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ESGCT 2010 Oral Presentations

Or 1
Homologous recombination
Toni Cathomen

See supplement

Or 2
Emerging use of transposons for gene therapy
Professor T VandenDriessche

Effective gene therapy requires robust delivery of the desired genes into the relevant target cells, long-term gene expression, and minimal risks of secondary effects. The development of efficient and safe non-viral vectors would greatly facilitate clinical gene therapy studies. However, non-viral gene transfer approaches typically result in only transient gene expression in most primary cells. The use of non-viral gene delivery approaches in conjunction with the latest generation transposon technology based on Sleeping Beauty (SB) or piggyBac transposons may potentially overcome some of these limitations. In particular, a large-scale genetic screen in mammalian cells yielded novel hyperactive SB transposases, resulting in robust and stable gene marking in vivo after hematopoietic reconstitution with cord blood-derived CD34+ hematopoietic stem/progenitor cells in mouse models. Moreover, the first-in-man clinical trial has recently been approved to use redirected T cells engineered with SB for gene therapy of B-cell lymphoma. Finally, induced pluripotent stem cells (iPS) could be generated after genetic reprogramming with transposons encoding reprogramming factors. Moreover, transposons can be used to "coax" the differentiation of iPS into transplantable cell types. These recent developments underscore the emerging potential of transposons in gene therapy applications and induced pluripotent stem generation for regenerative medicine. (VandenDriessche et al., Blood. 2009;114:1461–1468; Mates, Chuah et al., Nature Genetics, 41(6):753–61, 2009; VandenDriessche et al., Hum Gene Ther. 20(12):1559–61, 2009; Belay, Matrai et al., Stem Cells, in press 2010).

Or 3
Antisense oligonucleotides mediated exon skipping therapy for Duchenne muscular dystrophy
Dr A Aartsma-Rus1, Professor GJB van Ommen2, Dr JCT van Deutekom3

Antisense-mediated reading frame restoration is presently one of the most promising therapeutic approaches for Duchenne muscular dystrophy (DMD). In this approach, antisense oligoribonucleotides (AONs) induce specific exon skipping during pre-mRNA splicing of mutated dystrophin transcripts. This is aimed to restore the disrupted open reading frame and allow synthesis of internally deleted, partly functional Becker-like dystrophin proteins. The approach is theoretically applicable to over 70% of all patients, with exon 51 skipping being applicable to the largest group of patients (13% of all mutations). Proof of concept has been achieved in cultured muscle cells from patients carrying different mutation types, in the mdx mouse model, and recently in DMD patients in clinical trials after local treatment of PRO051, a 2'-O-methyl phosphorothioate modified AON targeting exon 51. In each case AON treatment resulted in skipping of the targeted exon and dystrophin restoration in the absence of adverse effects. For therapeutic application, long term systemic delivery would be preferred. Therefore, current work focuses on different systemic delivery methods and long term treatment of dystrophic animal models. Following encouraging results in mice, a subsequent trial where patients are treated systemically has recently been completed successfully and a 6-months follow-up trial using the most effective dosage is underway by Prosensa.

Or 4
Cancer immunotherapy with genetically modified lymphocytes
Dr R Morgan

Adoptive cell transfer using anti-tumor antigen reactive T cells has proven to be a useful strategy for the treatment of metastatic melanoma, with objective response rates of up to 72%, with 16% of patients rendered disease free. These studies involved the extraction and ex-vivo expansion of naturally occurring tumor-infiltrating lymphocytes (TIL). Only one-half of patients are able to receive this therapy due to lack of harvestable tumors, inability to grow TIL, or a lack of cellular reactivity. As an alternative approach to TIL therapy, high-affinity T cell receptors (TCR) can be introduced into normal T cells and the adoptive transfer of these cells into the lymphodepleted patients has been shown to
mediate cancer regression. Adoptive transfer of TCR-transduced PBL targeting melanoma differentiation antigens like MART-1 and gp100 resulted in objective cancer regression in up to 30% of patients. However, patients also exhibited significant toxicity associated with destruction of normal melanocytes in the skin, eye, and ear. In a similar TCR gene therapy trial targeting carcinoembryonic antigen (CEA), 1 of 3 colorectal cancer patients obtained an objective cancer regression, but transient colitis was observed in all patients. Recent efforts to overcome the on-target toxicities associated with immunotherapies directed against antigens expressed on normal tissues are focused on generating TCRs targeting cancer testis antigens (CTA). CTA are immunogenic proteins and their expression is highly restricted to tumors and non-MHC expressing germ cells of testis, thus they may represent an ideal target for tumor immunotherapy. Therefore, targeting T cells against tumor associated CT antigens might selectively eliminate tumor cells avoiding toxicity to normal tissue. In a trial targeting CTA NY-ESO-1, in patients with melanoma and synovial cell carcinoma, long-term partial regressions and two complete regressions have been observed without associated toxicities. It should be emphasized, that in over one hundred patients receiving TCR gene therapy, there been no toxicities similar to graft-versus-host disease reported in murine studies. In our current clinical trials only a minority of patients with advanced cancer are eligible for TCR gene therapy protocols, as they must express human leukocyte antigen (HLA)-A*0201 and have tumors that express a common tumor-associated antigen. Unlike a conventional TCR, a chimeric antigen receptor (CAR) is capable of binding excitatory signals to T cells in a non-MHC-restricted manner. These hybrid proteins, composed of an extracellular antigen recognition domain fused to an intracellular T-cell activation domain, may therefore be used in patients regardless of their HLA genotype. The non-HLA-restricted antigen recognition is achieved by harnessing the antigen-binding properties of monoclonal antibodies (mAb); this recognition is also independent of antigen processing, thus bypassing a potential mechanism by which tumor cells can evade the immune system in vivo. Several clinical trials using CAR-transduced T cells have been reported, and the Surgery Branch’s efforts targeting CD19 have lead to the effective elimination of lymphoma in one patient. Efforts aimed at improving the response rate and minimizing on-target toxicity are the key to progress in T cell gene therapy for cancer.

Cancer Vaccines; Results and Perspectives

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A strong development of cancer vaccines occurred during the last 10 years since several clinical studies were performed with vaccines based on, a) peptide expressed by human tumors; b) tumor cells genetically modified or dendritic cells pulsed with protein/peptide antigens; c) recombinant vectors containing DNA or RNA sequences coding for antigens of different tumors. While new immunobiological information has been collected through these translational studies, the tumor response only rarely exceeded 20% while vaccine- or tumor-specific T cell responses varied from 30 to 80%. However, the increased knowledge of molecular mechanisms of immunological escape by neoplastic cells suggests that, by counteracting these inhibitory circuits, anti-tumor vaccines can become effective particularly in association with immune-modulatory antibodies. By using gene-transduced autologous T cells as vector of MAGE-3, we were able to show that metastatic melanoma patients can mount a specific T cells response that was often accompanied by a clinical response. Examples of phase III randomized clinical studies of vaccination with therapeutic efficacy have been recently presented in metastatic melanoma patients, treated with, a) the immune-modulating antibody to CTLA4, b) a gp100 peptide-based vaccine in combination with high dose IL-2, and c) in prostate cancer patients receiving a dendritic cell-based vaccine (Dendreon). In addition, a phase II randomized trial in NHL patients has shown a survival benefit in the group of subjects receiving the vaccine. These new clinical trials will be presented in terms of both clinical and immune responses and the reasons for their success will be discussed.

Hematopoietic stem cell transplantation: a platform for cell and gene therapy

David Klatzmann

See supplement

Gene therapy of thalassemia

Dr G Ferrari1

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Thalassemias are a heterogeneous group of inherited anemias, which collectively represent the most common mono- genic disorders. The beta-thalassemias are characterized by reduced or absent production of hemoglobin beta-chains. The most severe form, beta-thalassemia major or Cooley’s anemia, is characterized by a profound anemia that, if not treated, leads to death in the first year of life. Conventional treatment is based on lifelong blood transfusion and continuous iron chelation therapy to prevent toxic iron overload. So far, the only available cure for beta-thalassemia is allogeneic bone marrow (BM) transplantation, which is however available for less than 30% of the thalassemic patients. Autologous transplantation of genetically corrected HSCs is considered an attractive therapeutic alternative to allogeneic BM transplantation for patients lacking a compatible donor. The development of lentiviral vectors (LVs) and the optimization of HSCs transduction conditions has provided a significant contribution to this field, leading to the recent application of LVs expressing the human beta-globin gene in preclinical murine models and in human thalassemic cells.
The molecular features of beta-globin LVs, the transduction efficiency of human hematopoietic stem/progenitor cells coupled with the level of engraftment and the therapeutic potential to correct thalassemia at safe, low vector copy number, are important issues to be considered and addressed for the evaluation of risk/benefit ratios. Different LVs expressing beta-globin have recently been developed. All possess variations of beta-globin locus control region hypersensitive site elements HS2, HS3 and HS4 and in the use of the chicken HS4 insulator element inserted into the viral vector long terminal repeats. A great effort is dedicated to improve the efficacy of beta-globin LVs, by using erythroid specific transcriptional control elements that enhance transgene expression without impairing of viral production. Once developed, the therapeutic potential of beta-globin vectors need to be tested in the most reliable disease models, represented by the thalassemic mutant mice and by the patients' hematopoietic cells. Now, the results from one patient treated in the first trial in France are available and show progress and limitation of gene therapy in humans. In this presentation the major recent contributions to the field, from development of vectors and preclinical models to the results in the patient, will be discussed.

Or 8
Gene therapy for lysosomal storage disorders
Nathalie Cartier

Lysosomal storage diseases (LSD) are over 40 inherited metabolic disorders due to the deficiency of a single enzyme activity that results in the accumulation of uncatabolized substrate in lysosomes. LSD have heterogeneous physiopathology and clinical manifestations, and can often affect several different organs. Lysosomal enzymes are trafficked by a mannose-6-phosphate receptor mechanism, that allows internalization of the enzyme. Thus, normal enzyme can be provided to deficient cells and prevent storage. Enzyme replacement therapy (ERT) has improved the outcome of some of these disorders; however, in many cases, enzyme replacement therapy or substrate reduction therapy are insufficiently efficacious or associated with complications. One of the greatest challenges is in developing effective therapies to treat the CNS manifestations of these complex disorders, since blood brain barrier limits the access of the recombinant enzyme to the nervous tissues. Gene therapy is an attractive therapeutic alternative. Different gene therapy strategies have been developed with encouraging preclinical and clinical results. Autologous transplantation of hematopoietic cells is currently evaluated for some diseases, allowing high level of enzyme expression and avoiding the high morbidity and mortality risk of allogeneic hematopoietic cell transplantation. Intravenous administration of viral vectors was effective in some preclinical models, particularly when delivery was performed at the neonatal period. The efficacy of direct in situ delivery of recombinant enzyme via the use of AAV vectors was demonstrated in several applications, particularly those with cerebral involvement. Several clinical applications are ongoing. The presentation will review the different approaches currently considered for treating LSD and the challenges that are raised.

Or 9
A Phase I/II cell therapy for duchenne muscular dystrophy
Dr G Cossu

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Mesoangioblasts are recently characterized progenitor cells, associated with the vasculature and able to differentiate into different types of solid mesoderm, including skeletal muscle (1). When both wild type or dystrophic, genetically corrected, mesoangioblasts were delivered intra-arterially to dystrophic muscle of a-sarcoglycan KO mice (a model for limb girdle muscular dystrophy), they resulted in a significant functional amelioration of the dystrophic phenotype (2). Intra-arterial delivery of wt mesoangioblasts, non DLA matched to GRMD dystrophic dogs resulted in a partial recovery of muscle morphology and function, dystrophin expression and clinical amelioration. Delivery of autologous mesoangioblasts expressing human micro-dystrophin did not cause a comparable amelioration, despite widespread micro-dystrophin expression (3). Human adult mesoangioblasts were isolated and expanded in vitro from muscle biopsies: they were shown to correspond to a subset of pericytes (4). Based on these results, a monocenter, prospective, non-randomised, clinical phase I/II study of cell therapy with HLA-matched donor human mesoangioblasts in DMD patients started in June 2009, with a one year preliminary study (involving 28 DMD patients, aged 5-10), required to validate outcome measures. Six out of these patients will undergo successive intra-arterial transplantations at escalating doses of cells under a continuous regime of immune suppression. Safety will be the primary objective of the study. However it is expected that transplantation of mesoangioblasts will result in a detectable increase in muscle strength and consequent clinical amelioration or stabilization. Problems still facing this approach and possible strategies to overcome them will be discussed.


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Or 10
Gene therapy for retinal degeneration
Robin Ali

See supplement

Or 11
Cell therapy for neuroinflammatory diseases
Gianvito Martino
Recent evidence consistently challenges the sole and limited view that neural stem/precursor cells (NPCs) may protect the central nervous system (CNS) from inflammatory damage leading to neurodegeneration exclusively through out-cell replacement. As a matter of fact, NPC transplantation may also promote CNS repair via intrinsic neuroprotective bystander capacities, mainly exerted by undifferentiated stem cells releasing, at the site of tissue damage, a milieu of neuroprotective molecules whose release is temporally and spatially orchestrated by environmental needs. This milieu contains molecules (e.g. immunomodulatory substances, neurotrophic growth factors and stem cell regulators), which are constitutively expressed by NPCs for maintaining tissue homeostasis either both during development and adult life. The intrinsic nature (pleiotropism and redundancy) of these molecules as well as their constitutive characteristics, may also reconcile data showing that other sources of somatic stem cells (e.g. mesenchymal stem cells), with very low capabilities of neural (trans) differentiation, may efficiently promote CNS repair. Thus, cell plasticity can also be viewed as the capacity of somatic stem cells to adapt their fate and function(s) to specific environmental needs occurring as a result of different pathological conditions (therapeutic plasticity). The challenging ability of transplanted NPCs to protect the brain from several types of injuries using different and/or articulated bystander strategies is of pivotal importance for the future of stem cell based therapeutic approaches.

**Or 12**

**Overcoming obstacles to effective T cell therapy of malignant disease**

Professor P Greenberg, Dr G Ragnarsson, Dr T Schmitt, Ms C Chou, Dr C Fowler, Dr A Schietinger, Dr I Strommes, Dr J Blattman

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The development of molecular methods to identify candidate tumor antigens that might be targeted for attack by T cells, in concert with improved understanding of T cell activation and generation, have encouraged efforts to modulate T cell immunity as a strategy to treat human malignancies. However, despite characterization of many immunogenic tumor antigens that might be targeted, including prionogenic proteins over-expressed by the tumor, clinical trials with demonstrable efficacy in diseases other than melanoma have been limited. Our lab has been assessing strategies to overcome the obstacles to achieving therapeutic success, and has used adoptive transfer of ex vivo expanded T cells in both mouse models and human clinical trials to obtain insights into the major impediments to effectively modulating immunity to treat patients with established malignancies.

Issues being addressed include overcoming the low avidity for the tumor of naturally isolated tumor-reactive CD8 T cells by isolating TCR genes, inducing and selecting mutants with high affinity, and re-introducing the TCR genes into syngeneic/autologous T cells. Additionally, CD8 T cells specific for self/tumor antigens, even if capable of efficiently recognizing tumor cells in vitro, may be rendered anergic/tolerant in vivo after transfer. As this represents a substantive obstacle to tumor therapy, recent studies from our lab have explored the mechanisms responsible for such tolerance, and we are designing strategies to circumvent these mechanisms by genetically modifying the T cells, including disrupting negative regulatory pathways operative in potentially responding T cells.

Studies in mouse models and human clinical trials will be described.

**Or 13**

**Dedifferentiation, transdifferentiation and reprogramming: three routes to gene and cell therapy**

Juan Carlos Izpisúa Belmonte

See supplement

**Or 14**

**Suicide gene therapy abrogates late transplant mortality in haploidentical stem cell transplantation**

Dr CB Bordignon

In allogeneic hemopoietic cell transplantation (allo-HSCT) most of the antileukemic potential resides in alloreactivity towards patient-specific antigens by donor lymphocytes. Unfortunately, alloreactivity is also responsible for the most serious and frequent complication: the Graft-versus-Host Disease (GvHD), that impairs quality of life and reduces survival expectancy of transplanted patients. The risk of GvHD associated to T cell infusions is prohibitive in patients transplanted from HLA-mismatched donors. Several cell and gene transfer approaches designed to enforce the graft-versus-tumor effect, promote a functional immune reconstitution and prevent/control GvHD are currently under clinical investigation. The majority of these approaches, based on selection of specific T cell subpopulations, aim at reducing the repertoire of infused donor lymphocytes, so to prevent alloreactivity. In contrast, suicide gene therapy aims at a selective control of GvHD in treated patients, thus permitting the full exploitation of alloreactivity against cancer. In a phase I-II clinical trial enrolling 54 pts undergoing haploidentical HSCT, we showed that the infusion of suicide gene-modified T cells prompted the renewal of thymic activity, which contributed to the early recovery of a polyclonal and functional T cell repertoire in patients of all ages. Contextually, the infused transduced cells mediated a direct antitumor effect, through their recognition of allogeneic determinants on leukemic cells. GvHD was successfully treated in all affected patients. Early immune reconstitution associated to control of GvHD resulted in the abrogation of late transplant related mortality. Efficacy of suicide gene therapy in the context of haploidentical HSCT for leukemia is currently being assessed in a phase III randomized multicentric clinical trial.
Or 15

Early clinical trial results following administration of a low dose of a novel self complementary adenovypen-associated viral vector encoding human factor ix in two subjects with severe Haemophilia B

Dr AC Nathwani, Dr J McIntosh, Dr P Rustagi, Dr B Golder, Dr M Kay, Dr J Allay, Dr J Coleman, Dr S Sleep, Dr K A High, Dr F Mingoza, Dr J Gray, Dr U Reiss, Dr AW Nienhuis, Dr AM Davidoff

We have developed a unique approach for the treatment of haemophilia B (HB) that is currently being tested in the clinic. This entails peripheral vein administration of a single dose of our novel self complementary AAV vector encoding a codon-optimised human FIX transgene (scAAV2/8-LP1-hFIXco) into adult subjects with severe HB. Vector is being administered in the absence of immunosuppression. Thus far, two subjects have received peripheral vein infusion of vector at the low dose, each without any adverse reactions including heptotoxicity. The longest follow-up is in the first subject, in whom plasma FIX levels increased from a baseline of <1% to between 1.5-2% of normal levels within 2 weeks. This level of transgene expression has been maintained for a period that extends beyond 6 months following vector infusion. Importantly, this subject has not received any treatment or prophylaxis with FIX concentrate over this period and remains free of spontaneous joint bleeds. The same dose has recently been administered to a second patient without toxicity. The FIX level in this subject, who is on regular prophylaxis, is currently 2% of normal, 13 days after his last dose of prophylaxis. His FIX levels continue to be monitored to more conclusively establish evidence of expression of transgenic FIX. These early data are highly encouraging and suggest that low doses of scAAV vector, when pseudotyped with serotype 8 capsid can mediate therapeutic levels of FIX for at least several months without provoking an immunological response of the type seen in the previous trial.

Or 16

Correction of Wiskott-Aldrich syndrome by hematopoietic stem cell gene therapy

Kaan Boztug, Manfred Schmidt, Adrian Schwarzer, Pinaki P. Banerjee, Marie Böhmi, Rita Beier, Inés Avedillo Diez, Ricardo Dewey, Claudia R. Ball, Ali Nowrouzi, Sonja Naundorf, Klaus Kühlicke, Rainer Blasczyk, Martina Rose, Chris Fraser, Mathias Liesl, Rudolf Ferrari, Miguel Abboud, Waleed Al-Herz, Irina Kondratenko, Marta Lázsló Marródi, Jordan S. Orange, Christof von Kalle, and Christoph Klein

Wiskott Aldrich Syndrome is a life-threatening immune disorder characterized by bleeding secondary to microthrombocytopenia, immunodeficiency, autoimmunity, and susceptibility to lymphoma. A clinical gene therapy protocol using transplantation of autologous hematopoietic stem cells transduced with a GALV pseudotyped MLV-derivated retroviral vector was developed at Hannover Medical School. We here present an analysis of ten patients treated in this trial between 2006 and 2009. Patients received 3 – 23 x 10^6/kg genetically modified hematopoietic progenitor cells upon partial myeloablative conditioning using busulfan (8 mg/kg). 9/10 patients received more than 10^6/kg progenitor cells and had evidence of sustained engraftment of WASP-positive hematopoietic progenitor cells. One patient received 2.4x10^6/kg progenitor cells, failed to show sustained engraftment and was eventually transplanted with haploidentical hematopoietic stem cells. In the remaining patients, WASP expression was determined in myeloid and lymphoid cells as well as in CD34+ hematopoietic progenitor cells using Western Blot and FACS analysis, respectively. While the percentage of WASP-positive myeloid cells was relatively stable over time (range 10 to 60%), a marked increase over time in the percentage of WASP-positive T lymphocytes and NK cells was observed, resulting in more than 80% of WASP-positive lymphoid cells 12 months after gene therapy. An increase in platelet counts was observed in all patients. Furthermore, the majority of platelets showed evidence of WASP expression, detectable as early as 3 months after gene therapy. Functional immune reconstitution was documented in dendritic cells (podosome formation), T cells (proliferation in response to CD3-signaling), and NK cells (formation of immunological synapse and NK cell killing activity). TCR V? spectratyping analyses showed in improvement of receptor skewing upon gene therapy in some patients. A clinical benefit was notable in all 9 patients with sustained engraftment: eczema, bleeding diathesis and immunodeficiency resolved. Autoimmune phenomena such as autoimmune hemolytic anemia and colitis also resolved after gene therapy. Repetitive bone marrow examinations did not reveal morphological or cytogenetic alterations. Comprehensive insertion site analysis using 454 pyrosequencing demonstrated vector integration that targeted multiple genes controlling growth, development and immunological responses in a persistently polyclonal hematopoiesis.

In sum, hematopoietic stem cell gene therapy for Wiskott-Aldrich Syndrome is feasible and effective at correcting the
various cellular defects implicated in this disease. Prospective monitoring will determine the long-term efficacy and safety profile of this experimental therapeutic approach in patients with Wiskott-Aldrich syndrome.

Or 17

HSC gene therapy trial for Metachromatic Leukodystrophy: first report on gene marking efficiency

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Metachromatic Leukodystrophy (MLD) is a demyelinating lysosomal storage disorder due to deficiency of arylsulfatase A (ARSA). In the absence of effective therapies, MLD is a disease with an urgent medical need. We previously showed that transplantation of lentiviral vector (LV)-transduced hematopoietic stem cells (HSC) efficiently delivers ARSA to the nervous system and prevents disease manifestations of MLD in the mouse model provided that functional enzyme is expressed above normal donors’ levels in the HSC progeny. In an extensive series of preclinical studies we showed the feasibility and safety of obtaining such ARSA over-expression in human bone marrow (BM) HSC upon transduction with an optimized ex vivo protocol reproducibly yielding >1.5 LV copy per genome (VCN) of GMP-manufactured LV. According to these data a Phase I/II clinical trial of HSC gene therapy was approved for the treatment of MLD patients and the first patient was treated in our Institute last May. Upon administration of a myeloablative dose of busulfan, the patient received autologous BM HSC freshly transduced with ARSA-encoding LV to VCN = 2 and efficiency of 95%. No serious adverse events were reported in the peri- and post-transplant phase up to 90 days follow up. The patient recovered from aplasia by day +28 with complete transfusion independence. A sustained engraftment of the transduced cells consistent with the in vitro data was observed in all blood lineages except CD3+T lymphocytes (as expected from the choice of a non-immunosuppressive conditioning) and in BM CD34+ cells, starting from 28 days after the transplant to the latest follow-up time (3 months). Preliminary vector integration analysis shows a highly polyclonal distribution. These marking levels far exceed those reported in previous trials using gamma-retroviral vectors or LV and, if maintained long-term, would suggest a substantial gain in gene marking efficacy. Sustained, above-normal ARSA activity was also measured in patient’s blood leukocytes. Thus far, the follow up of this patient demonstrates the short-term safety and feasibility of the procedure. Long-term evaluation will establish safety and assess efficacy.

Or 18

Phase I clinical trial of allogeneic T cells transduced with inducible caspase 9 suicide gene

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Adaptive T-cell transfer after haploidentical CD34+ stem cell transplantation (haplo-CD34+ SCT) is often complicated by graft versus host disease (GvHD). We have previously demonstrated that allogeneic CD34+ cells transduced with the inducible caspase 9 suicide gene (iCasp9) can be killed by administering a chemical inducer of dimerization (CID-AP1903) leading to T-cells apoptosis. Contrarily to viral based suicide genes our system is likely not immunogenic, killing is not restricted to dividing cells and ganciclovir administration is not precluded. Briefly, donor PBMCs are co-cultured with recipient irradiated EBV-transformed lymphoblastoid cells (40:1) for 72 hrs, allografted with a CD25 immunotoxin and transduced with the iCasp9 suicide gene and the inducible caspase 9 suicide gene. We developed an inducible caspase 9 suicide gene T cells, (dose levels from 1x106 to 3x106 cells/kg). Expression of iCasp9 did not preclude in vivo expansion of infused T cells, which became detectable by flow cytometry (CD3+ iCASP9+ cells) and by qPCR (for iCasp9) within 7 days of infusion. Three patients developed grade I-II aGVHD. Administration of the dimerizer drug produced >90% ablation of CD3+ iCASP9+ cells, within 30 minutes of infusion, with resolution of aGvHD within 24hrs. The residual
allogeneically reconstituted T cells were reactive to viruses (CMV) and fungi (Aspergillus fumigatus) (IFN-γ production) but no longer produced GVHD. (Supported by an NHLBI grant: U54HL081007)

Or 19

ProSavin® a Gene Therapy Approach for Parkinson Disease: Phase I Clinical Trial Update

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L-Dopa and dopamine agonists provide the primary standard of care for Parkinson’s Disease (PD). Although highly efficacious in the early stages of disease, their long term use is associated with severe motor side effects that seriously impact on the quality of life. Such side effects are believed to be caused by the fluctuating nature of dopaminergic stimulation that arises from oral drug administration. We have developed a lentiviral vector (ProSavin®) derived from the equine infectious anaemia virus expressing the three key dopamine biosynthetic enzymes (tyrosine hydroxylase, aromatic L-amino acid decarboxylase and GTP cyclohydrolase-1), with the aim of providing a continuous source of dopamine in the striatum. ProSavin was previously demonstrated to mediate dopamine production and behavioural correction in rat and non human primate models of PD. A phase I, open label clinical study has been initiated in which six PD patients have received ProSavin at one of two dose levels (3 patients per dose level). All the patients have completed at least 12 months follow up and ProSavin has been demonstrated to be safe and well tolerated at both doses. There were no “OFF” state dyskinesias, no immune responses to ProSavin and no serious adverse events. The average improvement in motor function observed at one year (UPDRS Part III “OFF”) was 28% relative to pre treatment scores. An update on the current status of the trial will be presented.

Or 20

TCR gene therapy for cancer

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TCR gene transfer is an attractive strategy to equip T-cells with defined antigen-specific TCRs using short-term in vitro procedures. Selection of host cells with a known specificity and introduction of a well characterized high affinity TCR may result in an off-the shelf therapy that combines potent anti-tumor reactivity with a minimal risk of GvHD after allogeneic stem cell transplantation. However, some potential drawbacks to TCR gene transfer exist. TCR transfer leads to lower expression of the introduced TCR compared to parental T-cell clone due to competition for cell surface expression with the endogenous TCR and mixed TCR dimers. High affinity TCR is necessary to overcome this problem. High affinity TCRs directed against minor histocompatibility antigens are available, however identification of high affinity TCRs specific for tumor associated self-antigens is a critical bottleneck in this strategy. In addition, TCR transfer may lead to the formation of mixed TCR dimers, composed of introduced TCR chains pairing with the endogenous TCRs or β chain, harbouring potentially harmful new reactivities. In this presentation the benefits and threats of TCR gene transfer and possible solutions will be presented.

Or 21

Stathmin expression and p53 status: a new rationale for the management of ovarian cancer

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Epithelial Ovarian Cancers (EOC) remains the most lethal of the gynecological cancers. Mutations of the p53 gene represent a frequent alteration found in EOC and it has been postulated as a putative determinant of EOC chemosensitivity. Evidences suggest that the Microtubules destabilizing protein, Stathmin, could represent a downstream effector of mutant p53. Accordingly, our preliminary data indicate that stathmin overexpression correlates with low survival in EOC and p53 nuclear accumulation (considered a marker of its mutation).

We have analyzed the effect of stathmin silencing on EOC cells survival and sensitivity to platinum (DNA damage) and taxanes (MT target) focusing on its relationship with p53 mutations. Stathmin silencing by adenoviral sh vectors, reduced EOC cells viability only in the presence of p53 while p53 null cells remained completely insensitive. A moderate sensitivity was observed in cells which carried a WT p53. Moreover stathmin silencing in MDAH 2774 (p53 wild type) enhanced of about 2-fold the sensitivity to platinum and taxanes (MT target) focusing on its relationship with p53 mutations. Stathmin silencing in MDAH 2774 (p53 wild type) enhanced of about 2-fold the sensitivity to platinum and taxanes while it has no effects on drug-induced cell death in p53 null cells. Results with MDAH 2774 were confirmed in vivo with a xenograft nude mice model.

In progress experiments of silencing stathmin and p53 in MDAH 2774 cells or overexpressing mutated p53 in p53 null cells will define whether or not stathmin expression cooperate with mutant p53 in the resistance of EOC cells to drug-induced cell death.

Collectively our data suggest that stathmin could represent a promising marker and/or therapeutic target for EOC carrying a mutated p53 gene.

Or 22

Harnessing CD1-restricted T cells for the immunotherapy of leukemia

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Epithelial Ovarian Cancers (EOC) remains the most lethal of the gynecological cancers. Mutations of the p53 gene represent a frequent alteration found in EOC and it has been postulated as a putative determinant of EOC chemosensitivity. Evidences suggest that the Microtubules destabilizing protein, Stathmin, could represent a downstream effector of mutant p53. Accordingly, our preliminary data indicate that stathmin overexpression correlates with low survival in EOC and p53 nuclear accumulation (considered a marker of its mutation).

We have analyzed the effect of stathmin silencing on EOC cells survival and sensitivity to platinum (DNA damage) and taxanes (MT target) focusing on its relationship with p53 mutations. Stathmin silencing by adenoviral sh vectors, reduced EOC cells viability only in the presence of p53 while p53 null cells remained completely insensitive. A moderate sensitivity was observed in cells which carried a WT p53. Moreover stathmin silencing in MDAH 2774 (p53 wild type) enhanced of about 2-fold the sensitivity to platinum and taxanes while it has no effects on drug-induced cell death in p53 null cells. Results with MDAH 2774 were confirmed in vivo with a xenograft nude mice model.

In progress experiments of silencing stathmin and p53 in MDAH 2774 cells or overexpressing mutated p53 in p53 null cells will define whether or not stathmin expression cooperate with mutant p53 in the resistance of EOC cells to drug-induced cell death.

Collectively our data suggest that stathmin could represent a promising marker and/or therapeutic target for EOC carrying a mutated p53 gene.
A subset of T cells recognise lipid antigens of either bacterial or self origin presented by CD1 molecules, a family of non-polymorphic antigen-presenting molecules. There are four human CD1 isoforms: CD1a-e. T cells specific for self lipids are autoreactive and could play a role in cancer immune surveillance, where self-antigens are the targets of immune responses. The lack of polymorphism of CD1 molecules is attractive for a clinical application of this system, while their expression only in haematopoietic cells targets of immune responses. The lack of polymorphism of CD1 molecules is attractive for a clinical application of this system, while their expression only in haematopoietic cells

pressurion of the BCR/ABL fusion oncogene. Although it is well known that CML cells are genetically unstable, the mechanisms accounting for this genomic instability are still poorly understood. Because the Fanconi anemia pathway is believed to control several mechanisms of DNA repair, we investigated whether this pathway was disrupted in CML cells. Our data show that CML cells have a defective capacity to generate FANCD2 nuclear foci, either in dividing cells or after DNA damage. Similarly, human cord blood CD34+ cells transduced with BCR/ABL retroviral vectors showed impaired FANCD2 foci formation, whereas FANCD2 monoubiquitination in these cells was unaffected. Considering that previous results have demonstrated that BRCA1 is responsible for FANCD2 translocation to the nucleus after DNA damage we studied the ability of a retroviral vector expressing BRCA1 to restore FANCD2 foci formation and to improve chromosomal stability of BCR/ABL expressing cells. Notably, both the impaired formation of FANCD2 nuclear foci and the genomic instability of BCR/ABL cells, measured as the percentage of cells with chromosomal aberrations and multiple centrosomes, were reverted by the ectopic expression of BRCA1. Taken together our data show for the first time that the ectopic expression of BRCA1 in BCR/ABL cells can restore the FA/BRCA pathway and improve the chromosomal stability of these cells. This data opens new perspectives in the management of CML.

Or 24
Specificity for a tumor-associated self-antigen drives the development of functional memory T cells in the absence of vaccination

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Vaccines against the tumor-associated self antigen Wilms’ Tumor antigen 1 (WT1) have been tested in cancer patients, with a number of studies ongoing. It is currently not known whether physiological levels of WT1 expression in stem and progenitor cells of normal tissue results in the deletion or tolerance induction of WT1-specific T cells. Furthermore, it has been suggested that specificity for self-antigen may drive the spontaneous generation of memory phenotype T cells, but direct evidence has not been provided. Using a non-tolerant repertoire, we have previously isolated HLA-class I restricted TCR specific for WT1. We used TCR gene transfer into purified haematopoietic stem cells to study the fate of WT1-specific T cells in the thymus and the periphery of HLA-A2Kb transgenic mice. Thymocytes expressing the WT1-specific TCR derived from high avidity human CD8+ T cells were positively selected into the single positive CD8+ population. In the periphery, T cells specific for the WT1-antigen differentiated into CD44-high memory phenotype cells, while T cells specific for a non-self viral antigen retained a CD44-low naïve phenotype. Only the WT1-specific T cells, but not the virus-
specific T cells, displayed rapid antigen-specific effector function without prior vaccination. Despite long-term persistence of fully functional WT1-specific memory T cells, the animals did not develop any signs of autoimmunity. The WT1-specific T cells in the BM without impairing stem cell function as demonstrated by engagement of secondary recipients. Our data demonstrate that T cell specificity for a tumour-associated self-antigen did not result in tolerance induction, but instead promoted the development of functionally competent memory phenotype T cells.

Or 25

Designing AAV Vectors for Translational Studies: Update on DMD Phase 1 Trial

Prof Richard Samulski

Initial AAV vectors for clinical studies have been derived from natural isolates. We have engineered and tested in Phase 1 DMD study chimeric vector derived from type 1 and type 2. Outcome of these studies and next generation of DMD viral vectors suited for efficient gene delivery will be described and pre-clinical data presented.

Or 26

Modification of Adenovirus Tropism with Designed Multi-Valent Targeting Adapters

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To achieve disease-site-specific gene delivery by adenovirus (Ad) vectors, the native tropism of Ads must be modified. To this end, we developed bspecific targeting adapters that bind to Ad5 fiber and Her2, an established biomarker of human cancers, and enable targeted transduction of Her2-expressing cells. Specifically, we applied the technologies of the Designed Ankyrin Repeat Proteins (DARPins) and ribosome display to design a DARPin that binds the knob domain of the Ad fiber with low nanomolar affinity, and then fused this protein genetically with the Her2-specific DARPin. Next, we gradually improved the stability of the complex formed by the adapter and Ad virion by increasing the valency of adapter-virus binding. We designed adapters that chelated the knob in a bivalent or trivalent fashion and showed that as the functional affinity of these molecules for Ads increased, so did the efficacy of gene transfer by the adapter-Ad complex, enabling efficient transduction. We confirmed the Her2 specificity of this transduction and its dependence on the adapters’ Her2-binding DARPin component. We noted that even the most complex adapter molecules with four copies of DARPins could be expressed in E. coli at very high levels and could be easily purified. Since DARPins can be generated in principle against any target, this approach provides a versatile strategy for developing a broad range of disease-specific gene vectors.

Or 27

Analysis of rAAV Integration Frequency and Persistence in Rat Liver after ssAAV and scAAV Delivery

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Long-term gene expression is achieved following recombinant adeno-associated virus (rAAV) vector delivery. Previously, we have shown that rare rAAV integration in hepatocytes after rAAV8 delivery may lead to long-term gene expression in liver. Thus, even rare integration raises concerns about the clinical safety of rAAV. Analysing rAAV integration frequency and persistence allows evaluating genotoxicity of different serotypes and vector doses. Therefore, we have adapted LAM-PCR to deep sequencing and characterized rAAV genomes in rat liver after single-stranded (ss)AAV or self-complementary (sc)AAV injection of different doses. 250-500 ng of DNA extracted from twelve ssAAV1 transduced animals 14 months post rAAV-injection and from six scAAV1 or six scAAV8 transduced ones 13 months post injection (partial hepatectomy, HP) and 3 months after HP were analysed. >68,000 LAM-amplicons revealed 67 insertion sites (IS) in total, up to eight per sample. In both vector-types around 40% of the IS were located in or upstream of gene while about 20% were located downstream. Animals transduced with 1.2E12 TU/kg ssAAV showed a higher average number of IS (4.6) compared to animals transduced with 8E12 TU/kg (0.5). Semi-quantitative measurements revealed that up to 100% of the analysed sequences were due to concatemers in animals transduced with a higher ssAAV dose while 3/4 were referred to IS in animals with a lower dose. Furthermore, the feasibility of full ITR sequencing in ssAAV and scAAV transduced rat liver was shown while partial ITR revealed preferred breakpoints in scAAV transduced animals. These insights allow assessing rAAV safety under different (pre)clinical settings.

Or 28

Engineering Acid-Responsive Artificial Envelopes around Adenovirus for Efficient Gene Transfer

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Gene therapy involves the delivery of a functional gene by a vector into target cells, resulting in a desired therapeutic effect. Adenovirus (Ad) has shown a great promise in gene therapy. However, in vivo studies have reported an immunogenic response and significant hepatotoxicity in liver. These issues currently inhibit the use of this vector for clinical genetic therapies. Such limitations have been overcome by engineering artificially enveloped Ad using zwitterionic and cationic lipid formulations. However, this resulted in a significant reduction of gene expression in vitro due to the poor release of the enveloped virus from the endosomal compartment. In this study, we have explored the use of pH-sensitive DOPE:CHEMS envelopes to enhance the virus release from the endosome. The surface engineered Ad were characterised by atomic force microscopy, dot blot, dynamic light scattering and zeta potential measurements. The gene expression of Ad encoding for beta-galactosidase reporter gene enveloped in DOPE:CHEMS showed high levels of gene expression when tested in different cell lines. These results were further confirmed by studying the intracellular trafficking of Cy3-labelled Ad using confocal laser scanning microscopy. Cy-3 Ad enveloped in DOPE:CHEMS showed a uniform fluorescence distribution within the cytoplasm indicating Ad endosomal release. Moreover, pH-sensitive enveloped Ad injected directly into human cervical adenocarcoma (C33a) xenografts grown on the flank of nude mice showed same level of gene expression to naked Ad. In conclusion, this type of artificially-enveloped Ad offers a promising tool in gene delivery since high level of Ad gene expression can be maintained with improved immunogenicity and hepatotoxicity in vivo.

Or 29
Unswerving Factor IX Expression in NAb-negative Macaques following IV Administration of AAV8 Vector
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Adeno-associated病毒 (AAV) vectors are promising for gene therapy approaches especially for hemophilia. In order to evaluate therapeutic efficacy of AAV vectors in hemophilia gene therapy, we have been working on preclinical studies using cynomolgus macaques. Earlier results indicate the efficacy of AAV8-based vectors in transducing macaque liver by intravenous injection. Nonetheless, inhibitory actions of pre-existing neutralizing antibody (NAb) against vector capsid, even at marginal levels, are also concerned, suggesting the necessity of more sensitive assay for NAb detection. To solve this issue, we have improved NAb detection system against AAV8 capsid through augmenting infection steps. Five sero-negative male cynomolgus macaques were selected based on this assay. AAV8-based vector encoding mutant macaque factor IX driven by liver-specific promoter was injected intravenously at 2 × 5 x 10^{12} vg/kg, which are relevant vector dose in human trials. All of the animals exhibited robust and sustained factor IX expression at the therapeutic window (3.0 ○ 20.2% of normal). No differences were found in factor IX concentration by the vector dose. High copy numbers of vector genome (12.9 ± 1.3 vg/dge) were detected in the liver biopsy specimen. Our results indicate a prospect of hemophilia gene therapy using AAV8 as well as the sufficient sensitivity of our improved NAb assay, which would be useful in human clinical trials using AAV vectors. This study was performed in collaboration with Tsukuba Primate Research Center, National Institute for Biomedical Innovation, and The Corporation for Production and Research of Laboratory Primates, Japan.

Or 30
Hematopoietic stem cell gene therapy with lentiviral vector in X-linked adrenoleukodystrophy
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Cerebral demyelination of X-ALD can be arrested in children and adults by allogeneic HSC transplantation (HCT) within 12-18 months, provided the procedure is performed at an early stage of the disease. The long term beneficial effects of HCT in X-ALD are likely due to the progressive turnover of brain microglia that are derived from myeloid progenitors in the bone-marrow. Despite the increased availability of cord blood, not all boys or adults with cerebral and who are candidate for HCT have a suitable HLA-matched donor. In addition, allogeneic HCT remains associated with significant mortality risk, particularly in adults. In two boys with cerebral ALD but without HLA-matched donor, we recently reported that HSC gene therapy with lentiviral vector has comparable effects to those seen after allogeneic HCT in arresting cerebral demyelination. Data on ALD protein expression as well as the identification of identical lentiviral insertion sites in myeloid and lymphoid lineages strongly suggested that HSCs were transduced in the patients. Hematopoesis remained polyclonal without evidence of clonal skewing or dominance. Data will be presented on longer follow-up of these two treated patients, as well as on a third
patient who has been treated more recently by HSC gene therapy.

**Or 31**

**Thalassaemia**

Philippe Lebouche

See supplement

**Or 32**

**GATA1 HS2 leads to position-independent transgene expression in LV-transduced hematopoietic cells**

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Persistent and position-independent expression is a fundamental requirement for the development of efficient transgene expressing vectors. We tested the role of an autoregulatory enhancer (HS2, hypersensitive site 2) of the erythroid-specific GATA-1 gene in protecting an expression cassette from the repressive effect of the surrounding chromatin. Human and murine hematopoietic stem cells (HSCs) were transduced with lentiviral vectors (LV) expressing GFP under the control of an internal CMV promoter. This transcriptional cassette was cloned in the framework of LV in which the viral enhancer region in the LTRs was deleted or replaced by two HS2 elements. Transgene expression derived from HS2-containing LV was persistent and less prone to repression, compared to expression from LV lacking the HS2 element, in the erythroid progeny of HSCs in vitro and in vivo. On the basis of these findings, we cloned the GATA-1 HS2 in a beta-globin expressing vector and demonstrated that this modification was able to markedly increase the probability of transgene expression in thalassemic mice transplanted with transduced HSCs, leading to correction of the thalassemic phenotype.

ChIP analyses showed that the transcription factor GATA-1 together with the histone acetyltransferase CBP, binds to HS2-containing LTRs. Consequently, CBP-mediated histone acetylation level increased significantly at the transgene promoter. Overall, our data support the hypothesis that the binding of GATA-1 to LV LTRs containing HS2 mediates the recruitment of CBP, resulting in the formation of an open chromatin structure at the proviral integration sites and thus reducing the chances of chromatin-mediated inactivation of transcription.

**Or 33**

**Long-Term Survival Of Primary Kupffer Cells After Transplantation Into Syngeneic Mouse Recipients**

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**Background:** Kupffer cells (KC) are the resident hepatic macrophages, constitute 5-15% of all liver cells and play a critical role in the liver physiology. Manipulation of KC has been helpful in altering disease-specific processes. Previous work from our and other groups showed that several liver cell types can be transplanted. This suggested that KC will be amenable to similar reconstitution and offer insights into the potential of resident or extrahepatic sources of cells.

**Methods:** KC were isolated by magnetic sorting from nonparenchymal cells after GFP or CD45.1 mouse liver perfusion with collagenase. KC were transplanted by tail or portal vein injection into DPPIV-/-, IL-6 KO or hemophilia A mice. Liver samples were collected 2h, 1, 3, 7, 15 d, 1 and 3 mo after transplantation. IL-6 KO mice were challenged with LPS to assess whether transplanted cells were functional. qPCR and FVIII immunostaining were performed to verify the presence of FVIII mRNA in KC.

**Results:** Immunofluorescence showed the presence of transplanted cells in the liver of recipient mice up to 3 months. RT-qPCR analysis for IL-6 confirmed the functionality of transplanted KC in IL-6 KO mice. Studies with KC isolated from healthy mice showed FVIII mRNA expression and we localized FVIII protein in cells by FVIII immunostaining. Finally, KC were able to rescue the bleeding phenotype of hemophilia A mice in short term transplantation assays.

**Conclusions:** As KC can be isolated and returned to the liver after injection in recipient mice with cell engraftment under suitable conditions. KC transplantation might have therapeutic potential, e.g., in hemophilia.

**Or 34**

**Monitoring and exciting reprogramming factors: a novel lentiviral expression system for reprogramming**

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Cellular identity is largely determined by transcription factor networks. The concerted action of 4 reprogramming factors (RFs: Oct4, Sox2, Klf4, c-Myc) generates induced pluripotent stem cells (iPSC), a promising resource for regenerative medicine and disease modelling. We constructed a versatile lentiviral vector system for mono- and polycistronic expression of murine, human or codon-optimized RFs under control of the strong retroviral promoter SFFV, which enables...
high expression in most somatic cells and is rapidly silenced in pluripotent cells. To avoid the risks of residual permanent RF expression and insertional mutagenesis, we included FRT, lox or rox sites for removal through transient recombinase expression. Proof-of-principle for the generation of RF-free iPSCs was obtained using FRT-flanked vectors and a novel approach of retroviral protein transduction (Voelkel et al., PNAS 2010). Using this system we generated murine and human iPSCs with high efficiency. To learn more about the kinetics of iPSC reprogramming and to understand the underlying mechanisms, we developed color-coded RF vectors to follow expression kinetics qualitatively and quantitatively in relation to an Oct4-GFP indicator allele. We documented the dynamic conversion of fibroblasts to pluripotent cells by fluorescence microscopy combined with long-term single cell tracking and alternatively high-definition structural analysis of single iPSC colonies. Filming the “birth” of iPSC, we obtained movies, which show the potential genetic mosaic of early iPSC colonies and also indicate the necessity of stochastic epigenetic changes during reprogramming. In summary, the described vector system supports “excisable”, efficient and relatively safe reprogramming strategies in regenerative medicine.

Or 35
Generation and hematopoietic differentiation of disease-free iPSCs in mouse models of Fanconi Anemia
Dr S Navarro, Ms V Moleiro, Ms R Chinchón, Mr FJ Molina, Ms ML Lozano, Dr E Samper, Dr B Schiedmeier, T, Maetzg, Dr M Gallà, A, Schambach, Dr G Mstoslavsky, Dr JC Segovia, Dr A Raya, Dr G Guenechea, Dr C Baum, Dr J C Izpisua-Belmonte, Dr J A Bueren

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In recent studies we have shown that upon correction of their genetic defect, somatic cells from Fanconi anemia (FA) patients can be reprogrammed to generate iPSCs which can be re-differentiated towards the hematopoietic lineage (Raya et al. Nature 2009). With the final objective of demonstrating the in vitro therapeutic relevance of cell reprogramming in FA, we aimed to reproduce our in vitro studies with samples from FA patients, using different mouse models of FA. In contrast, to data obtained with human FA-A cells, iPSCs clones could be generated from uncorrected mouse FA-A fibroblasts, but not from fibroblasts from FA-D1/BRCA2 mice, which show a more severe phenotype. In this mouse model, the genetic complementation with BRCA2-LVs was required to generate stable iPSC cell clones. Aiming to improve the safety of the cell reprogramming approach, excisable reprogramming lentiviral vectors were used to co-transduce FA-D1 fibroblasts with the therapeutic BRCA2-LV. Thereafter, stable iPSC clones were transduced with Cre-expressing IDLVs to excise the reprogramming cassette, and thus to limit leukemogenesis risks after transplantation. Because BRCA2 is required for the generation of Rad51 foci after DNA damage, we confirmed the disease-free nature of iPSCs clones generated from FA-D1 mice by their ability to generate Rad51 foci after exposure to DNA cross-linking drugs. Using different in vitro differentiation models, genetically-corrected iPSC cell clones were capable of generating hematopoietic progenitor cells. Our next objective aims the replacement of the defective hematopoiesis of FA-D1 mice by means of the transplantation of disease-free HSCs generated from corrected FA-D1 iPSCs.

Or 36
“Taming” iPSCs: Coaxed differentiation of iPSCs into myogenic precursors using hyperactive transposons expressing the myogenic transcription factor PAX3
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Induced pluripotent stem cells (iPS) hold great promise for regenerative medicine. The development of robust non-viral approaches for gene transfer into iPS would facilitate functional studies and potential clinical applications. We have previously generated hyperactive transposases derived from Sleeping Beauty, using an in vitro molecular evolution and selection paradigm (Mates, Chuah et al., Nature Genetics, 41(6):753–61, 2009; VandenDriessche et al., Blood, 114(8):1461–8, 2009; VandenDriessche et al., Hum Gene Ther. 20(12):1559–61, 2009). We now demonstrate that these hyperactive transposases resulted in relatively robust stable gene transfer efficiencies and expression in iPS. Nucleofection of iPS with a transposon encoding GFP and an expression construct encoding hyperactive SB100X transposases, yielded a net transposition efficiency of 25% eGFP expression in iPS. Nucleofection of iPS with a transposon encoding GFP and an expression construct encoding hyperactive SB100X transposases, yielded a net transposition efficiency of 25% eGFP + iPS cells. In contrast, iPS transfected with an inactive mutant failed to yield any stably expressing GFP + cells. Transposed iPS that had undergone 20 successive passages continued to express high GFP levels for at least 70 days after nucleofection, consistent with the presence of canonical transposon genomic integrations. The genetically modified iPS expressed pluripotency markers and retained their ability to differentiate along cardiac, hepatic and neuronal lineages and displayed a normal karyotype. Most importantly, transposon-mediated delivery of the myogenic PAX3 transcription factor into iPS coaxed their differentiation into MYOD + myogenic progenitors and multinucleated myofibers, indicating that PAX3 may serve as a myogenic “molecular switch” in iPS. Hence, this
hyperactive transposon system represents an attractive non-viral gene transfer platform to coax differentiation of iPS into transplantable cell types with broad implications for regenerative medicine.

**Or 37**

A novel protein-based approach to induced pluripotent stem (iPS) cell generation.

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Induced pluripotent stem (iPS) cell technology holds great promise for regenerative medicine. Forced retroviral expression of four or fewer transcription factors (Oct-4, Klf-4, Sox2 & c-Myc) is typically needed to generate iPS cells. Retroviral vectors risk residual viral genominc integrations which, while epigenetically repressed in iPS cells, have oncogenic potential. Furthermore, it has recently been shown that human iPS cells without viral integrants show a more desirable primitive phenotype closer to embryonic stem (ES) cells. Because the reprogramming factors need only be present for 2 weeks, transient delivery as protein would represent an ideal alternative approach to iPS cell generation. However, the required transcription factors are difficult to produce as soluble, bioactive proteins. Zhou et al. reported direct reprogramming of somatic cells (murine embryonic fibroblasts) with proteins made membrane permeant by an N-terminal short TAT sequence (Cell Stem Cell 4:381–384, 2009). However, that experiment has not been reproduced in murine or human cells. We now report a novel technique employing a Fast Protein Liquid Chromatography-based refolding protocol generating large quantities of soluble Oct-4, Klf-4, & Sox2, which, when added to the culture media of murine adult stem cells or human CD34+ cells, target the nucleus and produce iPS cell colonies. Human and murine colonies continue to stain strongly positive for Oct-4 and appropriate stage specific embryonic antigenic markers for at least four weeks after induction. Baculoviral-produced Oct-4 protein shows even greater bioactivity. These protein-based approaches have the potential to transform iPS cell technology by significantly enhancing safety and function.

**Or 38**

Pbx1 restrains myeloid maturation and maintains lymphoid potential in hematopoietic progenitors.

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Hematopoiesis is sustained by hematopoietic stem cells (HSCs), which are endowed with life-long self-renewal and multipotent differentiation capacity. In addition to HSCs, the hematopoietic hierarchy is critically dependent on various progenitors, including multi-potent progenitors (MPPs), common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs), characterized by progressively reduced self-renewal potentials and increasing lineage-restriction. However, little is known about factors that may restrain progenitor maturation to allow for transient proliferative expansion, including rapid rescue of bone marrow following myeloablation for transplantation or gene therapy purposes.

In this study we aimed at characterizing the role of the proto-oncogene Pbx1 in myeloid and lymphoid progenitors. To this purpose, we employed Pbx1 conditional-ko mice, that we have previously shown having a significant reduction in CMPs and CLPs, in addition to a profound defect in HSC self-renewal. Through analysis of progenitors’ proliferation, differentiation capacity and transcriptional profiling, we demonstrate that in the absence of Pbx1 the MPP and CMP progenitor pools are reduced due to aberrantly rapid myeloid maturation, associated with decreased expression of Meis1 and its targets including Flt3. Importantly, we found that Pbx1 maintains proto-oncogenic pathways in myeloid progenitors including leukemia stem cell programs. The CLP reduction, conversely, results from a defect in lymphoid priming arising as early as the HSC stage, present also in CMPs, that surprisingly still express lymphoid genes underlying a previously un-appreciated lineage promiscuity.

These results highlight a role for Pbx1 in restraining myeloid maturation in part through maintenance of a transcriptional program containing its dimerization partner Meis1.

**Or 39**

In vivo selection of hematopoietic stem cells by a truncated erythropoietin receptor (tEpoR)

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In the gene therapy of diseases affecting the hematopoietic system, low frequency of hematopoietic stem cells (HSCs) and their quiescent nature are major obstacles to a successful therapeutic outcome. Transgenes that do not provide an intrinsic selective advantage require to be associated with a selection marker. A potential selection strategy is the use of modified growth factor receptors, conferring a ligand-dependent selective advantage to transduced cells. We showed that a truncated form of the erythropoietin receptor (tEpoR) is able to provide a competitive advantage to murine and human HSCs upon transplantation into suitable recipients. Cellular and molecular mechanisms of tEpoR activity in transduced cells are unknown. We show that cord blood-derived CD34+ cells expressing tEpoR are significantly protected from apoptosis induced by cytokine starvation. Affymetrix gene expression profiling identified a specific set of increased and decreased transcripts in cells expressing tEpoR, which include genes involved in the control of apoptosis. To test if the in vivo competitive engraftment is due to expansion
of the incoming stem cells pool, we performed a competitive repopulation experiment in transplanted mice. We observed that LV-mediated expression of tEpoR leads to competitive advantage of transduced HSCs in reconstituted irradiated recipients promoting a 10-fold in vivo expansion of transduced HSCs compared to untransduced cells. Experiments of transplantation using HSCs transiently expressing the tEpoR are ongoing. The hypothesis that tEpoR promotes engraftment of transduced repopulating cells, increasing their in vivo survival rather than inducing their proliferative expansion, could have important safety implications for gene therapy.

**Or 40**

**Development and application of zinc-finger nucleases**

Dr T Cathomen†

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Zinc-finger nucleases (ZFNs) are a powerful tool to edit the human genome *ad libitum*. The technology has experienced a remarkable development in the last few years with regard to technology platforms to generate these designer nucleases. As a result, ZFNs have been successfully employed to knock out or correct disease-related genes in primary human cells, including T cells, hematopoietic precursor cells, and induced pluripotent stem cells. In my talk, I will summarize the technological innovations that have successfully catapulted ZFNs into the genome engineering arena and provide an overview of parameters that determine ZFN activity and ZFN-associated toxicity, both key qualities in any therapeutic application involving designer nucleases. As a final point, I will present preliminary data that introduce TAL-based nucleases as a valuable alternative to ZFNs.

**Or 41**

**Targeted integrations in stem cells**

Luigi Naldini

See supplement

**Or 42**

**Mechanisms of retroviral silencing**

Didier Trono

Epigenetic processes play a central role in the regulation of gene expression, whether from endogenous loci or from exogenous vectors. KRAB-containing zinc finger proteins (KRAB-ZFPs) are tetrapod-restricted, sequence-specific DNA-binding transcriptional repressors that act by triggering the formation of heterochromatin via their cofactor KAP1. While the functions of these regulators long remained unknown, recent evidence indicate that KRAB/KAPI-mediated transcriptional control is a master regulator of mammalian homeostasis, partaking in events as diverse as stem cell pluripotency, genomic imprinting, hematopoietic differentiation and control of behavioral stress. We discovered that KAPI and KRAB-ZFPs mediate the early embryonic silencing of endogenous retroelements, and our newest data indicate that this system is also at play in modulating the expression of exogenous retroviruses such as HIV. I will discuss these results and other aspects of KRAB/KAPI-mediated control that seem particularly relevant for gene therapy.

**Or 43**

**Genome-Wide Determination of Double-Strand Breaks reveals High Specificity of Zinc Finger Nucleases**

Mr R Gabriel1, A Lombardo2, A Arens1, JC Miller3, P Genovese2, C Kaeppel1, A Nowrouzi3, J Wang2, G Friedman1, MC Holmes3, PD Gregory3, H Glimm1, M Schmidt1, L Naldini2, C von Kalle1

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Zinc finger nucleases (ZFN) facilitate precise and efficient gene editing in living cells by targeting DNA double strand breaks (DSB) to predetermined genomic loci. However, rapid repair of DSBs has prevented detection of ZFN activity at off-target loci. To date ZFN off-target analysis has had to rely on *in vitro* binding assays and bioinformatic predictions and therefore remains poorly understood. Here we show that integrase-defective lentiviral vectors (IDLV) are captured and ligated into genomic DSBs by non-homologous end-joining (NHEJ), thereby stably marking these otherwise undetectable transient events. Mapping integration sites (IS) of IDLV by LAM-PCR followed by high-throughput sequencing enables the determination of target specificity of a given ZFN. Analysis of >1500 unique IS revealed that the intended ZFN target site was by far the most frequently targeted genomic locus, demonstrated by IDLV clustering around the anticipated site of ZFN cleavage. LAM-PCR further revealed clustered IDLV IS at a small number of distinct off-target loci. These off-target hotspots were validated by a mismatch-selective endonuclease assay allowing detection of NHEJ repair at preselected genomic sites after ZFN treatment. Deep-sequencing further validated these results revealing that off-target activity was restricted to genomic loci harboring high homology to the intended ZFN target that permit binding of two ZFNs in specific spatial arrangements on DNA. High-throughput IS mapping established a consensus binding site for actual ZFN cleavage *in vivo*. This analysis confirms the high specificity of ZFN and represents an important tool in the further development of nucleases for use in a clinical setting.

**Or 44**

**T cell engineering for the treatment of infectious diseases**

Professor C June1

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We are exploring the use of engineered T cells bearing enhanced receptors and strategies to augment their resistance to HIV infection in patients with chronic HIV-1 infection. HIV therapy with antiretroviral drugs is effective to control viremia, however it has not shown promise to eradicate the viral reservoir, leading to the necessity for lifelong therapy. The unique observation that allogeneic stem cell transplantation with a CCR5 deficient stem cell preparation led to eradication replication competent HIV raises the question as to whether autologous T cells or stem cells rendered CCR5 deficient could lead to a similar reduction in the HIV reservoir. We are testing the feasibility of zinc finger nucleases to create CCR5 deficient CD4 T cells in an ongoing clinical trial. As a separate approach, we are reprogramming CD8 T cells using lentiviral vectors to express affinity enhanced alpha and beta chains of TCRs derived from potent HIV specific CTLs, with the long term goal of generating an immune system that has potent adaptive immunity and innate resistance to HIV infection.

References

Or 45

Zinc finger nucleases drive efficient gene disruption and site-specific gene addition in human HSC

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CCR5 is the major cellular co-receptor for HIV-1 entry and an important target for anti-HIV approaches. CCR5 targeted zinc finger nucleases (ZFN) have been used previously in transformed and primary human CD4 T cells to disrupt the CCR5 locus and provide long-term protection against R5-tropic HIV. Incubation of CD34+ HSC with the CCR5 ZFNs results in the modification of up to 34% CCR5 loci (mean 21%). Evaluation of these cells by transplantation into NSG mice and subsequent challenge with HIV-1 revealed that mice receiving ZFN-treated HSC displayed strong selection for CCR5-disrupted human cells in multiple organs and the elimination of detectable HIV-1 from the peripheral blood. This data supports the use of the CCR5 ZFNs for the modification of autologous HSC harvested from HIV-infected patients. The high degree of sequence specificity achieved with ZFNs can also be exploited to achieve site specific gene addition through homology-directed repair. Potentially, such a locus could provide a well-characterized ‘safe harbor’ for gene addition, allowing long-term and stable ectopic expression of therapeutic genes with reduced potential for insertional mutagenesis when compared to randomly integrating vectors. The high levels of ZFN activity we achieved in human HSC prompted us to evaluate gene addition at the CCR5 locus. A plasmid donor template containing a GFP reporter flanked by CCR5 sequences was co-delivered to CD34+ cells with CCR5-specific ZFNs, and resulted in sustained GFP expression. These data suggest that the co-delivery of ZFNs and donor template is sufficient to enable site-specific gene addition in CD34+ HSCs.

Or 46

LEDGF/p75 as antiviral target for HIV gene therapy

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Background: The treatment of HIV-infected patients with a combination of antiretroviral drugs allows long-term suppression of HIV, but no cure. Hence, development of novel strategies such as gene therapy is imperative. During the last decade, the insight has grown that HIV engages cellular proteins (co-factors) to complete its replication, which are attractive targets for antiviral therapy. We originally identified Lens Epithelium-Derived Growth Factor (LEDGF/p75) and validated it as an important co-factor of HIV integration. We now aim at developing a T-cell directed gene therapy for HIV, targeting the LEDGF/p75-HIV integrase interaction. A strategy that targets an essential co-factor instead of a viral protein may be less prone to viral resistance development.

Methods: LEDGF/p75 contains an N-terminal chromatin binding domain and interacts with IN via the C-terminal integrase-binding domain (IBD). Primary human CD4+ T-cells were stably transduced with lentiviral vectors driving IBD overexpression (LEDGFp75), LEDGF/p75 knockdown or a combination of both strategies. The effect on HIV replication was measured in a breakthrough experiment using p24 ELISA. Selective advantage of transgenic PM1-cells over the endogenous population after HIV challenge was evidenced by flow cytometry.

Results/Conclusion: LEDGFp75 overexpression by itself or in combination with knockdown inhibited HIV replication 20- to 40-fold, thus protecting primary T-cells from HIV infection. Furthermore, a 2- to 4-fold enrichment of transgenic cells was observed in PM1-cells upon HIV challenge, corroborating the selective advantage. We are currently validating this concept in vivo in a humanized mouse model by transplanting transgenic T-cells in Rag2-/-γc-/- mice and challenging them with HIV.

