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6th AUSTRALASIAN GENE THERAPY SOCIETY MEETING

Date

April 29 – May 1, 2009

Venue

Kerry Packer Education Centre
Royal Prince Alfred Hospital
Sydney, NSW, Australia

The Australasian Gene Therapy Society (AGTS) held its sixth biennial meeting between 28th April and 1st May 2009. 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 6th meeting of the society were:

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KEYNOTE PRESENTATIONS

KEYNOTE 1: DEVELOPMENT OF GENE THERAPY FOR DUCHENNE MUSCULAR DYSTROPHY USING SYSTEMIC DELIVERY OF AAV VECTORS EXPRESSING TRUNCATED DYSTROPHINS

Jeffrey S. Chamberlain,[1] Brian Schultz,[1] Guy Odom,[1] Zejing Wang,[2] Stephen Tapscott[1,2] and Paul Gregorevic[1,3], [1]*Dept. of Neurology, Muscular Dystrophy Research Center, University of Washington Medical School, Seattle, USA;* [2]*Division of Human Biology, Fred Hutchinson Cancer Research Center,* [3]*Baker Heart Institute, Melbourne, Australia.*

Duchenne muscular dystrophy (DMD), is caused by mutations in the dystrophin gene. We are developing methods to deliver therapeutic genes to muscles throughout the body to either replace the missing dystrophin gene or to help compensate for the lack of dystrophin. We show that shuttle vectors derived from adeno-associated virus type 6 (rAAV6) are able to deliver genes to muscles throughout the body of adult mice when injected directly into the bloodstream. rAAV6 delivery results in highly efficient gene expression in skeletal and cardiac muscle that persists for the lifespan of the mouse. However, the AAV shuttles have a limited carrying capacity, and as a result we have also been developing truncated versions of the dystrophin gene that can be carried by AAV yet retain sufficient functional capacity to halt dystrophy. Subtle modifications to the design of micro-dystrophin result in dramatic differences in properties such as force development, neuromuscular junction maturation, myotendinous junction fragility and formation of ringed myofibers. A single injection of an AAV6/micro-dystrophin vector into the vasculature of adult, dystrophic mice results in elimination of dystrophic histopathology for the lifespan of the mouse. We are also focused on scaling up the procedures in the dog model of DMD. These studies revealed a cellular immune response directed against the AAV capsid proteins, but which could be blocked by short-term immune suppression, leading to long-term dystrophin expression. We have therefore begun testing whether AAV6 vectors can be delivered via the vasculature to dogs. We have observed that delivery of AAV6 vectors into various veins and arteries of the dog results in efficient gene transfer to downstream muscles, but does not lead to whole body gene transfer. Instead, it appears that vector will need to be delivered into multiple vascular sites to target muscles body wide. These results suggest that a combination of intravascular AAV delivery coupled with transient immune suppression could lead to an effective therapy for DMD.

KEYNOTE 2: SPLICE SWITCHING THERAPIES AS PERSONALISED GENETIC TREATMENTS: APPLICATIONS TO MUSCULAR DYSTROPHY, THALASSEMIA AND SPINAL MUSCULAR ATROPHY

Steve Wilton and Sue Fletcher, *Centre for Neuromuscular and Neurological Disorders, University of Western Australia*

Protein-truncating mutations in the dystrophin gene result in Duchenne muscular dystrophy (DMD), the most common and severe form of childhood muscle wasting. The entire dystrophin protein is not necessary for near normal function, as some Becker muscular dystrophy patients with in-frame dystrophin gene deletions present with minor symptoms. A phosphorodiamidate morpholino oligomer designed to excise dystrophin exon 51 from the mature mRNA was evaluated in a Phase I clinical trial. The test compound was injected into the *extensor digitorum brevis* muscle of non-ambulant DMD patients, and subsequent analysis showed "an unequivocal, widespread and robust response in terms of dystrophin positive fibres". Systemic trials are now underway. This is good news for the estimated 10% of DMD individuals who may benefit from exon 51 skipping, what about other DMD mutations? While 10–12 AOs should restore the reading-frame in the common genomic deletion hotspots, scores of AOs will be needed to by-pass the many different protein-truncating mutations spread across the gene.

Multiple gene transcripts are generated from at least 75% of human genes through alternative splicing. Although this greatly increases our genetic plasticity, the additional levels of splicing control offer multiple opportunities for things to go wrong. An estimated 15% of all gene mutations induce abnormal pre-mRNA splicing. Splice switching AOs were first shown to suppress abnormal splicing by masking of activated cryptic splice sites that otherwise lead to aberrant mRNA, such as β-globin mutations causing thalassemia.

In addition to inducing exon skipping or suppressing cryptic splice sites, some situations require an exon to be incorporated in the mature gene transcript. AO targeting of silencing elements in a pre-mRNA can promote exon inclusion, a mechanism relevant to the second most common autosomal recessive disorder, Spinal muscular atrophy. If exon skipping can be successfully applied to DMD, great opportunities lie ahead for splice switching therapies for other different genetic or acquired disorders.

KEYNOTE 3: TARGETED AND ARMED ONCOLYTIC POXVIRUSES: A NOVEL MULTI-MECHANISTIC THERAPEUTIC CLASS FOR CANCER

Caroline Breitbach, David H Kirn, *Jennerex Biotherapeutics Inc., San Francisco, CA – USA and Department of Clinical Pharmacology, University of Oxford (UK); dkirn@jennerex.com*

Engineered viruses have been developed for cancer therapy both as non-replicating gene therapy agents and as cancer vaccines. Oncolytic viruses, in contrast, were developed to replicate within, and subsequently lyse, cancer cells. Clinical efficacy to date with each of these approaches has been limited by multiple factors including the inability to infect enough tumor cells *in vivo* locally within a tumor or systemically, and resistance of complex advanced tumors to a single mechanism-of action (MOA). Over the last several years, however, a novel therapeutic class has emerged that combines the best features of all three approaches: targeted and armed oncolytic poxviruses. Recent preclinical and clinical results demonstrate convincingly that products from this therapeutic class can achieve highly selective and potent cancer destruction systemically through a multi-pronged MOA. Given recent clinical validation, we expect this therapeutic class to expand rapidly.

KEYNOTE 4: ADENO-ASSOCIATED VIRAL VECTORS IN CLINICAL TRAILS OF LIPOPROTEIN LIPASE DEFICIENCY

Anthony J. Gringeri and Sander van Deventer, *Amsterdam Molecular Therapeutics, P.O. Box 22506, 1100 DA Amsterdam, The Netherlands.*

Over the last twenty years, gene therapy has become a practical clinical tool partly because of advances in vector technology. Adeno-associated viral vectors (AAV) are non-pathogenic, replication-deficient parvoviruses that transfer DNA most efficiently to tissues with a low turnover rate, e.g., muscle, brain, liver, and retina. The transgenes then remain mainly episomal. Individual AAV serotypes display distinct tissue tropisms, allowing the transduction of target tissues with great specificity. Therapeutic effect with a variety of transgenes has persisted lifelong in laboratory animals. AMT has performed two clinical trials using the transgene encoding lipoprotein lipase (LPL), the enzyme catalyzing triglyceride (TG) catabolism. Patients with LPL deficiency have abnormally high serum TG concentrations and chylomicronemia, which may cause life-threatening pancreatitis (PT). In these clinical trials, the majority of patients showed a reduction in serum TG as well as improvement in other symptoms like eruptive xanthomas and lipemia retinalis. Analysis of muscle biopsies showed that expression of active LPL protein correlated well with the number of genome copies administered. Furthermore, tissue staining revealed LPL-mediated lipid uptake in injected muscle, whereas no uptake was seen in non-injected muscle from the same patients. Most importantly, treated patients showed a nearly seven-fold reduction in the incidence of pancreatitis. Treatment was well tolerated, with an acceptable safety profile. Thus, AAV are a safe and effective means to mediate gene therapy in humans.

KEYNOTE 5: APPLICATION OF MOLECULAR IMAGING IN GENE AND CELL THERAPY

Shahriar S. Yaghoubi Ph.D., *Molecular Imaging Program, Department of Radiology, School of Medicine, Stanford University, USA*

Molecular imaging is enabling non-invasive analysis of therapeutic transgene (TG) or therapeutic cell (TC) pharmacokinetics through time in living subjects, including humans. The expression of a TG can be imaged either directly using a specific imaging probe that detects the products it encodes, or indirectly by linking its expression to that of an imaging reporter gene (RG). Indirect TG expression can be accomplished by co-vector administration or designing a fusion TG-RG genetic construct, a bicistronic construct containing the TG and RG under control of the same promoter, but separated by an internal ribosomal entry site, a single genetic construct containing both RG and TG with double identical promoters, and a bidirectional transcriptional approach. All of these techniques have been studied successfully in pre-clinical models. The direct approach has been used to monitor suicide gene therapy with

Herpes Simplex Virus 1 thymidine kinase (HSV1-tk, or its mutants) in rodents with implanted tumors and in patients with hepatocellular carcinoma. Three different techniques have so far been used, evaluated or envisioned for imaging TCs in living subjects. Direct labeling of cells with imaging probes is the simplest technique and has been demonstrated in humans using radionuclide or MRI based probes. Another technique that involves genetically engineering TCs with a RG and then imaging them with a reporter probe (RP) can allow long-term imaging of all aspects of TC pharmacokinetics (biodistribution, survival, status) at multiple time points in living subjects. We recently reported the first case of imaging cytolytic T cells homing to glioblastomas, using the HSV1-tk RG and the positron emission tomography RP, [^{18}F]FHBG in a patient (Nature Clinical Practice Oncology 6(1):53–58 (January 2009)). Finally, one can envision developing a new probe that can specifically detect a receptor associated with the therapeutic cells. Molecular imaging should help optimize cell/gene therapy protocols for patients.

The Greg Johnson Memorial Oration

A PERSPECTIVE ON CANCER GENE THERAPY

Professor Pamela J Russell, *Oncology Research Centre, Prince of Wales Hospital Clinical School, The University of New South Wales, Randwick NSW 2031, Australia*

Thirty years after its initiation, human gene therapy is now in an exciting new area of medical research, due to clearer principles of biology, progress in genomics and proteomics and developments in genetic engineering. Strategies for cancer gene therapy are based on knowledge of the biology of the particular cancer, and include targeting a defective gene, such a tumour suppressor gene, upregulation of drug resistance genes on normal tissues in conjunction with chemotherapy, down-regulation of specific genes through the use of siRNA, or targeted killing within a non-essential organ, such as the prostate through gene-directed enzyme drug therapy (GDEPT). Alternatively, strategies are developed to upregulate anti-cancer immune responses. With respect to cancer, development of delivery vehicles that are efficient at allowing the gene to reach target tissues at adequate levels and specific to only target cancer tissues with appropriate regulation to protect the normal tissues, have formed the main thrust of current research. Thus, improving the potency and specificity of therapeutic delivery and increasing safety profiles remain major challenges in clinical oncology. Delivery by viruses is currently the most effective. Some viruses, such as Adenoviruses can be targeted more specifically to cancer cells both transcriptionally, through the use of regulatory elements to target the appropriate tissue/cancer, as well as transductionally, through the use of tropism modified viruses that can enter cancer cells through tumour-specific ligands. However, despite progress, even targeted gene therapy suffers from inefficient gene transfer especially in the clinical scenario and the immunogenicity of Adenoviruses remains as an obstacle to their use. Given the inadequacies often encountered with individual therapy regimens, improved results have been obtained when some of these strategies are used in combination, such as different combined GDEPT strategies plus immune- or radio-therapy, or GDEPT and chemotherapy. Novel approaches include the combination of viral/nonviral gene vectors with nanotechnology which is based on preferential accumulation of nanoparticles within tumour cells. The versatility of such systems stems from their prospects in targeted therapeutics and concomitant imaging; some formulations of iron-oxide nanoparticles are now FDA approved for cancer imaging and drug delivery. Cellular therapies provide the perfect arena to allow systemic and targeted delivery of gene vectors or even better, nanaoparticles together with gene vectors. New directions that will lead to the final culmination of gene therapy as stand alone or a potent adjuvant therapy to current therapies will be highlighted.

ORAL PRESENTATIONS (* Invited)

O1: INDUCED NON-PRODUCTIVE SPLICING TO STUDY MUSCLE GENE EXPRESSION

Sue Fletcher, Abbie Adams, Russell Johnsen, Kane Greer and Steve Wilton, *Molecular Genetic Therapy Group, Centre for Neuromuscular and Neurological Disorders M518, University of Western Australia, Crawley 6009, WA*

RNA silencing has been applied to suppress gene expression, with varying degrees of specificity and efficiency reported. Endogenous alternative splicing can regulate gene expression through a process called Regulated Unproductive Splicing and Translation (RUST), by either incorporating an exon carrying a nonsense mutation, or excising an exon to disrupt the reading frame. As a result, the mature gene transcripts cannot be translated into functional products. We show that it is possible to efficiently disrupt the normal dystrophin mRNA reading frame and ablate dystrophin expression. Total suppression of dystrophin gene expression can be induced and maintained for several weeks *in vivo*, and a severe dystrophic pathology observed within 4 weeks of commencing treatment in neonatal normal mice. This approach to gene down-regulation is very efficient and specific. Disruption of gene expression by selected exon exclusion could be applied to many different genes, and offers the opportunity to induce transient mouse models to study the consequences of gene suppression *in vivo*. In addition, selected exon removal to yield in-frame transcripts can allow mapping of functional protein domains, based upon exon boundaries, and provide a possible alternative to transgenic mouse models for the study of muscle gene expression. We are applying this approach to defining dystrophin functional domains, to optimise exon-skipping strategies for the treatment of Duchenne Muscular Dystrophy.

