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5th AUSTRALASIAN GENE THERAPY SOCIETY MEETING

Date

April 18–20, 2007

Venue

Shine Dome Academy of Science, Canberra,

ACT, Australia

The Australasian Gene Therapy Society (AGTS) held its fifth biennial meeting on 18–20th April 2007. AGTS acknowledges and thanks the Journal of Gene Medicine for making the abstracts presented during the meeting available to the international research community.

The members of the Organising Committee for the 5th meeting of the society were:

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PLENARY LECTURES

Plenary 1 Genetic Complexity in a Monogenic Gene Therapy Model/Progress in Human Gene Therapy

Theodore Friedmann, M.D.,, *School of Medicine, University of California San Diego, La Jolla, California, U.S.A. 92093-0634*

*GENETIC COMPLEXITY IN A MONOGENIC GENE THERAPY MODEL

The effective application of gene transfer methods to gene therapy requires detailed understanding of pathogenic mechanisms. Monogenic diseases have represented important models for the development of many concepts and tools of gene therapy, and gene therapy approaches in those disorders have been based on a presumed direct and linear relationship of the primary genetic defect to the resulting disease phenotype. We have used transcriptional profiling methods to examine mechanisms of gene expression in a mouse model of the human HPRT-deficiency disorder Lesch Nyhan Disease. We have found that underlying primary HPRT defect produces secondary aberrations in the expression of a number of downstream genes, and we propose that such genetic aberrations may play a role in pathogenesis. If so, this kind of complex genetic interaction may convert a "simple" monogenic disease into a more complex "multigenic" disease and identify additional surrogate genes for potential genetic or pharmacological intervention. Such mechanisms may also play a role in other genetic disorders.

*PROGRESS IN HUMAN GENE THERAPY

The history of human gene therapy has seen enormous advances in the concepts and tools of gene transfer but has also been beset with numerous technical setbacks. This evolution is typical for the birth and development of many new areas of biomedicine including bone marrow and other tissue transplantation, cancer chemotherapy, clinical applications of monoclonal antibody technology and others. All of those therapeutic breakthroughs went through decades-long periods of failure before becoming indispensable parts of modern medical practice. Some, such as bone marrow transplantation, were declared in their early stages to be failed concepts. Gene therapy has been in the clinic for 17 years and has produced admittedly imperfect but undeniably effective new forms of treatment for immunodeficiency, several forms of cancer and possibly other disorders. Progress in human gene therapy is proceeding at a rate comparable to that of other novel therapies and is poised to deliver broader and more effective therapies for a number of rare and common diseases.

*Combined presentation.

Plenary 2 Production Requirements for Recombinant Adeno-Associated Virus Gene Therapies

Robert M. Kotin, *Laboratory of Biochemical Genetics, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD USA*

Large vector doses are necessary for developing successful therapies employing non-replicating viral vectors. This can be especially important in the treatment of many catastrophic diseases, such as cancer or the muscular dystrophies. Among non-replicating viral vectors, recombinant adeno-associated virus (rAAV) has numerous advantages. Typically, rAAV is produced by co-transfection of adherent HEK 293 cells. Although relatively efficient at small scale, expansion of adherent cells is not feasible beyond a certain size. In contrast, volumetric expansion of suspension cells provides a means for increasing cell number over several orders of magnitude, limited by the available vessel volume. To produce rAAV in suspension cells, we engineered three recombinant baculovirus expression vectors (BEVs) providing the cis and trans elements required for efficient rAAV production in Sf9 insect cells. Improved culture conditions increased the yield of rAAV recovered from a bioreactor to approximately 2×10^{14} DNase-resistant particles per liter.

Our advances in large-scale production of rAAV have allowed us to address the treatment of solid tumors and Duchenne muscular dystrophy (DMD). For cancer therapy, we have used shRNA-expressing rAAV to target Hec1, an essential kinetochore protein. Hec1 depletion prevents chromatid attachment to microtubules; as a result, the affected cells arrest in mitosis and die, whereas non-dividing cells are unaffected. To treat DMD, we are investigating an exon skipping strategy to ameliorate the disease via restoration of functional dystrophin. We believe that rAAV production in insect cells will provide the scalable, cost-effective, and current good manufacturing practice (cGMP)-compliant process required to support clinical trials in these, and other, important areas.

Plenary 3 Obstacles to Tumor Targeting with Adenovirus Vectors

Robert Strauss,[1] Pavel Sova,[2] ZongYi Li,[1] Ying Liu,[1] Daniel Stone,[1] André Lieber[1,2], [1]*Department of Medicine,* [2]*Department of Pathology, University of Washington, Seattle, USA*

i) Unspecific sequestration limits tumor cell transduction with tumor-targeted adenoviruses (Ad) after systemic administration. The vast majority of Ad serotype 5 based vectors is sequestered in the liver by Kupffer cells. Ad uptake into Kupffer cells is not mediated by the coxsackie-adenovirus receptor but involves blood protein mediated interaction between the Ad fiber and cellular heparansulfate proteoglycans. This mechanism of Ad uptake is less efficient if the Ad5 fiber shaft is replaced with a shorter shaft such as that of the subgroup B serotype Ad35 (Ad5/35). In addition to soluble blood factors, we have found that platelets mediate Ad uptake by Kupffer cells and that platelet depletion before Ad injection reduced liver sequestration and improved transduction of liver metastases with a tumor-targeted Ad vector.

ii) Another obstacle to tumor targeting is the genetic instability of malignant cells resulting in phenotypic heterogeneity of cells in a given tumor. In studies with clonal cell cultures derived from ovarian cancer biopsies, we found uniformly high level expression of the Ad35 receptor, CD46, and efficient binding of Ad5/35 vectors to tumor cells. Nevertheless, clonal ovarian cancer cultures displayed great variability in transgene expression and killing upon infection with an Ad5/35-based oncolytic vector. Expression array based comparison of resistant vs susceptible cultures revealed that pathways, which regulate tight junctions and control actin polymerization are involved in conferring resistance to viral oncolysis at several levels. We are currently testing approaches to interfere with these resistance mechanisms.

iii) Tumor stroma often tightly surrounds tumor nests and therefore limits the access of oncolytic viruses to malignant cells. Because bone marrow contributes different cell types to

the tumor stroma, including inflammatory cells, fibroblasts and vascular endothelial cells, we tested whether genetically modified human CD34+ stem cells can provide progenitors that home to tumor stroma upon engraftment of CD34+ cells into bone marrow. We further modified CD34+ cells to either inducibly express gene products that are able to degrade stroma proteins (for example relaxin) or that are able to activate an inactive prodrug into a cytotoxic drug at the tumor site.

Plenary 4 Clinical Trials of Replication-competent Adenovirus-mediated Suicide Gene Therapy

Kenneth N. Barton, Svend O. Freytag and Jae Ho Kim, *Dept. of Radiation Oncology, Henry Ford Hospital, Detroit, Michigan, USA*

Radiation therapy is a primary choice for the treatment of many cancers. Even with the most advanced treatment algorithms, toxicities to normal tissue limit the total effective radiation dose that can be delivered to patients. Unfortunately, this limit to the amount of radiation delivered is typically below the dose necessary to totally eradicate the tumor. To provide improved tumor control in the clinical setting, we have developed a novel trimodal approach utilizing a replication-competent adenovirus expressing cytotoxic chemo-radiosensitizing genes in combination with external beam radiation therapy. After validating the trimodal approach in preclinical models, we have moved it to the clinic for the treatment of prostate cancer. In our first two clinical studies, the trimodal approach was shown to be safe with only minor or self correcting adverse events reported. There were indications of efficacy as well. Biopsy results of 23 patients were encouraging with 78% negative and 22% positive at 2 years. In a five year follow-up analysis of the patients from our first trial ($n = 14$), the mean PSA doubling time increased from 16 to 31 months ($p = 0.014$). Recently we have imaged gene expression in a prostate cancer patient 72 hours following the administration of a therapeutic adenovirus expressing the sodium iodide symporter. Based upon these encouraging results we have or are in the process of applying the trimodal gene therapy approach to other sites including pancreas and brain.

Plenary 5 The use of clostridial spores in the combat against solid tumors

Jozef Anné[1] Willy Landuyt,[2] Sandra Nuyts[2] & Lieve Van Mellaert[1], [1]*Laboratory of Bacteriology, Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium.* [2]*Department of Radiation Oncology, Leuvens Kanker Instituut (LKI), University Hospital Gasthuisberg, B-3000 Leuven, Belgium*

Despite advances in the field of prevention and treatment of cancer, morbidity and mortality remain high. The efficacy to control cancer is limited by several major parameters, including the presence of a poor oxygenation status within regions of the tumor. Tissue hypoxia occurs in solid tumors as a result of an inadequate supply of oxygen, due to a rapid exponential cellular proliferation and an inefficient vascular supply. Hypoxia is an adverse prognostic indicator in cancer as it is associated with tumor progression and resistance to therapy. The development of novel cancer therapies that are selective for cancer cells, and thus with limited toxicity to normal tissues, is a challenge for oncology researchers.

To overcome intrinsic resistance to therapy, many attempts have been made to restore or mimic oxygen supply. Alternatively, hypoxia can be exploited by, for example, non-pathogenic bacterial systems that are active only in the hypoxic/necrotic regions of the tumor. Anaerobic bacteria including *Clostridium* with selective growth in solid tumor regions with pathophysiologic characteristics of hypoxia and anoxia have been investigated by us and others as potential tools.

Recently, genetically-modified non-pathogenic bacteria emerged as potential antitumor agents, either to provide direct tumoricidal effects or to deliver tumoricidal molecules. Several attempts with varying success showed the potential of *Clostridium* in antitumor therapy, when it was equipped with prodrug-converting enzymes, with cytokines or bacterial toxins. They are also recognized as immunomodulators to boost the host immune response against tumor cells. Our most recent research indicated that the antitumor effect could be increased by combining radiotherapy with *Clostridium*. This increased antitumor activity likely originates from an enhanced immune response against cancer cells.

Plenary 6 Modification of Genomic DNA Following Microinjection and Nucleofection of Large Oligodeoxynucleotides

Babak Bedayat,[1] Hamid Emamekhoo,[1] Alireza Abdolmohammadi,[1] David DeSemir,[2] Rosalie Maurisse,[1] Dieter C Gruenert[1,3,4], [1]*California Pacific Medical Center Research Institute,* [2]*Department of Dermatology,* [3]*Department of Laboratory Medicine, University of California, San Francisco, CA, Department of Medicine,* [4]*University of Vermont, Burlington, VT*

Recent studies have shown that oligodeoxynucleotides can be used to directly modify genomic sequences. Large (>300-bp) oligodeoxynucleotides (LODNs) or small DNA fragment (SDFs) have been shown to be effective at modifying the cystic fibrosis transmembrane conductance regulator (CFTR) *in vitro* and *in vivo* as well as the human β-globin gene *in vitro*. These studies have been intriguing in the context of gene therapy, but have not been readily quantified in terms of the efficiency of gene conversion. Impediments to achieving readily quantifiable levels of sequence-specific modification relative to oligonucleotide dose have been the method of delivering the LODN/SDF into the cells as well as the genomic DNA target. Most mass transfection methods have limited precision and it is generally not possible to determine the number of LODN/SDF that are required to facilitate a homologous exchange. The targeting of the hypoxanthine phosphoribosyl transferase (HPRT) makes it possible to directly quantify the efficiency of modification based on the number of per cell exposure. However, the actual number of LODN/SDF that enter the nucleus and are available to facilitate the homologous exchange cannot be readily determined. The ability to microinject a known volume of LODN/SDF containing solution into the cell nucleus greatly enhances that ability to establish a dose relationship between the number of LODNs/SDFs delivered to the cell nucleus and the number of converted cells. Previous studies have indicated that the minimum number of LODNs/SDFs required to facilitate modification of the human β-globin gene at an efficiency of $\sim$1–10% was in the range of 2,500 to 5,000 per microinjected nucleus. HPRT mutations in human lymphoblasts were corrected via nucleofection (electroporation) at a frequency ranging from 0.1 to 2%.

