THE JOURNAL OF GENE MEDICINE
J Gene Med 2005; **7**: 1113–1143.
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/jgm.808

The Australasian Gene Therapy Society

4th Society Meeting

27–29 April 2005

Rydges Hotel, Carlton, Melbourne, Victoria, Australia

The Australasian Gene Therapy Society (AGTS) held its fourth biennial meeting on 27–29 April 2005, providing an opportunity for groups across the Australasian region to present their latest contributions in gene therapy research. AGTS acknowledges and thanks the Journal of Gene Medicine for making the abstracts presented during the meeting available to the international research community.

The Organising Committee for the 4th meeting of the society included:

Panos Ioannou, Melbourne
John Rasko, Sydney
Ming Wei, Brisbane
Ann Simpson, Sydney
Ian Alexander, Sydney
Gerald Both, Sydney
Jane Fleming, Sydney
David Jans, Melbourne
Colin Pouton, Melbourne
Martin Pera, Melbourne
Bob Williamson, Melbourne
Alan Trounson, Melbourne
Jim Vadolas, Melbourne
Robert Kapsa, Melbourne
Chee Kai Chan, Melbourne
Sylvia Metcalf, Melbourne
Joseph Sarsero, Melbourne
Heidi Peters, Melbourne
Mary Anne Aitken, Melbourne
Steve Jane, Melbourne

The meeting was sponsored by:

Murdoch Childrens Research Institute, Melbourne
Children's Medical Research Institute, Sydney
St Vincent's Hospital, Melbourne
National Muscular Dystrophy Research Centre, Melbourne
Howard Florey Institute, Melbourne
Biotechnology Australia
Invitrogen
Roche
GE Healthcare/Amersham
Chemicon Australia, Upstate
Corbert Research
Davies Collison Cave
Genzyme
The Journal of Gene Medicine,
John Wiley & Sons, Ltd.
Proligo
Scientifix

Abstracts communicated by Joseph P. Sarsero.

For further information, please refer to the AGTS webpage: http://www.agts.org.au

PLENARY LECTURES

Plenary 1. *In vivo* Selection and Chemoprotection of Haematopoietic Stem Cells: Implications for the Treatment of Genetic and Acquired Diseases

Hans-Peter Kiem, *Fred Hutchinson Cancer Research Center, Markey Molecular Medicine Center, University of Washington School of Medicine, 1100 Fairview Avenue N., D1-100, Seattle, WA 98109, USA.*

The therapeutic potential of hematopoietic stem cell gene therapy has clearly been demonstrated with the successful treatment of patients with severe combined immunodeficiency (SCID). A critical limitation to stem cell gene therapy has been the low gene transfer efficiency with available vector systems in clinical trials or clinically relevant large animal models, and more recently the risk of insertional mutagenesis. In vivo selection has been proposed as a strategy to increase the level of in vivo gene marking by conferring a selective survival advantage to the transduced cells. Since stem cell gene transfer studies in the mouse have generally not been very predictive of outcomes in humans we have studied stem cell gene transfer and selection in clinically relevant large animal models. We have studied the use of a drug resistance gene O^6-methylguanine-DNA methyltransferase (MGMT) to select transduced cells in large animal models. We have used the P140K mutant of MGMT, which allows for enhanced in vivo selection when BCNU and temozolomide are combined with O^6-benzylguanine, an inhibitor of wild type MGMT but not P140K. We have demonstrated that this strategy can be used in dogs and monkeys to significantly increase gene-marking levels in multilineage repopulating cells with marking levels of greater than 90% in granulocytes. We have also shown that MGMT-expressing cells can provide protection from BCNU- or temozolomide-induced myelosuppression which should have significant implications for the treatment of patients with malignant diseases. Drug treatment resulted in polyclonal selection and protection of hematopoietic stem cells and so far there have been no signs of monoclonality or hematological abnormalities in the animals. These data demonstrate that durable, therapeutically relevant in vivo selection and chemoprotection of gene-modified cells can be achieved in large animal models and support the evaluation of this strategy in clinical trials.

Plenary 2. Novel Episomal Replicating Vectors for Gene Therapy

Hans J. Lipps, *Institute of Cell Biology, University Witten/Herdecke, 58448 Witten, Germany.*

Currently used viral vectors for gene therapy suffer from a number of limitations including integration into the host genome which may lead to insertional mutagenesis and silencing of the transgene, expression of viral proteins leading to immunological reactions of the recipient organism or only transient expression of the transgene. There is increasing agreement that the ideal vectors for gene therapy should exclusively contain chromosomal elements and replicate episomally in the recipient cell. Based on the observation that the binding of an origin of replication to the nuclear matrix precedes the onset of S-phase in mammalian cells, we constructed a vector containing a scaffold/matrix attachment sequence (S/MAR). This vector replicates at low copy number episomally in a variety of mammalian cells, including primary cells. It is mitotically stable in the absence of selection by binding to the nuclear matrix through an interaction with the matrix protein SAF-A. We show that its function depends exclusively on a transcription unit linked to the S/MAR. This allowed us to construct a synthetic episomally replicating vector. Possible application of this vector system will be discussed and to overcome the problem of low transfection efficiency results to deliver it as a pseudovirus are presented.

Plenary 3. Human Embryonic Stem Cells and Gene Therapy – A Vision Shared with Panos

Alan Trounson[1] and Panos Ioannou[2], [1]*Monash Immunology and Stem Cell Laboratories, Monash University, Wellington Road, Clayton, Victoria 3800, Australia.* [2]*Cell and Gene Therapy Research Group, Murdoch Children's Research Institute, Royal Children's Hospital, Flemington Road, Parkville, Victoria 3052, Australia.*

Human embryonic stem (ES) cells are pluripotential, continuously multiplying cells derived from early stage human IVF embryos that are donated for research when excess to patients' requirements to resolve their infertility. It is possible to introduce genes into human ES cells in a targeted fashion (Zhang *et al*., 2001), and it was Panos Ioannou's vision that it would be possible to introduce BAC constructs containing genes of interest into human ES cells using modifications of methods used for mouse ES cells.