O2: USING RAAV-VECTOR MEDIATED GENE TRANSFER TO STUDY AND POTENTIALLY TREAT MUSCLE WASTING

Paul Gregorevic[1,2] & Jeffrey S. Chamberlain[2], [1]*Laboratory for Muscle Research & Therapeutics Development, Division of Metabolism and Obesity, BakerIDI Heart and Diabetes Institute. Melbourne 3004, Australia.* [2]*Department of Neurology, The University of Washington, Seattle 98195, USA*

Many medical conditions are caused or aggravated by the loss of skeletal muscle function. The prevention and treatment of muscle-related illness could significantly improve human health, but requires a more complete understanding of the mechanisms that govern muscle adaptation in health and disease. To this end, gene delivery technologies could potentially revolutionize the study of muscle, and accelerate the development of novel interventions for muscle-related disease. As an example, recombinant vectors derived from

adeno-associated viruses can readily and rapidly achieve sustainable expression of transgenes and transcription-regulating elements in mammalian skeletal muscle, via local and intravascular administration. These vectors offer the opportunity to dissect the intracellular mechanisms underlying the phenotypic adaptation of muscle with degrees of precision and speed otherwise unachievable.

To better understand acute muscle growth and wasting, we have been studying effects of rAAV6-mediated follistatin expression in models of muscle adaptation and disease. We have observed that rAAV6 : Fst administration promotes muscle fiber hypertrophy to increase murine skeletal muscle mass and force producing capacity by 100% and >50% respectively within 6 weeks. Hypertrophic effects mediated by this intervention are constrained to the region of transduction, but intravascular rAAV6 : Fst administration to mice promotes body-wide muscle hypertrophy and increases contractile capacity on a similar time scale, for over 24 months. We have ascertained that interventions incorporating rAAV6 : Fst administration can increase muscle mass and function in murine models of wasting including cancer cachexia and aging-associated sarcopenia, which suggests the cellular events affected by this intervention may be novel targets for prospective therapies. Our findings are being used as a basis for further investigation into the cellular events of muscle wasting and the potential to ameliorate disease via novel acute and long-term genetic interventions.

O3: EFFECTS OF DOSE AND EMPTY CAPSIDS ON RAAV6 GENE DELIVERY TO STRIATED MUSCLE IN MICE

Brian R. Schultz,[1] Paul Gregorevic,[2,3] James M. Allen,[2] Eric Finn,[2] Jeffrey S. Chamberlain[1,2], [1]*Molecular and Cellular Biology, University of Washington, Seattle, WA, USA* [2]*Department of Neurology, University of Washington School of Medicine, Seattle, WA, USA* [3]*Baker IDI Heart and Diabetes Institute, Melbourne, Victoria, Australia*

Neuromuscular diseases such as Duchenne muscular dystrophy may require therapeutic gene delivery to all striated muscles of the body, including cardiac, diaphragmatic, and limb skeletal muscles. While whole-body gene delivery to mouse muscles via recombinant adeno-associated viral (rAAV) vectors has proven to be relatively simple at adequate doses, translating such results to larger animals and to human patients is more challenging. In order to further understand systemic rAAV gene delivery properties and potentials for enhancement, we examined rAAV6 reporter gene transduction in mouse. In a dose response study of IV-injected rAAV6, we found that once an apparent vector genome dose threshold is surpassed, transgene expression levels increase logarithmically over a linear range of doses. Furthermore, each muscle responds uniquely to the dose increases. We also determined that including empty capsids (carrying no vector genome) at a sub-threshold vector genome dose enhances transgene expression levels on a muscle- and capsid serotype-specific basis. We observed expression increases of 2-fold in heart, 30-fold in diaphragm, and 70-fold in soleus. While AAV1 and AAV6 empty capsids produce similar expression augmentation, neither AAV2 nor AAV8 empty capsids enhance transgene expression by rAAV6 vectors. An equivalent addition of genome-carrying rAAV6 capsids increases transgene expression above that produced by empty capsids, but only 2–4 fold. We therefore conclude that much of the logarithmic expression increase beyond the dose threshold is due to capsid concentration. Although AAV6 empty capsids increase transgene expression by an order of magnitude in some muscle tissues, vector genome concentration in the same tissues increases less than 4-fold. Thus, empty capsids appear to effect expression increases largely by intracellular mechanisms in target cells. We expect these findings will lead to an increased understanding of the processes involved in rAAV vascular administration to target tissues and will assist the design of vector delivery protocols.

O4: EXCEPTIONS TO THE READING FRAME RULE IN DMD: WILL EXON SKIPPING BE RELEVANT?

Kane Greer, Russell Johnsen, Steve Wilton, Sue Fletcher, *Molecular Genetic Therapy Group, CNND, University of Western Australia, Perth*

Duchenne Muscular Dystrophy (DMD) is a severe muscle wasting disease caused by the absence of a functional dystrophin protein. The majority of the mutations in the dystrophin gene cause the protein to be prematurely truncated and therefore non-functional. Mutations that result in an internally deleted protein lead to a milder allelic condition, Becker muscular dystrophy. However, not all dystrophin mutations comply with the reading frame rule. We report two small in-frame exon duplications that, while expected to show a BMD phenotype, were found to cause DMD. This genotype: phenotype inconsistency could arise from disruption of an essential functional domain, or compromised transcription across the massive intron 2, which is in excess of 170 kb. Here we describe the application of phosphorodiamidate morpholino oligomers coupled to a cell penetrating peptide (PMO) to remove selected exons during mRNA processing. Myoblasts prepared from two DMD patients, one with a duplication of exon three, and the other, exons three and four, which are both in-frame re-arrangements, were transfected with PMOs. Oligomers were targeted at either exon three or four, or the two exons together in a cocktail. RNA was analysed by RT-PCR to monitor exon skipping and RNA levels were quantitated using real-time PCR. Preliminary studies have failed to detect protein in untreated cells, suggesting a defect in gene transcript processing.

O5: SIRNA-MEDIATED REDUCTION OF α-GLOBIN: APPLICATIONS IN β-THALASSAEMIA

Jim Vadolas, Hady Wardan and Hsiao Voon, *Cell and Gene Therapy Research Group, The Murdoch Children's Research Institute, The University of Melbourne, Royal Children's Hospital, Flemington Road, Parkville 3052, Melbourne, Australia*

β-Thalassaemia arises when α-globin is synthesised at levels exceeding the binding capacity of available β-globin chains, usually due to mutations affecting the β-globin locus, which reduce β-globin expression. Excess α-globin chains precipitate in erythroid progenitor cells resulting mechanical damage to membrane structures and trigger premature apoptosis in erythroid progenitor cells, leading to ineffective erythropoiesis. The key role of globin imbalance in contributing to thalassaemia severity is most clearly illustrated in individuals who inherit an abnormal number of functional α-globin genes along with β-globin mutations.

Individuals who co-inherit α-thalassaemia with homozygous β-thalassaemia have an improved phenotype and suffer less severe anaemia than if either set of mutations were inherited alone. The degree of correction is correlated closely with the degree to which globin balance has been restored. One mutated copy of α-globin generally has minimal impacts but two or three mutated α-globin genes can improve thalassaemic phenotypes significantly. Hence, alterations in α-globin chain synthesis have considerable effects on β-thalassaemic phenotypes. This raises the possibility of targeting α-globin as a therapy for β-thalassaemia. We have explored the feasibility of utilising short-interfering RNA (siRNA) to mediate reductions in α-globin expression. A number of siRNA sequences targeting murine α-globin were tested in haemoglobinised murine erythroleukaemic cells. One highly effective siRNA sequence (si-α 4) was identified and reduced α-globin by approximately 65% at both the RNA and the protein level. Electroporation of si-α 4 into murine thalassaemic primary erythroid cultures restored α : β-globin ratios to balanced wild-type levels and resulted in detectable phenotypic correction. These results indicate that siRNA-mediated reduction of α-globin has potential therapeutic applications in the treatment of β-thalassaemia. A number of studies have demonstrated phenotypic improvements by utilising the RNA interference (RNAi) pathway to reduce expression of disease causing genes. These include both vector-expressed shRNAs and siRNA-based approaches and it is likely that both approaches will have their place in clinical medicine.

O6: CATIONIC LIPOSOME-CARBONATE APATITE CONJUGATES: NOVEL HYBRID CARRIER FOR MRNA-BASED GENE THERAPY

Fatema T. Zohra, Ezharul H. Chowdhury, Toshihiro Akaike, *Department of Biomolecular Engineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Japan*

The employment of mRNA as a gene therapy tool is very beneficial for the majority of cells *in vivo* which are mostly non-dividing or slowly dividing. mRNA transfection strategy is also applicable for cancer vaccination or immunotherapy. We designed a novel hybrid carrier for mRNA through decorating inorganic carbonate apatite onto cationic liposome of DOTAP {N-[1-(2,3-dioleoloxy)propyl]-N,N,N-trimethyl ammonium chloride} and analyzed the processes involved in the transfection potency. Transmission electron microscopic (TEM) observation clearly indicated the presence of inorganic carbonate apatite particles on mRNA-liposome complex and demonstrated the structure of the new hybrid carrier material. Hybrid-particle-associated cell membrane contact of fluorescein labeled luciferase mRNA was significant as observed under confocal microscope. Consequentially cellular uptake of labeled mRNA was also quite notable for DOTAP-apatite carrier compared to DOTAP and lipofectamine 2000. Flow cytometric analysis further revealed cell associated higher fluorescence level for DOTAP-apatite carrier in comparison with DOTAP and lipofectamine 2000. Endocytosis mediated enhanced internalization was confirmed through evaluating tranfection efficiency in the presence of endocytosis inhibitors and labeling cell endosome with observation under confocal microscope. Enhanced gene expression (10 to 20 times) of in vitro transcribed luciferase mRNA was observed for DOTAP-apatite mediated transfection in HeLa cell, NIH3T3 cell (dividing cell) and mouse primary hepatocytes (non-dividing). We propose that the way of internalization of the carrier bound gene just after binding on cell membrane is the determinant factor for final gene expression. Although internalization way was evaluated as endocytosis, more specifically, use of amiloride which is a specific inhibitor of macropinocytosis showed a drastic reduction of the uptake of fluorescein labeled mRNA through DOTAP-apatite carrier not for only DOTAP. We compared transfection efficiency of mRNA and pDNA in Human Umbilical Vein Endothelial cell (HUVEC) to demonstrate advantages of mRNA – based gene therapy. Taken together, this carrier showed potentiality over the existing mRNA carriers of cationic liposomes and could be very effective for future application of mRNA – based gene therapy.

O7*: THE TRIUMPHS AND TRIBULATIONS OF A GENE THERAPY TRIAL

Elizabeth Rakoczy, *Centre for Ophthalmology and Visual Science, University of Western Australia*

Age Related Macular Degeneration (AMD) is the major cause of blindness in the developed world. The severe "wet" or neovascular form of AMD has a rapid progressive course and is difficult to treat. AMD is a complex disease with a strong life style factor. Regardless of the causative agent, patients with "wet" AMD present with elevated levels of vascular endothelial growth factor (VEGF) in the eye. Thus, neutralising this excessive VEGF was expected to be a useful strategy.

The concept of secretion gene therapy was developed and a recombinant adeno-associated viral (rAAV) vector that expressed sFlt-1, a soluble form of the VEGF receptor was produced and tested in a wide range of *in vitro* and *in vivo* studies. Preclinical studies have demonstrated that following a single injection into the eye, rAAV.sFlt inhibited some of the ocular pathological changes associated with VEGF activity in a mouse model of retinal neovascularisation and in a primate model of "wet" AMD. Encouraged by the lack of toxicity and an 18 months of efficacy both in mouse and primate models we started to pursue regulatory approval for a Phase I clinical trial in 2005. Finally, in January 2009 we received a conditional approval from the Therapeutics Goods Administration. If the trial is successful we hope that a single injection of rAAV.sFlt-1 will be sufficient to treat "wet" AMD and prevent the recurrence of blood vessel formation.

O8: A STEROID-INDUCIBLE PROMOTER FOR LENTIVIRUS-MEDIATED TRANSDUCTION OF THE CORNEA

DGA Parker, HM Brereton, S Klebe, DJ Coster, KA Williams, *Department of Ophthalmology, Flinders University, Adelaide, Australia*

Kaplan-Meier corneal graft survival within Australia is 51% at 15 years, and the most common cause of failure is immunological rejection. *Ex vivo* gene therapy of the donor cornea can modulate corneal transplant rejection in animal models. Our aim was to investigate a steroid-inducible promoter for use in corneal gene therapy. A 320 base pair sequence encoding five glucocorticoid response elements (GRE) was cloned into a non-replicative,

self-inactivating, VSVG-pseudotyped lentiviral vector (LV) carrying the ovine interleukin 10 (IL10) gene (LV-GRE-IL10). A similar vector carrying the simian virus 40 (SV40) promoter was used for comparative purposes (LV-SV40-IL10). A549 cells, ovine, and human corneas were cultured *in vitro* in the presence or absence of dexamethasone. Transgene expression was assessed by an ovine IL10-specific ELISA. Expression of secreted IL10 in A549 cells transduced with LV-GRE-IL10 was 35-fold higher in the presence of 50 nM and 1000 nM dexamethasone than in its absence. Dexamethasone withdrawal resulted in return of secreted IL10 to control levels. Ovine corneas transduced with LV-GRE-IL10 showed low, basal expression of IL10 in the absence of dexamethasone. After 9 days, supernatants from corneas transduced with LV-GRE-IL10 and cultured with glucocorticoid contained 9 times more IL10 than control corneas, and 3 times more IL10 than corneas transduced with LV-SV40-IL10 at the same MOI. Levels of IL10 in supernatants from human corneas transduced with LV-GRE-IL10 were approximately 10-fold higher than in transduced corneas cultured without dexamethasone. Thus, transduction with a lentiviral vector containing a steroid-inducible promoter resulted in marked upregulation of transgene expression in A549 cells and in ovine and human corneas. Transgene expression levels were higher than levels achieved by a vector containing the SV40 promoter. The GRE promoter exhibited low leakiness in the absence of dexamethasone, and shows promise for controlled transgene expression in the cornea.