Plenary 7 Significant Airway Transduction After *In Utero* Administration Of A Gp64 Pseudotyped Lentiviral Vector

Suzanne Buckley[1], Steven Howe[2], Vicky Sheard[3], Donald Anson,[4] David Parsons,[4] Adrian Thrasher,[2] Charles Coutelle,[5] Simon Waddington[1], Tristan McKay[3], [1]*Department of Haematology, Royal Free and University College Medical School, London, United Kingdom.* [2]*Molecular Immunology Unit, Institute of Child Health, UCL, London, United Kingdom.* [3]*Cancer Research UK, Department of Medical Oncology, Paterson Institute, Christie Hospital, Manchester, United Kingdom.* [4]*Department of Pulmonary Medicine, Women's and Children's Hospital. Adelaide, Australia.* [5]*Department of Molecular and Cellular Medicine, Imperial College, London, United Kingdom*

Various viral vector systems have been used to target the developing airways as a gene therapy strategy for the pulmonary manifestations of Cystic Fibrosis (CF) without success. Early intervention gene transfer may be necessary in CF where the airway epithelium becomes largely inaccessible in adult life due to accumulation of thick, sticky mucus. In addition, CFTR expression is significantly higher *in utero* than it is in adult life therefore expression at an early stage may be beneficial to the developing lung. Fetal or neonatal instillation of a gene therapy vector may also enhance transduction of stem cells in the developing airways. Previously, we have shown that adenoviral vectors can be used to transduce the fetal/neonatal airway but this transduction is short lived due to the non-integrating nature of the vector. VSV-G pseudotyped lentiviral vectors transduce murine airways with relatively low efficiency however vector pseudotyped with the baculovirus gp64 envelope has recently been shown to efficiently and durably transduce the nasal epithelium of adult mice (Sinn, Burnight *et al*. 2005). Therefore, we compared gp64 and VSV-G pseudotyped HIV-SFFV-eGFP virus when administered intra-nasally to MF1 adults, neonates (1 day old) and via injection into the amniotic fluid of fetuses (E16). Lung tissue was harvested after 2 weeks. Fluorescence stereomicroscopy revealed widespread and substantial GFP fluorescence throughout the majority of lungs of mice injected in the adult and neonatal periods but this was greatly reduced in the mice injected *in utero*. This result was confirmed by GFP ELISA where expression levels after intra-amniotic injection were approximately 10-fold lower than after neonatal instillation. Most interestingly, the pattern of cell-type specific expression varied greatly with age at administration and with virus pseudotype. With VSV-G pseudotyped virus, the transduced cell-type was almost exclusively alveolar cells and transduction efficiency was ordered adult>neonatal>fetal. In stark contrast, administration of the gp64 pseudotyped lentivirus resulted in extensive airway epithelial transduction of the order fetal>neonatal>adult. In conclusion, we provide compelling evidence that fetal administration of gp64 pseudotyped lentivirus efficiently and specifically targets the airway epithelium of mice. This pseudotype combined with administration at this developmental stage could be beneficial in early treatment of CF airway disease.

Sinn PL, ER Burnight, *et al*. (2005). "Persistent gene expression in mouse nasal epithelia following feline immunodeficiency virus-based vector gene transfer." *J Virol* **79**(20): 12818–27.

The Greg Johnson Memorial Oration. Translational Research in new Areas: Lessons From Papillomavirus Immunotherapy

Professor Ian Frazer FAA,, *Director, Diamantina Institute for Cancer Immunology and Metabolic Medicine, The University of Queensland, 4th Floor Research Extension, Building 1, Princess Alexandra Hospital, Ipswich Road, Woolloongabba Q'land 4102 Australia*

Translation of basic knowledge about physiology and pathophysiology of diseases into new therapy, and taking that therapy from the lab to the clinic is the goal of most biomedical research. For tumor immunotherapy, as for gene therapy, converting basic knowledge into approved therapy has proven challenging. Modelling benefits from therapy in animals is expected. However, animal models are not always available, and when they are, their relevance is usually controversial. Where safety data are sought, and where the optimal dose and route of delivery of new agents is to be determined, animal data are often misleading. However, Phase I clinical trials are expensive, patient recruitment is difficult, and repeated studies to determine optimal scheduling for intervention are often unfeasible or undesirable. Further, all patients must be managed by accepted standard care, which makes detection of therapeutic benefits from early phase II clinical trials expensive as studies need to be large and may only show equivalent benefit where improvements are required. Lessons learnt will be illustrated from experience in developing immunotherapy for cancer over 25 years.

ORAL PRESENTATIONS

O1 Microdystrophin Expression in Mouse Models of Duchenne Muscular Dystrophy Reveals Novel Roles for Full-Length Dystrophin at the Myotendinous Junction

Glen B Banks,[1] Luke M Judge,[1] Paul Gregorevic,[1] Sheng Li,[1] Caitlin Doremus,[1] Leonard Meuse,[1] Eric E Finn,[1] James M Allen,[1,2] Stanley C Froehner,[3] Jeffrey S Chamberlain[1,2,4], [1]*Departments of Neurology, Senator Paul D Wellstone Muscular Dystrophy Cooperative Research Center,* [2]*Medicine,* [3]*Physiology and Biophysics and* [4]*Biochemistry, University of Washington, Seattle, Washington 98195, USA*

The myotendinous junction (MTJ) is a major site of force transmission in skeletal muscle. It is the weakest region of the musculotendinous unit being the most common site for sporting injuries. Many of the proteins that protect muscles from contraction-induced injury are concentrated at the MTJ. However, tendon failure has never been observed in any mammalian model of muscular dystrophy or myopathy. In the present study we examined the effect of truncated dystrophins on the MTJ in mouse models of Duchenne Muscular Dystrophy (DMD). We delivered a microdystrophin (ΔR4-R23) into the gastrocnemius muscles of mdx mice using adeno-associated virus pseudotyped with serotype 6 capsids (AAV6-microdystrophin). Microdystrophin caused overt tendon failure and ringbinden where the outer myofibrils encircle the inner myofibrils. These results suggest full-length dystrophin maintains tensile strength through the myotendinous junction. The broad incidence of ringbinden throughout all types of muscular dystrophies and myopathies suggests tendon failure could be a common pathological feature in human muscle diseases. We also

provide evidence that the phenotypic changes brought about by microdystrophin were caused by its domain composition rather than its small size. By defining novel roles for dystrophin we have generated a new microdystrophin for gene therapy of DMD.

O2 An Insulin Secreting Liver Cell Line, TAO, is Resistant to the Cytotoxic Effects of Pro-inflammatory Cytokines via NF-KB-Dependent Pathways

Janet Biady, Bronwyn A. O'Brien and Ann M. Simpson, *Department of Medical and Molecular Biosciences, University of Technology, Sydney, Australia*

To be a potential cure for Type 1 diabetes surrogate beta cells must synthesise, store and release insulin in response to metabolic stimuli and avoid autoimmune destruction. Previously we engineered an insulin-secreting liver cell line, Huh7ins, which stored and released insulin, however insulin secretion began at sub-physiological levels of glucose (2.5 mM). Therefore, this cell line was further modified and resultant cell line (TAO) is responsive to glucose within the physiological range (4.5 mM). TAO cells are resistant to the pro-inflammatory cytokines [IFN-γ (384 ng/mL), TNF-α (10 ng/mL) and IL-1β (2000 pg/mL)], which destroy normal beta cells during the pathogenesis of Type 1 diabetes. TAO cells did not become apoptotic after cytokine treatment as compared to a beta cell line (MIN-6). TAO cells also produced lower levels of nitric oxide than MIN-6 cells when challenged with cytokines. TAO cells expressed receptors for the pro-inflammatory cytokines used thereby eliminating the absence of receptors as being the cause of resistance. To elucidate a role for nuclear factor-kappa B (NF-κB) in the resistance of TAO cells to the effects of cytokines, quantitative real-time-PCR was used to determine expression levels of the NF-κB inhibitors (IκB-α, IκB-β and IκB-ε). Expression levels IκB-α, IκB-β and IκB-ε were decreased as compared to the untreated cells. Fas was also down-regulated while iNOS gene expression remained unchanged relative to untreated cells. These characteristics make TAO cells attractive candidates for surrogate beta cells and provide important information as to the molecular machinery necessary to create insulin-secreting cells from hepatocytes.

*O3 AAV2/8-Mediated Delivery to The Juvenile Mouse Liver

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Adeno-Associated Virus vectors (rAAV) are capable of achieving highly efficient gene transfer to the adult mouse liver with relatively stable long-term transgene expression. In the developing mouse liver, however, where hepatocytes are undergoing rapid proliferation, relatively little is known. Many genetic-metabolic liver diseases potentially amenable to gene therapy will require therapeutic intervention early in life. The current study therefore focused on gene transfer to the developing liver. An AAV2/8 vector encoding eGFP under the control of a liver-specific promoter was administered to neonatal mice by intraperitoneal injection (5×10^{10} vg/mouse), and the liver analysed for transgene expression over time. Up to 2 weeks approximately 70% of hepatocytes were eGFP-positive, but this dropped rapidly to 2–3% between 2–3 weeks and was stable thereafter. At later time-points, eGFP-expressing cell clusters appeared, indicative of vector integration. Quantitative PCR analyses showed a dramatic loss in vector genomes from 1–2 weeks, coinciding with rapid liver growth. Southern analyses of Hirt DNA suggested the underlying mechanism was loss of episomal vector genomes.

Intraperitoneal injection of juvenile mice was effective at all ages, however, subsequent fall-off of transgene expression was greater in younger mice. Vector readministration was investigated as a strategy for overcoming loss of vector genomes as a consequence of liver growth. At all ages, however, humoral anti-AAV8 capsid responses limited the efficacy of vector readministration. This loss of expression in the developing mouse liver and immunological constraint to vector readministration will need to be addressed for rAAV to realise its therapeutic potential in the growing liver.

*O3 and O4 will be given as a combined presentation.

*O4 AAV2/8-Mediated Correction of OTC Deficiency in the Adult and Neonatal *spf^{ash}* Mouse

Cunningham SC,[1] Spinoulas A,[1] Dane AP,[1] Carpenter K,[2] Wilcken B,[2] Kuchell P[3] and Alexander IE[1,4], [1]*Gene Therapy Research Unit, The Children's Hospital at Westmead and Children's Medical Research Institute, NSW, Australia.* [2]*Biochemical Genetics, The Children's Hospital at Westmead, NSW, Australia.* [3]*School of Molecular and Microbial Biosciences, University of Sydney, NSW, Australia.* [4]*Discipline of Paediatrics and Child Health, University of Sydney, NSW, Australia*

Recombinant adeno-associated virus (rAAV) vectors have significant promise in the treatment of genetic-metabolic liver disease in infants and children, where conventional therapies have many shortcomings. Severe neonatal ornithine transcarbamylase (OTC) deficiency is an ideal model for liver gene therapy as low levels of correction are likely to be therapeutic, and the risk-benefit profile is favourable. We therefore investigated the possibility of treating OTC deficiency in the mouse with an AAV2/8 vector.