Or 47

Lentiviral mediated delivery of HIV by TRIM-Cyclophilin fusion proteins

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Background: Antiretroviral therapy has proven highly effective against HIV. However, problems with availability, cost and resistance are common-place and alternative approaches to confer resistance against the virus are under development. Natural evolutionary phenomena have rendered certain non-human primates resistant to infection with HIV-1. For example, LINE-1 retrotransposition of Cyclophilin A (CypA) DNA into the TRIM5 gene in New World Owl monkeys gave rise to an in-frame fusion between exons 1-7 of TRIM5 and replacement of the B30.2 domains by CypA. The resulting fusion protein potently restricts HIV-1 infection. We have investigated whether expression of similar proteins in human T cells could allow protection against HIV infection.

Methods: Human TRIM5 and TRIM21-Cyclophilin variants were generated with different inter-gene linker lengths, and their expression linked to enhanced green fluorescent protein (eGFP). The factors were delivered to cell lines and primary cells using a HIV-1 derived self-inactivating vector. Transduced cells were challenged using HIV-1 virus modified to express yellow fluorescent protein (YFP).

Results: Expression of YFP was restricted up to 100 fold compared to non-transduced controls or cells expressing eGFP alone. In the presence of cyclosporin A, this effect could be competitively inhibited. In experiments using replication competent wild type HIV-1, viral replication was quantified by p24 ELISA and found in TRIM-Cyp modified cultures to be competitively inhibited. In experiments using replication incompetent HIV-1, viral replication was quantified by p24 ELISA and found in TRIM-Cyp modified cultures to be reduced to less than 1% of control wells.

Conclusions: Human mimics of simian TRIM-Cyp fusion proteins, delivered by lentiviral vectors potently restrict HIV-1. Expression in human haematopoietic stem cells and/or T cells could allow reconstitution of immunity in patients infected with HIV.

Or 49

Editing the genome of stem cells with Zinc Finger Nuclease

Philip Gregory

Sangamo BioSciences Inc

The ability to engineer precise genetic modifications into human stem cells would both accelerate research and extend the range of potential therapeutic applications. This possibility is now being realized via the use of zinc finger nucleases (ZFNs). ZFNs are customizable, sequence-specific endonucleases that can be designed to introduce a discrete cleavage event at any user-chosen location within the stem cell genome. By adjusting conditions under which the cleavage event is subsequently repaired, one may efficiently and precisely disrupt or edit the targeted locus, or integrate a larger, gene-sized DNA fragment. This technology – which is portable to any eukaryote – has been used for diverse applications, including therapeutic gene modification in primary cells, trait stacking of producer cell lines for improved manufacture of biologics, and gene targeting in previously refractory species such as nematodes, rabbits and rats. The talk will focus on recent applications of this technology in human stem cells. Examples include gene tagging in embryonic stem cells, gene targeting in induced pluripotent stem cells, and gene addition at safe harbors in a variety of stem cell types. Preclinical proof-of-concept studies towards the development of autologous, CCR5-disrupted CD34 stem cells as a treatment for HIV will also be presented.

Or 50

Combining Targeted Integration and Cassette Design for Robust and Benign Transgene Expression without Impacting Endogenous Gene Transcription

Mr A Lombardo1, Mrs D Cesana1, Mr P Genovese3, Mrs E Provasti2, Mrs M Neri1, Mr B Di Stefano5, Mrs Z Magnani4, Mr O Pello5, Mr MC Holmes6, Mr PD Gregory5, Mr V Broccoli3, Mrs A Grittì5, Mrs C Bonini1, Professor L Naldini1

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Site-specific integration may overcome major hurdles of current gene transfer approaches, namely insertional mutagenesis and unpredictable transgene expression. However, little is known about the impact of the insertion on the targeted locus and vice versa. Here we address this question by targeting a panel of transgene expression cassettes into the CCR5 gene or AAVS1 locus, and evaluate: i) permissiveness for transgene expression and ii) transcriptional perturbation of the locus. We found efficient zinc finger nucleases-mediated insertion in both sites across a panel of human primary cell types, including T lymphocytes, NSC and iPSC cells. Transgene expression was always higher from the AAVS1 locus. Next, we measured the expression level of 26 genes present in the 400kb region centered either on the gene-modified CCR5 or AAVS1 locus, and evaluate: i) permissiveness for transgene expression and ii) transcriptional perturbation of the locus. We found efficient zinc finger nucleases-mediated insertion in both sites across a panel of human primary cell types, including T lymphocytes, NSC and iPSC cells. Transgene expression was always higher from the AAVS1 locus. Next, we measured the expression level of 26 genes present in the 400kb region centered either on the gene-modified CCR5 or AAVS1 locus, and found transcriptional up-regulation to be locus-dependent i.e. absent at AAVS1. Integration into AAVS1 targets intron 1 of the PPP1R12C gene with the expression cassettes added in the sense transcriptional orientation. Thus, insertion of new cis-acting elements could interfere with PPP1R12C expression. Indeed, analysis of cell clones containing mono- or bi-allelic insertion of the cassettes revealed
that PPP1R12C transcription was negatively affected by the presence of splicing acceptor (SA) sites present in some of the cassettes. Eliminating these SA sites or inserting the cassettes in opposite orientation allowed for robust transgene expression while maintaining normal PPP1R12C expression. Overall, comparison of these different sites and cassette designs uncover essential features of the locus and the cassette that allow for benign targeted insertion in human primary cells.

Or 51
Retargeting Sleeping Beauty transposon insertions by engineered zinc finger DNA binding domains recognizing human L1 repeats
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The Sleeping Beauty (SB) transposon is a nonviral, integrating vector system with proven efficacy in preclinical models. We mapped large numbers of SB insertions by a LAM-PCR protocol and Illumina sequencing. The data show a close-to-random insertion profile that could lead to genotoxic effects. We evaluated zinc finger (ZF) DNA binding domains to introduce a bias into SB’s target selection properties. We chose to target human LINE1 (L1) repeats, because i) they are relatively AT-rich, and therefore represent attractive targets for SB, and ii) they are present in the human genome in large numbers, which could increase the chances of targeted transposition events. One 6-finger ZF protein recognizing the 3’-region of L1, ZFB, showed specific binding to an 18-bp site represented by >12,000 copies in the human genome. We generated >6000 SB insertions by the SB transposase and a ZFB/SB fusion. As random reference, a computer-generated dataset was used. An enrichment in transposon insertions in L1 elements was seen in the ZFB/SB dataset, which peaked at a 4-fold enrichment of transposon insertions in a 400-bp window around ZFB binding sites. Statistically relevant differences in transposon insertions in genes (39.4 % for ZFB/SB versus 42.1 % for SB) was also found. Thus, the data suggest that L1 repeats act as a sponge that retarget a fraction of SB insertions away from genes. Further improvements in ZF technology and a careful choice of targeted genomic regions may improve the safety profile of SB for future clinical applications.

Or 52
Meganucleases for gene therapy
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There is a growing interest in the use of targeted approaches that allow the precise repair of a deleterious mutation at its endogenous locus as well as the insertion of a functional coding sequence at a chosen locus. Such targeted approaches depend on specific tools, including sequence specific endonucleases that can induce high frequencies of homologous gene targeting in the vicinity of their cleavage site. Meganucleases, the most specific natural endonucleases, represent ideal tools for genome targeting. Natural as well as engineered meganucleases can be used to induce up to 20% of gene insertion into chosen human genes. Eventually, the use of these proteins for therapeutic applications will depend on their intrinsic properties (activity/specificity) as well as on the use or identification of appropriate vectorization methods. To assess the properties and potential of these proteins, we have evaluated their activity and toxicity in several assays. Meganucleases presenting a good activity/toxicity ratio could be used to induce targeted recombination or cleavage in different cell types, including stem cells. We have also developed an approach to select for potential safe harbors that could be used for targeted integration. The combination of an efficient and very specific meganuclease with an appropriate insertion site should provide a platform for addressing several different diseases affecting the same cell lineage.

Or 53
A transient cell cycle arrest enhances AAV and ZFN-mediated gene targeting
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Vectors based on adeno-associated virus (AAV) are efficient tools to modify genomes by gene targeting. Gene targeting is based on the homology-directed repair (HDR) pathway, which can be stimulated several 1000-fold by creating a DNA double strand break (DSB) in the target locus. Here, we aimed to assess the effect of the cell cycle on AAV and DSB-mediated HDR. To this end, we established three human cell lines that carry a mutated eGFP gene flanked by recognition sites for the meganuclease I-SceI and zinc-finger nucleases (ZFNs). After treatment with different cytostatic drugs, cells were transduced with AAV vectors that encode the nuclease and a repair donor with the purpose of rescuing eGFP expression by HDR. The cell cycle profile, the extent of cytotoxicity and the frequency of HDR were assessed by flow cytometry. We show that a transient cell cycle arrest in the G2 phase increased AAV-mediated HDR up to 6-fold and we achieved targeted gene editing in up to 12% of transduced cells. A significant increase in HDR was also observed upon plasmid-based eGFP rescue, implying that drug-induced cell cycle arrest is a general means to augment DSB-mediated HDR. Noteworthy, the transient cell cycle arrest had only minor effects on the ratio of AAV vectors used for HDR vs. illegitimate vector integration.
In conclusion, the combined effects of site-specific DSBs with a transient cell cycle arrest allowed us to lower the vector dose - and hence illegitimate integration of the vectors - without compromising on the HDR frequency.

**Or 54**

**Delivery issues for oncolytic viruses**

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While there are several examples where gene therapy approaches are looking promising, they all reflect situations where the gene vectors can be introduced efficiently into target cells - either by direct injection or by transduction ex vivo. Indeed, cancer gene therapy can be very effective if tumour-killing viruses are injected directly into tumours.

Unfortunately three quarters of people who develop cancer in the West go on to die from metastatic disease. In this situation it is not possible to inject gene therapy vectors into all the tumour nodules, and intravenous 'systemic' therapy is required. However the human bloodstream represents a very aggressive environment for most gene therapy vectors. Therapeutic microbes delivered via the bloodstream encounter many host defences and anatomical barriers that must be surmounted to enable their access to disseminated cancers. This is particularly true for adenovirus type 5 in humans, where most recipients have powerful pre-existing adenovirus-neutralising activity. We have recently shown that human (but not murine) erythrocytes provide an additional barrier by sequestering adenovirus onto the Coxackie and Adenovirus Receptor and (via antibodies) complement receptor 1. Coating adenovirus with a layer of hydrophilic polymer can prevent this interaction and allow virus to circulate free in the plasma, showing passive targeting to disseminated tumours and mediating good anticancer efficacy. Entry of polymer-coated virus particles into the tumour mass is a product of fluid transfer, and is directly proportional to the area under the plasma concentration-time curve. Increasing extravasation of fluid through tumour-associated endothelium using permeability-enhancers such as Tumour Necrosis Factor alpha can improve virus particle entry into tumours over 100-fold, reaching as high as 10% injected dose (virus particles) per tumour. This provides the possibility for highly efficient targeting to tumours and good anticancer efficacy.

An alternative approach is to target agents to infect tumour-associated vasculature. However while this provides a vulnerable target to traditional gene therapy approaches, endothelial cells do not normally support 'oncolytic' viruses. One approach to overcoming this problem is to encode syncytium-forming proteins within the endothelial, to enable trans-complementation of virus replication by tumour-associated factors.

**Or 55**

**Replicating Retrovirus Encoding a Prodrug Activator (Toca 511) Enters Clinic for the Treatment of Glioblastoma Multiforme (GBM)**

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A central problem for gene-mediated cancer therapies has been delivery of therapeutic genes to the tumor and not elsewhere. To address this issue, we are developing a replicating gammaretrovirus (amphotropic MLV) that delivers a therapeutic gene throughout a cancer mass by preferentially infecting and spreading through dividing cancer cells, without causing cell lysis. Toca 511 encodes an improved cytosine deaminase (CD), and is administered with the prodrug, 5-fluorocytosine (5-FC) that is converted locally to the anticancer drug 5-FU. In previous publications an earlier generation replicating vector preferentially infected intracranial human xenografts in nude mice (Tai et al. 2005 Mol Ther 12:842) and led to increased survival in treatment groups compared to controls. We improved the vector and CD gene, and manufactured and characterized concentrated purified vector lots. In two immune-competent mouse brain cancer rising-dose models (BALB/c-CT26 and B6C3F1-Tu2449), a single vector administration by intracranial injection, followed by systemic 5-F, improved median survival up to at least 6 months (the duration of the experiment) while controls died within approximately 30 days. Toca 511 infected the brain tumor tissue efficiently after intracranial injection, expressed the CD gene and 5-F was rapidly metabolized to 5-FU in vivo. Biodistribution studies in mice and dogs showed that there is almost no detectable spread or survival of viral vector over 6 months, outside of tumor, except in permissive BALB/c mice that lack the APOBEC3 MLV restriction factor. A phase 1/II dose escalation study is now underway in patients with recurrent GBM.
binding domains of receptor-blind MV hemagglutinin (H); these H-proteins allowed targeting of lentiviral vectors.

Insertion of a gene encoding a DARPin-H attachment protein into the MV genome allowed the generation of recombinant replicating MVs carrying one out of 6 different HER2/neu specific DARpins as targeting domain, respectively. All DARpin-targeted MV variants revealed HER2/neu-specific targeting, genomic stability, efficient viral replication and oncolytic activity in receptor-positive target cells in vitro. We found evidence that the target cell killing efficiency of the targeted viruses was dependent on the receptor density in target cells and the affinity of the DARpins to the target receptors. In receptor-high target cells, DARPin-targeted MVs displayed the cytotoxicity of parental, non-targeted oncolytic MV, killing more than 90% of target cells in three days. In vitro experiments analysing the oncolytic efficacy in appropriate Xenograft animal models are under way, as well as the side-by-side comparison of the DARPin-MV with published scFv-MV targeted against HER2/neu.

In conclusion, DARpins were found to be effective in targeting oncolytic MV and DARPin-targeted OVs will therefore complement existing targeting approaches.

**Or 57**

**MicroRNA Regulation of Adenovirus Afford Control of Oncolytic Activity Without Changing Endogenous MicroRNA Levels**

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MicroRNA regulation of therapeutic viruses is an emerging field which allows the selective destruction of essential viral RNA molecules in sites of potential toxicity. We have previously demonstrated that the hepatic toxicity caused by intravenous injection of wild type adenovirus type 5 (Ad5WT) in mice can be prevented by incorporating 4 binding sites for the liver specific microRNA, mir122, into the 3’ UTR of E1A mRNA. This virus, termed Ad5mir122, is safe at doses 10-fold above the LD50 of Ad5WT. Here we show that Ad5mir122 maintains wild type oncolytic potency and address the potential toxic effects of microRNA regulation on the host cell.

Complete microarray profiling of mRNA from Ad5WT-infected livers showed the levels of >3900 mRNAs were changed >2-fold, however, less than 600 were altered by infection with Ad5mir122. Comparison of these mRNAs with predicted mir122 targets showed no overlap, suggesting they might be affected by capsid proteins or residual viral gene expression. RT-QPCR for E1A 13S transcripts and western blot for E1A showed that both mRNA and protein were significantly knocked-down when compared to Ad5WT. RT QPCR for mir122 in infected livers showed that the quantity of mir122 was unchanged by Ad5mir122. In order to assess the anti-cancer activity of Ad5mir122, CD1 nude mice bearing HepG2 tumours were administered 2x10³⁰⁷ viral particles (X4, intravenous) and showed significantly reduced tumour growth and extended survival without toxicity. These results demonstrate that microRNA regulation can control viral replication without affecting endogenous mRNA targets, or the level of the microRNA.

**Or 58**

**Personalized oncolytic adenovirus disorder for treatment of chemotherapy refractory solid tumors**

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Given the ease of construction of oncolytic adenoviruses with several modifications, the approach lends itself well to individually personalized medicine. This has been recognized also by legislators and patient-by-patient treatments are regulated by the EU Advanced Therapies directive, which has allowed us to treat more than 200 patients in an Advanced Therapy Access Program. Following extensive preclinical testing, 9 different viruses have been used. The optimal virus capsid, tumor specific promoter and arming device are selected based on preclinical and clinical data, taking into account the nature of the clinical problem in each patient (local vs systemic), while sero-switching has been utilized to enhance systemic delivery. Three schedules of low-dose cyclophosphamide have been used to reduce regulatory T-cells and induce TH2->TH1 switch. Injections have been performed in ultrasound, visual or CT guidance, intratumorally, intracavitary and/or intravenously on an individual basis. Pretreatment tumor samples have been studied for selecting the optimal virus and for prediction of efficacy. Overall, treatments have been remarkably safe with no mortality. Serious adverse events are seen in circa 5% of treatments, while mild to moderate fever, flu-like symptoms, tumor pain and fatigue are common. Evidence of possible efficacy (radiological stable disease or better) has been seen in 48% of patients overall and up to 78% with the best virus and optimized schedule. With the best schedule, more than half survive for a year or longer which is unusual in this difficult patient population and compares well to historical controls. A clinical trial is in progress.

**Or 59**

**Gene therapy for muscular dystrophy: current progress and future prospects**

George Dickson

**Or 60**

**Shifting heart to skeletal muscle: miR669 functions as a cell fate switch between cardiac and skeletal muscle lineages**
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Resident progenitor cells in the heart are able to differentiate in one or more cardiac cell types. While surface marker expression and ability to generate cardiomyocytes have been characterized for several types of these progenitors1–3, little is known about how cardiac differentiation is regulated. Recent works have highlighted an important role for miRNAs in regulating skeletal and cardiac myogenesis4,5. In chronic cardiac diseases resident progenitors appear unable to counteract progressive degeneration, possibly because they may come exhausted in repeated attempts to regenerate the failing heart. Beta sarcoglycan (Sgcb) null mice are a model for limb girdle muscular dystrophy type 2E and undergo progressive diastolic cardiomyopathy. In this study we isolated and characterized cardiac progenitors from Sgcb null hearts and unexpectedly observed their aberrant skeletal muscle phenotype both in vitro and in vivo. This is due to the absence of two key regulatory microRNAs, miR669a and miR669p, capable of suppressing skeletal myogenesis by directly targeting the MyoD 3’UTR. According to our findings, miR669p, capable of suppressing skeletal myogenesis by directly targeting the MyoD 3’UTR, is absent in Sgcb-null progenitors due to the homologous recombination of one or more cardiac cell types. While surface marker expression and ability to generate cardiomyocytes have been characterized for several types of these progenitors1–3, little is known about how cardiac differentiation is regulated. Recent works have highlighted an important role for miRNAs in regulating skeletal and cardiac myogenesis4,5. In chronic cardiac diseases resident progenitors appear unable to counteract progressive degeneration, possibly because they may come exhausted in repeated attempts to regenerate the failing heart. Beta sarcoglycan (Sgcb) null mice are a model for limb girdle muscular dystrophy type 2E and undergo progressive diastolic cardiomyopathy. In this study we isolated and characterized cardiac progenitors from Sgcb null hearts and unexpectedly observed their aberrant skeletal muscle phenotype both in vitro and in vivo. This is due to the absence of two key regulatory microRNAs, miR669a and miR669p, capable of suppressing skeletal myogenesis by directly targeting the MyoD 3’UTR. According to our findings, the absence of Sgcb gene abolished miR669p and reduced miR669a expression. miR669p, embedded in Sgcb gene, is absent in Sgcb-null progenitors due to the homologous recombination with neomycine cassette. miR669a, encoded by Sfmbt2 gene, is strongly downregulated due to a signal cascade involving intracellular calcium, calpain proteases and degradation of YY1, positive regulator for Sfmbt.

This finding opens a new scenario on the fate choice of mesoderm progenitors and makes the task of utilizing endogenous cardiac stem cells more complex in certain pathologies.

4) van Rooij E. et al. Science (2007)
5) van Rooij E. et al. Dev Cell (2009)

Or 61
Prevention of cardiac function in mdx mice after AAV9-mediated microdystrophin gene transfer
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Dystrophin plays an important role in muscle contraction linking the intracellular cytoskeleton to the extracellular matrix. Mutations within the dystrophin encoding sequence may lead to a complete loss of the protein causing Duchenne muscular dystrophy (DMD), frequently associated with severe cardiomyopathy. While clinical studies for gene therapy of skeletal myopathy are performed, therapies for cardiac muscles have barely been evolved.

Aim of our study was to establish an efficient long term treatment for DMD-associated cardiomyopathy in a mouse model for dystrophin-deficiency (mdx). Mdx mice were treated with 1×10^12 viral genomes of AAV9, containing a microdystrophin-cDNA (μDys), transcriptionally controlled by either CMV or CMV enhanced myosin light chain (CMV-MLC) promoter. Mice were challenged by voluntary wheel exercise over 8 month while left ventricular fraction of shortening, a cardiac performance indicator, has been monitored. Finally, heart, quadriceps femoris muscle (MQF) and liver were dissected and analysed for expression of μDys. We found (1) an increased cardiac μDys expression with the CMV-MLC- in comparison to CMV-promoter, (2) a preserved fraction of shortening with the CMV-MLC- and CMV-promoter, indicating, that even a minor μDys expression leads to a physiological benefit, and (3) absence of μDys expression in MQF and liver. These findings demonstrate a high specificity and efficiency of the chosen delivery approach for cardiac muscle resulting in a sustained therapeutic effect.

In conclusion, we established an efficient and specific AAV9-mediated gene transfer of microdystrophin-cDNA in mdx hearts, which may become a tool to develop treatment strategies for DMD-associated cardiomyopathy.

Or 62
Rescue of dystrophic muscle by stem cell-mediated transfer of a HAC containing the dystrophin locus
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Human artificial chromosomes (HACs) have many advantages over conventional gene therapy vectors, such as episomal maintenance and the ability to carry large genetic regions with their own regulatory elements. There is currently no evidence of efficacious therapy using HACs in any genetic disorder. We show here the rescue of a mouse model of Duchenne Muscular Dystrophy (DMD) by a novel strategy, combining HAC-mediated gene replacement with mesangioblast (MABs: vessel-associated stem cells) transplantation. DMD is a devastating myopathy for which no therapy exists, despite many experimental and clinical trials. To this aim, we corrected MABs from dystrophic mdx mice (mdxMABs) with a HAC vector containing the large (2.4Mb) human dystrophin gene (DYS-HAC). DYS-HAC expressing mdxMABs robustly engrafted and produced many dystrophin positive fibers in dystrophic mice upon intra-muscular and intra-arterial transplantation, leading to significant morphological and functional amelioration of the dystrophic phenotype. These data provide the first functional evidence of HAC-mediated rescue in a pre-clinical model of a genetic disease and set the conditions for future clinical translation of this experimental strategy for Duchenne Muscular Dystrophy.

**Or 63**

**Effective limb transduction with phenotypic correction after injection of rAAV8-U7snRNA in GRMD dogs**

Dr P Moullier, Dr C Le Guiner, Dr M Montus, Dr L Servais, Dr L Garcia, Dr Y Fromes, Dr Y Cherei, Dr T Voit, The « AFM-sponsored Duchenne Consortium »

In the Duchenne Muscular Dystrophy (DMD) the selective removal by exon skipping of exons flanking an out of frame mutation in the dystrophin messenger can result in in-frame mRNA transcripts that are translated into shorter but functionally active dystrophin.

The goal of our project is to determine in GRMD, the effective dose of our therapeutic product defined as a recombinant Adeno-Associated Virus serotype 8 (rAAV8) expressing a modified U7 snRNA specific for the exon skipping of the dystrophin transcript. The mode of delivery is the locoregional high-pressure intravenous (IV) injection in a forelimb.

Three groups of GRMD dogs were exposed to 3 different rAAV8-U7snRNA doses. Each dog was followed ≥3 months after injection. The primary outcomes are the restoration of dystrophin expression and the improvement of the tissue pathology in the injected limb compared to the contralateral. The secondary outcomes are the muscle strength correction, the biodistribution and shedding patterns as well as the immune response against rAAV8 capsid and dystrophin.

We built a unique network of laboratories with complementary skills to deliver a GLP-compliant set of preclinical data to further define the regulatory toxicology studies. The organization of our network and the results obtained in our GRMD dogs study will be presented.

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

**Or 64**

**Lethal Graft-versus-Host Disease in mouse models of T Cell Receptor Gene Therapy**

Dr G Bendle, Mr C Linnemann, Ms A Hooijkaas, Ms L Bies, Dr M de Witte, Dr A Jorritsma, Dr A Kaiser, Dr N Pourn, Professor R Debetz, Dr E Kieback, Professor W Uckert, Dr J Song, Professor J Haanen, Professor T Schumacher

The antigen-specificity of a T cell is solely determined by the T cell receptor (TCR) alpha- and beta-chains. Therefore, the transfer of TCR genes into patient T cells can be used to induce immune reactivity towards defined antigens to which the endogenous T cell repertoire is insufficiently reactive. This approach, which is called TCR gene therapy, is currently being developed to target tumors and pathogens and the clinical testing of this approach has commenced in cancer patients.

However, we have observed the occurrence of lethal cytokine-driven autoimmune pathology in mouse models of TCR gene therapy under conditions that closely mimic the (intended) clinical setting. This therapy-induced autoimmune process results in a fatal destruction of the hematopoietic compartment accomplished by more general aspects of Graft-versus-Host Disease. Autoimmune pathology is seen with a panel of different murine TCRs and when various strategies are used to promote in vivo T cell function. We have defined the molecular mechanism that underlies the development of this phenomenon, showing that the pairing of introduced and endogenous TCR chains in TCR gene modified T cells leads to the formation of self-reactive TCRs that are responsible for autoimmune pathology.

Our data show the development of strategies to limit the formation of self-reactive TCRs in TCR gene modified T cells will be essential for the safe clinical implementation of TCR gene therapy. To this end we demonstrate that adjustment in the design of gene therapy vectors and target T cell populations can be utilized to ameliorate the risk of TCR gene therapy-induced autoimmune pathology.
**ESGCT 2010 ORAL PRESENTATIONS**

**Or 65**

T-cell engineering for cancer applications
Dr M Pule

Through the application of gene-therapy methods, it is now possible to generate large numbers of engineered T-cells with novel specificities and properties for cancer therapy. I will discuss three practical advances to this approach: (1) Administration of T-cells expressing antibody-derived Chimeric Antigen Receptors (CARs) against disialoganglioside has shown efficacy in refractory neuroblastoma. We have optimized our original retroviral cassette to build on this clinical success: We have refined the CAR by identifying the optimal format for surface expression and antigen recognition, replacing murine antigen-recognizing anti body single chains with a humanized version and introducing compound signalling endodomain. In addition, we have optimized co-expression of the iCasp9 suicide genes with this CAR, allowing deletion of transduced T-cells in the face of unwanted toxicity. This cassette is ready for clinical testing (2). A compact marker/suicide gene which relies on off-the-shelf reagents/pharmaceuticals would be of considerable practical utility. We have generated a highly compact (136 amino acid) marker/suicide gene construct by identifying and joining the minimal epitopes required for anti-CD34 mAb QBEND10 and Rituximab binding on a CD8 stalk. This allows facile detection of transduction efficiency, clinical grade sorting with Miltenyi CliniMACS beads. Exposure of transduced T-cells to rituximab and complement results in >95% killing (3) Finally, we have engineered several calcineurin mutants which engender tacrolimus and/or cyclosporine resistance in T-cells. We anticipate these will allow cellular immunotherapy of EBV driven lymphoma in the face of ongoing immunosuppression.

**Or 66**

Adaptive immunotherapy with TCR-transferred lymphocytes
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T cell receptor gene transfer has been used successfully to redirect the specificity of human and murine T cells. The first clinical studies have been completed and shown feasibility of this approach and persistence of TCRexpressing cells in patients. We have explored the possibility of using TCR gene transfer to generate antigen-specific regulatory T cells. Using a murine model system we found that antigen-specific regulatory T cells can be generated by TCR gene transfer into purified Treg, or by co-transfer of TCR and FoxF3 genes into purified CD4+ T cells. Upon adoptive transfer, the gene-modified T cells were able to suppress arthritis in an antigen-specific fashion. This provides a rationale to use TCR gene transfer to control unwanted immune responses in vivo. Recently, we have started to explore if TCR gene transfer into haematopoietic stem cells will result in the generation of naive and central memory T cells with specificity for a tumour-associated self-antigen. We have transferred the genes of an HLA-A0201-restricted TCR with specificity for the tumour-associated Wilm’s Tumour 1 (WT1) antigen into haematopoietic stem cells of HLA-A0201-transgenic mice followed by stem cell transplantation into syngeneic recipients. The results indicate that this experimental system provides a powerful model to study the thymic development, peripheral phenotype, persistence and function of WT1-specific T cells in vivo.

**Or 67**

Immunotherapy for Prostate Cancer: Current Indications and Future Prospects
Eric J Small, MD

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Sipuleucel-T (Provenge) is an autologous active cellular immunotherapy product for the treatment of metastatic castrate resistant prostate cancer (CRPC). In the pivotal study, in men with asymptomatic or minimally symptomatic metastatic CRPC, treatment with Provenge improved overall survival compared with control, 25.8 months compared with 21.7 months (P = .032; HR = 0.775). The adverse events reported more frequently with Provenge treatment were chills, fever, headache, influenza-like illness, myalgia, hyperhidrosis, and groin pain. An integrated analysis of 3 randomized trials included 737 patients (488 sipuleucel-T: 249 placebo) revealed a significant sipuleucel-T treatment effect (HR = 0.735, 95% CI:0.613, 0.882, P < 0.001), and the treatment effect was found to be homogeneous across the 3 trials. A positive treatment effect was observed in subgroups, including those defined by age, race, ECOG performance status, number of bone metastases, and previous chemotherapy use. The mechanism of action of Sipuleucel-T is being studied in a pre-prostatectomy study.

CTLA-4 is an inhibitory molecule expressed on activated T cells. CTLA-4 blockade with ipilimumab augments T cell responses and anti-tumor immunity in animal models. Combination therapy with GM-CSF has demonstrated anti-cancer activity and a dose-response relationship between ipilimumab dose and effector T cell activation was observed. Future directions include a randomized phase 2 trial of Ipilimumab with and without GM-CSF, and a combination trial of Ipilimumab and Sipuleucel-T. An ongoing trial of radiation to a metastatic lesion with and without Ipilimumab is underway.

**Or 68**

Editing Human Lymphocyte Specificity for Safe and Effective Adaptive Immunotherapy of Leukemia
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T cell receptor (TCR) gene-transfer is an attractive strategy for the adoptive immunotherapy of tumors. However, the full potential of this approach is limited by the co-expression in one lymphocyte of endogenous and exogenous chains, resulting in reduced expression of the introduced tumor-specific TCR, and in acquisition of two novel and unpredicted specificities due to TCR mispairing. To address these issues, we designed and transferred Zinc Finger Nucleases (ZFNs) targeting the constant region of the TCR alpha and beta chain genes by viral vectors into T lymphocytes, thus promoting the genetic disruption of the endogenous TCR. Lymphocytes targeted by each ZFNs set failed in expressing the CD3/TCR complex on cell surface. Once sorted, CD3+ cells proved stable and permissive to lentiviral transduction. For a complete editing of T cell specificity, we selected a TCR specific for the WT1 tumor antigen and set up a protocol of sequential disruption of the TCR chains followed by lentiviral transfer of the WT1-specific TCR chains. At the end of the procedure we obtained a population of TCR-edited lymphocytes, carrying only the tumor-specific TCR that, in the absence of competition, was expressed at high and physiological levels. Accordingly, TCR-edited lymphocytes were superior to conventional TCR-transferred cells in promoting specific recognition of WT1+ targets, including primary leukemias, and, most importantly, were devoid of residual reactivity including alloreactivity. These data demonstrate that genetic re-programming of T cell specificity in primary lymphocytes is feasible and functional and might improve the therapeutic outcome of cancer immunotherapy.

Regulatory T (Tr) cells are a specialized subset of T cells that control immune responses and promote and maintain immune-tolerance. We established protocols to generate homogeneous populations of Tr cells using lentiviral vector (LV)-mediated over-expression of the tolerogenic molecules, IL-10 or FOXP3, into CD4+ T cells. Results demonstrate that stable over-expression of either FOXP3 or IL-10 in human CD4+ T cells confers Tr phenotype and functions. These results open the possibility to generate antigen-specific Tr cells for cellular therapy to promote specific tolerance in patients with immune-mediated diseases.

An alternative approach to modulate the immune response consists in de-targeting transgene expression from hematopoietic cells by miR142-regulated LV. Prevention of immune-mediated clearance of gene-modified cells and induction of robust tolerance to the encoded-antigen, mediated by transgene-specific Tr cells, was observed using miR142-LV in vivo. Not only integrase-competent LV but also integrase-defective LV (IDLV), despite low transgene expression, can promote immunological tolerance to foreign antigens.

These findings have important implications for developing future gene therapy strategies based on Tr cell therapy and on microRNA-regulated vector approach to restore/promote tolerance toward specific antigens/transgenes and prevent undesired immune responses.

**Or 70**

**Gene transfer leads to B cell reconstitution in a murine model of Wiskott-Aldrich Syndrome**

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Wiskott-Aldrich Syndrome (WAS) is an X-linked primary immunodeficiency characterized by thrombocytopenia, eczema, autoimmunity and lymphomas. Transplantation of hematopoietic stem cells (HSC) from HLA-identical donors is curative, but not available to all patients. Therapy based on the transplant of genetically corrected autologous HSC could represent a valid alternative approach for patients lacking a suitable donor. We have developed a protocol of gene therapy (GT) for WAS using a lentiviral vector encoding for human WASp promoter/cDNA and demonstrated efficacy and safety of our gene transfer approach. Because of the importance of analyzing B cell reconstitution in an immunodeficiency with a strong autoimmune phenotype, we fully evaluated this aspect in a new series of experiments. We observed that WASp+ B cells were present in all tissues isolated from GT treated mice, with the highest levels detected among splenic marginal zone B cells. These data were confirmed by the restoration of B cell follicle and marginal zone architecture in spleen of GT treated mice. To test functionality of B cells in GT treated mice, we performed a challenge with pneumococcal antigens four months after GT by injecting i.p. P23

**Or 69**

**Strategies to overcome immunity against transgenes**

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Immune responses are a major hurdle to successful gene therapy. Existing approaches to overcome immune responses employ non-specific methods of immune suppression, including steroids, and compounds that block T-cell signaling, expansion, and function. Developing strategies for inducing antigen-specific tolerance can provide a way to overcome the limitations of the existing immune-suppressive agents.
We could demonstrate an improved antibody response to challenge with pneumococcal antigens in GT treated mice. In addition, we observed the reduction of serum autoantibodies against dsDNA, which are present in majority of Was−/− mice. These observations contribute to the implementation of our future clinical GT trial for WAS providing further evidence of the efficacy of our gene transfer approach.

**Or 71**
Genetically Engineered Cells Target CNS and Cure Experimental Autoimmune Encephalomyelitis

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Multiple Sclerosis (MS) is an autoimmune disease of the central nervous system (CNS). In the mouse model of MS (EAE) treatments with either T regulatory (Treg) cells or multipotent mesenchymal stromal cells (MSC) have proven beneficial. However, systemic administration of such cells may cause problems with infections. Further, few cells may reach the CNS to hamper the pathological immune reaction. We hypothesized that Tregs or MSCs can be targeted to the CNS using gene transfer of a CNS-targeting chimeric artificial receptor (CAR) to provide a local immunosuppression.

A scFv was cloned from the hybridoma (8.18C5) producing anti-human myelin oligodendrocyte glycoprotein (MOG) antibodies. The scFv was cloned into a CAR and transferred into a lentiviral vector. CD4+ T-cells were transduced with the CAR expressed in trans with murine FoxP3 which promotes Treg differentiation, while MSC were modified using the CAR only. Engineered Tregs and MSCs were analyzed for their suppressive ability. Further, engineered cells were stained with a fluorescent dye and injected into mice which were sacrificed and the cells were tracked in vivo. Finally, the engineered cells were used to treat EAE mice.

By inserting the genes for FoxP3 and CAR we developed Tregs that potently suppressed T-cells in vitro. Similar results were obtained with the CAR engineered MSCs. Further, both Tregs and MSCs were able to locate to the brain. The engineered suppressor cells hampered CNS inflammation and cured mice with EAE.

In conclusion, genetically engineered suppressor cells are able to localize to the CNS and cure EAE mice.

**Or 72**
Integrate-Defective Lentiviral Vectors Enable Tolerogenic Expression of Bioactive Molecules in the Liver With Minimal Genotoxic Risk

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Lentiviral vectors are attractive tools for liver-directed gene therapy by virtue of their ability for stable gene expression and lack of pre-existing immunity in most human subjects. However, the use of integrating vectors may raise some concerns regarding the potential risk of insertional mutagenesis. Here we investigated integrate-defective lentiviral vectors (IDLV) containing an inactivating mutation in the integrase (D64V) and show that hepatocyte-targeted IDLV support a prolonged window of transgene expression from the liver upon intravenous injection in mice. Whereas this expression attains lower levels as compared to that obtained with the integrate-competent vector counterparts, it can result in relevant biological effects such as sustained therapeutic coagulation factor IX expression levels in hemophilia B mice and induction of transgene-specific regulatory T cells and immunological tolerance to foreign antigens. Deep sequencing of transduced livers showed that the majority of IDLV genomes remain episomal. Rare genomic integrations show no preference for gene coding regions and occur mostly by a mechanism inconsistent with residual integrate activity. Thus, IDLV provide an attractive platform for tolerogenic expression of bioactive molecules with minimal genotoxic risk.

**Or 73**
Liver gene transfer by integrate-defective vectors induces antigen-specific regulatory T cells

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Integrate-Defective Lentiviral Vectors (IDLV) are emerging as an attractive gene delivery system. These vectors harness the pantropism and proficiency of lentiviral vector transduction without relying on integration and permanent modification of the cellular genome. The extent and duration of IDLV expression, however, can vary substantially with the target tissue and it is not known whether IDLV can achieve relevant transgene expression levels following hepatic gene delivery. Here we report a detailed characterization of IDLV performance in primary human hepatocytes.
demonstrate that Treg induction for the correction of monogenic diseases. Our new findings depend on high levels of vector integration and transgene expression within hepatocytes, which would limit application of this finding outside of gene replacement strategies for the correction of monogenic diseases. Our new findings demonstrate that Treg induction in vivo can now be obtained in a safer way opening up new therapeutic opportunities for immune modulation.

Relevance of an Academic GMP Pan-European Vector Infra-structure (PEVI)
Odile Cohen-Haguenauer
On behalf of the GeneCellPro consortium (see below). CimitGene and LBPA at ENSC, F-94235 Cachan Cedex and Hospital St-Louis F-75475 Paris

In the past 5 years, European investigators have played a major role in the development of clinical gene therapy. The provision of substantial funds by some individual member states to construct GMP facilities makes it an opportune time to network available gene therapy GMP facilities at an EU level. The integrated coordination of GMP production facilities and human skills for advanced gene and genetically-modified (GM) cell therapy, can dramatically enhance academic-led ‘First-in-man’ gene therapy trials. Once proof of efficacy is gathered, technology can be transferred to the private sector which will take over further development taking advantage of knowledge and know-how. Complex technical challenges require existing production facilities to adapt to emerging technologies in a coordinated manner. These include a mandatory requirement for the highest quality of production translating-gene-transfer technologies with pharmaceutical-grade GMP processes to the clinic. A consensus has emerged on the directions and priorities to adopt, applying to advanced technologies with improved efficacy and safety profiles, in particular AAV, lentivirus-based and oncolytic vectors. Translating cutting-edge research into ‘First-in-man’ trials require that pre-normative research is conducted which aims to develop standard assays, processes and candidate reference materials. This research will help harmonise practices and quality in the production of GMP vector lots and GM-cells. In gathering critical expertise in Europe and establish conditions for interoperability, the PEVI infrastructure will contribute to the demands of the advanced therapy medicinal products regulation and to both health and quality of life of EU citizens.

Large scale manufacturing of transduced CD34+ cells using lentiviral vectors for Metachromatic Leukodystrophy and Wiskott Aldrich Syndrome clinical trials
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MolMed Spa is a biotechnology company focused – inter alia on cellular and gene therapy products. It is formally authorised for the production and release of medicinal products for human use and it is acting as GMP manufacturing site for two innovative gene therapy clinical trials (Metachromatic Leukodystrophy, MLD and Wiskott Aldrich Syndrome, WAS) sponsored by Telethon. The large scale transduction process has been designed in collaboration with Tiget-HSR: CD34+ cells are transduced by 2 hits of ARSA or WASP GMP-grade
LV at MOI 100 respectively, in RetroNectin® coated bags in presence of cytokines. For GMP validation, three transduction runs for each type of vector were performed using CD34+ cells from healthy donor bone marrow. Validation results indicate that the GMP transduction process guarantees the quality of the product in terms of identity/potency, purity and safety. Recently, the GMP process has been used to prepare cells for the first two patients, one for each trial. CD34+ purification yield was 49% and 41% and 12 x 10⁶ and 9 x 10⁶ of transduced CD34+/kg were obtained for MLD and WAS patient, respectively. High transduction efficiencies, in the range of the data obtained in validation phase, were observed. Moreover, ARSA activity and WAS protein were present in transduced cells. Clinical lots were sterile, free from mycoplasma and RCL; purity analyses demonstrated the absence of host cell DNA sequences integrated in cell genome.

Or 76
Micro RNA knockdown significantly enhances adenovirus replication and vector production
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The use of micro RNAs (miRNAs) fundamentally improved the generation of viral vectors for gene therapy. In this study we used our recently established RNAi knockdown cell line B6 based on the RNAi suppressor protein P19 (Rauschhuber and Ehrhardt, submitted) to analyze the influence of the RNAi pathway on adenoviral vectors. We investigated replication of wildtype adenovirus 5 (wtAd5) and first generation adenovirus (FgAd) on a genome level. An up to 10-fold increase in viral genome copy numbers could demonstrate that there is significantly enhanced viral DNA replication under RNAi knockdown conditions. To analyze whether we can also achieve higher viral titers in the B6 cell line, we infected with a common FgAd expressing firefly luciferase (FgAdluc). After re-infection of HEK293 cells measured 2 days later. We show that within the first minute most of the viral vectors (88%) from the bone grafts were released.

A murine critical size fracture model was used for in vivo studies. Allografts coated with AAV-luciferase were implanted and bioluminescence imaging was used to assess luciferase activity. New bone formation was stimulated by VEGF and bFGF2 and evaluated after 10 weeks using micro-CT and histomorphometry.

In conclusion allografts with freeze-dried AAV vectors were placed onto confluent layers of HEK 293 cells. Subsequently the grafts were moved to a new cell layer every minute, and GFP positive cells measured 2 days later. We show that the grafts were moved to a new cell layer every minute, and GFP positive cells measured 2 days later. We show that within the first minute most of the viral vectors (88%) from the bone grafts were released.

Bone allografts are widely used clinically although they often heal insufficiently leading to risk of fracture. These problems may be alleviated using allografts coated with freeze-dried AAV vectors carrying genes for osteogenic stimuli. We have evaluated the kinetics of the gene delivery from bone allografts and potential effects of stimulation of new bone formation.

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Bioluminescence imaging showed localized gene expression reaching a plateau from 51 to 70 days. Interestingly VEGF coated allografts lead to an approx. 5 fold increase in total bone volume compared to bFGF2, a combination of VEGF and bFGF2 and controls, although with large variation within each group.

In conclusion allografts with freeze-dried AAV vectors on the surface is a promising new tool for enhancement of the formation of new bone. The positive effect of VEGF on bone formation points to new blood vessels as a critical step in the healing process.

Or 78
Insect cell-based AAV manufacturing—robust technology for clinical and commercial supply
Mrs M Koefoed¹, Miss A.G Pedersen¹, Professor K Soballe², Professor TG Jensen¹, Dr M Ulrich-Vinther²
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HIV-derived lentiviral vectors (LV) have shown good efficacy and safety data in preclinical models and in a recent Hematopoietic Stem Cell (HSC)-based clinical trial for X-linked adrenoleukodystrophy (ALD). However, a careful analysis of LV integration sites in ALD patients’ derived cells showed that a relevant number of Common Insertion Sites (CIS) were present. This observation raises concerns because the detection of CIS is a well-established hallmark of insertional mutagenesis in mice and clinical trials. Indeed, in clinical trials for X-linked severe combined immunodeficiency and chronic granulomatous disease, several patients developed malignancies triggered by γ-retroviral vector (γ;RV) integrations at CIS recurrently targeting the same proto- oncogenes. Thus, it is possible that the occurrence of CIS in the ALD clinical trial is a still silent effect of genotoxicity caused by LV integrations that confer a selective advantage. To understand if CIS generated by LV integrations are the product of genotoxicity we generated our own dataset of LV integrations in human HSC and their progeny after engraftment in immunodeficient mice and studied the integration pattern and the clonal repertoire of vector marked cells in in vitro culture and in vivo. Our integration profile was extensively compared to LV integrations found in the ALD clinical trial, in other gene therapy trials that reported insertional leukemogenesis, as well as in retroviral and transposon-mediated oncogene tagging studies in mice. Interestingly, we found that our integration profile had the same CIS reported in ALD patients. More strikingly however, most CIS in our experimental model and in ALD patients cluster in megabase-wide chromosomal regions of high LV integration density. Conversely, cancer-triggering integrations at CIS found in tumor cells from clinical trials and oncogenes tagging studies in mice, do not form clusters, target always a single gene and are contained in narrow genomic intervals. These findings imply that LV CIS are produced by an integration bias towards specific genomic regions rather than by oncogenic selection.

**Or 81**

**Gamma-Retroviral Salvage Gene Therapy for Two Children with Chronic Granulomatous Disease**

Dr J Reichenbach, Dr U Siler, Dr A Paruzynski, Dr M Schmid, Professor CH von Kalle, Dr M Grez, Professor R.A Seger

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Two children with X-linked CGD and therapy-refractory aspergillosis, age 5 (P1) and 8 (P2) years, received gammaretroviral salvage gene therapy after submyeloablative chemotherapy with Busulphan IV. Invasive Aspergillosis was eradicated within 8 weeks after expressing therapeutic gp91phox and reactive oxygen metabolite levels (15% and 30% oxidase positive cells, respectively). The children recovered from life-threatening paraparesis (P1) and respiratory insufficiency (P2). Subsequent myeloid expansion of transduced cells resulted from insertional mutagenesis by the vector and upregulation of Evi-1 expression. Expansion of gene transduced cells was seen in P1 after 1 year and up to 40% by one dominant MDS clone and in P2 after 3 months and up to 90% by two dominant clones (MDS1/CAMTA1 and MDS1/Stat3). While P2 showed beginning myeloid dysplasia (MDS) after 2.4 yr (up), P1 remains clinically well (no MDS after 5yr (up)). Allogeneic haematopoietic stem cell transplantation has recently been performed for P2 and is planned for P1. A new SIN gamma-retroviral vector with an internal c-fes myeloid enhancer/promoter has been developed to avoid genotoxicity in the next CGD gene therapy study.

**Or 80**

**Lentiviral Vector Common Integration Sites in Preclinical Models and a Clinical Trial Reflect a Benign Integration Bias and not Oncogenic Selection**

Eugenio Montini
San Raffaele Scientific Institute; San Raffaele – Telethon Institute for Gene Therapy (HSR-TIGET); Milan, Italy

HIV-derived lentiviral vectors (LV) have shown good efficacy and safety data in preclinical models and in a recent Hematopoietic Stem Cell (HSC)-based clinical trial for X-linked adrenoleukodystrophy (ALD). However, a careful analysis of LV integration sites in ALD patients’ derived cells showed that a relevant number of Common Insertion Sites (CIS) were present. This observation raises concerns because the detection of CIS is a well-established hallmark of insertional mutagenesis in mice and clinical trials. Indeed, in clinical trials for X-linked severe combined immunodeficiency and chronic granulomatous disease, several patients developed malignancies triggered by γ-retroviral vector (γ;RV) integrations at CIS recurrently targeting the same proto-oncogenes. Thus, it is possible that the occurrence of CIS in the ALD clinical trial is a still silent effect of genotoxicity caused by LV integrations that confer a selective advantage. To understand if CIS generated by LV integrations are the product of genotoxicity we generated our own dataset of LV integrations in human HSC and their progeny after engraftment in immunodeficient mice and studied the integration pattern and the clonal repertoire of vector marked cells in in vitro culture and in vivo. Our integration profile was extensively compared to LV integrations found in the ALD clinical trial, in other gene therapy trials that reported insertional leukemogenesis, as well as in retroviral and transposon-mediated oncogene tagging studies in mice. Interestingly, we found that our integration profile had the same CIS reported in ALD patients. More strikingly however, most CIS in our experimental model and in ALD patients cluster in megabase-wide chromosomal regions of high LV integration density. Conversely, cancer-triggering integrations at CIS found in tumor cells from clinical trials and oncogenes tagging studies in mice, do not form clusters, target always a single gene and are contained in narrow genomic intervals. These findings imply that LV CIS are produced by an integration bias towards specific genomic regions rather than by oncogenic selection.
The infusion of donor lymphocytes transduced with a retroviral vector expressing the HSV-TK suicide gene in patients undergoing hematopoietic stem cell transplantation for leukemia/lymphoma promotes immune reconstitution and prevents infections and graft-versus-host disease. Analysis of the clonal dynamics of genetically modified lymphocytes in vivo is of crucial importance to understand the potential genotoxic risk of this therapeutic approach. We used linear amplification-mediated PCR and pyrosequencing to build a genome-wide, high-definition map of retroviral integration sites in the genome of peripheral blood T cells from two different donors, and used gene expression profiling and bioinformatics to associate integration clusters to transcriptional activity and to genetic and epigenetic features of the T cell genome. Comparison with matched random controls and with integrations obtained from CD34+ hematopoietic stem/progenitor cells showed that integration clusters occur within chromatin regions bearing epigenetic marks associated with active promoters and regulatory elements in a cell-specific fashion. Analysis of integration sites in T cells obtained ex vivo two months after infusion showed no evidence of integration-related clonal expansion or dominance, but rather loss of cells harboring integration events interfering with RNA post-transcriptional processing. This study shows that retroviral transduction has a very low genotoxic potential for adult T cells.

**Or 83**

**Exploiting viral integration to analyse stem cell dynamics**

Professor C von Kalle, Christof von Kalle

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Understanding the regulation of self-renewal and differentiation of stem cells in hematopoiesis as well as in normal and cancerous solid tissues is pivotaly important to develop safe and efficient genetically modified stem cell therapeutics and to eradicate malignant cells in human cancer. Hematopoietic stem cells are an easily accessible source of adult stem cells for therapeutic interventions including their genetic correction.

Increasingly effective modalities have been developed to genetically modify human cells ex vivo. Clinical gene therapy studies demonstrated that insertionional activation of regulatory genes may lead to a selective advantage in hematopoiesis. We hypothesize that preferred integration loci mark candidate genes and regulatory genomic elements of hematopoietic stem cells whose deregulation could represent an early event in leukemogenesis. Additional identification of candidate stem cell regulatory genes can be accomplished by analyzing the complete integrations site repertoire in a clinical gene therapy study. The impact of our pharmacokinetic analyses will shed new light on stem cell dynamic and biology.

**Or 84**

**Delineating the cellular pathways and molecular determinants of normal and leukemic hematopoiesis**

Sten Eirik Jacobsen

Haematopoietic Stem Cell Laboratory, Weatherall Institute of Molecular Medicine, Oxford University, United Kingdom

The classical model for hematopoiesis predicts that the commitment to a single hematopoietic lineage requires that the first and obligatory lineage commitment step of hematopoietic stem cells (HSCs) results in a strict separation between the lymphoid and myelo-erythroid lineages. Although this model has been strongly supported by the identification of common lymphoid progenitors (CLPs) and common myeloid progenitor (CMPs), more recent data from us and others support the existence of alternative commitment pathways. The current status as for our understanding of the lineage commitment pathways in normal hematopoiesis in mouse and man will be reviewed.

As for malignant hematopoiesis, the existence of leukemic stem cells (LSCs) as distinct and rare populations of cells capable of reconstituting leukemia has recently been questioned. A related but distinct question, is whether there exists rare populations of LSCs which are uniquely resistant to conventional therapeutic targeting, and therefore also the likely source of relapses following such treatments. In a subgroup of myelodysplastic syndromes (MDS), the del(5q) syndrome, we have pinpointed the primary cellular target for the 5q deletion to a rare population (typically less than 0.1% of all BM cells) of multipotent (lympho-myeloid) stem or progenitor cells, which displays phenotypic and functional stem cell properties, and efficiently displaces the normal HSC compartment. Notably, a high fraction of otherwise transfusion-dependent del(5q) MDS patients go into complete clinical, morphological and molecular remission in response to Lenalidomide treatment. Whereas our investigation of the bone marrow of these 5q- patients before and after treatment with Lenalidomide, confirm that Lenalidomide efficiently eliminates the 5q- clone at the progenitor level, we can in all patients demonstrate that the 5q- MDS stem cell compartment is selectively resistant to Lenalidomide. Over time, lenalidomide resistance develops in most of the patients in partial and complete remission, with recurrence or expansion of the del(5q) clone and clinical and cytogenetic progression. Thus, these studies establish the existence of rare and phenotypically distinct malignant stem cells that are selectively
resistant to therapeutic targeting at the time of complete clinical and cytogenetic remission.

Or 85

Regeneration Next: Towards Heart Stem Cell Therapeutics

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Recent advances in stem cell biology hold great promise for a new era of cell-based therapy, sparking considerable interest amongst scientists, clinicians, and their patients. However, although our understanding of the biology of stem cells has expanded exponentially in the past few years, the translational arm of stem cell science is in a relatively primitive state. While a number of clinical studies have been initiated with autologous cells with a variety of delivery systems, the early returns point to several inherent problems. In this regard, the clinical potential of stem cells can only be fully realized by the identification of the key barriers to clinical implementation, including scalability, delivery, grafting, survival, and safety issues. In this discussion, we examine experimental paradigms to address the critical steps in the translation from stem cell biology to regenerative medicine, utilizing cardiovascular disease as a case study. Towards this goal, we will review a new promising system for cardiovascular regenerative medicine developed in the Chien lab. Recent studies in the mouse embryo have identified a multipotent, “master” cardiac progenitor, which contributes to all the major cell types in the murine heart. In contrast to murine cardiogenesis, human heart development is associated with a longer onset of heart cell lineage diversification and expansion, suggesting the possibility of potentially divergent pathways. We have recently identified a diverse set of human fetal ISL1þ cardiovascular progenitor populations that give rise to the cardiomyocyte, smooth muscle, and endothelial cell lineages. A subset of these progenitor populations expressing ISL1 but not NKX2-5 or cardiac differentiation markers is localized to the fetal atria and proximal/medial OFT, and a diverse set of lineage-restricted progenitors and intermediates is marked by ISL1 in combination with NKX2-5 or cardiac differentiation markers. Using two independent transgenic and gene-targeting approaches in human embryonic stem (hES) cell lines, we demonstrate that purified populations of ISL1þ primordial progenitors are capable of self renewal and expansion prior to differentiation into the three major cell types in the heart: cardiomyocyte, smooth muscle, and endothelial cell lineages. Recent studies have documented the ability to purify a rare, novel ventricular progenitor in the islet lineage that can self expand and that can then spontaneous assemble into a mature strip of ventricular muscle, representing a convergence of tissue engineering and stem cell technology. Recent studies in the Chien lab have established a novel approach for the generation of human model systems for cardiovascular disease and open novel approaches for human regenerative cardiovascular medicine. A new direction for heart stem cell therapeutics is on the horizon and joint ventures with the private sector are in a position to accelerate this transition from stem cell biology towards true regenerative medicine.

Or 86

Uncovering haematopoietic system dynamics in gene therapy treated patients by retroviral tagging

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Upon retroviral gene transfer, transduced cells are univocally tagged by vector insertions. We previously showed that ADA-SCID gene therapy (GT) with CD34þ cells and reduced intensity conditioning resulted in, multilineage engraftment, in the absence of aberrant expansion. Long-term studies in these patients provide a unique model to study in depth haematopoietic clonal dynamics by retroviral tagging. We performed a comprehensive multilineage longitudinal insertion profile of bone marrow (BM) (CD34þ, CD15þ, CD19þ, Glycoporphin+) and peripheral blood (PB) (CD15þ, CD19þ, CD4þ, CD8þ cells, naive and memory T cell subpopulations) cells in 4 patients 3-6 years after GT, retrieving to date 1055 and 1999 insertions from BM and PB cell lineages respectively. We could shape the insertional landscape of each lineage through a tri-factorial analysis uncovering the effects of selective advantages in periphery and the frequency of identical integrants in different haematopoietic compartments. BM cells displayed the highest proportion of shared integrants (up to 58.3%), reflecting the real-time repopulating activity of gene-corrected progenitors. Strikingly, we found “core integrants”, shared between CD34þ cells and both lymphoid and myeloid lineages, stably tagging active long-term multipotent progenitors overtime. Tracking two of these integrants (inside a fragile site of MLLT3 gene and downstream the LRRC30 gene) by specific PCRs we confirmed the multilineage contribution to haematopoiesis of these progenitor clones, showing fluctuating lineage outputs over 4 years. The application of mathematical models to our datasets is allowing to uncover new information on the fate and activity of gene corrected haematopoietic progenitors years after transplantation in humans.

Or 87

Function of microRNA-126 in hematopoietic stem cell--implications for gene therapy

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Little is known about miRNA function in hematopoietic stem and progenitor cells (HSPC). Using a functional reporter assay, we identified miR-126 as an HSPC-specific miRNA which is rapidly down-regulated upon differentiation, offering significant potential to engineer stem-cell protective, regulated expression cassettes for HSC gene therapy. To shed light on the biological function of miR-126, we generated a stable miR-126 knock-down (kd) or knock-in (ki) in mouse HSPC using lentiviral vectors. Kd or ki cells were competitively transplanted along with congenic, control vector-transduced cells, and hematopoietic chimerism was followed for >1 year in both primary and secondary recipients. No hematopoietic tumors were observed in >30 mice with long-term follow-up, miR-126 kd HSPC displayed enhanced myeloid and B cell contribution, particularly during the early phases of reconstitution, while subsequently reaching steady state. This enhanced contribution could be reproduced after secondary transplantation, again reaching a steady state, with some mice showing signs of exhaustion. On the other hand, miR-126 overexpression resulted in a competitive disadvantage in vivo involving all hematopoietic lineages, paralleled by a complete depletion of miR-126 ki HSC in the BM. While showing normal clonogenic activity in vitro, miR-126 ki cells were decreased as early as 3 days after bone marrow transplantation. Interestingly, KSL ki cells had an increased proliferative index as judged by EdU incorporation, and hematopoietic contribution temporarily recovered before exhaustion. These data suggest that miR-126 ki favors HSC commitment at the cost of self-renewal. We conclude that a precise level of miR-126 is required for robust HSPC function.

Or 88
Specific gene transfer to CD133-positive hematopoietic Progenitor cells
Dr S Kneissl¹, Mr T Abel¹, Dr E Verhoeven², Professor U Koehl³, Dr I Johnston⁴, Professor FL Cosset², Professor K Cichutek¹, Professor CJ Buchholz²
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We developed a flexible and highly specific targeting method for lentiviral vectors (LVs) relying on the specificity of single-chain antibodies (scFv) recognizing cell surface antigens. For the generation of such LVs the modified measles virus (MV) glycoproteins hemagglutinin (H), responsible for receptor recognition and fusion (F) protein were incorporated into LVs. The H protein was blinded for the MV receptors and displayed a scFv. Using an anti-CD20 scFv, we could demonstrate targeted gene transfer into primary human CD20-positive lymphocytes. Now, we show the versatility of this technology by targeting CD133-positive human hematopoietic stem cells (HSCs). This population is of extraordinary interest for gene therapy as correction of genetic defects in these cells will be implemented in the whole hematopoietic system. CD133-LV selectively transduced human G-CSF mobilized peripheral blood CD133/CD34-positive cells and stably transferred the reporter gene to all hematopoietic lineages assessable by a colony forming assay, whereas VSV-G pseudotyped vectors also transduced CD34-negative cells. To determine the targeting capacity of CD133-LV for strongly underrepresented target cells in presence of other blood cells such as granulocytes, monocytes and lymphocytes, primary human CD133-positive cells were mixed with peripheral blood. CD133-LV transduced the CD133-positive cells with unimpaired efficiency even when only 5% target cells were present, while the surplus of non-target cells remained largely untransduced. These results are promising for targeted gene transfer into HSCs that might become possible by simply adding CD133-LV to bone marrow harvests or mobilized peripheral blood samples making the purification of HSCs prior to gene transfer obsolete.

Or 89
European databases: what can we learn from the “promise” EBMT database?
Carmen Ruiz
The European Group for Blood and Marrow Transplantation (EBMT) is a non-profit organisation established in 1974 in order to allow scientists and physicians involved in clinical bone marrow transplantation to share their experience and develop co-operative studies. From the 70’s onwards, clinical data started being collected into what is now known as the EBMT Registry. When the EBMT started collecting data, transplantation was not a main stream procedure. Today it is for some indications and we believe that the continuous collection of Registry data has widened the experience and improved the outcome of transplanted patients. The Registry currently contains clinical data on more than 315,000 patients, submitted from more than 50 countries. There is a growing desire on the part of donor registries, health departments, cord blood banks, study groups and other outcome registries to be able to access the content of the EBMT Registry and we are looking to develop closer collaborations. The EBMT overlaps with the community involved in cellular therapies. A Cellular Therapy Med-A (“minimum essential data”) was designed by the Cellular Therapy committee and has been in use for the last 2 years. We believe that the EBMT could offer its expertise on data collection and part of the Registry infrastructure to kick start a similar data collection exercise in the Cellular Gene Therapy community. We are looking to further enhance our data collection system, to better serve our centres, improve collaboration with existing partners and create new
partnerships. An invitation to do just that is offered to the Cellular Gene Therapy community.

Or 90

Long-term follow-up of patients treated with Gene Therapy Medicinal Products

Christina Galli
See supplement

Or 91

From patient association sponsored research to pharmaceutical development

Lucia Monaco
Telethon
See supplement

Or 92

The Hospital Exemption Regulation – New Options for Gene Therapy?

Dr C Buchholz

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Until recently, there was only a single option available to bring gene therapy medicinal products to the market, i.e. run through clinical trials to demonstrate safety and efficacy for your product, then apply for marketing authorisation in the centralised procedure at the European Medicines Agency (EMA). With Regulation (EC) No 1394/2007 which amends Directive 2001/83/EC coming into force, Advanced Therapy Medicinal Products (ATMP’s) including gene therapy products will be excepted from the obligation of obtaining marketing authorisation via the centralised procedure under certain conditions (so called “Hospital Exemption”). While by this regulation cell and tissue products can be excepted from the ATMP Directive came into force and are produced in small scale for individual patients, it may well apply also to certain types of gene therapy products currently under development. Such products could then be put onto the market on the basis of a national authorisation, but without centralised marketing authorisation. This presentation will discuss the implementation of this regulation into national law, the requirements to be fulfilled to fall under this exception as well as its impact on future gene therapy product development.

Or 93

Intra-Cerebral Administration of Viral Vectors via MR Guided Convection Enhanced Delivery; Translational Studies

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Optimal viral vector delivery into the brain is challenging and brain distribution of viral vectors is uncertain. To address this issue we developed viral vector delivery system that permits direct MRI monitoring of vector distribution within the brain in real-time. This significant advance allows for the first time to adjust parameters of vector infusion while delivering gene therapy, giving surgeon full control over gene transfer technology.