O9: TY3 YEAST RETROTRANSPOSON INTEGRASE ACTIVITY IN HUMAN CELLS

Nicholas Casey, Greg Woods, *Menzies Research Institute, University of Tasmania, Hobart, Australia*

Integrating gene therapy vectors, while providing efficient and stable transgene expression, carry a risk of insertional mutagenesis. Consequently, there has been much research into improving their safety.

Retrotransposons, while related to retroviruses, lack the machinery for lateral transfer between cells. In the Ty3 yeast retrotransposon, selection pressure for minimal disruption to the host genome has resulted in a targeting mechanism that integrates the retrotransposon immediately upstream of Pol-III class, tRNA genes.

The retention of such characteristics in human cells would make this targeting mechanism very attractive for gene therapy purposes. Ty3 integrase associates with human tRNA genes expressed in yeast cells, and a chimeric form is active but non-specific in human cells.

We tested the activity of the complete or 'wild type' integrase in human cells.

We constructed a vector co-expressing the Ty3 Integrase with a growth-selection marker. The control vector lacked the integrase gene. We also constructed a marker 'integrant' of a Green Fluorescent Protein (GFP) gene flanked by Ty3 retrotransposon Long Terminal Repeats. This integrant and one or other vector were co-transfected into 293T cells by Calcium Phosphate precipitation transfection.

Initial GFP expression was widespread in both groups, indicating successful transfection of the transgenes. As expected, fluorescence was lost from some or all cells of many colonies. This was ascribed to transient expression. Similarly, a certain rate of 'passive integration' (integration not mediated by integrase) was expected in the control group. Importantly however, where the Ty3 integrase was expressed, the proportion of colonies exhibiting stable, ubiquitous expression of the GFP marker was many-fold higher than in controls.

These results indicate that 'wild-type' Ty3 integrase is active in human cells and can facilitate stable transgene expression.

O10: SUPPRESSION OF CLONAL DOMINANCE IN CULTURED HUMAN LYMPHOID CELLS BY ADDITION OF THE CHS4 INSULATOR TO A LENTIVIRAL VECTOR

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Lentiviral vectors efficiently transduce quiescent stem cells and are being evaluated for gene therapy of blood disorders and other genetic diseases. Reducing the risk of genotoxicity due to insertional mutagenesis is an important safety consideration. The 5′ chicken beta-globin hypersensitive site 4 (cHS4) insulator inherently possesses chromatin barrier and enhancer-blocking activities. In the context of evaluating various vector designs, we inserted the cHS4 insulator into a self-inactivating lentiviral vector backbone expressing green fluorescent protein (GFP) under the control of a truncated MSCV promoter. Using human T-cells (Jurkat), GFP-positive single-cell isolates were obtained nine days post-transduction and evaluated by Southern blot for vector copy number and integrity, as well as integration site analysis via LM-PCR. Isolates containing an intact single-copy control vector genome were compared to those containing a vector with cHS4 insulator elements flanking the GFP expression cassette. All chromosomes, including the previously identified hot-spot region, 11q13, were targeted similarly by both vectors. The same consensus oligonucleotide palindrome was also identified at the LTR-genomic junction for both vectors. The insulator had minimal effect on mean fluorescent intensity and only modestly reduced the variability of GFP expression in a sub-set of individual single cell isolates cultured for four months. For both vectors, most unique integration sites were intragenic but the insulator-containing vector had a moderate predilection to integrate near transcriptional start sites (TSS). Clonal dominance was evident in the population of cells containing the integrated control vector genome, as reflected by the recovery of multiple single-cell isolates containing the same integration site. We infer that certain integrations conferred a proliferative or survival advantage by affecting gene expression via insertional mutagenesis leading to this clonal dominance. Despite the preference to integrate near the TSS, this dominant effect was significantly diminished by incorporating flanking insulators in the vector genome. Our data support the incorporation of the cHS4 insulator to lentiviral vectors used in gene therapy trials to enhance safety.

O11: SITE-SPECIFIC INTEGRATION OF FUNCTIONAL GENOMIC LOCI: APPLICATIONS IN GENE THERAPY

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Vectors based on the adeno-associated virus (AAV) have attracted much attention as potent gene-delivery vehicles, mainly because of the persistence of this non-pathogenic virus in the host cell and its sustained expression of therapeutic gene(s). Moreover, the ability of wild-type AAV, or recombinant AAV (rAAV), to integrate site-specifically in chromosome 19, designated AAVS1, in the presence of the Rep protein has been considered as potentially useful for gene therapy since in principle, it should be possible to direct integration events to a known site. However, the over expression of wild type Rep induces apoptosis, which limits the therapeutic application of targeted integration of AAV vectors. We have developed a novel strategy for the targeted integration of large DNA plasmids containing the P5 integration efficiency element (P5IEE) derived from AAV. This was achieved using *in vitro* transcribed mRNA to transiently express the Rep proteins in human cells, which may circumvent the toxicity and chromosome instability associated with long-term expression of Rep. We show that the co-transfection of a 21 kb BAC containing P5IEE with Rep mRNA facilitated the targeted DNA integration to the AAV1 site in up to 30% of all analysed integration sites. No targeted integration events were observed following transfection of cells with BAC DNA alone. Using the above approach, the site-specific integration of a 200 kb BAC, containing P5IEE and carrying the entire -globin locus, modified to express the EGFP reporter under the control of the -globin promoter, was used to transfect K562 cells. We identified several K562-derived clones, up to 19% efficiency, that contained the EGFP-modified -globin locus integrated at the AASV1 site. Our study suggests that Rep mRNA transfection can be used to facilitate the site-specific integration of an entire genomic locus. We propose that this non-viral gene therapy strategy may be used in conjunction with patient-derived stem cells to facilitate persistent and stable transgene expression while avoiding the risks associated with random integration.

O12: HIGH-INCIDENCE OF LYMPHOMAGENESIS IN SCID-X1 MICE FOLLOWING LENTIVIRAL VECTOR-MEDIATED PHENOTYPE CORRECTION INDEPENDENT OF INSERTIONAL MUTAGENESIS AND γC EXPRESSION

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The development of lymphoproliferative illnesses in infants following treatment in two related gene therapy trials for X-linked severe combined immunodeficiency (SCID-X1) has prompted substantial research effort into designing vectors with improved safety profiles. Underpinning vector design is the need to induce sufficient γc expression for immune reconstitution while avoiding strong viral enhancer sequences to reduce the risk of inadvertent gene activation upon integration. This is a particular challenge as we have previously demonstrated that there is a threshold for γc expression that must be achieved for reconstitution of both T- and NK cell compartments. In this study we explore the capacity of lentiviral vectors that encode the human γc transgene to reconstitute γc deficient mice. We observed incomplete T- and B cell development in mice transplanted with progenitors expressing γc from the PGK promoter. In contrast, functional T- and B-cell compartments were restored in animals receiving an equivalent vector containing the EF1α promoter. Interestingly, lymphoma developed in four of the mice reconstituted with the EF1α-γc vector but extensive analyses revealed that the underlying mechanism did not involve insertional mutagenesis or γc over-expression. These findings indicate that malignancies observed following gene therapy may not necessarily be due to insertional mutagenesis, highlighting the need for careful analysis and interpretation of adverse events observed in preclinical animal models.

O13: A COMBINATION OF MOLECULAR CHEMOTHERAPY AND TRADITIONAL CHEMOTHERAPY: PROSPECTS OF SYNERGIES AGAINST CANCER

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In this study, we have explored the combination of a novel Purine Nucleoside Phosphorylase mediated Gene Directed Enzyme Prodrug Therapy (PNP-GDEPT) with chemotherapeutics, Taxotere and/or Carboplatin to target prostate and ovarian cancer (PC&OC). PNP converts the prodrug (fludarabine-phosphate) to a toxic purine, 2-fluoroadenine (2FA) that inhibits RNA/DNA synthesis. Taxotere is active against late-stage PC whilst carboplatin is first line therapy for OC. Neither modality is adequately effective. We expect that a combination will target heterogeneity via cytotoxicity to diverse cancer cell populations leading to effective synergies, which may improve efficacy and quality of life.

For **PC**: *Synergy between Ad-PNP-GDEPT and Taxotere were assessed in vitro and in vivo.* Cell killing effects of combination led to significant synergistic killing of human PC3 & murine RM1 PC cells accompanied by enhanced apoptosis. A lower individual dose (by up to 8 fold) led to enhanced efficacy. *In vivo*, the combination regimen given at the suboptimal doses led to reduction in *local tumour (PC3 & RM1) growth in* nude and in C57BL/6 mice, respectively. A significant reduction in lung RM1 colony numbers indicated enhanced *systemic efficacy. Combination treated mice also displayed significantly improved survival* (25 days vs 15 days for control mice). Importantly, the condition of combination treated mice (e.g. weight loss) was better than those given individual treatments. The possible involvement of the immune system in this enhanced effect is under investigation. For **OC**, three-way synergy between Ad-PNP-GDEPT, Taxotere and carboplatin was effectively demonstrated in SKOV-3 and OVCAR-3 cells. This was significantly greater than bimodal or individual treatments. A 10–50 fold dose reduction of individual

treatments was effective when combined, accompanied by enhanced apoptosis. Western-blotting analyses revealed a shift in the expression of anti-apoptotic and proapoptotic proteins upon treatment with various combinations. *This is the first demonstration of synergy between these modalities.*

O14: ADULT STEM-CELL BASED DELIVERY OF A PROSTATE CANCER GENE THERAPY

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Mesenchymal stem cells (MSC) display a tumour-tropic property that has been exploited for the targeted delivery of therapeutic genes to various metastatic carcinomas in animal models. This study explores the utility of MSC to deliver reporter/suicide genes to prostate cancer (PCa) using the syngeneic RM1 mouse model.

We recently optimised the genetic modification of murine MSC using nucleofection (Amaxa), a novel non-viral transfection method allowing plasmid DNA delivery to the nucleus of primary cells (see abstract O'Han M, *et al.*). MSC were nucleofected with reporter GFP and firefly luciferase (fluc2) and selected in hygromycin to produce stable expression. In a pilot study, when MSC-GFP cells were implanted into the mouse prostate with or without RM1 prostate tumour cells and examined on day 18 using IVIS 100 imaging (Caliper), MSC persisted only within the tumour-bearing prostate ($p<0.05$). To test their systemic homing ability, MSC-GFP were infused every second day via the tail vein of mice in the presence or absence of RM1 lung pseudometastases. MSC persisted within the lungs of RM1 tumour-bearing mice alone ($p<0.01$, compared to non-tumour bearing mice). These results suggest MSC can engraft organ-confined PCa and home to metastatic PCa.

Suicide gene therapy involves the transfer of a suicide gene into tumours to render them sensitive to prodrugs that are otherwise non-toxic to normal tissues. Prostate tumours established from RM1 tumour cells stably transfected with suicide genes cytosine deaminase and uracil phosphoribosyltransferase (CDUPRT) followed by systemic treatment with prodrug 5-fluorocytosine, showed significant reductions in tumour growth (1). Our work showed that stable nucleofection of MSC with CDUPRT (MSC-CDUPRT) prior to implantation into established RM1 prostate tumours gave similar levels of PCa killing (~75%; $p<0.05$) observed in our published experiment in the presence of prodrug. Thus MSC deliver suicide genes which convert prodrug into a diffusible toxin and show promise as an adult cell-based suicide gene therapy for PCa.

(1) Khatri A *et al.*, 2006, J Gene Medicine, 8(9): 1086–96.

O15: RNA INTERFERENCE FOR THE TREATMENT OF CERVICAL CANCERS: SOLVING THE DELIVERY AND EFFICACY ISSUES

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RNA interference (RNAi) is a novel means to silence specific gene expression. While RNAi has become a routine tool in laboratory studies, the real promise of RNAi is in its potential for the treatment of infectious diseases, cancers, and genetic disorders. In terms of cancer therapy most studies have relied upon targeting mutated or overexpressed oncogenes and while there are promising *in vitro* results efficacy data from *in vivo* small animal models has been poor. The major issues are efficacy and delivery. We will present data on the development of *in vivo* delivery systems including viruses and liposomes and their use in small animal models to deliver RNAi. We have developed a simple and effective liposome preparation technique for *in vivo* use that gives excellent delivery to tumours, is easy to prepare and is stabile for at least 4 weeks. In terms of efficacy we have discovered a novel means by which to improve the effect of RNAi via activation of the innate and adaptive immune systems. Remarkably, we show that injecting mice with tumour cells pre-treated with E7-specific shRNA resulted in protection from subsequent injections of untreated tumours cells. We can use RNAi to unmask hidden cancer tumour antigens so as to gain not only a direct killing of tumour cells but also elimination of both treated and untreated tumours by the immune system. This is the first demonstration of tumour-specific immunity generated via RNAi and has major implications for the potential clinical use of RNAi and implies that one does not need to deliver RNAi to every cancer cell.

O16: NEW APPROACHES IN DEVELOPING GENE TRANSFER VECTORS FOR THE THERAPY OF SOLID TUMOURS

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Of diagnosed cancers, 90% are solid tumours. A high percentages (up to 75%) of these cancers are diagnosed at a late stage when available conventional therapies are ineffective. Gene therapy is a new modelity that has shown promises in preclinical experiments, but the theaputic potential has yet to be realised in the clinic.