Adult and neonatal *spf^{ash}* male mice were treated by intraperitoneal injection with a rAAV2/8 vector expressing the mOTC cDNA under the transcriptional control of a liver-specific promoter. Adult mice were injected with 1.5×10^{12} vg/animal, resulting in a 17-fold increase in OTC activity above physiological levels, with associated normalisation of urinary orotic acid levels consistent with complete biochemical correction. Even with a vector dose 10-fold less, 3-fold physiological enzyme activities were achieved, again with complete correction. By *in situ* staining, close to 100% of hepatocytes were shown to be transduced with this dose.

Since ultimately therapeutic intervention early in life is desired, gene transfer to the developing liver was investigated. Neonatal mice injected ip with 3×10^{11} vg/animal showed a 2-fold increase in enzyme activity 2 weeks after injection, followed by a dramatic reduction from 3 weeks. These results show that massive OTC over-expression and biochemical correction was achievable with a modest vector dose following intraperitoneal delivery in both adult and neonatal mice. The challenge will be maintaining

long-term correction given loss of transgene expression over time in the developing liver.

*O3 and O4 will be given as a combined presentation.

O5 Analysis of Whole Unselected Cellular Genomes Shows that wild-type AAV Integrates into Many Human Chromosomes; Implications for new Vector Design

Horace R. Drew, Linda J. Lockett and Gerald W. Both, *CSIRO Molecular and Health Technologies, North Ryde, NSW, Australia, 2113*

Wild-type adeno associated virus (AAV) has been shown to undergo preferential Rep-mediated integration into the AAV-S1 region of human chromosome 19 during latent infection, based on analyses of cells derived from long-term culture or neomycin selection. In contrast, recombinant AAV (rAAV) vectors carry substantial deletions and lack site-specificity. Integration by retro- and lentiviruses has recently been examined at the level of the whole genome using ligation-mediated PCR (LM-PCR). Here we used LM-PCR to examine integration by wild-type AAV2 in *unselected* human cells shortly after infection. We found that AAV joined to many different sites in human chromosomal DNA, rather than just preferred sites in AAV-S1. In fifty-two authentic clones AAV was joined to a variety of human chromosomes at sites where micro-homology matched one of several "GGTC" motifs within the p5 promoter. Under these unselected conditions, canonical integration events within AAV-S1 on human chromosome 19 constituted only 1–2% of the total, as measured by Southern blot or AAV-S1-PCR. As a control, LM-PCR was also used to analyse integration by p5-ITR plasmids in the presence of AAV Rep in a neomycin-*selected* cell system. There 22% of LM-PCR clones were found to lie in the vicinity of AAV-S1 but junctions with other chromosomes were also identified. No "GGTC"-type clones were observed. Our data therefore suggest that the specificity of chromosomal integration by new rAAV vectors, especially those that include a p5 promoter for Rep-mediated integration, should be monitored by whole-genome analysis in unselected cells.

O6 Systemic Delivery of 'Stealth' Virus Particles for the Treatment of Cancer

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Targeting disseminated cancer via the blood stream represents a formidable challenge for viral vectors. Multiple interactions with blood components, non-target tissues and the rapid clearance by macrophages combine to limit the effectiveness of vectors in humans. Coating adenovirus particles with hydrophilic polymers (pc-virus) creates a physical barrier between the virus and the environment, decreasing unwanted interactions. Pc-virus demonstrates an increased plasma $t_{1/2}$ in mice from 2 minutes to 2 hours, reflecting a decrease in Kupffer cell scavenging efficiency from 99.7% to 3.6%. This improvement in virus circulation time is directly related to the concentration of particles accumulating passively in tumours by the enhanced permeability and retention effect (EPR). Active targeting of pc-virus with tumour specific ligands such as EGF results in highly selective and efficient infection *in vitro*. *In vivo*, active targeting substantially improves the specific activity of virus particles in tumours but does not contribute to particle accumulation by the EPR effect. In a peritoneal model of ovarian cancer, EGF targeted Ad5*wt* shows improved animal survival without the unwanted inflammatory events characteristic of unmodified virus.

Evaluation of viral vectors in animal models is far from ideal due to the numerous differences in important interactions with blood components and the immune system. For example, Ad5 binds to human erythrocytes *in vitro* (and in patients) preventing infection of target cells whereas no interaction murine erythrocytes is observed. We are therefore developing improved pre-clinical models of virus pharmacokinetics based on human blood samples and liver perfusion.

*O7 Exon Skipping Prevents the Onset of Dystrophic Pathology in the *mdx* Mouse

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Duchenne and Becker muscular dystrophies are allelic disorders arising from mutations in the dystrophin gene. Duchenne muscular dystrophy is characterised by an absence of functional protein, while Becker muscular dystrophy, commonly caused by in-frame deletions, shows synthesis of partially functional protein. Antisense oligonucleotides can induce specific exon removal during processing of the dystrophin primary transcript, whilst maintaining or restoring the reading frame, and thereby overcome protein-truncating mutations. The *mdx* mouse has a nonsense mutation in exon 23 of the dystrophin gene that precludes functional dystrophin production, and this model has been used in the development of treatment strategies for dystrophinopathies. A phosphorodiamidate morpholino oligomer has previously been shown to exclude exon 23 from the dystrophin gene transcript and induce dystrophin expression in the *mdx* mouse, *in vivo* and *in vitro*. A cell-penetrating peptide-conjugated oligomer, targeted to the mouse dystrophin exon 23 donor splice site, was administered to *mdx* mice by intraperitoneal injection. We demonstrate dystrophin expression and near-normal muscle architecture in all muscles examined, except for cardiac muscle. The cell penetrating peptide greatly enhanced uptake of the phosphorodiamidate morpholino oligomer, resulting in widespread dystrophin expression. Treatment of neonatal *mdx* mice induced dystrophin expression and averted the onset of the dystrophic process that normally begins shortly before 3 weeks of age.

*O7 and O8 will be given as a combined presentation.

*O8 A Personalised Genetic Treatment for DMD

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Duchenne muscular dystrophy (DMD), the most common severe childhood muscle wasting disease, arises from protein truncating mutations in the dystrophin gene. DMD males are restricted to a wheelchair by age 12 and, until the introduction of steroid treatments, more effective physiotherapy and nocturnal assisted ventilation, the majority did not live past 20 years of age. Becker muscular dystrophy also arises from dystrophin mutations, but these are typically in-frame resulting in a dystrophin that may retain near-normal function. Large segments of the dystrophin gene can be deleted without seriously compromising protein function.

We are developing antisense oligonucleotide (AO) induced exon skipping strategies that have the potential to redirect DMD dystrophin pre-mRNA processing, so that an internally deleted, but still functional protein can be generated. Clinical trials are underway in Europe to demonstrate proof of concept, after intramuscular injection of antisense compounds to induce skipping of exon 51. If safety concerns are met, systemic administration will follow as soon as permissible. However, mutations can occur across the entire dystrophin gene. In order to provide a therapy for all suitable patients, it will be essential to apply appropriate AOs to different exons. We have developed a panel of AOs that induce efficient skipping of more than 55 dystrophin exons. Clinical testing of this number of AOs is not feasible, but if a class of compounds that exhibit an excellent safety profile can be used, a personalised genetic therapy approach may be considered.

*O7 and O8 will be given as a combined presentation.

O9 Limiting γc Expression Differentially Affects Signaling via the Interleukin (IL)-7 and IL-15 Receptors

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X-linked severe combined immunodeficiency (SCID-X1) results from mutations in the gene encoding the common gamma chain (γc) of the receptors for IL-2, 4, 7, 9, 15 and 21. Affected infants typically, lack T and NK cells as a consequence of loss of signaling via the IL-7R and IL-15R, respectively. In a minority of infants, however, autologous NK cells are observed despite failure of T cell ontogeny. The mechanisms by which mutations in γc differentially impact T and NK cell ontogeny remain incompletely understood. We used SCID-X1 patient-derived EBV-transformed B cells, containing only trace amounts of correctly spliced message, to test the hypothesis that IL-15R-mediated signaling is preferentially retained as γc expression becomes limiting. Signal transduction via the IL-15R was readily detected in control EBV-transformed B cells, and via the IL-7R when modified to express IL-7Rα. Under the same experimental conditions patient-derived EBV-transformed B cells expressing trace amounts of γc proved incapable of signal transduction via the IL-7R while retaining the capacity for signal transduction via the IL-15R. An equivalent result was obtained in a human T-cell line (ED-7R) modified to express γc by transduction with lentiviral vectors containing heterologous promoters of varying strength. Together, these results confirm that signal transduction via the IL-15R, and hence NK ontogeny, is preferentially retained relative to the IL-7R as γc expression becomes limiting. This result has implications for the treatment of SCID-X1 by gene therapy; as promoter strength is reduced this phenomenon has the potential to skew reconstitution towards the NK compartment.

O10 Promising Gene-Based Interventions to Enhance Contractile Performance in Models of Severe Skeletal Muscle Dysfunction

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Severe loss of skeletal muscle strength associated with conditions such as muscular dystrophies, aging, cancer, and chronic obstructive pulmonary disease can be lethal. Interventions that enhance muscle function in patients therefore may extend lifespan and also considerably improve quality of life. We have established that an intervention utilizing recombinant vectors derived from Type 6 Adeno-associated Virus (rAAV6 vectors) carrying a construct encoding a human follistatin isoform promotes muscle fiber hypertrophy to increase murine skeletal muscle mass by 100% and force producing capacity by $>$50% within 6 weeks of intramuscular administration. Hypertrophic effects mediated by this localized intervention are constrained to the region of transduction, but intravascular administration of this vector to mice elicits body-wide skeletal muscle hypertrophy and increases contractile capacity similarly, without loss of effect for over 16 months. This intervention represents a novel model system to study the biology of skeletal muscle adaptation, and may also prove applicable as a therapeutic approach where rapid loss of muscle strength is a serious health concern. We have determined that interventions incorporating rAAV6-Fst administration can restore muscle mass and function in murine models of muscle disease including aging-related sarcopenia, where the muscles of treated animals approach the functional capability of muscles from young adult animals. This communication shall review our ongoing work utilizing rAAV6-mediated follistatin expression to study skeletal muscle adaptation, and to establish the therapeutic potential for treatment of conditions caused or complicated by loss of skeletal muscle strength.

O11 Transient Expression of AAV-Derived Rep Protein Facilitates Site-Specific Integration of a 205 kb Bacterial Artificial Chromosome Containing the Entire B-globin Locus

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The risk of insertional mutagenesis is a major issue when techniques that rely on random integration of

exogenous DNA into the human genome are used for gene therapy. Adeno-associated virus (AAV) integrates preferentially into a defined site on chromosome 19, AAVS1, which is mediated by the Rep68/78 proteins and the Rep-binding elements located within the AAV genome and AAVS1. We developed a novel strategy for the targeted integration of large DNA plasmids containing the AAV-derived P5 integration efficiency element. This was achieved by transiently expressing the Rep68/78 proteins in human cells using *in vitro* transcribed mRNA, which can avoid the cytotoxicity issues associated with long-term Rep expression. We show that the co-transfection of a 21 kb plasmid with Rep mRNA facilitates site-specific DNA integration into AAVS1 in up to 30% of all analysed integration sites, while no AAVS1 integration events were observed following transfection of cells with plasmid DNA alone.