To allow for precise intracerebral delivery of biologics for therapy of neurological disease we developed a skull-mounted aiming device (SmartFrame) and integrated software platform (ClearPoint) for interventional MRI guided placement of deep brain stimulators. In anticipation of upcoming gene therapy clinical trials, we adapted this device for real-time convection enhanced delivery (RCD) of therapeutics via a custom designed infusion cannula. Based on real-time MRI data, this system allows selection of brain targets, provides instructions for cannula insertion along a chosen trajectory, and permits visual monitoring of infusions. The targeting accuracy of this delivery system and infusion cannula performance were validated in nonhuman primates (NHPs). Infusions of an MRI tracer (Gd-DPTA) were delivered to the thalamus (n = 4), subthalamus (4), substantia nigra (n = 1), hippocampus (n = 2) and enthorinal cortex (n = 4), and the targeting error was determined for each cannula placement.

We found that the average absolute targeting error for all targets (n = 15) was 0.8mm (95% CI = 0.14mm). No infusions in any target produced cannula reflux or leakage from the targeted structure, even when infusions occurred in close proximity to a previous needle tract. No hemorrhages occurred during cannula placement and no signs of unexpected tissue damage were seen on histology.

The ClearPoint system allows RCD to be performed with a high level of precision, predictability, and safety. This method may improve the success rate for clinical trials involving intracerebral drug delivery by direct injection.

Our advanced gene delivery system is currently tested for delivery of therapeutic genes in Parkinson’s (PD), Huntington’s (HD) and Nieman-Pick, AADC deficiency in children and brain tumors. Data will be provided to demonstrate promises and challenges in successful clinical translation of gene transfer technology for CNS disorders.

Or 94

Current Advances in Gene Therapy for Spinal Muscular Atrophy

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SMA, a recessive autosomal disorder, is one of the most common genetic causes of death in childhood. It is caused by
mutations of the survival motor neuron (SMN) gene. We previously reported that Lentiviral vector expressing SMN was successfully used to increase the life expectancy by 5 days. The marginal efficacy of this therapeutic approach, however, prompted us to explore different strategies for gene delivery to motor neurons to achieve a more clinically relevant result.

The first part of this presentation will describe the efficiency of scAAV9 mediated SMN gene replacement in SMA mouse model. We report that a single systemic injection of SMN-expressing scAAV9 vector reversed the phenotype of SMN−/−mice, a well established animal model of SMA. Most notably, SMN replacement led to substantial increase in the life expectancy, thereby achieving one of the highest therapeutic effects reported in the field to date. The second part will discuss the role of PTEN, a negative regulator of the target of rapamycin (mTOR) pathway, in SMA. AAV6 expressing siPTEN into hind limb muscles at postnatal day 1 in SMN−/− mice leads to significant PTEN depletion and robust improvement in motor neuron survival. These studies report promising advances in AAV-based therapy for SMA. We are currently planning to initiate clinical trials using scAAV-based SMN replacement in the very near future.

References

Or 95
Critical factors affecting microglia reconstitution after hematopoietic stem cell transplantation
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The occurrence of progressive myeloid infiltration and microglia reconstitution by donor cells in the brain following transplantation of hematopoietic stem progenitor/cells (HSPC) was demonstrated under specific conditions in several reports. However, the mechanisms of microglia reconstitution after the transplant are not well understood. We investigated the modalities, the kinetic and the factors that could affect this phenomenon in mice. In particular, we performed this study in the mouse model of Metachromatic Leukodystrophy (MLD), a lysosomal storage disorder characterized by the accumulation of the un-degraded substrates in glial cells and neuron, leading to microglia activation and neuroinflammation. After the transplantation of HSPC labeled with magnetic resonance contrast media and upon transduction with lentiviral vectors encoding GFP, we tracked the kinetic of the myeloid infiltration into the brain, in basal conditions or after the administration of different preparatory regimens, using magnetic resonance imaging, cytofluorimetry, tissue pathology and immunostaining. Independently from the conditioning regimen applied, we observed a short-term infiltration of the brain by labeled cells, with a preferential location within areas of neurogenesis or at disease sites. However, in order to obtain a long-term persistence of the transplanted cells into the brain and mature microglia reconstitution the use of conditioning regimens capable of exerting a direct CNS “myeloblastic” effect was required. Interestingly, the extent of the microglia reconstitution was proportional to the extent of removal of the endogenous microglia by the conditioning regimen and to the disease burden which renders these cells more susceptible to the regimen-related toxicity.

Or 96
AAV9-Based sulfamidase gene transfer reverses somatic and neurological pathology in mpsiiia mice
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Mucopolysaccharidosis type IIIA (MPSIIIA) is an inherited lysosomal storage disease caused by the deficiency of the sulfamidase enzyme, resulting in intralysosomal accumulation of the glycosaminoglycan (GAG) heparan sulfate. Severe neurodegeneration and other non-neurological alterations lead to death of the affected subjects during adolescence. No effective treatment for MPSIIIA is currently available. Here we used AAV serotype 9 to deliver the sulfamidase gene to somatic tissues as well as the central nervous system of MPSIIIA mice at 2 months of age, when high levels of GAG accumulation are already present. Intravenous administration of sulfamidase-expressing AAV9 vectors resulted in a widespread reversal of lysosomal accumulation in all brain regions, which was parallel to a reduction of neuroinflammatory markers and an improvement of neuromotor alterations of MPSIIIA mice. Intravenous AAV9 treatment also corrected GAG accumulation in all somatic tissues. We also locally delivered AAV9 vectors carrying the sulfamidase gene to the cerebrospinal fluid of MPSIIIA mice, by direct injection into the cisterna magna. This treatment mediated normalization of GAG storage in all brain areas, concomitant with a complete reversal of the lysosomal accumulation in neurons, astrocytes and microglia, and the absence of neuroinflammatory process. Intracisternally treated animals showed also significant correction of GAG accumulation in the somatic tissues, due to leakage of the vector to systemic circulation. In summary, these results may constitute the basis for the development of a novel non-invasive therapeutic alternative for MPSIIIA patients.

Or 97
Towards a Safe and Effective Gene Therapy Approach for Globoid Cell Leukodystrophy
Globoid cell leukodystrophy (GLD) is a lysosomal storage disorder due to the deficiency of the enzyme Galactocerebroside (GALC). The enzymatic deficiency results in the storage of undegraded metabolites in the nervous systems, leading to progressive dysmyelination. We are developing a gene therapy strategy based on hematopoietic stem/progenitor cells (HSPC) and lentiviral vectors (LV) in the murine models of GLD. Our initial efforts were affected by the finding that GALC supra-normal expression in HSPC is responsible for apoptosis and functional impairment of transduced cells. Importantly, mature hematopoietic cells, including the myeloid HSPC progeny infiltrating the nervous system, are not affected by GALC supra-normal expression following gene transfer, suggesting a unique sensitivity of HSPC to enzyme toxicity. According to this evidence, we exploited miRNA-regulated LV for GALC gene transfer: the use of a LV containing the target sequence of an miRNA exclusively expressed in HSPC (miR126), allowed safe transduction of HSPC and GALC expression only in their differentiated progeny. Importantly, this approach led to a significant amelioration of the survival and overall phenotype of the transplanted GLD mice, also as compared to affected animals transplanted with wild type HSPC. Of notice, gene therapy reconstituted the enzymatic activity in the brain of the affected mice and led to correction of the metabolic defect in resident neuronal and glial cells. Overall, our findings provide the proof-of-principle of a novel gene therapy approach, which might provide GLD patients with a new treatment option more efficacious than transplantation of normal donor’s HSPC.

Or 100

WT1-targeted dendritic cell vaccine elicits clinical and immunological responses in AML patients

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Background: Active immunization using tumor antigen-loaded dendritic cells holds promise for the adjuvant treatment of cancer to eradicate or control residual disease, but so far most dendritic cell trials have been performed in end-stage cancer patients with high tumor loads.

Methods: in a phase I/II trial, we investigated the effect of autologous dendritic cell vaccination in 10 patients with acute myeloid leukemia (AML). The Wilms’ tumor 1 protein (WT1), a nearly universal tumor antigen, was chosen as an immunotherapeutic target because of its established role in leukemogenesis and superior immunogenic characteristics.

Results: Two patients in partial remission after chemotherapy were brought into complete remission following intradermal administration of full-length WT1 mRNA-electroporated dendritic cells. In these 2 patients, and in 3 other patients who were in complete remission, the AML-associated tumor marker returned to normal following dendritic cell vaccination, compatible with the induction of molecular remission. Clinical responses were correlated with (miRNAs) that could regulate the proangiogenic and protumoral activity of TEMs, for searched for miRNAs differentially expressed in TEMs vs. TIE2- (or inflammatory) TAMs. Because miRNAs are sometimes lodged within intronic gene regions, we searched for intronic miRNA consensus sequences within genes that we previously found to be upregulated in TEMs vs. inflammatory TAMs. We found one gene highly expressed by TEMs but not by inflammatory TAMs that contains a previously undescribed intronic miRNA (int-miRNA). We validated the expression of the int-miRNA by qPCR and found it to be significantly upregulated in tumor-derived TEMs vs. inflammatory TAMs. By using multiple strategies, including intronic microRNA overexpression, we identified the active strand of this miRNA and measured its activity in vivo by using a lentiviral vector-based reporter system. Our data indicate that the int-miRNA is specifically active in TEMs among the circulating and tumor-infiltrating myeloid cells. Finally, in vitro data as well as bioinformatic analyses suggest the int-miRNA may be involved in regulating TEM motility in tumors. We are currently analyzing predicted target genes that could be regulated by the int-miRNA at the post-transcriptional level in TEMs that overexpress the miRNA sequence. These data identify a novel miRNA, which is characterized by a remarkably specific expression pattern among TAMs and that might be targeted to improve anticancer therapies.

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Genetically Modified Lymphocytes for Active Immunotherapy

Vincenzo Russo

See supplement

Or 99

Modulating microRNAs in tumor macrophages to inhibit tumor angiogenesis

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TIE2-expressing macrophages (TEMs), an antigenically and functionally defined subpopulation of tumor-associated macrophages (TAMs), have been implicated in the promotion of tumor angiogenesis. In order to identify microRNAs (miRNAs) that could regulate the proangiogenic and protumoral activity of TEMs, we searched for miRNAs differentially expressed in TEMs vs. TIE2- (or inflammatory) TAMs. Because miRNAs are sometimes lodged within intronic gene regions, we searched for intronic miRNA consensus sequences within genes that we previously found to be upregulated in TEMs vs. inflammatory TAMs. We found one gene highly expressed by TEMs but not by inflammatory TAMs that contains a previously undescribed intronic miRNA (int-miRNA). We validated the expression of the int-miRNA by qPCR and found it to be significantly upregulated in tumor-derived TEMs vs. inflammatory TAMs. By using multiple strategies, including intronic microRNA overexpression, we identified the active strand of this miRNA and measured its activity in vivo by using a lentiviral vector-based reporter system. Our data indicate that the int-miRNA is specifically active in TEMs among the circulating and tumor-infiltrating myeloid cells. Finally, in vitro data as well as bioinformatic analyses suggest the int-miRNA may be involved in regulating TEM motility in tumors. We are currently analyzing predicted target genes that could be regulated by the int-miRNA at the post-transcriptional level in TEMs that overexpress the miRNA sequence. These data identify a novel miRNA, which is characterized by a remarkably specific expression pattern among TAMs and that might be targeted to improve anticancer therapies.
vaccine-associated increases in WT1-specific CD8+ T-cell frequencies, as detected by peptide/HLA-A*0201 tetramer staining, and elevated levels of activated natural killer (NK) cells post-vaccination. Furthermore, vaccinated patients showed increased levels of WT1-specific IFN-γ-producing CD8+ T-cells and features of general immune activation.

Conclusions: These data support the further development of vaccination with WT1 mRNA-loaded dendritic cells as a post-remission treatment to prevent full relapse in AML patients.

Or 101

Immunotherapy for prostate cancer with tumor and minor histocompatibility antigen-specific TCR-transferred T cells
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We have demonstrated that the concomitance of minor histocompatibility (H) antigen- and high-affinity tumor-specific T cell responses found in allogeneic hematopoietic transplant recipients cause the eradicating of autochthonous prostate mouse adenocarcinoma (Hess Michelini, 2010). As in most cases donors might lack high affinity T cells specific for tumor-associated antigens, we have investigated the possibility to supplement donor T cells with sizeable frequencies of high-affinity tumor-specific TCR-transferred lymphocytes. Furthermore, as graft-versus-host disease (GVHD) is the major complication of allotransplantation, we also investigated whether combining tumor- and minor H antigen-restricted TCR-transferred T cells might confer graft versus tumor without evoking GVHD. We report that when allogeneic donor T cells or high affinity tumor-specific TCR-redirected CD8+ T cells per se are insufficient in causing disease eradication, their concomitant infusion promotes tumor regression. TCR-transferred cells promptly respond to tumorspecific vaccines and, although showing limited persistence in peripheral lymphoid organs, promote tumor infiltration and eradication. Likewise, the infusion of tumor-specific TCR-transferred T cells together with a limited number of minor H antigen-specific TCR-engineered T cells reveal therapeutically effective, in the absence of signs of GVHD. Thus, together these data support the value of combining TCR-transferred T cells specific for tumor and minor H antigens for the immunotherapy of prostate cancer.

Or 102

Generation of monoclonal TCR-expressing human T-lineage cells from induced pluripotent stem cells of single peripheral T-lymphocyte origin
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Adoptive immunotherapy is a promising approach to cancer immunotherapy that circumvents some of the limitations of active immunotherapy. However, its effectiveness is often hampered by exhaustion of antigen-specific T cells. To address this issue, we utilized induced pluripotent stem cells (iPSCs) generated from human peripheral T cells (T-iPSCs). Rearrangement of T-cell receptor (TCR) genes was maintained in these T-iPSCs which, because T-iPSCs have unlimited self-renewal capacity, could be expanded ex vivo. These T-iPSCs were then re-differentiated into CD3+TCRαβ+ functional T-lineage cells in vitro with higher efficiency than was possible for embryonic stem cells, iPSCs derived from fibroblasts, or cord blood cells. Complete monoclonality of productive TCR transcripts, transcribed from the genome inherited from the original progenitor T cell, was observed in re-differentiated T-lineage cells: No signature of de novo TCR gene rearrangement could be identified. The technology described opens a way for effective T-cell therapy with antigen-specific monoclonal TCR.

Or 103

Searching for genes that induce myocardial protection, myocardial repair or neoangiogenesis by in vivo gene transfer using AAV vectors
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The identification of novel genes and pathways controlling prenatal cardiomyocyte proliferation, regulating cardiomyocyte survival during the adult life or promoting new blood vessel formation in ischemic conditions holds paramount interest in view of developing new therapeutic approaches for patients with ischemic cardiomyopathy and heart failure. Over the last several years, we have exploited the potential of viral vectors based on the Adeno-Associated Virus (AAV) to explore the therapeutic potential of several genes in small and large animal models of cardiovascular disorders. AAV vectors have the capacity to transduce myocardial cells in vitro and in vivo with high efficiency and to promote the expression of their transgenes for indefinite periods of time.

These studies have led to the identification of a few novel regulators of cardiomyocyte function (among these, VEGFB, a selective activator of cardiomyocyte-expressed VEGFR1, which protects against pacing-induced heart failure in dogs) and cardiomyocyte proliferation (e.g. Jagged1, which induces neonatal cardiomyocyte expansion through the Notch1 pathway), as well as disclosing the role of defined mononuclear cell populations in the regulation of blood vessel formation.

More recently, we have undertaken an exhaustive approach in the identification of factors with beneficial effect upon myocardial damage by the construction of library of AAV vectors delivering the whole mouse secretome (1500 + mouse secreted proteins). Preliminary screening of a
subset of this library has led to the identification of peptide hormone ghrelin as a powerful cardioprotective agent.

Or 104

Coordinated co-expression of PDGF-BB accelerates stabilization of VEGF164-induced angiogenesis
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Background: VEGF induces normal or aberrant angiogenesis depending on its expression level in the microenvironment around each transduced cell. Therefore even rare hotspots of high expression should be avoided, making it difficult to achieve safety and efficacy with direct gene delivery. Further, 4 weeks of sustained expression are required to achieve vascular stabilization. We found that co-expression of PDGF-BB from a single bicistronic construct induced only normal angiogenesis despite high and heterogeneous VEGF levels. Here we tested its effects on vascular stabilization.

Methods: We expressed mVEGF164, hPDGF-BB or both in muscle implanting retrovirally transduced myoblasts. VEGF signaling was abrogated at defined time-points by systemic VEGF-Trap treatment.

Results: Two weeks after implantation, VEGF-induced vessels all regressed, whereas 50% of induced vessels already stabilized with PDGF-BB co-expression.

To investigate the role of factor dose, we implanted clonal populations homogeneously producing specific levels of VEGF, with or without PDGF-BB.

At low VEGF levels, 2% and 30% of new vessels were stable after 2 and 3 weeks respectively. PDGF-BB moderately improved these stabilization rates to 10% and 50%. However, at high levels, VEGF alone caused aberrant vessels that never stabilized, whereas PDGF-BB induced normal capillaries of which 50% and 80% were already VEGF-independent after 2 and 3 weeks.

Conclusions: PDGF-BB co-expression both prevented aberrant angiogenesis by heterogeneous and high VEGF levels and accelerated vascular stabilization in a time-frame compatible with short-term expressing viral vectors. Therefore, PDGF-BB co-expression could overcome the intrinsic limitations of direct VEGF gene delivery.

Or 105

Potent cardioprotective effect exerted by AAV-VEGF-B in small and large animal models of myocardial
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VEGF-B, a member of the vascular endothelial growth factor family, is emerging as a major cardiovascular protecting factor.

We have recently observed, in a rat model of acute myocardial infarction, that the intramyocardial injection of AAV2-VEGF-B167, which selectively binds VEGFR-1, significantly improves both regional and global cardiac contractility and maintains favorable tissue remodeling in the absence of a significant angiogenic response. We found that cardiomyocytes expressed physiologically active VEGFR-1, VEGFR-2 and Neuropilin-1.

Histological and molecular analysis revealed that VEGF-B is capable to elicite a compensatory, hypertrophic response and a marked antiapoptotic effect both in vitro on isolated neonatal cardiomyocytes and in vivo after induction of ischemia.

Consistent with this conclusion, we found that VEGF-B exerts a similarly striking, angiogenesis-unrelated cardioprotective effect in a pacing-induced model of non-ischemic heart failure in chronically instrumented dogs. Control animals subjected to 4 weeks of pacing suffered from an overt congestive heart failure; on the contrary, at the same time point, intramyocardial AAV9-mediated VEGF-B expression maintained heart contractility and prevented LV wall thinning.

Also in this model, apoptosis was significantly reduced in myocardium expressing VEGF-B. Consistently, protecting signaling through Akt was superphysiological in AAV-VEGF-B treated tissue, while there was a reduction or normalization of the activity of the pro-apoptotic intracellular mediators GSK-3β and FoxO3a. Cardiac VEGF-1 expression was reduced four-fold in paced dogs, suggesting that exogenous VEGF-B exerted a compensatory receptor stimulation.

Together, these results point toward VEGFR-1 signaling in the heart as an important mediator of cardiomyocyte function, with clear therapeutic implications.

Or 106

Selective gene transfer to endothelial cells using lentiviral vectors
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Endothelial cells lining the inner side of blood vessels form an attractive target cell population for a number of different gene therapy concepts. To generate endothelial cell specific vectors we applied a re-targeting approach recently demonstrated for B lymphocytes via CD20 (Funke et al., 2008). This technology relies on the pseudotyping of lentiviral vectors with engineered measles virus glycoproteins displaying a single-chain antibody (scAb) for target recognition. For the targeting of endothelial cells a human endoglin (CD105) and a murine CD105 specific scAb were applied in the system.

The human CD105-targeting vector efficiently transduced CD105-positive HT1080 cells and, interestingly, was almost 10-fold more efficient on different types of primary human endothelial cells. Gene transfer was stable in HUVEC cells and did not alter their phenotype as demonstrated by long-
term cultivation and matrigel tube formation assays. Selectivity was demonstrated in cocultures of endothelial cells and lymphocytes. While VSV-G pseudotyped vectors did not discriminate between both cell types, the endoglin targeting vector selectively transduced HUVEC cells cocultivated with primary human lymphocytes (PBMC). The murine CD105-targeting vector was similarly specific and efficiently transduced primary CD105+ murine cells isolated from heart tissue. In vitro applications of this targeting vector transferring a luciferase reporter gene are currently ongoing.

The data suggest that lentiviral endoglin-targeting vectors allow the specific genetic modification of endothelial cells and may thus improve the specificity of in vivo gene transfer applications.

**Or 107**

**Apollipoprotein B100 knock-down by AAV-delivered shRNA lowers cholesterol in mice**

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Serum low-density lipoprotein cholesterol (LDL-C) levels are proportionate to the risk of atherosclerotic cardiovascular disease. In order to reduce serum total cholesterol and LDL-C levels, we used RNA interference to inhibit expression of the structural protein of LDL-C, apolipoprotein B100 (ApoB). We developed and screened 19 short hairpin RNAs targeting conserved sequences in human, mouse, and macaque ApoB mRNAs (shApoBs) and subsequently narrowed our focus to one candidate for in vivo testing in wild-type mice. Recombinant adeno-associated virus (AAV) was used for long-term transduction of murine liver with shApoB. We observed a strong dose-dependent knock-down of ApoB mRNA and protein levels, which correlated with a reduction in total cholesterol levels, without obvious signs of toxicity. Furthermore, shApoB was found to specifically reduce LDL-C in diet-induced dyslipidemic mice, while high-density lipoprotein cholesterol (HDL-C) remained unaffected. Finally, we demonstrated elevated lipid accumulation in murine liver transduced with shApoB, a known phenotypic side-effect of lowering ApoB levels. These results demonstrate a robust dose-dependent knock-down of ApoB by AAV-delivered shRNA in murine liver, thus providing an excellent candidate for development of RNAi-based gene therapy.

**Or 108**

**AAV vectors for gene therapy of retinal degeneration**

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AAV vectors are safe, efficient and versatile tools for gene transfer to the retina. The availability of dozens of different serotypes with distinct transduction characteristics, appropriate regulatory elements and strategies to overcome the vector limited cargo capacity allow to design efficient protocols for retinal gene transfer in small and large animal models.

Recently, the safety and efficacy of AAV-mediated gene transfer to the retina of patients with a severe form of inherited childhood blindness has been shown, paving the way for additional trials for other blinding conditions.

**Or 109**

**Clinical trial of gene therapy for early onset severe retinal dystrophy resulting from defects in RPE65**

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Early-onset severe retinal dystrophy caused by defects in the gene encoding the retinal isomerase RPE65 is associated with poor vision at birth and complete loss of vision in early adulthood. In a phase I/II dose-escalation trial, we have delivered subretinally recombinant adeno-associated virus (rAAV) vector expressing RPE65 under the control of an RPE65 promoter in 9 human subjects with early onset severe retinal dystrophy associated with mutations in RPE65. We have examined systemic vector dissemination and immune responses following vector delivery, assessed visual function pre- and post-vector delivery using a range of psychophysical techniques, and performed detailed electrophysiology and retinal imaging studies.

There have been no serious adverse effects of surgical delivery of vector in the subjects enrolled to date. We have detected no systemic dissemination of vector genome. Although we have detected an increase in systemic neutralising antibodies to AAV capsid in two subjects but there have been no evidence of immune responses to RPE65 protein. We have measured significant improvements in retinal sensitivity by microperimetry and dark-adapted perimetry, and improved performance in a test of visually-guided mobility. The outcomes in the first 9 subjects to date suggest that subretinal delivery of rAAV vector can be safe in humans in the short term and can improve retinal sensitivity. These findings support further clinical studies in subjects with RPE65 deficiency and the development of gene therapy for other inherited retinal disorders.

**Or 110**

**Limbal Stem-Cell Therapy and Long-Term Corneal Regeneration**

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**Background:** Corneal renewal and repair are mediated by stem cells of the limbus. Ocular burns may destroy the limbus, causing limbal stem-cell deficiency. We investigated the long-term clinical results of cell therapy in patients with burn-related corneal destruction associated with limbal stem-cell deficiency, a highly disabling ocular disease.

**Methods:** We used autologous limbal stem cells cultivated on fibrin to treat 112 patients with corneal damage, most of whom had burn-dependent limbal stem-cell deficiency. Clinical results were assessed by means of Kaplan–Meier, Kruskal–Wallis, and univariate and multivariate logistic-regression analyses. We also assessed the clinical outcome according to the percentage of holoclone-forming stem cells, detected as cells that stain intensely (p63-bright cells) in the cultures.

**Results:** Permanent restoration of a transparent, renewing corneal epithelium was attained in 76.6% of eyes. The failures occurred within the first year. Restored eyes remained stable over time, with up to 10 years of follow-up. In post hoc analyses, success — that is, the generation of normal epithelium on donor stroma — was associated with the percentage of p63-bright holoclone-forming stem cells in culture. Cultures in which p63-bright cells constituted more than 3% of the total number of clonogenic cells were associated with successful transplantation in 78% of patients. In contrast, cultures in which such cells made up 3% or less of the total number of cells were associated with successful transplantation in only 11% of patients. Graft failure was also associated with the type of initial ocular damage and postoperative complications.

**Conclusions:** Cultures of limbal stem cells represent a source of cells for transplantation in the treatment of destruction of the human cornea due to burns.

**Or 112**

**Functional benefit in a mouse model for dominant retinitis pigmentosa following gene therapy**

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The development of gene therapies, targeting the primary genetic lesion, for many dominantly inherited diseases has been severely impeded by their inherent mutational heterogeneity. This is clearly demonstrated by the blinding disorder rhodopsin-linked autosomal dominant retinitis pigmentosa (RHO-adRP) in which well over 100 different mutations in the rhodopsin gene (RHO) have been characterised. In this study we have developed a mutation-independent AAV therapy for RHO-adRP administered to a murine disease model, Pro347Ser.

AAV2/5 vectors express a short hairpin RNA (shRNA), which targets both human and murine rhodopsin transcript. In addition, vectors express a replacement RHO gene, which is resistant to suppression due to five nucleotide alterations at the shRNA target site, all of which are at degenerate positions of the RHO coding sequence. Hence replacement genes encode wild type protein. Suppression and Replacement AAVs were subretinally injected into 5-day old Pro347Ser mice. Therapeutic benefit was determined by histology and electrophysiology.

The approach outlined in this study is potentially applicable to many dominantly inherited disorders where mutational heterogeneity demands a mutation-independent therapy.

**Or 113**

**Gene Therapy for the Treatment of wet-AMD**

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VEGF plays a critical role in neovascular age-related macular degeneration and proliferative diabetic retinopathy. VEGF antagonists are useful for treating such disorders; however current treatments require monthly intravitreal injections. We have designed a soluble anti-VEGF molecule (sFLT01) and delivered it by intravitreal injection of an AAV
vector. We have shown that AAV2-sFLT01 inhibits neovascularization in murine disease models. In the eyes of cynomolgus monkeys, AAV2-sFLT01 gives expression levels persistent for at least one year. We also performed laser-CNV experiments 5 months after vector administration in non-human primates and show that sFLT01 is effective at inhibiting neovascularization. We have now completed a 12 month safety study of AAV2-sFLT01 administered intravitreally in cynomolgus monkeys. Animals were dosed with vehicle, 2x10^9 or 2x10^10 vector particles/eye. Electroretinograms, fluorescein angiograms and tonometry were assessed, and no test article-related findings were observed in any group. Indirect ophthalmoscopy and slit lamp exams revealed a mild to moderate vitreal haze and cells in the high dose group only, beginning at 1 month with most animals in this group affected by 3 months. This response resolved over time in the majority of animals. Histological evaluation of the eyes in this study found no structural changes in any part of the eye including the retina. In conclusion we have demonstrated long term efficacy with minimal side effects following intravitreal delivery of AAV-sFLT01 in rodents and non-human primate models. These results suggest an alternate method of long term treatment for diseases of ocular neovascularization without the need for repeated intraocular injections.

**Or 114**

**Intrathecal injection of HDAd vectors results in long-term transgene expression in neuroepithelial cells and neurons**

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Helper-dependent adenoviral (HDAd) vectors are devoid of all viral genes and result in long term transgene expression in the absence of chronic toxicity. Because of their ability to infect post-mitotic cells, including cells of the central nervous system (CNS), they are particularly attractive for brain-directed gene therapy. However, the blood-brain-barrier limits transduction of brain cells by HDAd vectors injected intravenously. Administration of viral vectors into the cerebrospinal fluid (CSF) through injection in the cisterna magna or lumbar puncture is an attractive approach to achieve widespread transgene expression in the CNS. Moreover, intrathecal injection is far less invasive than intracerebral injection. We show that intrathecal injection of HDAd vectors results in mice in extensive transduction of neuroependymal cells, minimal toxicity, and sustained transgene expression for up to one year post-injection. We also demonstrate for the first time that HDAd delivered by intrathecal injections results in transduction of neuronal cells. These findings are significant because neuronal cells are important targets for correction of neurodegenerative diseases and long term expression is crucial for the treatment of genetic diseases. HDAd vector-mediated transduction of neuroepithelial cells and secretion of the therapeutic gene into the CSF may allow delivery of the transgene product to the whole CNS through the ventricular circulation and phenotypic correction of brain diseases with diffuse involvement, such as lysosomal storage disorders.

**Or 115**

**Recruitment of Neuropilin-1-expressing Mononuclear Cells (NEMs) by AAV2-Sema3A contributes to vessel stabilization and inhibits tumor growth**

Dr A. Carrer¹, Dr S. Zacchigna¹, Dr S. Moimas¹, Dr L. Zentilin¹, Professor M. Giacca¹

Initially discovered for their role in axon guidance, class 3 semaphorins, along with their neuropilin receptors (Nrp1 and Nrp2) have recently been implicated in angiogenesis. We have recently reported the ability of AAV-Sema3A to recruit a population of mononuclear cells expressing Nrp1 (CD11b+/Nrp1+/Gr1-), which are crucial for vessel maturation (Nrp1-Expressing Mononuclear cells, NEMs; J. Clin. Invest. 2008, 118, 2062). In addition, AAV-Sema3A exerted marked antitumoral activity by improving pericyte coverage of tumor blood vessels and restoring tissue normoxia (J. Clin. Invest. 2009, 119, 3356).

In order to characterize the anti-tumor role of NEMs, CD11b+/Nrp1+/Gr1- cells were isolated either from the bone marrow or from AAV-Sema3A-injected muscles, and administered to tumor-bearing animals. In both models, NEMs counteracted tumor growth. Consistent with the above findings, NEM-treated tumors exhibited a more mature vascular network, associated to a higher degree of α-SMA + /NG2 + mural cell coverage (3-fold) and decreased vascular leakiness (30% reduction), suggesting a role for NEM in triggering vessel normalization.

In addition, Sema3A expression inhibited the recruitment of pro-angiogenic tumor-associated macrophages (TAM), as unveiled by F4/80 staining of tumor sections. Phenotypic characterization of NEMs, purified by cell sorting, magnetic beads or laser microdissection, revealed that these are a unique population of bone marrow-derived cells, characterized by an expression profile reminiscent of M1 polarized macrophages. NEMs were in no case found incorporated in the vasculature, nor they displayed angiogenic properties, however, they contributed to vessel maturation through a paracrine effect, ensuing in the activation and proliferation of tissue-resident mural cells.

**Or 116**

**Targeting of myelogenous leukemia stem cells through genetic redirection of T cells against CD44v6**

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Myelogenous leukemia is organized as a hierarchy dominated by leukemia stem cells (LSC). LSC are resistant to radiochemotherapy and ultimately responsible for relapse. The results of allogeneic hemopoietic cell transplantation suggest that LSC are sensitive to adoptive T-cell therapy. Nevertheless, the frequent occurrence of HLA-loss variants after transplantation recommends the targeting of antigens crucial to LSC biology. LSC self-renewal depends on signals provided within the bone marrow (BM) stem-cell niche, among which those mediated by CD44 molecules. With the aim of targeting LSC, we constructed a second-generation chimeric antigen receptor (CAR) based on the fusion of the single-chain fragment of a CD44v6-specific mAb with the signaling domains of the TCR z chain and CD28 (CD44v6CAR.28z). Retroviral-mediated gene transfer after stimulation with beads coupled to anti-CD3 and anti-CD28 mAb allowed robust and stable transgene expression and selective elimination of CD44v6-expressing cells, including primary leukemias, but not of healthy hemopoietic stem cells. Redirected T cells were T central memory cells and expressed the BM “addressin” CXCR4. During culture with IL-7 and IL-15, we observed the selective enrichment of CD44v6-directed T cells, possibly through cross-stimulation by CD44v6 low-expressing activated T cells. When cultured with autologous monocytes, which also express CD44v6, redirected T cells expanded significantly. Co-transfer of the suicide gene HSV thymidine kinase enabled the elimination of redirected T cells, providing a safety switch in case of unexpected toxicity. Altogether these results warrant the clinical implementation of this novel strategy of T-cell gene therapy for the safe and effective eradication of LSC.
ESGCT 2010 Poster Presentations

P 1
Increased AAV transduction in postmitotic tissues correlates with the downregulation of DNA-damage response machinery
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Despite the broad success of rAAV vectors, several aspects of their biology still remain obscure. In particular, limited information is available on molecular determinants of AAV tissue permissivity. Previously, we have demonstrated that the host cell DNA damage response machinery and, in particular, the proteins involved in double-stranded DNA break repair (DSB) (Mre11-Rad50-Nbs1 (MRN) complex), interact and negatively regulate incoming rAAV genome processing.

Since in vivo AAV transduction is particularly efficient in postmitotic tissues such as heart, muscle, brain and retina, we envisioned elucidating the molecular mechanisms underlying this high rAAV permissivity and to specifically understand whether it could be mediated by the diminished expression of proteins involved in DSB repair in terminally differentiated cells.

In a comprehensive analysis using tissue extracts from adult mice, we observed decreased expression levels of MRN complex in postmitotic tissues in comparison with tissues with high proliferative activity known to be poorly permissive of AAV transduction.

Accordingly, in vitro, in a cell model of myoblast differentiation and in primary cultures of neonatal murine cardiomyocytes, we also demonstrated that permissivity to rAAV transduction increased with the cell differentiation status. Consistently, we observed a gradual decrease in the expression of the MRN complex along the process of myoblast differentiation.

In vivo, the increased cardiac transduction upon systemic delivery of AAV correlated with abrupt decrease of MRN expression from day 7 after birth.

These results indicate that the downregulation of the DNA damage response machinery in postmitotic cells results in the stabilization of rAAV genomes leading to increased functional transduction.

P 2
Deciphering AAV replication strategy in the presence of HSV-1
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Recombinant AAV vectors are currently produced using either adenovirus (Ad), baculovirus, or herpes virus (HSV) helper activities. Understanding how AAV replicates under these different conditions is critical to further optimize vector production. This study is focused on the analysis of AAV replication in the presence of HSV-1, a powerful helper virus for vector production. We previously showed that nine HSV-1 factors were able to support AAV rep gene expression and genome replication (Alazard-Dany et al. PLoS Pathogens 2009). To further elucidate the strategy of AAV replication in the presence of HSV-1, we have recently undertaken the proteomic analysis of cellular and HSV-1 factors associated with Rep proteins during AAV replication. This study resulted in the identification of approximately 60 cellular proteins among which factors involved in DNA and RNA metabolism represented the largest functional categories. Finally, several HSV-1 factors were also found associated to Rep among which UL12. We demonstrated here for the first time that this protein plays a role during AAV replication by enhancing the resolution of AAV replicative forms. Altogether these results indicate that, in the presence of HSV-1, AAV may replicate using a basal set of cellular DNA replication enzymes but also extensively relies on HSV-1-derived proteins for its replication, among which UL12 a newly discovered helper factors. They also suggest that AAV may be able to differentially adapt its replication strategy to the nuclear environment induced by the helper virus.

P 3
Transduction profiles of FX-binding ablated Ad5 vectors in non-human primates (Microcebus murinus)
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The mechanism underlying Adenovirus type 5 (Ad5) entry into hepatocytes has been recently described. We
demonstrated that coagulation factor X (FX) binds to Ad5-hexon protein at high affinity and this interaction mediates hepatocyte transduction following intravenous (i.v.) delivery into rodent models. FX-binding ablated Ad5 vectors have been genetically engineered to ablate the interaction with FX resulting in substantially reduced hepatocyte transduction following i.v. administration in mice and rats. However, many other interactions and anatomical features, including fenestrae size in liver sinusoids and interactions with key tropism-determining capsid proteins can differ between different species. The effect of FX-ablation on liver gene transfer in non-rodent models has not been reported. Here, we document the transduction profiles and viral genome accumulation of Ad5 and FX-binding ablated Ad5 vector in non-human primates (*Microcebus murinus*) following the i.v. delivery of 1×10¹¹ viral particles/animal. Transduction profiles analyzed at 48 h showed FX-binding mutated Ad5 vectors precluded liver transduction while Ad5 showed efficient liver gene transfer compared to FX-binding ablated Ad5 vectors (p < 0.05). Viral genome accumulation at 48 h showed FX-binding ablated Ad5 vectors presented lower accumulation of viral genomes in most organs analyzed compared to Ad5. Of note, higher amounts of FX-binding ablated Ad5 viral genomes were found in the spleen, as previously observed in rodents, but at considerably lower levels than those observed for Ad5. In summary, FX-binding ablated Ad5 vectors showed similar biodistribution profiles in non-human primates than in rodent models.

**P 4**

**A non-replicating adenoavirus expressing Hey1 is cytotoxic to prostate cancer cells**

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Androgen receptor (AR) cell signalling is constitutively active in most hormone-refractory prostate cancer (PCa) tumours and suppression hypothesized to impede PCa cell proliferation. Hey1, a co-repressor of the AR is being investigated as a therapeutic transgene for treatment of late-stage PCa. A replication-defective recombinant adenoivirus deleted for E1 and E3, expressing Hey1 was constructed (Ad5Hey1). A luciferase reporter system demonstrated it repressed AR activity in a dose dependent manner in AR-expressing PCa 22Rv1 cells. Knock down of AR verified cell death by AR silencing. AR-specific siRNA suppressed expression and produced 37 ± 2.2% cell death compared to 13 ± 1.9% for control siRNA. Ad5Hey1 cytotoxicity in PCa cells assessed by MTS viability assays found AS5Hey1 alone in 22Rv1 cells produced EC₅₀ values comparable to the cytotoxic EIA-12S gene expressed from an E1-E3 deleted virus. In combination with docetaxel and mitoxantrone, Ad5Hey1 sensitised the cells in a Hey1-dependent manner. In these cells, protein expression of p53 increased with increasing virus doses. p21 levels also increased over time at low doses of virus however disappeared at higher. This suggests cells are being driven towards cycle arrest or apoptosis. Verified further by cell cycle analysis, at low virus doses the number of cells in G2/M-phases increased compared to untreated cells and at higher doses a larger proportion of cells were in sub-G1 indicating cells are being pushed towards apoptosis. Data will be presented to suggest that Hey1 can be expressed from a potent replication selective adenoivirus to further improve on oncolysis anti-tumour efficacy both in vitro and in prostate cancer xenograft models.

**P 5**

**Promoter targeting is sufficient to cause oligodenrogial tropism of adeno-associated virus vectors**

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**Background:** Recombinant adeno-associated viral vectors (rAAV) have become a versatile tool for gene transfer to the central nervous system (CNS). However, established AAV-serotypes do only transduce neurons, while efficient and widespread transduction of oligodenroglia has not been observed. Retargeting the natural AAV tropism is a prerequisite for a gene therapy of leukodystrophies, a group of disorders caused by oligodenrocyte dysfunction. Canavan disease (CD), a leukodystrophy caused by mutations in the gene encoding the enzyme aspartoacylase (ASPA), is ideally suited for a gene-replacement therapy, because the genetic defect is a null mutation.

**Objectives:** Identification and characterization of AAV-based vectors with oligodenrogial tropism and the application of these vectors for CD gene therapy.

**Methods:** We packaged AAV2 pseudotyped AAV1, AAV1/2, AAV8 vectors for expression of genes encoding green fluorescent protein (EGFP) or ASPA under the control of the chicken-beta-actin (CBA) promoter or the myelin-basic protein (MBP) promoter. Transduction efficacy and tropism was assessed by double immunohistochemistry after transduction of enriched oligodenroglia cultures and delivery to the mouse brain.

**Results:** All vectors carrying the CBA promoter transduced neurons *in vitro* and *in vivo*. The MBP promoter was sufficient to shift transgene expression exclusively to cultured oligodenrocytes for AAV1/2 and AAV8, while AAV1 showed some additional astroglial transduction. However, only AAV1/2-MBP-injection resulted in robust, yet not exclusive, transduction of oligodenroglia *in vivo*. Transduction efficacy was unaffected by the transgene or the purification method.

**Conclusions:** Our findings suggest that AAV1/2-MBP is a viral vector for CD gene therapy and for the treatment of other leukodystrophies.
**P 6**

**Intrathecal administration of AAVrh10 efficiently transduces motor and sensory neurons in mice**

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**Background:** Adeno-associated viral (AAV) vectors are one of the most promising gene therapy vectors for human clinical trials as they are able to drive long-term expression, mostly as an episomal form. No toxicity is associated with wild type virus, which increases safety issues, and they can easily be produced at pure high-titer. Different AAV serotypes can efficiently transduce neurons both in central and peripheral nervous systems using various administration routes. Until now the transduction of motoneurons in spinal cord has been poorly achieved by intramuscular injection of serotypes 1, 6 and 9. Intravenous injections of AAV9, though, obtained efficient motoneuron transduction but rised safety concerns due to spread biodistribution.

**Results:** In vitro infection of DRG organotypic cultures with AAV1 and AAVrh10 showed preferential infection of small neurons. Among them, AAV1 mainly infected non-peptidergic small neurons and AAVrh10 transduced peptidergic small neurons. After intrathecal injection of AAV vectors in mice, GFP positive neurons were counted. In DRG slices 15% for AAV1 and around 50% of neurons for AAVrh10 were infected with similar neuronal pattern as observed in vitro. In spinal cord slices, AAVrh10 infected around 30% of motoneurons all along the spinal cord, while no infection was observed with AAV1.

**Conclusion:** Intrathecal injection of AAVrh10 is able to infect motoneurons in spinal cord, which hadn’t been reported until now. Thus, this AAV serotype can be a promising tool to design gene therapy approaches for diseases affecting motor and sensory neurons.

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**P 7**

**Evaluation of a novel AAV/transposase hybrid-vector system for somatic integration in therapy and in vivo**

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Recombinant adeno-associated viral (rAAV) vectors predominantly persist as extra-chromosomal genomes. However, in dividing cells, rAAV vector genome copy numbers and transgene expression levels decline rapidly. Herein, we inserted the hyperactive Sleeping Beauty (SB) transposase variants HSB5 and the novel SB100 displaying 10- and 100-fold increased integration efficiencies compared to wild-type SB into our AAV-based two-viral-vector system.

Previous work showed that transposition only works sufficiently from circular substrates. However, once inside the cell rAAV genomes form various episomal DNA forms including circular and linear DNA molecules. However, our initial experiments suggested that Flp-mediated circularization may not be required for AAV-delivered transposase activity significantly reducing the complexity of our system.

In order to avoid co-transduction of two AAV-vectors we generated a stably SB100 expressing cell line. Integration efficiencies were analyzed and we found that after infection with a rAAV encoding a neomycin transposon, the integration efficiencies in SB100-cells was 5-fold increased compared to cells with inactive SB (mSB) indicating for the first time that transposition works from rAAV Analysis of insertion sites after SB100-mediated integration into the host genome is ongoing.

To address whether the new version of the rAAV hybrid-vector-system also results into stable transgene expression levels in vivo, we co-injected C57Bl/6 mice with a rAAV transposon donor vector expressing the human coagulation factor IX and an adenoirialis-vector encoding HSB5. After induction of rapid cell cycling in mouse liver, transgene expression levels were stabilized in mice which received HSB5 compared to the control mSB group indicating that transposition occured.

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**P 8**

**Influence of surface-exposed tyrosine phosphorylation on AAV2 capsid processing and presentation**

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Reducing the levels of capsid antigen presentation on MHC class I (MHCI) may represent a successful approach to reducing immunogenicity of adeno-associated viral (AAV) vectors. Upon entering the cell, AAV capsid gets processed and presented on MHCI molecules in amounts sufficient to trigger cytotoxic T lymphocyte (CTL) killing of transduced cells. Here we compared levels of antigen presentation and CTL killing of the recently described tyrosine-mutant capsids (PNAS 2008;105:7827) with unmodified AAV2. Mutation of tyrosine residues on the AAV capsid that are target for ubiquitination could potentially result in decreased MHCI antigen presentation. To test this hypothesis, a human hepatocyte cell line was transduced with an unmodified AAV2 vector, a vector carrying the Y370F mutation (SM), or a vector with the Y444F, Y500F, and Y730F mutations (TM). Antigen presentation was assayed using a Jurma/VP1 T cell line recognizing a conserved epitope within the AAV capsid and expressing luciferase upon T cell receptor engagement. No difference in antigen presentation over a wide range of MOI was noted between the unmodified or mutant vectors in this assay. This correlated with equivalent levels of CTL-mediated killing.
Treatment with proteasome inhibitors reduced antigen presentation in vitro and enhanced transgene expression in vivo of unmodified and mutant vectors. While these results suggest that both unmodified and tyrosine mutant AAV vectors follow similar antigen processing and presentation pathways, the use of mutant capsids with higher transduction efficiency may allow to reach therapeutic efficacy at lower vector doses, thus decreasing the total vector antigen load.

**P 9**

**PEGylated helper-dependent adenoviral vector for gene therapy of Hyperlipidemias**

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Helper-dependent adenoviral (HD-Ad) vectors are very promising for gene therapy application; however, their administration induces an acute toxicity that impairs the possibility of a safe clinical administration. We previously observed that PEGylation of HD-Ad vectors strongly reduces acute inflammation both in murine and in primate models. In order to evaluate if PEGylated HD-Ad vectors (PEG-HD-Ad) can combine safety with the correction of the pathological phenotype, we treated a mouse model of hypercholesterolemia (LDLR-deficient mice) with a vector expressing apolipoprotein A-I (HD-Ad-ApoA-I), already proven successful in a previous study. In this study, we treated LDLR-deficient mice with high doses of HD-Ad-ApoA-I, the PEGylated version of the same vector (PEG-HD-Ad-ApoA-I) and PBS as control. After 12 weeks, mice treated with both HD-Ad-ApoA-I and PEG-HD-Ad-ApoA-I showed reduction in triglycerides and LDL-C, and elevation of HDL-C. Moreover, they showed significantly smaller areas of atherosclerotic plaques compared to untreated mice. We confirmed the increased safety of PEGylated vectors, analyzing cytokine response after treatment: PEGylated vector induced a lower increase in IL-6, IL-12, KC and TNF-alpha compared to the naive vector. This data indicates, for the first time, that the reduction of toxicity due to the PEGylation of HD-Ad vectors is independent from the transgene expressed and that treatment with PEGylated vectors can correct pathological phenotypes. In conclusion, these data further support the possibility the clinical application of PEGylated HD-Ad vectors for correction of genetic diseases.

**P 10**

**AAV mediated shRNA expression in human skin**

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shRNA delivery by recombinant adeno-associated viral (rAAV) vectors has successfully been applied to several tissues in vivo. However, RNAi in human skin by rAAV-delivered shRNAs has never been reported. In a previous study using lentiviral vector delivery, we demonstrated that TNF-a can be downregulated in inflammatory psoriatic skin in the xenograft transplantation model by RNA interference with resulting clinically and histologically improvements (Mol Ther 2009;17:1743–1753).

We are now using the same model for comparisons between rAAV vectors and lentiviral vectors for shRNA delivery into human skin in vivo. This is done using a highly potent TNF-a-directed shRNA (shTNFa3) which we designed and used in our previous study.

Currently, we are testing the efficiency of TNF-a knock-down in vitro by shTNFa3 when delivered by rAAV vectors with different designs and comparing these results to results obtained with shTNFa3 delivered by lentiviral vectors. Additionally, we are comparing different AAV serotypes in human skin grafted onto mice to determine the optimal serotype. This is done using luciferase as an in vivo marker.

**P 11**

**A novel adenoviral hybrid-vector system carrying a plasmid replicon for gene therapy**

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In dividing cells the two aims a gene therapy should accomplish are defined as the nuclear distribution and retention of therapeutic DNA. Because monosystems fail to fulfil both tasks with equal efficiency, hybrid-vector systems are promising in facing the challenges of molecular medicine. Our hybrid-vector system HADV-pEPito synergizes the helper-dependent adenoviral vector(HADV) with the plasmid pEPito containing the therapeutic gene and special DNA sequence SMAR(Scaffold/Matrix-Attachment-Region) for episomal retention and replication. Our technique provides a powerful tool for stable maintenance of therapeutic DNA reducing the risk of insertional mutagenesis. HADV-pEPito and the SMAR deleted control (HADV-pEPito-ASMAR) were generated with our novel BAC-technology. Both contain promoter hCMV/EF1 upstream of an eGFP-IRES-BSD-cassette. 83% of A549-cells infected with MOI of 50 were positive for eGFP-expression. pEPito has to be released from linear adenoviral genomes with Flpe recombinase to function as a plasmid. PCR proved this recombination for both constructs. Episomal status of recombinant plasmids was verified with rescue experiments at day 4 after co-infecting A549-cells with our constructs and Flpe-expressing virus HADV-mSB-Flpe. More bacterial colonies grew after transformation of genomic DNA containing HADV-pEPito with Flpe (n = 281) than of DNA containing HADV-pEPito-ASMAR with Flpe (n = 25), suggesting replication of excised plasmids.
Ongoing experiments compare HDAdV-pEPito and HDAdV-pEPito-ASMAR concerning their long-term persistence in mice and their in-vitro establishment in a colony forming assay (CFA). Previous CFAs with the original plasmids inserted into our viruses seemed to show a tendency towards SMAR-positive pEPito (48 surviving colonies) compared to SMAR deleted pEPito-ASMAR (27 surviving colonies) after 4 weeks of selection.

**P 12**

Induction of MX1 Gene Expression across Species by Mouse and Human Interferon Beta

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Towards our development of gene therapies to treat rheumatoid arthritis an assay was needed for the measurement of interferon beta (IFNβ) activity after rAAV5 mediated gene delivery. Our pre-clinical vector is a rAAV5 that will deliver a human IFNβ gene under the control of a nuclear factor kappa B (NF-kB) promoter. The myxovirus resistance protein 1 (MX1) is a key mediator of innate immunity against pathogens. MX1 gene expression is induced by type 1 interferon and is currently used to evaluate IFNβ activity and anti-IFNβ neutralizing antibodies in multiple sclerosis patients. These tests determine the cross-species ability of mouse and human IFNβ to induce MX1 gene expression.

Mouse fibroblast like synoviocytes (mFLS) and human 2V6.11 cells were infected with AAV5 expressing either mouse or human IFNβ (moi 10). After 72 hours IFNβ expression was measured by ELISA and MX1 gene expression was determined by real-time PCR.

Mouse FLS infections produced more than 10 ng/ml of both mouse and human IFNβ whereas human 2V6.11 infections produced more than 100 ng/ml of both mouse and human IFNβ. In mFLS, human IFNβ induced 21% of the level of MX1 gene expression compared to mouse IFNβ. In human 2V6.11 cells, mouse IFNβ induced 49% of the level of MX1 gene expression compared to human IFNβ.

Infection of mouse FLS with AAV5 expressing human IFNβ induced a modest level of MX1 gene expression. Additionally, mouse IFNβ induced a moderate level of MX1 gene in human cells. Induction of MX1 gene expression across species may be a useful tool for the evaluation of IFNβ activity during pre-clinical testing.

**P 13**

Incorporation of a Metallothionein Fusion into the Adenovirus Protein IX for Non-invasive Imaging

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Introduction: Adenovirus (Ad) vectors have been exploited for a wide range of gene therapy applications. However tracking Ad biodistribution in vivo is limited to invasive procedures such as biopsies, which are error prone, non-quantitative, and do give a full representation of the pharmacokinetics involved. Also, the major drawback to approaches using reporter genes is that these systems require initial viral infection and subsequent cellular expression of reporter gene expression to allow quantification. To overcome these limitations, we incorporated the human metallothionein (MT) protein as a fusion to the Ad minor capsid protein pIX. The pIX protein is a locus capable of presenting incorporated ligands on the virus capsid surface, and MT has high affinity for heavy metals, including radiometals such as 99mTc.

Methods: After incorporating the MT coding sequence as a C-terminus fusion to pIX, we used the E. coli recombination system to generate a non-replicative vector containing an E1A expression cassette with GFP under control of the CMV promoter (Ad5-CMV5-tGFP-pIX-MT). We tested whether Ad5-CMV5-GFP-pIX-MT could be radiolabeled by 99mTc binding. After radiolabeling the Ad5-CMV5-GFP-pIX-MT, we determined whether SPECT imaging could be used to monitor Ad biodistribution and uptake in vivo.

Results: Our results strongly demonstrate imaging capability in vivo using pIX-MT, visualizing the biodistribution of Ads.

Conclusions: These results demonstrate the feasibility of 99mTc binding to the pIX-MT fusion on the Ad capsid using a simple transchelation reaction. The genetic Ad capsid labeling system offers noninvasive dynamic imaging of biodistribution that can be monitored by existing nuclear medicine imaging.

**P 14**

Use of non-clinical and clinical data for the environmental risk assessment of Glybera (alipogene tiparvovec)

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Purpose: Glybera (alipogene tiparvovec) is an AAV-1 based gene therapy medicinal product developed for long term correction of lipoprotein lipase deficiency (LPLD), a seriously debilitating inherited lipid metabolism disorder. The environmental risk assessment (ERA) forms an important part of the submission in the European Union but gene therapy specific experimental assessment protocols are not available. Glybera is the first AAV-based gene therapy medicinal product filed in Europe for Market Authorization.

Methods: We screened the non-clinical and clinical studies to select data that should be included in the ERA. This data was supplemented with use scenarios from clinical trials and literature on the biology of wild-type AAV and modified AAV-based gene therapy vectors, to assess magnitude and likelihood of potential adverse events.

Results: All essential experimental data for the ERA were generated as part of the non-clinical and clinical development. The clinical vector shedding from different excreta
Betamethasone delays the Egr-1 promoter response to UV light of Ad-Egr1-Luc7 on human and mouse fibroblasts

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Background: Egr1 is a finger zinc transcriptional factor involved closely with transcriptional activation of collagen type1 on skin. Transcriptional activation is induced by UV light, serum, hormones and drugs. Hereby, we describe the effect of Betamethasone on human primary fibroblast (HPF) transduced with Ad-Egr1-Luc-7, exposed to UV, in order to propose as betamethasone like repressor of Ad-Egr1-Luc-7.

Methods: 5×10 3 3T3 mouse fibroblasts and HPF cells were seeded and infected at 75 MOI with Ad-Egr1-Luc, and AD-CMV-Luc as reference control. Cells were deprived of serum (FBS) 24 hours before induction assay. The Induction assay was performed in 1).SFB(+); 2).FSB(−); 3).FBS(−)/betamethasone(+). All conditions were grouped in A).UV(−) and, B) UV+(+). Reporter activity was measured by luciferase activity assay at 3 and 6 hours.

Results: Transcriptional activity of Egr1, was induced by UV light en both cell lines at 3 hours, however, the maximum activity was observed at 6 hours (10 Folds of luminescence). Activity of Egr1 not shows significant change in presence of serum. However, Egr1 activity increases at ~7 fold times (46.2/10 3 LC) compared to (5.2/10 3 LC) of CMV in presence of UV. When cells are exposed to UV and Betamethasone, luciferase activity are in 3T3 cells but in PHF the egr1 level is lower than CMV, this correlation is inverted at 6 hours 3 fold times.

Conclusion: In this model of UV induction, the egr1 activity in PHF is 10 times over 3T3 and early delayed by betamethasone. Betamethasone could be proposed as temporal repressor of Ad-Egr1-Luc-7.

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P 17

Development of a reliable blood test for the detection of rAAV-mediated gene doping

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The goal of our project is to develop a test for the detection of the presence of a vector delivered transgene of interest encoding a protein capable of enhancing athletic performance. Several in vivo studies have shown that vectors derived from Adeno-Associated Virus (AAV) are able to provide long-term expression of a transgene after a single
administration. It has been demonstrated especially after one intramuscular injection of a recombinant AAV (rAAV) vector that vector genomes are detectable in blood cells several months or years after injection. These results suggested that a sensitive-PCR technique can be used to detect rAAV-mediated gene doping from a simple blood sample. Our project consisted of a single intramuscular injection of macaques with two different rAAV serotypes known for their efficiency to transduce skeletal muscle cells: the rAAV1 and rAAV8. Various clinically relevant doses were tested. Each vector carried the expression cassette CMV-cmEpo-SV40polyA that codes the cynomolgous Erythropoietin gene. Our initial goal was to establish the minimal dose of vector that can cause an increase of ≥ 15% of the hematocrit compared to the normal level. Once the minimum dose was determined, an optimized PCR method was used to highlight the presence of the transgene in blood. We validated Taqman real-time PCR sensitive conditions that allow us to detect at least 5 copies of the rAAV Epo transgene in the background of endogenous genomic DNA.

Data generated in our nonhuman primates will be the basis for developing a legally defensible commercial real-time PCR assay.

**P 18**

**Different types of cell death in HEK293 in response to Ad5 infection and environmental factors**

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**Background:** HEK293 cell line and its derivatives are widely used in the production of viral vectors and recombinant proteins. Nevertheless, information of mechanisms of cell death are limited and controversial. Studies related to cell death are important in the design and optimization of strategies to improve the production process. This study aims to identify the type of cell death developed by HEK293 in a static culture in response to different stress conditions.

**Methods:** HEK293 cell line were maintained in DMEM-F12 media supplemented with 10% FBS. There were 3 trials of cell death induction: Temperature (47 °C), lack of growth factors and infection with Adenovirus 5 (Ad5). Analysis of cell growth, morphology by epifluorescence microscopy and transmission electron microscopy, determination of cell cycle by flow cytometry and the presence of p53 protein by Western blot were performed.

**Results:** Cell death related to autophagic features were observed during the experiments at high temperature (42 °C) and in those related to the lack of growth factors and control cultures. Meanwhile, HEK293 cells presented apoptotic characteristics in response to adenoviral infection with Ad5 at MOI of 50. An independent pathway of p53 was developed by this cell death because it did not show a specific pattern of DNA fragmentation and changes in the expression of it.

**Conclusion:** HEK293 culture has a mixed pattern of programmed cell death depending the type of stimulus presented in the culture. This knowledge helps to improve the infection process and the strategies of viral vector production.

**P 19**

**Robust recombinase-free system Production of HD adenoviral vectors with negligible contamination**

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Helper dependent adenoviral vectors (HDAd) are devoid of all viral coding sequences and retain only minimal viral cis-acting sequences required for vector propagation. The Cre/loxP system is the classical and most widely method for producing HDAds. In this system, the helper adenoviral packaging signal is excised rendering the helper virus unpackageable but able to trans-complement HDAd propagation. However high activity of the Cre recombinase needed for low Ad helper contamination is associated to high toxicity. To better address this issue, we have cloned attB/attP-C31 sequences flanking the packaging signal of both, human and canine adenovirus (Ad-5 and CAV-2) vectors to develop a new system based on delayed packaging of Helper-Ad respect to HDAds. Surprisingly, the mechanisms causing this delay were not related to the presence of the recombinase. Subsequent band-shift studies suggested that an unknown cell factor (in both, permissive human 293 and canine DK cells) interacts with the attB sequence and affects the correct functioning of the packaging complex. Viral cycle studies indicated a clear delay in both canine and human attB-Ad, showing its potential in different adenoviral serotypes.

In addition, production assays in 293 suspension cells showed that human HDAd vectors can be produced at high titer with negligible helper contamination levels.

Future experiments will be performed to identify the unknown factor and to optimize large-scale production/purification of HD-Ad vectors using both human and canine adenovirus for long-term gene therapy strategies.

**P 20**

**Complexation of recombinant adeno-associated virus with polyelectrolytes for gene delivery**

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**Background:** This study attempts to study the effect of complexation of recombinant adeno-associated virus (rAAV) with polyelectrolytes on gene delivery.

**Method:** A previously constructed recombinant adeno-associated virus vector, containing the furin-mutated human insulin gene downstream of rat insulin 1 promoter and the enhanced green fluorescent protein (EGFP) gene driven by the cytomegalovirus immediate early (CMVIE) promoter, was employed to investigate the roles of reactive oxygen species (ROS) in rAAV-mediated gene transfer. Huh7 hepatoma cells were transduced with the virus vector complexed with polyethyleneimine (PEI), a cationic polyelectrolyte, followed by flow cytometric analysis, and the generation of reactive oxygen species (ROS) was determined with 2,7'-dichlorodihydrofluorescein diacetate (H2DCFH-DA) or dihydrothidine (DHE).

**Results:** Flow cytometric analysis showed that complexation of rAAV with polyethyleneimine (PEI), a cationic polyelectrolyte, resulted in an enhanced uptake of rAAV. To examine the virus uptake by the cells, rAAV was labeled with the carboxyfluoresceine dye Cy3, and the enhancement of rAAV binding by PEI was confirmed by confocal fluorescence microscopy. Treatment of Huh7 cells with NaClO, on the other hand, reduced the transduction efficiency as compared to the cells treated with rAAV alone, suggesting the roles of proteoglycans in gene transfer. Complexation of rAAV with PEI increased the generation of ROS, including superoxide and hydrogen peroxide, in the rAAV-treated cells, and pretreatment of the cells with the anti-oxidants reduced the transduction efficiency of the rAAV-PEI complexes.

**Conclusion:** Results obtained in this study demonstrated the roles of ROS generation in gene delivery mediated by the rAAV-PEI complexes.

**P 22**

**Cytotoxic adenoviruses delivered into the common bile duct induce tumor regression in Ela1-myc mice**

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Gene-based anti-cancer therapies delivered by adenoviral vectors or oncolytic adenoviruses are commonly applied in vivo through intratumoral injections or by systemic delivery. However, intratumoral administration results in a limited spread of the viruses and systemic injections are often accompanied by toxic effects. In the current work we have explored the feasibility of targeting pancreatic tumors by adenoviruses through a loco-regional route. We have retrogradely injected adenoviruses through the common bile duct of wild type (wt) and Ela1-myc mice, a transgenic model of pancreatic cancer, by a technique very similar to the established endoscopic retrograde cholangiopancreatography procedure applied in humans and studied tumor targeting and anti-cancer effects. Administration of AdCMVluc or AduPARluc in wt mice revealed a peak of luciferase activity at 4 days post-injection that localized in the pancreas with no expression in other abdominal tissues such as liver or intestine. Viral administration into Ela1-myc mice resulted in lower luciferase activity when injected with AdCMVluc but not after AduPARluc administration, suggesting increased activity of the uPAR promoter in the tumors. Furthermore we evaluated the impact of AduPARTat8TK/GCV therapy in Ela1-myc mice. Primary carcinoma cells from Ela1-myc pancreatic tumours displaying a terminal cysteine, were coupled to LPEI via a heterobifunctional polyethylene glycol linker. Cellular binding and internalization was monitored by flow cytometry and laser scanning microscopy. In vitro transfections and intratumoral injections in a clinically relevant orthotopic prostate cancer model were performed with luciferase encoding plasmid.