Recent understanding of tumour biology demonstrated the difficult and disappointing nature with conventional therapies. As the tumour masses enlarge, the vasculature becomes inadequate to meet the demands of the fast growing tumour cells. Consequently, angiogenesis and hypoxia occur, which in turn induced pathological changes in the tumour, ie.: the emergence of hypoxic and necrotic regions, the heterogeneity of tumour cell population, and the anaerobic metabolism. All these played a fundamental role in the treatment failure. The unique patho-physiology of solid tumour can, in fact, be turned to an advantage

as hypoxia and necrosis provided a haven for certain species of anaerobes. Amongst them, clostridia were found to have the ability of spontaneous colonization, apparent selectivity and considerable oncolysis. Clinical trials used wild-type clostridia for the treatment of solid tumours, such as malignant glioma had showed efficacy and safety 50 years ago, but the life span of the patients did not extend due to incomplete tumour lysis, allowing regrowth and reoccurrence. This is the result of the well-nourished proliferative rim of the tumour that is not conducive to growth of oxygen-sensitive clostridia.

The advance of recombinant DNA technology reignited the field, enabling genetic improvement of clostridia's innate oncolytic capability. This presentation will summarise the progress in the use of clostridia for oncolytic cancer therapy as well as rationales and strategies in the development of clostridia-based gene delivery system using gene therapy technologies. It is expected that new clostridia-based gene therapy vectors will be of great use for the therapy of primary solid tumours as well as metastasis, the fatal cause of most cancer death.

O17: PRODUCTION AND RESCUE OF A SEVERE PHENOTYPE OF ORNITHINE TRANSCARBAMYLASE DEFICIENCY IN THE *SPF^{ASH}* MOUSE MODEL USING ADENO-ASSOCIATED VIRAL VECTORS AND RNAI TECHNOLOGY

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Severe neonatal ornithine transcarbamylase (OTC) deficiency is an ideal model for liver gene therapy. Conventional therapies have many shortcomings, and low levels of correction are likely to be therapeutic. The *spf^{ash}* mouse model, while an excellent tool in which to functionally validate vectors for treatment of OTC efficiency, suffers in that it represents only a mild phenotype in which 5–7% normal enzyme activity precludes the severe neonatal hyperammonemia typically seen in infants. In initial studies in adult *spf^{ash}* male mice, using an rAAV2/8 vector encoding mOTC (rAAV2/8.mOTC), we have achieved levels of OTC activity up to an impressive 17-fold above wild-type levels. Orotic aciduria, the most sensitive indicator of OTC deficiency, was normalised at all vector doses tested and correction was life-long. Neonatal mice showed similarly high levels of gene transfer and OTC expression at early time-points with markedly reduced orotic acid levels. However, in concert with liver growth there was a decline in OTC activity and a steady rise in orotic acid levels. OTC activity stabilised as mice reached adulthood at approximately two-fold above levels in untreated *spf^{ash}* mice. Importantly, this residual level of expression is theoretically sufficient to confer substantial therapeutic benefit. As discussed above, defining the minimal level of stable gene transfer required to convert a severe to a mild phenotype is not directly testable in the *spf^{ash}* mouse. We have therefore developed an RNAi-based strategy for converting the mild phenotype to a severe clinically symptomatic hyperammonaemic phenotype. A select shRNA sequence was delivered to adult *spf^{ash}* male mice using a rAAV2/8 vector. Injected mice exhibited neurological impairment with highly elevated plasma ammonia levels and reduction of OTC activity to less than 2% of wild-type. The minimum dose required for rescue of the severe hyperammonaemic phenotype using rAAV2/8.mOTC is currently being determined.

O18*: FACING THE BLEEDING OBVIOUS: AAV-MEDIATED GENE THERAPY FOR HAEMOPHILIA B

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We have completed a Phase I dose escalation study of liver-directed AAV2-FIX in humans with severe hemophilia B (*Nature Medicine*, 2006 Mar;12(3):342-7). The rationale for the clinical trial was based on studies in mice, hemophilic dogs, and non-human primates demonstrating long-term (>4 yrs) expression of Factor IX (FIX) after infusion of an AAV vector expressing FIX into the portal vein or the hepatic artery. Two subjects treated at a dose of 5×10^{12} vg/kg showed detectable circulating levels of FIX (maximum levels of 11.8% and 3% respectively), but expression was transient and accompanied in one case by a reversible asymptomatic transaminitis. There was no evidence of a FIX inhibitor at any point. An additional subject was treated at a dose of 1×10^{12} vg/kg but transaminases also rose after vector injection and resolved spontaneously. Assessment of the CD8+ T cell immune response to AAV-2 supported a model in which a briefly detectable response to AAV capsid epitopes resulted in elimination of the transduced cells (*Nature Medicine*, 2007;13(4):419-22 IF: 26.382). We concluded that AAV-2 vectors can transduce human hepatocytes in vivo, but that long-term expression was prevented by a specific immune response. The discrepancy between the long-term efficacy in animal studies versus the short-term expression observed in humans could be explained. In order to circumvent this limitation, we are now initiating a trial involving transient immune suppression – although other approaches are also under consideration. The rationale and risks and for this safety and dose escalation study will be discussed. The study will evaluate potential efficacy in each dose group. Pre-existing immunity to AAV and its relationship to gene expression (or transgene activity) will also be assessed, as will the potential for germline transmission of vector administered into the liver.

O19: AN ASTROCYTE-DORSAL ROOT GANGLION CO-CULTURE MODEL TO ASSESS NEURITE OUTGROWTH FROM LENTIVIRAL VECTOR EXPRESSED NEUROTROPHIN-3

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Traumatic injury to the CNS results in an inability to regenerate axons through an inhibitory environment such as the glial scar. This cellular environment is composed predominantly of reactive astrocytes and oligodendrocyte precursor cells (OPCs) and inhibitory molecules such as chondroitin sulphate proteoglycans (CSPGs) and myelin inhibitory proteins. The aim of this study is to assess the tropism of a HIV-1 based lentiviral vector in the injured CNS and develop a combined gene therapy approach based on Neurotrophin 3 growth factor expression and RNAi mediated knockdown of the inhibitory CSPG, NG2.

Lentiviral vector (GFP) transduction of rat mixed glial cultures resulted in labelling of up to 75% of GFAP +ve astrocytes and NG2+ve OPC cells to a lesser extent. Stereotaxic delivery of viral vector to an injured cortical region of the brain resulted in a transduction area of up to 3 mm^3, almost the entire glial scar area, with significant transduction of astrocytes and OPCs.

Having demonstrated transduction of the major cellular components of the glial scar we established an *in vitro* co-culture model of either primary astrocytes or an NG2 secreting cell line (Neu7) together with dorsal root ganglion cells. Lentiviral expressed NT3 from astrocytes resulted in significant DRG outgrowth, whereas NT3 expressed from the Neu7 cell line resulted in no significant axon growth, suggesting NT3 alone does not overcome the inhibitory properties of NG2. Similarly, addition of exogenous recombinant NT3 was only effective in the astrocyte-DRG co-culture. Finally when NG2 expression in Neu7 cells was reduced by vector mediated shRNA expression, significant DRG outgrowth was observed.

Our *in vitro* experiments demonstrate that the NG2 proteoglycan is a major inhibitor of the growth promoting effects of NT3 and we propose that a composite vector including both growth factor and shRNA expression will be a rational approach to axon regeneration

O20*: GENE-MODIFIED LEUKOCYTES FOR CANCER THERAPY

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Immunotherapy is a promising means of cancer therapy, although responses to therapy are generally limited to only a proportion of patients with either viral-induced malignancies, melanoma or some lymphomas. A major reason for these limited effects is the lack of tumour specific lymphocytes due to tolerance induction or a limited immune repertoire. To overcome these limitations, we have used genetic modification of several leukocyte types to provide specificity for cancer cells. Using viral and non-viral vectors we have genetically modified T cells, NK cells and dendritic cells with cell surface chimeric receptors consisting of extracellular erbB-2-specific antibody linked to intracellular signalling molecules. Leukocytes expressing these chimeric receptors were demonstrated to exert various functional activities against erbB-2-expressing tumour cells, including cytokine secretion, cytotoxicity and chemokine release in vitro. Adoptive transfer of gene-modified T cells or NK cells was also demonstrated to inhibit tumour growth in mice. Using genetic modification to generate large numbers of tumour-reactive leukocytes for adoptive transfer into patients may provide new options for cancer treatment.

O21: GENE THERAPY FOR THE INDUCTION OF IMMUNOLOGICAL TOLERANCE IN AUTOIMMUNE DISEASE

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Autoimmune diseases affect 5–6% of the population and characterized by a chronic adaptive immune response that target self-antigens; leading to clinical pathology. This includes a wide range of diseases such as multiple sclerosis, type 1 diabetes and rheumatoid arthritis. There are no cures; with treatment restricted to immunosuppressive regimes or replacement therapy. The development of autoimmunity is often associated with a breakdown in immunological tolerance and activation of self-reactive clones. Exposure to self-antigens during development in the thymus is a major mechanism associated with reducing the burden of autoreactive T cell clones. We have previously shown that ectopic expression of autoantigen can render mice resistant to autoimmune disease induction. Tolerance can be transferred by the bone marrow compartment, thus confirming the major role that bone marrow-derived cells, such as dendritic cells, have in tolerance induction. Our recent studies have focused on utilizing gene therapy strategies aimed at utilising bone marrow haematopoietic stem cells (HSC) to drive *in vivo* ectopic expression and promote immunological tolerance. Using a mouse model of experimental autoimmune encephalomyelitis (EAE), we demonstrate that the transfer of retrovirally transduced HSCs encoding MOG (myelin oligodendrocyte glycoprotein) promotes deletional tolerance and render mice resistant to MOG-induced autoimmune disease. We have also shown that this strategy can be used in a curative manner to reverse established EAE and maintain long-term remission. These findings provide a solid framework for the potential clinical application of gene therapy to treat autoimmunity.

O22: MODULATION OF CENTRAL TOLERANCE MECHANISMS BY INTRATHYMIC GENE TRANSFER AMELIORATES AUTOIMMUNE-MEDIATED CNS DAMAGE

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A rational approach to treat autoimmune diseases such as multiple sclerosis (MS) requires the restoration of self-tolerance. We hypothesized that over-expression of myelin oligodendrocyte glycoprotein (MOG), a key autoantigen in MS, in the thymus of C57BL/6 mice will reinstall immune tolerance, avoid autoimmunity and ameliorate the signs of experimental autoimmune encephalomyelitis (EAE) – an animal model for MS. Animals were intrathymically injected with concentrated lentiviral vectors engineered to express MOG (LV-MOG) or the hydrogen potassium ATPase – an irrelevant autoantigen (LV-HKATPase) or PBS. As a further control aged-matched naive animals were also used. Animals were subsequently immunized with the encaphalotogenic MOG_{35-55} peptide 12 weeks post treatment and monitored for a further 6 weeks. Thereafter, animals were humanely killed and tissues and organs analyzed. Neurological assessment indicated that untreated and control animals injected with LV-HKATPase or with PBS, developed fulminant symptoms of EAE after immunization. In contrast, animals receiving the therapeutic LV-MOG vector manifest significantly reduced symptoms. Histological analysis of the brain and spinal cords from control animals showed inflammatory lesions that correlated with severe areas of demyelination and axonal loss. Notably LV-MOG-treated animals demonstrated significantly fewer cellular infiltrates that corresponded with a lower number of demyelinating areas with little, or no axonal loss. In LV-MOG-treated animals, a reduction in the number of antigen-specific $CD4^+$ T-cells was demonstrated. Functionally, this translated to a 34% to 50% decrease in MOG_{35-55}-stimulated T cell proliferative responses in comparison to controls. Furthermore, a significant reduction in Th1 and Th17 cytokine levels was also shown in these cultures. Collectively these data indicate that intrathymic delivery of a LV-MOG represents a novel form of tolerance induction for the treatment of MS. Our approach further adds to the armamentarium of non-toxic gene-based strategies for the treatment of this debilitating disease.

O23*: LIVER-DIRECTED GENE THERAPY RESULTS IN THE PERMANENT REVERSAL OF AUTOIMMUNE DIABETES IN NON OBESE DIABETIC MICE

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Type I diabetes (TID) mellitus results from the T-cell mediated autoimmune destruction of the insulin-producing pancreatic beta cells. Gene therapy is one strategy being explored to cure TID. Previously, we used a novel surgical technique to express furin-cleavable human insulin (INS-FUR) in the livers of streptozotocin diabetic-Wistar rats using the HMD lentiviral vector, resulting in permanent reversal of diabetes. The aim of the present study was to determine if our methodology could reverse diabetes in the non obese diabetic (NOD) mouse (blood glucose >14 mM). Mice were monitored for body weight and blood glucose levels. An intravenous glucose tolerance test (IVGTT) was performed after a 14h fast in untreated (n= 6), empty vector-transduced and INS-FUR-transduced NOD mice, together with non-obese resistant (NOR) mice (n= 10). Liver, pancreas, kidney, spleen and lung were harvested at the experimental endpoint (5 months). Expression of insulin was determined by RT-PCR, immunohistochemistry, and transmission electron microscopy. Immunofluorescent staining was used to identify $CD4^+/CD25^+$ T cell populations within the spleen.
We have achieved 371.9% (n= 5) transduction efficiency of hepatocytes *in vivo*. Mice treated with INS-FUR, exhibited reversal of hyperglycaemia within 48h and normoglycaemia was maintained for 5 months, without the induction of hypoglycemia. IVGTTs revealed that insulin-transduced mice showed similar responses to elevated blood glucose concentrations as nondiabetic NOR mice. The transduction procedure resulted in expression of several beta cell transcription factors, pancreatic hormones glucagon and somatostatin and significant hepatic insulin storage in granules characteristic of beta cells in HMD/INS-FUR-treated mice. Liver function tests remained normal and there was no evidence of lymphocytic infiltration in the livers of HMD/INS-FUR-treated animals. Numbers of $CD4^+/CD25^+$ T cells, significantly increased ($P<0.01$) in HMD/INS-FUR treated NOD diabetic mice. Importantly, pancreatic exocrine transdifferentiation did not occur. Our data suggest that this regimen may ultimately be employed clinically to cure TID.

