vitro, Hep3B cells were transduced with the AMLV-CD, GALV-CD, AMLV-TK, GALV-TK, respectively or in combination. The resultant cells were used to evaluate the cytotoxic effect of RRV-mediated prodrug activator gene therapy with CD and TK in the presence of their respective prodrugs, 5-fluorocytosine and Ganciclovir. In vitro cytocidal effects obtained by combining different prodrug activator genes were significantly greater than when the same prodrug activator genes were delivered with two different vectors. These data indicate the potential utility of dual-vector prodrug activator gene therapy using two different RRVs carrying different prodrug activator genes.

P269

Bioinformatic pipeline for identification and quantification of integration sites for clonal tracking studies

L Leonardelli[1] D Pellin[3] S Scala[1] F Dionisio[1] L Basso Ricci[1] D Cittaro[4] C Di Serio[3] A Aiuti[1 2] L Biasco[1]

1: San Raffaele Institute for Gene Therapy (OSR TIGET) 2: Vita-Salute San Raffaele University 3: University Centre of Statistics in the Biomedical Sciences (CUSSB) 4: Centre of Translational Genomics and Bioinformatics, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS)

An established system to track gene-corrected cells in gene therapy (GT) treated patients is based on the analysis of vector integration sites (IS). Importantly, since IS univocally tag each transduced cell, we exploited these molecular marks to perform clonal tracking studies to address basic biological questions on the fate of engineered clones. To this aim, we designed a customizable and tailored pipeline to analyse LAM-PCR amplicons sequenced by Illumina MiSeq/HiSeq technology. The sequencing data are initially processed through a series of quality filters and cleaned from vector and linker cassette sequences with customizable settings. De-multiplexing is then performed according to the recognition of specific barcodes combination used upon library preparation and the sequences are aligned to the reference genome. Hg19 human genome assembly is composed of 93 contigs, among which the mitochondrial genome, unlocalized and unplaced contigs and some alternative haplotypes in chr6. While previous approaches aligned only to the ''official'' 24 human chromosomes, using the whole assembled genome allowed improving alignment accuracy and concomitantly increased the amount of detectable IS. Another novel aspect of our pipeline consists on the detection, validation and quantification of random barcodes that we recently encoded in our linker cassette. This methodology avoids the use of sequencing reads count for clonal assessment and improves the accuracy of IS quantification since genomic fragments are marked before PCR amplification steps. We validated this approach by assays performed on serial dilutions of individual clones and on clinical samples from Wiskott Aldrich Syndrome GT patients.

P270

Targeted alphavirus SFV4-miRT124 overcomes type I interferon response and shows oncolytic efficacy in mouse CT-2A glioma model

M Martikainen M Niittykoski M von und zu Fraunberg A Immonen S Koponen M Vähä-Koskela E Ylösmäki J E Jääskeläinen K Saksela A Hinkkanen

1: Department of Biotechnology and Molecular Medicine, A.I.Virtanen Institute, University of Eastern Finland, Kuopio, Finland 2: Neurosurgery of NeuroCenter, Kuopio University Hospital, Kuopio, Finland 3: Department of Virology, University of Helsinki, Helsinki, Finland 4: Institute of Biotechnology, University of Helsinki, Helsinki, Finland

Malignant glioma is a devastating central nervous system disease with no effective treatment available. Oncolytic viruses provide a novel candidate therapy. However, current generation viruses have shown poor response in clinical settings. This may be partly due to use of type I interferon (IFN-I) sensitive vectors. Neuroattenuated Semliki Forest virus (SFV, alphavirus) vector VA7 has shown oncolytic potency in human glioma xenografts, but replicates poorly in syngeneic gliomas due to type IFN-I mediated antiviral response. Here we utilized SFV4, a neurovirulent prototype strain that replicates robustly and causes lethal encephalitis in mice. To attenuate the neurovirulence we engineered a novel SFV4-miRT124 vector bearing 6 targets for neuronally expressed miR-124. Both SFV4 and the attenuated SFV4-miRT124 replicated in mouse CT-2A-Fluc mouse glioma cells despite presence of IFN-beta in drastic contrast to VA7. IFN-beta also inhibited VA7 replication in human glioblastoma-derived cell lines while having only moderate effect on SFV4-miRT124. A single intraperitoneal dose of SFV4-miRT124 to CT-2A-Fluc tumor-bearing C57BL/6 mice resulted in significantly reduced growth and loss of established gliomas in 50% of mice, as analyzed with bioluminescence imaging and MRI. As indication of viral replication in the tumor, SFV glycoproteins were detected by IHC only in SFV4-miRT124-treated gliomas. Neurological symptoms correlated with the presence of viral antigen in the brain in a fraction (12.5%) of SFV4-miRT124 treated mice. Our results indicate that robust viral replication is required to overcome tumor IFN-I in syngeneic glioma tumors. Strict guidance of enhanced oncolytic vectors to malignant cells might enhance therapy efficacy.