We also report the targeted integration of a 205 kb BAC carrying the entire β-globin locus, which contains an EGFP reporter under the control of the γ-globin promoter. We have generated, identified and characterised two K562-derived clones which likely contain a single copy of the modified β-globin locus integrated into AAVS1. These clones have a virtually identical EGFP expression profile which has been stably maintained for over six months in continuos culture, in the absence of selective pressure. Our data suggest that the site-specific integration of an entire genomic locus is a feasible approach to facilitate persistent and stable transgene expression while avoiding the risks associated with random integration of exogenous DNA.

O12 A Low Intensity Conditioning Bone Marrow Transplant Model Based on MGMT Gene Transfer and *In Vivo* Selection

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Gene transfer of O^6-methylguanine-DNA-methyltransferase (MGMT) into haematopoietic stem cells (HSC) offers the possibility of providing chemoprotection against alkylating chemotherapy to patients being treated for malignant brain tumours. It may also provide a mechanism for *in vivo* selection of donor cells in allogeneic HSC transplantation. Our aim is to develop an allogeneic transplant model based on MGMT gene transfer into donor HSC, and we hypothesise that transplantation using low intensity conditioning should allow engraftment of drug resistant HSC that can be expanded to reach significant levels of donor/recipient chimerism. The benefits of low-intensity allogeneic transplantation would be in a reduction of treatment-associated risk, and in a widening of both the patient population and range of diseases that might be treatable using this therapy. We have tested our hypothesis in a murine major mismatch model in which donor-HSC from C57BL/6-MGMT(P140K) transgenic mice were transplanted into BALB/c recipients following conditioning with Busulfan and immunosuppressive antibodies. *In vivo* selection of donor cells was imposed by administration of O6-Benzylguanine (O6BG) and the alkylating agent BCNU to recipient mice. The initial level of engraftment achieved was related to both Busulfan and cell dose. O6BG/BCNU administration was successful in producing high levels of donor/recipient chimerism (>50%) from a small number of engrafted cells (<1 %). Conditions for engraftment and *in vivo* selection established using this model will be a useful tool in studies investigating the development of donor/recipient tolerance prior to solid organ transplantation and for developing strategies to treat genetic diseases using allogeneic transplantation.

O13 Inhibition of the Innate Immune Response to Lentivirus Mediated Gene Transfer with Leptomycin B

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Introduction: There are many barriers inhibiting gene delivery *in vivo*, with the innate immune response to infection with micro-organisms playing an important role in host defence. Infection by HIV-1 invokes such an immune response. The nuclear proteins integrase interactor protein 1 (INI1) and promyelocytic leukaemia protein (PML), both enhance HIV-1 infection. The innate anti-retroviral immune response relocates these proteins to the cytoplasm, decreasing HIV-1 infectivity. Leptomycin B (LMB) interrupts this relocation, inhibiting this anti-retroviral response (1). Here we investigate whether LMB can enhance lentivirus mediated gene transfer to the murine nasal airways.

Methods: C57Bl/6 mice received 4 μL of LPC pre-treatment (1% w/v in PBS). 1 hour later each group received 20 μL of a lentivirus vector carrying the LacZ gene. LMB was added at concentrations of 5, 50 and 500 ng/mL to both the LPC and the lentivirus. In a second study mice received the optimal concentration of LMB (determined previously) in either LPC or virus alone. Mice were sacrificed at 1 week post-treatment, and gene transfer assessed by histological analysis of LacZ gene expression.

Results: Analysis of initial study showed that the optimal concentration of LMB was 500 ng/mL with gene transfer 4-fold higher than seen with our standard treatment (63.3 ± 41 cells compared to 17 ± 7.9 cells for the standard). The results of the second study are currently being analysed.

Conclusion: The innate anti-retroviral immune response can be successfully inhibited by the use of LMB resulting in increased gene transfer to the airway epithelium.

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O14 Lentiviral Mediated gene Therapy for Murine Mucopolysaccharidosis type IIIA

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Mucopolysaccharidosis type IIIA (MPS IIIA) is a heritable glycosaminoglycan (gag) storage disorder which is characterised by lysosomal accumulation of heparan sulphate, secondary to a deficiency of sulphamidase. There is currently no treatment for affected individuals, who experience progressive CNS deterioration and hepatosplenomegaly prior to an early death [1].

As a first step towards developing gene therapy as a treatment for MPS IIIA, an MPS IIIA mouse model [2] was used to examine the efficacy of intravenous lentiviral-mediated gene therapy. Five week old mice were injected with virus expressing murine sulphamidase and analysed 6 months after treatment. Transduction by the lentiviral vector was highest in the liver and spleen of treated animals, and sulphamidase activity in liver averaged 68% and 186% of normal activity respectively.

Storage was assessed using histochemical, chemical and mass spectrometric analyses. Storage in most somatic tissues was largely normalized, although chondrocytes were an obvious exception. Visually, improvement of lysosomal storage within the brain was variable. However, β-hexosaminidase activity, which is abnormally elevated in MPS IIIA [2], was significantly reduced in every treated tissue, including the brain. Total uronic acid was also significantly reduced in the brains of treated mice. The levels of a disaccharide marker (hexosamine-*N*-sulfate[α-1,4]hexuronic acid; HNS-UA) of heparan sulphate storage were also decreased, albeit non-significantly.

These results suggest that lentiviral mediated somatic gene transfer is a promising treatment option for treating not only the somatic, but also the CNS pathology found in MPS IIIA.

1. Neufeld EF Muenzer J. *The Mucopolysaccharidoses*. 2001. p. 415–450.
2. Bhaumik M, *et al*. *A mouse model for mucopolysaccharidosis type IIIA (Sanfilippo syndrome)*. *Glycobiology*, 1999 **9**(12): p. 1389–1396.

O15 Mesenchymal Stem Cells as Gene Delivery Vehicles for Cancer Gene Therapy

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Mesenchymal stem cells (MSC) have attracted attention as cellular vehicles for therapeutic gene transfer largely due to their ability to: self-renew; differentiate into mesenchymal tissue and their long-term engraftment *in vivo*. MSC also possess the machinery to secrete therapeutic proteins and most importantly are not rejected by the immune system, which is a major hurdle for most gene delivery mechanisms currently being investigated. This study explores the utility of MSC to deliver therapeutic genes to organ-confined and metastatic prostate cancer (PCa) using the RM1 mouse model. Cell-based gene therapy requires genetic modification retained by daughter cells. After examining several vectors, vesicular stomatitis virus pseudotyped lentiviral vectors showed sustained enhanced green fluorescent protein (eGFP) reporter gene expression in murine MSC (MSC-eGFP) for 50 passages *in vitro* by flow cytometry. When MSC-eGFP (10^6) were implanted into the mouse prostate with or without RM1 tumour cells on day 0, examination by fluorescence microscopy showed persistent eGFP expression within the prostate/bladder up to day 18. No MSC were detected in any other organ examined. In another experiment, MSC-eGFP (10^6) were infused every second day (2–14) *via* the tail vein of mice with or without RM1 lung metastases. When sacrificed on day 18, MSC-eGFP cells persisted within the lungs of metastases-bearing mice with no visible MSC in any other organ examined. The level of MSC tissue infiltration quantified from genomic DNA extraction and eGFP Q-PCR will be presented. These preliminary results suggest MSC may selectively home to and engraft prostate cancer.

O16 Non-Invasive X-ray Imaging of Mouse Airways And Organs At Very High Resolution – A New Option For Gene Expression Studies?

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Although the diagnostic and monitoring capabilities of X-rays are recognized in humans, its use in small laboratory animals is prevented by inadequate spatial and temporal resolution, and poor contrast. Phase-contrast synchrotron X-ray imaging can overcome these limitations. Anaesthetised C57Bl/6 mice were imaged 2-D at the SPring-8 synchrotron in Hyogo, Japan, using CCD detectors with 1.1 µm pixels (90 cm phase-contrast propagation distance, 25 keV, at 15 or 30 sec intervals using 100-300 ms exposures, for up to 45 mins). The 3-D CT slice images (12.3 um apart) were collected from intact humanely-killed mice (12 um pixel-based detector, 60 cm propagation distance, 17 keV) for later volume reconstructions.

Time-lapse videos in 2-D showed objects spontaneously transiting the airway surface at rates consistent with mucociliary clearance under barbiturate anaesthesia; full-width tracheal width changed dynamically during imaging. Altered airway-surface architecture could be detected after saline aerosols was delivered. In 3-D CT reconstructions, airway surface electrical-potential (dummy) electrode positions could be accurately mapped in the medio-dorsal regions of respiratory epithelium. The 3-D reconstructions of bony structures, cochlea, and middle-ear displayed striking clarity and resolution. The non-invasiveness of this technique suggests its use as a novel outcome measure of physical changes in small animal models, and suggests long-term monitoring is possible in individual animals. Phase-contrast synchrotron X-ray can provide new options for the acute and longitudinal study of

disease pathophysiologies where successful gene expression produces physical change in organ structure.
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O17 Gene Therapy for Eye Diseases. A Success Story

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Recombinant adenoassociated virus (rAAV) mediated gene therapy has been successfully used to deliver a variety of genes into the eye. In our laboratory we targeted Leber's Congenital Amaurosis (LCA) and Age Related Macular Degeneration (AMD). There are two animal models available for LCA; the RPE65−/− mouse and the Briard dog model. We delivered rAAV.RPE65 into the eyes of RPE 65−/− mice and Briard dogs and convincingly demonstrated the recovery of vision in both animal models. The extent and longevity of the visual function improvement varied but in general it lasted for up to 10 months in mice and up to 4 years in the Briard dogs. In another study we developed and applied Secretion Gene Therapy to inhibit neovascularisation in the adult eye. Subretinal neovascularisation is associated with AMD and it is the major cause of blindness in the developed world. We delivered rAAV.sFLT into the eyes of animal models. We used laser photocoagulation induced mouse, rat and monkey models for choroidal neovascularisation, a transgenic mouse model for retinal neovascularisation and an induced rat model for corneal neovascularisation. The sflt protein, a VEGF antagonist, inhibited neovascularisation without repeated injections for up to 18 months in the mouse and monkey eyes (when the study was terminated) without any damage caused to retinal function or morphology. These high levels of efficacy and the lack of toxicity provided sufficient basis to start preparations for Phase I/II gene therapy trials for AMD in humans in Australia.

O18 Killing Activity of HSV-TK/GCV Suicide Gene Therapy for Cancer Stem Cells Derived from Malignant Glioma

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Despite many efforts to develop effective therapy, the outcome of malignant glioma remains poor. Gene therapy for this disease using retroviral vector is attractive, because the virus can infect only mitotic cells. We established the packaging cell line PAMP51 producing high-titer retrovirus expressing suicide gene HSV-TK and concentrated the virus with titers as high as $1–10^{11–12}$ c.f.u./ml. Using this retroviral vector, mouse glioma model was completely cured by administration of ganciclovir (GCV). Recently, several investigators identified a small population of cancer stem cells in brain tumor tissues and cell lines. These cancer stem cells form spheres and maintain self-renewal capacity, tumorigenecity and multiple drug resistance. Therefore, we investigated the therapeutic efficacy of suicide gene therapy for the cancer stem cells derived from glioma cells. The cancer stem cells isolated from glioma cells by sphere culture preferentially expressed stem cell marker, CD133. Compared with parental cells, a lower efficiency of transduction with retrovirus vector was observed in cancer stem cells. The activity of MBP promoter driving HSV-TK expression was not different in parental and stem cells, but the stem cells appeared to be more tolerant of the treatment of GCV. However, cancer stem cells expressed connexin43, a component of gap junction as well as parental cells in both in vivo and in vitro. This result suggests that gap junction mediated bystander effect is an essential factor for killing the non-transduced cancer stem cells.