The prospect of using human ES cell derivatives for the correction of serious genetic disease is possible as derivatives of human ES cells do not appear to induce a serious rejection response following transplantation, or they may be immunologically compatible when derived from patients with genetic disease, by nuclear transfer of their somatic cells into enucleated host oocytes (patient specific embryonic stem cells). The proof-of-concept experiments that demonstrate the latter approach have been published using mouse ES cells (Rideout *et al*., 2002). In this case, a defect in the Rag2 gene which causes severe immune deficiency was corrected by deriving mouse ES cells from the mutant mice, correcting the mutation in one of the $Rag2^{-/-}$ alleles by homologous recombination and induction of lymphoid differentiation by introducing the HoxB4 gene and culture of the ES cells on OP9 cells. When transplanted into irradiated mutant $Rag2^{-/-}$ mice, the immune deficiency was corrected when the ES cell hematopoietic derivatives colonised the recipient's bone marrow.

Stem cells may be an optimal vehicle for delivery of gene products into diffuse targets such as the central nervous system. Examples for this include the correction of Tay-Sachs disease in mice using a mouse neural progenitor transfected with the gene encoding enzymatically active β-hexosaminidase (Lacorazza *et al*., 1996). Mucopoly saccharidosis VII, a lysosomal storage disorder caused by β-glucuronidase deficiency, has also been corrected using a neural progenitor cell line (Snyder *et al*., 1995).

It is possible that embryonic stem cells that have been lineage restricted by directed differentiation are the ideal vehicle to deliver tissue-specific gene therapy. The vision shared with Panos may one day be a method of choice for treatment of inherited genetic disease. He would be pleased if we continue his work in this direction.

References

Lacorazza, *et al*., 1996; *Nat. Med.* **2**: 424–429.
Rideout, *et al*., 2002; *Cell* **109**: 17–27.
Snyder, *et al*., 1995; *Nature* **374**: 367–370.
Zhang, *et al*., 2001; *Nat. Biotechnol.* **19**: 1129–1133.

Plenary 4. Differentiation and Genetic Manipulation of ES Cells – Creating Tools for Regenerative Medicine and Therapy of Genetic Diseases

Andrew Elefanty, *Monash Immunology and Stem Cell Laboratories, Monash University, Clayton, Victoria 3800, Australia.*

In order to realize the therapeutic potential of human embryonic stem cells (hESCs), it will be necessary to regulate their differentiation in a uniform and reproducible manner. Also, in order to better understand and possibly correct genetic diseases, it would be advantageous to have the ability to manipulate the ES cell genome.

Murine ES cell lines were generated in which reporter genes were inserted by homologous recombination into the loci of genes marking key stages during ES cell differentiation. These included the *Mixl1* gene (*Mixl1*$^{GFP/w}$ cells) whose expression is restricted to precursors of mesoderm and endoderm in the primitive streak and the *Pdx1* gene (*Pdxl1*$^{GFP/w}$ cells) that is expressed in the developing pancreas. GFP expression from the *Pdx1* locus has been used as a reporter for foregut endoderm and pancreatic development, in order to improve the in vitro culture conditions for pancreatic islet cell generation. The *Mixl1*$^{GFP/w}$ cell line has been used to quantify the effects of growth factors on mesendodermal differentiation. We have recently developed a new method for efficient mesodermal differentiation of hESC, showing that human embryoid bodies (hEBs) expressed similar molecular markers to developing mouse EBs. In the presence of hematopoietic growth factors, blood cells formed in over 90% of hEBs.

The ability to identify readily hESCs and their differentiated progeny in transplantation experiments will facilitate the analysis of hESC potential and function in vivo. We have generated a hESC line, designated "*Envy*", in which robust levels of GFP are expressed in stem cells and all differentiated progeny. We are complementing these experiments by generating hESCs which carry reporter genes inserted into specific gene loci, analogous to the mouse lines that we have described. The success of these experiments will lay the foundations for the generation of disease models and future successful therapy of genetic lesions.

Plenary 5. Modification of Genomic Targets by Small Fragment Homologous Replacement (SFHR): Development of Genetic Therapies for Inherited Disease

Rosalie Maurisse,[1] Babak Bedayat,[1] Alireza Abdolmohammadi,[1] J. Patrick O'Neill,[2] Janice A. Nicklas,[2] Kaarin K. Goncz,[3] Dieter C. Gruenert[1,3,4], [1]*California Pacific Medical Center Research Institute, Cell Biology Division, San Francisco, CA 94107, USA; Departments of* [2]*Pediatrics and* [3]*Medicine, University of Vermont, Burlington, VT 05405, USA;* [4]*Department of Laboratory Medicine, University of California, San Francisco, San Francisco, CA 94143, USA.*

Gene therapy has opened a new arena for clinical management of inherited disease. Genetic therapies now in clinical trials are based on introducing therapeutic cDNA into the cells of an organ that manifests a specific inherited disease-related pathology. While most gene therapy clinical trials involve the treatment of cancer, there appears to have been some success in the treatment of at least one inherited disorder using a cDNA-based gene therapy. Patients with X-linked severe combined immune deficiency (X1-SCID) have been successfully treated with retroviral vectors carrying the γ_C subunit transgene of the IL-2 receptor. Unfortunately, several of the patients treated with this transgene system have acquired T-cell leukemia that appears to be the result of transgene induced insertional mutagenesis. Alternative methods that use oligonucleotides for genetically modifying cellular DNA and phenotype have been developed over the past several years. The ability to modify genomic sequences has been particularly appealing in that it can modify a gene, either by correcting or introducing a mutation, and yet maintain the integrity of the gene by ensuring that the relationship between the protein coding sequences and the cell-specific gene regulatory elements is not disrupted. The predominant genomic DNA modification strategies using DNA oligonucleotides that have been developed include small fragment homologous replacement (SFHR), triplex-forming oligonucleotide (TFO) modification, RNA-DNA oligonucleotide (RDO) modification, zinc-finger (ZNF) enzymatic cleavage-mediated oligonucleotide modification. Of these approaches, SFHR has been particularly promising in that it has been effective at not only inducing single base transitions, but it has also shown the capacity to insert and delete multiple bases simultaneously. *In vitro, in vivo*, and *ex vivo* studies have demonstrated sequence-specific modification of the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) gene, the sickle cell anemia locus of the human β-globin gene, and the hypoxanthine-guanine phosphoribosyltransferase• (HPRT) gene associated with Lesch-Nyhan syndrome. The studies undertaken have indicated that SFHR can occur at efficiencies that might be therapeutic. Furthermore, SFHR has shown potential for development of transgenic animal models of inherited disease. Ultimately, a combination of SFHR and stem cell based therapies might prove to be efficacious in the repair of organs damaged by the pathology associated with the course of an inherited disease. This work has been supported by NIH grant DK066403 as well as grants from the Cystic Fibrosis Foundation, Pennsylvania Cystic Fibrosis, Inc, and the California Pacific Medical Center Research Foundation.