P271

AAV9 vector-mediated expression of a soluble coxsackievirus-adenovirus receptor inhibits coxsackievirus B3-induced chronic myocarditis in a therapeutic approach in mice

S Pinkert[1] R Klopfleisch[4] B Dieringer[1] M Schmidtke[3] K Savvatis[2] H Fechner[1]

1: Technische Universität Berlin 2: Charité University Medicine 3: Friedrich Schiller Universität 4: Freie Universität Berlin

Coxsackie-B-viruses (CVB) are prototype agents of acute myocarditis and chronic dilatative cardiomyopathy (DCM) but an effective targeted therapy is still not available. Soluble receptors as a decoy have been found to efficiently inhibit infection of a broad range of viruses. Previously we demonstrated that adenovector-mediated expression of a soluble coxsackievirus-adenovirus receptor protein (sCAR-Fc) as a virus receptor trap reduced efficiently acute cardiac CVB3 infection in mice under therapeutic conditions. In this study we analyzed the therapeutic potential of sCAR-Fc against CVB3-induced chronic myocarditis in a mouse model. To achieve high and long lasting sCAR-Fc expression in vivo, we generated a dimeric AAV9 vector (scAAV9sCAR-Fc). Initially investigations showed that intravenous (i.v.) application of 1x1011 scAAV9-sCAR-Fc vector genome copies per mouse led to persistent and high sCAR-Fc serum levels of 5-9 μg/ml between day 5 and day 28 after transduction. To analyze the therapeutic efficiency of scAAV9sCAR-Fc for treatment of chronic CVB3-induced myocarditis, NMRI mice were infected with CVB3 and 24 h later transduced with scAAV9sCAR-Fc. The animals were analyzed 28 days after CVB3 infection. Heamodynamic measurement showed a significantly improved cardiac contractility and diastolic relaxation compared to the control animals which were transduced with scAAV9-sCAR-Fctrunc expressing a truncated version of sCAR-Fc. We further found, that virus induced myocardial inflammation and remodeling was significantly reduced by sCAR-Fc. Thus, here we show for the first time that early therapeutic employment of sCAR-Fc after infection with CVB3 is able to inhibit development of chronic CVB3-induced myocarditis.

P272

Expression of an engineered soluble Coxsackievirus and Adenovirus Receptor by a dimeric AAV9 vector inhibits Adenovirus infection in mice

C Röger[1] T Pozzuto[1] R Klopfleisch[2] J Kurreck[1] H Fechner[1] S Pinkert[1]

1: Technische Universität Berlin 2: Freie Universität Berlin

Immunosuppressed patients, such as recipients of hematopoietic stem cell transplantation, occasionally develop severe and fatal adenovirus (Ad) infections. Here, we analyzed the potential of a virus receptor trap based on a soluble coxsackievirus-adenovirus receptor (sCAR) for inhibition of Ad infection. In vitro, a dimeric fusion protein, sCAR-Fc, consisting of the extracellular domain of CAR and the Fc portion of human IgG1 and a monomeric sCAR lacking the Fc domain were expressed in cell culture. sCAR was stronger secreted into the cell culture supernatant than sCAR-Fc but had lower Ad neutralization activity than sCAR-Fc. Further investigations showed that sCAR-Fc reduced the Ad infection by a 100-fold and Ad-induced cytotoxicity approximately 20-fold. Not only was Ad infection inhibited by sCAR-Fc applied prior to infection, it also inhibited infection when used to treat ongoing Ad infection. In vivo, sCAR-Fc was delivered to immunosuppressed mice by an AAV9 vector resulting in persistent and high ($>40\,\mu$g/ml) sCAR-Fc serum levels. The sCAR-Fc serum concentration was sufficient to significantly inhibit hepatic and cardiac wild-type Ad5 infection. Treatment with sCAR-Fc did not induced side effects. Thus, sCAR-Fc virus receptor trap may be a promising novel therapeutic for treatment of Ad infections.