**Results:** EGFR targeted polyplexes specifically enhanced transfection on EGFR overexpressing glioblastoma and hepatoma tumor cells, mEGF, CMY and MYI polyplexes induced EGFR activation leading to phosphorylation of Erk and Akt. In contrast, C-GE11 polyplexes led to highest transfection levels without activation of EGFR. mEGF polyplexes diminished cell surface expressed EGFR for up to four hours, while this effect was absent with C-GE11 polyplexes. Intratumorally injected C-GE11 polyplexes with a plasmid optimized for in vivo transfections were superior in inducing luciferase transgene expression as compared to untargeted ones.

**Discussion:** With this fully synthetic system, targeted, safe and efficient transgene expression is achieved in vitro and in vivo paving the way for further therapeutic studies.
P 23
Bioreducible polymer-conjugated hepatoma-specific oncolytic adenovirus for systemic cancer gene therapy
Professor Chae-Ok Yun1, Mr Pyung-Hwan Kim2, Mr Tae-il Kim3, Mr James W. Yockman3, Professor Sung Wan Kim3
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3Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah

Systemic administration of adenovirus (Ad) is hampered by a multitude of problems including immediate accumulation in the liver, resulting in a very short circulatory half-life and induction of immune responses. An alternative to overcome the limitations of Ad in vivo is to engineer a hybrid gene transfer vector system combining viral and non-viral elements. In this study, we have generated a hybrid polymer-virus vector through chemical conjugation. The surface of Ad was chemically conjugated to cationic polymer ABP (arginine-grafted bioreducible polymer) via the cross-linker, DTSSP (3,3’-Dithiobis[sulfosuccinimidylpropionate]). The size distribution and zeta potential of Ad-linker-ABP complex were increased depending on polymer ratio compared with those of naked Ad. The transduction efficiency of Ad-linker-ABP complex was enhanced compared with that of naked Ad in various cancer cell lines. In particular, a higher efficacy was observed in coxsackie and adenovirus receptor (CAR)-deficient cancer cells showing 4-fold increase of transduction efficiency. Earlier accumulation in endosomes was identified by Lysotracker staining when Ad was conjugated with ABP. When hepatoma-specific oncolytic Ad was conjugated with ABP, the Ad-linker-ABP complex elicited an enhanced cell killing effect towards liver cancer cells (Huh7 and HepG2) expressing z-fetoprotein (AFP) while sparing non-liver cancer cells. Moreover, the level of interleukin-6 (IL-6) cytokine released from macrophage cells was significantly reduced when Ad was conjugated with ABP compared with that of naked Ad, suggesting that Ad-linker-ABP complex can elude the innate immune response. Taken together, these hybrid Ad vectors may provide an interesting option for the delivery of therapeutic viruses to disseminated tumor masses by systemic administration.

P 24
miR-183 functions as a potential oncogene by targeting EGR1 and promoting tumor cell migration
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Background: MicroRNAs (miRNAs) are ~22nt non-coding RNAs that post-transcriptionally regulate gene expression. Decreased levels of many common tumor suppressors, for example, Early growth response 1 (EGR1) as well as PTEN, are implicated in a wide range of tumor types. We hypothesize that transcriptional perturbations in specific miRNA(s) observed across multiple tumor types may regulate the level of important tumor suppressors like EGR1 and PTEN in these tumor types.

Method: An integrative network analysis was used to identify potential microRNA candidates shared in synovial sarcoma (SS), rhabdomyosarcoma (RMS) and colon cancer. In vitro target validation was conducted using Luciferase reporter systems. Western blotting was used to verify protein level. Further analyses including cell migration/invasion, proliferation, cell cycle, apoptosis and live/dead assay were performed to reveal function effects of anti-miR-183.

Results: We identified that miR-183 is significantly over-expressed in all tumor types being tested (SS, RMS, and colon cancer) and their corresponding cell lines. We proved that EGR1 is a bona fide target of miR-183, at least in the 3 tumors we tested. miR-183 knockdown with anti-miR rescued the protein level of EGR1 and PTEN. Functional analyses showed that miR-183 is an important contributor to cell migration/invasion in all the cell lines tested. Interestingly, different cell lines showed different response in cell cycle shift after anti-miR-183 treatment.

Conclusion: miR-183 is a potential oncomir through regulating two tumor suppressor genes EGR1 and PTEN. The deregulation of miR-183-EGR1-PTEN network may be central to many tumor types. miR-183 may have a therapeutic role to regulate cancer metastasis.
Efficient gene transfer in human malignant B cells by a Measles virus gp pseudotyped lentiviral vector

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B-lymphocytes are attractive targets for gene therapy of diseases associated with B-cell dysfunction and also for immunotherapy by their potential to induce specific immune activation or tolerance. Moreover, malignant B-cell transduction would allow the design of gene transfer based therapies as well as biological studies. However, up to now efficient lentiviral transduction of resting healthy or malignant B-cells had not been reported.

Recently, we generated lentiviral vectors pseudotyped with measles virus glycoproteins (MV-LVs) that allowed efficient transduction of quiescent human T-cells and B-cells after a single exposure. Indeed, MV-LVs can efficiently transduce either BCR-activated as well as quiescent human B-cells. Additionally, MV-LVs are the first vector tools that allow efficient stable gene transfer into leukemic cancer B-CLL cells, one of the most prominent adult blood cancers that are blocked in G0/G1 phase of the cell cycle. MV-LVs proved also to be the candidate of choice for stable gene transfer into marginal zone lymphoma (MZL) B-cells, another B-cell malignancy and were highly superior to VSV-G-LVs. Interestingly, efficient transduction by MV-LVs co-incided with signaling-activating-molecule (SLAM) expression, one of the measles virus receptors. A poor gene transfer profile for VSV-G-LVs was also evident when the cells were pre-stimulated through the B-cell receptor while MV-LV transduction of B-cells augmented significantly for the 12 B-CLL and 6 MZL donors evaluated. Summarizing, MV-LVs overcome the intrinsic inability to infect quiescent post-mitotic cells and susceptibility to innate antiviral defenses that exist in normal cells but are inactivated in many cancers, MLV-based RCR vector replication has proven to be highly tumor-selective. We have previously demonstrated that RCR vectors are capable of highly efficient replication in tumor cells, and we have found significantly enhanced survival benefit in a variety of cancer models in vivo when RCR vectors are employed for delivery of produg activator genes. In the present study, we tested a newly developed RCR vector with modified virus backbone sequences and a codon-optimized cytosine deaminase produg activator gene (Toca511), which has now entered Phase I/II clinical trials for glioblastoma. We examined the transduction efficiency of this newly developed RCR vector in U87 human glioblastoma models and confirmed that >99% tumor transduction could be achieved over time. Next, therapeutic efficacy was evaluated, and the minimum effective doses of 5FC produg and Toca511 vector determined. Significant survival benefit was seen after 5FC treatment compared to control groups, even after injecting only 1x10⁶ total units of RCR vector (p = 0.0058). These data support the use of Toca511 to treat highly aggressive solid tumors such as glioblastoma. Now, with the use of RCR vectors, the original promise of retrovirus-mediated gene therapy strategies for cancer may finally be fulfilled.

Preclinical Studies Support the Clinical Utility of RCR Vectors for Cancer Gene Therapy

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Unique among replicating viruses being developed as oncolytic agents, replication-competent retroviruses (RCR) replicate without immediate lysis of host cells and maintain viral persistence through stable integration. Due to its intrinsic inability to infect quiescent post-mitotic cells and susceptibility to innate antiviral defenses that exist in normal cells but are inactivated in many cancers, MLV-based RCR vector replication has proven to be highly tumor-selective. We have previously demonstrated that RCR vectors are capable of highly efficient replication in tumor cells, and we have found significantly enhanced survival benefit in a variety of cancer models in vivo when RCR vectors are employed for delivery of produg activator genes. In the present study, we tested a newly developed RCR vector with modified virus backbone sequences and a codon-optimized cytosine deaminase produg activator gene (Toca511), which has now entered Phase I/II clinical trials for glioblastoma. We examined the transduction efficiency of this newly developed RCR vector in U87 human glioblastoma models and confirmed that >99% tumor transduction could be achieved over time. Next, therapeutic efficacy was evaluated, and the minimum effective doses of 5FC produg and Toca511 vector determined. Significant survival benefit was seen after 5FC treatment compared to control groups, even after injecting only 1x10⁶ total units of RCR vector (p = 0.0058). These data support the use of Toca511 to treat highly aggressive solid tumors such as glioblastoma. Now, with the use of RCR vectors, the original promise of retrovirus-mediated gene therapy strategies for cancer may finally be fulfilled.

P 28

Cell-specific targeting using a Sindbis-pseudotyped lentiviral vector expressing anti-FITC scFv

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A crucial factor for successful gene therapy is the efficacy of specific gene transfer, which is usually done by lentiviral vectors. Fusion and specificity of lentiviral vectors must be provided by envelope glycoprotein domains. Sindbis envelope can pseudotype lentiviral particles and display exogenous protein domains. Previous results demonstrated that Sindbis envelope can accommodate anti-receptor single-chain antibodies (scFv) and target cell-specific viral infection. Additionally, it was shown that protein A-chimeric Sindbis envelope can specifically target cells immunolabelled with anti-receptor IgG via Fc recognition. However, these strategies might present some problems for in vivo applications, since there may be non-specific reactions with plasma antibodies and the need for cloning a receptor specific antibody each time a new molecule is to be targeted. To overcome these problems we developed a new lentiviral vector capable of
transducing several cell types in a specific manner without
the above constraints, that consists of a chimeric scFv-Sindbis
virus envelope that binds fluorescein (FITC) with high affin-
ity and consequently recognize FITC-conjugated proteins.
Using this targeting strategy we were able, in vitro, to target
efficiently and specifically cells expressing a receptor labelled
by a FITC-conjugated antibody. Moreover, we could specifi-
cially kill those transduced cells using an HSVtk/GCV suicide
gene strategy. We are currently testing the in vivo efficiency of
this gene therapy proposal in a mouse model of T-cell acute
lymphoblastic leukaemia, although the strategy herein pro-
posed has the potential to be applied to a broad range of
diseases.

P 29

Human prostate transglutaminase, a prostate specific gene
with potential applications for gene therapy
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Background: Targeted gene therapy offers an alternative
means to kill prostate cancer cells. One method to achieve
efficient targeting is the use of tissue specific promoters that
drive expression of therapeutic genes only in the desired
tissue. To make use of such regulatory sequences more
knowledge about their control is needed. Human prostate
transglutaminase (hTGp) is a highly prostate specific gene,
but the mechanisms that control its expression are not well
understood.

Results: Androgen had previously been shown to have a
positive effect on hTGp expression, but the hTGp pro-

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ter contains not only multiple androgen receptor

binding sites but also retinoic acid responsive elements
(RAREs). atRA exerted a major effect on hTGp expression
while androgen played a minor and indeed negative role,
in contrast to previous reports. The RAREs that activate
hTGp transcription were located within the promoter se-
c
t
quence in a region of 4.5kb. Cells were treated with

TTNPB, a synthetic retinoid that

activates RAR’s, showing a

major role for RAR gamma in hTGp transcription after

atRA treatment.

Conclusion: hTGp transcriptional regulation is dependent
primarily on atRA but not on androgen. Control is exerted by
the RAR’s, mainly by RAR gamma. This study shows that
even when most prostate specific genes are androgen regu-
lated, there are more elements capable of controlling their
regulation. By investigating the whole set of factors that
control prostate specificity, we will be able to make use of
these regulatory elements to express therapeutic genes in the
prostate.

P 30

Clinical Effect of rAd-p53 Combined with FOLFOX4
on the Prognosis of Advanced Colorectal Cancer
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110042, Liao-ning Province, China

Background: This research was to determine the safety and
prognosis of recombinant adenovirus p53(Gendicine®) in-
jection combined with FOLFOX4 on the patients with ad-
vanced colorectal cancer.

Methods: Twenty-two patients were recruited and ana-
lyzed in the study from July 2008 and April 2010. Gendicine®
(1x10 12VP) was injected into tumorally, intravenously or
intraperitoneally, to all the patients once a week for two
weeks, and then followed by systemic chemotheraphy
(FOLFOX4 regimen) as one cycle. 12 cases with local ad-
vanced disease then received operation.

Results: All patients were alive during the follow-up
(68 ~ 766 days). KPS Score were evaluated in the patients
after treatment as 68.2%(15) in 90-100, 22.7%(5 cases) in 90-
70, and 9.1%(2 cases) in 70-50, compared with that of 95.1%
(13 cases) in 90-70, 18.2%(4 cases) in 70-50, 18.2%(4 cases) in
50-30, and 4.5%(1 case) in 30-10 before the treatment. Self-
limiting fever was reported in 10 patients (45.5%) and
nasea and diarrhea in 1 (4.5%) after Gendicine® administra-
tion. 1 patient was assessed as complete respose and 4 as
partial response in the 5 patients with recurrent disease after
the treatment, while 1 was assessed as partial response and 2
as stie disease in the 3 patients with liver metastasis. No
recurrent symptom were dignosed in the palliative opera-
tion group.

Conclusion: Recombinant adenovirus-p53 combined with
FOLFOX4 provides a safe and effective choice of treatment for
prolonging progression-free survival time and improves the
quality of life of patients with local recurrent and/or meta-
static colorectal cancer.

P 31

Adenoviruses expressing interferon-lambda induced
apoptosis to the infected human tumors and produce
anti-tumor effects in vivo
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Recently identified cytokines, IL-28A, IL-28B and IL-29,
belong to type III interferon (IFN), IFN-lambda, but the pre-
cise biological properties remain uncharacterized. IFN-
lambda could have a distinct activity other than type I IFN,
IFN-alpha and IFN-beta despite the similar signal cascades.
We found that human esophageal carcinoma cells, which
expressed the receptor complex consisting of IL-10Beta and

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IL-28Ralpaha, were susceptible for IFN-lambda due to either cell cycle arrest or apoptosis. The cell cycle arrest was accompanied by up-regulated p21 expression and dephosphorylated pRB protein. Apoptosis was evidenced by increased sub-G1 populations and annexin-V-positive cells. We constructed adenoviruses bearing the IFN-lambda gene with a fiber-knob modification (Ad/IFN-lambda) and investigated the anti-tumor effects. Transduction of the carcinoma cells with Ad/IFN-lambda increased expressions of cleaved caspase-9, Bax and cytoplasmic cytochrome C, suggesting that IFN-lambda activated the mitochondria-mediated apoptosis pathway. We observed loss of tumorigenicity of esophageal carcinoma YES-2 cells in nude mice when they were infected with Ad/IFN-lambda. Human fibroblasts, negative for the IL-28-lambda expression, were resistant to IFN-lambda but Ad/IFN-lambda-infected fibroblasts induced apoptosis of co-cultured YES-2 cells and retarded the tumor growth in vivo. Further analyses showed that increased NK cells activity and anti-angiogenesis were not involved in the anti-tumor effects. These data collectively suggest that Ad/IFN-lambda induce apoptosis of the infected receptor-positive tumor cells and fibroblasts-mediated delivery of IFN-lambda is a possible strategy for cancer therapy by inducing direct cell death of the target carcinoma.

**P 32**

**Use of the minimalistic nonviral MIDGE expression system for improved in vitro and in vivo transfer**

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Great efforts are made for improvement of nonviral vectors to reduce size, proportion of unnecessary sequences to increase transfer- and expression efficiencies. The minimalistic immunologically defined gene expression (Mologen, Berlin, Germany) vector system provides a nonviral linear DNA molecule that is depleted of bacterial origin, the replication backbone sequences, CpG motifs and the antibiotic resistance sequences. Therefore, MIDGE vectors are significantly reduced in size. In our in vitro experiments using equimolar amounts of luciferase expressing plasmid or MIDGE vector we observed 3 to 13-fold increased expression by the MIDGE vector in transducted human colon carcinoma cell lines HCT116 and SW480, and melanoma cell lines A375, MeWo and SKME-28. FACS analysis demonstrated improved transfection efficiency of MIDGE vectors in these tumor cell lines. To analyze applicability of MIDGE-based vector for therapeutic gene expression, we analyzed the tumor necrosis factor alpha (TNF) expressing MIDGE in human HCT116 and A375 cells in vitro. The MIDGE vector generated significantly higher TNF-levels in comparison to plasmid-mediated expression. The use of the TNF-expressing MIDGE vector for nonviral in vivo gene therapy approved its effectiveness. The intratumoral nonviral jet-injection in vivo transfer of the TNF-expressing MIDGE vector into A375 melanomas resulted in efficient high level cytokine expression. We show, that this high level TNF-expression leads to chemosensitization of melanoma in vivo for improved chemotherapy with vinde-sine. This is reflected by significant tumor growth inhibition. The study demonstrates applicability of MIDGE vector based gene therapy for local gene therapy approaches.

**P 33**

**Self-inactivating retroviral vectors expressing human antiangiogenic genes for cancer gene therapy**

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Most of the solid tumors growth and metastasis critically depends on angiogenesis. Endostatin and angiostatin are potent angiogenesis inhibitors, which suppress endothelial cell proliferation and migration, tumor vascularization and slow down the growth of tumors. Previously we have successfully used mesenchymal stromal cells isolated from human adipose tissue (AT-MSCs) as delivery vehicles for tumor-targeted suicide gene therapy of mouse xenograft. Now the anti-angiogenic genes were used as therapeutic genes.

In order to improve and prolong the expression of angio-static genes in tumor area, new safer self-inactivating bicis-tronic retroviral vectors were constructed. The human endostatinXVIII and the synthetic fusion endostatin-angios- tatin genes were cloned into these vectors by standard cloning techniques. Replication-deficient retroviral particles prepared by transient transfection in appropriate packaging cell line were used for infection of target cells, human HT-29 colon carcinoma cell line and AT-MSCs. HT-29-Endo, HT-29-Endo-Angio, AT-MSCs-Endo and AT-MSCs-Endo-Angio cell cultures were prepared. Integration of provirus containing therapeutic genes into the cell genome and gene expression was confirmed by real-time PCR. Biological effect of secreted antiangiogenic proteins was confirmed against the endothelial cell proliferation. Therapeutic cells – genetically engineered AT-MSCs-Endo and AT-MSCs-Endo-Angio were tested for inhibitory effect on tumor cells in vivo on mouse xenograft tumors. Endostatin secretion was proved by ELISA and tumor growth inhibition was achieved.

**P 34**

**rAd-p53 combining transcatheter arterial embolization with gelatin sponge particles in HCC**

Professor ZH Yue Wei1

**Background:** Hepatocellular carcinoma(HCC) is one of the most common cancer worldwide. This study aims to evaluate the efficacy of rAd-p53 gene therapy combining transcatheter arterial embolization using gelatin sponge (GS) particles in treatment of HCC.
**P 35**

**Development of non-viral gene delivery system using CXCR4 ligand-conjugated cross-linking peptides**

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Application DNA as therapeutics requires cell-specific targeting which can be achieved by modification of vehicles with a ligand for certain receptor. CXCR4 is a receptor of chemokine SDF-1 and is expressed on some types of cancer and stem cells. Cystein-flanked peptides which are capable to form DNA condensates because of cross-linking are considered to be a perspective group of non-viral vehicles. The aim of this project is to characterize a CXCR4 ligand-conjugated cross-linking peptides as a receptor-mediated gene delivery system.

We studied four types of DNA/peptide complexes with different ratio between cystein-flanked arginine-rich peptide modified with N-terminal sequence of the chemokine SDF-1 (residues 1–17) and peptide (CHRRRRRRHC) as 100%, 50%, 10% and 0% (ligand-free control). The peptides modification with histidine residues facilitates the escape of DNA from endosomes. Template polymerization of cross-linking peptides was used to form DNA/peptide complexes. EtBr exclusion assay proved peptides ability to condense DNA. Transfection activity was studied in two CXCR4(+) cell lines (A172 and HeLa) and CXCR4(-) cell lines (CHO) with lacZ as a reporter gene. Transfectional efficacy of ligand-conjugated vehicles in CXCR4(+) HeLa and A172 cells was 10-times higher compared to control peptide. The level of transgene expression with ligand-conjugated peptides in low N/P ratios was comparable with the efficacy of PEI. Otherwise transfection efficacy of ligand-conjugated peptides on CXCR4(-) CHO cells was lower than in control PEI. Thus these results demonstrate that ligand-conjugated vehicles reported can be a perspective approach for effective gene delivery to CXCR4 expressing cells.

This work was supported by Carl Zeiss fellowship and RFBR grant 10-04-01236-a.

**P 36**

**Inclusion of miR122 targets in wild-type serotype 5 adenovirus to ablate liver replication**

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Adenovirus-based oncolytic therapies present important limitations regarding tissue selectivity and toxicity, an issue well-documented for the commonly used adenovirus serotype 5 (Ad5).

Basic research has shown a specific micro-RNA (miR) pattern for many tissues, like the virtually exclusive expression of miR122 by liver. We have shown that this feature can be utilized to selectively ablate adenovirus replication in cells of hepatic origin by genomic insertion of miR122 targets to reduce E1A gene expression.

In this study we have examined in more detail the optimal number of miR122 targets, studied the cell type-specific inhibitory potential of miR122 via careful cell viability analyses, and extended these studies to more relevant experimental systems.

These results will provide a rational basis for proceeding towards ex vivo and in vivo studies to test the applicability of this system for clinical gene therapy purposes.

**P 37**

**Comparative evaluation of replicating retroviral vectors carrying a suicide gene in multiple preclinical in vivo models of glioblastoma**

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Adenovirus-based oncolytic therapies present important limitations regarding tissue selectivity and toxicity, an issue well-documented for the commonly used adenovirus serotype 5 (Ad5).

Basic research has shown a specific micro-RNA (miR) pattern for many tissues, like the virtually exclusive expression of miR122 by liver. We have shown that this feature can be utilized to selectively ablate adenovirus replication in cells of hepatic origin by genomic insertion of miR122 targets to reduce E1A gene expression.

In this study we have examined in more detail the optimal number of miR122 targets, studied the cell type-specific inhibitory potential of miR122 via careful cell viability analyses, and extended these studies to more relevant experimental systems.

These results will provide a rational basis for proceeding towards ex vivo and in vivo studies to test the applicability of this system for clinical gene therapy purposes.
Targeting therapeutic drug using Superparamagnetic iron oxide nanoparticles (SPION) as carrier systems gained increased attention in the biomedical field. Generally, particles carrying different core sizes and various coatings were used in both vitro and in vivo. According to their desired purpose, they were also additionally functionalized with drugs, peptides or proteins of interest. The p53 gene is a tumor suppressor and genome defense. It plays an important role in proliferation control, apoptosis, and cell death in a model of non-small cell lung cancer. Plasmid delivered S. typhi flagellin induces inflammation and cell death in a model of non-small cell lung cancer.

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Magnetic field derived aerosol delivery (MagnetoSol) facilitates targeted gene delivery to distinct lung regions

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Severe lung diseases, such as lung cancer, are usually treated by intravenous administration of drugs. This application route however requires relatively high drug amounts in order to maintain a therapeutically effective drug level in the lungs. This circumstance leads to severe side effects, especially when cytotoxic drugs are applied to the patient. Aerosol application however has been shown to increase drug levels in the lungs, while reducing systemic side effects, because less of the therapeutic agent is needed. Nevertheless the lungs represent a delicate organ, predisposed for inflammation and irritation. In order to reduce side effects and coincidentally increase drug concentrations in distinct regions of the lungs we applied Superparamagnetic Iron Nanoparticles (SPIONs) and Fluorescein-Natrium as an aerosol to conscious mice, which were exposed to a magnetic field during inhalation. This experiment resulted in a 3-fold increase of Fluorescein-Natrium in the lungs in total and to a 3-fold increase of iron in the right lung lobe, when the mice were exposed a magnet field on the right chest during inhalation. Moreover we demonstrated magnetic field gene expression in one distinct lung lobe under the influence of a magnetic field during the inhalation procedure. Summarizing we postulate that MagnetoSols could be an effective tool for the gene therapeutic treatment of lung diseases, such as lung cancer or tuberculosis.

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**P 40**

Plasmid delivered S. typhi flagellin induces inflammation and cell death in a model of non-small cell lung cancer

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Tumor cells, including those of non-small cell lung cancer, developed numerous mechanism impairing maturation of dendritic cells (DCs). The delivery of stimulatory signals to dendritic cells in the tumor microenvironment is believed to be an effective means to break tumor-induced tolerance. The work presented here evaluates the immunostimulatory properties of a TLR5 ligand Salmonella typhi flagellin (FlfC). We developed a novel plasmid vector containing a cell membrane-displayed humanized FlfC ORF under control of tumor-specific two-step transcriptional activation system. In vitro, transduction of A549 tumor cells with plasmid coding for humanized Salmonella flagellin induced the maturation of human monocyte-derived DCs in co-cultures.

Two groups of C57BL/6 mice bearing a subcutaneous syngenic Lewis lung cancer (LLC) tumor were intratumorally injected with in vivo-JetPEI and FlfC plasmid or as a control with in vivo JetPEI and phosphate-buffered saline. Four administrations were performed within 1 week. Tumor volumes and survival were monitored for 6 weeks. Plasmid injections delayed the growth of implanted LLC tumors and significantly improved survival rates. Histological examination of plasmid-injected tumors revealed lymphocyte infiltration and significant necrosis. These results suggest that plasmid-delivered FlfC could be effective immunoadjuvant for cancer immunotherapy.

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Randomized and controlled clinical trial for thyroid cancer treated by rAd-p53

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Background: TCs are insensitive to radiotherapy and chemotherapy. Surgery is the main options for TC. Studies show there are p53 genetic mutations in TC with various manifestations. This study aims to discuss the safety and efficacy of rAd-p53 gene therapy to treat TC in combination with surgery and other traditional methods.

Method: This is a randomized and controlled clinical trial. 22 patients were enrolled into the Trial Group and received rAd-p53 plus surgery, or surgery and 131I or surgery and radiation, or surgery, radiation and 131I. 18 patients received p53 local injections, 2 received intervenous infusion, and 2 received administration by the both methods. Another 22 patients were enrolled into the Control Group.

Results: All patients in the Trial Group got fever and muscular soreness after the 1st injection of rAd-p53, which were all tolerable and disappeared after 2-3 times injections without special treatment. Other side effects related to rAd-p53 injection were arthralgia in 4, nausea in 2, and numbness of tongue tip in 1, which all dissapeared without special treatment. ECOG Scores decreased in different degree in all the patients in the Trial Group, which were better than that of the control group. 2 patients in the study were sensitive to radiotherapy, which might be sensitized by rAd-p53. In some patients, tumors were softened after 2 times injections.

Conclusion: The study shows that it is safe to use rAd-p53 in TC patients with main adverse reaction of flu symptoms like fever and muscular soreness. It suggests that rAd-p53 may change the insensitivity of TC cell to external radiotherapy.

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Chitosan Mediated VEGF shRNA Transfer for the Treatment of Breast Cancer

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Background: Angiogenesis is an exciting and promising area of anti-cancer drug research. VEGF and its receptors is the most extensively investigated system in tumor angiogenesis. RNA interference includes strategies that result in the down-regulation of the expression of a target gene at the post-transcriptional level using RNAi agents. The aim of this study was to investigate whether intratumoral injection of chitosan/shVEGF complexes could suppress VEGF expression in the breast cancer model of rats.

Method: Chitosan/shVEGF complexes were prepared in 2/1 ratio. For tumor formation, Sprague Dawley rats in the experiment were injected intraperitonally 50 mg/kg NMU (Sigma) on the 45-50 day of age. After tumor formation, chitosan/shVEGF complexes were injected into tumor-bearing rats. Tumor progression was reported. VEGF expression were examined by immunohistochemistry, RT-PCR and western blot.

Results: Tumor volume decreased at the end of experiments after chitosan/shVEGF complexes treatment (96%). VEGF immunexpression was reduced approximately 67.4% by i.t applied complexes. VEGF-A mRNA expression was also reduced approximately 84.60%. Free shRNA injection indicated lower tumor suppression. The western blotting results correlated with the RT-PCR and tumor volume measurements.

Conclusion: The data suggest that chitosan/shVEGF complexes can be used to inhibit tumor growth and VEGF expression in breast carcinoma model of rats. Chitosan is very suitable and effective gene delivery system for VEGF shRNA in breast cancer models.

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Non-pathogenic bacteria as cell therapy vectors for cancer treatment

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The ideal cancer treatment would eradicate tumour cells selectively with minimum side effects on normal tissue. Bacteria have emerged as gene vectors with natural tumour specificity, capable of homing to tumours and replicating locally to high levels when systemically administered. Pathogenic, invasive species have been utilized to deliver plasmids intracellularly to tumour cells, and some genera possess oncolytic capability, such as Clostridium and Salmonella. However, their inherent toxicity has outweighed therapeutic responses in patients, despite efforts to reduce toxicity through genetic modification.

A promising alternative exploits non-pathogenic bacteria. Non-invasive, apathogenic species expressing heterologous genes can secrete therapeutic proteins locally within the tumour (analogous to Mesenchymal Stem Cell therapeutic strategies.) We are investigating a range of commensal bacteria (natural inhabitants of the human GIT, and often employed as probiotics). Our group has developed imaging systems using luminescent reporter gene (lux) tagging of various bacteria permitting real-time visualization of vector, in subcutaneous and orthotopic murine models (melanoma, breast, lung, glioma).

We have also demonstrated that certain species (Bifidobacterium breve) are capable of trafficking to systemic tumours following oral administration, with equal efficiency to intravenous injection. Our studies indicate a low level, delayed anti-vector immune response within tumours, with no bacterial clearance from tumours for up to 1 month post administration. Through engineering of secreting constructs, these replication competent bacteria can mediate high-level production of soluble agents within tumour masses, presenting a powerful and safe approach to specific gene/cell therapy of primary tumours and secondary metastases.

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p53 Gene Have a Role in Phenotype Reversion of Melanoma in vitro

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Background: When accompanied with abnormal p53 expression, Melanoma suffered a higher risk of recurrence and metastasis. We evaluate the optimum multiplicity of infection (MOI) and transfection efficiency of recombinant Ad-p53 gene to malignant melanoma cell line?A375?in vitro, and observe the effect of exogenous p53 gene on biologic phenotype of A375 cells.

Methods: A375 cells in logarithmic growth phase were seeded onto six-well plate. After 24h, the cells attached and Ad-GFP solution were added into the medium with MOI 0 to 500. The transfection efficiency of rAd-p53, expression of exogenous p53 gene, proliferation inhibition rate of each group, cell cycle and apoptosis, as well as the expression of P21CIP/WAF, bcl-2, MMF-9 before and after the transfection, were all detected or determined.

Results: Exogenous p53 gene was successfully transfected into A375 cells with the transfection efficiency more than 95% and inhibited A375 cell proliferation significantly with MOI ranged from 100 to 500. G1 stage increasing and S stage decreasing were detected after transfection. The percentage of apoptosis increased 24h after transfection and apoptotic peak was obvious (P < 0.01). The expression of p53 mRNA, P21CIP/WAF mRNA and P21CIP/WAF protein increased obviously, while bcl-2 mRNA and protein decreased 72h after transfection when compared with the control group.

Conclusions: Exogenous wild type p53 gene could be effectively transfected into malignant melanoma cell line and strongly inhibited cell proliferation, altered the cell cycle and induced cell apoptosis. p21CIP/WAF and bcl-2 were partly responsible for the inhibition of A375 cells growth.

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Chitosan nanoparticles containing shRNA targeting VEGF as cancer therapeutic

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Background: RNAi is a highly promising technology for gene therapy application in treatment of different cancers and preliminary results of trials with siRNAs targeted against VEGF for treatment of breast cancer are encouraging. However, RNAi is challenged by the method of delivery and stability in vivo. Therefore, the efficiency of carrier system or delivery is an important subject in this technology. The aims of this study are to evaluate shRNA-loaded (shRNA-expressing pDNA targeting vascular endothelial growth factor-A (VEGF-A)) chitosan nanoparticles for cellular uptake and to investigate the silencing effect of siRNA using human breast cancer cells in vitro.

Method: Chitosan nanoparticles were prepared using ionotropic gelation method. Physicochemical and morphological characterization of nanoparticles were studied. Cell uptake of nanoparticles were investigated using fluorescence microscope. The VEGF silencing activity of shRNA-loaded chitosan nanoparticles were measured by ELISA method in different cell lines; MCF-7, MDA-MB-435, Hela, HEK293.

Results: Nanoparticles protected the shRNA against the degradative effects of serum and enzyme. As a result of in vitro transfection studies made in different cell lines with nanoparticles containing shRNA in different amounts, the highest gene inhibition (56%) was measured in MCF-7 after transfection while the lowest gene inhibition (48%) was observed in MDA-MB435.
Conclusion: Chitosan nanoparticles show high potential as carrier for safer and cost effective shRNA delivery in breast cancer.

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Comparison of Bystander Effect Mediated by MSC Expressing Cytosine Deaminase or HSV Thymidine Kinase Towards Tumor Cells

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Background: Adipose tissue-derived mesenchymal stromal cells (AT-MSC) retrovirally transduced with either cytosine deaminase-phosphoribosyl transferase (CD-MSC) or Herpes simplex virus thymidine kinase (TK-MSC) in combination with appropriate prodrug can be used for cancer gene therapy. We aimed to compare the efficiency of the above mentioned systems and to mechanisms responsible for sensitivity of different tumor cells to cell-mediated prodrug therapy. We used a fluorometric assay in order to determine the extent of bystander effect mediated by engineered AT-MSC toward various tumor cells.

Method: Tumor cells stably expressing the green fluorescent protein were cocultured with therapeutic AT-MSC in the presence of ganciclovir (GCV) or 5-fluorocytosine (5-FC). Gap junctional intercellular communication (GJIC) was examined using flow cytometry and expression of genes responsible for drug metabolism and efflux was detected by RT-PCR.

Results: We observed significant differences in sensitivity of tested tumor cell lines. Both therapeutic systems were effective on glioblastoma cells 8-MG-BA, CD-MSC exhibited strong bystander effect on melanoma A375 cells. GJIC defective HeLa cells responded neither TK-MSC/GCV nor CD-MSC/5-FC therapy. In spite of functional GJIC between A375 cells and AT-MSC, this cell line was resistant to TK-MSC/GCV treatment. Cell line MDA-MB-231, resistant to CD-MSC/5-FC system, was very sensitive to TK-MSC/GCV treatment.

Conclusion: Taken together, neither CD-MSC/5-FC nor TK-MSC/GCV approach should be considered a universal tool for cancer therapy. Although additional studies are necessary, it is obvious that intercellular communication, multidrug resistance proteins and enzymes included in drug metabolism contribute to bystander effect efficiency of cancer gene therapy mediated by AT-MSC.

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Histidine-rich amphipathic peptides promote efficient delivery of nucleic acids into mammalian cells

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Besides being a useful tool in research, gene transfer has a high potential as treatment for a variety of genetic and acquired diseases. However, in order to enable a gene to become a pharmaceutical, efficient and safe methods of delivery have to be developed. We found that cationic amphipath histidine-rich peptide antibiotics can efficiently deliver DNA into mammalian cells. Our lead compound, LAH4 (KKAL LALIHHLALHLALALKKA), demonstrated in vitro transfection efficiencies comparable to those of commercially available reagents. Synthesis and evaluation of LAH mutants provided evidence that the transfection efficiency depends on the number and positioning of histidine residues in the peptide as well as on the pH at which the in-plane to transmembrane transition takes place. Our results also suggest a mechanism of selective destabilization by LAH4 of anionic lipids in the membranes of cells during transfection. Further results indicate that acidification of the endosome results in high local concentrations of free peptide in this organelle. These peptides become then available to interact with the endosomal membranes and thereby are responsible for the delivery of the plasmid DNA complex to the cytoplasm. When these data are taken together, they indicate a dual role of the peptide during the transfection process, namely DNA complexation and membrane permeabilization. Finally, we will report that peptides of the LAH family are efficient siRNA delivery vehicles.

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A Case Report of Recurrent and Metastatic Rectal Cancer Responding to rad-p53 and FOLFOX4

Professor ZH Zhang Guo

Background: Locally recurrent and metastatic disease carries a poor prognosis and remains the most frequent cause of death due to colorectal adenocarcinoma. Although aggressive operation is the only curative operation, it is only feasible in a few cases. Patients with nonresectable recurrent and metastatic tumors are always treated with palliative modalities, and long-term survival is limited. Gene therapy has been used as a possible treatment modality for various malignant diseases.

Methods: The patients presented here is a 57-year-old man who was operated with APR for rectal cancer 3 years ago, and was diagnosed locoregional recurrence and multiple lung metastases, accompanied with ureteric dilatation, pelvis effusion, acute renal failure, hyperkalaemia, oedema of lower extremity (KPS < 20). The patient was treated with p53 gene (totally 20 × 1015VP) (Gendicine®, Shenzhen Sibino Gentech, China) combined with FOLFOX for ten cycles.

Results: During a 13 months follow-up period, the patient was alive in good condition (KPS > 90). Obstruction of urinary passage was relieved and a steady improvement in

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plasma biochemistry toward normal was observed. We also found that the locally recurrent disease was necrosis and marked lung metastatic tumor shrinkage or disappeared, and sustained normal CEA and liver function. In the patient treated with Gendicine + FOLFOX, there was no severe side effect.

Conclusion: It can be concluded that Gendicine® combined with FOLFOX chemotherapy is an effective and safe strategy for the treatment of patient with advanced rectal cancer.

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Enhancing the Efficacy of TCR Gene Therapy by Co-Transfer of CD3 Molecules

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T cell receptor (TCR) gene transfer is an efficient strategy to redirect the specificity of T cells. Efficient cell surface TCR expression requires the formation of a stable TCR-CD3 complex. CD3 may be rate-limiting for the expression of TCR, which may result in reduced cell surface expression of the introduced TCR. The increased expression of TCR may improve the functional avidity of the genetically modified T cells. Co-transfer of CD3 and TCR into a murine T cell line enhanced expression of an Influenza-specific TCR (NP-specific), resulting in increased NP tetramer binding compared to cells transduced with TCR only. We found that transduction of both CD8+ and CD4+ T cells with MHC class I restricted TCR results in antigen-specific function of these cells in vitro. The co-transfer of CD3 into these cells increases expression of the introduced TCR and the functional avidity of these cells to their specific peptide. Data suggest that TCR-CD3 co-transduced T cells are preferably expanded compared to the TCR-only transduced T cells in response to an NP-expressing tumour, and are capable of memory response formation upon secondary challenge with tumour in vivo. Furthermore, TCR-CD3 T cells eradicate tumour faster than TCR-only transduced cells. Using in vivo imaging, we found that increased efficiency in tumour eradication may be linked to faster tumour infiltration by TCR-CD3 co-transduced T cells compared to TCR-only transduced T cells. The data suggest that co-transduction of CD8+ with TCR and CD3 enhances functional avidity and anti-tumour immunity of cells without resulting in toxicity in vivo.

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Integration-Deficient tetracistronic Lentiviral vector engineered SMART-DC vaccine against Melanoma

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Background: Novel approaches facilitating consistent and potent Dendritic cell (DC) production for large scale vaccination trials are warranted. We previously demonstrated, human and mouse DC precursors programmed with lentiviral vectors (LV) encoding growth factors (GM-CSF, IL-4) and tumor antigen (TRP-2) led to induction of highly viable and therapeutically potent “SMART-DCs” (self-differentiated Myeloid-derived Antigen-presenting-cells Reactive against Tumors) in a B-16 melanoma mouse model (Koya et al, 2007). Their biosafety was enhanced 2-fold by introducing a suicide gene (HSV-TK) and a cell-specific MHCII promoter (Pincha et al, submitted). In this study, we tested, integration-deficient (ID) packaging system to program even safer SMART-DCs with state of art tetracistronic LVs (HSV-TK, GMCSF, IL-4, TRP2) under both CMV and MHCII promoters. Additionally, we explored preconditioning with Flt3L to expand and direct hematopoietic stem cell precursors toward DC differentiation.

Methods and Results: Mouse bone marrow cells were preconditioned (8 hours) + / – Flt3L and transduced with tetracistronic ID-LVs (CMV and MHCII), lead to autonomous self differentiation into DCs. Cytopsin and FACS analysis of surface markers: CD11c, CD11b and MHCII were performed every 7 days to confirm DC phenotype over a period of 3 weeks. Highest purity was obtained at day 14 (upto 65%). C57BL6 mice vaccinated with all SMART-DC groups protected (upto 40%) mice against melanoma. Flt3L (+) groups doubled SMART-DC yield in vitro and prolonged survival in vivo.

Future: In summary, we demonstrate rationale programming of SMART-DCs with tetracistronic ID-LVs capable of protecting mice against melanoma. We are currently exploring intravenous administration of tetracistronic ID-LVs as direct vaccines.

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Targeting the epidermal growth factor receptor (HER) family by T cell receptor gene-modified T lymphocytes

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HER2 is over-expressed in 25–40% of all breast cancers and in a variety of other tumors. Due to the selective over-expression in malignant tissue, HER2 is considered one of the most attractive targets for therapeutic interventions.

In this study, we isolated the TCR genes of a HER2-reactive, allo-HLA-A2-restricted CTL clone and introduced the genes into a retroviral vector. Efficient cell surface expression
of the TCR and improved functional avidity of the gene-modified T cells were achieved after murinization (replacement of the human TCR constant regions by mouse counterparts), codon-optimization and application of the P2A gene linker (HER2-TCR-opt). Thus, the TCR expression in transduced T cells increased from 1.5% to 41% measured by A2/HER2-multimer staining. The ability to secrete IFN-γ of HER2-TCR-opt-transduced T cells was comparable to that of the CTL clone, which showed a half maximum IFN-γ secretion at $10^{-7}$ M towards HER2 peptide-loaded T2 cells. Furthermore, HER2-TCR-opt-transduced T cells lysed HER2-expressing tumor cell lines as efficient as the parental clone (lysis of 58% at an E:T of 30:1). The TCR showed a cross-reactivity to HER3 and HER4 that was similar to the parental CTL clone. Extensive testing with HER2/3/4 negative cell lines did not reveal any further epitopes that were recognized by the TCR.

Our results contribute to the development of a TCR-based approach for the treatment of HER2-positive breast cancer, as well as of other malignancies expressing HER2, HER3 and/or HER4.

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**Adjuvant immunomagnetofection prolongs relapse-free survival of fibrosarcoma bearing cats**

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Feline fibrosarcoma is an everyday challenge in veterinary practice. Despite aggressive pre- or post-operative treatment it has a high relapse rate of approx. 75% within 6 months after surgical resection. To obtain a better therapeutic outcome, novel strategies are necessary. Hence, we established immunostimulatory therapy by magnetofection. Here we report preliminary results from a comparative clinical study where the genes for feline GM-CSF, IFN-γ and IL-2, feline GM-CSF alone or human GM-CSF were administered. The study design is prospective, randomized, placebo-controlled (= standard therapy) and includes four arms: (1) standard therapy, i.e. surgery alone; (2) nonviral magnetofection of the triple-combination of feline GM-CSF, IFN-γ and IL-2 genes into the tumor before surgery or nonviral administration by magnetofection of feline (3) or human GM-CSF (4) gene alone. Preliminary clinical endpoints of the studies are relapse-free survival. Nonviral magnetofection, a procedure developed in our laboratory, is gene therapy by plasmids associated with magnetic nanoparticles under the influence of a magnetic field. The magnetic field was applied to achieve improved retention of the injected vector dose in the tumor. All gene-therapeutic treatments were well tolerated and led to significantly prolonged relapse-free survival. Recent results from FACS-analysis of the primary tumor cells from the treated groups showed increased MHC-II expression compared to those of control cats. This is encouraging concerning future use in veterinary practice as this treatment can be easily administered. The results are promising with respect to their potential in human medicine, as they have been obtained in real patients instead of experimental tumor models.

**P 53**

**Tumour Immune Evasion Enforced by Viral cDNA Library Vaccination Can Be Exploited for Ambush of Emerging Tumour Cell Variants**

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The tumour associated antigens (TAA) most relevant for tumour rejection remain largely unidentified in most tumor types. We have shown previously that autoimmune T cell reactivity, which also mediates tumor rejection, can be activated in vivo by inflammatory killing of normal cells. In addition, expression of a TAA in an immunogenic virus generates potent T cell responses against the TAA. Therefore, we hypothesized that it would be possible to use a cDNA library from a normal tissue, expressed from an immunogenic viral platform, to vaccinate tumor bearing hosts against a wide range of TAA expressed on tumors of the same histological type. We show here that a cDNA library from normal human prostate expressed by Vesicular Stomatitis Virus cured established murine prostate tumors. However, under suboptimal vaccinating conditions, tumor escape was possible, but only by the acquisition of a radically new tumor cell phenotype. Recurrent tumors could then be treated by sequential vaccination with a cDNA library from the recurrent tumors or by exploiting a novel acquired sensitivity to chemotherapy. This two-step ‘trap-and-ambush’ strategy uses initial vaccination to trap tumor cells into a very restricted evolution to ensure escape from the selective pressure imposed against the very broad antigenic repertoire encoded by the cDNA library. Thereafter, a highly targeted second line treatment against the new, enforced tumor phenotype can ambush these emerging escape variants. This combination represents a novel way to exploit the ability of tumor cells to escape powerful selective pressures in vivo for therapeutic advantage.

**P 54**

**Memory T cells masquerading as naive cells: Implications on adoptive T cell immunotherapy**

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Ex vivo T cell manipulation often induces their terminal differentiation, resulting in poor persistence and activity of transferred cells. We previously showed that costimulation
and culture with gchaint cytokines generates gene modified T cells with a functional central memory (TCM) phenotype superior to effector/effector memory (TEM) counterparts for expansion potential and antitumor activity. Here we investigated the consequence of initial targeting of selected T cell subpopulations. We activated and efficiently transduced FACS-sorted T naïve (TN), TCM and TEM cells by viral vectors. In contrast to TCM and TEM, TN had a greater expansion potential and more sustained expression of IL7-Ra. Strikingly, manipulation of TN resulted in a predominant population of post-mitotic lymphocytes expressing the CD45RA+/CD62L+CCR7+/naïve phenotype. These cells expressed markers common to early differentiated cells (CD27+CD28+CD127+Bcl2+) and markers proper of memory lymphocytes (CD45RO+CD122+CXCR3+). Post-mitotic TN produced lower levels of IFNγ and Granzyme A, expressed higher levels of c-Kit and CXCR4, and lower levels of HLA-DR, CCR5 and PD1 than memory counterparts. To verify their self-renewal and differentiation potential upon antigen stimulations, TN, TCM and TEM were transduced to express a WT1-specific TCR. Upon multiple stimulation TN expanded at higher numbers and were unique in the ability to generate a mixed population of CD127+/CD127- lymphocytes. When infused in immunodeficient mice, transduced TN proved higher engraftment and persistence potential than memory counterparts, reconstituted a mixed CD45RA+/CD62L+/CD127+/naïve phenotype and highly xenoreactive. These results suggest that gene transfer into TN lymphocytes might increase the efficacy of cancer immunotherapy.

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Expansion of NK cells for cancer immunotherapy: from process optimization to clinical evaluation

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Natural killer (NK) cell based immunotherapy is a prominent approach for the treatment of cancer. We have previously reported a GMP-compliant method for the expansion of NK cells from healthy donors or cancer patients. These cells showed cytotoxic activity against primary tumor cells in vitro and in experimental models, a response that stimulated evaluation in clinical settings. Infusion of ex vivo expanded NK cells in five cancer patients following allogeneic transplantation has been evaluated. The treatment was safe whether administered alone or with subcutaneous interleukin-2. No signs of acute GvHD developed. Although tumor response was not a primary goal, the decreasing alpha-fetoprotein levels and autopsy results in a patient with hepatocellular carcinoma indicated possible anti-tumor effect.

As the ex vivo expansion of NK cells under GMP conditions are crucial for facilitating large clinical trials, we also aimed to optimize a large-scale, feeder-free, closed system. In this study, the cells were cultured for 3 weeks in bags as well as an automated bioreactor and compared to flask-based cultures. Significant expansion of NK cells was obtained in all systems. The bioreactor yielded a final product rich in NK cells engraftable in a patient with hepatocellular carcinoma indicated possible anti-tumor effect.

The large-scale use of Dendritic Cells (DCs) to boost antitumor responses in the clinics require methods easy to standardize and consistent cell viability. A novel concept for DC production consists of overnight lentiviral vector (LV) transduction of growth factors and full-length antigens into monocytes that results into induction of “SMART-DCs” (Self-differentiated Myeloid-derived Antigen-presenting-cells Reactive against Tumors). This concept has been validated in the pre-clinical B16 melanoma mouse model (Koya et al, 2007). Third generation self-inactivating tricistronic lentiviral vectors containing interposing 2A elements co-expressing human GM-CSF, IL-4 and MART-1 or TRP-2 were constructed. Cytokine preconditioning and 16h transduction of CD14+ monocytes with high titer LVs resulted in persistent (3 weeks) viability, DC immunophenotype (CD209+, MHCIi+, CD80+CD86+), secretion of GM-CSF and IL-4 and expression of MART-1 or TRP2. Monocytes that were transduced with LVs overnight in a closed GMP-grade bag system, washed, frozen and thawed also resulted in effective SMART-DC recovery and differentiation. PBMCs primed/boosted in vitro with autologous SMART-DCs/MART-1 demonstrated the induction of MART-1-specific T cell responses assayed by IFNγ-ELISPOT-Assay. A consortium for clinical development of SMART-DCs has been formed for additional preclinical testing (optimal vector dose, toxicity studies), development of Standard Operating Procedures (for vector production and cell transduction) and establishment of identity and potency markers for a phase I immunotherapy clinical trial.
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Thymic renewal in adults after haploidentical hematopoietic stem cell transplantation and suicide gene therapy

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Background: In haploidentical Hematopoietic Stem Cell Transplantation (HSCT), the infusion of donor lymphocytes transduced to express the Herpes Simplex Virus Thymidine kinase (HSV-Tk) suicide gene allows to control GvHD, to mediate GvL, and to rapidly provide an effective and polyclonal anti-infective T cell repertoire. In haplo-SCT, the infusion of donor lymphocytes circulating in treated patients. Therefore, we investigated the putative role of HSV-Tk+ cells in promoting thymic activity and T cell development from graft progenitors.

Methods: Twenty-eight adult patients received genetically modified donor T cells in the TK007 study. In selected patients, post-transplantation thymic function was assessed by qPCR amplification of single joint T cell Receptor Excision Circles (qTREC) and immunophenotype analysis of CD31+ recent thymic emigrants (RTEs) in CD4+ naive T cells. Thymic volume was assessed by CT scans.

Results: Post-transplant recovery of naïve T cells not carrying the HSV-Tk transgene occurred. The newly constituted CD4+ naïve T cells were almost entirely comprised by CD31+ RTEs. Comparative analysis with a cohort of patients undergoing haplo-SCT in the absence of TK+ cells, suggested a direct role of Tk+ cells in promoting thymopoiesis. Accordingly, CT scans documented an increase in thymic volume and TREC counts rose following Tk+ cell add-backs.

Conclusions: These data show that the infusion of suicide gene-modified T cells prompts the renewal of thymic activity and the recovery of a polyclonal T cell repertoire.

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Improvement of anti-leukemic activity of CIK cells through CD33-specific chimeric receptors (CARs)

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CIK cells are ex-vivo expanded effector cells with potent antitumoral activity. We showed that CIK cell infusion in AML patients is well tolerated, but with limited clinical responses. To improve their functions, CIK cells were transduced with anti-CD33-z or anti-CD28-OX40-z CAR (mean CAR expression, 65%). Transduced CIK cells acquired potent cytotoxicity against AML targets: after 4 h we observed, at E:T ratio 5:1, a mean lysis of HL-60 cells of 79% and 75%, for anti-CD33-z and anti-CD33-CD28-OX40-z cells (p < 0.005 vs untransduced CIK cells). Analogous lytic efficiency was observed against the KG-1 cell line and primary AML blasts and in long-term killing experiments. In these assays, anti-CD33-CD28-OX40-z-CIK cells eliminated almost all leukemic cells after 6 days co-culture on human stromal layer w/o IL2 at E:T ratio 1:200, with 16% mean residual primary AML cells compared to 31% (p ≤ 0.05) of anti-CD33-z CIK cells and 91% (p ≤ 0.005) of untransduced CIK cells. Moreover, a prominent CD33-specific proliferative activity was observed, with a mean proliferation index of 3.7 (p ≤ 0.005) in anti-CD33-CD28-OX40-z and of 2.4 (p ≤ 0.05) in anti-CD33-z-CIK cells after primary AML cell-mediated stimulation compared to 1.4 for untransduced CIK cells.
cells. In addition, when stimulated with irradiated HL-60, anti-CD33-CD28OX40-z and anti-CD33-z, CAR-transduced CIK cells secreted 10-fold and 11-fold higher amount of IFN-g, 180-fold and 250-fold more TNF-a, 600-fold and 1400-fold more IL2 (p ≤ 0.05 for anti-CD33-CD28-OX40-z and for anti-CD33-z) compared to unmanipulated cells. Importantly, anti-CD33-CAR-expressing CIK cells showed toxicity against normal hematopoietic progenitors, but a consistent number of clonogenic progenitors could be recovered in colony forming-assays. Our results indicate that anti-CD33 CAR strongly enhances anti-leukemic CIK functions, suggesting that CD33.CAR-expressing CIK cells might represent a promising tool for AML immunotherapy.

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A conditional system for the activation of lymphocytes expressing activating and inhibitory CARs
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The use of Chimeric Antigen Receptors (CAR) represents a new strategy to redirect cytotoxic T lymphocytes specificity toward tumor cells. The data generated by the clinical utilization of CARs evidenced their potential for cancer treatment and to cause off-target effects. In an attempt to circumvent the off-target effects, we tested a system based on conditional responses to two CARs: the anti CD19scFv-4-1BB-zeta acti-

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T cell-based immunotherapy for renal cell carcinoma
Professor W Ucket1, M Leisegang2, A Turqueti-Neves3, T Blankenstein2, B Engels2, DJ Schendel3, E Noessner3

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have been tested for their sensibility to the corresponding activator in vitro by evaluating residual CD34+ cells. iCasp9-transduced cells were rapidly killed with high efficiency by CID (mean survival, 11% after 24 hours, and 5% after 7 days; n = 7). Gancyclovir treated HSV-TK-expressing cells showed similar levels of efficacy only after 3 days and CD20 and mTMPK-transduced cells showed only minimal killing at all time points (mean survival after 7 days, 84% and 32%).

The same results were obtained by analyzing apoptosis induction through Annexin-V-AAD staining. In fact, after 24 hours of incubation with CID, nearly 100% iCasp9+ cells were apoptotic, whereas a significant lower % of apoptotic cells was observed with the other suicide genes. Altogether our results suggest that the faster activity of iCasp9 might be advantageous in case of occurring toxicity, and, together with its lack of immunogenicity and the absence of side-effects of CID, support the clinical applicability of iCap9-based suicide strategy.

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The next generation TCR gene therapy using siTCR vector and RetroNectin expansion method
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Tumor antigen-specific T-cell receptor (TCR) gene therapy has been shown as an attractive strategy to treat cancer patients. However, the introduced TCR has been shown as an attractive strategy to treat cancer patients. Thus far, the clinical translation of TCR gene therapy has been largely limited due to the potential toxicity induced by the introduction of TCRs into T cells. In order to address these challenges, we have developed a novel method for the generation of TCR-transduced T cells using a combination of siRNA and RetroNectin technology.

In this study, we developed novel "siTCR" retroviral vectors encoding both siRNA constructs that knockdown endogenous TCR subunits, resulting in insufficient formation of heterodimers of therapeutic TCR. Moreover, a serious safety concern on the generation of T cells with unexpected specificities, including self-reactive T cells caused by TCR mispairing has been postulated. Therefore, we advocate that the transient redirection approach is well suited to meet safety aspects for early-phase studies, prior to trials using stably transduced cells once the CAR has been proven safe. The simplicity of this methodology also facilitates rapid screening of candidate targets and novel receptors in preclinical studies.

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Transiently redirected T cells for adoptive transfer; co-electroporation of chimeric antigen receptors
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Background: Clinical trials are underway investigating the safety and efficacy of adoptively transferred T-cells expressing Chimeric Antigen Receptors (CAR). Recent experiences with on-target/off-organ toxicity call for caution. Transient rather than permanent expression might represent a safer alternative in first-in-man studies with novel CARs and additionally in applications where long-lasting transgene activity is undesired.

Methods: We describe the preclinical evaluation of a method based on transient modification of bioreactor expanded T-cells with a CD19-CAR directed against B-cell malignancies. CAR mRNA is generated under cell-free conditions in a scalable process using recombinant RNA polymerase. Efficient and non-toxic square-wave electroporation is used to load the mRNA into the cytoplasm of T-cells with no risk of insertional mutagenesis.

Results: After transfection >80% of T-cells are viable with 94% CAR-expression. Transfected cells are cytolytic to CD19+ targets and produce IFN-γ, even at day 8 post-electroporation with undetectable CAR-expression. Increased mRNA concentration results in higher CAR-expression, improved killing and more IFN-γ release but at the expense of increased activation-induced cell death. Finally, we demonstrate that a second transgene can be introduced by co-electroporation of XCR4 or CCR7 with the CAR to facilitate migration.

Conclusions: We advocate that the transient redirection approach is well suited to meet safety aspects for early-phase studies, prior to trials using stably transduced cells once the CAR has been proven safe. The simplicity of this methodology also facilitates rapid screening of candidate targets and novel receptors in preclinical studies.
Background: B-cell chronic lymphocytic leukemia (B-CLL) is characterized by a progressive accumulation of mature CD19+CD5-CD20- B-lymphocytes that over-express the B-cell activation marker CD23. Here we cloned and expressed in T-lymphocytes a CAR targeting the CD23 antigen (CD23.CAR) on B-CLL cells.

Methods: Cytotoxic activity was measured using a 51Chromium release assay. Co-culture experiments of non-transduced (NT) and CAR+ T-cells with viable LCLs have been performed to assess expansion capability and soluble CD23(CD23)-mediated inhibition of the CD23.CAR+ T-lymphocytes. IFN-g, TNF-a, TNF-b and IL2 release were monitored twice a week for tumor growth. Animals were monitored twice a week for tumor growth.

Results: CD23.CAR+ T-cells showed specific cytotoxic activity against CD23+ tumor cell lines (average lysis 54%) and primary CD23+ B-CLL cells (average lysis 58%). This effect was obtained without relevant toxicity against normal B-lymphocytes. Moreover, CD23.CAR+ T-cells released 4-fold more IFN-gamma, 157-fold more TNF-alpha and 1445-fold more TNF-beta in response to CD23+ target cells. IL-2 was also released (average release 2681 pg/mL) and sustained the antigen-dependent proliferation of CD23.CAR+ T-cells without the addition of any exogenous cytokines. Preliminary in vivo data showed CD23.CAR+ T-cells can exert their cytotoxic activity toward the B-CLL derived MEC1-cell line, reducing tumor diameter and prolonging mice survival as compared to NT-cells-treated mice.

Conclusions: Altogether these data suggest gene modification of T-cells to express the CD23.CAR represents a selective immunotherapy approach to eliminate CD23+ leukemic cells, while sparing normal B lymphocytes, in patients with B-CLL.

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Validation of anti-tumor effects mediated by anti-CD19CAR T-cells for B cell lymphoma

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In order to develop a new therapeutic strategy for refractory B-cell non-Hodgkin lymphoma, we evaluate the efficacy of an adoptive immunotherapy using genetically engineered autologous T lymphocytes that express a CD19-antibody fragment fused to the TCR-z/CD28 receptor (19-28z) in model experiments. Here we examined whether 19-28z-transduced T lymphocytes could eradicate CD19+ tumors in vitro. Human peripheral blood T lymphocytes from healthy volunteers were activated by immobilized anti-CD3 antibody/Retinoicin, and transduced with retrovirus vectors encoding 19-28z prepared from producer cells, established by stable transduction of the 19-28z plasmid into the PG13 packaging cells. Transduced T lymphocytes were expanded ex vivo in the presence of NIH/3T3 fibroblasts expressing human CD19. Following antigen stimulation, 19-28z-transduced cell numbers increased about 1000-fold, and the surface expression of 19-28z positive CD3+ T lymphocytes was approximately 95%, as assessed by flow cytometry. Expanded 19-28z+ T lymphocytes efficiently lysed CD19+ tumor cell lines (Raji and Daudi Burkitt lymphoma cell lines) in 51Cr release assays. These results indicate that functional 19-28z + T lymphocytes would be effective for the treatment of refractory B-cell non-Hodgkin lymphoma.
Adoptive T-cell immunotherapies are safe and clinically effective in cancer patients. Clinical responses strongly correlate with in vivo T-cell survival. Systemic administration of recombinant IL-2 is used to sustain T-cell persistence. However, although effective, IL-2 induces side effects and regulatory T-cells (Tregs) expansion. We have now explored whether IL-15, able to sustain T-cell expansion and function, shares with IL-2 the unwanted effect on Tregs. We used Epstein-Barr-Virus (EBV)-specific cytotoxic T-lymphocytes (CTLs) as a model. Treg inhibitory activity was assessed using CSFE-based assays (to evaluate inhibition of proliferation), and co-culture experiments with lymphoblastoid-cell lines (LCs) (to assess inhibition of effector function), with or without IL-2 (25U/mL) or IL-15 (2.5ng/mL).

T-cell proliferation was inhibited by Tregs alone (from 56% ± 6% to 20% ± 5%, p < 0.05) or IL-2 + Tregs (from 76% ± 3% to 53% ± 3%, p < 0.05), but not by IL-15 + Tregs (from 81% ± 3% to 74% ± 3%, p = 0.1). Similarly, the anti-tumor activity of EBV-CTLs was significantly impaired by Tregs alone (residual LCs increased from 31% ± 13% to 55% ± 13%, p < 0.05) or IL-2 + Tregs (from 16% ± 8% to 36% ± 14%, p < 0.05) but not by IL-15 + Tregs (from 7% ± 6% to 11% ± 7%, p = 0.2).

Our study suggests that IL-15, unlike IL-2, can relieve effector cells from Tregs inhibition. We are currently exploring potential mechanisms of this observation by studying the effects of IL-2 and IL-15 on each component (Tregs vs effector cells). Preliminary data suggest that IL-15 does not directly revert Tregs inhibitory properties, but preferentially enhance T-cell proliferation and anti-tumor activity. Transgenic production of IL-15 by tumor-specific CTLs could help in overcoming Tregs effects while maintaining their ability to kill tumor cells.

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Targeting and retention of antigen to the endoplasmic reticulum enhances immune tumor protection

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Background: Endoplasmic reticulum (ER) is the main place where the MHC I are loaded with epitopes to elicit an immune cellular response. Most of the protein antigens are degraded in the cytoplasm to aminocids and very few epitopes reach the ER. Antigen targeting to this organelle by means of fusion to Calreticulin, an ER living protein, avoid this degradation problem enhancing the immune response. In order to simplify this strategy and to determine if most of the antigen properties conferred by Calreticulin are dependent on its ability to be retained in the ER, we engineered a new version of E7 gene bearing the signals required for ER targeting (SP) and ER retention (KDEL: Lys-Asp-Gln-Leu).

Method: We constructed recombinant adenovirus to express the E7 antigen with the SP and KDEL signals. E7 targeting was demonstrated by immunofluorescence on cell cultures transfected with these Sp-E7-KDEL and Calreticulin-E7 adenovirus. IFN-g induction and tumor protection assay were performed on C57B6 mice.