O24: INCORPORATING 2A PEPTIDE SEQUENCES INTO LENTIVIRAL VECTORS FOR EFFICIENT MOLECULAR IMAGING *IN VIVO*

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Several viruses, including members of the *Picornaviridiae* family, use 2A peptides or 2A-like sequences to mediate protein cleavage. The incorporation of a 2A peptide linking

sequences between genes encoded in the same open reading frame (ORF) results in near-complete separation and stoichometric production of encoded proteins via a ribosomal skipping mechanism. Cleavage occurs at a highly conserved consensus sequence at the C-terminal end of the 2A peptide encoded within the vector. We have developed a vector platform for the assembly of multicistronic ORFs to facilitate shuttling into mammalian expression vectors, retroviral and lentiviral vectors. Importantly, selection of cassettes containing tandem arrays of reporter genes or markers is tightly linked to the expression of our genes of interest.

We have established an *in vivo* tumour model in NOD/SCID mice to allow us to non-invasively examine the effect of tumour suppressor genes on tumour progression. The MCF7 breast cancer cell-line was transduced with lentiviral vectors containing a cassette with eGFP 2A-linked to luciferase. GFP-positive cells were sorted by FACS, clonally isolated, expanded and then further transduced with lentiviral vectors containing the mCherry fluorescent protein with or without a 2A-linked cistron containing CTCF, a putative tumour suppressor gene. GFP and mCherry 'double positive' cells were isolated by FACS and then injected orthotopically into opposing mammary fat pads of NOD/SCID mice. Tumour development was then tracked in mice following the injection of luciferin substrate intravenously and the mice were anaesthetised before imaging using the Xenogen IVIS 100 biophotonic imaging system. We report here a 'toolbox' of 2A-linked multicistronic vectors enabling the analysis and imaging of gene expression *in vitro* using multi-parametric flow cytometry or confocal microscopy; and *in vivo* using molecular imaging.

O25*: REJUVENATION OF THE THYMUS COMBINED WITH GENE MANIPULATION OF STEM CELLS: A NOVEL APPROACH TO TREATING AUTOIMMUNITY AND AIDS

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Diseases such as AIDS represent one of the greatest challenges to modern medicine primarily because of the ability of the virus to paralyse the very forces within the immune system, which are required for its removal. While anti-retroviral therapies are becoming progressively more efficient, they do not deplete residual viral reservoirs and they certainly do not induce resurrection of the engine room of the immune system, the thymus, which undergoes severe atrophy with aging – this being manifest as early as puberty. Similarly, age-related deficiencies in function also occur in the bone marrow, compounding the thymus problem because the latter is dependent on the former for provision of progenitor cells. Hence even if viral load can be reduced, the capacity for naïve T cell (particularly CD4+) regeneration becomes severely compromised with age. Ironically even if this could be achieved, there are two conflicting outcomes. On the one hand new T cells may be generated to restore immune competence, but in doing so there are theoretically more T cells as targets for renewed HIV infection. A two-pronged approach is thus required. First there needs to be a means of rejuvenating thymic function to reverse its age-induced atrophy, and secondly the new T cells need to be HIV resistant. We have developed strategies to address the former, primarily focussing on the intervention of sex steroid-induced inhibition. By giving LHRH, in both animal models and humans undergoing immune system destruction with chemotherapy, we have found significant improvement in thymus function. Furthermore this induces greatly improved bone marrow function leading to higher levels of HSC and also greatly improved engraftment of exogenous HSC. This platform is now poised to be combined with those involving genetic manipulation of HSC with anti- HIV genes. This dual approach of thymus and bone marrow rejuvenation coupled with the anti-HIV gene transfected HSC should enable the successful recovery from HIV infection and ultimately establish a "resistant" immune system which should provide not only better defence against opportunistic infections, but also provide a rational platform for making HIV vaccines more effective.

We have also shown the ability to induce thymic rejuvenation combined with more efficient donor HSC transplantation can also be applied to restoring self-tolerance in autoimmune diseases such as multiple sclerosis, and inducing donor specific tolerance in the transplantation setting.

Recently we have been pursuing the identity of thymic epithelial stem cells which provide another potential target for genetic manipulation and downstream modulation of T cell differentiation and function.

O26*: PHASE II SAFETY AND EFFICACY OF OZ1: RETROVIRAL VECTOR ANTI-*TAT/VPR* RIBOZYME FOR HIV-1

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A phase II randomized, double blind, placebo-controlled, gene transfer clinical trial assessed the safety and efficacy of OZ1 delivered in autologous CD34+ cells. 74 HIV-1 positive adults received an infusion of autologous CD34+ cells transduced with either OZ1 (a murine oncoretroviral vector with a *tat/vpr* specific anti-HIV ribozyme.) or mock. Over 100 weeks, antiretroviral therapy was interrupted twice (at weeks 24–28 and 40–100). In addition to safety measures including those specific to gene transfer studies, viral load, lymphocyte count and gene marking were assessed.

No adverse event was attributed to OZ1, and there was no evidence of insertional mutagenesis or other gene therapy related safety parameter. The primary endpoint of difference in viral load at weeks 47/48. was not met. However, secondary endpoints including time-weighted area under the log HIV viral load curve (TWAUC), were significantly different in the OZ1 group compared to the Control group including: TWAUC for weeks 40–48 and weeks 40–100; median difference $\log_{10} - 0.34$ ($p = 0.024$) and -0.37 ($p = 0.034$), respectively. The number of participants with plasma viral load <4 $\log_{10}$ copies/ml at weeks 47/48, or with a TWAUC in the lowest quartile for weeks 40–100 as well as the median time to increase to $4\log_{10}$ viral load copies/ml after interruption were statistically different. Moreover, in those participants with OZ1 expression beyond week 48, the weeks 47/48 viral load ($p = 0.003$) and median TWAUC for both weeks 40–48 and weeks 40–100 were lower than in the Control group. While not statistically significant, the

OZ1 group had higher CD4+ T-lymphocyte counts, absolute and percentage, and lower CD8 percentage over time. No evidence of resistance to OZ1 or to the patients' standard antiretroviral therapy was seen.
This study provides the first indication that cell-delivered gene transfer can reduce HIV viral load, and may preserve immune function avoiding the toxicities associated with antiretroviral therapy.

O27: DEVELOPMENT OF HYBRID SYSTEMS FOR CELL-MEDIATED MUSCLE REGENERATION: ADVANCED ELECTROMATERIALS AND WET-SPUN BIODEGRADABLE FIBERS

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In myo-dystrophic processes such as Duchenne muscular dystrophy (DMD), onset of disease pathology is associated with significant loss of regenerative precursor cells. In the face of such significant cell loss, it is likely that even with the rectification of the genetic defect, some measure of cell replacement therapy will still be required to replace the large amounts muscle tissues and precursors lost during the dystrophic process. Effective cell-mediated myo-restoration has been difficult to achieve largely due to contra-regenerative effects arising within the host (dystrophic) tissue environment, which results in the eradication of transplanted cells soon after implantation. A number of studies have indicated that to some extent, this issue may be overcome by preconditioning of cells prior to implantation. Likewise, control of the cyto-molecular environment into which the donor cells are placed has significant potential to improve cell transplantation outcomes in damaged and/or dystrophic muscle. This study describes a novel bio-synthetic platform for ex vivo growth and pre-treatment of muscle precursors in an aligned linear orientation on fibres spun from biodegradable polymer formulations on a conducting polymer platform. This hybrid growth platform allows the controlled development of myogenic precursors to optimal myogenic capacity via electrical stimulation, stimulated release of pro-myogenic factors and forms the basis of a multi-modal polymer system that may be co-implanted with precursor cells to improve cell transplantation outcomes in damaged or dystrophic muscle.

POSTER PRESENTATIONS

P1: RAAV TRANSDUCTION IS REGULATED BY β-ADRENORECEPTOR SIGNALING IN MUSCLE CELLS

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Recombinant adeno-associated virus (rAAV)-based gene therapy represents a promising approach for the treatment of muscle diseases, but the molecular mechanisms that direct rAAV transduction remain unclear. Since many target tissues, including muscle, are refractory to rAAV transduction, increasing the efficiency of gene delivery is fundamental toward developing therapies using these vectors. Here we demonstrate that β-adrenergic receptor stimulation with isoproterenol (ISO) markedly increased muscle tissue transduction of rAAV *in vitro* and *in vivo*. Conversely, chronic β-adrenergic receptor downregulation significantly suppressed rAAV transduction. Pretreatment with calcium signaling cascade inhibitors including calcineurin inhibitory peptide (CNIP) strongly suppressed the positive effects of ISO on rAAV transduction. Additionally we document that ISO treatment led to a significant increase in double-stranded (ds) DNA synthesis of the rAAV genome and an increase in promoter activity. Consistent with increasing promoter activity, we found by chromatin immuno-precipitation that the rAAV genome physically interacts with a calcineurin-dependent transcription factor, NFAT. Moreover, stimulation with ISO did not affect rAAV transduction in calcineurin nullizygous mice. Collectively, we conclude that a calcium-dependent pathway regulates rAAV vector transduction at a number of stages that may include vector mobilization, DNA conversion, and transcription activity. Modulating this pathway through β-adrenergic signaling enhances rAAV-mediated gene delivery to myocytes, and may provide a significant improvement to current therapeutic approaches for muscle diseases.

P2: EXON SKIPPING STRATEGIES TO ADDRESS DMD-CAUSING MUTATIONS: PERSONALISED GENETIC THERAPIES

Penny Meloni, Leah Stone, Sue Fletcher and Steve Wilton, *Molecular Genetic Therapy Group, CNND, University of Western Australia, Perth*

Duchenne Muscular Dystrophy (DMD) is a severe and progressive muscle wasting disease, caused by protein truncating mutations in the dystrophin gene. Becker Muscular Dystrophy (BMD), an allelic condition is generally caused by in-frame deletions within the dystrophin gene, resulting in the production of an internally deleted but variably functional protein. Antisense-Oligomer (AO) induced-exon skipping has the potential to by-pass protein truncating mutations and restore functional dystrophin

expression. We have designed and optimised a series of AOs to excise individual dystrophin exons 2 to 78 during processing of the dystrophin pre-mRNA. Here we describe exon skipping strategies to rescue dystrophin expression in patient cell lines where more than one exon-skipping strategy may restore the reading frame. A genomic deletion of exon 44 may be addressed by the excision of exons 43 or 45. The resultant dystrophin isoforms may have different functionality. A splice acceptor defect in exon 21 leads to disruption of the reading frame, and this may be restored by one of three different strategies. It will be crucial to identify which AO treatment will result in the most functional dystrophin isoform for those cases where more than one treatment option is applicable.

P3: NORMALISATION OF GENE EXPRESSION IN *MDX* MICE MUSCLE

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Duchenne muscular dystrophy (DMD), a recessive X-linked form of muscular dystrophy caused by mutations in the dystrophin gene, is characterised by rapid progression of muscle degeneration, eventually leading to loss in ambulation, paralysis, and death.

The *mdx* mouse model of muscular dystrophy has a nonsense mutation in exon 23 of the dystrophin gene. We have previously shown a morpholino antisense oligomer could remove this single exon, resulting in an in-frame mRNA transcript encoding a shortened but functional dystrophin protein. In-order to further investigate the consequences associated with this protein restoration, we use Genechip Exon Arrays to compare expression levels in the diaphragm of 10 week old treated *mdx* mice to that of age matched *mdx* and C57BL control mice. Quality control and statistical analysis were necessary to normalise microarray data. A one-way ANOVA and fold changes were used to compile gene lists. These genes were categorised in three groups, Molecular function, Biological process and Pathway, based on gene ontology classification e.g. signal transduction, ion transport etc, and then analysed in more detail. The data arising from this study will provide insight into gene interactions and their affects on pathways that are involved in muscle regeneration, which may provide additional therapies for DMD patients.

P4: NUCLEOFECTION IS AN EFFICIENT METHOD FOR THE GENETIC MODIFICATION OF MURINE MESENCHYMAL STEM CELLS

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Multipotent mesenchymal stem cells (MSC) have been utilised for gene therapy based on their ability to home to tumours or sites of injury while maintaining their ability to self-renew. For genetically-modified MSC to be successfully used in the clinic, an efficient mode of gene delivery must be determined. MSC are known to be somewhat resistant to transfection using lipid-based methods. Hence MSC gene modification using nucleofection (Amaxa), a novel non-viral transfection method based on a unique combination of electrical parameters and solutions allowing plasmid DNA delivery to the nucleus was tested.

Murine bone marrow cells were harvested and fluorescence activated cell sorting was used to enrich the adherent fraction for absence of expression of lineage markers and high-level expression of Sca-1. A pilot study utilising the 4.7 kb plasmid pEGFP-N1 was performed to determine the most favourable nucleofection electrical parameters. Based on GFP expression 96 hours post-nucleofection, transfection efficiency ranged between 20–88%. A more comprehensive optimisation was then undertaken to obtain the optimal cell number (1×10^6), amount of DNA (5-10 μg) and specific nucleofection program to be used for large scale studies involving the pVITRO2 plasmid. pVITRO2 (Invivogen) allows the constituitive co-expression of two genes of interest and a hygromycin resistance gene which is active both in *E.coli* and mammalian cells allowing for stable selection. Nucleofection conditions were optimised for pVITRO2 carrying GFP and firefly luciferase (fluc2) reporter genes (pVITRO2-GFP/fluc2) and control vector pVITRO2-GFP/LacZ. Using these optimised conditions, the nucleofection efficacy of three separate mMSC isolations was examined. Viability, GFP expression before sorting and GFP^+ cell recovery following sorting was assessed using Trypan Blue exclusion and FACS analysis, respectively. *In vitro* bioluminescent imaging (IVIS100, Caliper) of MSC transiently or stably expressing fluc2 showed a linear correlation between cell number and luciferase signal following incubation with D-luciferin. *In vivo* tracking studies will be performed once stable populations of these genetically modified MSC are obtained. Understanding the localisation of genetically modified MSC will assist in their development as cellular therapies in the future.