O19 Delivery of Furin-Cleavable Insulin to Diabetic Rat Livers Resulted in Long-term Correction of Diabetes and Partial Pancreatic Transdifferentiation of the Liver

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Type I diabetes mellitus results from the autoimmune destruction of pancreatic beta cells. Exogenous insulin therapy cannot achieve precise physiologic control of blood glucose concentrations and debilitating complications develop. Gene therapy is one strategy being examined to cure Type I diabetes. Lentiviral vectors are promising tools for liver-directed gene therapy of diabetes. However, to date, transduction rates *in vivo* remain low in hepatocytes, without the induction of cell cycling.
We introduced furin-cleavable human insulin (INS-FUR) into streptozotocin diabetic-Wistar rats using the HMD lentiviral vector via the portal circulation. Following delivery of the insulin gene or empty vector by a unique surgical technique, rats were monitored for changes in body weight and blood glucose levels. Intravenous glucose tolerance tests (IVGTT) were performed after an 8 h fast. Expression of insulin was determined by RT-PCR, immunohistochemistry and transmission electron microscopy.
We have achieved up to $60\% \pm 5.0\%$ transduction of hepatocytes *in vivo*. Rats treated with INS-FUR, exhibited normalization of blood glucose levels within 48 h and normoglycaemia was maintained for 500 days. Insulin was expressed only in the liver of insulin-transduced rats. IVGTT revealed that insulin-transduced rats showed similar responses to normal rats. The procedure resulted in expression of several beta cell transcription factors, some pancreatic endocrine transdifferentiation, significant hepatic insulin storage in granules characteristic of beta cells. Liver function tests remained normal. Importantly, pancreatic exocrine transdifferentiation did not occur. Our data suggest that this regimen may ultimately be employed for treatment of Type I diabetes.

O20 Reconstituted Chromatin Containing Engineered Histones Is An Efficient Gene Delivery Vehicle

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Non-viral mediated gene therapy is inefficient, largely due to the significant barriers of the plasma and nuclear membranes, with nuclear delivery of DNA shown to be the most rate-limiting step. The DNA binding and compacting core histone proteins have recently been shown to enter intact cells in receptor- and energy-independent fashion, consistent with protein transduction. Here, we demonstrate, for the first time, the ability of chromatin, reconstituted using engineered histone H2B proteins, to deliver DNA into intact cells by protein transduction and transport it to the nucleus, resulting in high levels of transfection efficiency ~6 fold that of commercial liposomal reagents. We report that the DNA is condensed and protected against degradation and recognised with high affinity by members of the Importin family of nuclear transport proteins. Inclusion of an additional optimised, efficient nuclear localisation signal from the SV40 large tumour antigen within the engineered histone H2B, significantly enhanced recognition by Importins, subsequent nuclear localisation, and ultimately gene expression. Reconstituted chromatin represents a new and efficient means to deliver DNA to a wide variety of cell types, with potential to treat a multitude of complex genetic disorders.

O21 Bugging Tumours to Deliver Anticancer Agents with Anaerobic Bacteria

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90% of all human cancers are solid tumours. Current conventional therapies are ineffective and insufficient. Recent understanding of the unique pathology of solid tumours has shed light on the difficult and disappointing nature of their clinical treatment.

Clearly, although the initial avascular mass is harmless, but when it grows to and exceeds about 2 mm in diameter, the local vasculatures of the surrounding normal tissues become inadequate to support the growing tumour mass, and thus undergo angiogenesis that results in biological changes and adaptive metabolisms, i.e.: formation of defective vessels, appearance of hypoxic areas, and emergence of an heterogeneous tumour cell population. This micro milieu provides a haven for anaerobic bacteria. In this presentation, we summarize the three major bacterial species that have been tested, and compared their advantages and disadvantages. Amongst these groups, the strictly anaerobic clostridia are superior over other facultative anaerobes such as salmonella or lactic acid-producing, Gram positive, obligate, anaerobic bifidobacteria. In combination with our own work, we will show you that pathogenic and non-pathogenic clostridia have been demonstrated to specifically colonise and destroy solid tumours. Others' work has also showed that trials of non-pathogenic strains in humans carried out in the early days had plausible safety. Genetic modifications and adaptation of pathogenic and non-pathogenic strains have further created improved features both for oncolysis and for gene delivery. However, these manipulations rarely generate strains that resulted in complete tumour control alone. Combined modalities of therapies with chemo and radiation therapies, on the other hand, often perform better, including "cure" of solid tumours in a high percentage of animals.

Considering that clostridia have unlimited capacities for genetic improvement, we predict that designer clostridia forecast a promising future for the development of potent strains for tumour destruction, incorporating mechanisms such as immunotherapy to overcome immune suppression and to elicit strong anti-tumour responses.

O22 Silencing AT1-R for the Control of High Blood Pressure

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Angiotensin II (Ang II) is a powerful vasoconstrictor that plays an important role in blood pressure regulation, cardiovascular and renal pathology. Its action is mediated through the G protein-coupled Ang II type 1 receptor (AT_1-R). Stimulation of AT1-R resulted in classic hormonal actions on blood pressure, and fluid homeostasis.

We have taken advantage of a new RNA interference (RNAi) technology and developed dsRNAs specific for the down-regulation of AT1-R. To enable *in vivo* gene delivery into animal models of hypertension, we have developed DNA plasmid-mediated expression of dsRNA (pPlasRi-AT1R). In this presentation, we will show you that a single intravenous injection of the DNA plasmid resulted in significant blood pressure reduction for 8 weeks in adult spontaneously hypertensive rat model. Blood pressure started to drop at the first week, went to lowest on the fourth week, and gradually returned to normal control level by 8 weeks. Most importantly, the blood pressure lowering effect correlated with the reduction in AT1-R expression. There was absence of any significant toxicity on animal growth or blood chemistry in the experimental animals.

Taken together, our results indicate that the gene therapy approach, based on silencing Ang II at a genetic level, can be an effective therapy for hypertension. The study lays the foundation for further studies of the technology and its applications for the treatment of other cardiovascular diseases and associated renal pathologies. Future directions will focus on combinational silencing by using a cocktail of therapeutic targets rather than a single particular gene.

O23 Lentivirus-Mediated gene Transfer to the rat, Ovine and Human Cornea

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Irreversible rejection is an important cause of clinical corneal transplant failure. Gene therapy shows promise

for modulating corneal transplant rejection, but the most appropriate vector for gene transfer to the cornea has yet to be determined. We investigated a lentiviral vector for its ability to transduce corneal endothelium. A lentivector expressing enhanced yellow fluorescent protein (eYFP) under the control of the Simian virus type 40 early promoter (LV-SV40-eYFP) transduced 80–90% of rat, ovine and human corneal endothelial cells, as detected by fluorescence microscopy. However, the kinetics of gene expression varied amongst species, with ovine corneal endothelium showing a relative delay in detectable reporter gene expression compared with the rat or human. This delay was confirmed at mRNA and protein levels using a lentivector carrying the secreted therapeutic protein, interleukin 10. Vectors containing the myeloproliferative sarcoma virus promoter or the phosphoglycerate kinase promoter were not significantly more effective than LV-SV40-eYFP. The stability of eYFP expression *in vivo* was assessed following orthotopic corneal transplantation in the inbred rat and the outbred sheep. Following transduction of the donor cornea *ex vivo*, eYFP expression was maintained in corneal endothelial cells for at least 28 days after corneal transplantation in the sheep, and for >60 days in the rat. Thus, rat, ovine and human corneal endothelial cells were efficiently transduced by the lentiviral vector, and gene expression appeared stable over weeks *in vivo*. The performance of the vector prompts its exploration as a means of achieving sustained expression of therapeutic transgenes in human corneal transplantation.

POSTER PRESENTATIONS

P1 Antisense Oligonucleotide-induced Exon Skipping Across the Human Dystrophin Gene Transcript

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Antisense oligonucleotides (AOs), directed at amenable splicing motifs across the dystrophin gene transcript, can be used as a potential therapeutic intervention during pre-mRNA splicing. Selected exons are excised using an AO to either remove nonsense mutations or restore the reading frame around frame-shifting mutations in the mature mRNA. In many cases, it should be possible to predict the level of functionality of the induced dystrophin protein by comparing it to similar rearrangements found in Becker muscular dystrophy, a less severe form of Duchenne muscular dystrophy, which arises from in-frame dystrophin deletions.
We describe a panel of AOs designed to induce skipping of every exon within the human dystrophin gene transcript, with the exception of the first and last exons. Every exon targeted in vitro was removed from the dystrophin mRNA, although some exons were more efficiently excluded than others. The efficiency with which the targeted exons were excluded could be improved by careful AO design and in some cases combining AOs targeted to a single exon.
We have classified the exons according to the ease with which they can be removed from the nascent transcript. No single motif has emerged as a universal AO annealing site for redirection of dystrophin pre-mRNA processing, although the general trend is that the most efficient compounds are directed at motifs in the first half of the target exon.

P2 Antisense Oligonucleotide Induced Exon Skipping and the Dystrophin Gene Transcript: Cocktails and Chemistries

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AOs have been used *in vitro* and *in vivo* to redirect dystrophin pre-mRNA processing in human and animal cells, thereby allowing protein-truncating mutations to be removed or bypassed, so that a shorter but still functional protein can be generated. Targeted exon skipping may be directed at the dystrophin gene transcript to remove nonsense or frame-shifting mutations that would otherwise have lead to Duchenne Muscular Dystrophy, the most common childhood cause of muscle wasting.
Although many dystrophin exons can be excised using a single AO, several exons require two motifs to be masked for efficient or specific exon skipping. Some AOs were inactive when applied individually, yet pronounced exon excision was induced when used in combinations, clearly indicating synergistic rather than cumulative effects on splicing.
We describe optimisation and excision of recalcitrant dystrophin exons from the mature mRNA using AO cocktails for enhanced efficiency and/or specificity. The use of either 2′-O-methyl modified bases on a phosphorothioate backbone (2OMeAOs) or phosphorodiamidate morpholino oligomers (PMOs) does not seem to influence exon skipping trends, indicating optimisation of AO design with 2OMe chemistry should be directly applicable to PMO.

P3 Induced Dystrophin Exon Skipping in Human Muscle Explants

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Antisense oligonucleotide (AO) manipulation of pre-mRNA splicing of the dystrophin gene shows potential in overcoming Duchenne muscular dystrophy (DMD)-causing mutations. AOs are used to alter dystrophin pre-mRNA splicing to reduce the consequences of nonsense or frame-shifting mutations that would otherwise prematurely terminate translation. We have developed a panel of AOs, targeting 77 out of 79 exons, to address mutations across the human dystrophin gene transcript that will restore the reading frame by either removing single or multiple exons.
The aim of this study was to demonstrate that AOs could be used to induce exon skipping in human muscle. To date, this approach has been limited to studies using animal models or cultured monolayers of human muscle cells. We used different AO chemistries to induce exon skipping in human muscle explants, derived from normal and DMD human tissue, with great success. We propose that inducing

exon skipping in human muscle explants is closer to *in vivo* conditions than cells in monolayer cultures, and that it is possible that explant studies may be used as a further tool to assist in the design of the AOs and evolution of different AO chemistries.