Results: The SP-E7-KDEL protein showed a similar pattern distribution as the control calnexin, an endogenous protein localized in the ER. Mice infected with the SP-E7-KDEL adenovirus showed an interferon induction and tumor protection response, similar to that provided by the adenovirus expressing the fusion protein Calreticulin-E7.

Conclusion: This work demonstrate that just by adding a signal peptide and KDEL sequence, the antigens can be targeted and retained in the ER with a consequent enhancement of immune response and tumor protection.
Adoptive transfer of tumor-specific cytotoxic T lymphocytes (CTLs) results in target cells lysis by activating the intrinsic apoptotic cell death program. Not surprisingly, deregulation of the apoptotic machinery is one of the central mechanisms by which tumor cells escape immune destruction despite specific CTL recognition. We show that treatment with the proteasome inhibitor bortezomib sensitizes previously resistant tumor cells for cytolytic T cell attack. Human T cells were redirected to target melanoma cells by engineered expression of an immunoreceptor with binding specificity for high molecular weight-melanoma associated antigen (HMW-MAA). Established melanoma cell lines as well as primary melanoma cells from tumor biopsies, which are notoriously resistant towards T cell lysis, became sensitive upon bortezomib treatment. Detailed analysis of the underlying molecular mechanism revealed that bortezomib-treatment induced the mitochondrial accumulation of NOXA which potentiated the release of mitochondrial SMAC in response to CTL effector functions including caspase-8 and granzyme B. The results of our study support the concept that proteasome inhibition increases the sensitivity of tumor cells towards a cytotoxic T cell attack by NOXA-mediated enhancement of mitochondrial release of SMAC.

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**Generation of antigen–specific cytolytic T cells for prostate cancer therapy**

Miss V Rashkova, Professor M Essand

Adoptive transfer of T cells specific for cancer antigens is a novel approach to treat cancer. Such immunotherapy has been successful in a setting where tumor infiltrating lymphocytes are isolated from patients, expanded ex vivo and given back to the patients in large numbers. However, in some patients and some malignancies such cells are not found or difficult to detect. An alternative approach would be to genetically modify T cells with a specific T cell receptor (TCR) to a given antigen. This approach has been used in the past for melanoma patients with encouraging results.

Using cytomegalovirus (CMV)-specific T cells against the pp65 antigen as a model we show that tetramer-specific T cells can be obtained, T cell clones can be generated and specific TCR sequence can be amplified. Our experiments confirm sequences that have already been published for the same peptide from pp65.

Using this validated protocol, we aim to develop cytolytic T cells against peptides derived from prostate antigens TARP, STEAP and PSA, which are only expressed in the prostate. With the help of HLA -A2 tetramers for TARP, STEAP and PSA peptides we can detect specific T cells and fluorophore directed beads can be used for the isolation of those cells. Our results indicate that such cells can be found in blood from prostate cancer patients. To deliver antigen specific TCR to T-cells we use a lentivirus vector with a self-cleaving peptide between the alpha and beta chain of the TCR to assure their equal expression.

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**Meganucleases : a novel anti-viral approach**

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The majority of current anti-viral treatments are based on the prevention of productive viral replication through the utilization of agents that inhibit essential virally encoded proteins. These anti-viral treatments, however, do not eliminate the viral genome from cells and thus removal of treatment or the presence of drug resistant mutations result in viral proliferation. Many chronic viral infections are due to double-stranded DNA viruses or viruses that involve a double-stranded DNA intermediate during their replicative cycle. Thus, an attractive alternative anti-viral strategy is to specifically cleave and either partially excise or eliminate viral DNA from infected cells and thus render them virus free.

Meganucleases are endonucleases that recognize large cleavage sites (>12bp) with a high specificity. We have shown that the expression of the meganuclease I-SceI, either before or after infection with a modified Herpes Simplex Virus (HSV) containing a meganuclease recognition site, results in a dramatic reduction of viral DNA. Meganucleases specific for viral DNA could thus represent a novel class of agents for the treatment of viral infections, targeting latent viral DNA in infected cells and rendering them virus free. Using a semi-rational approach, we have used a two step strategy to produce meganucleases cleaving several different viral genomes, including HIV, HSV and HBV. We will present data concerning the use of virus specific meganucleases for the development of a new anti-viral approach and therapeutic strategies for certain persistent infections.

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**Development of CMV-specific T cells for the management of transplantation patients**

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Development of CMV-specific T cells for the management of transplantation patients

**Background:** Bone marrow transplantation is a curative approach for the management of leukaemia patients. However, the requisite patient immunosuppression can lead to reactivation of viruses such as CMV or EBV, causing significant morbidity and mortality. Adoptive transfer of donor-
derived CMV-specific CD8⁺ CTL clones has proven effective in the prevention and treatment of viral infections that are unresponsive to antiretroviral therapy. But, this procedure has not been widely adopted due to the technical and financial demands of extensive T cell isolation and expansion **ex vivo**, and moreover is impossible when the donor is seronegative for the virus. Thus, new strategies are needed. We report here the development of CMV-specific CTL via T cell receptor (TCR) gene transfer.

**Methods:** CMV-specific TCR genes were cloned and transduced into human T cells. The antigen-specific activity of these engineered T cells was tested **in vitro** by CTL killing and cytokine secretion assays, and **in vivo** using a NOD/SCID mouse tumour model.

**Results:** We show that CMV-TCR can be expressed on the surface of human T cells, and these TCR engineered T cells both recognize and kill CMV viral antigen-expressing target cells. Using a K562 leukaemia cell line expressing HLA-A2 and the CMV antigen pp65, we demonstrate that the engineered human T cells can inhibit tumour growth in NOD/SCID mice.

**Conclusion:** This is the first report that CMV-TCR engineered human T cells can inhibit tumour development **in vivo**, and paves the way for using CMV-TCR engineered T cells for the management of transplant patients.

**P 75**

Conditioning therapy with lentivirally reprogrammed dendritic cells accelerate the expansion and bio-distribution of CMV pp65-reactive human T cells

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We evaluated the impact of conditioning therapy with genetically programmed dendritic cells (DCs) in order to enhance the engraftment and stimulation of donor T cells reactive against human cytomegalovirus (CMV). **Ex vivo** transduction of human monocytes with lentiviral vectors (LV) expressing GM-CSF and IL-4 induced their self-differentiation into "SMART-DCs" that were stable antigen-presenting cells viable for three weeks **in vitro** and in immunodeficient NOD.Rag1-/- IL2rg-/- (NRG) mice. SMART-DCs co-expressing the full length pp65 CMV antigen also maintained high viability and a stable DC immunophenotype. We analyzed the **in vitro** effects of SMART-DC-pp65 pre-administration on the engraftment and stimulation of anti-pp65 autologous T cells obtained from sero-positive CMV reactive donors using optical imaging, flow cytometry, immunofluorescence microscopy and immune analyses. "Conventional" DCs pulsed with a pp65 overlapping peptide pool were used as controls. NRG mice pre-conditioned s.c. with SMART-DC-pp65 showed: 1. Superior expansion of CD4⁺ and CD8⁺ human T cells in the spleen and peripheral blood; 2. Massive recruitment of lymphocytes to the DC injection site; 3. Stimulation of human CD8⁺ T cells against different pp65 epitopes. Thus, conditioning the host with engineered DCs prior to transfusion with donor T cells is a potential protective approach for CMV immunotherapy.

**P 76**

Nonclinical studies for the treatment of wet age-related macular degeneration for a first in man clinical trial using direct administration of an EIAV lentiviral vector (RetinoStat)

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RetinoStat® is currently being developed at Oxford BioMedica (OBM) for the initiation of a first in man (FIM) clinical study for the treatment of wet age-related macular degeneration (AMD). AMD is the leading cause of blindness in patients over 60 years in the developed world, resulting from neovascularisation in the retina. RetinoStat, expressing endostatin and angioptatin, will be administered by subretinal administration, which should result in the long-term expression of these angiostatic factors and suppression of neovascularisation. RetinoStat is an equine infectious anaemia virus (EIAV)-based Lentiviral vector, utilising the same platform technology that is currently being used in a Phase I/II clinical trial for the treatment of Parkinson’s disease (ProSavin®).

GLP non-clinical combined toxicology, shedding and biodistribution studies have been conducted in two species to support the regulatory submission of RetinoStat, using subretinal administration of RetinoStat. A wide range of biological samples were taken both in-life and at necropsy to evaluate the bio-distribution of vector, vector shedding and vector persistence and included a variety of target and non-target sample types. Samples were assessed for the presence of vector by quantitative real-time reverse transcriptase PCR (qRT-PCR) for vector genomic RNA. Persistence of vector was assessed by quantitative real-time PCR (qPCR) for vector associated DNA. The most striking general observation from these studies was the fact that vector and vector associated sequences were highly restricted to target tissue (eye). An overview of the data from these studies will be presented.

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Investigation of novel gene-based therapies for retinitis pigmentosa 17

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Retinitis pigmentosa is a highly heterogeneous form of inherited blindness. The RP17 form of the disease is caused by an arginine to tryptophan (R14W) mutation in the signal sequence of carbonic anhydrase IV (CAIV). While CAIV is expressed in the choriocapillaris of the eye and renal epithelium, the R14W
mutation results in an exclusively ocular phenotype in affected individuals. We show using immunocytochemistry, western blot analysis and flow cytometry experiments that the R14W mutant form of CAIV is misfolded and mis-trafficked in COS-7 and HT-1080 cells. In HEK-293 cells, which are of kidney origin, we have shown that the R14W mutant form of CAIV is correctly folded and targeted, perhaps explaining the lack of kidney phenotype in RP17 patients, despite high expression of the mutant protein in these cells. We have investigated the expression of ER-specific chaperones in HEK-293 cells expressing R14W mutant CAIV, in order to determine whether high expression of these proteins induces correct folding of the mutant protein in these cells. We believe that over-expression of these chaperones in COS-7 and HT-1080 cells expressing R14W mutant CAIV will result in correct folding of the protein, as is seen in HEK-293 cells. In addition, we have also designed allel-specific shRNA to silence R14W mutant CAIV, but not the wildtype form of the protein. We believe that by using these approaches, we have explored two possible means of providing therapy for RP17.

P 78
Allele-specific silencing of mutant Huntingtin in HD neural stem cells and in vivo
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Huntington’s disease is a progressive and fatal neurodegenerative disorder, characterized by disturbances in mood and deterioration in cognitive and motor functions. This dominant and inherited disease is caused by the expression of mutant Huntingtin (Htt) protein with a CAG repeat expansion. Suppression of Htt expression, using RNA interference represents an interesting potential therapeutic strategy for this untreatable pathology. Nevertheless, the loss of normal Htt function might also contribute to the pathogenesis of HD. The reduced physiological activity of Htt in HD might also affect the development of therapeutic strategies with RNAi. Allele-specific silencing of the mutant allele using siRNAs targeting heterozygous single nucleotide polymorphisms SNPs that distinguish between the two alleles represents a very promising therapeutic strategy for HD.

In this study, we show the efficacy of shRNA targeting three SNPs covering the majority of HD patients. Lentiviral-mediated delivery resulted in efficient and selective in vitro silencing of a chimeric mutant Htt reporter system consisting of the sequence of the first 171 amino acids of the mutant human Htt fused to the Htt exons containing the SNP. Furthermore, the defect in the vesicular transport of BDNF along microtubules was corrected in HD neural stem cells.

In vivo, siSNP efficiently degraded the mRNA of fully matched chimeric mutant Htt expressing the various SNP and prevented the appearance of neuropathology. On the contrary, the presence of one mismatch in the targeted mRNA prevented its degradation in almost all cases, leading to the accumulation of Htt aggregates and the appearance of striatal pathology.

P 79
Therapeutic potential of genetically modified neural stem cells (NSCs) in a mouse model of Globoid Cell Leukodystrophy (GLD)
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GLD is a rare genetic disorder caused by the deficiency of the lysosomal enzyme β-galactocerebrosidase (GALC), characterized by progressive central and peripheral demyelination. We evaluated the potential of Neural Stem Cell (NSC)-based approach to correct the metabolic defect and to ameliorate CNS pathology in Twitcher (Twi) mice, a true model of GLD. NSC lines derived from neonatal Twi and WT mice were efficiently transduced with bidirectional lentiviral vectors encoding for GALC and GFP, achieving supraphysiological GALC activity. In order to obtain a stable source of GALC-secreting cells in the brain we transplanted 5×107 WT or GALC over-expressing NSCs into the telencephalic lateral ventricles of neonatal Twi mice. Forty days after transplant we found that 1-3% of the total injected NSCs were engrafted and distributed into the brain, some of them expressing neural lineage markers. Engrafted NSCs robustly produced and secreted the GALC protein, as assessed by immunofluorescence and western blot analysis. Circulation of the enzyme in the cerebrospinal fluid flow coupled to active cross-correction resulted into restoration of GALC activity up to 50% of WT levels in the brain and spinal cord tissues of NSC-transplanted Twi mice. Metabolic correction resulted in partial clearance of glycosphingolipid storage in CNS tissues and improvement of walking ability in NSC-treated mice as compared to untreated controls.

These results warrant further consideration of NSC gene therapy for the treatment of GLD, likely in combination with other approaches ensuring enzymatic reconstitution in visceral organs and in the PNS.

P 80
Systemic delivery of scAAV9 expressing SMN prolongs survival in a mouse model of SMA
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Background: Spinal Muscular Atrophy (SMA) is one of the most common genetic causes of death in childhood. It is caused by mutations of the survival motor neuron (SMN) gene, leading to depletion in SMN protein levels. To date there are no effective drug treatments for this disease. SMN replacement by gene therapy seems therefore a promising strategy for successful treatment of the disease. Consistently, we previously reported that intramuscular administration of a lentiviral vector expressing SMN lead to a small but significant increase in the lifespan of SMNΔ7 mice. However, the marginal efficacy of this original therapeutic approach prompted us to explore different strategies for gene therapy delivery to motor neurons to achieve a more clinically relevant result. Self complementary adeno-associated virus 9 (scAAV9) mediates efficient and sustained transgene expression in cells of the nervous system following systemic administration. Therefore, we evaluated the efficiency of scAAV9-mediated SMN gene replacement in a mouse model of SMA.

Method: A single injection of SMN-expressing scAAV9 vector was performed into the facial vein of neonate SMNΔ7 mice. The pups were evaluated on a daily basis for survival and their motor function was assessed by behavioural tests.

Results: scAAV9-SMN gene therapy restored SMN protein expression and mediated correction of the motor deficits in these animals resulting in a substantial extension in their lifespan.

Conclusion: These data demonstrate a clinically relevant extension of survival in SMNΔ7 model and provide evidence for the most efficacious therapy observed in this field to date.

P 82

Transduction of choroid plexus epithelia is crucial for gene therapy rescue in a murine model of Menkes disease

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The mottled-brindled (mo-br) mouse manifests a lethal (dies by 14d) abnormality in copper transport to the brain caused by mutation of atp7a, a P-type ATPase, and is a model for Menkes disease. The mutant allele, a small in-frame deletion, results in a loss of residual copper transport, and rescue is possible by intraperitoneal copper administration depending on genetic background. We documented that mo-br mice on C57BL/6J were not rescued with intraperitoneal or intravenous copper, and exploited this model to gain insight concerning normal copper delivery to the CNS, using a brain-directed gene therapy approach.

We treated neonatal mo-br C57BL/6J mice with either intracerebroventricular recombinant adeno-associated virus serotype 5 (AAV5) harboring a human atp7a homolog, intracerebroventricular copper, or both. Only combination (AAV5 plus copper) treatment rescued mo-br, with tripling of mean survival and 31% living beyond 110d. As expected from prior characterization, AAV5 transduction occurred primarily in choroid plexus epithelia, without detectable transgene expression in neurons or brain capillary endothelial cells. Survival was associated with higher brain copper and increased activity of dopamine-beta-hydroxylase, a copper enzyme mettallated in the trans-Golgi compartment. In contrast, activities of cytosolic and mitochondrial copper enzymes did not differ across treatment groups. At 300d,
electron microscopy showed no overt ultrastructural brain abnormalities in combination treated mutants.

Our findings suggest that 1) transduction of choroid plexus epithelia in mo-br enhances brain copper retention and utilization, 2) copper enzymes processed in the trans-Golgi contribute to the rescue, and 3) ATP7A gene therapy may have future clinical applications.

P 83

High-throughput transcriptional analysis of gene therapy viral vectors effects on brain cells

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The gene therapy approach using viral vectors currently represents one of the best hopes for treating numerous genetic and acquired brain disorders. Different viral vector platforms have been extensively studied and utilised in clinical trials on the Central Nervous System (CNS), by taking advantage of the specific viral features. However, the improvement of viral systems to mediate safe and long-lasting expression of therapeutic transgenes in brain is particularly challenging due to the post-mitotic nature of nervous cells, the high level of compartmentalisation of the CNS, the potential toxicity and the alteration of the neuronal physiology triggered by the virus. Although many studies have proved the efficacy of the viral sources in transducing the brain in vivo, little is known on neuronal cells perturbations following the vector interaction. To address this issue, we have analysed the global transcriptome of differentiated midbrain-derived human neuronal progenitor cells transduced in vitro with HIV-1-, AAV9-, Helper Dependent human adenoviral (HD hAd)- and Helper Dependent canine adenoviral (HD CAV-2)- vectors, at early and late time points. In particular, canine adenovectors have proved to be an interesting alternative to the human Ad, because of their efficiency in transducing human cells and the absence of CAV-specific neutralizing antibodies in human serum, that inhibit the vector effect. This study intends to provide insights in vector development for CNS, consisting in the ability to predict the neuronal functions altered by the vectors and the possibility to act on these with tools aimed at improving the efficacy and reducing the toxicity.

P 84

Activation of autophagy rescues behavioral and neuropathological cerebellar deficits in a lentiviral mouse model of Machado-Joseph disease

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Machado-Joseph disease (MJD), also known as spinocerebellar ataxia type 3 (SCA3) is a fatal, dominant neurodegenerative disorder. MJD results from an abnormal increased repetition of the trinucleotide CAG in the MJD-1 gene, translating into an expanded polyglutamine tract that confers a toxic gain of function to the ataxin-3 protein. Clinical manifestations include cerebellar ataxia and pyramidal signs culminating in severe neurodegeneration.

Even though we previously reported a lentiviral (LV)-based model for MJD (Alves et al., 2008), a LV-model displaying the neuropathological features in the cerebellum, one of most severely attained brain regions in the disorder, had not yet been produced.

In this study, we generated a new mouse model of Machado-Joseph disease by injection of lentiviral vectors encoding human mutant ataxin-3 to the mouse cerebellum. Expression of the mutant protein with 72 glutamines in this region induced a severe behavioral phenotype starting at 4 weeks and progressing until 10 weeks post-injection. At this time-point animals were sacrificed and analyzed at by immunohistochemistry. Accumulation of ubiquitinated inclusions, neuropathological abnormalities and neuronal death were observed in animals expressing mutant ataxin-3 but not in animals expressing GFP (control). Importantly, overexpression of the autophagy-related protein beclin-1 in this model mitigated behavioral deficits and pathological hallmarks.

These data suggest that a) lentiviral-mediated expression of mutant ataxin-3 in the mouse cerebellum provides a quick and cost-effective model of MJD and b) that stimulation of autophagy is a promising therapeutic strategy for this disease.


P 85

Gene therapy for diabetic neuropathy by intrathecal NRG1typeIII administration

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Background: Diabetic neuropathy is one of the most common long-term complications of diabetes mellitus. The discovery of an effective treatment requires a greater understanding of the causes leading to the development of diabetic neuropathy. Among several proposed pathogenic mechanisms, the process of myelination might be affected since patients have significant deterioration of peripheral nerve function. In this context, NRG1 type III has been described as an important factor involved in myelination and is vital for this process in the peripheral nervous system.

Results: We analyzed the effect of high concentration of glucose in vitro and in vivo in various tissue cultures and mice models of type I diabetes. The results showed Schwann cell alteration in both, myelin proteins and transcription factors involved in myelination, already at prediabetic stage. Moreover, neurons showed a significant decrease in NRG1 type III expression in vitro and in vivo already at prediabetes.

Sensory and motor neuron transduction was achieved in diabetic mice by delivering intrathecally AAVrh10 coding for NRG1 type III. Electrophysiological studies showed an improvement in motor and sensory conduction tests, compared to untreated or mock-treated diabetic animals. Electrophysiological data correlated with a decrease in the number of degenerated axons, with an increase in the diameter of myelinating fibres and with the recovery of myelin-related protein levels.

Conclusions: Our results suggest that AAV vectors coding for NRG1 type III might be good candidates for gene therapy treatment of diabetic neuropathy and other de-myelinating neuropathies but treatment may be necessary since the very first days of hyperglycemia.

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Magnetic field-assisted transduction of neuronal and glial cells with magnetic virus vectors

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We aim at using magnetic viral vectors and a magnetic field-assisted transduction to enhance the expression of neurotrophic factors that prevent the degeneration and enhance recovery of the neurons at the target site in the brain. We used RAd-(TK/GFP) fus, LVsFeGFP and LVsFds-Red2 vectors coding for reporter genes and in house synthesized magnetic nanoparticles (MNPs) with the an average core size of 10 and 80 nm. We quantified the association of the vectors with both MNPs and internalization of the vector using radioactively labelled adenovirus. We evaluated the magnetophoretic mobility of the complexes from the time course of the turbidity of the virus-MNP complex suspensions in defined magnetic fields. The optimal MNPs-to-virus particle ratio ensured almost complete vector association with MNPs into the complexes stable enough in CSF. TEM and AFM data showed structurally intact viruses “decorated” by multiple MNPs. In B92 glial and Neuro2A neuroblastoma cells magnetotransduction with optimized formulations of the magnetic viral complexes resulted in considerably improved efficiency compared to standard infection. The complexes possess magnetic responsiveness high enough to allow trapping of the vectors at specific target sites of the rat brain after intracerebroventricular (ICV) injection, thus achieving therapeutic effect at relatively low vector and MNP dosage and decreasing virus associated toxicity and immune response. Additionally, the ICV route should provide a minimally invasive approach for gene therapy in the brain. In further experiments we will use optimized magnetic vectors in order to implement neuroprotective gene delivery by magnetotransduction in the brain of aging rats with dopaminergic neurodegeneration.

P 87

Systemic injection of AAV type 9 vector in utero facilitates global gene expression in the CNS

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Background: Since both the immune system and the blood brain barrier (BBB) are developmentally immature, systemic delivery of viral vector during the perinatal period may be a potential strategy to treat genetic neurological diseases. In this work, we examined biodistribution of adeno-associated viral vector serotype 9 (AAV9) following intraperitoneal injection into prenatal and postnatal mice.

Method: AAV9 vector encoding GFP (5x10⁴ viral genomes/g) was injected into the peritoneal cavity of fetal (embryonic day 15; n = 10) or neonatal (postnatal day 1; n = 14) C57BL/6 mice. The mice were sacrificed at 8 weeks of age and the tissues were analyzed by real-time PCR for biodistribution of vector and by immunohistochemistry for GFP expression.
Results: GFP expression was detected in all organs including the brain. The copy number of the AAV genomes in fetal injected mice were significantly lower than those of neonatal injected mice in all visceral organs. In the brain of neonatal injected mice, GFP expression was detected mainly in the olfactory bulbs. In contrast, global gene delivery occurred in the whole brain after fetal injection. The higher copy number of AAV was observed in the areas of the cerebrum, hypothalamus and hypocampus, compared to neonatal injected mice.

Conclusion: Our data suggest that systemic injection of AAV9 in utero is an effective strategy to cross the BBB for gene transfer into the CNS, which is tightly protected from viral infection in adult mice, and an important option for treatment of early onset genetic neurological diseases.

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Neurotropic viruses tropism and spread in the CNS
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Background: Neurotropic viruses, such as HSV-1 and Rabies virus (RV), may infect the CNS causing severe diseases, while carry the potential to serve as vectors in gene therapy to the nervous system.

Method: In order to study neurotropic virus tropism and spread in the brain, an ex vivo system of organ culture was established.

Results: i) HSV-1 infection was confined to leptomeningeal, periventricular and cortical brain regions. ii) HSV-1 did not preferentially infect proliferating cells, although it had a predilection to ventricular zones and the infection was localized to early progenitor cells. iii) The infection pattern of lentivirus pseudotyped by VSV-G and RV-G was compared to that of HSV-1. While the tropism of the VSV-G pseudotyped vector had a striking resemblance to HSV-1, the RV-G pseudotype infection was weak and more diffuse in the entire parenchyma. VSV-G pseudotype widely infected neurons, sparing astrocytes, while the RV-G pseudotype infected astrocytes, but not neurons. iv) The infection and tissue spread of HSV-1, expressing the GFP protein, was evaluated after injection into the brain ventricular zone. Within a day, infection was observed as a focal site and subsequently the virus spread along distinct anatomical structures. Neutralizing antibodies failed to inhibit viral spread, suggesting that the mechanism of spread is not mediated by extracellular re-infection.

Conclusions: Taken together these results indicate unique patterns of lentiviral and HSV-1 infections in the brain and furthermore, HSV-1 spread in the brain is determined both by anatomical neuronal networks as well as by intracellular factors.

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Efficient gene expression in the spinal cord from integrase-deficient lentiviral Vectors
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Gene transfer to spinal cord cells may be crucial for therapy in diseases including Spinal muscular atrophy and Amyotrophic lateral sclerosis, as well as in spinal cord injury. Methods are required that combine high gene transfer efficiency with enhanced bio-safety. Lentiviral vectors are efficient for transduction of a variety of cell populations, but like all integrating vectors they pose a risk of causing insertional mutagenesis. We have recently developed integrase-deficient lentiviral vectors (IDLVs) that remain episomal and yet retain the transduction efficiency of standard integrating lentivectors. IDLVs are particularly adequate for applications in non-dividing cells, where the episomes, which lack replication sequences, are not diluted out through repeated cell division. Following work in the eye and brain, we have now applied IDLVs for transduction of spinal cord in vitro, in explants and in vivo. Our results demonstrate similar efficiency of pGFP expression from integrating lentivectors and IDLVs in most cell types tested, including motor neurons, interneurons, dorsal root ganglia neurons and astroglia. Microglia is less efficiently transduced by IDLVs in pure cell cultures but not in explants. After intra-parenchimal injection in vivo, transduction is mainly neuronal, with both motor and interneurons being efficiently targeted. We have also demonstrated that IDLV-mediated expression of a growth factor rescues motor neuron cultures from death caused by removal of exogenous trophic support, and efficient IDLV-mediated RNA interference and SMN expression. These results suggest that IDLVs could be efficient and safer tools for cord transduction in therapeutic strategies, particularly for Spinal muscular atrophy.

P 90
Cultivation of human MSC in a closed bag cultivation system modified by dielectric barrier discharge
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Eating well in the laboratory: using a closed cultivation system with dielectric barrier discharge (DBD) to cultivate human bone marrow derived mesenchymal stem cells (MSC) in an efficient, sterile and scalable system. While cultivating MSC in bioreactors, the risk of contamination with endotoxins and other pathogenic factors is in evitable. Addition of a closed, sterility guaranteed high-throughput cultivation system with an integrated endotoxin cleaning process is one solution to this problem. In addition, cultivating MSC in an aseptic environment is a requirement for the production of the therapeutic products such as stem cell therapies, therapeutic proteins or other biologics (e.g. to treat diabetes, cancer, MS, PD). In this presentation, we will show how to cultivate human MSC with dielectric barrier discharge (DBD) and how to achieve a high cell density in the closed cultivation system. The cultivation system is based on a closed, sterile and scalable cultivation system and is equipped with an integrated endotoxin cleaning process. The system is suitable for high-throughput cultivation of human MSC with dielectric barrier discharge (DBD) and can be used for the production of therapeutic products such as stem cell therapies, therapeutic proteins or other biologics.
For safe, GMP compliant production of therapeutic cells a closed disposable cell culture bag system has many advantages. We have previously shown that the closed cell culture bag is suitable for the cultivation of suspension cells but did not support growth of adherent cells. However, when the inner bag surface is functionalized by an atmospheric-pressure plasma process in the presence of suitable film forming agents to create an amino group or silane coating, adherently growing cells could be expanded, whereas the unmodified cell culture bag had cell repellent properties. Biocompatibility of the surface treated cell culture bag was assessed with adherently growing cell lines and primary human bone derived mesenchymal cells (MSC). Cellular properties of MSC cultivated in bags were compared to standard culture conditions. We evaluated cell morphology, expression of cell surface markers, attachment, cell proliferation and osteogenic and adipogenic differentiation. Both osteogenesis and adipogenesis of the MSC could be induced in the modified bags. Gene transfer by adenoviral vectors allowed efficient modification of bag cultivated cells. Also in FCS free cultivation conditions expansion was effective. Further, we successfully cryopreserved adherent cell layers in situ on the modified culture bag surface. The influence of bag cultivation on the cells was analyzed by expression profiling in comparison to classic cultivation in polystyrene flasks. In conclusion we have shown that adherent cell expansion, differentiation and cryopreservation is possible on surface adapted cell culture bags with potential application as a disposible closed bag system for GMP compliant cell cultivation.

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Transfection Efficiency and Safety Profiles of Dextran-Spermine/Plasmid DNA in Mouse Lungs

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Generation of an efficient gene delivery vector with low toxicity profile is required to achieve successful gene delivery for pulmonary diseases. Dextran-spermine (D-SPM), a cationic-based gene delivery vector, has been shown to be capable of transfecting cells and tissues in vitro and in vivo. However, no study has been performed to determine the efficiency and safety of this delivery agent exclusively in the mouse lungs via intranasal delivery to date. In this study, we determined the optimal conditions for gene expression of D-SPM/plasmid DNA (D-SPM/pDNA) in the lungs of BALB/c mice by varying the weight-mixing ratio of D-SPM to pDNA. The levels of transgene expression were also measured at different time points. Reporter gene expression levels were observed highest at weight-mixing ratio of 16 with 13.5μg pDNA and at day 1 post-administration. Re-administration with similar conditions of D-SPM/pDNA was performed at 24 hr post initial administration, but it did not augment the first administration to enhance or even to restore the transgene expression. Quantifications of cytokines and neutrophil inductions in bronchial alveolar lavage fluids were performed to assess the safety profile of the complex in the lungs. Administration of D-SPM/pDNA resulted in modestly elevated level of IL-12 with no difference in the levels of IFN-γ when compared to the untreated mice, although an increased in the neutrophil count was observed. These results demonstrate that D-SPM has the potential to be further developed into an efficient and safe gene delivery vector to the lungs.

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Silencing host cell proteins associated with retroviral particles

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Background: Retrovirus and Lentivirus derived vectors have already demonstrated their potential as gene transfer tools in gene therapy protocols, in vaccinology and as onco-lytic agents for cancer treatment. HEK 293 cell lines have showed to be an excellent platform for the production of these viruses. Retroviral particles incorporate several host cell proteins namely membrane proteins that retain function and may participate in the infection process, but on the other hand can be also immunogenic reducing the efficiency of the viral particles.

Methodology: In this work we assess the feasibility of reducing host cell protein incorporation in retroviral particles by knockdown RNA interference. The CD81 was selected since it is a membrane protein virtually present in all cells and known to be incorporated on retroviral and lentiviral particles surface and, being highly immunogenic can lead to vector inactivation in vivo, reducing its therapeutic potential. A 293 derived cell line producing retrovirus like particles from MLV (RVLPs) was stably silenced using short hairpin RNAi.

Results: CD81 was successfully silenced up to 90-95% of clone variability was found regarding to cell growth and metabolism but clones with normal patterns of growth without impaired RVLP production were successfully obtained. The analyses of RLPs showed low or no detection of CD81 protein.

Conclusion: The work herein presented constitutes a proof-of-concept, showing that it is possible to manipulate the incorporation of host cell proteins in the produced viral particles either for studying the effect of their incorporation on vector efficiency and safety or to improve the producer cell lines.

P 93

Magnetic microbubbles as mediators of gene delivery

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Shake flasks in bioprocess development.

In recent years microbubbles technology has gained lot of interest in the field of gene and drug delivery. Based on the “Magnetofection” concept, besides the development of magnetic acoustically active lipospheres [1], we have been able to prepare lipid monolayer shelled microbubbles loaded with highly positively charged naked magnetic nanoparticles (composed of iron oxide) through electrostatic and matrix affinity interactions. These magnetic microbubbles show strong ultrasound contrast.

Treatment of cancer cells with these microbubbles using ultrasound exhibited strong dose-dependent cytotoxic effects, although ultrasound alone, lipid microbubbles alone, magnetic nanoparticles or magnetic microbubbles alone at the corresponding concentrations did not affect the cell viability. On the other hand, when these magnetic microbubbles were mixed with plasmid DNA encoding a reporter gene, we achieved gene delivery to cultured adherent cells only when ultrasound was applied. Gene transfer efficiency was strongly dependent on the application of a gradient magnetic field to sediment the microbubbles on the target cell membranes.

From the preliminary experiments we conclude that magnetic microbubbles could be used as magnetically targeted diagnostic agents for real-time ultrasound as well as magnetic resonance imaging. At the same time, such magnetic microbubbles may be useful for therapeutic purposes such as in cancer therapy, vascular thrombolysis and gene therapy. However, further improvements are required to control their cytotoxicity.

References

P 94
pO2 and pH profile evolution in shake flasks during 293 cell growth and infection with adenovirus
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The metabolic state of the culture is essential for quality assurance at the early stages of a process. Key parameters in cell culture are the partial oxygen pressure (pO2) and the pH, both of which decisively affect the culture quality. Continuous monitoring of oxygen consumption and pH is advantageous for optimization of cell culture processes.

Shake flasks are used in the biotechnology industry for a variety of tasks however, the data obtained from the cultures, have not been so far translated to scale-up due to the lack of knowledge of the conditions under which they are performed. The new tendency to use modern optical sensors and bioprocess monitoring devices will increase the relevance of shake flasks in bioprocess development.

Adenoviruses have increasingly been used as vectors in strategies for gene therapy and vaccination. Since the process analytical technologies initiative, where it is encouraged to design, analyze, and control manufacturing as early as possible in the development phase, it become important for all proceedings, even for well characterized processes as producing adenovirus, to increase the information at the early stages for quality assurance.

In this work, the new Shake Flask Reader from PreSens, which measure the pO2 content and pH of the medium accurately and non-invasively, was used to monitor the production of adenoviruses in 293 cells. Data on the metabolic characterization during cell growth and infection at different cell concentrations will be presented. These results should aid the scaling-up process to bioreactors providing more information to develop process control strategies.

P 95
Lentiviral vectors to generate blood samples expressing selected blood group antigens: an innovation
Dr C Bagnis, Dr S Chapel, Dr J Chiaroni, Dr P Bailly

To avoid Haemolytic Transfusion Reaction (HTR), blood transfusion safety and efficiency relies on analysis of donor blood and patient sera, with some very rare blood group phenotype being difficult to obtain from donor population. An in vitro process to generate blood samples expressing preselected rare blood group phenotypes as control for diagnosis purposes remains an important issue to address. Our projects focuses on genetic manipulation to mediate silencing or expression of blood group system antigens. CD34+ hematopoietic stem cells were transduced with a lentiviral vector expressing a short hairpin RNA directed against UT-B mRNA, or the cDNA coding for the UT-B protein (Jk/b Kidd blood group system). Cells were then cultivated to promote terminal erythroid differentiation prior to be analyzed for phenotype being difficult to obtain from donor population. Whether this strategy can be considered as a tool to generate rare blood group control samples for diagnosis remains to be discussed.

P 96
The biosafe pFAR vectors display superior expression efficiency in skin, liver and tumours
Dr C Marie, Mr M Quiviger, Dr G Vandermeulen, Dr M Richard, Professor V Préat, Professor D Scherman
Minicircle – an overview

Nonviral gene therapy requires the production of plasmid DNA that meets defined criteria such as biosafety and high quality.

To produce biosafe plasmids, we have designed a novel antibiotic-free selection system that is based on the suppression of an amber mutation by a plasmid-borne function. The nonsense mutation was introduced into the essential chromosomal thyA gene of *Escherichia coli*, resulting in thymidine auxotrophy. In parallel, pFAR4, a small plasmid vector carrying a suppressor t-RNA gene, was entirely synthesised. The introduction of pFAR4 into the thyA mutant restored normal growth to the auxotrophic strain and allowed, after optimisation of the bacterial producer strain, an efficient production of predominantly monomeric supercoiled plasmids.

The potentiality of pFAR4 as an eukaryotic expression vector was first assessed by monitoring luciferase activities after electrottransfer of LUC-encoding plasmids into various tissues. In muscle, high luciferase expression levels were observed with the pFAR4 derivative. Interestingly, tumour cells transfected with the pFAR4 derivative and the thiomyA mutant restored normal growth to the auxotrophic strain and allowed, after optimisation of the bacterial producer strain, an efficient production of predominantly monomeric supercoiled plasmids.

Minicircle – an overview

Dr M. Schleef1, Dr M. Schmeer, Dr M. Blaesen1, Dr R. Baier

For future gene therapy and genetic vaccination approaches it is crucial to develop safe and efficient vector systems. Currently, viral and non-viral vectors are used, having their advantages and limitations. The dissemination of antibiotic resistance genes, as well as the uncontrolled expression of backbone sequences present in plasmid DNA may have profound detrimental effects. Additionally, CpG motifs have been shown to contribute to silencing of episomal transgene expression. Hence, removal of bacterial backbone DNA can greatly improve safety and efficiency of DNA used in gene therapy and vaccination.

Here, we give an overview on earlier and recent approaches for the production, purification, and application of such minimal constructs. Different approaches have been described so far, from plasmids where the antibiotics resistance gene has been replaced by another marker to minicircle DNA consisting almost only of the gene of interest. Minicircles can be produced by *in vivo* site-specific recombination of a so-called parental plasmid resulting in a miniplasmid and the minicircle. This recombination can be achieved using different enzymes which need specific target sequences as recombination sites. The most important difference between these approaches is the efficiency of the recombination step as well as the purification procedure used in order to remove the miniplasmid (with the bacterial sequence motifs) and residual amounts of the parental plasmid if necessary.

In addition to their improved safety profile, minicircles have been shown to increase the efficiency of transgene expression *in vitro* and *in vivo* studies. Scale-up was recently possible in fermentation and results in an extremely pure DNA product.

P 98

Unraveling the transduction mechanism used by MV-LVs for quiescent lymphocyte gene transfer

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Gene transfer into quiescent T and B cells is of importance for gene therapy and immunotherapy approaches to correct multiple hematopoietic disorders. We generated LVs pseudotyped with measles virus glycoproteins (MV-HEd-LVs), which represent the first tool allowing efficiently transduction of quiescent human T and B cells. Indeed, MV-HEd-LVs allow an efficient transduction of primary target cells since they express CD46 and SLAM, the natural receptors of the vicinal MV-Edmonston strain. Interestingly, LVs pseudotyped with MV H gps, blind for the CD46 binding site, were completely inefficient for resting lymphocyte transduction. Similarly, SLAM-blind H mutants give rise to particles that recognize only CD46 as entry receptor, were also inefficient for transduction. We revealed that SLAM-blind LVs accomplish cell entry and reverse transcription, at similar levels as MV-HEd-LVs, but unlike for MV-Ed-LVs, this proviral DNA was not translated into efficient proviral integration. In contrast, only background levels of proviral DNA were found with CD46-blind-LVs. Moreover, our results indicate that both CD46- and SLAM-binding sites need to be present in cis in the H gp to allow successful engagement of both receptors resulting in a stable transduction of quiescent lymphocytes. The mechanism of entry seems to be crucial to determine the fate of these LVs in quiescent lymphocytes and our results point out that macropinocytosis may be involved. Altogether, our data suggest that even if vector entry can occur through CD46 receptor, SLAM-triggered signaling may be needed for an efficient transduction.

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A non-invasive animal in vivo imaging system to investigate MSC homing in rheumatoid arthritis

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Introduction: Despite the common use of Collagen Induced Arthritis (CIA) as a model for rheumatoid arthritis (RA) in mice, homing of mesenchymal stem cells (MSCs) to diseased joints has not being demonstrated definitively to date. The Bazooka-SPECT, a low cost small animal in-vivo imaging system, allows spatial resolution of radioactively labeled cells administered to live animals. In this study, MSCs were engineered to express human sodium iodide symporter (NIS) which allows uptake of the gamma ray emitter technetium ($^{99m}$Tc) in these cells. Localisation of NIS expressing MSCs was determined using the Bazooka-SPECT imaging platform.

Materials & Methods: CIA was induced in ten male DBA/1 mice aged 8-10 weeks. MSCs from a GFP transgenic mouse (FVB strain) were transduced with adenovirus expressing NIS. AdNIS MSCs, MSCs transduced with empty adenovirus (AdNull) and untransduced MSCs were administered via IV and IP routes. All MSC injections were performed 42 days post immunization and 3-5 days post MSC injection. $^{99m}$Tc was administered by IP followed by in vivo imaging. As a backup to the imaging data, all joints were harvested for DNA extraction and detection of GFP genomic DNA by PCR.

Results: $^{99m}$Tc uptake was detected in mice with high clinical RA scores which received IV adNIS MSC and IP AdNIS Tc injections, while mice receiving adNull and untransduced MSCs did not show signal following in vivo imaging. PCR for GFP genomic DNA, confirmed the in vivo imaging platform results.

Conclusion: Using the Bazooka SPECT we determined intravenous administration of MSC to be the optimal route of delivery in the CIA model.

P 100

Characterisation of the replication kinetics of infectious EIAV in permissive cell lines: Application

Dr DC Farley, Dr R Bannister, Mrs M Carlucci, Dr J Miskin, Dr K Mitrophanous

We have developed a minimal gene therapy lentiviral vector system based on Equine Infectious Anemia Virus (EIAV), which is being utilised in a number of indications, including Parkinson’s disease. Safety testing for RCL for manufacturing of RCL in batches of clinical product and end-of-production cells is a prerequisite for drug release. Since wild type EIAV dose not replicate in human cells, we previously developed an assay using murine leukemia virus (MLV) as a surrogate RCL positive control and HEK293 cells (human) as an indicator cell line to amplify RCL/MLV from a minimal infectious dose (M.I.D.) [Miskin et al., 2006]. Detection of reverse transcriptase (RT) within supernatants by qRT-PCR provides the assay end-point, with a read-out differential of 4-5 logs between RT-positive and RT-negative samples.

As part of the ongoing characterisation of EIAV vectors we evaluated the replication kinetics of the wild type virus on a number of cell lines reported to be permissive for infection. EIAV grew maximally in 92BR (donkey) cultures. Accessory gene knock-out strains were generated to assess growth of attenuated EIAV. No amplification of EIAV lacking either Tat or Rev was observed in 92BR cultures over a period of at least 40 days, but EIAV lacking an intact S2 open reading frame replicated with similar kinetics as EIAV. In addition we will present empirical data supporting the use of Poisson distribution equations to determine the theoretical minimal infectious dose required to set confidence levels for detection of an RCL in a cell based amplification assay.

P 101

Cell therapy of DMD: development of GMP manufacturing and testing methods for clinical application

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 MolMed S.p.A. has extensive expertise on cellular and gene therapy product development. Our GMP facility is formally authorized for the production and release of medicinal products for human use. MolMed will act as GMP manufacturing site for an upcoming clinical trial for Duchenne Muscular Dystrophy (DMD), conducted at HSR, based on the use of human mesoangioblasts (hMAB), cells showing myogenic potential and ability to negotiate vascular walls.

Methods for hMAB isolation, expansion and characterization were scaled-up, optimized and adapted to GMP.

A good cell expansion (range 2.2-3.6) was obtained in flasks and cell factories up to p20. As expected, cultured hMAB expressed the mesenchimal stem cell/hMAB markers CD44 and CD13, while they did not express the endothelial/hematopoietic cell markers CD31, CD34, CD45; satellite cells (CD56+) were <5%. The senescent cells were <6% up to p29, no chromosome abnormalities were detected.

Spontaneous differentiation into myotubes was observed up to p16. Thawed cultured cells were maintained for 4 days, and then formulated as required for patient treatment. Viability was >90% and cell expansion was ~3.

The following GMP process was designed: hMAB from dedicated donors will be expanded in cell factories (~1 ± 0.3x10^7 total cells at p5-p11), then frozen constituting the Intermediate Product (IP); IP cells will be thawed, briefly cultured, then formulated in saline solution/heparin, constituting the fresh Medicinal Product (MP).

IP and MP will be characterized for Identity/Potency, Safety, and Purity.

P 102

Large scale LV production and purification: process optimization for in vivo gene therapy studies

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Molmed S.p.A. is a biotech company focused – inter alia – on development of cell/gene therapy products. A GMP process for LV production and purification was developed in collaboration with HSR-TIGET and Génethon and is currently used for LV manufacturing for ex-vivo gene therapy clinical trials sponsored by Telethon.

Based on such process, the production of high quality purified LV for in-vivo haemophilia B gene therapy in large animal models was designed.

Vector production is based on quadri-transfection of 293T cells with a third generation LV system in 12 ten-trays cell factories (CF10), followed by purification through endonuclease treatment, anion exchange chromatography, concentration, gel filtration and final sterilizing filtration.

The current process has an overall yield around 30% in terms of total viral particles and results in 99% reduction of key contaminants such as plasmid DNA, host cell DNA and proteins.

To obtain a product suitable for in-vivo application in dogs, the following parameters have been considered:

- Selection of protein-free formulation buffer (PBS) and evaluation of vector stability in storage conditions (-80°C)
- Increase of LV production efficiency using strategies improving transcription and translation of packaging proteins and viral RNA
- Improvement of vector purity by further reducing DNA contaminants

Results indicate that PBS as protein-free formulation buffer is suitable and guarantees vector stability for at least 12 months, without significant variations in infectious titer and infectivity.

Up to 5x increase in LV production has been obtained at 1 CF10 scale.

The addition of a second endonuclease treatment is under evaluation.

P 103

Histidine-rich amphipathic peptides promote efficient delivery of nucleic acids into mammalian cells

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Besides being a useful tool in research, gene transfer has a high potential as treatment for a variety of genetic and acquired diseases. However, in order to enable a gene to become a pharmaceutical, efficient and safe methods of delivery have to be developed. We found that cationic amphiphatic histidine-rich peptide antibiotics can efficiently deliver DNA into mammalian cells. Our lead compound, LAH4 (KKAL-LALALHLHLALALALKKA), demonstrated in vitro transfection efficiencies comparable to those of commercially available reagents. Synthesis and evaluation of LAH mutants provided evidence that the transfection efficiency depends on the number and positioning of histidine residues in the peptide as well as on the pH at which the in-plane to transmembrane transition takes place. Our results also suggest a mechanism of selective destabilization by LAH4 of anionic lipids in the membranes of cells during transfection. Further results indicate that acidification of the endosome results in high local concentrations of free peptide in this organelle. These peptides become then available to interact with the endosomal membranes and thereby are responsible for the delivery of the plasmid DNA complex to the cytoplasm. When these data are taken together, they indicate a dual role of the peptide during the transfection process, namely DNA complexation and membrane permeabilization. Finally, we will report that peptides of the LAH family are efficient siRNA delivery vehicles.

P 104

VEGF receptor binding peptide linked amphiphilic peptide micelles for targeting delivery of plasmid

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Angiogenic endothelial cells are important target for cancer gene therapy. In this research, an angiogenic endothelial cell-targeting carrier was developed with VEGF receptor binding peptide (VRBP) and amphiphilic peptide micelles. The amphiphilic peptide was RRRVVVVVV (R3V6), which formed peptide micelles in aqueous solution. In the previous study, it was proved that V3R6 delivered plasmid DNA (pDNA) to various types of cells and did not have any toxicity. VRBP was a previously identified short peptide, whose sequence was ATWLPPR. VRBP-linked R3V6 was synthesized chemically for targeting gene delivery. In gel retardation assay, pDNA was completely retarded at a 2:1 weight ratio (peptide:pDNA). Heparin competition assay suggests that VRBP-R3V6/pDNA complex was dissociated more easily than poly-L-lysine (PLL)/pDNA complex. In transfection assay to calf pulmonary aortic endothelial (CPAE) cells, VRBP-R3V6/pDNA had the highest transfection efficiency at a 30:1 weight ratio (peptide:pDNA). In addition, VRBP-R3V6 had higher transfection efficiency than PLL and R3V6. In cytotoxicity assay, VRBP-R3V6 did not show any detectable toxicity to cells. Considering higher transfection efficiency to CPAE and lower cytotoxicity than PLL, VRBP-R3V6 may be useful for targeting gene delivery to angiogenic endothelial cells.
Development of a High Throughput Immunoassay for Screening ProSavin® Producer Cell Line Clones

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ProSavin® is an Equine Infectious Anaemia Virus (EIAV) based lentiviral vector for the treatment of Parkinson’s disease that encodes three enzymes required for dopamine synthesis: aromatic amino acid decarboxylase (AADC), tyrosine hydroxylase (TH) and GTP-cyclohydrolase 1 (CH1). Producer cell lines (PCLs) are being developed for the large scale manufacture of ProSavin®. Such cell lines contain a regulatory protein (Tet repressor) and the vector components (EIAV Gag/Pol, VSV-G envelope and the EIAV ProSavin® vector genome) stably integrated into the cellular genomes. To identify cell lines that are capable of producing ProSavin® at high titre a large number of clones have to be screened. This process is labour intensive, involving clonal cell expansion, preparation of a clone cryobank, manipulation of clones for screening, and assaying of vector-containing cell culture supernatants to assess titres. For this reason a high throughput titration assay would significantly expedite the selection of high vector producing clones.

Currently, ProSavin® is quantified by transduction of HEK293T cells cultured in 12-well plates. Transduced cells are determined using a flow cytometry based method to quantify fluorescently labelled cells, or by DNA integration assay which uses a quantitative PCR approach. Both methods are time consuming and are not considered high throughput. Here we discuss the development of a number of 96-well plate based ProSavin® titration assays which employ the use of immunodetection for TH. The applicability of these assays as a general screening strategy will be discussed in greater detail.

Progress in minicircle manufacturing and performance testing

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The dissemination of antibiotic resistance genes, as well as the uncontrolled expression of backbone sequences present in plasmids may have profound detrimental effects. Additionally, unmethylated CpG motifs have been shown to silence episomal transgene expression. Therefore, an important goal in vector development is to produce supercoiled DNA lacking bacterial backbone sequences: minicircle DNA. PlasmidFactory®s technology facilitates the production of highly pure minicircle DNA for applications in gene therapy and vaccination as well as virus production.

The production technology is based on two processes:

1) An inducible, sequence specific, and very efficient in vivo recombination process

2) A chromatographic purification technology for the isolation of the minicircle DNA.

The resulting minicircle DNA only consists of the gene of interest and a tiny residual sequence stretch including one of the two recombination sites. The chromatographic purification results in an exceptional purity, proven by various QC tests, which is extremely important since even small amounts of contaminants can produce dramatic experimental artefacts.

For first efficacy studies, reporter genes for different types of analyses within various tissues, cells, animals and for testing the mode of administration (e.g. electro gene transfer, sonoporation, lipofection, magnetofection etc.) have been used. Also biodistribution studies using these constructs have been performed. Additionally, minicircle constructs for vaccines, virus production etc. are currently investigated.

The results demonstrate a significant increase of gene expression of minicircle DNA in comparison to a standard plasmid.

As a result of the work presented here, PlasmidFactory has launched several commercially available minicircle DNA products.

RAG1 and RAG2 as Potential Mediators of Targeted Gene Integration

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Gene therapy is a promising method for treating a number of genetic and non-genetic diseases. The most straight forward strategy to treat individuals with deleterious mutations is by inserting a functional version of the gene in the genome. However, the occurrence of vector-related side effects has highlighted the requirement for improved vector designs.

The present project explores an up until now ignored possibility of using the naturally occurring recombinases, RAG1 and RAG2, to introduce the wanted transgene specifically into the human immunoglobulin (lg) gene loci. These enzymes recognize unique Recombination Signalling Sequences (RSS) that could act as potential integration sites for the transgene.

Different B cell lines were tested for their RAG1 and RAG2 expression and their integration sites were mapped. Molecular cloning was used to generate an expression vector containing the integration mediating enzymes, Rag1 and Rag2. We are currently in the process of establishing an in vivo assay for testing the functionality of exogenously expressed Rag1 and Rag2’s. We will observe their ability to execute recombination both on artificial substrate introduced into a B cell line but also directly within the Ig Loci.

After succeeding with the in vivo assay, it is our ambition to use the RAG1/RAG2 expression vector as mediator of targeted integration of a reporter gene.
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**Distribution of Lentiviral Vectors in Gene Therapy for X-Adrenoleukodystrophy**

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Gene therapy using retroviral vectors has been successful in treating monogenic diseases, but insertion of functional retrovirus LTRs can lead to severe side effects. Lentiviral vectors with self-inactivating (SIN) configuration are a promising alternative showing a reduced genotoxic risk. Here, we report on the first clinical trial using HIV-1 lentiviral SIN-vector to treat monogenic diseases. The treatment of the genetic defect in cerebral adrenoleukodystrophy (X-ALD) showed stable and sustained restoration, and is so far not accompanied by signs of clonal dominance or even premalignant disproportionational distribution of cellular contributions. We performed a large scale vector integration sites analysis on ex vivo transduced cells prior to reinfusion and on engrafted cells by LAM-PCR and subsequent pyrosequencing on samples from the first three patients treated by now. Our results revealed the expected insertions profile for lentiviral vectors, showing gene coding regions as preferred targets for lentiviral vector integration. Interestingly, we observed insertion of the lentiviral vector as common integration sites in same genes or gene regions common in all three patients, likely reflecting a preference of lentiviral vector insertion for particular genes. Furthermore, the occurrence of identical IS identified in myeloid and lymphoid lineages indicated a successful ex vivo transduction of early hematopoietic progenitors. High throughput distribution analysis of the IS repertoire indicates that lentivirus vectors offer great promise for safe and effective correction of human stem and progenitor cells.

**P 109**

**Transplantation of gene-modified HSCs for HIV patients with malignant diseases indicating H SCT**

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Highly active antiretroviral therapy (HAART) treatment has shown great efficacy in suppressing HIV replication and AIDS, but is still not available to all patients. Moreover, HAART does not lead to virus elimination and is often accompanied by severe side effects. Additional limiting factors include patient compliance and appearance of multiple-resistant HIV strains. On the other hand, transplantation of genetically protected haematopoietic stem cells (HSC) may lead to complete suppression of viral replication, as convincingly demonstrated by Hütter and colleagues who transplanted an HIV-positive leukaemia patient with HSC from a donor homozygous for the CCR5<sup>32D12</sup> mutation. However, due to severe side effects allogeneic H SCT would be an option for high-risk patients, and approx. 1% of Caucasians have a CCR5<sup>32D12</sup> donor, only. Therefore, gene therapy may represent a promising alternative. We have recently introduced a novel gene therapy principle based on expression of a membrane-anchored entry inhibitor (maC46) in HIV target cells transduced with the retroviral vector M870. maC46 has successfully been tested in numerous pre-clinical systems and a first clinical study using autologous T cells from HIV patients (van Lunzen et al 2007). We have now developed a new clinical protocol involving the genetic modification of HSC with M870. Based thereon, HIV target cells (T cells, monocytes) derived from gene-modified HSC are expected to be protected from HIV entry. The clinical protocol is open for patients in need of autologous or allogeneic H SCT, depending on the underlying disease. Study outline and preliminary patient data will be presented.

**P 110**

**Two Patients of a Successful WAS Gene Therapy Trial Show a Polyclonal Integration Site Distribution**

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In a German clinical gene therapy trial two WAS patients were successfully treated by receiving autologous CD34+ cells that were transduced with a MLV based retroviral vector. LAM-PCR analysis combined with high throughput sequencing showed a polyclonal and lineage-specific integration site distribution with >5700 unique integration sites (IS) for patient 1 (P1) and >9500 unique IS for patient 2 (P2). The strongest integration clones show an IS upstream of CCND2 for P1 and an IS within the MD5 locus for P2. Both clones contribute continuously to the gene-corrected hematopoiesis until the latest time point analyzed. We further detected a high clustering of up to >90 IS within the proto-oncogenes MDS1-EVI1 (P1: 81 IS; P2: 94 IS), PRDM16 (P1: 10 IS; P2: 28 IS), LMO2 (P1: 13 IS; P2: 29 IS) and CCND2 (P1: 11 IS; P2: 18 IS). The analysis of those IS in sorted cell fractions showed a distinct distribution. Thus, IS within or close to the gene loci MDS1-EVI1 and PRDM16 were predominantly detected in the myeloid fraction whereas IS within or close to LMO2 and CCND2 occurred preferentially in the lymphoid fraction. Despite the detection of IS within or close to already known proto-oncogenes, we found a highly polyclonal reconstitution of the hematopoietic system until 3 years after gene therapy (1108 and 1071 days after gene therapy, respectively). Further analysis of the clonal composition and a high throughput sequencing analysis will help to further study the efficacy and safety of the used vector system.

P 111

Pilot phase I/II clinical trial with autologous mesenchymal stem cells in patients with multiple sclerosis

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Mesenchymal stromal cells (MSC) have shown neuroprotective and immunomodulatory effects. A phase I/II clinical trial was designed to evaluate the feasibility and safety of intrathecal and intravenous administration of autologous MSCs in patients with multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS). Fifteen MS (mean EDSS = 6.7 ± 1.05) and 19 ALS patients (mean ALSFRS = 20.26 ± 8.56), were enrolled. Following culture, a mean number of 63.2 ± 2.5x10⁶ MSCs was injected intrathecally (n = 35) and intravenously (n = 14). In 9 cases, MSCs were magnetically labeled with the superparamagnetic iron oxide, Feridex®. In 21 patients there were injection-related side effects consisting of transient fever and headache. No major side effects were reported during a follow up period of up to 25 months. The mean ALSFRS remained stable during the 6 first months of observation, whereas the mean EDSS score improved from 6.7 ± 1.05 to 5.9 ± 1.6. MR imaging visualized the MSCs in the occipital horns of the ventricles, and indicated a possible migration of Feridex®-labeled cells in the meninges, the subarachnoid space and the spinal cord. Immunological analysis, revealed an increase in the proportion of CD4 + CD25 + regulatory T-cells, and a decrease in the proliferative responses of lymphocytes, the IFNγ and IL-17 production and of the expression of CD40, CD83, CD86, and HLA-DR on myeloid dendritic cells, at 24hrs following MSC transplantation. Our findings suggest that intrathecal and intravenous injection of MSCs in patients with MS and ALS, is a clinically feasible and relatively safe procedure. Controlled studies are needed to evaluate the long term safety and the potential clinical efficacy of MSC treatment.

P 112

Therapeutic efficacy of hematopoietic stem cell gene therapy for Hurler Type 1 Mucopolysaccharidosis

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Type 1 Mucopolysaccharidosis (MPS I) is an inherited lysosomal storage disorder caused by the deficiency of α-L-iduronidase (IDUA), which is responsible for systemic glycosaminoglycans (GAGs) accumulation, leading to skeletal and joint disease, visual and auditory defects, cardiac insufficiency, hepatosplenomegaly and mental retardation. The available treatment options (enzyme replacement therapy and hematopoietic cell transplantation -HCT) are poorly effective on skeletal and brain disease manifestations. In order to improve the therapeutic efficacy of HCT on these skeletal and neurological manifestations, we are developing a gene therapy (GT) strategy based on IDUA supra-normal expression in hematopoietic stem cells (HSC) and their progeny by lentiviral vector (LV) gene transfer. Transduction of murine HSC with a LV encoding the human IDUA cDNA led to enzyme over-expression up to 150 fold above normal levels in their differentiated progeny, which could thus more abundantly correct affected tissues upon infiltration. Indeed, when these cells were transplanted into Idua-/- mice, supra-normal
enzyme expression was observed in circulating hematopoietic cells and in all tested diseased tissues, including the brain, in which wild type HCT failed to deliver the functional enzyme. Importantly, GT corrected all major disease manifestations, decreasing GAGs storage and improving auditory impairment and retinal structure. Moreover, GT demonstrated a greater efficacy than wild type HCT in correcting the MPS I-related skeletal and behavioral defects. Thus, these results demonstrate that enzyme over-expression renders HSC GT effective in tissues refractory to correction following transplantation of normal donors’ HSC, paving the way for future clinical testing.

**P 113**

Update on a Japanese clinical trial of stem cell gene therapy for ADA-deficiency

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We report an update of ~6 year follow-ups of our ADA-SCID gene therapy (GT) trial carried out without cytoreductive conditioning. Pt 1 received BM CD34+ cells transduced with the RV vector GCsapM-ADA in Dec 2003, and so did pt 2 in Feb 2004. At present, both patients remain off PEG-ADA with toxic metabolite levels in erythrocytes being kept low, indicating continuous production of substantial amounts of ADA from hematopoietic compartments reconstituted by gene-corrected cells. Immunity still stays protective in both patients, although their lymphocyte counts are currently low requiring replacement of IVIG. Hematopoietic gene marking has been evident with the highest levels in T cells. These cells showed supra-normal ADA activity, demonstrating functional correction in circulating T lymphocytes. Despite this, recent thymic emigration has not been demonstrable, indicating that full T cell reconstitution in ADA-GT likely necessitates measures to enhance thymic repopulation, such as mild preconditioning. Gene insertion analysis revealed long-term engraftment of gene-marked primitive hematopoietic cells sustaining lympho-myeloid reconstitution with common RV integration sites shared between lineages. No serious adverse events have been observed. Overall, GT for ADA-deficiency without cytoreductive conditioning may be considered to be a safe treatment capable of providing patients with sustained generalized detoxification. Immune reconstitution, however, may not be achieved with the current protocol at the levels comparable to those observed in pioneering GT trials utilizing mild preconditioning. Thorough improvement in strategies is necessary in a way that enables enhancement in reconstitution abilities of hematopoietic stem/progenitor cells and/or T cell precursors.

**P 114**

Integration Site Analysis and Deep Sequencing for Vector Biosafety Assessment in CGD Gene Therapy

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Insertional activation of MDS1/EVI1 has led to the clonal expansion of gene marked myeloid progenitor cells, triggering the development of monosomy 7 and a myelodysplastic syndrome in both patients enrolled in a clinical trial for the treatment of X-linked chronic granulomatous disease (X-CGD). To decrease vector-host interactions and associated side effects a new self-inactivating (SIN) gammaretroviral vector (SF91sfes.gp91s) was developed. The internal cellular promoter and a codon-optimized transgene aim to improve the safety and efficacy of gene delivery (Manuel Grez, Georg-Speyer-Haus, Frankfurt). We performed high-throughput integration site (IS) analysis in combination with deep sequencing (454/Roche) for vector biosafety assessment on mice transplanted with gene modified hematopoietic stem cells. The full LTR SFFV-promoter driven vector which was used in the previous trial was analyzed as a control (SF91eGFP).

We could detect a total of 1159 unique IS in SF91eGFP (n = 4) and 2261 in SINfes.gp91s mice (n = 5). In average, we retrieved 281 unique IS in SF91eGFP mice, showing a less polyclonal IS pattern than SINfes.gp91s transplanted mice (442 unique IS/mouse). Moreover, (pre)dominant clones were found more frequently in mice transplanted with the SF91eGFP vector than with SINfes.gp91s. Furthermore, in SINfes.gp91s mice no CIS formation occurred in/near EV1/MDS1 while in SF91eGFP mice 5 IS formed 2 CIS upstream of MDS1 and EV1, respectively. Finally, SF91eGFP secondary recipients revealed an increased in vivo skewing towards cancer related genes compared to SINfes.gp91s (p = 2.9*10−6). Overall, the SINfes.gp91s vector showed no obvious signs of vector-induced side-effects.