P273

Generation of high-titer retroviral and lentiviral vectors encoding microRNAs: Escaping vector self-targeting

A. F. Rodrigues[1] N. Schmolka[4] B. Silva-Santos[4] P. M. Alves[1 2] A. S. Coroadinha[1 2]

1: Instituto de Biologia Experimental e Tecnologica 2: Instituto de Tecnologia Química e Biológica - UNL, Oeiras, Portugal 3: Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Portugal

MicroRNAs hold vast therapeutic potential and are undergoing clinical evaluation for the treatment of cancer, infectious diseases and chronic conditions. Retroviral vectors, including gamma-retrovirus and lentivirus, provide efficient delivery vehicles that can help translating microRNA-based therapeutics into reliable and long-lasting Gene Therapy treatments. However, the production of retroviral vectors encoding microRNAs faces the inherent challenge of self-targeting: the viral transgene is targeted to the RNA interference pathway during vector production, reducing the titer and quality of the viral preparations. Herein, we studied self-targeting in retroviral vector encoding different microRNA sequences. We show that self-targeting translates into reduced availability of viral vector transgene during the production, generating empty viral particles devoid of transduction (infectious) potential. While total particles content remained nearly identical, infectious vector titers were reduced from -100 fold to total abolishment. In the clinical context this output is particularly problematic since, in the absence of reporter genes, microRNA-encoding vectors are difficult to titrate and indirect measurements of physical particles are often used. Thus, we evaluated the effect of inhibiting the RNA interference pathway during vector production. A new cell line was developed stably expressing a gene promoting the inhibition of this pathway: 293mr (microRNA resistant). 293mr cells allowed rescuing infectious vector titers to the same order of magnitude of the empty vector control, both in transient and in stable production. 293mr is thus a valuable cell line for the production of retroviral and lentiviral vectors for microRNA-based Gene Therapy applications.

P274

Different degrees of in-vivo enhancer-blocking activity and safety of insulated SIN LVs

M. Volpin[1 2] D. Cesana[1] A. Calabria[1] E. Tenderini[1] F. Benedicenti[1] K. Mitra[3] J. Lenz[3] G. Stamatoyannopoulos[4] E. Montini[1]

1: Telethon Institute of Gene Therapy (HSR-TIGET) 2: Università Vita-Salute San Raffaele 3: Albert Einstein College of Medicine 4: University of Washington

We evaluated the in-vivo insulation ability and safety profile of four validated CCCTC-binding-factor (CTCF)-based chromatin insulators (CI) (three from Liu and Muraro et al. 2015; one unpublished CI) by including them into Self-Inactivating (SIN) Lentiviral-Vectors (LVs) harboring a strong enhancer-promoter and tested them in the Cdkn2a-/- and Cdkn2a+/- in-vivo genotoxicity assays. With minor differences, all insulated-LVs slightly improved the median survival-time vs. uninsulted-LV when injected in Cdkn2a-/- mice (193-203 days vs. 186

days, respectively). By analyzing > 4700 vector integration sites (IS) from tumors we identified striking differences in the common integration sites (CIS) targeted by the different insulated-SIN LVs: Two insulated-vectors activated predominantly Map3k8 oncogene, a CIS-gene that we previously described in this model being activated by an interplay of enhancer-mediated and truncation-transactivation with un-insulated vectors, thus indicating inefficient insulating-ability of these CI in-vivo. Inclusion of a different insulator in the vector construct hampered enhancer-mediated activation of Mpa3k8 accompanied by increased targeting of Pten tumor-suppressor, as escape genotoxicity mechanism on which CI cannot act. Importantly, for the last insulated-LV no CIS could be identified, indicating ability to perform enhancer-blocking insulation and safety-improvement in vivo. We furthermore assayed the best-performing insulated-LVs in heterozygous mice. Herewith, we observed for the first time a significant improvement (p value = 0.0116) in median survival-time vs. uninsulated-LV when injecting the latter insulated-LV. Our data demonstrate that the ability of CI to block the crosstalk between powerful vector-enhancers and cellular regulatory elements in-vivo increases the safety of SIN LVs and validate their employment in future gene therapy applications.