P5: RESTRICTION OF RD114 PSEUDOTYPED VECTORS IN BABOON $CD34^+$ HSC

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The feline endogenous virus RD114 envelope is routinely utilised to pseudotype retroviral vectors for gene therapy applications. Previous studies have shown the ability of these vectors to transduce human $CD34^+$ haemopoietic stem cells (HSC). Interestingly, baboon $CD34^+$ cells have been shown to be resistant to transduction by RD114 pseudotyped vectors (Horn *et al*2004, Mol. Ther. 10, 417–31). We have developed a baboon model of HSC gene transfer and mobilisation (Larsen *et al*2008, Stem Cells 26, 2974–80). This study aimed to determine if the resistance of baboon HSCs was due to endogenous expression of baboon endogenous virus (BaEV) envelope causing receptor interference. Primary baboon cells (HSCs; mesenchymal stromal cells; and dermal fibroblasts) were transduced. In contrast to the two other baboon cell types, HSCs were unable to be transduced by RD114, yet transduction of $CD34^+$ cells was achieved by both GaLV and VSV-G pseudotyped retroviral vectors. To determine if this cell type specific restriction was

due to expression of the BaEV envelope, Q-PCR was performed to measure envelope expression. All baboon cell types tested expressed BaEV envelope at different levels. Furthermore, transduction efficiency variation was found between MSCs isolated from different baboons. The level of RD114 transduction efficiency did not correlate to the relative expression of BaEV envelope. In addition, RDR was shown to be expressed in the baboon HSCs and was able to mediate transduction by RD114 in a heterologous cell line. These data suggest the existence of an envelope-dependent restriction mechanism unique to baboon $CD34^+$ HSC that is unrelated to BaEV envelope expression.

P6: EXON SKIPPING THERAPIES TO ADDRESS DMD-CAUSING MUTATIONS TARGETING EXON BLOCKS

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Duchenne Muscular Dystrophy (DMD) is an X-linked disorder resulting in severe muscle wasting, beginning in the lower limbs and rapidly progressing to all voluntary muscles of the body. The disease is generally caused by protein truncating mutations in the dystrophin gene and results in the absence of a functional protein, known to provide a structural link between the muscle cytoskeleton and extracellular matrix to maintain muscle integrity. Becker Muscular Dystrophy (BMD), a less severe form of muscular dystrophy, is generally caused by in-frame dystrophin exon deletions resulting in a shorter protein with variable functionality, depending upon the region and/or amount of the dystrophin gene lost. We have developed and optimised a series of antisense oligomers (AO's) targeting the isolated removal of exons 2 to 78 of the 79 exon dystrophin gene, with the current focus upon the removal of in-frame exon blocks. Targeted removal of exon blocks would minimize the number of oligomer preparations required to address clustered dystrophin mutations and may facilitate establishment of dosage regimes. We report the targeted excision of dystrophin exons 17 and 18, with an oligomer cocktail to address three different mutations in this region. At the dystrophin transcript level, there appears to be variable responses to the oligomer cocktail, with the exon 18 duplication mutation being most amenable to splice intervention.

P7: ENHANCEMENT IN TRANSGENE EXPRESSION AFTER GENETIC FUSION WITH THE CH2/CH3 DOMAIN OF IGG_1

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We have been investigating the utilization of rAAV as a platform for the delivery of genetic vaccines using malaria as a disease model and muscle as a target tissue. In an effort to augment responses to the secreted transgene product, we have modified vector encoded antigen by fusion to the C-terminus of CTLA4-Ig (**C**ytotoxic **T**-**L**ymphocyte **A**ctivation antigen-**4** and the hinge domain of human IgG isotype 1). This strategy augmented humoral responses to low vector doses but induces long-lived antigen-specific tolerance at higher doses of vector (Logan *et al*. 2009 Gene Therapy). This finding was unexpected as rAAV delivery to muscle, unlike liver, has demonstrated this tissue provides a highly immunogenic environment for induction of humoral immunity against rAAV encoded transgene products.

We are currently investigating the mechanism underlying this novel observation. *In vitro* studies show that antigen fusion to CTLA4Ig leads to approximately a one log enhancement in antigen expression. Theoretically, this phenomenon could contribute to tolerance in our model. A domain swapping experiment indicated that enhanced expression was not associated with CTLA4 or antibody dimerization but correlated with the presence of the IgG isotype 1 hinge and Fc domain. We hypothesized that the neonatal Fc receptor, a molecule that prolongs antibody half-life through transcytosis, was enhancing antigen expression. However, mutation of the Fc domain of the IgG molecule to prevent this interaction did not affect antigen expression. While the mechanism underlying IgG-enhanced expression is yet to be delineated, this observation may be valuable for improving transgene expression in those studies where the presence of an N-terminal molecule does not interfere with molecule function.

P8: SUSTAINED RELEASE OF DRUG LOADED WITHIN BIOTINYLATED PERFLUOROCARBON NANOPARTICLES AND THE USE IN MAGNETIC RESONANCE IMAGING

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Keywords fluorocarbons; nanoparticles; tunica intima; magnetic resonance imaging; sustained release

AIM To investigate the *in vitro* release profile of loading drugs encapsulated within the perfluorocarbon (PFC) nanoparticles (NPs) and its ability of enhancing magnetic resonance imaging (MRI). METHODS Dexamethasone sodium phosphate (DxP) or dexamethasone acetate (DxA), and Gadopentetate Dimeglumine (Gd-DTPA) were loaded within biotinylated PFC NPs were constructed by high pressure homogeneous processing method. The morphology and size of NPs were examined by scanning electron microscope (SEM) and laser particle size analyzer. Drug loading and *in vitro* releasing were assessed by high performance liquid chromatography (HPLC). MRI was used to observe the imaging and signal intensity of contrast within the NPs. RESULTS The particle size of DxP-NP and DxA-NP were ($224 \pm s$ 6) and (236 ± 9) nm respectively. The encapsulation efficiency (EE) of DxP-NP were (66.4 ± 1.0)%, with an obviously bursting phenomenon, the initial releasing rate were 77.2%, while the EE of DxA-NP were (95.3 ± 1.3)%, the initial releasing were 23.6%. Both of the two NPs could release persisted over one week. The NPs loading Gd-DTPA could enhance the signal intensity of Gd-DTPA about 16% detected by MRI. CONCLUSION PFC NPs loaded with hydrophilic drug have relatively high encapsulation efficiency and sustained release pattern. Contrast loaded within NPs could enhance the signal intensity detected by MRI.

P9: TARGETING OF PLASMID DNA-LIPOPLEXES TO CELLS WITH MOLECULES ANCHORED VIA A METAL CHELATOR LIPID

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The ability to target the delivery of plasmid DNA (pDNA) to specific cells *in vivo* is an important first step in increasing the transfection efficiencies of non-viral vectors. We have previously used PEGylated (stealth) liposomes containing the chelator lipid 3(nitrilotriacetic acid)-ditetradecylamine (NTA_3-DTDA) to target the delivery of antigen and cytokines to immune cells *in vivo*. In this work we utilized stealth liposomes containing NTA_3-DTDA and the ionisable aminolipid 1,2-dioleoyl-3-dimethylammonium-propane (DODAP) to incorporate pDNA into lipoplexes for targeting to cells. The liposomes were acidified (pH 5.5) and mixed with pDNA to allow complex formation; and the mixture then neutralized to produce neutral lipoplexes. Our studies show that such lipoplexes can be engrafted with histidine-tagged molecules and targeted to specific receptors on cells. The transfection efficiency of these lipoplexes was assessed using the vector pEGFP-N1, which encodes the reporter gene enhanced green fluorescent protein (EGFP). Initial transfections of HEK-293 cells using a histidine-tagged peptide derived from the arginine-rich motif of the HIV-1 TAT protein (T2) resulted in a low percentage of cells expressing EGFP at 48 hr post-transfection. Optimization of the liposome formulation and pDNA incorporation process, however, yielded substantial improvements in transfection efficiency. In particular, the inclusion of an endosome-destabilising peptide and an inhibitor of DNase I increased transfection by 2-fold. Transfection efficiencies obtained were ~50% for HEK-293 cells targeted with T2-engrafted pEGFP-N1-lipoplexes, and 30–40% for HepG2 cells targeted with pEGFP-N1-lipoplexes engrafted with a peptide specific for the vascular endothelial growth factor receptor Flt-1. pDNA-lipoplexes produced by this method are ~250 nm in diameter and target cells in serum-containing media. The results show that NTA_3-DTDA can be used to target pDNA-lipoplexes to cells, and could provide a convenient approach for targeting pDNA to cells *in vivo* for therapeutic applications.

P10: ANALYSIS OF RETROVIRAL INTEGRATION SITES USING LAM-PCR TO SUPPORT THE INITIATION OF A PHASE I CANCER GENE THERAPY TRIAL

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A phase I cancer gene therapy clinical trial for the treatment of paediatric brain tumours is currently being initiated at the Children's Hospital at Westmead (CHW). In this trial, which targets the haematopoietic stem cell (HSC), retroviral mediated gene-transfer of a mutant O6-methylguanine-DNA-methyltransferase (MGMT(P140K)) gene is predicted to confer drug resistance to alkylating chemotherapy, to allow for escalating doses of chemotherapy to treat the tumour. This strategy may overcome the problem of tumour chemo-resistance without leading to increased toxicity in the bone marrow.

Since the first report of a genotoxic adverse event (Hacein-Bey-Abina *et al*. 2003) associated with retroviral gene-transfer targeting the HSC, extensive analysis of integrating vectors has been performed to investigate vector safety. Linear amplification mediated PCR (LAM-PCR) is the most commonly employed method to analyse sample clonality and identify vector insertion sites. In order to establish this technique as a means of analysing patient samples following gene-transfer, clones from a human $CD34^+$ cell line (TF1) with 1, 2, or 3 integration sites were used for LAM-PCR methodological work-up. Genomic DNA from an un-selected, transduced HT1080 cell population was also used to analyse complex integration patterns and identify insertion sites.

In order to validate the technique using a clinically relevant sample, genomic DNA extracted from the T cells of a patient treated for SCID-X1 at CHW, in collaboration with the team from Hopital Necker-Enfants Malades in Paris (Ginn *et al*. 2005) was also subjected to LAM-PCR analysis. Five vector integration sites were identified which corresponded to four previously published for this patient (Diechmann *et al*2007), in addition to an independently discovered insertion site on chromosome five. These data support the use of LAM-PCR for analysis of both retroviral integration and monitoring of the clonality of gene-modified cell populations in patients being treated in the up-coming trial.

P11: PATTERNS OF RAAV PERSISTENCE IN THE MOUSE LIVER: SEXUAL DIMORPHISM AND HEPATOCELLULAR PROLIFERATION

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Genetic metabolic liver diseases are collectively common and often difficult or impossible to treat. Liver transplantation is often required, but carries its own set of risks and limitations. Alternative approaches, such as gene therapy, are urgently required. Adeno-associated virus (rAAV) vectors have shown exceptional promise for liver directed gene therapy, with numerous reports in small and large animal models. However, translating this research to humans requires further understanding of host-vector interactions, especially those controlling the efficiency of initial gene transfer and subsequent long-term persistence of gene expression.

In this study mice were dosed with a rAAV2/8 vector and long-term transgene expression and vector genome persistence examined. Interestingly it was discovered that adult female and male mice, while initially showing similar patterns of transgene expression across the hepatic lobule, show distinctly different patterns of transgene persistence. Female mice retain a predominantly perivenous expression pattern while male mice loose expression in this region. Upon further investigation evidence of a correlation between these changing patterns of expression and underlying hepatocellular proliferation was found.

These observations are of significance to the gene therapy field, particularly in the context of rAAV-mediated gene

transfer for the correction of liver functions that show metabolic zonation.

P12: A PHASE I CANCER GENE THERAPY TRIAL TO AID IN THE TREATMENT OF MALIGNANT BRAIN TUMOURS

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Translation of laboratory based pre-clinical gene therapy research into the clinic requires the input and expertise of scientists, clinicians, and other staff skilled in administration and project management.

At The Children's Hospital at Westmead (CHW), a Phase I cancer gene therapy trial is being initiated. The primary objective of this trial is to test the safety and feasibility of delivering a drug resistance gene to haematopoietic stem cells of patients being treated with alkylating chemotherapy for malignant brain tumours. Evidence comes from small and large animal models that chemoprotection of the bone marrow allows for dose escalation to treat drug resistant tumours.

Translation of this strategy into the clinic has required a significant collaborative effort between the Oncology and Gene Therapy Research Units at CHW. To date, this effort has culminated in the manufacture of clinical grade retroviral vector supernatant in CHW's newly commissioned Cleanroom facility. It has also involved submission of the trial protocol and the manufacturing protocol for gene-modified haematopoietic stem cells for scrutiny by the CHW Ethics Committee, the Therapeutics Goods Administration (TGA) and the Office of the Gene Technology Regulator (OGTR). This process has been informative for the CHW team in terms of planning for other gene therapy or cell based therapies which may require examination by regulatory authorities in the future.