Plenary 6. Human Engineered Chromosomes for Gene Therapy

Lee H. Wong, Richard Saffery, Danielle Irvine, Julie Quach, Melissa Anderson, Elizabeth Earle, Kerry Fowler, and K. H. Andy Choo, *Chromosome Research Laboratory, Murdoch Children's Research Institute, Royal Children's Hospital, Flemington Road, Parkville, Victoria 3052, Australia.*

The human engineered chromosome (HEC) technology represents a potentially exciting gene delivery system for the correction of human genetic disorders. However, for the full potential of HECs as chromosome-based gene delivery vectors to be realised, a number of key obstacles must be overcome. Five essential milestones must be satisfied before clinical studies can commence. These milestones are: to be able to clearly demonstrate (1) production of stable and fully characterisable HECs; (2) insertion and proper expression of desired genes on HECs; (3) purification of

HECs to homogeneity; (4) delivery of purified HECs into different cell types; and (5) HEC stability and expression in different tissues in animal models. Although research to achieve these outcomes continues to encounter significant challenges, considerable progress has been made in the past few years. We will review the current status of progress and obstacles in each of the five areas as we attempt to move the HEC technology forward as a possible gene therapy tool.
Our own study has been based on the use of neocentromere-based HECs. These neocentric HECs differ from HECs generated by other groups in a significant way in that unlike all other HECs that used repetitive centromeric DNA to confer mitotic stability, our HECs do not rely on non-repetitive centromere DNA. As such our HECs offer the advantage of ease of full characterisation and manipulation. A review of our recent data, including those in transchromosomal mice, will be presented in the light of those of the other workers.

Plenary 7. Antisense Induced Redirection of Splicing: Will Duchenne Muscular Dystrophy be the Thin Edge of the Wedge?

Steve D Wilton and Sue Fletcher, *Experimental Molecular Medicine Group, Centre for Neuromuscular and Neurological Disorders, QE II Medical Centre, Nedlands, 6009 Perth, Australia.*

Alternative splicing is a major source of diversity of gene expression. Multiple products, some of which have antagonistic activities, are generated from the same gene. Redirecting gene expression through antisense oligonucleotide (AO)-induced modification of splicing patterns could be applied to a range of conditions including: (a) correcting gene defects arising from splicing mutations, (b) by-passing serious disease-causing mutations through induced exon skipping, and (c) redirecting gene transcript splicing patterns as a potential anticancer therapy.
We are evaluating the potential of AOs to by-pass defects in the dystrophin gene that preclude the synthesis of a functional protein and result in the severe Duchenne muscular dystrophy. In-frame dystrophin gene re-arrangements typically result in a milder form of muscular dystrophy, clearly demonstrating that some regions of the dystrophin protein can be lost with relatively minor consequences. The huge size of the dystrophin gene, its complex expression patterns and the fact that the predominant isoforms are expressed in non-dividing cells, have hindered gene replacement therapies for DMD, but render the dystrophin gene an ideal candidate to evaluate AO therapies directed at splicing intervention.
An international consortium between researchers in the Netherlands, United Kingdom and Australia is planning clinical trials to evaluate AO intervention in DMD. In a relatively short space of time, exon skipping has progressed from some interesting *in vitro* experiments to the planned commencement of first-time-in-human trials later this year. Despite much optimism in this area, caution must be advised. Exon skipping cannot "cure" DMD but may reduce the severity; exon skipping cannot be applied to all cases of DMD and complete coverage of many dystrophin mutations would require a large battery of different AOs. Each AO must be regarded as a different drug and the extensive safety and toxicology testing will be a massive undertaking that must be addressed.

Plenary 8. Systemic Delivery of Adenovirus: Use Of Polymers to Mask Unwanted Infection and Enable Intravenous Delivery

Leonard W. Seymour, *et al*. (Len.Seymour@clinpharm.ox.ac), *University of Oxford and Hybrid Systems Ltd., Oxford UK.*

We have previously reported that active uptake of adenovirus into hepatocytes *in vivo* can be ablated through the conjugation of a polymer, poly-[N-(2-hydroxypropyl)methacrylamide] (PHPMA) to the amino groups on the virus surface (pcVirus). This results in an increased level of adenovirus in the plasma (ca. 10% of the input dose following i.v. administration of a dose of 1×10^{11} particles) and a reduction in the levels of serum transaminases detectable in the blood [1].
Intravenous administration of clodronate liposomes to mice is known to destroy tissue macrophages in the spleen and the liver [2] and so we hypothesised that clodronate liposomes could further increase the circulation of pcVirus. Twenty-four hours following i.v. administration of clodronate liposomes, 5×10^{10} particles of non-coated virus and pcVirus were administered i.v., blood samples taken and analysed for the amounts of viruses present using Q-PCR. The data shows that 100% of the administered dose of pcVirus was present in the bloodstream, compared with only 1% of the non-coated virus.
As an alternative to using a pharmacological agent to suppress phagocyte activity we fractionated the dose of pcVirus, as it has been reported that the scavenging of adenovirus by Kupffer cells results in their destruction [3]. Four hours following pre-doses of 1×10^{10} and 1×10^{11} particles of pcVirus, a further dose of 1×10^{11} particles was administered (the chase dose), blood samples taken and virus content analysed using Q-PCR. The level of pcVirus detectable in the blood 30 min after the chase dose was clearly influenced by the size of the pre-dose, with a predose of 1×10^{11} particles resulting in 100% of the chase dose being detected. Taken together these data indicate that ablation of the natural tropism of adenovirus can be coupled with temporary suppression of host scavenging mechanisms to permit efficient systemic delivery of therapeutic adenovirus.