P4 A Hybrid Episomal-Based Vector System for gene Therapy

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Epstein-Barr virus (EBV) based vectors contain components derived from human EBV. These vectors are capable of long-term episomal maintenance in many mammalian cells. Epstein-Barr nuclear antigen 1 (EBNA1) and oriP are the only viral elements necessary for stable episomal maintenance of these vectors in cells. A major drawback of EBV-derived vectors is their inability to replicate in mouse and hamster cells. This causes problems when validating these EBV-derived vectors in many animal models for gene therapy. A recently developed small circular vector, pEPI-eGFP [scaffold/matrix attachment region (S/MAR)-based vector], containing sequences from the 5′-region of the human interferon β-gene was shown to stably maintain episomally over more than 100 generations without selection pressure in CHO cells. Active transcription upstream of the S/MAR running into this sequence has been shown to be required and sufficient for episomal replication for this vector. We have successfully modified a 21.3 kb EBV-based BAC (bacteria artificial chromosome) by replacing the EBNA1 gene with a S/MAR cassette isolated from pEPI-eGFP. This cassette contains the S/MAR sequence with active upstream transcription of the EGFP gene and an antibiotic selection marker. This novel S/MAR-based BAC vector, pS/MAR-160G, contains no virally encoded trans-acting factor. The oriP and the S/MAR sequence on this vector provide the potential of episomal maintenance and replication in different mammalian cells including mouse cells. This hybrid episomal-based vector system could be a useful non-viral gene delivery system for gene therapy approaches.

P5 Vector Distribution in the hepatic lobule following rAAV2/8 delivery

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AAV8 vectors are capable of achieving high levels of transduction in the mouse liver and therefore show great promise for liver-directed gene therapy. The current study focused on determining the level of long-term transgene expression in the liver but brought to light an important factor that should be considered when targeting the liver. Across the liver lobule zonation of metabolic pathways occurs. Many of the urea cycle enzymes, for example ornithine transcarbamylase (OTC), are most highly expressed in the periportal region. Therefore it is important, when using liver gene therapy for metabolic disease, to ensure that the transgene is expressed in the area where the natural pathway occurs.

A rAAV2/8 vector encoding eGFP under the control of a liver-specific promoter was administered via intraperitoneal or portal vein injection to adult mice. Liver was harvested at various time points from 2 weeks to 12 months. Distribution of vector genomes and transgene expression was analysed by laser microdissection and fluorescence microscopy, respectively

AAV8 transduction of mouse liver is highly efficient, with close to 100% of hepatocytes transduced initially and maintained at about 60% for at least 12 months. At early time points the highest levels of eGFP expression were observed around the central vein. Q-PCR revealed higher numbers of vector genome present in these areas compared with periportal regions. Assessment of the zonal appearance of transduction at later time-points and its potential effect on liver-directed gene therapy are currently being undertaken.

P6 A Novel Cryptic Splice Site in IVSI-110 B-Thalassemia

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The IVSI-110 β-thalassemia mutation was first described in 1981 and is one of the most common β-globin splicing mutations found in patients of Greek or Cypriot origins. This mutation is the result of a G to A substitution at position 110 in the first intron. This mutation has previously been reported to generate an aberrant 3′ acceptor splice site, which is preferentially recognised by the spliceosome over the normal 3′ acceptor splice site. The IVSI-110 mutation leads to a 90% reduction in normal β-globin chain synthesis causing transfusion-dependent disease in homozygous patients. Our group recently reported the creation and characterisation of 'humanised' transgenic mice containing the IVSI-110 human β-globin locus. Using human β-globin-specific RT-PCR, we noted two human β-globin-specific aberrant spliced products. We observed quantitative differences in the aberrant β-globin specific RT-PCR spliced products in peripheral blood and bone marrow-derived cells from 'humanised' IVSI-110 transgenic mice and IVSI-110 human patient-derived peripheral blood cells. We attribute the relative differences in the level of aberrant splice products to mRNA instability. In this study, we confirm by cloning of RT-PCR products and DNA sequencing the identity of the novel activated cryptic 3′ acceptor site in mice and also in humans containing the IVSI-110 mutation. We conclude, that the identification of novel cryptic splice site indicates that IVSI-110 splicing is more complex than previously reported, and is worthy of further investigation.

P7 Lack of dominant-negative effects of mutant ornithine transcarbamylase *in vivo*

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Recombinant adeno-associated virus (rAAV) vectors are capable of achieving high-level gene transfer to the mouse liver and, therefore, show significant promise for the treatment of metabolic liver diseases. In a murine model for ornithine transcarbamylase (OTC) deficiency our group has demonstrated that delivering a rAAV2/8 vector expressing *mOTC* under the transcriptional control of a liver-specific promoter by intraperitoneal injection can attain liver-wide transgene expression. This resulted in OTC enzyme activities over an order of magnitude above physiological concentrations and normalisation of urinary orotic acid levels consistent with complete biochemical correction.

In gene therapy applications for OTC, mutant proteins have the potential to show dominant negative effects against the wild-type protein introduced into the patients' cells by gene transfer, thereby limiting therapeutic efficacy. Indeed, *in vitro* studies have suggested dominant negative effects; but these have not been demonstrated *in vivo*. In this study we used a murine model to determine whether co-expression of wild-type and mutant OTC proteins would interfere with OTC activity and thus exert a dominant negative effect. Using site-directed mutagenesis we constructed three mutant OTC proteins (K88N, R92Q and R141Q) and delivered these to the liver by using rAAV2/8 gene transfer. Treated mice showed no increase in urinary orotic acid levels or reduction in OTC activity despite high-level expression of the mutant proteins. This suggests that these mutations have little effect on endogenous OTC expression and is favourable for the development of human clinical trials for OTC.

P8 Induced Exon Skipping: AO design is more important than chemistry

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Duchenne muscular dystrophy (DMD), a genetic disorder that arises from protein truncating mutations in the dystrophin gene, is characterised by severe and progressive muscle wasting. Mutations causing DMD can occur in any of the 79 exons or introns of this massive gene (2.4 Mb), and antisense oligonucleotide (AO) induced exon skipping is emerging as a novel potential therapy, with two separate trials underway.

Our rationale in AO design to address protein truncating dystrophin mutations by induced exon skipping is to prepare 2′-O-methyl modified bases on a phosphorothioate backbone that will anneal to potential splice motifs and alter dystrophin pre-mRNA processing. These compounds can be prepared in-house and, when transfected as a cationic lipoplexes, are readily taken up *in vitro*. Overlapping 2OMeAOs are prepared to pre-mRNA motifs shown to influence splicing, in an attempt to optimise AO design. Titration studies then allow identification of the most effective AOs, those that induce pronounced exon excision at low transfection concentrations. Although the 2OMeAOs are being used in clinical trials in the Netherlands, we have found that phosphorodiamidate morpholino oligomers (PMOs) are better suited for *in vivo* applications. However, PMOs are not taken up efficiently *in vitro*, impeding design of optimal compounds. We present data showing that the hierarchy of exon skipping efficiency generated with overlapping 2OMeAOs was identical to that observed with the corresponding PMOs.

P9 Establishment and Validation of a LAM-PCR assay for Retroviral Integration site Analysis During a Clinical trial

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The O6-methylguanine-DNA methyltransferase (MGMT) gene encodes a DNA repair enzyme that removes alkyl groups from guanine nucleotides after exposure to alkylating agents. MGMT is expressed in tumour cells thereby providing a mechanism for resistance to chemotherapeutic agents. The drug O6-benzyl-guanine (O6BG) can effectively inhibit MGMT activity, leaving tumours susceptible to alkylating drugs. Myelosuppression is a significant side affect because MGMT is expressed at low levels in the bone marrow. A retroviral vector expressing a MGMT P140K mutant (MGMT(P140K)) has been constructed. This vector has been shown to effectively confer chemoprotection from alkylating agents in non-human primate models. Our aim is to transduce haematopoietic stem cells with the MGMT(P140K) vector from paediatric patients with brain tumours. The expected result is chemosensitivity of the tumour and chemoprotection of the bone marrow.

In the French-based clinical trial for X-linked severe combined immunodeficiency (SCID-X1) eleven children have been treated with a retroviral vector expressing γc. Four of these children have gone on to develop leukaemia-like illnesses as a result of vector integration in or near oncogene loci, such as *LMO2*. This phenomenon is known as insertional mutagenesis. As a consequence, the analysis of retroviral integration sites following gene delivery is a requirement of regulatory authorities. We have transduced a human $CD34^+$ cell line (TF1) with the MGMT(P140K) vector and isolated clones containing 1, 2 or 3 proviral copies as determined by quantitative PCR. These clones have been used to optimise the LAM-PCR procedure before analysing clinical samples containing more complex integration site patterns.

P10 The Use of Anion Exchange Chromatography for the Purification of Lentiviral Vectors

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The use of anion exchange chromatography for purification of lentiviral vectors was investigated. Virus supernatant was clarified by 0.45 μm filtration and concentrated *via* ultrafiltration. The virus was then applied to different anion exchange columns. Initial investigations compared 7 different chromatography matrices for their ability to bind and elute virus. Two were chosen for further assessment; Sepharose QFF resin (Amersham Biosciences) and MustangQ Membrane (Pall Life Sciences).

The capacity of QFF was determined to be ≈50 μg p24/mL resin, recovery of infectious virus was 84%. Gradient elution resulted in two peaks of virus. The first peak contained 23% of the virus with a 5 fold increase in specific infectivity and a 2 fold decrease in infectious units (IU)/ng protein. The second peak contained 45% of the virus with a 15 fold increase in specific infectivity and a 15 fold increase in IU/ng protein. Elution with a step gradient resulted in improved purification.

The capacity of MustangQ was at least 175 μg p24/25 mm unit with 100% of virus recovered. Again, elution with a gradient of 0-2M NaCl resulted in two peaks of virus. The first peak contained 56% of the virus whilst the second peak contained 45% of the virus. The specific infectivity of both peaks was unchanged (from the starting material) while IU/ng protein increased 4 fold in the first peak and 6.5 fold in the second peak. The use of a step gradient for the elution of virus from the MustangQ membrane is currently being investigated.

P11 Improving the Safety of HIV-1 Derived Gene Therapy Vectors: Reducing the Rate of SIN Repair and Polyadenylation Signal Readthrough

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Recent events have highlighted the need to increase the safety of retroviral vectors by minimising the probability of proviral mediated gene activation. Two processes that will have a major impact on this phenomenon are self-inactivation (SIN) repair [1] and polyadenylation (pA) signal readthrough [2, 3]. In order to reduce the rate of these processes we made a number of modifications to our vector. The 3'LTR was modified by deleting all of U3 except the *att* sequence and substituting the HIV-1 pA signal for the Simian Virus 40 late (SV40) pA signal. A marker rescue assay showed that the rate of SIN repair in the modified vector was significantly less than the unmodified vector (Chi Squared, $p \leq 0.05$). Vector titre was unaffected.