**P 115**

LV mediated Gene therapy for Wiskott-Aldrich Syndrome: in vitro and in vivo preclinical studies

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Wiskott-Aldrich Syndrome (WAS) is an X-linked immunodeficiency characterized by thrombocytopenia, infections, autoimmunity and lymphomas. We previously demonstrated that a lentiviral vector (LVV) encoding for human WAS under the control of endogenous 1.6 kb promoter efficiently corrected
human and mouse cells. We then set up a clinically applicable and efficient transduction protocol based on 60 hours of culture and 2 hits of gene transfer (MOI 100) on CD34+ cells from normal donors. The selected protocol was applied to WAS patients bone marrow CD34+ cells for the validation of the GMP grade LVV. Transduced cells showed a vector copy number per cell of 1.4±0.3 and about 80% of transduced colonies. WAS cells proliferated less than normal donors (with or without LVV exposure) but there was no toxicity of LVV on clonogenic progenitors. Following gene transfer, WASp expression was restored in patients’ differentiated cells, including megakaryocytes. Analyses of vector integrations on in vitro transduced CD34+ cells showed polyclonal integrations with the expected bias of LVV for transcriptional units. Pharmacokinetic of transduced CD34+ cells was studied by injection in sub-lethally irradiated neonate Rag2−/−/c−/− mice. Transduced cells showed a normal biodistribution to hematopoietic organs and germline transmission. In conclusion, we demonstrate that our GMP grade LVV allows robust and reproducible transduction of CD34+ cells leading to restoration of WASp expression with no toxicity. A phase I/II gene therapy protocol based on infusion of transduced CD34+ cells combined with a reduced intensity conditioning has recently started.

P 116

Acceleration of osseointegration by nanoparticle loaded dental implants: A study in mini pigs

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The loss of dental implants is a major obstacle in reconstructive surgery and is often caused by an insufficient initial formation of a tight bone implant interface. Using a large animal model i.e. the Göttingen mini pig we examined whether nanoparticle loaded implants could be used to accelerate the contact between bone and the metallic surface of the implant. Nanoparticles were generated using plasmid DNA/branched polyethylenimine complexes shielded with a protective polyethylene glycol polymer which were lyophilized and dispersed in an organic poly(D,L-lactic acid) solution to obtain stable biodegradable films of the implant surface. A new loading procedure “template guided coating” resulted in uniform films on the metallic surface as seen in raster electron microscopy with nanoparticle loading efficiencies of about 60% as estimated by 125I labeled DNA. Ex vivo studies were performed by drilling coated radioactive implants into pieces of bone which resulted in an abrasion of about 50% of the nanoparticle layer. For the animal study each mini pig (n=18) received six implants coated either with BMP-2 or Luciferase plasmid (used as control) into the maxilla in a split-mouth-design with a DNA concentration of 6 µg and 12 µg respectively. Additional implants were coated with 50 or 150 µg recombinant BMP-2 protein. Exact evaluation of the bone implant contact area performed by µCT analysis using a new algorithm demonstrated that plasmid BMP-2 nanoparticle loaded implants lead to a significant greater bone regeneration and osseointegration.

P 117

Angiogenic gene therapy with VEGF and HGF

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Background: Critical limb ischemia (CLI) is an ideal research target, in that its treatment outcome is clear-cut, ending in either limb amputation or functional limb salvage. Surgical and endovascular revascularization is the treatment of first choice for CLI, but with current therapy, it can be expected that >25% of CLI patients will require major limb amputation within 12 months. Therapeutic angiogenesis is a new strategy that attempts to improve the perfusion of ischemic vascular beds by promoting the formation of new blood vessels using gene therapy.

Methods and Results: A double-blind, placebo-controlled, vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) trial was conducted in patients with critical limb ischemia (with claudication, rest pain, ulcer lesions and an ankle/brachial index < 0.5). Patients were randomly assigned to placebo (n=10) and to drug administration with 10^5 copy of both VEGF and HGF pBLAST plasmids (n=10). Study drug was injected directly into the muscles of the ischemic limb. Intramuscular administration was safe and well tolerated. The clinical evolution has been monitored by ankle/brachial index, walking distance and the relief of rest pain. Six months after therapy 7 patients show partial or complete relief of rest pain, improvement of ischemic ulcer lesions and increased walking distance on the rolling carpet. The improved perfusion to the distal ischemic limb was demonstrated by a mean increase of 0.1 in the ankle/brachial index.

Conclusions: Therapeutic angiogenesis with VEGF and HGF gene transfer is a safe and non-inflammatory procedure with a good effectiveness in the revascularisation of the ischemic limb.

P 118

A study of purity test for keratinocyte therapy products

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Many keratinocyte therapy products are in development or have recently been introduced onto the market. Recently new
products are constantly produced and approved for clinical use in burn surgery and other chronic wounds. The purpose of this study is to suggest the purity test method for keratinocyte therapy products. In this study, we investigated the cellular impurity test and in-process impurity test for keratinocyte therapy products. For cellular impurity test, the ratio of inactive cell (fibroblast, endothelial cell and melanocyte) to against active cell was detected using real-time PCR and FACS (fluorescent activated cell sorter). Because of unavailability of specific surface marker, cellular impurity results by real-time PCR was more objective than FACS. For in-process impurity test, the amount of residue gentamicin and FBS were detected. A sensitive and robust high-performance liquid chromatographic method with fluorescence detection (HPLC-FLD) was developed for the determination of gentamicin residues in keratinocyte therapy product. Gentamicin is consists of four major components, C1, C1a, C2, and C2a. BSA (bovine serum albumin) residues of FBS were detected by commercial test kit based on ELISA (Enzyme-Linked Immunosorbent Assay).

P 119
Overview of Regulations and Standards for Stem Cell-Based Therapies: An International Perspective
Ms TM Nguyen1

As stem cell research continues to move towards more clinical applications, it is important to question how these stem cell-based products will be regulated. Stem cell-based products are novel and have complex characteristics due to their different sources, potency or uses, and so may be defined and classified in different ways. Even then, these products may not fit perfectly within existing categories leaving them potentially unmonitored or unregulated. As a result, this exposes challenges with respect to ensuring safety and quality in the processing, distribution and international circulation of human cell and tissue products. Stem cell-based therapy regulation varies between jurisdictions according to product characterization. However, there is a trend towards ensuring proper oversight of these therapies through different regulatory requirements and product approval processes.

This study seeks to illuminate particular barriers to harmonizing international laws and policies related to clinical translation of stem cell and to investigate potential methods for overcoming these challenges. This study will provide a comparative analysis of the regulatory frameworks surrounding the clinical translation of stem cells in various jurisdictions.

It is concluded that while the challenges to harmonization are diverse and important, so too are the benefits of establishing uniformity in the regulation of stem cell-based therapies worldwide. There is still much to learn and discover about stem cell-based therapies but it is important that the infrastructure is in place to determine the safety and efficacy of these therapies and products.

P 120
Gene therapy trial with lentiviral vector transduced CD34+ cells for the treatment of Wiskott-Aldrich Syndrome

Wiskott-Aldrich Syndrome (WAS) is an X-linked immunodeficiency characterized by thrombocytopenia, infections, autoimmunity and lymphomas. Gene therapy with autologous hematopoietic stem cells (HSC) could represent a valid alternative to allogeneic transplant for patients lacking an HLA-identical donor or who are at high risk of complications. We have developed a novel approach based on a self-inactivating lentiviral vector (LVV) encoding for human WAS under the control of the endogenous 1.6 kb promoter. We previously showed that this vector is safe and efficiently corrects the WAS defect in the murine model of the disease and in human cells. A highly purified, GMP grade, LVV transduced at high efficiency human CD34+ cells from healthy donors and patients, allowing restoration of WASp expression in multiple lineages without toxicity. A phase I/II protocol aimed at studying safety, biological activity, and efficacy of gene therapy in 6 WAS patients was opened in April 2010. Patients will receive preconditioning with anti-CD20 monoclonal antibody and reduced intensity busulfan and fludarabin; ATG will be included in case of grade 3 or 4 toxicities. Patients will be transplanted twice, first with LVV transduced human CD34+ autologous hematopoietic stem cells (HSC) and then with LVV transduced human CD34+ autologous hematopoietic stem cells (HSC).

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gene therapy for WAS patients using lentiviral-vector transduced HSC in combination with reduced intensity conditioning.

**P 121**

Lentiviral vector-mediated gene transfer of FOXP3 into CD4+ T cells isolated from patients with IPEX Syndrome generates potent suppressor regulatory T cells

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IPEX (Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-linked) is a genetic disease caused by mutations of the transcription factor FOXP3, the master gene for naturally occurring (n) CD4+CD25+ regulatory T (Treg) cell fate and function. It is well established that nTreg cells play a central role in the control of immune responses to auto-antigens, allergens, and tumor antigens. In IPEX patients the lack of functional nTreg cells leads to the development of early onset life-threatening systemic autoimmunity. Current therapies for the cure of patients with IPEX are limited. The majority of patients are treated with immunosuppressive drugs, with only partial control of the clinical manifestations. At present, haematopoietic stem cell transplantation (HSCT) is the only definitive cure, but it is available for a limited number of patients.

An alternative strategy to restore tolerance could aim at generating high numbers of human Treg cells by lentiviral-vector- (LV-) mediated gene transfer of FOXP3 (LV-FOXP3). We previously showed that transduction of human CD4+ T cells by LV-FOXP3 can efficiently inhibit Teff cell responses in vivo. In vitro we demonstrated that high an stable FOXP3 expression can be induced in CD4+ T cells isolated from patients carrying different FOXP3 mutations. FOXP3-mutated effector T (Teff) cells isolated from severely affected patients could be converted into functional Treg cells displaying potent in vitro suppressive activity. Preliminary results further suggest that LV-FOXP3 transduced cells can efficiently inhibit Teff cell responses in vivo in a xenogeneic graft versus host disease model (xeno-GvHD).

These results demonstrate that suppressive capacity is conferred to human T cells upon high and stable FOXP3 expression even in the presence of FOXP3 mutations, as in the case of IPEX patients. Overall, these findings pave the way for the development of a gene therapy approach using adoptive transfer of Treg-converted mature lymphocytes for the treatment of patients with IPEX Syndrome.

**P 122**

Enhancement of Ad-CRT/E7-mediated antitumor effect by preimmunization with L. lactis expressing HPV-16 E7

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**Background:** Developing effective vaccines that target HPV-16 E6 and/or E7 has become a necessity, because current prophylactic vaccines against HPV infection do not protect against cervical cancer already established. We previously showed the effectiveness of adenovirus expressing CRT/E7 (Ad-CRT-E7) in a cervical cancer animal model. We also demonstrated that intranasal immunization of Lactococcus lactis encoding HPV-16 E7 (Li-E7) anchored to its surface, induced significant HPV-16 E7-specific immune response. In this study we assessed the combination of both approaches in a cervical cancer animal model.

**Method:** Groups of mice were immunized intranasally with Li-E7 on days 0, 14, and 28. One week later, the mice were challenged with HPV-16 E7-expressing murine tumour cells (TC-1) in the right leg. Ad-CRT-E7 vector was injected intratumorally once they reached a diameter of 6 mm. Tumor growth and survival were monitored. Infiltration of CD8+ cytotoxic T lymphocytes and the presence of apoptosis within the tumour were analyzed.

**Results:** A single dose of Ad-CRT/E7 was able to reduce the tumour size 60%, with a 20% of survival, compared with the controls. Interestingly, this antitumor and survival effect were increased to 80% and 70% respectively by the intranasal preimmunization with Li-E7. Significant CD8+ cytotoxic T lymphocyte infiltration was detected in the tumours of mice treated with Li-E7 + Ad-CRT/E7. In situ TUNEL apoptosis analysis showed a strong correlation between tumour regression and a higher number of apoptotic cells.

**Conclusion:** Preimmunization with Li-E7 enhances the antitumour and survival effect of the Ad-CRT/E7 in a cervical cancer animal model.

**P 123**

Targeting lentiviral vectors to dendritic cells by the Nanobayer display technology

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Professional antigen-presenting cells (APC), such as dendritic cells (DCs) and macrophages, are targets for immunotherapy. Lentiviral vectors (LVs) have been successfully used to modify APC in vitro. However, their broad tropism limits their use in the clinic. As selective targeting will improve safety and efficacy, we developed the “Nano-body (Nb) display technology”. Herein, LVs are pseudotyped with a fusogenic, but binding-defective envelope together with a DC- and macrophage-specific Nanobody (DC2.1) or an irrelevant control (BCII10). We demonstrated the expression of Nbs on virus producing cells, their incorporation in the viral surface, and the production of high titer LVs. Flow cytometry revealed that BCII10 LVs didn’t mediate infection in vitro, whereas DC2.1 LVs selectively transduce primary DCs and macrophages. Broad tropism LVs additionally transduced T- and B-cells. We further evaluated the biodistribution of the LVs by in vivo bioluminescence imaging and flow cytometry demonstrating a similar transduction profile of the respective LVs in vivo as in vitro. In conclusion, we report for the first time on the “Nb display technology” for targeting of LVs to DCs and macrophages, a strategy that can be exploited in future applications, such as diagnostics and immunomodulatory therapies.

P 124

Modulation of dendritic cell maturation and function with small interfering RNAs targeting indoleamine 2,3-dioxygenase

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Background: Among the described mechanisms involved in the failure of immunotherapy, is the expression of indoleamine 2,3-dioxygenase (IDO) by dendritic and tumour cells. Therefore, strategies to silence IDO gene expression may improve anti-tumour immunity.

Methods: Human monocytes or monocyte-derived DCs were transfected with anti-IDO siRNAs and the effects of IDO gene silencing on DC maturation and function were investigated using standard methods.

Results: The designed siRNA totally inhibited IDO gene expression without impairing DC maturation and function. Depending on the design and chemical modifications, it is possible to design either monofunctional siRNAs devoid of immunostimulation or bifunctional siRNAs with gene silencing and immunostimulatory functions. Inhibition of IDO expression with both classes of siRNAs inhibited DC immunosuppressive function on T cell proliferation. Immature monocyte-derived DCs that had been transfected with siRNA-bearing 5'-triphosphate activated T cells, indicating that, even in the absence of external factors such as TNF-a, those DCs were sufficiently mature to initiate T-cell activation.

Conclusions: The inhibition of IDO expression and deliberate induction of cytokine with a single siRNA in immune cells should provide an important new immunotherapeutic option. The activation of DC maturation with 5'-triphosphate-bearing siRNA opens the possibility of generating mature DCs without the addition of external cytokines. This strategy of DC maturation may be extended into a therapeutic vaccination setting.

P 125

Bivalent Measles Virus Vaccines Presenting Different Forms of a Lentiviral Glycoprotein

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Live attenuated vaccines against measles virus (MV) are among of the safest vaccines and recombinant MVs that express foreign proteins have been shown to elicit significant immune responses and protection against the respective pathogens in animal experiments. Interestingly, all but one candidate vaccine published elsewhere expressed a soluble form of the foreign antigen. We therefore ask here whether the soluble, membrane-bound or other forms of the foreign antigen presented by recombinant MVs is critical for the induced immune responses.

We report the construction of soluble or stabilized (i.e. non-shedding) forms of the envelope (Env) glycoprotein of simian immunodeficiency virus SIVsmmPBj1.9, a model virus strain inducing acute viral pathogenesis. To this end, the SIV Env protein was genetically truncated to express only the ectodomain. Alternatively, the protease cleavage site was modified to prevent cleavage and allow expression of a stabilized gp160-Env. Bivalent recombinant MVs each encoding a different form of the SIVsmmPBj1.9 Env protein were rescued, successfully tested for Env expression, and shown to retain similar growth rates as parental MV with titers of up to 1x10⁸ TCID₅₀/ml. The bivalent vaccines were inoculated twice intraperitoneally into transgenic IFNARko-CD46Ge mice (1x10⁵ TCID₅₀/dose). Transgenic CD46 expression allows MV replication in these mice in vivo. We are measuring the quality of the cellular and humoral immune responses to different forms and amounts of the Env proteins presented by MV in mice. These studies will be used to select candidate MV-SIV vaccines for the analysis of the immune response and levels of protection in monkeys.

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Altered properties of BCR-ABL-Transformed mouse cells expressing endostatin

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Background: Among the described mechanisms involved in the failure of immunotherapy, is the expression of endostatin among of the safest vaccines and recombinant MVs that express foreign proteins have been shown to elicit significant immune responses and protection against the respective pathogens in animal experiments. Interestingly, all but one candidate vaccine published elsewhere expressed a soluble form of the foreign antigen. We therefore ask here whether the soluble, membrane-bound or other forms of the foreign antigen presented by recombinant MVs is critical for the induced immune responses.

We report the construction of soluble or stabilized (i.e. non-shedding) forms of the envelope (Env) glycoprotein of simian immunodeficiency virus SIVsmmPBj1.9, a model virus strain inducing acute viral pathogenesis. To this end, the SIV Env protein was genetically truncated to express only the ectodomain. Alternatively, the protease cleavage site was modified to prevent cleavage and allow expression of a stabilized gp160-Env. Bivalent recombinant MVs each encoding a different form of the SIVsmmPBj1.9 Env protein were rescued, successfully tested for Env expression, and shown to retain similar growth rates as parental MV with titers of up to 1x10⁸ TCID₅₀/ml. The bivalent vaccines were inoculated twice intraperitoneally into transgenic IFNARko-CD46Ge mice (1x10⁵ TCID₅₀/dose). Transgenic CD46 expression allows MV replication in these mice in vivo. We are measuring the quality of the cellular and humoral immune responses to different forms and amounts of the Env proteins presented by MV in mice. These studies will be used to select candidate MV-SIV vaccines for the analysis of the immune response and levels of protection in monkeys.
Background: Neangiogenesis plays an important role in chronic myeloid leukemia. We investigated whether gene modification of bcr-abl – transformed mouse cells resulting in endostatin production changed their oncogenic potential.

Method: Mouse B210 cells expressing BCR-ABL fusion protein and inducing leukemia and very rarely intraabdominal solid tumors after intravenous administration, were used. The cells were transfected with a plasmid carrying genes for mouse endostatin and resistance to blasticidine. Transduced cell clones were isolated in the presence of blasticidine. Production of endostatin was determined by Western blotting. BALB/c mice were inoculated intravenously and were followed for at least 75 days. The materials from the autopsied animals were tested histologically, immunohistologically and by the FISH technique.

Results: When compared with the original B210 cells, the capability of the gene-modified cells to induce leukemia was somewhat lower. However, mice which did not succumb to leukemia, later on developed aggressively growing intraabdominal and intrathoracic solid tumors. In the autopsied animals hepatosplenomegaly tended to produce solid tumors. FISH revealed the presence of bcr-abl fusion gene both in the tumours and spleens. In cultivated tumor-derived cells endostatin production was detected.

Conclusion: Surprisingly, B210 cells producing the angiogenesis inhibitor endostatin tended to produce solid tumors in intravenously inoculated mice much more frequently than the parental unmodified cells. This oncogenic activity was not associated with the loss of endostatin production.

Acknowledgement MZCR IGA: NS10634

P 128

mRNA-based vaccine: Dendritic cells targeting with Mannosylated and Histidylated lipopolypelexes and induction of antitumor vaccine

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Background: The transfection of dendritic cells (DCs) with mRNA encoding tumour antigen is a promising anti-cancer vaccine strategy that offers advantages over DNA transfection in terms of efficiency and safety. We report the preparation of mannosylated and histidylated lipopolypelexes (Man11-LPD100) as nanovectors that enhance mRNA transfection of DCs in vivo and induce anti-B16F10 melanoma vaccine effect in mice.

Method: Man11-LPD100 was prepared by addition of mannosylated and histidylated liposomes containing β-D-mannopyranosyl-N-dodecylhexadecanamide (11 mol %) on PE/ Glylated histidylated polylysine/mRNA polypelexes. Liposomes contained a cationic [O,O-diisoeyl-N-(3N-(N-methylimidazolium iodide)propylene) phosphoramidate] and a neutral [O,O-diisoeyl-N-histamine Phosphoramidate] co-lipid. The imidazole groups of the co-lipid and polymer favour endosome destabilisation and nucleic acids delivery in the cytosol.

Background: Granulocyte-macrophage colony-stimulating factor (GM-CSF) is strong stimulator of the immune system.

Methods: Mouse bcr-abl-transformed 12B1 cells which induce leukemia and also subcutaneous tumors in syngeneic animals were transfected with mouse GM-CSF gene. Groups of mice were inoculated subcutaneously with either 12B1-GM-CSF cells or parental 12B1 cells. At intervals two mice from each group were sacrificed. Blood sample was taken for cytological investigation and for determining the GM-CSF level in sera. At autopsy several organs were taken for histopathological, immunohistological, and cyto genetic investigation.

Results: After administration of 12B1 GM-CSF cells the first clinical symptoms were observed on day 6, i.e. about one week prior to appearance of subcutaneous tumors. Simultaneously a raise of GM-CSF level in serum was detected, which gradually increased until day 16. On day 6 the first signs of damage to lungs, heart, spleen, liver and kidneys were detected. These progressed in the subsequent days. Except of hepatosplenomegaly similar changes were not seen in mice inoculated with parental 12B1 cells. Starting on day 8 a marked increase of myeloid-derived suppressor cells was seen in 12B1-GM-CSF inoculated mice. Their numbers steadily increased and on day 18 they surpassed more than 10 times their counts in 12B1-inoculated mice and control animals. On the other hand, similar counts of T-reg cells were detected in both 12B1- and 12B1-GM-CSF inoculated animals.

Conclusion: The data strongly suggest that the extensive organ damage seen was caused by the overproduction of GM-CSF by the gene-modified cells.

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ESGCT 2010 POSTER PRESENTATIONS
Results: By scintigraphy, we showed that upon IV injection of Man11-LPD100 with 99mTc-DNA, 9% of the dose was accumulated in the spleen, a representative lymphoid tissue. We demonstrated that spleen from mice injected with EGFP mRNA Man11-LPD100 contained 4-fold more DCs expressing EGFP than the spleen from mice injected with LPD100. This better transfection of DCs was correlated with a better inhibition of B16F10 melanoma growth and an increased survival time when mice were immunized with Man11-LPD100 made with MART-1 mRNA.

Conclusion: These results indicate that Man11-LPD100 is an efficient system to deliver tumour antigen mRNA in splenic DCs for inducing antitumor vaccine. This project is developed in the frame of Ligue contre le Cancer (Nationale and Région Centre) and Cancéropole Grand Ouest.

P 129
Codon-optimized HC gene of Clostridium botulinum neurotoxin serotype E is an effective botulism DNA vaccine candidate
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Botulinum neurotoxins (BoNTs) produced by Clostridium botulinum are the most toxic substance known and act by preventing the release of the neurotransmitter acetylcholine at neuromuscular junction. BoNT consists of two polypeptide chains which are linked by a disulfide bond. The most commonly used human botulism vaccine is prepared from culture supernatant from C. botulinum. However, disadvantages associated with this vaccine, including simplicity of production and purity of product. Therefore, new generation botulism vaccines are required. In the present study, immunogenicity of plasmid DNA vaccine encoding the heavy chain (HC) domain of C. botulinum neurotoxin E was investigated. Using codon-optimized genes, DNA vaccines which expressed the carboxyl-terminal 50 kDa fragment of HC fused to IgM signal peptide were prepared. We transfected 293T cells with constructed plasmids and performed Western and ELISA assay. Transfection studies proved that fragment of HC protein was expressed from plasmid-transfected cells and was secreted into culture medium. By employing DNA vaccine in Balb/c mice, the immune response and corresponding neutralizing antitoxin titers were markedly increased. Our results suggest that our plasmid DNA vaccine can elicit strong immunogenicity and may be a potential alternative strategy to conventional protein vaccines against C. botulinum neurotoxin serotype E.

P 130
A Novel Adjuvant Ling Zhi-8 Enhances the Efficacy of DNA Cancer Vaccine by Activating Dendritic Cell
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DNA vaccine has been suggested to use in cancer therapy, but the efficacy remains to be improved. The immunostimulatory effect of a fungal immunomodulatory protein Ling Zhi-8 (LZ-8) isolated from Ganoderma lucidum has been reported. In this study, we tested the adjuvanticity of LZ-8 for HER-2/neu DNA vaccine against p185<sup> onc </sup> expressing tumor MBT-2. We first examined the effect of LZ-8 on mouse dendritic cells (DCs) because DCs play a key role in initiating vaccine responses. Recombinant LZ-8 activated mouse bone marrow-derived DCs and the stimulatory effect of LZ-8 was not due to any microbe contaminant. As shown by the poor responses of TLR4 mutant DCs to LZ-8, TLR4 was involved in LZ-8-induced DC activation. In addition, LZ-8 enhanced the ability of DCs to induce antigen-specific T cell activation <i>in vitro</i> and in a subunit vaccine model <i>in vivo</i>. Surprisingly, co-delivery of LZ-8 strongly improved the therapeutic effect of DNA vaccine against MBT-2 tumor in mice. This increase of anti-tumor activity was attributed to the enhancement of vaccine-induced Th1 and CTL responses. Consistent with the results from DCs, the promoting effect of LZ-8 on DNA vaccine was diminished when the MBT-2 tumor cells were grown in TLR4 mutant mice. Thus, we concluded that LZ-8 may be a promising adjuvant to enhance the efficacy of DNA vaccine by activating DCs via TLR4.

P 131
Prevention of streptozocin-induced diabetes mellitus via embryonal cell therapy
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In our research experimental plan we investigated the new property of glycoprotein of embryonal genesis (PEG) which is created by my scientific consultant prof.L.n.mkchyan and before it has been proved its anticancer effects as a new bio-compound of embryonal cell origin that could prevent tumor progress and it is known as antitumor modulator (EATM). Due to some observations in cancer patients who had simultaneously diabetes, this study was designed. We have a very interesting result as other biimmunological activity and preventive role in diabetic models, and could show decrease of glucose and modulation in some clinical biochemistry factors in hyperglycemic and diabetic condition. By means of a new combination of embryonal proteins as one of the natural fetus cell resource, this can be considered as a new anti-diabetic biodrug or diabetes new vaccine in future after complementary study and clinical evaluations.

P 132
AAV-mediated in vivo knock-down of luciferase using RNAi and U1i

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RNA interference (RNAi) has been successfully employed for specific inhibition of gene expression; however, safety and delivery of RNAi remain critical issues. We investigated the combinatorial use of RNAi and U1i to enhance target gene knock-down in vitro. Co-transfection of RNAi and U1i constructs in the HEK293T cell line reduced luciferase expression by 95%, which was more than observed for either inhibitor alone. Furthermore, we were able to attain similar knock-down when RNAi and U1i constructs were hydrodynamically transfected in mice, demonstrating for the first time the in vivo application of U1i. Moreover, we demonstrated long-term gene silencing by AAV-mediated transduction of murine muscle with RNAi/U1i constructs targeting firefly luciferase. In conclusion, these results provide a proof of principle for the combinatorial use of RNAi and U1i to enhance target gene knock-down in vivo.

P 133
Induction of long-term tolerance in IgE-mediated allergy by a gene therapy approach
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Background: Robust strategies for the prevention of allergy are currently not available. In a preventive murine model employing myeloablative irradiation we showed that tolerance towards an allergen can be induced through engraftment of retrovirally transduced syngeneic bone marrow (BM) expressing a membrane-anchored allergen (i.e. molecular chimerism). In the present studies we investigated whether tolerance can be induced in a non-myeloablative protocol.

Methods: BALB/c BM-cells (BMC) were transduced (VSV-Phl p 5) in vitro to express Phl p 5 (a major grass pollen allergen) (transduction efficiency: 7%). Non-myeloablated (6 Gy TBI) BALB/c mice received 2×106 Phl p 5-transduced BMC iv and were challenged with recombinant Phl p 5 and the major birch pollen allergen Bet v 1 (specificity control) at multiple time points post-BM transplantation (BMT).

Results: Non-myeloablated mice (n=10) transplanted with Phl p 5-transduced BMC developed low levels of white blood cell molecular chimerism (10/10) (approx. 1%). Serum levels of Phl p 5-specific IgE and IgG1 remained undetectable in 8/10 chimeras throughout follow-up (44 weeks post-BMT). Phl p 5-specific IgG2a and IgG3 was undetectable in any of ten chimeras at late time points (week 39/44), while in contrast Bet v 1-specific antibodies were detectable in all mice. Basophil degranulation assays revealed the absence of Phl p 5-specific degranulation in all chimeras. In T-cell proliferation assays chimeras showed specific non-responsiveness to Phl p 5.

Conclusion: This gene therapy approach demonstrates that even low levels of molecular chimerism are sufficient to maintain tolerance in allergy at the B-cell, T-cell and effector-cell levels long-term.

P 134
Restoration of dendritic cell functionality in gene therapy treated was−/− mice
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Wiskott-Aldrich syndrome (WAS) is a rare X-linked primary immunodeficiency caused by defective expression of the WAS protein (WASP) in haematopoietic cells. Several immune cell functions are altered in WAS patients, thus resulting in a complex clinical phenotype. Increasing evidences show the contribution of DC defects in WAS pathogenesis. Reduction of WASP expression impairs cytoskeleton reorganization and inhibits podosome formation in DCs, dramatically reducing their motility and functionality. Our group has previously demonstrated the efficacy of a gene therapy (GT) protocol in was−/− murine model. Haematopoietic stem cells from was−/− mice can be efficiently transduced with a lentiviral vector carrying human WAS cDNA driven by the endogenous promoter and reinfused in nonlethally irradiated recipient was−/− mice. As we showed correction in T cell proliferation and cytokine production, we are now interested in evaluating DC functionality in GT treated mice. We
detected normal DC count in secondary lymphoid organs and WASP positive DCs in spleen (~35%), lymph nodes (~22%) and thymus (~29%) of GT treated mice. To test DC functionality we differentiated them from bone marrow (BMDCs) of GT treated mice. We found that although only 20% of BMDCs from gene therapy treated mice expressed WASP, in vitro phagocytosis, in vivo migratory capacity and T cell priming are ameliorated compared to was<sup>−/−</sup> BMDCs. These results suggest that GT can efficiently restore DC functionality despite a reduced frequency of WASP expressing DCs.

P 135
Structural components of AAV capsids induce cell autonomous immune responses in human non-parenchymal liver cells
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Clinical efficacy of adeno-associated viral vectors (rAAV) is limited by humoral and cytotoxic T cell responses towards the viral capsid. Since adaptive immune responses are a consequence of pathogen recognition by the innate immune system, we investigated cell autonomous immune responses towards rAAV2 and rAAV8, which are both used for hepatic gene transfer. The liver consists of hepatocytes and non-parenchymal cells. While the former were successfully transduced by rAAV2 and rAAV8 in the absence of innate immune activation, in primary human non-parenchymal liver cells an NFκB-mediated inflammatory response was induced. Interestingly, neither serotype elicited type I interferon, which was in contrast to anti-AAV immune responses in plasmacytoid dendritic cells (pDC). In line, pDC and human non-parenchymal liver cells differed in the mechanism of recognition since viral capsids, but not genomes, were identified as the main pathogen associated molecular pattern (PAMP). The pathogen receptor of this newly identified PAMP is likely located at the cell surface, but seems non-functional in murine LSEC as neither vectors nor empty capsids have mounted cytokine up-regulation in murine LSEC. Hence, mechanism and consequences of the innate immune response in human liver cells differ from known responses and are key to understand the limitations of gene therapy using rAAV.

P 136
Lentiviral gene therapy of RAG1 SCID: risks and implications for treatment
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Recombination activating gene 1 (RAG1) deficiency results in severe combined immunodeficiency (SCID) due to complete lack of T and B lymphocytes. Consequently, these patients succumb from recurrent infections.

Aim of the study was to develop lentiviral gene therapy for RAG1 SCID. Constructs containing the viral SF or cellular PGK, EFS and UCOE or more pathway-restricted promoters of the RAG1, RAG2, IL2RG and TCRß7 gene driving sequence optimized RAG1 were compared for efficacy and safety in Rag1<sup>−/−</sup> mice. A standard 5×10<sup>6</sup> transduced lineage negative bone marrow cells containing on average 1-3 integrations per cell were transplanted in mice subjected to 6 Gy (γ-rays) conditioning.

Peripheral blood (PB) CD3<sup>+</sup> T cell reconstitution was achieved with all promoters (n = 54, 191 ± 199 cells/mL at month 5), but 8-fold lower than wildtype Rag1<sup>−/−</sup> transplanted mice (Rag1-WT, n = 7; 1479 ± 251 cells/mL). However, IL-2 mediated T cell responses, T cell receptor diversity and T-cell dependent immune responses were restored. In contrast, reconstitution of mature PB B220<sup>+</sup>IgM<sup>−</sup>IgD<sup>−</sup> B-cells was low for the SF vector (42 ± 47 cells/mL) as compared to Rag1-WT (596 ± 233 cells/mL). Although PB B-cell numbers were low, plasma immunoglobulin isotype levels were detected in the majority of the mice.

Two months onward, GT treated mice developed rashes with cellular tissue infiltrates, activated PB CD4<sup>+</sup>CD69<sup>+</sup> T-cells and high IgE plasma levels. Although immunological functions were restored in a proportion of these mice, these results underline that further development is required for successful RAG1 gene therapy with an inherent potential risk to develop autoimmune symptoms.

P 137
Therapeutic application of selective ERK activation for the treatment of inflammatory arthritis
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Rheumatoid arthritis is an autoimmune disease characterized by chronic joint inflammation and destruction. However, arthritogenic antigens are currently unknown and most therapeutic treatments rely on immunosuppressive drugs which have side effects. We demonstrate that co-delivery of a specific ERK activator with a model antigen, using lentivectors, induces antigen-specific immune suppression by differentiation
of antigen-specific regulatory T cells (Tregs) and inhibition of inflammatory T cell subsets. Differentiated Tregs strongly proliferate after antigen re-encounter in inflammatory conditions and exhibit antigen-dependent suppressive activities. ERK activation causes mouse and human dendritic cells (DCs) to secrete bioactive TGF-beta, which is required to suppress T cell responses and differentiate antigen-specific Tregs. In vivo administration of the ERK activator with antigen inhibits inflammatory arthritis partially through Treg activity if this antigen is administered during arthritis induction. Thus, antigen-specific tolerance was re-directed to circumvent the need for targeting an arthritogenic antigen. We also demonstrate equivalent mechanisms in human DCs, setting the scene for rapid translation to patients with rheumatoid arthritis. This strategy resulted in an effective therapeutic protocol with substantial advantages over DC or T cell vaccination.

P 138

Keratin 14 mRNA trans-splicing in dominant Epidermolysis bullosa

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Mutations in the K14 gene underly Epidermolysis bullosa simplex (EBS), a hereditary skin disease characterized by erosions and blistering of the skin and mucous membranes after minor trauma. One subgroup of EBS (Dowling-Meara) is inherited in an autosomal dominant way, rendering it a challenge for conventional cDNA-based gene therapy. In our model of EBS-DM, the heterozygous missense mutation R125P in the KRT14 gene leads to protein misfolding and subsequent self-aggregation. We replaced exons 1 to 7 of the K14 gene using “Spliceosome-Mediated RNA Trans-Splicing”, thereby reducing levels of the mutated allele and increasing the amount of functional protein. Essential trans-splicing components were brought in by an RNA-trans-splicing molecule (RTM). Besides the wildtype gene-portion to be replaced and important splicing features, the RTM incorporates a binding domain (BD), crucial for the trans-splicing specificity and efficiency. In a reporter based screen we identified best BDs for an RTM specifically replacing exons 1 to 7 of the K14 gene. Therefore, a library consisting of RTMs with random BDs was screened, resulting in the isolation of a highly functional RTM, which was adapted for endogenous trans-splicing. We showed specific trans-splicing into exon 8 of the endogenous K14 gene and a decrease of cis-spliced K14 transcripts by 40 to 60%. Scratch assays revealed a reversion of the migratory behaviour of RTM-transfected cells versus wildtype. Finally, we detected a 51kD protein band representing trans-spliced K14 by western blotting. We conclude that successful trans-splicing in this model constitutes a novel approach to treat autosomal dominant diseases.

P 139

Vector Biosafety in a Mouse Model of TCR Gene Therapy Developing Graft-Versus-Host Disease

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Engineering T cell specificity with the genetic introduction of antigen-specific T cell receptor (TCR) represents a promising tool for the treatment of tumors and viral infections. We applied our developed strategies for comprehensive genome wide retrieval of retroviral integration sites (IS) to analyse IS distribution and the clonal dynamics of programmed T cells in a mouse model of TCR gene therapy developing Graft-versus-Host Disease (GvHD).

Mouse T cells were transduced with a gammaretroviral vector encoding an ovalbumin-specific OT-I TCR and the cohort receiving a subsequent IL-2 administration developed GvHD. By performing linear amplification-mediated PCR (LAM-PCR) combined with high-throughput sequencing on CD4 and CD8 T cells sorted from the different mice cohorts, we were able to retrieve 841 unique IS in OT-I transduced mice and 1063 unique IS in control GFP transduced mice. The analysis of the clonal composition revealed in OT-I transduced samples a high clustering of IS in genes involved in the immune response and an enrichment of IS in specific gene classes, Hematological Disease and Inflammatory Response, compared to the GFP transduced cells (p = 7.84*10^-4 and p = 2.13*10^-2, respectively). Furthermore, the IS analysis of samples from mice developing GvHD revealed an increased distribution of IS located in genes and in the surrounding 10 kb (73.60% and 65.34%, respectively; p = 3*10^-2) and an increased frequency of (pre)dominant clones compared to the control cohort.

Our results highlight the importance of high-throughput IS analyses for monitoring the efficacy and the safety of vectors used in TCR gene transfer.

P 140

Axonal Transport of AAV Vector Provides Widespread Correction in Murine Globoid Cell Leukodystrophy

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Globoid cell leukodystrophy (GLD) is a devastating lysosomal storage disease caused by a deficiency of galactocerebroside (GALC). This leads to accumulation of toxic psychosine in the CNS, and progressive neurodegenerative demyelination. Given the global involvement of CNS and rapidity of neurological deterioration in the GLD, the treatment calls for a rapid and robust distribution of therapeutics throughout the CNS before the disease progresses. The high
efficiency with which AAV vectors are transported antero- and retrograde axonally opens the possibility of targeting a transgene to neurons population remote from the injection sites and difficult to access after a relative localized intracranial injection. We investigate the feasibility and efficacy of axonal transport of therapeutics to diseased CNS globally by inoculating AAV vector directly into brain structures with defined axonal connections with other structures. Broad distribution of AAV vector is seen in the entire brain, cerebellum, brain stem, and throughout spinal cord. Neuroanatomical mapping of transduced cells in CNS are consistent with the pattern of major axonal circuit in CNS. Widespread restoration of functional GalC activity in CNS leads to reduction of toxic substrate accumulation, correction of CNS inflammation, axonal damage, and demyelination, and amelioration of devastating phenotype. Our results show that targeting the brain structures with divergent axonal connection is an effective approach for achieving widespread therapeutics distribution throughout the CNS. This therapeutic strategy with axonal transportation might be promising as gene therapy for GLD, but also disorders indications with global neurodegeneration in future clinical trials.

P 141

Enforced IL-10 expression confers Tr1 cell phenotype and functions to human CD4+ T cells

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Background: Type 1 regulatory T (Tr1) cells are an inducible subset of regulatory T cells producing high levels of interleukin-10 (IL-10) and suppressing antigen-specific effector T cells via cytokine-dependent mechanisms. Several protocols have been developed to expand Tr1 cells in vitro. However, in most cases the resulting population still includes a consistent fraction of contaminating effector T cells that might represent an obstacle for the development of therapeutic adoptive transfer strategies.

Method: We constructed a bidirectional lentiviral vector (LV) encoding for human IL-10 and carrying GFP as marker gene (LV-IL-10=GFP). A detailed characterization of resulting LV-IL-10=GFP-transduced human CD4+ T cells in terms of phenotype and functions will be reported.

Results: LV-IL-10=GFP efficiently transduced activated human CD4+ T cells (CD4IL-10=GFP). CD4IL-10=GFP T cell lines express higher levels of ICOS, ICOS-L, IL-10R and HLA-G antigen compared to cells transduced with control LV-GFP (CD4GFP). CD4IL-10=GFP T cell lines secrete high amounts of IL-10 and low levels of IL-4, displaying a Tr1 cytokine production profile, are anergic, and suppress the proliferation of allogeneic CD4+ T cells in vitro and in vivo. CD4IL-10=GFP T cell lines express high levels of granzyme B and lyse target cells of myeloid origin.

Conclusion: Constitutive over-expression of IL-10 in human CD4+ T cells results in a stable cell population which recapitulates the phenotype and function of Tr1 cells.

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Spontaneous type 1 diabetes in NOD mice is related to an increase in DcR1 expression

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Background: Type 1 diabetes in non-obese diabetic (NOD) mice is the result of a complex interaction between genetic background and environment. Understanding the mechanisms underlying the development of this experimental model of type 1 diabetes will help to identify potential new therapeutic targets. Overexpression of the decoy receptor 1 (DcR1) has been described in the peripheral blood lymphocytes of patients with type 1 diabetes. We analysed expression of DcR1 in the pancreatic islets of streptozotocin (STZ)-induced diabetic NOD mice compared to non-diabetic littermates.

Method: NOD mice were induced with STZ and sacrificed at 8 weeks of age. Pancreatic islets were isolated and RNA was extracted. DcR1 expression was measured by quantitative real-time polymerase chain reaction (Q-RT-PCR).

Results: DcR1 expression was significantly increased in the pancreatic islets of diabetic NOD mice compared to non-diabetic littermates.

Conclusion: The increased expression of DcR1 in the pancreatic islets of diabetic NOD mice suggests a potential role of DcR1 in the pathogenesis of type 1 diabetes.
Background: Type 1 Diabetes (T1D) results from selective autoimmune destruction of the pancreatic beta islets by the infiltrating inflammatory cells. There is evidence for both destructive and protective roles for TNF-Related Apoptosis-Inducing Ligand (TRAIL) in T1D [1],[2]. We aimed to detect the expression profiles of TRAIL ligand and receptors in spontaneous disease development in NOD mice for implications on the role of these molecules in the developmental course of T1D.

Materials and Methods: Pancreatic beta islets at tissue sections prepared from a total of 10 three days diabetic NOD mice were immunohistochemically analysed for TRAIL ligand and receptor expressions. The results were compared with those of pre-diabetic NOD mice and age-matched Non-Obese Diabetes Resistant (NOR) mice. TUNEL was used for detection of the related apoptotic profile.

Results: DcR1 expression levels displayed a significant increase in diabetic mice, while the expression levels for the other two membrane-bound receptors or the TRAIL ligand did not change. Apoptotic cell count was highest at day 7.

Conclusion: Increase in the DcR1 expression levels in the beta islets in three days diabetic mice may reflect a protective strategy against the infiltrating cells expressing TRAIL.

References

P 144

Immunoresponses limit in utero hematopoietic engraftment of transduced fetal liver progenitors

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In utero cell and gene therapies constitute alternative strategies to the postnatal treatment of inherited diseases. Fetal hematopoietic progenitors could be a potential source of donor cells for this strategy. In the present study, hematopoietic lineage-negative fetal liver cells from 14.5 days old fetuses were transduced using different cytokine and culture combinations with a lentiviral vector expressing the enhanced green fluorescent protein (EGFP). Up to 70% of granulo-macrophage colony forming cells expressed the EGFP reporter gene when cells were transduced for 6 hours in presence of mSCF, hTPO and FLT3-L in retrometin-coated dishes at an MOI 10 TUs/cell. In utero transplantation experiments showed that conditions leading to high transduction efficiencies did not result in high engraftment levels of EGFP-expressing cells in the syngeneic recipients. In these animals we detected the presence of antibodies and cellular responses against EGFP, indicating immunoreaction against the transduced cells. Analysis of mother’s and pups’ immunoresponses showed that the first events of the immunoreaction came from the operated pregnant females, as early as two days after in utero transplant. Our results show that fetal liver hematopoietic progenitors can be transduced very efficiently after short periods of incubation with low lentiviral vector doses and demonstrate for the first time that in utero transplanted animals can develop both humoral and cellular immunoresponses against exogenous transgenes. These observations should be considered in future protocols of prenatal gene therapy.

P 145

Trail profile of synoviocytes and peripheral blood lymphocytes of rheumatoid arthritis patients

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Background: Rheumatoid arthritis (RA) is a common, progressive, destructive inflammatory disease modulated by death ligand/receptor expression both on T cells and RA synoviocytes. TNF Related Apoptosis Inducing Ligand (TRAIL) appears to play major roles during the development of autoimmune diseases such as diabetes [1]. However, its role in RA development remains unknown. For this reason, the primary objective of this study was to explore TRAIL and TRAIL receptor composition in peripheral blood lymphocytes of RA patients and compare it to that of synoviocytes [2].

Methods: Flow cytometry (FC) analyses were utilized to characterize the expression pattern of TRAIL and its receptors...
on synoviocytes and peripheral blood lymphocytes isolated from RA patients.

Results: Our results suggested that while DRS5 was the predominant receptor type expressed in RA synoviocytes, other TRAIL receptors exhibited a heterogeneous expression profile. On the other hand, the expression of TRAIL and its receptors both death and decay were increased on the peripheral lymphocytes of RA patients, compared to healthy controls.

Conclusion: Differential alteration of TRAIL and TRAIL receptor expression on T cells and synoviocytes indicates that the profile might be important in decision-making process of cell death or survival.

References

P 146
Effect of mesenchymal stromal cells on GVHD and GVL reactions in a CML relapse mouse model
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To study factors that affect Graft v. Host Disease (GVHD) and Graft vs. Leukemia effect (GVL) in haplo-BMTs, first, we generated a CML mouse model. For this, B6D2F1 (H2b/b) bone marrow lin+ cells were transduced with p210-NGFR retroviral vector that encodes BCR/ABL oncogene and INGFR. Cells were infused into lethally irradiated syngeneic mice, generating a CML-like syndrome that killed mice around day 18. To mimic CML-relapse after BMT, BM cells from haploidentical C57Bl/6 mice (H2b/b) were infused, producing a slower development of CML with < 28-72 day deaths. In both models, necropsies detected splenomegaly, hepatomegaly, hemorrhagic lungs, all with NGFR+ leukemic infiltration, as well as in hypercellular bone marrows. To develop a GV effect, haploidentical splenocytes from C57Bl/6 mice were additionally infused. The animals died of GVHD +13-36 days after co-transplants, similarly to the GVHD control group. No organomegaly or NGFR+ cells were detected in the analyzed tissues. In a previous work we demonstrated that MSCs infusion on days +0, +7 and +14 prevented GVHD in 60-80% of the mice in haplo-BMT (Yañez, 2006). Now, MSCs were additionally infused to study MSCs’ immunosuppressive effects on GV. This infusion did not increase mice survival, although it prolonged survival until day +55. Low values of NGFR+ cells were detected in bone marrow and spleen, and the death of the animals was attributed to a moderately delayed GVHD. Our results show that, in this CML-relapse/GV mouse model, MSCs did not induce an evident increase in leukemic relapses but had a modest efficacy in preventing GVHD.

P 147
Artificial Envelopment of Adenovirus Dramatically Alters Viral Liver Tropism and Tissue Distribution
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Human adenovirus serotype 5 (Ad5) is the most frequently used gene delivery vector in clinical studies. Following intravenous administration, Ad is rapidly cleared from systemic blood circulation with a half life of 2 minutes, and more than 99% of the injected dose is sequestered in liver resulting in significant hepatotoxicity. These issues currently restrict the use of Ad-based vectors, particularly for clinical gene therapy protocols that involve systemic administration. We propose that such limitations can be overcome by engineering artificial lipid envelopes around Ad. Liver tropism of artificially enveloped Ad vectors in cationic non-pH-sensitive DOTAP:Chol or anionic pH-sensitive DOPE:CHEMS lipid bilayers was studied after intravenous (tail vein) administration to Balb/C mice. Real-time PCR and transgene expression levels showed that the type of lipid envelope dramatically changed the uptake of viral particles in parenchymal cells (PC) and non-parenchymal cells (NPC) of the liver compared to naked Ad. Artificial lipid bilayers around Ad prevented PC transduction in the following order DOTAP:Chol >DOPE:CHEMS. Whole-body imaging of luciferase expression using an IVIS camera at 24 hr after administration of enveloped Ad showed an overall decrease in liver transduction similar to that observed for naked Ad injected in warfarin-treated mice. Furthermore, DOTAP:Chol envelopment significantly increased lung accumulation compared to DOPE:CHEMS enveloped or naked Ad. These data suggest that artificial lipid envelopes for Ad offer an approach to significantly divert viral particles from their natural liver tropism and may allow safe delivery to target organs in vivo.

P 148
Brain gene therapy for metachromatic leukodystrophy: towards clinical trial
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Brain gene therapy seems to be a more suitable option to treat patients affected with the more severe forms of Metachromatic leukodystrophy (MLD), a lethal lysosomal storage disorder due to a deficiency in arylsulfatase A (ARSA) enzyme, leading to sulfatide storage, demyelination and neuronal degeneration.

Proof of efficiency and safety of intracerebral delivery of adeno-associated-vector serotype 5 (AAV5) encoding human ARSA was established in MLD mice and non-human primates (NHP).

We demonstrated that, in comparison with AAV5/ARSA vector, intracerebral injection of AAVrh.10/ARSA resulted in a more rapid and complete correction of sulfatide storage and neuropathological lesions in symptomatic MLD mice. ARSA enzyme was detected in oligodendrocytes and oligodendrocyte-specific sulfatide isoforms accumulation was completely corrected.

Safety and efficiency study was performed in non-human primates. AAVrh.10/ARSA vector was injected in one or both hemispheres (1.1e11 PFU/hemisphere) in white matter areas, targets selection based on brain MRI. In a clinical perspective, surgical device was optimized to allow simultaneous infusion of 12 deposits, thus reducing time of surgical procedure. Animals were analyzed 3 months post-injection. We documented diffusion of the vector in up to 73% and up to 31% increased activity of ARSA enzyme in the whole injected hemisphere. As compared to our previous results with AAV5/ARSA, the use of a 20-fold lower dose of AAVrh.10-derived vector led to increased amounts of vector in the non-injected hemisphere, and increased level of ARSA activity in the injected hemisphere. Toxicological evaluation is currently ongoing prior to a phase I/II clinical trial for rapidly evolutive forms of MLD.

P 149

Electrical parameters for ex vivo plasmid electrotransfer to dermis and epidermis

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Previous studies have shown the ability to culture and to ex-vivo transducer organ fragments such as lungor skin. One of these studies showed that an intact miniature biopsy of human dermis transduced ex vivo by a viral vector encoding a gene for the therapeutic protein could be implanted into SCID mice and produced the protein for several months.

Gene electrotransfer (EGT) is used to internalize DNA plasmids in cells without causing irreversible damages on plasma membranes. A combinations of two types of electric pulses are used: (i) one 100 μs duration, high voltage pulse (HV) which transiently permeabilizes the cell membrane, and (ii) one or several 100 ms duration, low voltage pulse (LV) which exert(s) electrophoretic forces on the DNA and bring(s) this large macromolecule (that does not move by diffusion because of its size) in contact to the electropermeabilized membranes.

In this study, we show that ex vivo skin transduction is possible using a non viral method namely electrotransfer. Whole skin or skin fragments dissociated into dermis and epidermis were electrotransferred with a plasmid containing reporter gene coding for the luciferase. We show that the effective electrical parameters depend on the tissue complexity (whole skin, dermis or epidermis). Interestingly when whole skin is exposed to the electric pulses, both combinations of electric pulses are efficient, however at a lower level than for the isolated dermis or epidermis. Results were confirmed with other reporter genes (β-galactosidase and GFP). A study on the cell types transduced using this non viral method is ongoing.

P 150

Comparison of minicircle production methods for delivering naked DNA and expressing genes in animals

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Minicircle-DNA vectors are supercoiled plasmids carrying a minimal expression cassette and are generated via intramolecular recombination to eliminate the undesired plasmid backbone sequences. The advantages of minicircle-DNA vectors include the reduction of CpG motifs and the bacterial backbone which have shown to contribute to silencing of episomal transgene expression and to trigger immunotoxic responses. In this study, we compared two methods for the production of minicircle-DNA with different bacterial recombine expression systems: A first method uses a bacterial strain (MM294) expressing Cre recombinase under the tight control of the araC regulon mediating the recombination between two partially mutant attP sites in the parental plasmid (Bigger et al. 2001, J Biol Chem 276: 23018). The residual parental plasmids and excised bacterial vectors are digested by restriction enzyme and the undigested supercoiled minicircle-DNA is purified subsequently by CsCl-gradient centrifugation. A second method uses a bacterial strain (ZCY10P3S2T) which genome contains ten copies of the phage ßC31 gene encoding the bacteriophage ßC31 integrase to mediate the recombination event between attB and attP sites. In addition, the genome contains three copies of the I-SceI gene which encodes the restriction enzyme that destroys the plasmid backbone circle. Both genes are co-expressed under the control of the araC/BAD system (Chen and Kay, unpublished). We found that the latter technology is more favourable due to the high yield of pure minicircle-DNA, reduced costs and time. In conclusion, this method is highly suitable for large-scale production, making minicircle-DNA attractive as non-viral vectors for human gene therapy and vaccination.
P 151

Improvement of RNA trans-splicing based gene correction using a RTM screening system
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Background: Since many genes, involved in various diseases such as epidermolysis bullosa, are too large for a therapy with whole gene insertion, the method of “Spliceosome Mediated RNA Trans-splicing” (SMaRT) can be used as an alternative gene repair strategy on pre-mRNA level. The trans-splicing reaction is achieved by “RNA Trans-splicing Molecules” (RTMs) that bind to a target pre-mRNA thereby inducing the gene correction.

Method: By using a fluorescence reporter system we are able to rapidly evaluate the effect of various RTM binding domains on trans-splicing functionality. The screening system is composed of an intron specific target molecule and a RTM library both including split parts of the reporter gene acGFP. Recombination of both pre-mRNAs by trans-splicing lead to the fusion of both RTM parts manifested in the expression of acGFP in RTM and target molecule transfected HEK293 cells.

Results: Fluorescence microscopy reveals the expression of the reporter molecule acGFP two days post transfection. The most efficient RTMs, identified by “Fluorescence Activated Cell Sorting” (FACS), are able to induce endogenous trans-splicing on pre-mRNA level manifested in the fusion of the RTM with the 3’ exonic sequence of the target gene PLEC.

Conclusion: The screening for most functional RTMs using a fluorescence based screening system should improve endogenous trans-splicing in efficacy and specificity. We anticipate that these experiments should lead us to promising tools for gene therapy in plentin deficient patients.

P 152

In vivo fluorescence imaging of xenogeneic islet graft survival
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Background: Pancreatic islet xenotransplantation is an appealing therapy for the treatment for type 1 diabetes because of the potential to provide unlimited donor tissue supply, but it provokes an intense immune response. Also, it is difficult to assess graft mass after transplantation [1]. The aim of the study was to follow up the survival of rat-islet xenografts overtime in diabetic mice using fluorescence imaging system [2].

Method: AdEGFP transduced rat pancreatic islets were transplanted under the renal capsule of streptozotocin induced diabetic mice and non-fasting blood glucose levels were followed. Fluorescence signal emanated from graft was quantified over time using the cooled charge-coupled device. Grafts were examined for morphology and insulin content.

Results: Adenovirual transduction or EGFP expression did not affect the function of islets. Transplantation of AdEGFP transduced islets reversed diabetes in recipients within two days. Fluorescence signals emitted from xenografts were detectable post-transplantation and the signal intensity was gradually decreased as a sing of graft rejection.

Conclusion: It appears that the fluorescence imaging is a useful tool for the quantitative assessment of islet cell viability post-transplantation and it allows earlier detection of graft rejection in rat-to-mouse xenogenic model.

References

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In vivo gene transfer by non-viral transfection reagents freeze-dried onto mouse femoral allografts
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Background: Structural bone allografts lead to limited bone healing and a tendency to fracture. Recombinant adeno-associated virus freeze-dried onto murine femoral bone surfaces can be used to mediate in vivo gene transfer of osteogenic factors, thereby inducing new bone formation. In order to evaluate the efficiency of in vivo gene transfer using non-viral methods, we have freeze-dried complexes of transfection reagents together with plasmids onto the surface of bone allografts.

Methods: DNA complexes were made by combining plasmids with either Lipofectamine 2000 or branched poly-
Development of a Tet-On inducible lentiviral system for gene therapy of autoimmune disorder

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Lentiviral vectors (LV) are powerful tools for gene therapy and hold great potential as a therapeutic gene therapy strategy for autoimmune disorder. However the controlled delivery of therapeutic genes represents one of the limiting factor of LV. The tetracycline-dependent transcriptional regulatory systems (Tet-systems) are one of the best studied systems in vitro and in vivo. In the present study, we aimed to develop a highly inducible LV system as a tool for the gene therapy of autoimmune disorder. For this purpose, we used vasoactive intestinal peptide (VIP) as a type-II cytokine with demonstrated therapeutic effect in animal models of several autoimmune diseases, as therapeutic gene model. And experimental autoimmune encephalomyelitis (EAE) as autoimmune disorder model.

We constructed (i) a binary lentiviral-based system (SFFV-TetR, CMV-TetOGFP/VIP) and (ii) several single vectors containing the represor (TetR) and the TetO-regulated transgene (eGFP/VIP) in the same vector. We compared our systems with the previously described inducible single LVs system (pLVCT-tTR-KRAB and pLVPT-tTR-KRAB) in term of leakiness, transgene expression levels and transduction efficiency after Doxycycline (DOX) treatment. The highest transgene expression levels in the presence of DOX and the highest basal activity (leakiness) in the absence of DOX were found in the binary system. However, the PGK-driven (pLVPT-tTR-KRAB) and the CAG-driven (pLVCT-tTR-KRAB) LVs achieved better results in terms of responsiveness to DOX. Both systems show very low leakiness and good inducibility by DOX. We are currently studying potential applicability of this systems on EAE disease and potential therapeutic benefits compared with unregulated system.

P 155
Turning expression into advantage: a new tool for regulatory T cell therapy

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Background: FOXP3 is a transcription factor with a pivotal role in the function of regulatory T cells (Tregs), which are responsible for immunetolerance. Although many surface antigens characteristic of Tregs have been described, the selection and expansion of these cells for clinical purposes are still a difficult challenge. The aim of this research is to give a selective advantage to cells expressing the transcription factor, to allow better selection and expansion.

Methods: We ideated a system in which the promoter sequence of TLR10, which is actively regulated by FOXP3, is cloned into a bidirectional promoter cassette. This system will couple the presence of FOXP3 with the expression of a surface protein (NGFR) to allow cell selection, and of a proliferative gene (to be selected) to allow cell expansion. Integration of the vector will be accomplished with the Sleeping Beauty transposon system® (SB). A FOXP3-expressing vector is used to test the system in Jurkat and 293T cells.

Results: We transfected Jurkat cells and 293T cells with the Foxp3-expressing vectors, the bidirectional promotor cassette or the SB. The transfection efficiency was about 5% on Jurkat cells and ≥ 70% in 293T cells, as measured by fluorescence microscopy and flow cytometry. We cloned the TLR10 promoter into the pGL3-basic vector in order to check the activation of the promoter sequence by luciferase assay.

Conclusions: These are very preliminary results. Jurkat cells are a suitable cellular model to test our expression system, since 5% transfection efficiency can allow us to select cells positive for the surface marker NGFR.

P 156
Intramuscular Delivery of Antisense Oligonucleotides by Chitosan Nanoparticles

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Background: Antisense oligonucleotides (ODN) provide a gene-targeted approach for the prevention and treatment of diseases due to over expression of genes. In the antisense approach, ODNs complementary to specific mRNA sequence can inhibit gene expression. However one of the major drawbacks in antisense therapy is the difficulty in finding an adequate delivery methods for ODNs in relation to their...
cellular uptake. A number of strategies have been developed to deal with this problem, and use of cationic polymers as carrier for ODNs one of them. Chitosan is potentially safe and useful cationic carriers for gene delivery.

The aim of this study is to investigate the therapeutic potential of ODN-loaded chitosan nanoparticles after intramuscular application.

**Method:** 15-nucleotide phosphorothioate oligonucleotides (MWG-Biotech) were used as ODN. ODN-loaded chitosan nanoparticles were produced based on the ionic gelation of TPP with chitosan. ODN-chitosan nanoparticles were intramuscularly injected to the Sprague Dawley rats (initially b-gal injected) in different doses 15, 30 and 60µg. After 1, 3, 6, 9 and 12 days of post-transfection, animals were sacrificed and samples were taken for b-gal assay. b-gal expression was measured enzymatically.

**Results:** Highest gene inhibition was measured at 6th day with nanoparticles. The better b-gal inhibition was obtained using multiple doses nanoparticles than single, and this value was around 89%.

**Conclusion:** Chitosan nanoparticles are suitable for intramuscular application for antisense oligonucleotides. High gene suppression may be obtained using ODN-loaded chitosan nanoparticles.

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**P 157**

**Increased efficiency of minicircles versus plasmids under gene electrotransfer suboptimal conditions**

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Electric pulses are known to permeabilize the cell membrane and they are routinely used to allow drugs or genes entering the cells. In the case of the nucleic acids electrotransfer, combinations of two types of electric pulses can be used: (i) one 100 ms duration, high voltage pulse (HV) which transiently permeabilizes the cell membrane, and (ii) one or several 100 ms duration, low voltage pulse (LV) which exert(s) electrophoretic forces on the DNA and bring(s) this large macromolecule (that does not move by diffusion because of its size) in contact to the electropermeabilized membranes. This electrophoretic effect is very important in vivo, where the extracellular matrix really constrains cell membrane. We explored whether similar effects can be observed in vitro. We compared the efficacy of plasmids and of the corresponding minicircles, which are circular DNA molecules derived from the plasmids, without the bacterial sequences. These minicircles are thus smaller than the plasmids. We found the minicircles more efficient than the plasmids at identical molar concentration, not only in vitro but also in vivo, as they result in the production of a greater quantity of the protein encoded by electrotransferred reporter genes. We are investigating the possibility that the greater efficiency of the minicircles could be due, at least in part, to an easier passage through the extracellular matrix because of their smaller size.

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**P 158**

**STZ administration leads to a significant rise in TRAIL and DcR1 levels in Non-Obese Diabetic (NOD)**

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**Background:** TNF-Related Apoptosis-Inducing Ligand (TRAIL) is an important component of the immune system [1]. It is well known for its preferential toxic effects on tumor cells. Yet its role in T1D development is not clear [2]. Therefore, we analyzed the effects of Streptozotosin (STZ) induction on TRAIL ligand and receptor expression levels in NOD mice.

**Materials and Methods:** An optimal single dose of 150 mg/kg STZ was administered to a total of 20 NOD mice. Pancreatic tissues collected before diabetes induction and at sequential steps of disease development following induction. Sections were analyzed by immunohistochemistry. Related apoptotic cell counts were detected with TUNEL.

**Results:** STZ induction provided a significant increase in TRAIL ligand and DcR1 levels at day 14 when 90% of the animals were diabetic. DcR2 and DR5 expressions were not altered significantly. Apoptotic count peaked at day 7, while it decreased in the following days.