Current translational activities include the development and institution of a quality management system in addition to the validation of the "manufacturing process" that provides gene-modified haematopoietic stem cells for infusion into patients. Scientifically, the development of methodology for measuring gene transfer efficiency and analysing vector integration has provided the means to measure trial outcomes in terms of both safety and feasibility.

P13: LENTIVIRAL-MEDIATED GENE TRANSFER OF ANTI-CD4 SCFV PROLONGS CORNEAL ALLOGRAFT SURVIVAL

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Lentiviral vectors integrate into chromosomal DNA and are relatively non- immunogenic. In contrast, adenoviral vectors produce transient transgene expression and are inflammatory, but are notably efficient. Our aims were to compare rat corneal allograft survival after *ex vivo* transduction of donor corneas with an anti-rat CD4 scFv transgene in lentiviral and adenoviral vectors, and to examine the effect of lentivector injection into recipient regional lymph nodes (LN) prior to transplantation. *Ex vivo* transduction of donor corneas was performed using lentivirus (MOI 400) or adenovirus (MOI 330), prior to transplantation of WF corneas into F344 rats. Some recipients received intranodal lentiviral injections into superficial cervical LN, two days prior to transplantation. Grafts were scored daily for clarity, corneal neovascularisation and inflammation. The presence of anti-CD4 scFv in supernatants from donor corneal rims cultured for 5 days was detected by flow cytometry via a HIS6 tag. Donor grafts transduced with LV-CD4scFv_F2A_eYFP ($n = 8$) survived for a median of 21.5 days, compared with 17 days for unmodified grafts ($n = 8$), and 17 days for corneas transduced with LV-eYFP ($n = 10$), $p = 0.015$. LV-CD4scFv_F2A_eYFP-transduced donor corneas showed prolonged graft survival compared with Adv-CD4scFv-transduced corneas ($n = 8$), $p = 0018$. Anti-CD4 scFv was detected at similar levels in supernatants of both LV-CD4scFv_F2A_eYFP and Adv-CD4scFv transduced corneas. Intranodal injection of LV-CD4scFv_F2A_eYFP in recipient rats prior to grafting ($n = 8$) had no effect on corneal graft survival compared with unmodified grafts ($n = 8$), or grafts in rats that received intranodal injections of LV-eYFP ($n = 7$), $p > 0.05$. In conclusion, donor corneas transduced *ex vivo* with LV-CD4scFv_F2A_eYFP showed significantly prolonged allograft survival compared to Adv-CD4scFv-transduced donor corneas and to unmodified controls. However, intranodal injection of LV-CD4scFv_F2A_eYFP two days prior to corneal transplantation had no effect on allograft survival. Sustained local production of anti-CD4 scFv within the eye was thus capable of modulating corneal graft survival.

P14: CMV IS THE CONSTITUTIVE PROMOTER OF CHOICE TO DRIVE THERAPEUTIC TRANSGENE EXPRESSION IN THE SHEEP CORNEA

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Corneal allografts have an average survival time of 12.4 years, with the major cause of failure being immunological rejection. Immune modulation of corneal allografts using gene therapy has been successfully demonstrated in animal models. Using a lentiviral vector, we aimed to compare two constitutively active promoters, controlling expression of a therapeutic transgene encoding interleukin-10 (IL-10). A549 cells and excised ovine corneas were transduced with a self-inactivating HIV-1 lentivirus expressing ovine IL-10 under the control of either the simian virus 40 (SV40) or cytomegalovirus (CMV) promoter. After ten days in culture, IL-10 was measured at protein and mRNA levels by ELISA and qRT-PCR respectively. Each treatment had an n-value of 3. Further ovine corneas were treated with lentivirus at varying multiplicities of infection (MOI): 20, 40 and 100. IL-10 expression was measured over ten days at the protein level by ELISA. In cell culture, CMV induced stronger initial transgene expression, being significantly higher than SV40 treatment at the mRNA level at day 2 ($p = 0.01$), with expression then declining until day 10 where SV40 expression was significantly higher than CMV ($p = 0.005$). SV40 gave more consistent expression over the ten day culture at the mRNA level. These trends were reflected in the protein levels. In the cornea culture at the mRNA level,

the SV40 promoter induced significantly higher transgene expression than the mock-treated corneas ($p = 0.03$), however CMV gave approximately 14-fold higher expression than SV40. The CMV promoter showed higher, stronger expression of IL10 throughout the culture at the protein level: 480× higher than untreated corneas and 70x higher than SV40 induced expression at day 10. Across all time points and at all MOIs, the CMV promoter induced significantly higher protein expression than SV40. These results indicate CMV is the constitutive promoter of choice to drive transgene expression for gene therapy in ovine corneas.

P15: RISK ACCEPTABILITY IN CLINICAL RESEARCH: IS GENE THERAPY TOO RISK-AVERSE?

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High profile adverse events in gene therapy clinical trials have increased the focus on risks in gene therapy. Patient deaths and other serious adverse events in clinical trials are not uncommon, yet in the context of gene therapy trials they have been highly publicised and attracted enormous media attention. Jesse Gelsinger's death in a gene therapy trial for ornithine transcarbamylase deficiency provides a cogent reminder of this phenomenon, as it has become axiomatic of the uncertainty of medical research and the power and risks of both gene therapy research and genetics in general. This requires that researchers and clinicians have a clear understanding of what risk is and why it must be taken seriously.

Risk is not a fixed concept. It is defined and perceived differently by different stakeholders. As all clinical research involves a balance between risk and benefits, researchers must determine what constitutes 'acceptable' levels of risk during the design and conduct of studies. However, these kinds of judgments are normative, not scientific, and should be subjected to critical discussion. Key questions in this regard include: who makes decisions about acceptable risk levels? Which categories of patient are the most appropriate to recruit to a trial? What sorts of values influence these decisions? What kinds of questions should be asked to both protect research subjects and allow research to occur? And how can information about risk be communicated to research subjects to ensure they have sufficient comprehension to give informed consent?

Clinical trials are the best available means of evaluating the risks associated with any novel therapy in humans. Increased attention to the role and impact of risk in the design and conduct of clinical trials will lead towards better protection of patient safety and communicate the gene therapy field's commitment to the ethical conduct of clinical research.

P16: THE COMMISSIONING AND RUNNING OF CLEAN ROOM FACILITIES AT CHW IN SUPPORT OF A PHASE I CANCER GENE THERAPY TRIAL

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The Human Applications Laboratory (HAL) at the Children's Hospital at Westmead (CHW) was commissioned in January 2008. The facility, constructed within PC2 certified research laboratory space, also meets PC2 bio-containment requirements, housing two aseptic suites and one aseptic bio-contained suite surrounding a central workroom.

To date, HAL has supported the early operations of a Phase 1 cancer gene therapy clinical trial for treating paediatric patients with brain tumours. Two 10 litre batches of retroviral vector supernatant were manufactured within the aseptic bio-contained suite in May 2008. Currently, trial runs of the manufacturing process for gene-modified haematopoietic stem cells are being carried out in HAL.

These activities have provided an opportunity to assess the suitability of Standard Operating Procedures to maintain the integrity of the facility during use. Results of environmental monitoring of air and surface cleanliness have validated the procedures instituted to date, in addition to the building and plant maintenance schedule that controls air handling within HAL. A building monitoring system, which incorporates data logging of room temperatures, humidity and equipment performance, provides real-time monitoring of critical parameters, and a comprehensive alarm system for out of specification events.

With the future use of the facility likely to be in support of gene and cell therapies that may require GMP compliance, our current focus is on developing a quality management system to provide the framework for GMP compliant manufacture. Although GMP certification is not a requirement for a Phase 1 clinical trial, the facility has been informally inspected by a TGA GMP auditor and subjected to a GAP analysis by RISS (Research Infrastructure Support Service) to identify areas in which improvements to the management system would be required to become more fully GMP compliant. As a consequence, relevant steps are being undertaken conscientiously in preparation towards meeting the standards for GMP certification.

P17: HIGH RESOLUTION ULTRASOUND SYSTEM FOR REAL-TIME IMAGING TUMOUR SIZE AND VASCULATURE

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Around 90% cancers form solid tumours. The major differences between tumour and normal tissue lie in the cancerous growth and angiogenic vasculature. Accurate real-time imaging of tumour size and changes in tumour vasculature is of great significance in the context of evaluating tumour growth and therapeutic effects during cancer therapy. While most real-time imaging systems can not fulfill both purposes simultaneously, high resolution

imaging ultrasound seems quite promising. Our laboratory has established the VisualSonics, Vevo770 ultrasound system for monitoring tumour size and scrutinizing its vasculature with a powerful Doppler function allowing real-time studying of deep, small tumour vessels. We used the system to monitor tumour size and vasculature of subcutaneous xenograft and synergic mouse tumours derived from injection of 4 different cancer cell lines including A549, A431, SW620, and CT26. For each specific cancer cell line, three animals were used. After 3 weeks, when tumours are palpable, we found the ultrasound system was very accurate in measuring tumour size as compared to the direct manual measurement of removed tumours using a calliper. Meanwhile, the Doppler function of the system was able to clearly display tumour vasculature in fine details. Our results suggest that high resolution ultrasound system is capable of accurate measurement of tumour size in real time and more importantly, it's a powerful and economic modality for observing tumour vasculature and for cancer gene therapy studies using bacterial-mediated oncolytic gene therapy.

P18: EXPLOITING PROAPOPTOTIC FACTORS IN COLON CANCER CELLS FOR THE DEVELOPMENT OF NOVEL THERAPIES

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Cancer cells die by either necrosis or apoptosis. Our laboratory is interested in the understanding of apoptotic pathways and examining factors that upregulate apoptosis in cancer cells. Hypoxia is a hall marker in colon cancers. We thus initially examined hypoxia-induced apoptosis and molecular changes in the apoptotic cells. Three human and one mouse colon cancer cell lines were treated under hypoxic condition. These cells were harvested after various time points and the level of apoptosis was evaluated by JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide) staining and Caspase Glo assay. JC-1 staining showed a higher percentage of human cells shifting to green colour (apoptosis) than that of mouse cells after 48 hours. Caspase assay also showed that Caspase 3 level fast increased and reached a peak after 6 hours in HCT116, whereas only a very slow and insignificant increase in Caspase 3 level was detected in HT29, SW480 and CT26. These results suggested that human colon cancer cells were more sensitive to hypoxia than mouse colon cancer cells. We therefore examined chemical-induced apoptosis in these cell lines. Cinobufagin, extracted from a classical Chinese medicine Chan-Su, was recently found to reduce cell growth in several cancer cell lines *in vitro*. Cytotoxicity study (MTT assays) in five human colon cancer cell lines showed that Cinobufagin strongly inhibited SW260 proliferation at the IC_{50} value as low as 0.04 μM, while the highest value was at 10 μM for T84, indicating that the efficiency of Cinobufagin varies significantly from cell to cell. Work is underway to investigate the mechanism that caused such a diference, or whetehr a specific signalling transduction in colon cancer cells is involved. Taken together, our research has established methods to study hypoxic apoptosis in colon cancer cells and current results suggest that Cinobufagin could be an effective pro-apoptotic agent for the therapy of colon cancer.

P19: RECOMBINANT CYTOTOXIN C-CPE-ETA' EFFECTIVELY KILLS CLAUDIN-4-EXPRESSING CANCER CELLS

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Claudin-4 is a natural receptor for *Clostridium perfringens* enterotoxin (CPE). It has recently been found to be overexpressed on the plasma membrane of a range of cancer cells, such as ovarian, breast, colon and prostate cancer. Hence, we hypothesised that a CPE-based cytotoxin may be of potential use in targeted cytolysis of claudin-4 positive cancers. We created such a novel toxin, C-CPE-ETA', by fusing C-terminal high affinity binding domain of CPE (C-CPE) with a truncated form of *Pseudomonas aeruginosa exotoxin* A (ETA'), aiming to examine whether C-CPE will specifically target claudin-4 molecule and the targeted toxin is cytotoxic. After cloning into an expression plasmid and transforming it into a bacterial strain, a 58 kDa recombinant protein was evident by Western blot and Coomassie blue staining. Flow cytometric analysis and immune assays were performed using recombinant C-CPE-ETA' in a range of claudin-4 positive and negative cancer cell lines. The proapoptotic and cytolytic activities were evaluated using JC-1 staining and MTT assay. Purified and recombinant C-CPE-ETA' bound with high affinity to claudin-4-positive cancer cells, including breast cancer MCF-7, colon cancer SW480, HT29, prostate cancer DU145, PC3 and ovarian cancer CAOV3. Interestingly, after initial binding, C-CPE-ETA' was sequentially internalized into claudin-4-positive cells within 60 mins, which resulted in significant apoptotic cell death. This apoptotic effects increased when the incubation was further extended to 24h. The IC50 of recombinant C-CPE-ETA' ranged from 0.7 ng/ml to 50 ng/ml in claudin-4-positive cancer cells. However, control claudin-4-negative cell lines, Hela and HUVEC, were not susceptible to the recombinant C-CPE-ETA' at concentrations up to 10μg/ml, suggesting the strict claudin-4 selectivity. Further study in a MCF-7 subcutaneous xerograft tumour model system showed that intratumoural administration of recombinant C-CPE-ETA' significantly inhibited tumour growth. Our results suggest that the C-CPE-ETA' exhibits remarkably specific cytotoxicity. Its therapeutic potentials for claudin-4-postitive cancer warrants further development.

P20: OPTIMISATION OF THE RCAS VECTORS FOR BOVINE MAMMARY EPITHELIAL CELL TRANSFORMATION

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Functional genomics approaches have the potential to improve our understanding of animal production traits yet there has been little research dedicated to understanding molecular mechanisms involved in mammary gland development and function in dairy cattle. The employment of

contemporary gene transfer tools has potential to improve our understanding of the molecular regulation of bovine lactation.