To reduce pA signal readthrough in transduced cells, the pA signal in the provirus must be modified. Accordingly, the pA signal in the R/U5 region of the 5'LTR must be modified. Substituting the HIV-1 pA signal with the SV40 pA signal resulted in a 15 fold reduction in virus titre. However, the substitution of the Bovine Growth Hormone (bGH) pA signal in place of the HIV-1 pA signal resulted in only a slight decrease in virus titre. The effect of this modification on pA signal readthrough is currently being evaluated. Other modifications we have incorporated into our vector include the deletion of part of 5'U3 and of part of *gag* to minimise viral sequence in the vector, and the mutation of the major and minor splice donor sites to prevent splicing.

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2. Zaiss A-K, Son S, *et al. J Virol*, **76**(14): 7209–7219.
3. An W and Telesnitsky A. *J Virol*, **78**(7): 3419–3428.

P12 Evaluation of Recombinant Adeno-Associated Virus (rAAV2/8) Vector for Gene Therapy in Rat Liver Transplantation Models

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High transduction efficiency is a prerequisite for any vector considered for use in transplantation gene therapy. We evaluated the transduction of the rat liver *in vivo* with AAV2/8 and describe the production of a potentially immunomodulatory AAV8 vector for use in allotransplant models.

An AAV2/8 vector expressing GFP under the control the human alpha 1-antitrypsin promoter (AAV8_GFP) was used to transduce PVG livers by injection into the portal vein at six, nine and eleven weeks of age. GFP expression was examined at 12 weeks of age. AAV8_IDO, an AAV2/8 vector encoding for rat indoleamine dioxygenase (IDO), was constructed and tested by its ability to induce tryptophan catabolism and kynurenine production *in vitro*.

At the time of maximal GFP expression, injection of 10^{13} and 10^{12} vector genomes transduced respectively 65+/−5.8 and 7.15+/−7.8 percent of hepatocytes. Vector administration was not accompanied by any derangement of serological tests of liver function. The AAV8_IDO vector was able to induce tryptophan catabolism and kynurenine production by a hepatoma cell line, *Huh7*. Investigations are under way to examine whether treatment of the donor with this vector promotes rat liver transplant tolerance. In conclusion, AAV2/8 vectors produced highly efficient hepatocyte transduction *in vivo* and *vitro* and may provide a gene therapy tool suitable for use in transplantation models.

P13 The Genetic Adjuvant $C3d_3$ Augments Immune Responses to Antigens Delivered by AAV Genetic Vaccine Vectors in a Transgene-Dependent Manner

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We have previously demonstrated that AAV encoding MSP4/5 (from *Plasmodium yoelii*) can stimulate antibodies to MSP4/5 but that these do not confer protection to a challenge with *P.yoelii* in a model where high titre antibodies to MSP4/5 correlates with protection (Logan *et al*. 2006 Vaccine). In an effort to therefore enhance antibody stimulation, the transgene was genetically fused with three tandem copies of the complement protein, C3d, a proven strategy for augmenting immunity in other vaccine vector systems. The cDNA encoding MSP4/5-$C3d_3$ was fused with a TPA leader sequence cDNA (for antigen secretion) and cloned downstream of the CMV immediate/early promoter within an AAV provirus (pTRUF-2). Vectors were injected (10^{12} vg) into the hindlimb muscle (im) of 8–10 week old female Balb/C mice and serum samples were assayed for antibody responses to MSP4/5. Unexpectedly, $C3d_3$-fusion markedly decreased antibody responses to MSP4/5 which contrasted with the 50-100 fold enhancement in responses when the $C3d_3$-fusion strategy was similarly applied to another AAV encoded model antigen, hen egg lysozyme (HEL). These data indicate that the $C3d_3$ strategy operates in an antigen-dependent manner. Additional data with the HEL-$C3d_3$ construct has shown that *in vitro* gene modification of cells induces protein expression of HEL fused with $C3d_1$, $C3d_2$, $C3d_3$ and $C3d_4$ as determined by protein size estimation. Theoretically, these events would also occur during vector packaging and transduction and will need to be addressed if this strategy is to be further exploited using recombinant viral technologies.

P14 Effects of LPC Pre-Dosing on Lentiviral gene Expression in Mouse Lung

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We have previously shown that the surfactant L-α-lysophosphatidycholine (LPC) enhances lentivirus-mediated gene transfer to mouse nasal-airway [1]. In this study we assess LPC enhancement of lentiviral-mediated gene transfer in mouse lung.

LPC (15 ul of 0.1%, 0.3% or 1.0% (w/v) in PBS) was administered transorally to lungs of anaesthetized C57L/B6 mice. One hour later 30 ul of a LacZ lentivirus vector was administered in the same manner. Lungs were processed at day 7 for X-gal staining and sections counterstained with Saf-O or H/E.

No X-gal staining was present in controls. In some mice that received 0.1% LPC and virus, scattered punctuate groups of stained cells or lines of stained cells were found in trachea, carina, and the large bronchi near the carina. All mice receiving the highest LPC dose (1.0%) showed extensive gene transfer in larynx, trachea, carina, as well as in large, middle and small airways of most lobes of the lung, reaching 79% transduction of cells of an airway perimeter in one animal. The intermediate of dose of LPC (0.3%) produced intermediate levels of cell staining to that found in the 0.1% and 1.0% LPC-treated animals. Transduced epithelial cells included non-ciliated columnar cells, ciliated cells, and basal cells.

We conclude that lentivirus vector gene transfer into mouse lung is enhanced by lung pretreatment with 0.1%–1.0% LPC, and this preliminary data suggests that the effect is dose-dependent. Gene transfer was variously present in all airways, from trachea to bronchioles, and affected all types of airway epithelial cells.

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P15 Antisense Induced exon Skipping Restores Dystrophin in the 4^{CV} Mouse Model of Muscular Dystrophy

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Duchenne Muscular dystrophy (DMD), a severe neuromuscular disorder, is caused by nonsense or frameshift mutations in dystrophin gene that results in an absence of functional dystrophin. Loss of functional dystrophin renders muscle fibres vulnerable to membrane damage during contraction. Antisense oligonucleotide (AO) induced exon skipping has been used to induce specific exon removal and by-pass the disease-causing mutation. Some dystrophin mutations will require removal of more than one exon. We are investigating multi-exon skipping in the B6Ros.Cg-*Dmd*$^{mdx-4Cv}$/J (4^{cv}) muscular dystrophy mouse model, which has a nonsense mutation in exon 53 of the dystrophin gene. This area is of relevance to the human dystrophin gene since this area corresponds to the major deletion hot spot of the human dystrophin gene. To restore the reading frame of the 4^{cv} dystrophin mRNA, both exons 52 and 53 must be excised from the mature dystrophin gene transcript. 2′-O-Methyl AOs on a phosphorothioate backbone (2OMe) have been designed to mask motifs involved in splicing to remove these exons during pre-mRNA processing. We describe the removal of single exon and multi-exons in cell culture and *in vivo*. The exploration of exon skipping events in the 4^{CV} mouse model will provide additional information in AO design, and will be a molecular model relevant to many DMD cases.

P16 Microarray Study of the Effect of Ad5 in a Medullary Thyroid Cancer cell Line

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Medullary thyroid carcinoma (MTC) is a malignancy arising from C-cells of the thyroid and is primarily treated by surgery. Many patients develop metastatic disease that responds

poorly to chemotherapy and radiotherapy. A targeted gene therapy using Ad5 to deliver the suicide gene PNP, together with the pro-drug Fludarabine to a human MTC cell line (TT cells), appeared to lead to an increase in the growth rate or metabolism of TT cells as measured by an MTS assay, rather than the expected toxic effect.
A microarray study looking at changes in TT cell gene expression when infected with Ad5 was carried out to understand this result.
TT cells were incubated with or without Ad5 empty vector (E1, E3 deleted) at 200 virus particles per cell and RNA was extracted. cDNA was hybridised to the Compugen H19K human oligonucleotide array.
We found that the principal difference in gene expression between TT cells infected with Ad5 compared to TT cells with no Ad5, was a massive upregulation of translation. Ten tRNA synthase enzymes and 8 amino acid synthase enzymes were overexpressed in Ad5 infected TT cells compared to uninfected TT cells. Mitochondrial translation appeared to be down regulated and cytoplasmic translation upregulated. There was no clear trend in the expression of cell cycle or apoptosis genes.

P17 Achieving GMP Compliance for Gene Therapy Clinical Trial Activities

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The Children's Hospital at Westmead (CHW) is embarking on a Phase I gene therapy clinical trial for treating patients with brain tumours. The newly built Human Applications Laboratory (HAL), located within the Research Building at CHW, will be the primary facility for our gene therapy trial activities. Whilst GMP certification is not required for Phase I trials, we are never-the-less working towards full GMP compliance which will be necessary for future progression to later phase trails. Aspects of future GMP compliance were addressed during the design and construction of this new cleanroom facility.
In keeping with the aim of gaining GMP certification we have appointed a Quality Officer to oversee the commissioning of the HAL. In addition, the Quality Officer will oversee the implementation of all trial related activities, including the manufacture of vector supernatant and manipulation of patient cells, to a standard necessary to meet the rigorous requirements of GMP certification.
We have undertaken to qualify and validate all of our activities throughout the commissioning period. Our current list of tasks includes: Equipment Qualification/Validation, Process Qualification/Validation, the design and implementation of environmental monitoring, implementation of Document Control software and the writing of Standard Operating Procedures (SOPs) for all activities to be undertaken in the HAL. Our aim is to use the activities associated with the planned Phase I clinical trial as a guide to establishing the rigorous procedures necessary to achieve GMP compliance.

P18 Antisense oligonucleotide delivery to cardiac muscle: Knocking on a locked door

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Cardiac involvement in Duchenne Muscular Dystrophy (DMD) is evident in nearly a third of all patients, with up to 40% succumbing to heart disease. Whereas DMD is caused by a lack of functional dystrophin, Becker Muscular Dystrophy (BMD) is characterized by expression of an internally truncated, functional dystrophin protein. Nearly 90% of BMD patients over the age of 40 have evidence of cardiac involvement in the disease process. The most widely used animal model, the *mdx* mouse, has similar skeletal pathology to DMD patients, however, the cardiac pathology is more representative of BMD patients (i.e. late onset of cardiac involvement).
Advances in gene therapy have demonstrated both *in vivo* and *in vitro* delivery of constructs to skeletal muscles leading to expression of a functional dystrophin protein. Cardiac delivery of gene medicines has proved more difficult and unrewarding. We have been able to demonstrate antisense-induced exon skipping in cardiac cell cultures derived young *mdx* mice. Multi-exonic skipping and novel delivery methods have improved *in vitro* results, however *in vivo* experiments still lack definitive success. Besides the morphologic and functional differences between skeletal and cardiac muscle, a potential reason for the lack of success maybe the absence of pathology in the younger animals, leading to decreased uptake of gene therapy *in vivo*. We are pursuing several lines of research to further enhance levels of exon skipping in cardiac cells *in vitro* and *in vivo*.