**Conclusion:** Increase in the TRAIL ligand may imply a protective role for TRAIL against the infiltrating lymphocytes in STZ-induced TID. In addition, increased DcR1 expression may reveal a protective strategy adapted by the pancreatic beta islets against the infiltrating lymphocytes expressing TRAIL.

**References**


such as the TNF-Related Apoptosis Inducing Ligand (TRAIL) [1]. TRAIL’s role in T1D is not yet clear and further studies are required [2]. Thus, we analyzed the expression profiles of TRAIL ligand and its receptors in CY-accelerated T1D in NOD mice.

Materials and Methods: All mice received a single dose of 200 mg/kg CY. Pancreatic tissues were collected from prediabetic and diabetic NOD mice in addition to Non-Obese Diabetes Resistant (NOR) mice prior to immunohistochemical analysis. TUNEL assay was used for the detection of the related apoptotic profiles.

Results: TRAIL expression was significantly decreased in CY-accelerated T1D in NOD mice while no change was observed in the age-matched CY-applied NOR mice. The apoptotic cell count was increased along with diabetes development.

Conclusion: Different effects exerted on the immune system by various diabetes-inducing agents should be taken into consideration in diabetes investigations.

References

P 160
Crown Ethers potentiate Transfection activity of Cationic Lipoplexes in Mammalian Cells
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Background: Non-viral gene delivery modalities compatible with application in man are receiving growing attention in light of safety issues surrounding viral vector systems. Amongst the more promising DNA vehicles are liposomes. Those derived from cationic lipids show much potential although transfection levels achieved in vivo are moderate. In this investigation two cholesteryl derived ionophores have been formulated into cationic liposomes and have been examined for their transfection enhancing potential.

Methods: Liposome formulations included the cytoketin 3/[N-(N,N'-dimethylaminopropane) carbamoyl] cholesterol (Chol-T), the co-lipid dioleoylphosphatidylethanolamine and 5% (mole/mole) of the cholesteryl crown ethers RUI-128 (aza-18-crown-6) and RUI-129 (aza-15-crown-5). The remaining preparations were developed for liver targeting by including 5% (mole/mole) cholesteryl-β-D-galactopyranoside/glucopyranoside. Liposome interaction with plasmid DNA was characterized by hand shift, ethidium displacement and serum nuclease digestion analyses. Both growth inhibition and transfection activities were determined in the human kidney HEK293 and human hepatoma HepG2 cell lines.

Results: All liposomes showed effective DNA binding and protection against serum nuclease degradation. Liposomes containing the crown ether preparations exhibited slightly higher cytotoxicity but enhanced transfection levels in both cell lines. The preparations containing the cholesteryl-β-D-glycopyranosides however showed poor targeting in the HepG2 cell line.

Conclusion: Liposome preparations showed generally low cytotoxicity levels in both cell lines. Crown ether liposomes may have potentiated transfection through endosome destabilization however the absence of targeting may be ascribed to molecular shielding.

β-thalassemia results from severely reduced or absent expression of the β-chain of adult hemoglobin (α2β2;HbA) causing precipitation of excess α-chains and apoptosis of erythroid precursors. However, increased levels of fetal hemoglobin (α2γ2;HbF), such as in hereditary persistence of HbF (HPFH), ameliorate the severity of β-thalassemia. Furthermore, recent data revealed BCL11A as a major repressor of γ-globin expression in adult erythroid cells (Sankaran et al, Science 322:1839, 2008). In the present study, we compared two, self-inactivating, γ-globin lentiviral vectors termed V5m3-400 (Pestina et al, Mol Ther 17:245, 2009) and GGHI (Papanikolaou et al, Mol Ther 16[Suppl.1]:S278, 2008) with a lentiviral vector comprising a U6-regulated shRNA for BCL11A knockdown (BCL11A shRNA), suggesting a significant rescue via correction of the globin chain imbalance. These novel data document that both approaches, i.e. gene addition or genetic reactivation, have the potential to provide
therapeutic levels of HbF to thalassemic patients with such a degree of β-globin deficiency.

P 162
Development of gene therapy for haemophagocytic lymphohistiocytosis (HLH) due to perforin deficiency
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Haemophagocytic lymphohistiocytosis (HLH) is a devastating disorder of early childhood arising from defects of T and NK cell cytotoxicity. The most common form of this disease is due to mutations in the perforin gene. Current treatment options of HLH are limited, thus the development of gene therapy may have great benefit for such patients.

Two lentiviral vector constructs were designed; with perforin expression being driven by the SFFV promoter or by the PGK promoter. Both vectors induced expression of perforin in the RBL1 mast cell line and confocal microscopy confirmed that perforin expression was confined to the secretory granules.

To test the functionality of the perforin protein, vectors were used to transduce RBL2H3 cells; both vectors transduced efficiently and were able to induce cytotoxicity in RBL2H3 cells.

Next, NK cells and CD8+ T cells derived from perforin deficient mice were transduced with both perforin vectors. Low transduction efficiencies were obtained, nevertheless both perforin vectors were able to restore cytotoxicity to NK and CD8+ T cells.

Perforin vectors were also used to transduce enriched hematopoietic stem cells from perforin deficient mice and cells were differentiated in NK differentiation culture conditions. More than 70% of these cells differentiated into NK cells and from this population 30% of the cells expressed perforin. These cells were also shown to have the cytotoxic activity restored.

The restoration of cytotoxicity of perforin deficient cells allows us to take these vectors to the next step: the correction of a mutant murine model of perforin deficiency.

P 163
Long-term amelioration of feline Mucopolysaccaridosis VI after AAV-mediated liver gene transfer
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Mucopolysaccaridosis VI (MPS VI) is caused by deficient arylsulfatase B (ARSB) activity resulting in lysosomal storage of glycosaminoglycans (GAGs). MPS VI is characterized by dysostosis multiplex, organomegalgy, corneal clouding, and heart valve thickening.

Gene transfer to a factory organ like liver may provide a life-time source of secreted ARSB. To this end, adeno-associated viral (AAV) vectors with serotype 8 capsids (AAV2/8) represent a valuable tool for efficient liver gene transfer. We show that intravascular administration of adeno-associated viral vectors (AAV) 2/8-TBG-felineARSB in MPS VI cats resulted in ARSB expression up to one year, the last time point of the study. In newborn cats, normal circulating ARSB activity was achieved following delivery of high vector doses (6x10e13 gc/kg) while delivery of AAV2/8 doses as low as 2x10e12 gc/kg resulted in higher than normal serum ARSB levels in juvenile MPS VI cats. In MPS VI cats showing high serum ARSB levels, independent of the age of treatment, we observed: i) clearance of GAG storage ii) improvement of long bone length iii) reduction of heart valve thickness, and iv) improvement in spontaneous mobility. Thus, AAV2/8-mediated liver gene transfer represents a promising therapeutic strategy for MPS VI patients.

To this end, we are evaluating the efficacy of systemic delivery of AAV2/8-TBG-humanARSB in a knock-out mouse made tolerant to human ARSB by transgenic insertion of an inactive human ARSB allele (MPS VI-C91S).

P 164
Sustained therapeutic hFIX levels in rhesus macaques following catheter-based delivery of HDAd
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Hemophilia B is a good candidate for gene therapy because factor IX (FIX) activity ≥1% results in clinically significant improvement of the bleeding diathesis. Helper-dependent adenoviral (HDAd) vectors hold tremendous potential because they mediate long-term transgene expression without chronic toxicity. Administration of first generation adenoviral (FGAd) vectors expressing human FIX (hFIX) in rhesus macaques resulted in short term hFIX expression and induction of high-titer, neutralizing anti-hFIX antibodies. To determine safety and efficacy of HDAd-mediated hemophilia B gene therapy, we administered an HDAd expressing hFIX into rhesus macaques through a balloon catheter strategy we have previously developed and resulting in greater hepatocyte transduction than systemic intravenous administration. In this approach a balloon catheter is percutaneously positioned in the inferior vena cava to occlude hepatic venous outflow
and 1×10^{10}, 3×10^{10}, 1×10^{11}, or 1×10^{12} vp/kg of an HDAd expressing hFIX was injected into the liver via a hepatic artery catheter. Animals injected with 1×10^{11} and 1×10^{12} vp/kg exhibited therapeutic levels of hFIX (≥5% of normal FIX activity) for at least 461 days and 793 days, respectively. Subtherapeutic levels corresponding to ~0.1-0.5% were achieved in the animal injected with 3×10^{10} vp/kg and 1×10^{10} vp/kg. Importantly, HDAd administration did not result in the development of neutralizing anti-hFIX antibodies. These results suggest that in contrast to FGAd, HDAd vectors can drive long-term FIX expression, do not result in neutralizing anti-hFIX antibody formation, and 1×10^{11} vp/kg is the minimal dose to achieve clinically beneficial levels of FIX. Therefore, HDAd are attractive vectors for hemophilia B gene therapy.

P 165
AAV8 Gene Transfer Rescues a Neonatal Lethal Murine Model of Propionic Acidemia
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Propionic Acidemia (PA) is an autosomal recessive disorder of metabolism caused by a deficiency of propionyl-CoA carboxylase. Despite optimal dietary and cofactor therapy, PA patients still suffer from lethal metabolic instability and experience multisystemic complications. A murine model of PA (Pcca−/−) that uniformly perishes within the first 48 hours of life was used to determine the efficacy of adeno-associated viral (AAV) gene transfer as a potential therapy for PA. An AAV serotype 8 vector was engineered to express the human PCCA cDNA and delivered to newborn mice via an intrahepatic injection. Greater than 70% of the Pcca−/− mice were rescued after AAV8 mediated gene transfer and survived until day of life 16 or beyond. Western analysis of liver extracts showed that propionyl-CoA carboxylase was completely absent from Pcca−/− mice but was restored to greater than wild-type levels after AAV gene therapy. The treated Pcca−/− mice also exhibited markedly reduced plasma levels of 2-methylcitrate compared to the untreated Pcca−/− mice, which indicates significant propionyl-CoA carboxylase enzymatic activity was provided by gene transfer. At the time of this report, the oldest treated Pcca−/− mice are over two months of age. In summary, AAV gene delivery of PCCA effectively rescues Pcca−/− mice from neonatal lethality and substantially ameliorates metabolic markers of the disease. These experiments demonstrate a gene transfer approach using AAV8 that might be used as a treatment for PA, a devastating and often lethal disorder desperately in need of new therapeutic options.

P 166
Bone marrow transplantation cures hemophilia A
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Identification of cells capable of synthesizing and releasing FVIII is critical for therapeutic development in hemophilia A. Recent studies indicated that endothelial cells, particularly liver sinusoidal endothelial cells, are a major source of FVIII, although the origin of endothelial cells is incompletely defined and FVIII could potentially be expressed in additional cell types. To determine whether endothelial cells, or other cells, derived from bone marrow could produce FVIII, we transplanted healthy mouse bone marrow into hemophilia A mice. We tracked donor cells by genetic markers and analyzed FVIII production, as well as correction of hemophilia by several assays. After bone marrow transplantation, mice survived induction of bleeding and hemophilia A was corrected, although despite hepatic and endothelial injury to recruit transplanted cells, donor bone marrow-derived hepatocytes or endothelial cells were extremely rare, and did not account for these benefits. We discovered that FVIII was produced in mononuclear cells and mesenchymal stromal cells derived from donor bone marrow, which expressed FVIII at mRNA and protein levels. Moreover, injection of healthy mouse Kupffer cells (liver macrophage/mononuclear cells), which predominantly originate from the bone marrow, or of healthy human bone marrow-derived mesenchymal stromal cells resulted in the appearance of FVIII in blood and protected hemophilia A mice from bleeding challenge. Therefore, we concluded that bone marrow transplantation cured hemophilia A through donor mononuclear cells and mesenchymal stromal cells. These insights in cellular origins of FVIII offer new mechanisms for understanding alterations in FVIII synthesis and production and for developing effective therapies in hemophilia A.

P 167
Sustained partial correction of hemophilia B in the dog by a microRNA-regulated lentiviral vector
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Gene therapy has long held the promise of a cure for hemophilic patients. We previously demonstrated phenotypic correction of hemophilia B after a single administration of a microRNA-regulated lentiviral vector (LV) in a mouse model. We have now tested our gene therapy strategy in hemophilia B dogs. We administered intraportal 1.5×10^{10} integration units of highly purified LV to an 8-month old, 20 Kg hemophila B dog. The infusion was well tolerated and uneventful with only minor self-limiting hepatocellular toxicity. At the current follow up (>1 year post-infusion) the dog is alive and
well. Whole blood clotting times have been stably shortened and anti-canine FIX inhibitors tested negative. We have administered a 4-fold higher dose in a second dog. The infusion was aborted due to an acute and severe hypotensive response, likely due to an allergic reaction to an unknown vector component or contaminant, which resolved after arresting the administration. Experiments are now underway in order to elucidate the reason for this. In parallel to those studies, we have validated a number of specific interventions which increased the potency of our strategy, including the use of a codon-optimized transgene, a single administration of a proteasome inhibitor prior to LV infusion or a different LV pseudotype (baculovirus gp64 envelope), which allow the use of lower LV doses. These results indicate that liver-directed gene therapy by LV can provide long-lasting benefit without inducing an immune response to the transgene.

P 168

A new mouse model for Crigler-Najjar syndrome and possible therapeutic approaches

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Crigler-Najjar syndrome is a recessively inherited disorder characterized by severe unconjugated hyperbilirubinemia caused by uridine diphospho-glucuronosyltransferase-1a1 (UGT1A1) deficiency. The disease is lethal due to bilirubin-induced brain damage unless phototherapy is applied since birth. However, the treatment becomes ineffective during adulthood due to increased thickness of the skin. As a result, liver transplantation is required. We generated a mouse model of the disease by introducing a frame shift mutation in UGT1A1 exon 4 creating a stop codon immediately after the deletion. Newborn homozygous mutant mice (UGT1A1−/−) developed severe jaundice 2 days after birth. All mutant animals died within day 7 showing severe neurological damage, mimicking all major features of the human Crigler-Najjar syndrome.

Recombinant AAV9 viral vectors carrying the human UGT1A1 cDNA were IP injected into P2 newborn mutant mice. We were able to rescue 100% of AAV-treated mutant mice, that reached adulthood and were indistinguishable from their control littermates. Bilirubin levels in mutant AAV-treated mice were significantly lower than untreated mutant mice although they did not reach those of heterozygous or wild type littermates. Behavioral and histological analysis confirmed that mutant mice treated with AAV-UGT1A1 had no obvious neurological damage. Real-time quantitative PCR showed presence of the AAV-genomic DNA in liver, heart and skeletal muscle after 5 months post injection. However, UGT1A1 protein was detectable only in skeletal muscle by western blot analysis. We are now testing different vectors with liver-specific promoters in order to improve the efficacy of the gene-targeting protocols.

P 169

The Sea Urchin Sns5 Insulator Improves Expression of Lentiviral Vector Encoded Human β-Globin Gene

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Globin gene therapy for the treatment of b-thalassemia requires sustained and stable expression of the human b-globin gene in the erythroid lineage. Transgene expression following viral-mediated gene transfer may be variable and subject to silencing. Chromatin insulators are genomic elements that may reduce chromosomal position effect and silencing affecting g-retro and lentiviral vectors.

We compared different insulators flanking the TNS9.3 globin lentiviral vector (LV). We constructed LVs flanked with the sns5 sea urchin insulator (in sense and reverse orientation), the 400 bp and 1.2 kb chicken HS4 insulator or a control sequence of identical size. In panels of single copy murine erythroleukemic (MEL) cell clones, we showed an increase in human b-globin transcript levels when the sns5 insulator flanks the LV in sense orientation. The sns5 insulator also reduced LV silencing. Unlike the 1.2 kb chHS4 insulator, the insertion of sns5 into the LV did not reduce vector titer.

In order to assess the in vivo effect of the sns5 insulator on b-globin expression, lethally irradiated C57BL6Hbb<sup>th3</sup>/−/− mice were transplanted with LVs transduced C57BL6Hbb<sup>th3</sup>/−/− bone marrow cells. Human b-globin expression was analyzed in peripheral blood over time in long-term bone marrow chimera. The sns5 insulator increased transgene expression when compared with the TNS9.3 LV, as well as with LVs flanked with the spacer sequence or the 400 bp chHS4 insulator.

Clonal globin expression analysis and microarray studies in CFU-S are currently being performed to evaluate the capability of sns5 to reduce endogenous gene trans-activation.

P 170

Factor IX gene transfer for the treatment of hemophilia A

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Recently, long-term expression following factor IX (FIX) gene transfer has been shown in the first human subject with hemophilia B. Clinical gene therapy for the more common FVIII deficiency (hemophilia A) or for patients with anti-FVIII antibodies, however, is still out of sight. Here, we report a new gene transfer strategy using FIX variants with activity in absence of their co-factor (FVIII). These variants could be easily adapted to any established FIX gene therapy approach.

In an initial in vitro screening, we identified a single mutant (K265T) with 6.6% clotting activity (FIX antigen 100%) and a triple variant (V181I/K265T/I383V) with 17% activity in absence of FVIII. Bypassing activity was confirmed in plasma of
patients with high titers of inhibitory antibodies. The muteins were introduced into a non-viral vector system and stably expressed in different mouse models. At FIX levels ranging from 7500 to 19000ng/ml partial normalization of the aPTT and of blood loss following tail clip assay (1.5 and 3 mm) were observed in all the variant groups (n = 5-9 mice/group, p < 0.05-0.005), while wild-type FIX expressing mice did not differ from untreated animals. Similar results were obtained in mice with high titers of anti-FVIII antibodies. Further, untreated mice and mice tolerant to wild-type FIX were challenged with wild-type and mutant FIXs. While all untreated mice exhibited a strong immune response against all forms of FIX, no immune response was observed in mice tolerant to the wild type protein. Therefore, the proposed mutants do not break tolerance to the FIX.

**P 171**

Preclinical modelling of ex-vivo skin gene therapy for Netherton syndrome

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Defective expression of Lympho-epithelial Kazal-type-related inhibitor (LEKTI) gives rise to the inherited skin disorder Netherton syndrome (NS). The disease is characterised by a defective skin barrier with ichthyosiform erythroderma and is associated with severe dehydration and a high risk of mortality in early infancy. LEKTI is a secreted protein and we have shown that HIV-1 based lentiviral vectors encoding the SPINK5 transgene can correct the abnormal architecture of human skin grafted onto immunodeficient mice. Previous results showed notable correction of NS epidermal phenotype even in grafts where only small numbers of LEKTI expressing keratinocytes were detectable, raising the possibility of wider bystander benefit from the secreted protein. We have now revised the vector design to substitute retroviral promoter elements with sequences derived from the human Invovlucrin promoter. We show in three dimension organotypic cultures that the Invovlucrin promoter directs gene expression to the granular layer of the epidermis, the same compartment where LEKTI is normally expressed. Grafting of gene modified human NS skin onto nude mice showed revealed robust and durable correction of skin sheets. Interestingly vectors encoding the SPINK5 transgene in combination with the Invovlucrin promoter were less prone to methylation mediated gene silencing compared to vectors incorporating SFFV. These experiments demonstrate the feasibility of ex-vivo tissue modification and the engineering of grafts to correct inherited skin diseases.

**P 172**

Restored SMN1 expression by lentiviral vector in murine Embryonic Stem Cells derived from Spinal Muscular Atrophy (SMAI) mice

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Spinal Muscular Atrophy (SMA) is an autosomal recessive neuromuscular disorder caused by the loss of Survival Motor Neuron-1 gene (SMN1). Despite its ubiquitous expression SMN reduction cause the selective death of the alpha motor neurons (MNs). We explored the possibility of transducing by lentiviral vector (LVVs-GFP-hSMN1) two novel cells populations: murine Embryonic Stem Cells derived from SMA I mouse model and Neural Stem Cells (NSC) derived from spinal cord SMAAΔ7 model. After 5 days of transduction, mES and NSCs cells were sorted by FACS, based on their GFP expression. Subsequently, molecular and biochemical analyses were performed such as RT-PCR, Western Blot and confocal immunofluorescence microscopy. The increase of SMN1 mRNA in each sample treated was estimated up to 2.5 fold in mES SMA cells and in SMA mouse NSCs respect to untreated cells. Immunoblotting and “gems” count (63 gems every 100 clones) confirmed these observations. Fluorescence-sorted cells (both mES and NSCs) were further differentiated in vitro into functional MNs, correctly expressing cell-specific markers (Pax6, Olig2, Isl1, Hb9) both in transduced and not transduced cells. Preliminary study in vitro and in vivo are in progress to implant mES and NSCs-derived motor neuron, lentiviral transduced, into SMA mice, in order to evaluate any possible recovery of the pathological phenotype. Teratogenic potential of these committed cells has been preliminary evaluated.

Lentiviral-mediated approach on stem cells represents an attractive source for cells replacement therapy in neurological disorders such as SMA.

**P 173**

A Human Model of IMO by Lentiviral Mediated shRNA Knock-down of Tcrg1 in CD34+ Cells

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The overall aim of this study was to derive functional osteoclasts from CD34+ human cord blood cells and investigate whether lentiviral vectors can be used to overexpress and/or knock down genes in these cells. First, human cord blood CD34+ cells were cultured with M-CSF, GM-CSF, IL-6, SCF and Flt3L for 2 weeks. They expanded 500 fold, and gradually lost CD34 expression while 50% of cells became CD14+. Non-adherent cells were then incubated with M-CSF and RANKL on bone for 10 days. Osteoclasts derived from these cells expressed TRAP, released calcium, exhibited an actin-ring and generated resorption pits on bone slices. Next, CD34+ cells were transduced on day 0 using a SIN lentiviral vector expressing GFP under a SFFV promoter at MOI of 5 resulting in GFP expression in 20-25% of the cells at day 2. Expression was retained throughout differentiation to functional osteoclasts.

Finally, a human model of infantile malignant osteopetrosis (IMO) was generated by transducing cord blood CD34+ cells using lentiviral vectors with the puromycin resistance gene and shRNAs targeting Tcrg1. After trans-
duction and selection with puromycin cells were differentiated to mature osteoclasts. qPCR analysis and western blot revealed decreased Tcrg1 mRNA and protein levels. Osteoclasts had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci- clast had a 40% increase in TRAP expression whereas calci-

In conclusion the knock-down of TCIRG1 in CD34+ cord blood cells provides a human model of IMO necessary for our on-going gene therapy studies.

P 174

Improved growth of Fanconi anemia hematopoietic progenitors by the expression of specific miRNAs

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MicroRNAs are important regulators of gene expression. Initial studies from our laboratory in peripheral blood samples from FA-A patients showed that four microRNAs: hsa-mir-133a, hsa-mir-135b, hsa-mir-139, hsa-mir-181c are down-regulated, compared to expression levels determined in cells from healthy donors. Based on in silico studies and previous description of genes up-regulated in FA patients we focused our study in hsa-mir-181c, which has as possible targets TNF-α and IL-1β both of them previously described to be up-regulated in FA cells. Significantly, we observed that the ectopic expression of miRNA181c in FA LCLs decreased the expression of TNF-α in these cells, as determined by qPCR analyses while this was not the case for IL-1β. Similarly, the ectopic expression of this microRNA in BM samples from FA patients induced a modest, though consistent decrease in TNF-α expression, as deduced from flow cytometry studies. Strikingly, a marked increase in the generation of hematopoietic colonies was observed when BM cells from FA patients were transplanted with microRNA181c. This effect was even higher to that observed after addition of thalidomide to these samples, suggesting that this microRNA may also interact with other negative regulators of the hematopoiesis in FA patients. Finally, luciferase activity assays showed that hsa-mir-181c can directly recognize the TNF-α 3’UTR, confirming the direct interaction between hsa-mir-181c and the TNF-α mRNA. Based on these observations we propose that the expression of miRNAs specifically down-regulated in these cells from FA patients may constitute a new approach to improve the hematopoiesis, and probably other pathological manifestations of FA.

P 175

Hematopoietic stem cell mobilization with Mozobil (AMD3100) + G-CSF in a thalassemic mouse model

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We have previously shown that G-CSF mobilization in the HBBh-3 thalassemic mouse model is less efficient compared to normal C57Bl6 strain, mainly due to increased trapping of hematopoietic stem (Lin-sca-1+ckit−LSK) and progenitor cells (CFU-GM) in the enlarged thalassemic spleen. We explored here, whether the novel mobilizer Mozobil, either alone or in combination with G-CSF improves the mobilization efficiency of thalasemic mice.

HBBh-3 mice received G-CSF (250μg/kgX7days) or AMD (5mg/kgX3days) or their combination (AMD starting at day 5 of G-CSF administration).

Mozobil-alone didn’t significantly affect the spleen size and increased the numbers of circulating CFU-GM as compared to G-CSF. The AMD + G-CSF combination resulted in significant improvement of the mobilization efficiency of HBBh-3 mice over the G-CSF- and AMD-treated group in terms of blood LSK and CFU-GM, completely restoring their mobilizing capacity at levels comparable to G-CSF-treated normal mice. AMD mobilized stem and progenitor cells by “detaching” them from the bone marrow and probably the spleen, in contrast to G-CSF-induced marrow and splenic hyperplasia. Although splenectomy, as expected, restored the impaired G-CSF mobilization of thal mice at levels comparable to normal mice, it didn’t further improve the AMD-or AMD + G-CSF mobilization thereby adding support to the concept that AMD may mobilize HSCs from extramedullary sites also.

The combination of AMD3100 + G-CSF restores the inefficient G-CSF mobilization in a thalassemic mouse model without further burden the hyperplastic bone marrow and spleen. This effect may prove beneficial in a gene therapy setting where high numbers of stem cells need to be safely collected for genetic correction.

P 176

Catheter design and procedures for in vivo hAAT gene transfer to pig liver

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Background: Gene transfer by hydrodynamic procedure in mice is now being translated to larger animals, like pigs, by means of a retrovirus injection with interventionist cathe- terism (hydrocatheterism). Many limitations are being found when transfecing the liver since this is a very big organ with
a particular ability to absorb and by-pass great quantities of liquid. This work tries to clarify the best conditions to achieve pig liver transfection with the therapeutic human a1-antitrypsine (hAAT) gene.

**Methods:** Pigs were anaesthetised and catheterized through the jugular vein testing two conditions: a) 11F fitted catheter with 125ml injections to different neighbour small areas, b) 9-11F balloon catheter with 1-3 injections of 200ml to larger areas. Plasmid containing hAAT gene in saline solution (20µg/ml) was injected at 20ml/s. Plasma samples were taken for ELISA to determine hAAT protein.

**Results:** In catheter fitted model, the best results were obtained with a plasmid total concentration of 15-20µg/ml, regarding the whole volume injected, achieving up to 1mg/ml of hAAT protein in pig plasma. With this total concentration, in the balloon catheter experiments we obtained (11F catheter, 2 injections with 8 total mg of plasmid), 1.6mg/ml hAAT protein, maintaining this level at least 15 days post-injection.

**Conclusion:** We settled the best conditions (catheter type, perfusion flow, size of the territories involved and total plasmid concentration) amongst our different experiments. Despite this, 500 times higher protein production is needed to achieve therapeutic levels, so more studies are required to identify the barriers for a better transfection. Supported by SAF-2007-64492.

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**P 177**

**A pre-clinical cell therapy approach for RDEB with COL7A1 revertant keratinocytes**


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**Revertant mosaicism, also referred to as “natural gene therapy”, has been described for a number of genetic diseases of haematopoiesis, muscle, liver and skin. Until recently revertant mosaicism had not been described for COL7A1. A Recessive Dystrophic Epidermolysis Bullosa (RDEB) patient homozygous for the frame shift mutation COL7A1:c.6527insC, a prevalent mutation with founder effect in Spanish RDEB families, exhibited presented with a patch of clinically healthy revertant skin. In revertant keratinocytes a second site mutation (c.6528delT) was present, resulting in correction of the reading frame and expression of wild-type type VII collagen polypeptide (Pasmooj et al, JID online 24 June 2010). Such keratinocytes can be used for autologous transplantation of “naturally corrected” cells to treat affected skin.

In this study we assessed the in vivo capacity of COL7A1 revertant keratinocytes to attain long-term skin regeneration in a pre-clinical setting. Transplantation of revertant keratinocytes as part of bioengineered skin equivalents onto immunodeficient mice resulted in the engraftment and sustained regeneration of disease-free human skin. Persistence of collagen VII protein at the dermo-epidermal junction 10 weeks post-grafting suggests that the therapeutic effect was achieved due to engraftment of long-lived revertant epidermal stem cells.

Our preliminary data represents a proof of principle of revertant cell therapy (natural gene therapy) for RDEB.

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**P 178**

**Gene delivery and gold nanoparticle distribution in human ex vivo liver segments after catheterism**

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**Background:** In the translation of hydrodynamic gene transfer from mice to human application, different problems must be adressed, including molecular characterization of gene transfer and histological differences between species, that represent a barrier for the plasmid entry into cells.

**Methods:** Human hepatic segments from chirurgically fresh resected livers were obtained and weighed. A catheter was introduced through the suprahepatic vein, reaching an area as large as possible and inflating the catheter balloon to avoid the flow back. A volume of approx.1/5 or 1/10 of the segment weight, of 20µg/ml plasmid solution containing the green fluorescent protein gene (EGFP) was injected at 10-20ml/s flow rate. The same procedure was performed, but injecting a solution of 15-20nm gold nanoparticles (1.8x1012 Au particles/ml) for electron microscopy observations.

**Results:** EGFP gene expression was confirmed by fluorescent microscopy. RT-PCR showed a better (more than one magnitude order) delivery index (exogenousDNA copies/100pg total DNA) at 20ml/s flow rate but equivalent results of transcription index (exogenousRNA copy/100ng total RNA) employing either 20 or 10ml/s, reaching a better intrinsc efficacy (exogenousRNA/exogenous DNA copies) with 10ml/s. Electron microscopy shows that after injection and fixation, the gold nanoparticles remain under or inside the endothelia but never reach the hepatocytes.

**Conclusion:** Milder conditions in flow rate during injection achieve better transfection results than stronger ones. However, the electron microscopy reveals that human endothelia does not behave like mouse and gold particles remain trapped in the vessels, not reaching the hepatocytes, due to its hermetic characteristics. Supported by SAF 2007-64492.
**Microarray-based analysis of phenylalanine metabolism in retrovirus-transduced human keratinocytes**

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The aim of this study is to characterize the overall gene expression of cells with high levels of phenylalanine metabolism. Phenylalanine can be converted to tyrosine by phenylalanine hydroxylase (PAH). PAH requires a cofactor, tetrahydrobiopterin (BH₄). The first step in the BH₄ synthesis is catalyzed by GTP cyclohydrolase 1 (GCH1). Primary human keratinocytes co-transduced with PAH and GCH1 are capable of phenylalanine hydroxylation, as shown in previous studies. Co-transduction with GCH1 was required because overexpression of PAH alone did not lead to significant phenylalanine clearance.

In the present study, expression profiles of keratinocytes with increased phenylalanine metabolism were obtained using Affymetrix GeneChip HG-U133 Arrays. The data were normalized and filtered using Bioconductor packages. Compared to controls we identified 102 differentially expressed genes. GCH1 is known to take part in other processes as well as the phenylalanine catabolism. Therefore, by excluding genes differentially expressed by GCH1 alone and PAH alone we obtained a group of genes differentially expressed as a result of metabolically active PAH. This resulted in 42 remaining genes. Gene Ontology analysis revealed immunological response genes and carbohydrate binding gene to be upregulated as a result of transduction with PAH and GCH1 compared to the neomycin-resistance (neo) expressing controls. This result will be verified by qPCR on selected genes and we will investigate the effect on the phenylalanine metabolism of knocking down expression of selected genes.

Our results are important for future attempts to improve gene therapy strategies of phenylketonuria (PKU) where phenylalanine metabolism is impaired.

**The importance of sTRAIL in patients with rheumatoid arthritis**

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Background: TRAIL is an apoptosis-inducing agent in transformed cells but not in normal tissues [1]. It has been reported that TRAIL and TRAIL receptors might be involved in the pathophysiology of autoimmune diseases [2, 3]. The secreted form of TRAIL namely soluble TRAIL (sTRAIL) has recently been linked to the severity of SLE [4]. The goal of this study was to investigate whether or not a similar connection could be established in RA patients as well.

Methods: Serum sTRAIL levels of 40 RA patients and 20 healthy controls were measured by ELISA.

Results: Only those DMARD treated RA Patients (n = 20) had elevated levels of sTRAIL compared to those newly diagnosed RA patients who did not receive DMARDS and control group.

Conclusion: Serum sTRAIL levels were useful in distinguishing newly diagnosed RA patients and healthy control individuals from those who received DMARD treatment.

References
The outcome of trail presentation on T cells in patients with multiple myeloma

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Background: Multiple Myeloma (MM) develops due to the accumulation of malignant plasma cells in the bone marrow. Since TRAIL and TRAIL receptors are involved in cell death/survival pathways leading to carcinogenesis, TRAIL and TRAIL receptor profiles on tumor cells might serve as a prognostic marker for many cancer types [1, 2]. Cytotoxic T lymphocytes (CTLs) exhibit anti-tumor activity and are one of the major sources of TRAIL in blood. Thus, we hypothesized that an alteration or a defect in TRAIL expression might influence MM progression.

Methods: TRAIL expression profiles of T lymphocytes were investigated via EpicsAltra Beckman-Coulter flow cytometer, and statistical analysis was performed using SPSS.

Results: We first analyzed surface TRAIL expression on T cells of 16 sex and age matched control individuals. TRAIL expression was detected on both primary CD4 + helper T and CD8 + cytotoxic T cells where TRAIL expression on CTLs was higher than that of helper T cells.

Conclusion: Our next step is to recruit MM patients to analyze surface TRAIL expression on CTLs to check if the profile is connected to MM prognosis.

References

Distinctive expression profiles of trail and trail receptors in patients with endometrial carcinoma

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Background: Endometrial cancer is the most common gynecological malignancy and the fourth most common cancer type in women. TRAIL and TRAIL receptor profile has recently been tested for its potential to be used as a prognostic marker in cancer [1, 2]. However, how TRAIL and TRAIL receptors contribute to endometrial carcinogenesis is not known. Thus, we investigated TRAIL and TRAIL receptor expression profile during endometrial carcinogenesis to evaluate its potential as a prognostic marker and to predict the feasibility of TRAIL-mediated gene therapy approach.

Method: We analyzed TRAIL and all four TRAIL receptors in 100 patients with endometrial carcinoma including 18 normal endometrial tissue using immunohistochemistry. TUNEL assays were deployed to determine the cell death.

Results: Patients with endometrial carcinoma displayed a distinctive expression profile of TRAIL and TRAIL receptors compared to healthy controls. A decrease in TRAIL and DR4 death receptor expression but an increase in both of the decoy receptor expressions was detected.

Conclusion: Distinctive expression profile of TRAIL and its receptor expression in patients with endometrial carcinoma suggests that TRAIL signaling might be important during the endometrial carcinogenesis process.

References


P 185
Targeted gene addition to a predetermined site in the human genome using a ZFN-based nicking enzyme
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Zinc-finger nucleases (ZFNs) drive highly efficient genome editing by generating a site-specific DNA double-strand break (DSB) at a predetermined site in the genome. Subsequent repair of this break via the non-homologous end-joining (NHEJ) or homology-directed repair (HDR) pathways results in targeted gene disruption or gene addition, respectively. Here we report that ZFNs can be engineered to induce a site-specific DNA single-strand break (SSB) or nick. This nicked target site stimulates gene addition using a homologous donor template, but fails to induce the small insertions and deletions characteristic of repair via NHEJ. Gene addition by such zinc finger nickases (ZFNickases) occurs in both transformed and primary human cells at efficiencies of ~1-20%. Introduction of a site-specific nick at an endogenous locus provides an important tool for the study of DNA repair. Moreover, the ability of a SSB to direct repair pathway choice (i.e. HDR but not NHEJ) may prove advantageous for certain therapeutic applications such as the targeted correction of human disease-causing mutations.

P 186
Transcriptome Sequencing of Lentivirally Transduced Unrestricted Somatic Stem Cells
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Gene therapy using integration-proficient retroviral vectors was found associated with flanking gene de-regulation. Expression analysis of genes located in genomic “windows” around insertion sites or transcriptional profiling using microarrays only focus on known or array-spotted genes, respectively.

To provide deeper insights into vector-related alteration of the transcription profile, we used mRNA of lentivirally transduced human unrestricted somatic stem cells (USSC) for ultra-deep shotgun sequencing (Genome Analyzer II; Solexa/Illumina).

Transcriptome sequencing yielded 2x10^6 of mRNA sequence reads per sample (untransduced and transduced USSC). Reads were unambiguously aligned to the human genome (GRCh37 draft) using the TopHat software at recovery rates of nearly 90%. Using the Cufflinks pipeline we detected a total of 42 345 individually transcripts in untransduced USSC and 42 555 in the transduced sample.

In parallel by linear amplification-mediated PCR (LAM-PCR) and 454 pyrosequencing we obtained a total of 34 924 insertion site sequences of the lentiviral vector in the genomic DNA of USSC. Insertion site analysis yielded a total of 5 528 non-redundant and 4 413 (78%) intragenic insertion sites. Thus, we were able to establish a detailed integration profile for lentiviral vectors in human mesenchymal stem cells.

One goal of further analysis is the assessment of the capability of lentiviral vectors activating adjacent genes by identifying vector-genome read-throughs. Thus, using the combination of transcriptome sequencing and high throughput insertion site analysis a detection of a vector-dependent alteration of the transcription profile lentivirally transduced versus non-transduced cells should be provided.

P 187
Non-Integrative Lentivirus Drives High-Frequency cre-Mediated Cassette Exchange in Human Cells
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Recombinant mediated cassette exchange (RMCE) is a second-order (two-step) process leading to genetic modification in a specific genomic target sequence. It is necessary for future development of regenerative and genetic therapies and for human developmental studies. The process involves insertion of a docking genetic cassette in the genome followed by DNA transfer of a second cassette. Although the minimal requirements are the presence of compatible recombination signals and expression of recombinase, efficiency relies at least on cell viability upon transfection, expression of the enzyme, and the ability to target cell types of different origins.

To overcome such drawbacks, we developed an RMCE assay that uses an integrase-deficient lentivirus (IDLV) in the second step, thus avoiding undesired random integration of the vector sequences. We show that combining the lentiviral vector with promoterless trapping of double selectable markers during RMCE makes for an efficient, safe, and simple strategy with expected wide applicability. Additionally, recombinase that is encoded in the exchangeable cassette is self-limiting as a result of its own recombinase, thus avoiding toxicity. Our approach is based on IDLV vectors.
and provides proof-of-principle of a novel strategy modeled on a human cell line with randomly integrated copies of a genetic landing pad. This strategy does not present foreseeable limitations for application to other cell systems modified by homologous recombination. Safety, efficiency, and simplicity are the major advantages of our system, which can be applied in low-to-medium throughput strategies for screening of cDNAs, non-coding RNAs during functional genomic studies, and drug screening.

P 188
Design of novel PhiC31 Integrase fusion proteins for improving efficacy and safety of transgene insertion
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Based on site-directed recombination the bacteriophage derived PhiC31 integrase system provides outstanding opportunities for transgene integration into the host genome. Due to unpredicted insertions, events mediated by PhiC31, the optimization of activity and directing integration into specific target sites of the genome are important goals for the usage in therapeutic applications.

We constructed several fusion proteins of the integrase and different DNA binding domains (DBD) like the synthetic polydactyl zinc finger E2C and the AAV/Rep protein. The motifs were either fused N- or C-terminal to the integrase separated by a short T5- or a long GGS5-linker. Immunoblot and flow cytometry analyses revealed that the fusion proteins were expressed in comparable amounts after transfection into 293-cells. Catalytic activity retained up to 80% of the wildtype integrase (WT) when binding motifs were attached to the C-terminus connected by the long interdomain. We also generated fusion mutants lacking the native DBD of the integrase (ΔDBD), which were inactive when measuring recombination efficiencies between WT PhiC31 integrase recognition sites. After performing initial colony forming assays to detect integration mediated by the ΔDBD fusion constructs, we observed 30% activity of the WT.

In our previous study we could identify critical amino acids of the integrase involved in the insertion process and also hyperactive variants showing 5.5-fold increased integration efficiency. Mutants lacking their native DNA binding properties may be restored by fusing to known DBDs. In combination with hyperactive PhiC31 variants this approach could lead to a more specific and efficient PhiC31 integrase system.

P 189
Site-specific genome cleavage by a vector-incorporated HIV-1 integrase fusion protein
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To meet the well established need for a gene transfer vector bearing a safe integration profile, several approaches are being studied, including the development of zinc finger nucleases and harnessing the mechanisms of DNA-modifying elements. Despite the good progress already achieved, a set of difficulties related to the safe yet efficient delivery of the DNA-modifying proteins and elements still remains. We have previously shown that lentivirus vectors (LVVs) can be modified to contain both cytotoxic and inert integrase (IN) fusion proteins. To test the feasibility of this cis-packaging system to promote cellular delivery of a site-specific mega-nuclease, we fused I-PpoI into the C-terminus of IN. The DNA cleaving ability of the IN-I-PpoI fusion protein was first studied in vitro, after which LVVs carrying the fusion protein were produced and tested in cultured cells. Vectors containing IN-I-PpoI were able to promote specific cleavage of I-PpoI’s target site found in the nucleolar rRNA genes in two studied human cell lines. The vector-contained IN molecule profile was next optimized with regard to fusion protein derived cytotoxicity and integration efficiency to allow integration site selection (ISS) studies. Our results show that by altering the IN-composition of LVVs it is possible to 1) deliver a functional dimerization-requiring endonuclease into transduced cells nuclei 2) retain integration proficiency of the modified LVVs 3) affect the ISS of LVVs. These findings may prove useful in the quest for safe site-specific gene addition and genome modification, and show the potential of LVVs as diverse genome modifying tools.

P 190
A novel assay for measuring gene repair efficiency in the mouse PAH gene
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Targeted gene repair of single-base mutations can be used as an alternative to cDNA-based gene transfer. Promising techniques for this type of gene therapy include the use of Zinc Finger Nucleases (ZFNs) and Adeno-associated Viral Vectors (AAVs). PCR-based assays are currently used to screen the efficiency of gene repair - however the reliability of such assays can often be questioned due to possible PCR artifacts. Therefore, a highly sensitive non-PCR based assay which can be employed in a variety of cells is important for reliable establishment of the repair efficiencies. We are working on ZFNs and AAV specifically targeting the missense mutation in exon 7 of the active site in the phenylalanine hydroxylase
(PAH) gene of phenylketonuria (PKU) mice (pah(enu2)). We have inserted 4 bp in exon 7 resulting in translational stop and disturbance of the reading frame. A fusion protein is produced where a GFP gene is fused to exon 1-6, the truncated exon 7 and a truncated exon 8 resulting in an assay where GFP is only expressed upon repair of the stop mutation by homologous recombination mediated by ZFNs or AAV. The construct is subsequently cloned into a FLP-vector which is applicable in a recombination mediated by ZFNs or AAV. The construct is expressed upon repair of the stop mutation by homologous recombination. The ZFN leads to specific double stranded breaks causing mutation found in more than 50% of the Danish PKU patients). The ZFN leads to specific double stranded breaks stimulating homologous recombination. The ZFNs are co-transfected with a donor plasmid containing the functional PAH gene. So far we have used the ZFNs to insert a selection marker gene in the PAH gene by non-homology end joining into the double stranded break in tissue cultured mouse cells. We are currently using H2AX antibodies to measure the amount of double stranded breaks in ZFN transfected cells.

**P 192**

**A quantitative luciferase assay for DNA double strand break-induced homologous recombination**

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Meganucleases (MN) are site specific endonucleases, with 12 to 45 bp DNA recognition sites. They generate double-strand breaks that can be repaired by homologous recombination if a DNA template is present. MN may be engineered for custom recognition of any genetic locus and used for gene targeting. Our interest is to develop efficient means of targeting transgenes at specific loci for the purpose of gene therapy.

Our working model is a single chain MN derived from I-Crel that recognizes a sequence within the human Rag-1 gene. Available systems for the detection of MN activity in mammalian cells are based on reporter genes (lac Z, GFP) inactivated by a short duplication. The MN target sequence is placed in the middle of the duplication and upon double strand break, the reporter is repaired by recombination. We have constructed such a recombination detection system using luciferase as a reporter, in order to obtain a more quantitative assay that could also be used for detecting the MN activity after delivery into live animals. A duplication-inactivated thermostable Firefly luciferase gene was constructed and used in different assay format in human 293 cells. This luciferase-based system is fast, sensitive and quantitative, and will be useful for the detection of MN activity into experimental animals.

**P 193**

**Chromosome-based vectors: Novel routes to predictable gene expression**

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The mammalian genome is organized into independently-regulated chromatin domains. Each domain is largely shielded from its surroundings and prevented from heterochromatization by bordering elements, most notably S/MARs. During recent years we could refine our gene transfer procedures to enable • Re-targeting of endogenous stable, highly expressed chromosomal loci after initial characterization (RMCE approach); increase efficiencies by the inclusion and strategic localization of S/MAR elements. • Design and verify novel sets of heterospecific 48 bp FRT sites to enable enhanced RMCE approaches like multiplexing (simultaneous targeting of two or more specified genomic loci) or the simultaneous deletion of several FRT-flanked (“flirted”) genomic inserts in the absence of unwanted genomic rearrangements. • Create replicating nonviral epimask units (minicircles, MCs) based solely on a transcribed gene and a single S/MAR. • Combine both procedures: create MC derivatives after their establishment in the nuclear architecture.

All our approaches obey the pFAR (free of antibiotic resistance) principle in a straightforward manner. We will discuss routes leading to the predictable establishment and differentiation of ES- and iPSC cells.

**References**


P 194

Suppression of Collagen-Induced Arthritis by Intraarticular Lentiviral Delivery of TLR7 shRNA

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Background: Knockdown or inhibition of the Toll-like receptor (TLR) pathway from the surface or intracellular receptors to downstream signaling molecules is a novel therapeutic strategy for the treatment of rheumatoid arthritis (RA). TLR7, the receptor of single strand RNA, is highly expressed in the RA joints. Here we examined the effects of lentiviral vectors expressing small hairpin RNA (shRNA) targeting TLR7 on amelioration of collagen-induced arthritis (CIA).

Methods: Lentiviral vectors expressing TLR7 shRNA (Lt.shTLR7) or scrambled TLR7 shRNA (Lt.scramble) were injected intraarticularly to rats with CIA and their treatment responses were monitored by measures of clinical, radiologic, and histologic changes. Microvessel density as well as levels of VEGF, pro-inflammatory cytokines, matrix metalloproteinases (MMPs), and T cell infiltrates in the synovial tissues of ankle joints were determined.

Results: Expression of TLR7 was upregulated in the ankle joint during the disease progression of CIA in rats. Treatment of Lt.shTLR7 significantly reduced ankle circumference, articular index scores, radiographic scores, and histologic scores, as compared with Lt.scramble treatment. Moreover, microvessel density, VEGF, IL-1β, and IL-6 contents, as well as T-cell infiltrates within the synovial tissue were significantly lower in the Lt.shTLR7-treated rats. Whereas the level of MMP-2 was unaffected by the Lt.shTLR7 treatment, the expression of MMP-9 was decreased in the Lt.shTLR7-injected ankle joints.

Conclusion: Our results demonstrate for the first time that disruption of intraarticular TLR7 signaling can suppress arthritis in rats with CIA. Our findings implicate manipulation of TLR7 molecule as a potential target in clinical therapy of RA patients.

P 195

Gene therapy with AAV9-Calsarcin-1 avoids Angiotensin-II mediated contractile dysfunction

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Cardiac remodelling caused by sustained pressure and/or volume overload leads to the development of cardiac hypertrophy. One molecular pathway that is involved in hypertrophic growth is mediated by the calcium- and calmodulin-dependent serine/threonine phosphatase calcineurin, which plays a key role in transducing calcium-dependent signals from the cytosol to the nucleus.

In previous studies we have shown that mice lacking Calsarc-in-1 (CS1), a calcineurin-interacting protein, are sensitized to calcineurin signaling. Conversely mice over-expressing CS1 blunt hypertrophic growth when exposed to chronic angiotensin-II (Ang-II) infusion.

To use the protective effect of CS1 we first generate an adeno-associated virus serotype 9 (AAV9) that encompasses the CS1 full length coding sequence under the control of the 0.26kb cardiac myosin light chain promoter fused to the cytomegalovirus immediate-early enhancer (CMV(enh)/MLC0.26). AAV9-CS1 or a Renilla-luciferase control-AAV9 (AAV9-Ren) where systemically injected via the tail vain (dose 2x1011 Vg) in 8-weeks-old male C57Bl/6 wild type (WT) mice. One week after intravenously AAV9 injection mice were subjected to long-term stimulation with Ang-II (1000ng/kg per min) or 0.9% NaCl respectively using subcutaneous minipumps for two weeks.

Ang-II stimulated and AAV9-Ren treated WT mice showed a contractile dysfunction with a significant reduction of the fractional shortening (FS) assessed by echocardiography and a strong trend to a dilation of the left ventricle. AAV9 mediated overexpression of CS1 leads in contrast to a complete removal of LV-dysfunction.

Taken together these results give a further hint that CS1 influence the cardiac remodelling after hypertrophic stimulation in a positive way.

P 196

Antisensehyphen;induced myostatin exon skipping leads to muscle hypertrophy in mice following Octa-guanidine oligomer treatment

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Myostatin is a negative regulator of muscle mass, and several strategies are being developed to knock down its expression to improve muscle wasting conditions. Use of anti-myostatin antibodies and natural binding partners, have resulted in increase in muscle mass in test animals but they have low sustainability. The strategy using short hairpin RNAs offers long term expression but is accompanied by viral vectors-associated risks of uncontrolled insertion into the human genome. In this study we report for the first time the use of antisense oligonucleotides (AOs) to manipulate myostatin pre-mRNA splicing by exon skipping. AOs of both 2’O-methyl RNA (with a phosphorothioate backbone) and Phosphorodiamidate morpholino (PMO) chemistries lead to the disruption of the reading frame of myostatin transcript in vitro through exon skipping as shown by RT PCR analysis of
RNA from transfected C2C12 cells. This was exemplified by increased cell proliferation in treated cultures. Preliminary intramuscular studies in vivo showed that 2'-O-methyl RNA and PMO AOs successfully induced exon skipping that was observed until after 4 weeks of treatment. Systemic in vivo studies using vivo-PMO (octa-guanidine PMO) showed myostatin exon skipping led to a significant increase in the mass and cross sectional area of soleus muscle of treated mice. AO-induced myostatin exon 2 skipping provides the potential for a controlled and safe therapy for the increase in muscle mass in muscle wasting conditions including diseases such as Duchenne Muscular Dystrophy as the dosage can be controlled, monitored and re-administered safely as the need arises.

**P 197**

Efficient muscle-specific expression after gene transfer with promoters derived from troponin I gene

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Gene replacement therapy for muscle diseases using viral vectors requires strong and muscle specific expression of a therapeutic gene product. To achieve this goal, we generated regulatory cassettes by linking three (ΔUSEx3) or four (ΔUSEx4) copies of the truncated upstream enhancer (ΔUSE) of the slow troponin I gene. To evaluate the activity of ΔUSEx3 and ΔUSEx4 in the context of viral vectors, we constructed helper-dependent adenoviruses (HD) expressing β-galactosidase (β-gal) regulated by ΔUSEx3, ΔUSEx4 or the very potent hybrid CMV enhancer/β-actin (CAG) promoter. We also constructed AAV and lentiviral vectors (LV) expressing the mature form of human VEGF-D (hVEGF-DΔNAC) under the constitutive human phosphoglycerate kinase (hPGK) promoter were produced. In order to study the targeted expression of VEGF-D in epithelium only, also transgenic animals expressing hVEGF-DΔNAC under the mouse endothelial promoter Tie1 were created. The transgenic mice were generated by lentiviral perivitelline injection technique where the zona pellucida covering the oocytes is left in place. This results to a significantly higher number of implanted embryos and transgenic founders compared to traditional plasmid microinjection.

Transgenic mice were followed up to F3-F5. The VEGF-D transgenic mice showed enhanced blood capillary density and improved post ischemic muscle regeneration. No major changes in lymphatic capillary density were found, which suggests that hVEGF-DΔNAC has no lymphangiogenic effect. The transgenic mice showed an increased susceptibility to tumor formation and a reduced life span. The expression of transgene was observed in several tissues; however the transgene expression decreased in advanced generations. When the expression was targeted to the epithelium only, no lymphangiogenic effects were detected either but more different tumor types were observed. Lentivirus perivitelline injection has proven to be a very efficient transgenesis method. VEGF-D could be a potent factor in gene therapy for treating ischemic diseases.

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VEGF-D transgenic mice created by lentiviral transgenesis method

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Gene therapy with human adenovirus type 5 (Ad5) has been extensively explored for the treatment of diseases resistant to traditional therapies. However, following systemic administration of Ad, the resulting innate and adaptive immune responses dramatically affect the kinetics and toxicity profile of the vector. Building on our previously reported technology of...
artificial envelopment of Ad, we designed a variety of artificial lipid bilayer envelopes around the viral capsid in order to develop safe and efficient Ad vectors for gene therapy. Considering the critical role of host blood cells and plasma proteins in systemic administration of Ad, we studied the interaction of enveloped Ad in cationic non-pH-sensitive (DOTAP:Chol) or anionic pH-sensitive (DOPE:CHEMS) lipid bilayers with several different blood components. In vitro experiments showed that uptake of DOTAP:Chol-enveloped Ad in HepG2 cells was not affected by the presence of any blood factors, whereas treatment with blood factor X, but not IX or XI, increased cellular uptake and gene expression levels of DOPE:CHEMS enveloped Ad. Different blood cells were isolated from human or Balb/c mouse blood and their interactions with artificially enveloped Ad were investigated ex vivo. Artificial envelopment of Ad with DOTAP:Chol bilayers resulted in significantly higher binding to human and murine red blood cells (RBC) compared to DOPE:CHEMS envelopes, which highlighted the importance of the characteristics of the lipid envelopes. These results suggest that artificial envelopment of Ad dramatically alters the affinity towards blood components that will affect the virus tissue-distribution following systemic administration.

P 200

Evaluation of AAV6-microutrophin expression in dystrophic mouse models and the non-human primate

P 201

Efficient transduction of human umbilical veins ex vivo using vectors selected from an AAV9 library

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In our previous studies we have shown the potential of an AAV2 random peptide display library to select for AAV2 vectors with improved efficiency for endothelial gene transfer. A lower prevalence of neutralizing antibodies in humans and a more efficient gene transfer in several animal models, however, might render AAV9 more suitable for this purpose than AAV2.

Hence, our goal was the generation of an AAV9-based randomized heptapeptide library to select for efficient and specific gene transfer vectors on human endothelium. Using a highly diverse plasmid library (1.25E + 9 motives) harboring randomized oligonucleotides in the AAV9 cap gene, we generated an AAV9 peptide display library, which ensures that the displayed peptides correspond to the packaged genomes. The library was biopanned for 4 rounds on human coronary artery endothelial cells (HCAEC) and transduction efficiencies of AAV9 vectors displaying the enriched peptide motives were compared to wtAAV9. While wtAAV9 resulted in a transduction efficiency of 2.3 ± 1.6%, motives selected from the AAV9 library enabled efficiencies up to 92.8 ± 3.1% (p < 0.001). In order to analyze the potential of endothelial-targeted vectors to transduce endothelial cells in the vascular context, we incubated human umbilical veins in situ with wt or mutant AAV9 vectors. FACS analysis of endothelial cells isolated from these umbilical veins (HUVEC) revealed an improvement of transduction efficiency from 0.4 ± 0.7% to 80.3 ± 11.9%, respectively (p < 0.001).

In summary, selection of our AAV9 library has yielded motives with strong transduction of HCAEC in vitro and HUVEC ex vivo, rendering these vectors candidates for future clinical approaches.

P 202

Amelioration of Collagen-Induced Arthritis through Apoptosis of CD4+T Cells and Reduction of Synovial IL-17 Production

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Background: Indoleamine 2,3-dioxygenase (IDO), one of the initial and rate-limiting enzymes involved in the catabolism of tryptophan, has immunomodulatory activities and is an emerging therapeutic target in autoimmunity-related arthritis. Here we investigated the therapeutic effect IDO gene delivery on collagen-induced arthritis (CIA) in rats.

Methods: The reatment responses of adenoviral vectors encoding IDO (AdIDO) in the arthritic rats were examined. The therapeutic effects of AdIDO on ankle circumferences,
articular index, and radiographic and histological scores were evaluated in the treated rats. We further investigated the underlying mechanism of action.

**Results:** Reduction of ankle circumferences, articular index, and radiographic and histological scores were observed in AdIDO-treated ankles, as compared with those injected with control vectors. Furthermore, IDO transfer led to decreased infiltrating CD4+ T cells with enhanced apoptosis, reduced CD68+ macrophage numbers, increased kynurenine (a downstream tryptophan metabolite) levels, lower IL-17 concentrations, and decreased expression of RORγt, a transcription factor important for the development of Th17 cells, within the ankle joints. In addition, IDO gene delivery diminished type II collagen-specific IL-17 production and RORγt expression in CD4+ T cells from draining lymph nodes of CIA rats.

**Conclusion:** Our results demonstrate for the first time that intraarticular delivery of IDO gene ameliorates arthritis. Further analysis of this promoter may have the potential to achieve certain gene expression in severely affected muscles of mdx mice. The Lv-mediated transgenic mouse may prove a useful tool for assessing the enhancing/promoter activity of a variety of different regulatory cassettes.

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**P 204**

**In vitro aided identification of AAV6 as the best vector for gene transfer into porcine myocardium**

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**Background:** Adeno-associated virus (AAV) mediated gene transfer into diseased myocardium holds high promises. While efficient transfer is achieved in rodents, gene delivery to the hearts of larger animals appears to be limited. Our aim was the development of *in vitro* models to facilitate vector development for cardiac gene transfer in large animals and for future clinical trials.

**Methods:** To identify suitable models, luciferase reporter constructs driven by the CMV-promoter have been cross-packaged into the capsids of AAV serotypes 1-6, 8 and 9. These constructs have been tested in cardiomyocytes and/or organotypic myocardial slices of mice, rats and pigs. We then analyzed porcine myocardial gene transfer with the two most promising vectors and AAV9 after application via retro-infusion into the coronary veins.

**Results:** Cardiomyocytes and organotypic slices gave very similar results showing strong luciferase activity and pronounced variation between the serotypes. Comparison with published *in vivo* data revealed a good correlation for AAV1 to 6 but not for 8 and 9 which partially failed *in vitro*. Comparison of reporter activities in the pig *in vivo* revealed that AAV6 enables the most efficient cardiac gene transfer followed by AAV5, while AAV9 was inefficient, confirming the predictions of our model.

**Conclusion:** We have developed predictive models for the efficiency of cardiac gene delivery, enabling strongly improved transgene delivery to the porcine heart. The correlation with *in vivo* data underlines the feasibility of both models for vector assessment. The predictions unveiled two AAV serotypes more suited for porcine myocardial gene transfer than AAV9.

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**P 205**

**Canine microdystrophin results in remarkable expression and improvement in muscle pathology in a canine model of Duchenne Muscular Dystrophy following AAV gene transfer**

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Duchenne muscular dystrophy (DMD) is a severe inherited muscle-wasting disorder caused by mutations in the dystrophin gene. Recombinant adeno-associated virus (rAAV)-mediated gene therapy to transfer dystrophin genes into
Peripheral artery disease; combined approach in therapeutic angiogenesis

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Peripheral artery disease (PAD) is one of leading causes of disability in Western societies. Gene and cell therapy can be used to induce angiogenesis in ischemic skeletal muscle to prevent major necrosis and amputations in affected patients. Benefit from gene transfer and cell transplantation was shown in many experimental and clinical studies but development of a novel combined approach in this field is still an important point in medical science.

Our group focused on animal testing of several plasmid constructs with human VEGF165, HGF, angiopoietin-1 and mouse uPA to find combinations which could amplify angiogenic efficacy of gene transfer. We found that co-transfer of 2 growth factors (VEGF + HGF, VEGF + Ang-1 or HGF + Ang-1) renders a higher angiogenic effect than sole plasmids, which was shown by laser Doppler imaging and histologic methods.

Another important point assessed in our studies was use of AAV-transduced adipose derived stromal cells (ADSC) for induction of angiogenesis in mouse ischemic limb and Matrigel plug model. Recombinant AAV was used to induce overexpression of VEGF165 in human ADSC and modified cells showed higher angiogenic efficacy than GFP-transduced or unmanipulated ones. Cell transplantation gave a higher and more stable increase in limb perfusion than plasmid gene transfer. Our findings indicate that gene and cell therapy efficacy in PAD might be increased using combined methods: 1) gene transfer of several growth factors, 2) modified cells transplantation or, possibly, using both which is still to be tested in appropriate animal models.

P 208
MYOD1-transduced placental MSCs: The feasible cell therapy of neuromuscular disorders

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Background: Mesenchymal stem cells are one of the somatic stem cells, which could be induced into several tissues derived from mesoderm. We have shown that human dystrophin was able to be expressed in the muscle of mdx-scid mice after local administration of human placenta derived mesenchymal stem cells (hpMSCs) (Kawamichi et al. 2010). In order to enhance the myogenic properties of hpMSCs, we introduced human MYOD1 cDNA into hpMSCs using the lentiviral vector, and examined how the muscle-specific gene expression was enhanced.

Methods: The MYOD1 cDNA and pLent6/V5-DEST were obtained from the RIKEN and Invitrogen, respectively. Amniotic mesoderm derived cells (AMCs) were isolated from human preterm placentas. AMCs were transduced with the MYOD1 vector, and total RNA was extracted after 7 days culture. The mRNA levels for MYOD1, MYF5, MYOG and DMD were determined by qPCR using the Universal Probe Library system (Roche). Relative gene expression levels were calculated using the normal human skeletal muscle myoblast cells (Lonza) as a control.

Results: The mRNA levels of untreated and MYOD1-transduced hpMSCs as follows: MYOD1, 0.101 ± 0.053% and 4.77 ± 2.02%; MYF5, 0.013 ± 0.001% and 5.61 ± 3.82%; MYOG, 0.00% and 0.125 ± 0.006%; DMD, 2.76 ± 1.01% and 19.9 ± 5.9%.

Conclusion: Myogenic factor 5, myogenic differentiation 1 and myogenin are the essential transcription factors during the skeletal muscle development, and designated as the myogenic regulatory factors (MRFs). In this study, we showed the simultaneous upregulation of the MRF genes as well as DMD in the MYOD1-transduced hpMSCs, suggesting that the MYOD1-hpMSCs seem to be a promising tool for cellular therapy for neuromuscular disorders.

References

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Targeted gene transfer to proliferating endothelium: viral versus non-viral approaches
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Specific transfer of “therapeutic genes” into proliferating endothelial cells would constitute a valuable tool for the treatment of disorders involving the sprouting of new blood vessels such as tumor angiogenesis. Thus, specific endothelial surface molecules induced during the process may serve to target gene transfer vectors to the proliferating endothelium. We have focused our approach on E-selectin (CD62E), an activation marker largely restricted to endothelial cells in angiogenic and inflammatory settings, and we have compared viral versus non-viral vector systems for targeted gene transfer. The viral approach involves a chimeric retrovirus,
incorporating a single chain antibody fragment directed against CD26E in the retroviral envelope protein to mediate host cell specificity and entry. Envelope proteins were derived from Mo-MLV, SNV and 4070A retrovirus. The non-viral gene transfer variant was based on particles of cationic liposomes and plasmid DNA covered by a chimeric S-layer-streptavidin fusion protein engineered to bind the E-selectin antibody via a biotin-streptavidin bridge. Both systems were tested in vitro with proliferating human microvascular endothelial cells (stimulated to express E-selectin) for selective transfer of a reporter gene. In either case, target specificity was attained but resulted in a considerable reduction of gene transfer efficiency as compared to unmodified viral or liposomal particles. Both systems are currently further tested for stable versus transient trans-gene expression and for modifications to increase transfer efficiency.