RCAS vectors are based on the avian specific Rous Sarcoma Virus and requires the presence of the receptor, *tva*, to permit transduction. While replication competent in avian cells the virus is replication incompetent in mammalian cells and thus provides a safe yet efficient method for production of viral particle. MacT cells (immortalised bovine mammary epithelial cells) were modified to express the *tva* gene (linked to the reporter EGFP) and FACS selected. The RCASBP(A) vector was utilised to perform viral dilution series experiments with successful expression of the reporter genes DsRed and Luc, and also for the transfer of functional shRNAs in transduced MacT(*tva*) cells.

An RCAS vector, RCASM2C, modified to utilise the amphotropic receptor for MLV (Pit-2) while remaining replication incompetent in mammalian cells, was found to transduce both immortalised and primary bovine mammary epithelial cells. An alternative RCAS vector, BBAN, allowed a larger gene insert due to the lack of an envelope gene on the vector. Pseudotyping of the vector BBAN with VSV envelope glycoprotein resulted in the production of viral particle infectious to bovine mammary epithelial cells and in particular to a functional model of primary bovine mammary epithelial cells.

The RCAS vector system of gene transfer has proved a promising tool for functional genomics in *in vitro* models of bovine mammary gland function, and exhibits the potential to extend into a safe and efficient *in vivo* bovine mammary transduction tool.

P21: NANOTECHNOLOGY AND MESENCHYMAL STEM CELLS – PROSPECTS IN CANCER IMAGING AND GENE-THERAPY?

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Clinical oncology suffers from two major drawbacks, lack of specificity of therapy and low sensitivity/accuracy of imaging. Nanotechnology, based on the use of submicronic nanoparticles (<100–200 nm), occupies a premium niche in the arena of cancer imaging and therapeutics. The success of this approach has now led to FDA approval of superparamagnetic iron-oxide nanoparticles for imaging and drug-delivery. However, a major obstacle *in vivo* is short blood circulation times due to their clearance through the reticuloendothelial system.

To enhance specificity, Adenovirus (Ad)-mediated targeted gene therapy is the most explored, but has limited clinical use because of low Ad-receptor expression on cancer cells and their immunogenicity.

The tumour-homing capacity of Mesenchymal stem cells (MSC) can potentially be harnessed to address these issues by combining with magnetic nanoparticles (MNPs) as contrast enhancers. Use of MSC as gene delivery carriers is hampered by their lack of permissivity to Ad-transductions.

In this study, we have used 'magnetotransduction' i.e. use of external magnetic fields to facilitate MNP-Ad-vectors entry into MSC, to overcome their non-permissivity to standard Ad transduction. Polyethylenimine coated MNP (PEI-MNP) conjugated to Ad containing a reporter gene (Luciferase or Green fluorescent Protein) were used successfully to magnetotransduce murine MSC (mMSC) leading to >4000-fold enhanced gene expression over standard transduction alone. This did not affect their viability, differentiation and migratory ability *in vitro*. Colocalisation of Ads and near infrared dye (IR820)-labelled MNPs was demonstrated using confocal-microscopy and flow-cytometry.

Simultaneously, a high degree of intracellular iron loading of mMSC, sufficient for MRI detection was achieved. Further, magnetotransduced mMSC in tumours could be detected by MRI *ex vivo*. As this manipulation had no adverse impact on MSC, these agents can now be further assessed in immunocompetent preclinical models for use in therapeutic gene delivery and simultaneous MRI imaging. This will support their future translation to the clinic.

P22: CO-EXPRESSION OF ARGININOSUCCINATE SYNTHETASE; A STRATEGY TO REDUCE THE THRESHOLD FOR THERAPEUTIC SUCCESS IN THE TREATMENT OF OTC DEFICIENCY

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Ornithine transcarbamylase (OTC) deficient *spf*^{ash} mice have been successfully treated with both adenovirus and adeno-associated virus (AAV) vectors. The task now is to translate this success in mice through to human therapy. Size presents a formidable challenge as the neonatal human liver is 200-fold larger than that of the adult mouse. In view of this we are exploring a strategy aimed at optimising the ureagenic capacity of transduced hepatocytes such that the level of gene transfer required to confer therapeutic benefit is reduced.

Argininosuccinate synthetase (ASS) is the rate limiting enzyme of the urea cycle. We hypothesise that by over-expressing ASS in concert with OTC gene transfer, the ureagenic capacity of transduced hepatocytes can be increased to supra-physiological levels thereby reducing the number of transduced hepatocytes required for therapeutic benefit. To test this hypothesis we have co-transduced *spf*^{ash} mouse hepatocytes *in vivo* with AAV vectors encoding murine OTC (rAAV2/8.mOTC) and ASS (rAAV2/8.mASS). rAAV2/8.mASS was purpose built for these studies and was function tested *in vitro* and *in vivo* to confirm over-expression of mASS. The respective doses of each vector used were selected to provide liver-wide supra-physiological expression of mASS, but correction of OTC deficiency in only a subset of hepatocytes. The extent of metabolic correction at this dose of rAAV2/8.mOTC was then compared in *spf*^{ash} mice in the presence and absence of co-transduction with the rAAV2/8.mASS. Urinary orotic acid levels and *in vivo*

ureagenesis were chosen as end-point measures. Experiments have shown that delivery of rAAV2/8.mASS alone reduces orotic aciduria but does not consistently enhance ureagenesis in *spf*^{ash} mice. These results suggest that enhancement of ureagenesis was not significant at the selected doses and further experiments will determine whether a threshold level of ASS and OTC activity is required. Subsequent studies could be performed using bi-cistronic vectors encoding both enzymes.

P23: THE GENERATION OF GENOMIC REPORTER ASSAYS FOR γ- AND β-GLOBIN GENES EXPRESSION

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It is well known that high level of γ-globin expression can ameliorate the clinical symptoms associated β-thalassemia and sickle cell disease and this is best demonstrated in individuals who co-inherit hereditary persistence of foetal haemoglobin (HPFH). Pharmacologic reactivation of HbF has therefore been pursued as a potential therapeutic strategy for the treatment of haemoglobinopathies. Several HbF inducers have been identified and used clinically, however these agents lack specificity and some are toxic and carcinogenic. Understanding the regulatory mechanisms of the globin gene expression, especially the switching process from γ- to β-globin, would undoubtedly help to identify new targets for the development of better therapeutic strategies. In order to identify and evaluate factors that may influence the globin switching process a novel genomic dual-reporter assay system was developed for the assessment of globin gene expression. Using homologous recombination in bacteria, a 183 kb bacteria artificial chromosome (BAC) containing the intact human β-globin locus was modified to insert the reporter genes DsRed under the control of either Gγ or Aγ-globin promoter, and EGFP under the control of β-globin promoter. These constructs were used to generate stable cell lines in both K562 (human erythroleukaemic cells) and MEL (murine erythroleukaemic cells) cell lines. These cellular genomic reporter assays closely recapitulate the γ- and β-globin genes expression in embryonic/foetal (K562) or adult (MEL) environments, allowing for rapid and simultaneous analysis of both globin genes expression at specific developmental stages. Our results show that these genomic reporter assays have applications in the identification and evaluation of new chemical inducers of HbF, as well as in the study of globin gene regulation in particular the γ- to β-globin switch.

P24: ANTISENSE OLIGOMER INDUCED SPLICE MANIPULATION OF SURVIVAL MOTOR NEURON EXON 7

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Spinal muscular atrophy (SMA) is the most common autosomal recessive neurodegenerative disorder of children with an incidence of 1 in 10,000 live births and a carrier frequency of 1 in 40–50 adults. SMA is attributable to a deficiency in the survival of motor neuron protein (SMN) caused in most patients by mutation of the SMN1 gene. Deficiency of SMN protein results in degeneration of anterior horn cells leading to hypotonia, symmetrical muscle weakness, fasciculation of the tongue muscles and tremors of the fingers and hands.

There are two genomic copies of the SMN gene (SMN1 and SMN2) and expression of the full length SMN2 gene product has been shown to partly compensate for the lack of SMN1 product. However, a single base difference (C/T) at the sixth nucleotide in exon 7 of the SMN2 gene promotes excision of that exon from the mature transcript leading to production of only a minimal amount of full-length protein.

The promotion of exon 7 inclusion in the SMN2 transcript by masking splice silencing motifs with antisense oligonucleotides (AO) is a potential intervention to increase the level of full-length SMN protein. Using a panel of modified 2′-O-methyl AO's (phosphorothioate backbone) targeted across exon 7 of the SMN2 pre-mRNA we aimed to identify possible splice silencing motifs that would lead to exon 7 inclusion during SMN2 expression in SMA fibroblasts.

While our results failed to demonstrate a strong exonic silencing motif which could be masked to promote exon 7 inclusion parallel experiments in normal control cells showed robust exon 7 skipping could be induced by AO's targeted near the exon 7 donor splice site. Therefore, this study has provided additional insight into exon splicing as it relates to SMN exon 7 providing a potential model with which to study the functionality of alternatively spliced SMN proteins.

P25: OPTIMISATION OF ANTISENSE OLIGONUCLEOTIDE COCKTAILS USING *IN SILICO* AND *IN VITRO* TECHNIQUES FOR TARGETED EXON SKIPPING IN THE DYSTROPHIN CENTRAL ROD DOMAIN

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Becker muscular dystrophy (BMD), an allelic disorder to the severe condition Duchenne muscular dystrophy (DMD) presents with variable severity and age of onset. BMD is caused by mutations in the dystrophin gene that maintain the reading frame, resulting in a shorter, but still partially functional protein. BMD mutations are commonly found in the central rod domain, which consists of 25 triple-helical repeats similar to spectrin, with recent reports suggesting these repeats are not equivalent. One spectrin repeat is encoded by approximately 2 exons, hence excluding a single exon will leave an imperfect spectrin repeat. Antisense oligomer (AO) cocktails are currently being optimized for targeted removal of exons 23 + 24 in the *mdx* mouse, an animal model of DMD with a nonsense mutation in exon 23, to compare the effect on phenotype of taking out a half or a whole spectrin repeat. *In silico* analysis of oligomer cocktails is consistent with our hypothesis that the annealing of one compound to the pre-mRNA facilitates binding of the second oligomer. Individually, components of some cocktails induce no or only low levels of exon skipping. Hence it appears binding of one oligomer to a region not involved in spliceosome formation may permit another oligomer to anneal and mask crucial splice-control motifs not otherwise accessible. Optimized AO cocktails are being used in mice to induce multiple dystrophin isoforms for detailed molecular

histological and physiological studies. Functional muscle testing in 12-week-old mice indicates that *mdx* muscles are weaker and more susceptible to stretch-induced damage when compared to normal mice, especially the diaphragm, which is most reflective of the human DMD phenotype.

P26: SCA-1 AND C-KIT AND MUSCLE REMODELLING BY NON-HEMOPOIETIC BONE MARROW CELLS

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Bone marrow-derived cells (BMCs) have been shown to display myogenic tissue remodelling capacity. However, confined to around 1- to 3% myo-remodelling of recipient muscle fibres in vivo, there remains much contention regarding the clinical relevance of bone marrow for therapeutic application in myo-degenerative conditionsThis study aimed to evaluate if any particular BMC population displayed enhanced myo-regenerative capacity as well as to explore selectable molecular markers for isolation of these cells. On the basis that BMCs and muscle precursors expressing stem cell antigen-1 (Sca-1) were proposed to be the optimal cells able to remodel dystrophic mdx muscle prompted this study of relative myo-remodelling contributions from BM cells (compared to muscle cells) on the basis of expression or absence of Sca-1. Our results showed that myo-regenerative activity does not differ in cells sorted solely on the basis of Sca-1 presence or absence from either muscle or BM. Further fractionation of BM with lineage and CD45 cell surface markers subsequently revealed a strong selectability of myo-regenerative capacity with Sca-1 and this was in fact further enhanced in cells that also expressed c-Kit. On the basis of these results, we propose that c-Kit may provide a useful marker in addition to Sca-1 for the selection of cells with myo-remodelling capacity from BM and possibly from other cell types.

P27: TARGETED CORRECTIVE GENE CONVERSION (TCGC): MOLECULAR SYSTEMS FOR IMPROVED CORRECTION OF GENE MUTATIONS IN MUSCLE

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Targeted corrective gene conversion (TCGC) holds much promise as a future therapy for many hereditary diseases in humans, but there still remain significant impediments to effective mutation correction and it is clear that significant work remains to improve TCGC to levels where it can be considered for translation to the clinical setting. Nevertheless, mutation correction frequencies varying between 0.0001% and 40% have been reported using chimeraplasty, oligoplasty, triplex-forming oligonucleotides, and small corrective PCR amplicons. We have investigated potential mechanisms that impede effective gene correction and potential methods by which to improve the efficiency of dystrophin gene mutation correction in the mdx mouse model of Duchenne Muscular Dystrophy (DMD). Arrest of cell cycle using Hydroxyurea and double Thymidine blockade significantly improved TCGC frequency in mdx myoblasts at the gene level, but it was clearly evident that use of transfection reagents rendered TCGC efficiency variable with some, albeit limited expression of the corrected loci. The latter aspect was investigated by adjustment of transfection conditions, resulting in more robust gene correction with reliable expression of the corrected locus in a proportion of the treated cells, but still affected by viability issues imparted by the toxic chemistries used to mediate improved gene correction. These studies have generated new methods for improvement of TCGC by cell cycle arrest using molecular methods less toxic than transfection reagents or chemical cell cycle inhibitors: Molecular technologies such as PNA chemistry, advanced electromaterials, and CNA motifs that present lesional signals to the DNA metabolic machinery are potential technologies that may impart levels of efficiency that translate more efficiently from gene to protein levels and facilitate consideration towards clinical application.