References

1. Green, *et al*. *Gene Ther.* 2004; **11**: 1256–1263.
2. Van Rooijen N, *et al*. *Cell Tissue Res.* 1990; **260**: 215–222.
3. Schiedner G, *et al*. *Hum. Gene Ther.* 2003; **14**: 1631–1641.

Plenary 9. Stem Cell Gene Transfer by Lentiviral Vectors: Promise, Challenges, and New Therapeutic Opportunities

Luigi Naldini, *San Raffaele Telethon Institute for Gene Therapy, Milano, Italy.*

Plenary 10. Oversight of Clinical Gene Therapy Studies in Australia

R. J. A. Trent, *NHMRC Gene and Related Therapies Research Advisory Panel (GTRAP). National Health and Medical Research Council, MDP 100, GPO Box 9848, Canberra, ACT 2615, Australia.*

GTRAP was set up in 1994 to advise HRECs (Human Research Ethics Committees) on matters relating to technical, medical

and scientific aspects of clinical trials that utilised somatic cell gene transfer ("gene therapy"). At the time it was considered by the NHMRC that gene transfer technology was new, there were potential problems associated with it, and so a centralised national committee of experts would be useful source of information for HRECs. GTRAP also provides advice to scientists and the NHMRC on matters related to clinical gene therapy. Although HRECs will give the final approval for clinical gene therapy studies in their institutions, the HRECs cannot provide this approval unless the study has been reviewed and approved by GTRAP. More recently the terms of reference for GTRAP have been expanded to include xenotransplants and cellular therapies with stem cells. The function of GTRAP in the latter is still evolving and is likely to focus on class 3 products as defined by the TGA in its proposed regulatory framework for tissues and biological therapies (see http://www.tga.gov.au/bt/prtisreg.htm). This new framework, if implemented, will impact on clinical gene therapy particularly in relationship to GMP. GTRAP works closely with the Therapeutic Goods Administration (TGA), the Office of the Gene Technology Regulator (OGTR), and the Australian Health Ethics Committee (AHEC), having members in common with these regulatory/advisory bodies. This can be helpful to scientists, clinicians or sponsors who are involved in clinical gene therapy and want to know more about particular regulatory issues. Information on GTRAP including membership and requirements for review of gene therapy studies can be found on http://www.nhmrc.gov.au/research/gtrap.htm.

Plenary 11. Gene Therapy for SCID-X1: Progress, Pain and Prospects

Ian Alexander,[1,2] Bruce Bennetts,[1] Julie Curtin,[1] Samantha Ginn,[1] Alyson Kakakios,[1] Belinda Kramer,[1] Margot Latham,[1] Grant Logan,[2] Geoffrey McCowage,[1] Peter B. Rowe,[2] Christine Smyth,[2] and Melanie Wong[1], [1]*The Children's Hospital at Westmead and* [2]*Children's Medical Research Institute, Locked Bag 4001 Westmead, NSW 2145, Sydney, Australia.*

It is now five years since Alain Fischer, Marina Cavazzana-Calvo and colleagues at the Necker Hospital in Paris reported the first successful treatment of a genetic disease (SCID-X1) by gene therapy: a true milestone in medical history. Initial excitement, however, was soon tempered by a succession of adverse events directly attributable to the retroviral gene transfer technology used. A molecular understanding of these events, and accumulating data and experience from treated infants, are providing an increasingly clear picture of the challenges that must be surmounted to realise the full potential of this exciting new therapeutic approach.

To date, eighteen infants have been treated world-wide, ten in France, one in Australia (in collaboration with the French) and seven in England. Excluding several treated too recently to evaluate therapeutic efficacy, all but one have exhibited partial or complete immunological reconstitution, with the extent of reconstitution appearing to correlate with the dose of gene-modified cells administered. Cell doses in excess of 3×10^6 $CD34^+/\gamma c^+$ cells/kg appear to be required for robust reconstitution. The Australian infant received 1.3×10^6 $CD34^+/\gamma c^+$ cells/kg with resultant partial reconstitution eventually requiring further treatment (BMT from a matched unrelated donor with resultant chronic graft versus host disease and poor immunological reconstitution). Three of eleven infants in the French trial have developed leukaemia as a consequence of vector-mediated insertional mutagenesis and, remarkably, in the first two of these infants activation of the LMO2 gene was involved. This does not appear to be the case in the third infant who developed leukaemia as recently as January this year.

With the efficacy of gene therapy for SCID-X1 looking promising, albeit yet requiring long-term follow-up, the immediate challenge is to develop safer integrating vectors and preclinical assay systems that can accurately quantify the safety gain achieved in advanced human clinical use.

Plenary 12. Lessons from a Clinical Trial of Liver-Directed AAV Gene Transfer in Hemophilia B

John Rasko,[7] Katherine High,[1,2,3] Michael Tigges,[4] Catherine Manno,[1,2] Denise Sabatino,[1] Michael Dake,[5] Mahmood Razavi,[5] Valder Arruda,[1,2] Roland Herzog,[1,2] Pradip Rustagi,[5] Jurg Sommer,[4] Margaret Ragni,[6] Barbara Konkle,[2] Ruth Lessard,[4] Alvin Luk,[4] Bertil Glader,[4] Glenn Pierce,[4] Linda Couto,[4] Haiyan Jiang,[4] and Mark Kay[5], [1]*The Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA;* [2]*University of Pennsylvania, Philadelphia, PA 19104 USA;* [3]*Howard Hughes Medical Institute, Philadelphia, PA 19104 USA;* [4]*Avigen, Inc., Alameda, CA 94502 USA;* [5]*Stanford University, Stanford, CA 94305 USA;* [6]*University of Pittsburgh Medical Center, Pittsburgh, PA, USA, and* [7]*Gene Therapy, Centenary Institute and Sydney Cancer Centre, Royal Prince Alfred Hospital, Sydney, Australia, 2042.*