P 211

Biotinylated AAV as tool for attaching nucleic acids on the capsid surface

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Background: Adeno-associated viruses (AAV) enable an efficient transduction of myocardium. Aim of our study was to develop an approach for cardiac delivery of nucleic acids attached to the AAV surface combining viral and non-viral gene transfer techniques.

Methods: We loaded plasmid DNA on polylysine coupled to streptavidin. These DNA/polylysine complexes were attached to AAV2 and AAV9 vectors genetically biotinylated using a biotin acceptor protein (BAP) motif within the capsid sequence of AAV and cotransfection of biotin ligase within the vector production.

Results: Complete biotinylation decreased vector tropism and efficacy. Thus, we determined the amount of biotinylation of capsids that enables the best compromise between preservation of AAV tropism and transfer efficiency of plasmid-DNA by comparing different ratios of wildtype (wt) and BAP-modified capsids for vector production. Plasmid DNA encoding a red fluorescent reporter protein was linked via BAP-modified capsids for vector production. Plasmid-DNA via biotinylated AAV2 in HEK293 cells. Genetically biotinylated AAV9 enabled a preserved cardiac transduction pattern of myocardium in vivo.

Conclusion: Conditions were established for transfer of plasmid-DNA via biotinylated AAV2 in HEK293 cells. Genetically biotinylated AAV9 enabled a preserved cardiac transduction pattern of myocardium in vivo.

P 212

Semliki Forest virus VA7 infects human glioma derived slice cultures and cell explants whereas having only limited replication in healthy macaque brain tissue

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Background: Semliki Forest virus (SFV) is a non-pathogenic alphavirus, which replicates efficiently in a number of different cancer cell lines. In our recent study intravenously administered SFV VA7 completely eliminated subcutaneous and intracranial U87 human glioma xenografts in nude mice. Here we demonstrate the susceptibility of a human malignant glioma biopsy derived tissue slice culture and a cancer stem cell explant culture to VA7 mediated infection and oncolysis, and limited replication in healthy Macaque brain tissue culture.

Method: Live tissue slice cultures were established from a human malignant glioma biopsy and a healthy macaque brain respectively, and they were infected with oncolytic SFV VA7 alphavirus vector. VSV A51-EGFP and wtVSV-EGFP viruses were used as controls in these infections. The CD133+ cancer stem cells were derived from another human malignant glioma biopsy using commercial isolation column (Miltenyi Biotec). The cells and spontaneously forming tumour spheres were infected with VA7 virus with or without human IFNα pretreatment.

Results: VA7 infected human malignant glioma tissue slice cultures and eradicated biopsy derived malignant glioma stem cell explants. The replication was either restricted or delayed in the cancer stem cell explant cultures after human IFNα pretreatment. VA7 had only limited and focal replication profile in healthy macaque brain tissue slice culture.

Conclusion: Here we demonstrate that SFV VA7 is capable of infecting live human malignant glioma tissue cultures and destroying human malignant glioma derived cancer stem cells. The limited replication profile in the healthy macaque brain supports the evidence of SFV safety for primates.

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Mechanisms of oncolytic measles virus-induced neutrophil-mediated cytotoxicity against tumour

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Numerous studies implicate neutrophils in eliminating tumour cells following cancer treatments, most prominently after antibody therapies. We previously showed that neu-
Replication selective oncolytic adenoviruses (Ad) are genetically engineered viruses that target cancer cells. We generated AdΔΔ (Ad5 with E1A?CR2 and E1B?19K) that exhibits cytotoxic potency in prostate cancer cells and significantly reduced replication in normal cells. Ad?? synergestically enhanced tumour cell killing in combination with chemotherapeutics (i.e. mitoxantrone and docetaxel). To further improve on efficacy, the prodrug-converting enzyme CD/UPRT was inserted into the E3B region generating AdΔΔCU. CD/UPRT converts the prodrug 5-fluorouracil (5-FU) to the cytotoxic 5-fluorouracil (5-FU) in infected cells, resulting in high local concentrations while systemic toxicity is avoided. The aim of this study was to identify cellular mechanisms involved in the enhanced cancer cell killing of AdΔΔCU and 5-FC. The AdΔΔCU virus was characterised for cell killing (MTS), replication (qPCR), infectivity (FACS) and in vitro efficacy. Combination treatments of AdΔΔCU and 5-FC greatly increased cell killing efficiency in prostate cancer cells compared with virus alone (set to 0%) (PC3: 84.5 + /-9.0%, 22Rv: 91.7 + /-0.6%). The time to tumour progression (1.44cm²) was significantly prolonged in a PC3 xenograft model in athymic mice from 27 + /-9 days (PBS control) to 41 + /-22 days (AdΔΔCU alone) and ≥97 days (AdΔΔCU + 5-FC). A potential mechanism for the improved anti-tumour efficacy is the increase in viral uptake induced by 5-FU followed by higher early viral protein expression (E1A). However, viral replication was attenuated in the presence of drug indicating a complex interaction between the virus and drug. In conclusion, AdΔΔCU is a promising mutant as a viral gene therapy treatment for prostate cancer.

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Design and application of a new HSV vector for treatment of Glioblastoma

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Oncolytic vectors (OV) are attenuated lytic viruses that depend on characteristic defects in the anti-viral response of cancer cells to preferentially replicate and spread in tumors. OV derived from herpes simplex virus type 1 (oHSV) have shown promise in the clinic, but their efficacy is limited due to poor replication in tumor cells. We sought to develop a new class of oHSV vectors which can replicate essentially as unattenuated virus in glioma cells, but are blocked for replication in normal neurons. To Achieve this goal, (i) we developed suitable methods for targeting infection of specific cell types by directing vector attachment to unique cell surface receptors; (ii) we engineered the essential viral proteins ICP4 and ICP27 which will be express only in the absence of face receptors; (iii) we engineered the viral genes to preferentially replicate and spread in tumors. Oncolytic vectors (OV) are attenuated lytic viruses that depend on characteristic defects in the anti-viral response of cancer cells to preferentially replicate and spread in tumors. OV derived from herpes simplex virus type 1 (oHSV) have shown promise in the clinic, but their efficacy is limited due to poor replication in tumor cells. We sought to develop a new class of oHSV vectors which can replicate essentially as unattenuated virus in glioma cells, but are blocked for replication in normal neurons. To Achieve this goal, (i) we developed suitable methods for targeting infection of specific cell types by directing vector attachment to unique cell surface receptors; (ii) we engineered the essential viral proteins ICP4 and ICP27 which will be express only in the absence of specific miRNAs that are down-regulated in gliomas. expression is strictly controlled by naturally occurring microRNAs (miR) that are differentially expressed in normal brain neurons, neural precursor cells (NPCs) and tumor cells. Unlike current oncolytic viruses, our vectors doesn’t have any defective genes and our preliminary data showed a dramatically improved virus replication in tumor cells without raising toxicity for normal tissue.

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Investigating a new potent and selective oncolytic adenovirus (AdΔΔ) in combination with chemotherapy

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Patients suffering from pancreatic cancer face a poor prognosis. The only treatment option currently available is Gemcitabine, but tumour resistance rapidly develops. Re-
plication-selective oncolytic adenoviruses (Ad) represent a novel anti-cancer approach with the additional advantage of improved anti-tumour efficacy when combined with chemotherapy. We previously demonstrated that the deletion in the adenovirus anti-apoptotic gene E1B19K greatly enhances Gemcitabine-induced apoptosis in pancreatic cancer cell lines and in tumour xenografts compared to the corresponding wild-type virus (Leitner et al., 2010). The E1B19K deletion was therefore included in the replication selective E1AΔCR2 mutant to generate AdΔΔ, a new oncolytic Ad. We demonstrate that AdΔΔ replicates as efficiently as wild-type virus on a panel of pancreatic cancer cells, whilst being severely compromised on normal primary cells (NHBE). BrdU incorporation confirmed that AdΔΔ has impaired S-phase induction in normal cells, explaining tumour selectivity. Importantly, AdΔΔ retains the capacity to enhance tumour cell-killing in combination with cytotoxic drugs (e.g. Gemcitabine). Specifically, we observed 2-4 fold reductions in EC50 values for AdΔΔ when combined with Gemcitabine, Irinotecan and Cisplatin, while no sensitisation was observed in NHBE cells. Ongoing studies are investigating the mechanism of action for the enhanced cell death; preliminary findings indicate that induction of aberrant mitosis is important to lead to the observed sensitisation. We suggest that the AdΔΔ virus is a promising new agent for the treatment of pancreatic cancer and potentially other forms of cancer, based on its specific replication and induction of cell death in cancer cells in combination with chemotherapy.

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Generation of a 5/3 adenovirus incorporating transcriptional and translational control elements

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Virotherapy employing conditionally replicating adenoviruses (CRAds) represents a promising tool for a wide array of neoplastic diseases. Critical for a therapeutic index is selective killing of tumor cells while avoiding killing normal cells. Herein, we introduce a novel CRAd targeting strategy for cancer, incorporating three control elements: 1) transductional, 2) transcriptional and 3) mRNA translational targeting. A modified CRAd (Ad5/3-CXC4R-UTR) incorporating a serotype 5/3 chimeric fiber, the CXC4R promoter and the 5’-untranslated region (UTR) from the Fibroblast Growth Factor-2 mRNA to regulate E1A transcription and translation, was generated. We hypothesized that this novel vector would improve the therapeutic index by selective targeting of cancer cells.

Methods: Cytotoxicity of the constructed CRAd, Ad5/3-CXC4R-UTR, was tested in several breast cancer cell lines and primary cells by MTT-assay and crystal violet analysis. E1A transcription and viral replication were assessed in cells by measuring E1A mRNA levels and E4 copy number. Expression of E1A protein was examined by immunoblot analysis.

Results: The Ad5/3-CXC4R-UTR virus demonstrated a gain of specificity from normal cell killing compared to wild-type and dual-level CRAds (two control elements). However, in breast cancer cells although the triple level virus showed similar oncolytic activity compared to wild-type, it was less oncolytic than the dual-level CRAds.

Conclusion: Our conceptually novel approach did not lead to the expected gain of oncolytic activity in breast cancer cells, suggesting that Ad5/3/cell binding may induce a change of the intracellular signal cascade influencing the translation activity of the 5’-UTR sequence. Further investigations will address this proposition.

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Genetic Retargeting of Oncolytic H-1 Farvovirus

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H-1 rat parvovirus (H-1PV) attracts high attention as an anticancer agent, because of its oncosuppressive properties while not showing obvious side-effects in humans. The viral oncotropism relies on a more efficient H-1PV replication in cancer cells but the virus can enter both normal and cancer cells. For viral oncotherapy this constitutes a problem as uptake by normal cells sequesters a significant portion of the viral dose administered away from the tumor target.

To increase the efficacy of parvovirus-based treatments and to provide additional safety against eventual side-effects on normal cells, we aim to genetically reprogram H-1PV to selectively target human tumor cells. By analogy with the closely related Minute Virus of Mice (MVM), we developed an in silico 3D model of the H-1 wt capsid. Based on this model, we identified the amino acids in H-1PV involved in cell membrane recognition and entry. In situ mutagenesis of these residues significantly reduces the binding and entry of H1 into its originally permissive cells. We further engineered the entry-deficient virus by inserting into its capsid a double cyclic RGD peptide that targets αvβ3 and αvβ5 integrins over-expressed in cancer cells and angiogenic blood vessels. While viral capsid and DNA packaging are not affected by these modifications, insertion of the RGD peptide rescues viral infectivity towards cells over-expressing αvβ3 integrins and infection with the reengineered virus efficiently kills these cells. This work demonstrates that H-1PV can be genetically re-targeted through modification of its capsid and holds strong promise for a more efficient use of this virus in cancer therapy.

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Oncolytic vaccinia virus expressing decorin as a novel anticancer agent

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We aim to enhance the anti-tumour efficacy of oncolytic vaccinia virus by expressing murine decorin from a selectively replicating Lister strain (Lister-mDCN). Decorin is a small leucine-rich proteoglycan whose protein core interacts with numerous extracellular matrix proteins, growth factors and their receptors. It is downregulated in many tumour cells, including breast, ovarian and pancreatic cancers, and has both autocrine and paracrine actions on cells to downregulate the EGFR, HER2 (ErbB2) and c-met receptor. Additionally, the interaction of decorin with collagen fibrils within the tumour microenvironment is proposed to enhance virus spread within the tumour.

Lister-mDCN and a control virus were constructed and characterised in prostate, pancreatic and ovarian cancer cell lines. Expression of decorin protein from the virus was confirmed by western blotting and ELISA. The effect of decorin on vaccinia virus replication, cell proliferation and virus cytoxicity was subsequently examined by TCID50 and MTS assay. Lister-mDCN was shown to replicate and cause cytoxicity to a similar or greater degree than both the wild-type and control viruses. Lister-mDCN was also able to transiently reduce tumour burden by >70%, and significantly reduce the rate of tumour growth thereafter, in an in vivo model of ovarian cancer. A variety of in vitro experiments suggest that the mode of cell death by Lister-mDCN is unaltered compared to infection with the control virus. Replication and cytoxicity abilities are also comparable. Current work therefore aims to elucidate the mechanisms behind the efficacy of Lister-mDCN in vivo, and its effects on the tumour microenvironment.

**Conclusion:** Our findings suggest that tumour cell infectivity in vivo and good response to systemically administered oncolytic VA7-treatment correlates to low sensitivity to vector-induced interferons in the targeted cells. Different outcome in our previous animal studies can be explained by difference in IFN-β sensitivity in the used glioma cells. Strategies to safely overcome vector immunity are needed.

**P 221**

Unveiling biological readouts of miRNA-regulated gene therapy vectors

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MicroRNAs (miRNAs) are a class of short non-coding RNA molecules that regulate gene expression by translational inhibition or degradation of target messenger RNA sequences complementary to their seed region. Since some miRNAs are expressed in a tissue-specific manner, recent gene therapy approaches have employed miRNA target (miRT) sequences to de-target transgene expression from unwanted tissues. However, there is concern that expressing excess miRT may saturate miRNA activity and offset the regulation of its natural targets. Because miRNA natural targets are mostly unknown and the regulation exerted on them is often subtle, these concerns are difficult to address without characterizing the changes induced by individual miRNAs on the cell transcriptome and proteome. To this aim, we titered up copies of lentiviral vectors overexpressing miRT sequences into cells expressing the cognate miRNA until saturation of miRNA activity and then analyzed the effects on previously validated targets and at the transcriptomic and proteomic level. To test the system we stably knocked-down miR-223 activity in U937 cell line and detected significant (p < 0.05) changes in the expression of 120 transcripts and 190 proteins. Functional annotation and network analysis revealed a consistent regulation of phagocytic activity, confirming a role for miR-223 in inflammatory response regulation. Another current study is characterizing the natural targets of miR-126, a miRNA with a promising role in stem cell gene therapy because of its selective expression in hematopoietic stem and progenitor cells. Our findings will provide relevant biological readouts of miR-126 activity to be used for validating the safety of miR-126 regulated vectors.

**P 222**

Transient cold shock enhances zinc-finger nuclease-mediated gene disruption - Rule or exception?

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Designer zinc-finger nucleases (ZFNs) are a promising tool for precise genome editing. They consist of the catalytic domain of the FokI endonuclease and an engineered DNA binding domain. Recently, Doyon et al. (Nat Methods 7, 459-60; 2010) reported that transient hypothermia robustly increases ZFN-induced gene disruption. Here, we aimed at working out the mechanism behind enhanced ZFN activity at 30°C and to elucidate whether hypothermia is a generally applicable strategy to increase ZFN-based genome editing. We employed six different ZFN pairs – Sangamo and OPEN-based ZFNs directed against the human AAVS1, CCR5, IL2RG, HOXB13 and CFTR loci as well as the EGFP gene – and compared their activities at 30°C and 37°C in vitro and in human cell lines. In vitro cleavage assays revealed no difference in ZFN activity when comparing ZFNs of both platforms at 30°C vs. 37°C, implying that neither DNA binding nor the catalytic activity is affected by the temperature shift. Incubation of human cells at 30°C induced an M phase arrest and increased ZFN protein levels in transfected cells. Although we observed increased gene disruption at the AAVS1 locus at 30°C, the higher enzyme levels did not correlate with increased ZFN activity at the other analyzed genomic loci in three human cell lines. In summary, our data suggest that although mild hypothermic conditions let to a robust increase in ZFN protein levels, enhanced gene disruption activity of ZFNs at 30°C may not be a general phenomenon but rather dependent on the cell type and the genomic target locus.

Background: A major factor for successful gene therapy is strong and enduring expression of the transgene. Commonly used promoters for expression of transgenes are the CMV-immediate early (CMV-IE), chicken β-actin, ubiquitin C or the EF1α promoter, each with their own advantages and disadvantages.

Method: Here we present the generation of novel synthetic hybrid promoter, based on the CMV-IE and the EF1α promoter. It was obtained by generating shuffled consensus sequences of both promoters, which were analyzed by promoter prediction software. One sequence, the shuffle CMV/EFlα promoter (SCE), was synthesized and tested in vitro and in vivo. The SCEP sequence features an identity of 83% with the CMV and 85% with the EF1α promoter. The sequence was CpG-free designed to circumvent inflammatory reactions towards CpG isles and promoter methylation leading to silencing.

Result: In several cell lines a 2-7 fold stronger expression of the SCE promoter compared to the EF1α was observed; when compared to CMV-IE, SCE promoter activity was almost similar. All promoters were within a CpG-free plasmid featuring a human CMV-IE enhancer and luciferase as transgene. In immunocompetent Balb/c mice we observed a ~3 fold stronger transgene expression of SCEP compared to the EF1α promoter after hydrodynamic gene delivery to the liver for a time period of 30 days.

Discussion: Hence the SCE promoter is qualified for further applications in biotechnology or gene therapy, when strong and enduring transgene expression and evasion of promoter silencing is needed.

Prevention and Reversion of Transgene Silencing in HSCs Transduced with Lentiviral Vector

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Lentivirus (LV) is an attractive tool for gene therapy because it can transduce hematopoietic stem cells (HSCs) efficiently. Unfortunately, few studies have shown that the duration of transgene expression by LV is transient. To substantiate this, we transduced HSCs with LV carrying GFP and the levels of expression were measured at different time points. As expected, GFP expression declined by day 2 post-transduction. This may due to the de novo methylation of the transgene and chromatin modifications at the transgene-integrated region. We hypothesized that 5-azacytidine (5-azaC), a DNA demethylating agent, and Trichostatin A (TSA), an inhibitor of histone deacetylase, would be able to improve the level of transgene expression. First, either 5-azaC or TSA was added to HSCs a day after transduction or on the day following GFP silencing. 5-azaC was able to prevent and reverse the silencing effect. However, TSA was unable to reverse the GFP silencing, but it prevented the GFP silencing at high concentration. Next, we investigated the effects of 5-azaC and TSA combinations on GFP expression. Both drugs were added a day after transduction or on the day following GFP silencing. Combination of both drugs prevented GFP silencing when high concentration of TSA was used but there was no effect in reversing the silenced GFP. Here we suggest that transgene silencing can be prevented and reversed by 5-aza-C, demethylation is a prerequisite for the reversion of silencing and the combination of both drugs did not have a positive synergistic effect on the reversion of silencing.

A method for protecting therapeutic cells and microenvironment containment in patients for gene and cell therapies: a clinically proven enabling cell encapsulation technology
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We have developed a cell encapsulation technology as an enabling, platform technology for the treatment of a wide variety of diseases. Cells producing therapeutic products such as enzymes, growth factors, antibodies etc are encapsulated in polymers of cellulose sulphate and implanted in the body thereby achieving long term release of the therapeutic. The cellulose sulphate is inert and biocompatible and the capsules are robust and stable. The capsule protects the cells from the immune system (even if they are from a different species) and localises them by physically confining them but is porous so that therapeutic products can be released. The capsule also protects the body from release of cells (such as stem cells) that may cause pathology if they become lodged at non target sites. Safety and efficacy has been demonstrated in clinical trials to treat pancreatic cancer, using a cell based suicide gene/prodrug strategy (Lohr et al., Lancet 357, 1591-2). Large scale GMP production of an encapsulated cell product has been achieved and approved by the German authorities (BioProcessing Journal 4, 36-43). Around 20 different cell types have been successfully encapsulated, including stem cells, hybriomas, fibroblasts, lymphoid and epithelial cells. Encapsulated cells have been successfully implanted at various sites in the body e.g. subcutaneous, intraperitoneal, intramuscular, into blood vessels, peri-tumoral etc. The technology can be used to treat diseases as diverse as cancer, cardiovascular and neurodegenerative diseases, enzyme deficiencies, diabetes and other metabolic diseases and data supporting a number of different uses of the technology will be presented.

P 226 WASp-promoter driven LVs specific expression during hematopoietic differentiation of human embryonic stem cells (hESCs)

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Human embryonic stem cells (hESCs) are useful tools for studying early human embryonic development. Using optimized conditions it is possible to promote hematopoietic differentiation that can be followed by the expression of different hematopoietic markers. The aim of the present work was to study the behaviour of WASp-promoter-driven LVs (WE and AWE) during hematopoietic differentiation of hESCs. We transduced AND-1 (hESCs line established at the Andalusian Stem Cell Bank) with WE and AWE LVs, expanded and induced to hematopoiesis differentiation by embryoid bodies (EBs) formation and with the addition of FL-3L, SCF, IL-3, IL-6, G-CSF and BMP4 for 22 days. eGFP transgene expression and endogenous wasp mRNA levels were analyzed at different time points. eGFP mRNA was only detected upon hematopoietic induction (day 3). This expression increased over time reaching plateau at days 15-22. Interestingly this expression pattern follows very closely the expression profiles of the endogenous was gene. FACS analysis demonstrated that all eGFP þ cells were also positive for CD45, CD33 and CD31, typical hematopoietic markers. Interestingly the all CD45 negative population didn’t express eGFP, indicating that both vectors were restricted to the hematopoietic cell population. This study contributes to the better knowledge of wasp expression during human hematopoietic differentiation and demonstrates the value of WE and AWE vectors backbone for hematopoiesis basic research. In addition, the demonstration that the WASp-promoter driven LVs follow endogenous WASP expression pattern in this relevant cellular model, point out their potential use as physiologically-regulated vectors for gene therapy of Wiskott-Aldrich Syndrome.

P 227 Lentinival vector mediated purification of hepatic progenitors differentiated from human embryonic stem cells

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Cell therapy is now an alternative to orthotopic liver transplantation for the treatment of life-threatening liver metabolic diseases. However this approach is restricted by the lack of donors and limited number of cells since hepatocytes cannot be expanded in vitro. Therefore new sources of stem cells need to be defined and human embryonic stem cells (hESCs) still appear as the gold standard to generate differentiated cells. We previously developed chemically defined culture conditions to differentiate hESCs into hepatic progenitors that could be amplified 6-9 times. We now define an approach to purify hepatic progenitors, using lentiviral vectors in which GFP reporter gene expression is driven by the human apolipoprotein A-II (APOA-II) promoter. Conditions for lentivirus transduction were set up using a EF1a-GFP vector on undifferentiated and differentiated cells yielding 60% transduced cells at MOI 10 and 70% at MOI 30. Cells transduced with APOA-II-GFP vector were differentiated for 16 days, and GFP þ cells, representing 30% of the population, were purified by Flow cytometry, yielding 99% GFP þ cells. CK19 and PCNA expression showed that sorted cells were proliferating progenitors. Amplification and differentiation conditions were tested after passing the cells on different matrices. After 23 days of differentiation, we found that most of the cells expressed albumin. The cells also produced high level of urea and were able to uptake and excrete indocyanin green.

Our data indicate that hepatic progenitors can be purified using hepatic specific promoters in lentiviral constructs. These cells also can be amplified and differentiated further into more mature hepatocytes.
**P 228**

**Engraftment capacity of human embryonic stem cells into bleomycin and silica lung-damaged mice**

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Respiratory diseases are the leading causes of morbidity and mortality worldwide. Until now no treatments resulted to be resolutive to cure or reverse the disease, consequently, there is a pressing need for the development of novel therapies that facilitate the regeneration of alveolar epithelium pneumocytes (ATIIcs) destroyed by acute and chronic lung diseases.

The objective of this study was to assess: (i) the capacity of human embryonic stem cells (HUES-3) to differentiate in vitro into ATIIcs; (ii) the ability of committed HUES-3 cells (named HUES-3-ATIIcs) to counteract in vivo lung damage in two pulmonary fibrosis disease models, obtained by Bleomycin or Silica inhalation in mice.

HUES-3-ATIIcs cells developed in vitro an alveolar phenotype, as evidenced by the presence of multilamellar body formation and by the expression of SP-B, SP-C, and CFTR markers.

Transepithelial conductance of CFTR channel was also measured, demonstrating the formation of a multilayered epithelial structure in which CFTR is functionally expressed.

After injection of HUES-3-ATIIcs into both Silica and Bleomycin-damaged mice, microscopic and biomolecular analyses revealed a significant recovery of inflammation process and fibrotic damage. Moreover, a normal blood arterial oxygen saturation and weight recovery confirmed these processes and fibrotic damage. Moreover, a normal blood arterial oxygen saturation and weight recovery confirmed these processes.

Molecular analysis further confirmed the presence of human DNA within murine lung.

In conclusion HUES-3 cells have to be considered a promising way to recover lung functionality by reducing inflammatory and fibrotic changes, through a cell therapy approach.

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**P 229**

**Non-Ablative Allogeneic Neonatal Transplantation and Correction of β-Thalassemia following In Vivo Positive/Negative Selection**

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Sibling or matched, unrelated allogeneic hematopoietic stem cell transplants remain the only curative therapy for many hereditary disorders. However, the toxicity of preparative regimens, risks of graft vs. host disease (GVHD), infectious complications of immunosuppression, and limited donor availability, restrict application of this approach. The feasibility of allogeneic transplantation, without myeloablative or post-transplant immunosuppression, was tested using *in vivo* chemoselection of allogeneic hematopoietic stem cells (HSC) after transduction with a novel tricistronic lentiviral vector (MAGIT). This vector contains P140K-O2-methylguanine-methyltransferase (MGMT<sup>P140K</sup>), HSV-thymidine kinase (TK<supHSV</sup>), and eGFP enabling *in vivo* chemoselection of HSC by conferring resistance to benzylguanine (BG), an inhibitor of endogenous MGMT, and to chloroethylating agents such as BCNU and, b) depletion of proliferating cells such as malignant clones or donor T cells mediating GVHD, by expression of the suicide gene TK<supHSV</sup> and Ganciclovir administration.

Non-myeloablative transplantation of transduced, syngeneic, lineage depleted (Lin<sup>−</sup>) BM in neonates resulted in 0.7% GFP<sup+</sup> mononuclear cells in peripheral blood. BG/BCNU, administered 4 and 8 weeks post-transplant, produced 50-fold donor cell enrichment. Transplantation and chemoselection of HSC mismatched MAGIT-transduced Lin<sup>−</sup> BM produced similar expansion for >40 weeks. The efficacy of this transplantation approach was then successfully demonstrated in Hbb<sup>−/−</sup> heterozygous mice by correction of beta-thalassemia intermedia, without toxicity or GVHD. Negative selection, by administration of Ganciclovir resulted in donor cell depletion. Re-expansion of donor cells could be achieved with additional BC/BCNU treatment. These studies show promise for developing non-ablative transplant approaches using *in vivo* positive/negative selection.

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**P 230**

**Tet-regulated, lentivirally mediated BMP-2 expression in mesenchymal stem cells**

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Mesenchymal stem cells (MSC) have gained considerable attention in view of their potential use in regenerative medicine and tissue engineering. The combination with gene therapy allows to provide MSC with new traits for enhanced/improved tissue repair. Bone marrow derived MSCs are able to differentiate into different cell types among them osteocytes and chondrocytes and are therefore considered for the treatment of osteochondral defects. Regulated gene expression of growth factor bone morphogenetic protein 2 (BMP-2) or WNT1-inducible-signaling pathway protein 3 (WISP3), a CCN-protein known to be involved in development, homeostasis and repair of mesenchymal tissues was achieved using the Tet-on system, delivered by VSV-G pseudotyped lentiviral SIN vectors (LV). Cells were infected...
with Tet-on constructs containing the BMP-2 or WISP-3 cDNA. The expression was induced by doxycycline (dox).

Transduction efficiency of rabbit MSCs by eGFP-expressing LV was 65%. Transduction of MSC with the BMP-2 Tet-on vector resulted in inducible BMP-2 expression with a 36-fold increase of BMP-2 expression on RNA level as well as a 113-fold increase on protein level compared to uninduced cells. BMP-2 induction level was 2-fold higher than in primary rabbit chondrocytes. After lentiviral infection cells retained differentiation potential with enhanced chondrogenesis after induction of BMP-2 expression. WISP-3 regulation was demonstrated by RT-PCR.

The lentivirally delivered Tet-on system allows for regulated expression of BMP-2 and WISP3 after induction with doxycycline in mesenchymal stem cells. Induction and switching-off of gene expression on demand will constitute new and exciting therapeutic option for cartilage defects.

P 231
VEGF-expressing MSC for rapid vascularization of tissue-engineered bone grafts
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Rapid vascularisation of tissue-engineered grafts is a major obstacle in the development of regenerative medicine approaches. Vascular Endothelial Growth Factor (VEGF) is a powerful angiogenic factor. However its dose must be controlled in the microenvironment around each producing cell to avoid toxic effects. To achieve controlled expression in vivo, we developed a high-throughput method to rapidly purify cytokine-secreting cells, which are rich in mesenchymal stem cells. Induction and switching-off of gene expression on demand will constitute new and exciting therapeutic option for cartilage defects.

MSC compared with control cells. Bone formation by the different populations is currently being determined.

Optimized transduction allowed the genetic modification of primary MSC with minimal manipulation and no loss of biological potential, leading to improved in vivo vascularisation.

P 232
Lentiviral transduction of MPS1 bone marrow cells results in high production of alpha-L-iduronidase
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Mucopolysaccharidosis type I (MPS1, Hurler syndrome) is caused by a deficiency of the enzyme alpha-L-iduronidase (IDUA) and is characterized by glycosaminoglycan (GAG) accumulation causing multi-organ failure. Aim of this study is to obtain complete phenotype correction of disease in MPS1 mice, including central nervous system and skeletal defects, by overexpression of IDUA in hematopoietic cells.

Lentiviral vectors with the spleen focus forming virus- (SF) and phosphoglycerate kinase- (PGK) promoters driving codon optimized human IDUA cDNA (IDUAco) expression were constructed. These vectors were used to transduce male lineage-negative MPS1 bone marrow cells, which were subsequently transplanted in 3-week-old 6 Gy irradiated MPS1 female mice. Blood and urine samples were collected monthly. Long-term stable, on average 80% male cell chimerism was observed in peripheral blood containing 30% IDUA positive cells up to 7 months after transplantation. IDUA levels in blood were 500- and 80-fold that of normal wild type levels for the SF-IDUAco and PGK-IDUAco, respectively. Analysis of GAG secretion in urine revealed normalization to wild type levels for all transplantation groups. At month 7, the mice were sacrificed and assessment of GAG levels in spleen, liver, lung, heart, and kidney demonstrated significant reduction of GAGs to normal levels. Most importantly, GAG accumulation was significantly decreased in brain as well. Skeletal characteristics are currently studied by CT analysis.

We conclude that the IDUAco vectors can provide long-term high expression and concomitant reduction in GAG secretion in urine, and other organs, including brain. This warrants further development towards clinical application.

P 233
Tracking Mesenchymal Stem Cell Homing to the Infarcted Heart after Intracoronary, Intravenous and Intramyocardial Delivery
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Acute myocardial infarction with subsequent ventricular remodeling is the leading cause of congestive heart failure and death in developed countries. Mesenchymal stem cell therapy is being investigated to promote regeneration of infarcted myocardium. Many aspects of the delivery protocol will critically influence the outcome, including transplantation route, dose and time of delivery post-infarct. The optimal method of stem cell delivery is currently unknown. The objective of this study was to radiolabel MSCs with $^{111}$In-oxine and assess myocardial homing of $^{111}$In-MSCs administered via three delivery routes in rats: intravenous (IV), intramyocardially (IM), and intracoronary (IC).

Myocardial infarction was induced in Fischer 344 rats by ligating the LAD artery for 45 minutes. $^{111}$In-MSCs were administered via IV, IC, and IM. Using FastSPECT II, scintigraphic images were acquired from 1 hour up to 7 days after $^{111}$In-MSC injection. Dual-isotope imaging of $^{111}$In-MSC/$^{99m}$Tc-sestamibi was performed 24-48 hours after MSC transplantation to assess location of transplanted cells within the myocardium.

IV delivery resulted in high lung uptake initially and at 24 hours the lung activity had shifted toward other organs. $^{111}$In-radioactivity remained locally detectable in infarcted hearts up to 6-7 days after IV, IMC and IC delivery. The radioactivity detected in the hearts that received $^{111}$In-MSCs via IM and IC delivery was significantly higher than that after IV delivery. The activity in the hearts after IC injection was more widely distributed in the ischemic areas. In conclusion, IC delivery of MSCs may offer advantages over IM or IV in terms of cell-homing and engraftment.

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The population of C-kit positive cells in the heart contains mast cells and cardiac progenitors

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Stem cell therapy is a new promising tool to regenerate damaged myocardium. Human heart contains stem cells showing cardiac regeneration potential in vitro. The aim of the present study is to characterize cardiac stem cells in the human heart tissue.

Methods: Tissue samples of appendix of the right atrium were harvested during coronary artery bypass grafting and autopsy samples of left ventricular tissue were analyzed using fluorescence-activated cell sorting and immunohistochemical staining for the presence of C-kit-positive cells, hematopoietic cells (CD34, CD45), blood lineage (Lin) and tryptase. C-kit cells were harvested using explant culture and anti-C-kit antibody. Co-culture of isolated c-kit cells with neonatal rat cardiomyocytes was used to prove their cardiomyogenic potential.

Results: C-kit + cells represented 0.79 ± 0.32% of the total cell population of appendix and were CD34- and Lin-. Two populations of C-kit cells were identified: 60% of C-kit cells were CD45+, assuming these cells were of bone marrow origin and about 40% of C-kit cells were CD45-. Immunohistochemical staining of autopsy samples of left ventricular tissue showed that majority of C-kit cells are positive for CD45 and tryptase, suggested that they are mast cells. Only a small population of C-kit (+) CD45(-) triptase(-) cells represented human cardiac stem cells.

Using magnetic cell sorting c-kit positive cells could be successfully isolated from human heart tissue and expanded in vitro. C-kit cells undergo cardiomyogenic differentiation when co-cultured with neonatal rat cardiomyocytes.

Conclusions:Appendix of the right atrium could be an alternative source of autologous cardiac stem cells.
Adipose-derived Mesenchymal Stem Cells Enhance Tissue Regeneration by Inducing Growth of Blood Vessels

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Transplantation of adipose-derived stem cells (ASCs) induces tissue regeneration by accelerating the growth of blood vessels and nerve sprouts. Previously we demonstrated that ASCs stimulate blood vessel growth by producing angiogenic growth factors as well as enhancing vessel maturation. However, mechanisms of their action on the growth of nerve fibers are only partially understood. Here we show that transplantation of ASCs stimulates a repair of motor and sensory nerves as well as induces nerve sprouts growth in matrigel implants. Transcriptional analysis has revealed that ASCs express mRNAs for growth factors and extracellular matrix proteins specific for the outgrowth of nerve sprouts and myelination. Exposure of ASCs to a combination of retinoic acid with 5-azacytidine up-regulates BDNF production in these cells as well as their ability to induce nerve fiber growth in matrigel implants. BDNF neutralizing antibodies have abrogated stimulatory effect of ASCs on the growth of nerve sprouts. These data suggest that ASCs induce nerve repair and growth via BDNF production and this stimulatory effect can be further enhanced by cell exposure to neural differentiation medium prior to transplantation.

Development of a meniscal substitute by combining an inert scaffold and mesenchymal stromal cells

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The objective of this Program is to develop a meniscal substitute by combining an inert scaffold and mesenchymal stromal cells (MSCs).

Methodology: The colonisation process of an inert scaffold is currently evaluated. To achieve this goal, various seeding options were tested to colonise the scaffolds with MSCs. The phenotype status of the cells was also considered to define if a differentiation of the MSCs occurs depending on the culture conditions. Immunohistochemistry and flow cytometry were used to characterise the cells. A scanning electron microscopy study was performed to observe cellular distribution inside the scaffold. In parallel, a transfection protocol of the MSCs was initiated to define the impact of the transgene on MSCs differentiation.

Results: Cell proliferation inside and on the surface of the matrix appears to be related to the culture media. Platelet lysate allows a better proliferation than fetal bovine serum can do. Some expansion factors and some molecules involved in differentiation process (TGF B3), also have a positive effect on the scaffold’s colonisation. Depending on culture conditions, an initiation of differentiation was observed, in particular with a collagen II expression.

Conclusion: These promising results are the first steps of the development of a cellularised meniscal substitute. The long term behaviour of the MSCs in the scaffold remains to be studied. Mechanical and physical resistances of the cellularised scaffold have also to be studied. This would allows to conclude on the improvement of the performance of the scaffold currently commercialised and implanted without cells.

Circulating endothelial progenitor cells (EPCs) in metabolic syndrome therapy

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Background: Circulating endothelial progenitor cells (EPC) are derived from more immature, undifferentiated circulating stem cells, being involved in repairing of the damaged endothelium. The aim of this study was to quantitate the circulating stem cells (CD34+/VEGFR2+) and EPC (CD34+/VEGFR2+), in two groups of metabolic syndrome patients (MetSyn), with and without specific therapy (statins), in order to evaluate the therapy efficiency.

Methods: The CD34+ cells and CD34+/VEGFR2+ were quantified in peripheral blood samples from 20 patients with MetSyn, divided in two groups: patients newly diagnosed with no therapy (n = 10, age 42 ± 10) and patients with at least 3 month of statins therapy (n = 10, age 47 ± 6). 10 mL venous blood sample was used for the isolation of mononuclear cells in Ficoll density gradient. CD34+ enriched populations were obtained from peripheral blood mononuclear cells by immunomagnetic techniques (Miltenyi Biotec GMBH). The isolated subpopulations of cells were labeled with antibodies: anti CD34-FITC human and anti VEGFR2/KDR-PE human according with the manufacturer's recommendations and analyzed by flow cytometry.

Results: The study has shown that the number of both CD34+ and CD34+/VEGFR2+ cells in the group of metabolic syndrome patients following statins therapy was higher comparative with untreated MetSyn patients: 31.73% increase for CD34+ cells (1283 ± 1232 vs. 786 ± 570) and 54.05% increase for CD34+/VEGFR2+ cells (407 ± 357 vs. 187 ± 159).

Conclusion: These preliminary results have shown that the statin therapy administration have improved endothelial repair capacity by increasing the number of circulating stem cells CD34+, the increase being more accentuated for EPC CD34+/VEGFR2+ cells.
**P 239**

**Differentiation and Characterization of Cardiomyocytes from Rat BM-MSCs For Myocardial Regeneration**

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**Background:** Cell transplantation which aims to introduce healthy myogenic cells into the myocardium of the diseased heart, is showing a bright future in myocardial regeneration therapy. The differentiation potential of bone marrow mesenchymal stem cells (BM-MSCs) can be key role for myocardial regeneration. (1). 5-Azacytidine (5-Aza) is a cytosine analogue and demethylating agent. Recent studies showed that 5-Azacytidine can be used as a differentiation factor for cardiomyocyte differentiation. The present study investigated the replication lifespan and chemical-induced cardiomyogenic differentiation of rat MSCs in vitro.

**Method:** BM-MSCs isolated from rats and characterized with immunofluorescence staining method for specific cell surface proteins CD13 and CD29. BM-MSCs at P2 and P3 were selected for cardiomyocyte differentiation. BM-MSCs were exposed to different concentrations of 5-Aza for 24 hours, untreated cells used as the control group. Differentiated cells were incubated for 4 weeks and stained with cardiomyocyte specific markers desmin and cardiac troponin-T by using TR and FITC fluorochroms and cardiac troponin specific markers were detected by using TR and FITC fluorochroms and cardiac troponin-I levels was measured with ELISA method for the cardiomyocyte characterization.

**Results:** BM-MSCs at different passages (P2 and P3) can differentiate into cardiomyocytes with 5-Aza treatment. Differentiated cells started to form cell colonies and especially these colonies stained with desmin and cardiac troponin-T. In respect of ELISA method results 3 μM and 5 μM 5-Aza dilution displayed the highest amount of cardiac troponin-I.

**Conclusion** Differentiated mesenchymal stem cells are considered to have significant applications in cellular approaches for regeneration of heart muscle losses.

**Reference**


**P 240**

**The EBMT Registry: A possible model for Cellular Gene Therapy**

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The European Group for Blood and Marrow Transplantation (EBMT) is a non-profit organisation established in 1974 in order to allow scientists and physicians involved in clinical bone marrow transplantation to share their experience and develop co-operative studies. From the 70’s onwards, clinical data started being collected into what is now known as the EBMT Registry.

When the EBMT started collecting data, transplantation was not a main stream procedure. Today it is for some indications and we believe that the continuous collection of Registry data has widened the experience and improved the outcome of transplanted patients. The Registry currently contains clinical data on more than 315,000 patients, submitted from more than 50 countries.

There is a growing desire on the part of donor registries, health departments, cord blood banks, study groups and other outcome registries to be able to access the content of the EBMT Registry and we developing closer collaborations.

The EBMT overlaps with the community involved in cellular therapies. A Cellular Therapy Med-A (“minimum essential data”) was designed by the Cellular Therapy committee and has been in use for the last 2 years.

We believe that the EBMT could offer its expertise in data collection and part of the Registry infrastructure to kick start a similar data collection exercise in the Cellular Gene Therapy community. We are looking to further enhance our data collection system, to better serve our centres, improve collaboration with existing partners and create new partnerships. An invitation to do just that is offered to the Cellular Gene Therapy community.

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**Genome Wide Bioinformatical Analysis of Vector Integration Sites**

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To address the biosafety and to uncover vector induced side effects of gene transfer approaches, genome wide integration site profiles of the different vector types used in preclinical and clinical studies need to be determined. Combining standard or non-restrictive (nr) linear amplification-mediated (LAM) PCR and next generation sequencing (454/Roche) provides a time- and cost-efficiency tool for integration site retrieval. Our analysis tools, Quickmap (www.gtsg.org) and HISAP (Paruzynski A et al. Nature Protocols 2010), use the sequencing data output to identify relevant integration sites characterized by the chromosomal locus and further genomic information such as distance and orientation to the next genes or retrieval frequency of individual integration sites.

Besides providing an automated, standardized and easy to handle output for non-bioinformaticans, integration site analysis software needs to scope with a vast amount of different vector types (e.g. high frequent integrating onc-retro and lentiviral vectors, low-frequent integrating integrase deficient lentiviral and AAV vectors as well as transposons such as sleeping beauties) used in various areas of gene transfer approaches. This requires a hitherto unknown flexibility of integration site analysis programs and relevant downstream programs to answer the specific scientific questions beyond.

Here, we will present some aspects of our ‘downstream’
Highly efficient lentiviral ex vivo gene transfer into primary liver cells

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Hepatocellular carcinoma (HCC) is the fifth common cancer and characterised by a very poor prognosis. Additionally for many metabolic liver diseases there is no curative therapy option other than liver transplantation. Efficient ex-vivo gene transfer into isolated hepatocytes facilitates both the investigation of HCC mechanisms as well as the development of potential gene therapy approaches for metabolic diseases. We used the novel LeGO vector platform developed in our lab to establish highly efficient short-term protocols for lentiviral transduction of cultivated and primary hepatocytes. For transplantation experiments, we made use of the uPA-SCID mouse model. We first transduced different liver cell lines, in particular human telomerase-immortalised foetal liver cells (FH-HTERT). We found high transduction rates and stable transgene expression in all cells tested indicating strong activity of the used SFFV promoter in hepatocytes. After transfection of transduced FH-HTERT cells in uPA-SCID mice we observed stable transgene expression for at least six months in vivo. We went on with the transduction of primary hepatocytes. In order to preserve vitality and prevent dedifferentiation a 1-hour ex-vivo transduction protocol was established. Using this protocol we obtained transduction rates of >60% for primary murine hepatocytes. Transduced murine hepatocytes engrafted at remarkable rates after in-transplenic transplantation in hemizygote uPA-SCID mouse. The new protocol allows high-efficiency transduction while preserving the phenotype and engraftment potential of primary hepatocytes. It thus will be a very useful tool for investigations of various genes in HCC genesis and the development of ex-vivo gene therapy approaches.

Characterization of mRNA splicing perturbation in genes targeted by lentivector integration

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Oncogenesis induced by insertional mutagenesis with gene therapy vectors occurs mainly by deregulation of proto-oncogenes found at or nearby the insertion site. Proto-oncogene activation occurs by an enhancer-mediated mechanism or by a process of splicing capture which generates chimeric transcripts comprising portions of vector and cellular mRNAs. Although the activation of oncogenes may be reduced by the use of self-inactivating design and moderate cellular promoters, how to reduce genotoxic splicing capture events and aberrant transcript formation triggered by vector integration is still unclear. In this perspective, we developed a new PCR technique named Linear Amplification-Mediated PCR on cDNA (cLAM-PCR) which allows retrieving aberrantly spliced mRNAs that contain LV sequences fused with cellular transcripts.

We applied cLAM-PCR on lentiviral vector (LV)-transduced cell lines and primary human HSCs and identified several established and previously unknown splice sites within the LV backbone that participate in the aberrant splicing process with variable efficiency. Preliminary results with different LV designs show that the integrated LV can perturb the processing of cellular transcripts by interacting with the cellular splicing machinery and fusing with its own splice sites to cellular splice sites both upstream and downstream the integration site. Moreover, qPCR on different LV portions allowed us identifying different splice sites as major or minor contributors to the aberrant splicing process. This strategy will allow characterizing the mechanism and genetic features that modulate vector-induced aberrant splicing. In a biosafety perspective, elimination of splice sites within the lentiviral backbone will be instrumental in enhancing the safety of LVs.

LV integration in human genes generates abnormal transcripts through the usage of HIV splice sites

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HIV-derived, self-inactivating (SIN) lentiviral vectors (LVs), depleted of LTR enhancer and promoter regions, provide an efficient and versatile gene delivery system that overcomes most of the risks of insertional gene activation observed with wild-type LTR, MLV-derived retroviral vectors. Nevertheless, since LVs integrate preferentially into active genes, they have the potential to de-regulate gene expression at the post-transcriptional level, by interfering with the normal splicing and polyadenylation of primary transcripts. To test this hypothesis, human T cells were transduced with a specifically designed, “splice trap” LV, and cloned for the expression of a promoter-less GFP gene placed downstream of the constitutive HIV GAG splice acceptor site. In parallel, T-cells and keratinocytes were transduced with a conventional SIN-LV and randomly cloned. Integration sites were mapped by LM-PCR in 70 T-cell and 31 keratinocyte individual clones, and chimeric
transcripts identified by RACE PCR and exon-specific RT-PCR. Abnormal, chimeric transcripts were identified in more than 50% of the LV target genes in both cell types. Semi-quantitative RT-PCR revealed that fusion transcripts were represented at low to high levels compared to constitutively spliced, wild-type transcripts. Fusion transcripts were generated through aberrant splicing caused by the usage of both constitutive and cryptic splice sites located in the viral intron and the U5 portion of the 5′ LTR. These data point out a potential risk of post-transcriptional genotoxicity of LVs in human cells.

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**Systemic injection in a tumor prone mouse unravels the residual genotoxicity of self-inactivating lentiviral vectors**

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Our previously validated Cdkn2a⁻/⁻ lineage- transduction/transplantation mouse model revealed that a lentiviral vector (LV) containing the spleen focus-forming virus (SF) enhancer/promoter sequences within the LTRs (LV.SF.LTR) was genotoxic with respect to mock-controls. Intriguingly, a self-inactivating (SIN)LTRs LV containing the SF sequences in internal position (SIN.LV.SF) did not cause any tumor acceleration. The lower genotoxicity of this design coupled to the background oncogenesis of this mouse model could hamper the detection of residual vector genotoxicity. Thus, alternative in vivo assays to stringently test the safety of SINLVs are still an unmet need. Here, we report that systemic injection in newborn Cdkn2a⁻/⁻ mice of LV.SF.LTR induces an earlier hematopoietic tumor onset with respect to mock-controls or mice transplanted with lineage- Cdkn2a⁻/⁻ cells transduced with LV.SF.LTR. From these tumors we identified 18 Common Insertion Site (CIS), among which Braf (68 integrations in a genomic region of 4 kb), Sfi1 and Met2c were already identified as CIS by retroviruses or transposon integrations studies. These data indicate that insertional mutagenesis is the mechanism responsible for the accelerated tumor onset. Remarkably, when we used this new system to reassess the putative genotoxicity of SIN.LV.SF, newborn Cdkn2a⁻/⁻ mice systemically injected with this vector developed tumors at a significantly earlier onset with respect to untreated mice. Our data show for the first time that by this approach it is possible to detect the residual genotoxicity of a SINLV containing an enhancer in internal position and establish a new in vivo genotoxicity assay to test improved versions of SINLVs.

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**Directed integration of insulated lentiviral vectors to the heterochromatin towards safer gene transfer to stem cells**

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We have investigated the improvement of integrating vectors safety in combining (i) new short synthetic genetic elements with (ii) longer ones using self-inactivating SIN.LV vectors. The SIN.LV.SF.LTR vectors are composed of an insulator element positioned in the LTRs. We previously showed that these vectors were less genotoxic than the SIN.LV.LTR vectors and that the insulator element contributed to reduce the genotoxicity. Here, we show that the introduction of an enhancer in the internal position of the SIN.LV.SF.LTR vector further reduces the genotoxicity to levels comparable to SIN.LV.SF vector. We used these vectors in a liver gene transfer to establish whether the use of an insulator element can improve the safety of lentiviral vectors used to transfer genes to stem cells and to explore the mechanism of insertion of these vectors into the human genome.
insulator elements (GIE) and (ii) directing integration to heterochromatin. We have previously identify a specific GIE combination in collaboration with Nic Mermod, which translates into high titers and boundary effect in both gammaretroviral and lentivectors (DCaro4). In target cells, the expression profile becomes homogenous; its level is strictly conditioned by the promoter. These data remain stable in both HeLa cells and cord blood HSCs for over three months, irrespective of the multiplicity of infection (MOI). Since GIEs are believed to shield the transgenic cassette from inhibitory effects and silencing, DCaro4 has been further tested and compared to its non-insulated counterpart, with chimeric HIV-1 derived integrases targeting heterochromatin through either histone H3 or methylated CpG islands (ML6 & ML10 chimeras, respectively). With DCaro4 and ML6 chimeras, a homogeneous expression is sustained over time. With the control, GFP expression is just over background double-mutant in catalytic and ledgf binding-sites while expression can be induced with HDAC. In CD34+ cells from cord-blood, these data are recapitulated with the ML6 chimera. High throughput integration sites analysis reveals a distinct profile with DCaro4-ML6. Our approach could significantly reduce integration into open chromatin sensitive sites in stem cells at the time of transduction, a feature which might significantly decrease subsequent genotoxicity.

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LEDGF/p75 fusion proteins to retarget lentiviral integration away from genes

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Correction of genetic diseases requires integration of the therapeutic gene copy into the genome of patient cells. Retroviruses are commonly used as delivery vehicles because of their precise integration mechanism, but their use has led to adverse events in which vector integration activated proto-oncogenes and contributed to leukemogenesis. Here, we show that integration by lentiviral vectors can be targeted away from genes using an artificial tethering factor. During normal lentivirus infection, the host cell-encoded transcriptional coactivator lens epithelium-derived growth factor/p75 (LEDGF/p75) binds lentiviral integrase, thereby targeting integration to active transcription units and increasing the efficiency of infection. We replaced the LEDGF/p75 chromatin interaction-binding domain with CBX1. CBX1 binds histone H3 di- or trimethylated on K9, which is associated with pericentric heterochromatin and intergenic regions. The chimeric protein supported efficient transduction of lentiviral vectors and directed the integration of lentiviral vectors outside of genes, near CBX1 binding sites, regions generally disfavored for integration in wild-type cells. Despite integration in regions rich in epigenetic marks associated with gene silencing, lentiviral vector expression remained efficient. Thus, these results establish LEDGF/p75 as the dominant targeting factor for lentiviral integration and demonstrate that engineered LEDGF/p75 fusion proteins provide technology for controlling integration site selection.

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Lentiviral vector induced leukemia in a murine bone marrow transplantation (BMT) model

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The induction of leukemias by insertional mutagenesis has been observed in mouse models and clinical trials when using gammaretroviral vectors with strong viral enhancer/promoters. Lentiviral insertional leukemias have only been described in tumor-prone mouse models. With the aim of evaluating lineage-specific expression, we transplanted C57Bl/6 mice with BM cells transduced with lentiviral vectors expressing EGFP from the human GPIba promoter. Seven months after BMT one mouse succumbed to leukemia with leukocytosis and hepatosplenomegaly. Leukemic cells were donor derived, EGFP + with a surface marker phenotype of early B cell progenitors (B220 + CD43 + IgM +). Southern blot analysis confirmed a clonal leukemia with two vector insertions one of which was in the 8th intron of the Early B cell factor 1 (Ebf1) in forward orientation. Splice sites in the GPIba promoter were involved in splice products from the Ebf exon 8 leading to an early transcriptional termination (5-8% of the allele’s RNA). Furthermore RNAs were detected that spliced from the vector into exon 9 and 10. B-cell differentiation was enhanced by overexpressed full length Ebf1 and unaltered by the truncated form. No chromosomal aberrations were detected by SKY. A further mouse developed granulocytic proliferation 5 weeks after BMT with cells transduced with a lentiviral SIN vector driving Ebf1 from the SFFV promoter. A single intronic insertion in the Pdgf C gene upregulated expression ~100x. Our observations represent rare events detected in <200 transplanted mice but demonstrate the potential of lentiviral vectors to insertionally regulate gene expression with significant functional consequences.
Alpha sarcoglycan is required for FGF dependent myogenic progenitor cell proliferation in vitro and in vivo

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Background: Mice null for α-sarcoglycan (Sgca-null) develop a progressive myopathy and serve as a model for human limb girdle muscular dystrophy 2D. In mice the myopathy is very severe; α-sarcoglycan expression can be restored by adenovirus-mediated gene delivery and the dystrophic phenotype has been rescued by cell therapy as shown by previous studies.

Methods: In an attempt to characterize the cellular basis of the disease, we cloned myogenic progenitor cells (mpc), the resident post-natal muscle progenitors from dystrophic and wt mice analyzing their proliferative potency and differentiation ability. We further examined the ability of donor mpcs to proliferate and properly colonize muscle in vivo.

Results: Mpc cells from Sgca-null mice gave rise to smaller clones than wt or mdx dystrophic mice. The proliferation impairment of Sgca-null myogenic precursors was confirmed by single fiber analysis. This difference correlates with the expression of Sgca during the proliferation of mpc: in the absence of dystrophin and associated proteins, which are only expressed after differentiation, α-SG forms a complex with and stabilizes the FGF receptor. Ablation of the gene leads to FGF receptor loss from the membrane and impaired proliferation of mpc. The low proliferation rate of Sgca-null mpc was not affected by basic FGF administration but rescued by transduction with Sgca-expressing lentiviral vectors. When transplanted in dystrophic muscle, Sgca-null mpc exhibited reduced homing and proliferation.

Conclusion: The reduced proliferation ability of mpcs explains the severity of this muscular dystrophy and why donor progenitor cells engrafted efficiently and consequently ameliorated the disease.

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Hyperactive glycoprotein B mutations augment fully retargeted HSV infection

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Entry of HSV-1 is initiated by the binding of glycoprotein D (gD) to one of its receptors, herpesvirus entry mediator or nectin-1. This interaction causes a conformational change in gD that induces activation of the fusion effectors gB and/or gH required for virus penetration. To retarget virus entry exclusively to epidermal growth factor receptor (EGFR)-bearing cells, we mutated or deleted gD residues essential for binding to the natural receptors and inserted EGF or an EGF-specific single-chain antibody (scFv) near the amino terminus. Although ablation of the natural tropism was achieved, entry through EGFR was inefficient. In an attempt to isolate gain-of-function mutations that could generally stimulate entry, we passaged a nectin-1-ablated gD-mutant virus repeatedly through cells harboring a gD-binding-impaired nectin-1 until plaques appeared. Surprisingly, each of the purified isolates contained the same double mutation in gB, D285N/A549T. The gB mutations in a wild-type virus background enabled entry into cells that are normally resistant to HSV infection due to the absence of authentic gD receptors. The gB-mutant virus was also capable of entering cells through nectins other than nectin-1, and entry into nectin-1-bearing cells was markedly accelerated compared to wild-type virus. We combined the gB mutations with the EGFR-retargeted gD alleles and found that these viruses entered EGFR-transduced cells 100-fold more efficiently than the wild-type-gB versions, without detectably raising off-target entry. These viruses entered various tumor lines expressing EGFR with similar efficiencies as wild-type virus utilizing the natural receptors, and the retargeted infection was completely blocked by an anti-EGFR antibody. We also inserted a carcinoembryonic antigen (CEA)-specific scFv into detargeted gD and observed efficient CEA-dependent entry, supporting the broader applicability of our retargeting system. Our observations demonstrate that hyperactivity of a downstream component of the entry cascade can greatly enhance the infectivity of retargeted HSV vectors.

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Splice switching in hematopoietic cells — a novel strategy to correct BTK deficiency derived from aberrant splicing

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X-linked agammaglobulinemia (XLA) is a primary immunodeficiency disease, where the gene, Bruton's tyrosine kinase (BTK), is mutated. XLA-patients suffer from recurrent infections due to a blockage of B-cell development resulting in almost absent antibody production. Current treatment options are consisting of life-long periodic administrations of immunoglobulines intravenously and antibiotics. This option...
is only partially effective, expensive and can lead to long-term complications. We have previously found a new mutation in intron 4 of the BTK gene responsible for an aberrant splicing pattern in a Swedish family with XLA subjects. The mutation, which causes a GAA\textsuperscript{GT} change, forms a novel 5’ cryptic splice site resulting in the creation of a pseudo-exon of 118 bp.

Splice switching by the use of antisense oligonucleotides (ONs) is a novel therapeutic method with great potential for several types of genetic disorders. Clinical trials are currently being conducted in Duchenne’s muscular dystrophy (DMD) with the aim of converting DMD to the milder Becker dystrophy. For XLA-patients, who incorporate BTK pseudo-exons, it is in theory possible to generate normal transcripts by treatment with antisense ONs blocking the aberrant cryptic splice site. We initially scanned a set of 2’-O-methyl-based splice switching ONs (SSONs) for their capacity to redirect the cryptic splice in intron 4 of BTK using different reporter constructs. A set of highly active SSONs was identified. These SSONs have been further optimized, in both length and composition, using substitution with Locked nucleic acid (LNA) bases. In order to develop a relevant model for experimental treatment, we have generated two splice switching ONs (SSONs) for their capacity to re-direct the cryptic splice site resulting in the creation of a pseudo-exon of 118 bp.

In Alzheimer’s disease (AD), neurofibrillary degeneration (NFD) is a brain lesion characterized by intraneuronal aggregation of Tau. It is also encountered in a number of neurodegenerative disorders referred to as Tauopathies. The available transgenic mouse models take advantage of mutations on tau gene found in rare forms of fronto-temporal dementia. However, in AD, no Tau mutation is found and NFD develops through specific cortico-cortical pathways, starting in the hippocampal formation, which is not reproduced in transgenic animal models. Tau spreading process remains unknown probably because the mechanism of Tau pathology differs between AD and Tau mutation cases.

**Methods:** Targeting brain regions usually affected by NFD in AD requires the induction of localized expression of Tau. Lentiviral vectors (LV) are suitable tools to deliver gene in a specific region as they can be easily injected into the brain to mediate expression in neurons. In the present work, we developed a simple and rapid system to construct any exon-trapping-type vectors, the targeting efficiency has jumped to more than 50%. We are currently trying to extend this technology to other human cell lines. We have also developed a simple and rapid system to construct any exon-trapping-type vectors within one week, without the need for ligation reactions or restriction mapping. Although such vectors may only be applicable to human genes with relatively high levels of expression in the cell, we anticipate that our technology will eventually make it possible to disrupt (or correct) a gene of interest with nearly 100% efficiency.
developed a new non-transgenic rat model after intracranial injections of LV encoding a wild-type (WT) or a mutated form of Tau into the hippocampal formation.

**Results:** The two constructs mediated an age-dependent NFD which is more pronounced with the mutated form and lead to altered neurochemical profile and subsequent cognitive impairments as soon as 8 months LV post-injection. Moreover, for both constructs, cortico-cortical Tau projections were observed but with different pathways suggesting that the spreading process are different. These results emphasized that development of models based on WT form of Tau is now crucial to understand its ‘prion-like’ propagation to the brain.

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**Hybrid superparamagnetic iron oxide-branched polyethylenimine magnetoplexes for gene delivery**

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The work demonstrated the development of thermally cross-linked superparamagnetic nanomaterial which possessed polyethylene glycol moiety and covalently linked branched polyethylenimine (BPEI), and exhibited highly efficient magnetofection even under serum conditioned media. The study showed its high anti-biofouling, cell viability and serum stability and thus revealed a potential magnetic nanoparticle-mediated targeted gene delivery system. This superparamagnetic particle mediated rapid and efficient transfection in primary vascular endothelial cells (HUVEC) successfully inhibits expression of PAI-1 which is responsible for various vascular dysfunctions such as vascular inflammation and atherosclerosis and thereby provides a potential strategy to transfect highly sensitive HUVEC. The sequential steps for the enhanced magnetofection had been studied by monitoring cellular uptake with the aid of confocal microscopy.

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**Herpes Oncolytic Virus Elicits Greater Anti-Tumor Immunity**

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Oncolytic virus therapy is becoming a promising anticancer therapeutic, and oncolytic viruses have been shown to elicit anti-cancer immunity. We evaluated the effects of the herpes oncolytic virus R3616 on the host immune response compared to a representative chemotherapy drug, 5-FU. R3616 or 5-FU was directly injected into the subcutaneous tumors of non-immunized mice. R3616 induced a greater number of tumor-infiltrating T cells, macrophages, and dendritic cells than 5-FU. MC26 cells plus R3616 or 5-FU as an adjuvant were frozen, thawed, and then used to immunize mice. After immunization, the adaptive immune response suppressed subcutaneous tumor growth and prolonged the survival rate of re-challenged mice. The group immunized with MC26 cells plus R3616 showed the greatest number of infiltrating T cells in the implanted tumor among the immunized groups. These results indicate that the oncolytic herpes virus R3616 has the potential to elicit host anti-tumor immunity. we will describe together with our clinical data of HF10 herpes oncolytic virus therapy human trial as a back ground date of anti cancer immunity that was elicited by oncolytic virus.
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