P275

Efficiency and function of three adenovirus VEGF vector constructs in endothelial cells for application in gene therapy for Placental Insufficiency

C A Rossi[1] V Mehta[1] M Lees[1] J Martin[1] I Zachary[1] D M Peebles[1] A L David[1]

1: University College London

Placental Insufficiency is insufficient blood flow to the placenta during pregnancy. We previously showed how local transient VEGF expression in the maternal uterine arteries mediated through adenoviral vectors (Ad.) can increase fetal growth velocity in sheep and guinea pigs. Magnus Life Science and the EU FP7 'EVERREST' programme are funding the translation of this therapy into the clinic. Following regulatory advice, we aim to compare transduction efficiency and function of the two pre-clinical study Ad. constructs (Ad.VEGF-A165, Ad.VEGF-DΔNΔC-FLAG TAG) with the intended clinical trial construct (Ad.VEGF-DΔNΔC). Our experiments involved four primary adult endothelial cell lines from human umbilical vein (HUVECs), pregnant sheep uterine artery (sUAECs), rabbit (rAECs) and guinea pig aorta (gpAECs). Cells have been infected with increasing multiplicity of infection (MOI, between 10 and 1000 MOI) for 24 or 48 hours of the three vectors, compared to a control Ad. vector containing the LacZ gene (Ad.LacZ). Our read-outs are VEGF expression efficiency, tube formation assay and major cellular pathway activation. Increase of VEGF expression was measured after transduction with Ad.VEGF-DΔNΔC-FLAG TAG and Ad.VEGF-DΔNΔC, compared to Ad.VEGF-A165 in all endothelial cell types. Ad.VEGF-DΔNΔC promoted high tube formation rate in human, sheep and rabbit endothelial cells, compared to the other constructs. Phospho-Erk and phospho-Akt assays displayed no differences between the three vector constructs, whose effect was, as in all other sets of experiments, higher than Ad.LacZ. In conclusion, Ad.VEGF-DΔNΔC promotes higher VEGF expression and neovascularization potential, with comparability in major pathway activation, supporting use of this vector in the clinical trial.

P276

Comparison of AAV serotypes 1,2,5,8 and 9 for efficient cardiac and skeletal muscle gene transfer

L Holappa E Hytönen A Määttä J Huusko G Wirth S Ylä-Herttuala

1: A.I.Virtanen Institute

Background: Recombinant Adeno-Associated viruses (AAVs) have been used for cardiac gene therapy applications for many years. However, there is no consensus which serotype is optimal and how delivery route affects the transduction efficiency and biodistribution. In this study five different serotypes and three delivery routes were compared placing specific emphasis on the myocardial and skeletal muscle transduction.

Methods: AAV vectors were generated carrying Enhanced Green Fluorescent Protein (eGFP) under control of the cytomegalovirus promoter. AAV serotypes 1, 2, 5, 8 and 9 (10^10 vg) were delivered either intravenously, into left ventricular wall or into peroneus muscle (4-6 mice in each group). Echocardiographic data was gained from intramyocardial-injected mice with ultrasound (Vevo 770, VisualSonics Inc., Toronto, Canada). Mice were sacrificed 30 days after injection. Transduction efficacy was determined by analyzing eGFP fluorescence from frozen tissue sections with microscope.

Results: AAV1 and AAV8 resulted in the highest eGFP expression in left ventricular myocardium (LVM) after intramyocardial injection. However, expression was restricted close to the needle tract. AAV9 enabled global but much lower transduction in LVM when delivered intravenously. AAV8 and AAV9 efficiently transduced liver regardless of the delivery route. Skeletal muscle was transduced efficiently but very locally by AAV5, AAV8 and AAV9 after peroneus muscle injection but not at all after intravenous or intramyocardial injection. 10^10 vg dose of any serotype delivered intramyocardially or intramuscularly induced significant local inflammation.

Conclusion: AAV9 delivered intravenously is relatively safe gene delivery method for mice and enables long-term moderate transgene expression.

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