In 2001 we initiated a phase I dose escalation study of AAV-FIX in humans with severe hemophilia B (FIX levels <1%). 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. The first two doses, 2×10^{11} and 1×10^{12} vg/kg in patients, were safe but subtherapeutic. 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 (subject E) by a reversible asymptomatic transaminitis. There was no evidence of a FIX inhibitor at any point. Two differences were hypothesized to contribute to the difference between the observed long-term expression in the large animal model of hemophilia B and short-term expression in humans with the disease. First, pre-existing immunity to wild-type AAV-2 occurs in humans, but not dogs; and second, prior infection with hepatitis B, C, or both, can be found in humans but not in dogs with hemophilia. To further assess the roles of hepatitis and of the immune response to AAV-2, we treated an additional subject (subject G) at a dose of 1×10^{12} vg/kg. This subject was 20 yrs. of age and had never been infected with hepatitis. Nevertheless, his transaminases began to rise 3 weeks after vector injection, peaked 6 weeks after injection, and resolved spontaneously as had been seen in subject E. Both of these subjects had similar and low baseline anti-AAV antibody titers. Assessment of immune response to AAV-2 was carried out by ELISpot at serial time points before and after vector injection in subject G. These results were consistent with a model in which a T cell response to AAV capsid epitopes results in elimination of the transduced cells. This response is only briefly detectable in PBMCs. These immune responses may limit use of standard serotypes of AAV for gene transfer

into human liver. Transient immunosuppression may prevent these responses.

THE GREG JOHNSON MEMORIAL ORATION

Gene Therapy for Prevention and Cure of Type 1 Diabetes

Leonard C. Harrison (harrison@wehi.edu.au), *The Walter & Eliza Hall Institute of Medical Research, Parkville 3050, Victoria, Australia.*

Type 1 diabetes (T1D) is an autoimmune disease in which genes and environment contribute to cell-mediated immune destruction of insulin-producing beta cells in the islets of the pancreas. Insight into pathogenic mechanisms opens the way for gene therapy strategies to prevent and cure T1D. The therapeutic Holy Grail for autoimmune disease prevention is 'negative' vaccination against autoantigens that drive immune-mediated pathology, to induce disease-specific immune tolerance. This can be achieved by administering autoantigen via a 'tolerogenic' route (mucosal), cell type (resting dendritic cell), mode (with blockade of T-cell co-stimulation molecules) or form (as an 'altered peptide ligand'). Proinsulin is a key autoantigen that drives beta-cell destruction in the non-obese diabetic (NOD) mouse model and probably in humans. We found that intranasal vaccination of NOD mice with plasmid DNA encoding proinsulin induces regulatory T cells that protect against diabetes. Allogeneic or mixed-allogeneic bone marrow transplantation strategies are being trialled for severe autoimmune diseases, but are unlikely to be suitable for T1D prevention due to the requirement for cytotoxic conditioning of the host, and the risks of graft rejection and graft-versus-host disease. An ideal, safe approach would be to use autologous, genetically-engineered hematopoietic stem cells (HSCs) or their progeny to introduce autoantigen into the immature hematopoietic compartment. We found that transfer into young, irradiated NOD mice of 1000 syngeneic HSCs encoding proinsulin expression in antigen-presenting cell progeny totally prevents diabetes. This dramatic effect appears to depend on proinsulin expression by 'resting' dendritic cells. The application of this 'cell therapy' to humans must overcome two obstacles – introducing genes into stem cells without the risk of oncogenesis and avoiding toxic conditioning regimens in the host. The use of adenovirus vectors for transducing human and mouse HSCs will be discussed. The cure of T1D requires replacement or regeneration of beta cells, while averting immune attack. Genes for growth and transcription factors that promote beta-cell development and genes for immune molecules that protect islet grafts locally against immune attack have been delivered directly into pancreatic/islet tissues, with promising results in rodent models. Gene therapy for the prevention and cure of T1D must now be translated to humans.

ORAL PRESENTATIONS

O1. Rep-Dependent Site-Specific Integration into the AAV-S1 Region by Synthetic Integration Elements

Horace Drew, B. Ann Dalton, Denise Lewy and Gerald W. Both, *CSIRO Molecular Science, North Ryde, NSW 2113, Australia.*

Achieving the specific integration of genes into a non-essential site on the human chromosome is a prime objective in the development of gene therapy. Much attention has been devoted to adapting the naturally integrating adeno-associated virus (AAV) as a delivery vector since wild-type AAV strongly prefers to integrate at a site (AAV-S1) on chromosome 19. This process is catalysed by the AAV protein Rep78 in conjunction with the Rep78 promoter, p5, an element that also carries a Rep78 binding site. It was recently shown that p5 is the minimal element for site-specific integration and it can promote this in the absence of the AAV inverted terminal repeat (ITR) sequence which is adjacent to it in the AAV genome. However, AAV must achieve other objectives to replicate successfully and its sequences may not be optimised for integration *per se*. Therefore, we designed a series of 20 cassettes carrying a selectable marker gene (NeoR) in which natural AAV and synthetic integration elements (SIEs) were assessed for their ability to promote gene integration into the AAV-S1 site in the presence of Rep78. Wild-type AAV p5 and ITRs were compared to SIEs in which the length of the ITR and the sequence and/or relative orientation of p5 were varied. Additional binding sites for Rep78 were also built into some SIEs to enhance the binding affinity of Rep78. Thus, by selection of NeoR clones and mapping of junctions by PCR, we identified novel SIEs that efficiently promote Rep-dependent integration at the AAV-S1 site, although the SIEs themselves do not resemble the AAV genome structure. The rationale for improved integration is thought to lie in the more efficient creation of single stranded Rep binding sites in the DNA by read-through transcription from the p5 promoter. These SIEs are currently best delivered to target cells via transfection but with further development may provide an alternative delivery system to AAV.

O2. Synergistic Enhancement of Cellular Delivery of Bacteria Artificial Chromosomes using Ultrasound and Nuclear Targeting

Elin Berger,[1] Megan Nolan,[1] David Jans,[2] Jim Vadolas,[3] Panos Ioannou[3] and Chee Kai Chan[1], [1]*Department of Genetics, La Trobe University, Bundoora, Australia;* [2]*Department of Biochemistry and Molecular Biology, Monash University, Clayton, Australia;* [3]*Cell and Gene Therapy Research Group, Murdoch Children's Research Institute, Melbourne, Australia.*