P19 Downstream Process of Lentivirus: An Improved Method

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Introduction: Lentiviral vectors provide robust and efficient gene delivery, as well as long-term gene expression. However, effective *in vivo* transduction requires large amounts of virus and large-scale production of a high titre, high purity stock of recombinant virus is demanding. Hence it is crucial to optimise all aspects of lentivirus production and purification. Collection of virus in serum free medium allows easier purification of virus. However, serum also appears to stabilise virus, meaning downstream processing of virus collected in serum free media can result in low recovery. Downstream processing methods commonly used for production of lentivirus include ultrafiltration and ultracentrifugation. Here we investigate methods to increase recovery of virus collected in serum free medium with these processing methods.
Method: Lentivirus expressing enhanced yellow fluorescent protein (LV-EYFP) was collected in serum free medium and clarified by 0.45 μm filtration.
A number of agents (protein stabilisers) were then assessed for their effects on recovery of viable virus via

ultracentrifugation. Recovery of viable virus assessed by FACSCan analysis
A subset of the same reagents was then assessed as additives during concentration of virus by ultrafiltration using a 750-kilo Dalton membrane.
Results: Several agents were found to increase recovery of viable virus from serum free medium after ultracentrifugation and ultrafiltration. It was also noted that an initial diafiltration of virus supernatant improved recoveries during ultrafiltration. We are currently developing and testing a large-scale purification method based on these results.

P20 Short Interfering RNA Mediated Knockdown of a-globin : Applications in β-thalassaemia

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β-thalassaemia is an inherited haemoglobinopathy arising from mutated β-globin genes, resulting in reduced β-globin synthesis. Much of the pathology arises due to excess α-globin forming toxic insoluble precipitates in erythroid progenitor cells resulting in premature cell death. Decreased α-globin expression leads to milder symptoms, exemplified in individuals who co-inherit α-thalassaemia and β-thalassaemia. Since numerous studies have shown promising results utilising siRNA *in vitro* and *in vivo,* we are developing an siRNA strategy for reducing α-globin as a therapy for β-thalassaemia. In this study, we investigated whether α-globin specific siRNA is able to mediate the destruction of α-globin mRNA in murine erythroleukemic cells (MEL). MEL cells were induced to haemoglobinise using 2% DMSO for six days then electroporated with synthetic α-globin specific siRNAs. Total RNA was harvested 24 and 48 hours after transfection and analysed using real-time PCR. Primers specific for α-globin were used to determine expression of α-globin mRNA relative to β-actin expression. β-globin mRNA expression was also monitored as a control. Four α-globin-specific siRNA sequences (siα-1, siα-2, siα-3 and siα-4) were tested in this system. Three constructs generated significant reductions in α-globin expression at the mRNA level, reducing α-globin mRNA by between 40–80% as determined by real-time PCR. β-globin expression remained unaffected. In this study, we have identified several α-globin-specific siRNA sequences effective in generating knockdowns of murine α-globin mRNA. Our initial results indicate that siRNA can effectively direct the destruction of α-globin mRNA, a result which supports our approach of developing an siRNA based therapy for β-thalassaemia.

P21 "Off Target" Effects in Designing dsRNAs for Silencing of the AT1-R

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RNA interference (RNAi) is a gene-silencing phenomenon that involves double-stranded RNA (dsRNA)-mediated cleavage of mRNA. It has emerged as a powerful technology to specifically knockdown genes in mammalian cells both for functional analysis and for therapeutic purposes. RNAi is considered to be a sequence-specific gene silencing mechanism that is dependent on a perfect match between the guide strand of the dsRNA and the mRNA target sequence. However, selection of the targeted region and design of dsRNA sequences are currently a trial-and-error process. Only about 25% of dsRNA sequences that have been selected are target specific and functional. Therefore, in a given experiment, several synthetic dsRNAs need to be generated and tested for every target gene. We now know that the difficulty in designing dsRNA sequences is the 'off-target' silencing effects of a given dsRNA duplex.
In this study, we have designed three sets of dsRNAs specifically targeted the mRNA of AT1-R and constructed three DNA plasmids encoding these dsRNA. The silencing effects against the target were assessed in the level of mRNA and receptor protein expression. We showed significant differences between the three sequences, i.e.: dsRNA-A silenced AT1-R to $22 \pm 1\%$ of its original level, where as dsRNA-B only to $70 \pm 2.08\%$ and dsRNA-C to $67 \pm 2.1\%$. Our results indicated that one of the three dsRNA targeted for the AT1-R was selective and efficient in silencing the AT_1R gene, once again confirming the existence of "off-target" in AT1-R silencing. We are in the process of testing more sequences.

P22 Optimisation Studies for Vector Supernatant Production Prior to the Initiation of a Phase I Clinical Trial

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Our group is working towards the implementation of a Phase I clinical trial to establish the safety/efficacy of using a mutant MGMT gene to protect the bone marrow from the toxicity of chemotherapy in the treatment of high risk brain tumours. This will involve retroviral transduction of donor haemopoietic stem cells with vector-containing supernatant from the packaging cell line PG13.
Our aim is the production of clinically viable volumes of high titre vector supernatant, which we intend to manufacture in our Human Applications Laboratory (HAL) at CHW. Of 56 PG13 producer clones derived, 4 were chosen, based on superior titre, for intensive testing. One was then chosen for the generation of a Master Cell Bank, and our current focus is on optimising culture conditions for large scale supernatant production using this clone. The variables being tested include passage number after revival from cryostore, medium composition, the ratio of medium volume to flask surface area, and foetal bovine serum batch. In addition, cell number for plating and time from plating to supernatant collection have been tested. Vector titre is determined using analysis of transgene expression in HT1080 cells post-transduction by flow cytometry.
The results of these experiments are being used as the basis for a series of scaling up experiments. Transduction efficiency by recombinant viruses in cell factories has been shown (Okada *et al*, 2005) to significantly increase when active gassing of the cultures is undertaken. Experiments will be undertaken to determine if this is the case with PG13 cells.

P23 Correction of Methylmalonic Acidopathy *In Vitro* and *In Vivo* using a Lentiviral Vector

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Introduction: Methylmalonic Aciduria (MMA) is a rare autosomal recessive disorder of organic acid metabolism most commonly resulting from a reduction of methylmalonyl CoA mutase activity [1,2]. Therapeutic options for MMA are limited and morbidity and mortality remain high. Therefore, the development of an effective therapy for MMA remains a high priority. Positive results from liver and combined liver/kidney transplantation [3,4], suggest that a metabolic sink therapy may be efficacious.
Gene therapy offers a more generally applicable approach to treatment of MMA than organ transplantation. Accordingly, we are developing lentiviral (LV) vector-mediated gene transfer in *in vitro* and *in vivo* models of MMA.
Results: A LV vector that expresses methylmalonyl CoA mutase under the transcriptional control of the elongation factor 1α gene promoter was constructed and used to transduce MMA CoA mutase knockout mouse fibroblasts. The incorporation of C^{14} into TCA precipitable material was then used to demonstrate metabolic correction of propionyl catabolism. Vector copy number was evaluated by real-time PCR.
The LV vector was then injected into the tail vein of an MMA mouse model (n = 3). Untreated mice (n = 2) and normal mice (n = 5) were used as controls. Blood and urine are being collected periodically and the level of methylmalonic acid directly quantified by mass spectrometry. Preliminary data suggest a gradual decrease in methylmalonic acid levels in the urine of treated mice compared to untreated controls.
Conclusion: These results suggest the lentiviral vector mediated gene therapy for MMA may offer a viable and widely applicable treatment for this disorder.

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P24 Pharmacological suppression of nonsense mutations – a therapy for Methylmalonic Aciduria

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Aminoglycoside antibiotics, including gentamicin and G418, function by disturbing codon-anticodon recognition in tRNAs, potentially resulting in translational readthrough of stop codons. This feature of aminoglycosides has been exploited to induce readthrough of premature stop codons in a number of studies investigating their potential as a treatment for genetic diseases arising as a consequence of nonsense mutations. Methylmalonic Aciduria (MMA) is an autosomal recessive disorder of organic acid metabolism resulting from a functional defect in the enzyme Methylmalonyl-CoA Mutase (MCM). Several mutations have been identified in the MCM locus of affected patients, a large proportion of which generate a premature stop codon. In order to investigate the potential of aminoglycosides as therapeutics for MMA, we generated HeLa cell clones stably expressing the entire human MCM locus, modified to include a nonsense mutation, together with an in-frame EGFP reporter gene. Cells were cultured in the presence of 500 μg/mL G418, 800 μg/mL gentamicin, or a combination of the two for a period of 72 hrs. An increase in human MCM mRNA expression was detected by Real Time RT-PCR. Flow cytometric analysis revealed a resultant increase in green fluorescence following treatment with G418 and gentamicin alone, with an additive effect when the two were combined, giving a 25–40% increase in MPF after 72 hrs. These *in vitro* data highlight the potential of aminoglycosides as pharmacological agents to alleviate the effects of the genetic defect causing MMA in some patients. Future plans involve further evaluation of these drugs in various existing mouse models of MMA.

P25 Effective Correction of Dystrophin Loci in *mdx* Mouse Myogenic Precursors

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Targeted Corrective Gene Conversion (TCGC) in a number of formats, involves the introduction of corrective nucleic acids (CNAs) to nuclei containing a mutation, and holds much promise as a future therapy for many hereditary diseases in humans. Mutation correction frequencies varying between 0.001% and 40% have been reported using Chimeraplasty (Cpy), oligonucleoplasty (OP) and small corrective PCR amplicons (CPA). Accurate detection of correction is straightforward in the case of phenotypic correction at the protein level, but in cases where either low frequencies of correction occur, detection of correction at the protein level is not possible, PCR technology used to detect correction has great potential to either falsely indicate or otherwise greatly exaggerate the presence of corrected loci. In all cases, potential artifactual exaggeration of TCGC can be controlled by tightly controlled experimental detection protocols which incorporate primer strategies designed to minimise possible participation in subsequent PCR-based detection steps, and by use of extraction procedures that minimise or eradicate the carryover of CNA into the nucleic acids extracted from cells/tissues subjected to TCGC. Using SFHR for correction of the exon 23 dystrophin (*DMD*) gene mutation in the *mdx* mouse model of DMD, we show a generic locus-specific detection protocol designed to eradicate residual CNA species and avoid the artifactual or exaggerated detection of gene correction, which allows the

accurate detection and quantification of very low proportions of genes and transcript corrected by the SFHR process.

P26 Identification of Muscle-Remodelling Non-Hemopoietic Bone Marrow Stem Cells Using The Stem Cell Factor Receptor, c-Kit

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Muscle disease, including the X-Linked Duchenne Muscular Dystrophy involves the loss of significant amounts of muscle tissue. For this reason, non-muscle (stem) cell therapy may provide a means by which therapeutic non-disease gene locus can be delivered whilst simultaneously "replacing" the lost cells in the diseased muscle. However, if such cells are used to deliver donor-derived nuclei, the cells will ultimately undergo immune rejection. Alternatively, if autologous non-muscle (stem) cells are used, they will carry the original mutation that gave rise to the tissue loss in the first place. Thus, in terms of stem cell therapy for hereditary muscle conditions such as DMD, autologous cells in which the mutation has been corrected at the chromosomal locus provide a means by which autologous cells can be used to rebuild lost muscle. This study investigates the possible presence of a bone marrow-derived cell (BMC) population with enhanced myo-regenerative capacity and the selectable marker(s) that can be used for autologous (corrected) locus delivery in dystrophic muscle. We show that myo-regenerative activity does not differ in cells FACSorted on the basis of Sca-1 antigen alone. Further co-fractionation of BM using lineage and CD45 cell surface markers in addition to Sca-1 however, did generate Sca-1 Positive cells with significant myo-remodelling capacity but also that the myo-remodelling capacity was in fact further enhanced in cells selected using c-Kit (Stem Cell Factor Receptor) antigen. These results suggest that c-Kit may provide a useful selectable marker for the selection of cells with myo-remodelling capacity from BM and possibly other non-muscle tissues.

(Numbers refer to abstract numbers)