Bacteria artificial chromosomes (BACs) are useful vectors for gene therapy capable of accommodating long stretches of foreign DNA within them. This allows for inclusion of not only the therapeutic gene but multiple genes, as well as the associated regulatory elements. Moreover, BACs that carry the necessary elements for it to be stably maintained as an episome are ideal as it is able to avoid the problem of insertional mutagenesis. However, due to their size, efficient delivery of such BACs to target cells and into the nucleus for expression is a great challenge. To overcome the various barriers to efficient delivery of such large DNA constructs we have complexed an episomally replicating BAC, harbouring a 185 kb β-globin genomic fragment and also encoding the green fluorescent protein, with polyethyleneimine (PEI) that has been conjugated to a nuclear localisation signal (NLS). Following complexation with PEI-NLS, transfection efficiency in COS-7 cells was enhanced 4-fold, compared to the BAC complexed with PEI alone or with PEI conjugated to a non-functional, mutated NLS peptide. Enhanced nuclear accumulation of the fluorescently labelled BAC complexed with the PEI-NLS conjugate could be observed by confocal microscopy. Ultrasound has been shown in the past to be able

to generate transient pores in the cells' plasma membrane. When COS-7 cells were subjected to ultrasound at a frequency of 1 MHz at 0.5 W/cm^2 for 30 s, and transfected with the BAC complexed with PEI-NLS, a synergistic enhancement of 25- to 30-fold was observed. With optimisation, such a synergistic combination of ultrasound and NLSs may enable highly efficient delivery of large DNA constructs for gene therapy and stem cell research.

O3. *In vivo* Restoration of Dystrophin Expression

Sue Fletcher, Kaite Honeyman, Abbie Fall, Penny Harding, Russell Johnsen and Steve Wilton, *Experimental Molecular Medicine Group, Centre for Neuromuscular and Neurological Disorders, QE II Medical Centre, Nedlands, 6009 Perth, Australia.*

Duchenne muscular dystrophy (DMD) is the most common, serious form of muscular dystrophy and is caused by mutations in the large dystrophin gene. We are evaluating the potential of antisense oligonucleotides (AOs) to by-pass defects in the dystrophin gene that preclude the synthesis of a functional protein. In-frame dystrophin gene rearrangements typically result in the milder allelic disorder, Becker muscular dystrophy, clearly demonstrating that some regions of the dystrophin protein can be lost with relatively minor consequences. The huge size of the dystrophin gene, its complex expression patterns and expression of the predominant isoforms in non-dividing cells, have hindered gene replacement therapies for DMD, but render the dystrophin gene product an ideal candidate to evaluate AO therapies directed at splicing intervention.

We report *in vitro* and *in vivo* studies, comparing morpholino and 2′-O-methyl AOs of identical sequence and peptide nucleic acids (PNAs) directed at the same dystrophin splice site, in a mouse model of muscular dystrophy. PNAs were inefficient at inducing specific exon skipping *in vitro* and *in vivo* under all delivery conditions tested. The 2OMeAO was able to restore dystrophin expression *in vitro* and *in vivo* when complexed with delivery agents. The ability of the uncomplexed morpholino to induce exon skipping was unexpectedly high *in vivo*, whereas this same preparation appeared essentially ineffective *in vitro*. Intramuscular administration of the morpholino to juvenile mice reduced central nucleation and resulted in near-normal dystrophin expression and muscle architecture. The efficient uptake of the uncomplexed morpholino *in vivo* may be considered an advantage when applying these compounds in a clinical setting. While testing in cell culture is a critical step in the design of AOs for induced exon skipping, delivery and the ultimate efficacy of AOs can only be evaluated in a relevant and appropriate animal model.

O4. Use of Nuclear Localisation Sequences to Enhance Non-Viral Gene Delivery

David A. Jans[1] and Chee Kai Chan[2], [1]*Nuclear Signalling Laboratory, Dept. of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia;* [2]*Department of Genetics, La Trobe University, Bundoora, Victoria, Australia.*

Viral-based gene delivery constructs provide highly efficient gene transfer but have significant shortcomings, including immunogenicity, pathogenicity and the limited size of therapeutic DNA which can be incorporated into the viral genome. Non-viral approaches are not subject to these problems but, thus far, have proven inefficient in delivering therapeutic DNA to the nucleus. We have used various approaches to optimise non-viral DNA delivery by including nuclear localisation signals (NLSs), sequences that confer interaction with the host cell nuclear import machinery, and in particular with the NLS-recognising importin (IMP) proteins. We have developed an optimised, enhanced form of the NLS of the simian virus SV40 large tumour antigen (T-ag), where the basic NLS (amino acids 126–132) is combined with the phosphorylation site-containing flanking amino acids (amino acids 111–125), including the nuclear transport facilitating protein kinase CK2 site, but with the nuclear transport inhibiting cyclin-dependent kinase site (threonine 124) inactivated by mutation. We have shown that this NLS (opT-NLS) can enhance nuclear delivery and thereby gene transfer of DNAs introduced into cells by calcium phosphate precipitation, lipofection, polylysine-mediated delivery, and even receptor-mediated delivery. In all cases, the NLS enhances transgene expression significantly, although other factors such as the size of the transfecting construct are clearly important. Overall, our results indicate that the considered use of NLSs can greatly enhance interaction of transfecting DNAs with the nuclear transport machinery of the cell, and thereby facilitate nuclear delivery and transgene expression.

O5. Engineered Modular Protein-DNA Carriers for Efficient and Specific Gene Delivery

Dominic J. Glover[1] and David A. Jans[1], [1]*Nuclear Signalling Laboratory, Department of Biochemistry and Molecular Biology, Monash University, Victoria 3800, Australia.*

Non-viral gene delivery methods have reduced pathogenicity and capacity for insertional mutagenesis compared to viral approaches; however, conventional non-viral vectors are hampered by inefficient delivery of therapeutic DNA to the nucleus and transient transgene expression. This is due, in part, to numerous cellular barriers that prevent efficient delivery of therapeutic genes to and maintenance within the cell nucleus. We aim to overcome these barriers by the use of engineered modular DNA carrier (MDC) proteins that mimic key functions of viruses, including DNA compaction through the sperm chromatin component protamine, targeted cell attachment and entry using α-melanocyte-stimulating hormone as an internalizable melanoma cell-specific ligand, endosomal escape via the endosomolytic translocation domain of diphtheria toxin, and nuclear transport mediated by the optimized nuclear localization sequence of the simian virus SV40 large T-antigen. Poor protein expression of MDCs due to *E. coli* codon bias was overcome by supplying the rare *E. coli* tRNAs, which increased MDC production 100-fold. The individual domains of the purified MDCs are functional, producing efficient compaction and protection of DNA from nucleases, binding to MSH-receptors on B16-F1 mouse melanoma cells, and interaction with components of the nuclear transport machinery. To visualize and perform kinetic analysis of the trafficking of transfecting MDC-DNA complexes, a three-colour system has been created that is compatible with CLSM. This involves fluorescently labeling the MDC and plasmid DNA, that itself expresses a fluorescent reporter gene to measure overall transfection efficiency. Furthermore, we are applying a novel technique of isolating nuclei from mammalian cells for use in flow cytometry analysis, which enables a direct correlation of reporter gene expression with

the amount of foreign DNA delivered to the nucleus. Since the domains of MDCs are interchangeable, self-assembling modular vectors may be tailored to specific applications, such as long-term transgene expression through inclusion of a site-specific integrase domain.

O6. VP22 Enhanced Suicide Gene Therapy Strategy for the Treatment of Ovarian Cancer *In Vivo*

Wang Wenxia,[1] Kong Beihua,[1] Liu Chunsheng,[1] Ma Daoxin[1] and Ming Q. Wei[2], [1]*Department of Obstetrics and Gynecology, Qilu Hospital, Shandong University Jinan 250012, P.R. China.* [2]*Gene Therapy Unit, Department of Medicine, University of Queensland, Prince Charles Hospital, Brisbane, Australia*

Objective: The use of herpes simplex virus thymidine kinase (TK) gene transfer for the treatment of ovarian cancer in clinical settings is limited because none of the existing vector systems could deliver the therapeutic gene to a sufficient number of cells. To overcome the problem, we have sought to amplify the cytotoxicity of the TK gene with co-transfer of a HSV-1 structural protein VP22 that has been shown to have a remarkable ability for intercellular trafficking.
Methods: Human ovarian epithelial cancer cell line 3AO was transduced with lentivirus encoding HSV-VP22-TK, with lentivirus vector encoding HSV-TK as a control. These transduced cells were then mixed with non-transduced 3AO cells at a ratio of 1 : 9 respectively and inoculated by intraperitoneally injection into nude mice. GCV treatment was followed 48 hours later.
Results: In the group of mice inoculated with a mixture of tumour cells, including cells transduced with HSV-VP22-TK, treatment with GCV significantly inhibited tumour cell proliferation, resulting in slowing down of tumour formation and prolonged survival times. The average survival time of animals inoculated with a mixture of tumour cells, including cells transduced with HSV-VP22-TK, when treated with GCV, was significantly longer than those of animals inoculated with a mixture of tumour cells, including cells transduced with HSV-TK ($p = 0.006$).
Conclusion: VP22, when fused with TK, enhanced intercellular trafficking of TK and amplified the TK/GCV killing effect. These results suggest that the use of VP22 to promote intercellular trafficking may offer a new strategy to enhance the effectiveness of suicide gene therapy for the treatment of cancers.

O7. Development of Fluorescence-Based Genomic Reporter Assay for Evaluation of Potential Fetal Hemoglobin Inducers

H. Wardan,[1] J. Vadolas,[1] P. Lourthai,[1,3,4] M. Orford,[2] R. Williamson[1] and P. A. Ioannou[1,2] (jim.vadolas@mcri.edu.au), [1]*Cell and Gene Therapy Research Group, The Murdoch Children's Research Institute, Royal Children's Hospital, Flemington Road, Parkville 3052, Melbourne, Australia.* [2]*The Cyprus Institute of Neurology and Genetics, PO Box 3462, Nicosia, Cyprus.* [3]*The Thalassemia Research Center, Institute of Science and Technology for Research and Development, Mahidol University,* [4]*Department of Biochemistry, Mahidol University, Bangkok, Thailand.*

Reactivation of fetal hemoglobin has been proposed as a potential therapeutic strategy for β-thalassaemia and other hemoglobinopathies. Many of the drugs currently used have low efficacy, specificity, and associated cytotoxicity while some are potentially carcinogenic. Therefore, there is an urgent need to identify new pharmacological agents that can induce HbF with greater efficacy and less toxicity.
In this study, we report the development of a genomic reporter assay (GRA) using the modified human erythroleukemic cell line K562-EBNA-1, stably expressing the enhanced green fluorescent protein (EGFP) gene placed under the control of the $^{G}\gamma$-globin promoter of the human β-globin locus and retaining all of the upstream regulatory elements. The cell lines maintain a uniform level of basal EGFP expression and profile while under continuous culture for over 3 years. In our GRA, hemin proved to be a potent inducer of EGFP expression resulting in a dose-dependent increase reaching a maximum of $764 \pm 145\%$, mirroring the induction levels of the endogenous globin genes. Protein analysis by SDS-PAGE showed EGFP levels following hemin induction constituted 5–10% of total cellular protein. Hydroxyurea and butyric acid resulted in lower induction levels ($75 \pm 10\%$ and $82 \pm 29\%$, respectively) but were noted to have an additive effect ($159 \pm 72\%$), suggesting independent mechanisms of action.
This genomic reporter assay based on the K562-EBNA-1 cell line containing the EGFP-modified human β-globin locus can be directly used for high-throughput screening in 384-well plates to identify and evaluate new compounds with the capacity to upregulate the expression of fetal hemoglobin under physiologically relevant conditions. The technology used in the development of this assay could be similarly implemented to other diseases to identify drugs that can modify the expression of any gene of interest in a therapeutically relevant manner.

O8. Accurate Humanised Mouse Models of Friedreich Ataxia

Joseph P. Sarsero, Timothy P. Holloway, Lingli Li, Marion Zanese, Lucille Voullaire and Panos A. Ioannou (joe.sarsero@mcri.edu.au), *Cell and Gene Therapy Research Group, Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, Victoria 3052, Australia.*

The neurodegeneration and cardiomyopathy in Friedreich ataxia (FA) is caused by a GAA trinucleotide repeat expansion in the first intron of the *FRDA* gene that results in insufficiency of frataxin protein, and leads to increased oxidative stress, progressive loss of large sensory neurons and hypertrophic cardiomyopathy. Knockout mouse models of FA do not accurately recapitulate the molecular basis of the disease. Our strategy is to generate accurate humanised mouse models of FA that contain the entire human *FRDA* genomic locus harbouring a long GAA expansion. Such mice should manifest the main symptoms of FA and also provide the correct underlying molecular cause of the disease. A major obstacle in the generation of constructs containing long GAA expansions has been the instability of such repeats in bacterial multicopy vectors. By using single-copy BAC vectors we have successfully cloned and stably maintained GAA expansions containing up to 500 repeats. Using homologous recombination we have introduced a $(GAA)_{500}$ expansion adjacent to an antibiotic-resistance gene into the first intron of the human *FRDA* gene present on a fully sequenced BAC clone containing the human *FRDA* gene and surrounding regions. The modified BAC clone was used to generate two lines of transgenic mice differing in their transgene copy

number. This is the longest GAA expansion to be stably maintained in transgenic mice. RT-PCR and Western blot analyses confirmed that the presence of the introduced GAA expansion results in decreased *FRDA* expression and in lower levels of frataxin. Breeding of transgenic mice with heterozygous *Frda* knockout mice demonstrated that the human transgene is able to rescue the embryonic lethality of the homozygous knockout mice in both lines. Rescue mice are being assessed by a series of behavioural, neurological and histological tests for phenotypic symptoms of FA. These transgenic mice should facilitate the evaluation of potential new therapies specifically targeted to overcoming the molecular effects of the GAA expansion.

O9. Rescue of the Neonatal Lethal Methylmalonic Aciduria (MMA) in Homozygous Knockout Mice using the Intact Human *MUT* Locus

Heidi Peters, Natasha Hamilton, Leonie Wood, Joseph P. Sarsero, James Pitt and Panos A. Ioannou, *Cell and Gene Therapy Research Group, Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, Victoria 3052, Australia. (heidi.peters@mcri.edu.au)*

Methylmalonic aciduria (MMA) is an autosomal recessive disorder of organic acid metabolism. A knockout mouse model for MMA was generated by a targeted deletion of exon 3 of the mutase locus. Homozygous knockout ($Mut^{-/-}$) mice appear phenotypically normal at birth, but rapidly develop early neonatal lethality within 24 hours of age as a result of acidosis. Biochemically homozygous mice demonstrate elevated urinary methylmalonic/methylcitric acid levels and elevated plasma C3 acylcarnitine levels. Enzymatic and RNA testing confirm the null phenotype.

Four separate transgenic mouse lines carrying the intact human mutase locus in a fully sequenced BAC clone have also been produced. Transgenic mice from a line carrying four copies of the transgene at a single integration site were mated with heterozygous knockout mice to generate mice homozygous for the knockout mutation ($Mut^{-/-}$) and hemizygous for the human transgene ($MUT^{+/0}$). Rescue of the neonatal lethality was observed, with rescued mice now surviving past 1 year of age. These mice are generally slightly smaller than sex-matched littermates. Biochemical correction is not complete, as methylmalonic acid levels in blood and urine remain elevated, however, at significantly lower levels than those observed in homozygous knockout mice. Real-time PCR analysis of mRNA expression confirms the human transgene is expressed in all tissues tested, however at much higher levels in the brain followed by the kidney relative to the endogenous mouse gene.

Currently, mice carrying two copies of the human transgene are being mated with knockout mice. It is anticipated mice with an intermediate phenotype may result. Such mice would enable evaluation of the long-term pathophysiological effects of elevated methylmalonic acid levels, in addition to providing a valuable model for the investigation of therapeutic strategies, such as cell transplantation.

O10. Development of Recombinant Adeno-Associated Virus (rAAV) as a Genetic Vaccine Vector

Grant J. Logan,[1] Maolin Zheng,[1] Ross L. Coppel,[2] Peter B. Rowe,[1] Lina Wang[2] and Ian E. Alexander[1], [1]*Gene Therapy Research Unit, Children's Medical Research Unit and The Children's Hospital at Westmead, 214 Hawkesbury Road, Westmead, NSW 2145, Australia.* [2]*Department of Microbiology, Monash University, Melbourne, Victoria, Australia, 3800.*

rAAV can transduce muscle cells with high efficiency and induce long-term transgene expression in immunocompetent animals. Our aim is to exploit this unique biology of rAAV to develop a genetic vaccine delivery system that will stimulate long-term immunity against encoded antigens.

An rAAV vector was constructed that encodes MSP4/5 (from *Plasmodium yoelli*) genetically fused with a TPA leader sequence thereby targeting expressed protein for secretion from transduced cells. Vector genomes (vg) were packaged into serotype 1 capsids in HEK293 cells using a helper virus-free system. Intramuscular injection of 10^{12} vg of this vector into the upper hind-limb muscle of 8–10-week-old Balb/C mice stimulates MSP4/5-specific antibody responses that plateau 4–6 weeks post-injection and remain stable for 6 months.

We have attempted to augment responses in rAAV1-MSP4/5 primed animals by boosting with the same vector genome packaged in serotype 3 capsids. This serotype was chosen because our *in vitro* experiments demonstrated rAAV1 neutralising antibodies did not cross-neutralise rAAV3. Intramuscular injection of 10^{12} vg of the capsid 3 packaged vector stimulated MSP4/5-specific antibody responses in naïve animals; however, antibody levels in rAAV1-primed mice remained unchanged after subsequent injection of the second vector. This observation is subject to further investigation though the failure of a secondary response cannot be explained by any tolerising effect from the initial rAAV1 vector as animals mounted secondary responses to MSP4/5 protein emulsified in adjuvant.

In an alternative augmentation strategy using the complement protein C3d, the cDNA for three tandem copies of this molecule were cloned in-frame with MSP4/5 within the rAAV-MSP4/5 vector (MSP4/5-$C3d_3$). In contrast to other studies using this strategy, intramuscular injection of rAAV-MSP4/5-$C3d_3$ failed to stimulate antigen-specific immunity. Supporting evidence in the literature indicates that this strategy operates in an antigen-dependent manner and experiments are in progress to understand the underlying biological basis for these observations.

O11. Engineered Histones and Reconstituted Chromatin can Effect Gene Transfer in Intact Cells

Kylie M. Wagstaff,[1] David J. Tremethick[2] and David A. Jans[1], [1]*Nuclear Signalling Laboratory, Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia.* [2]*Chromatin and Transcriptional Regulation Laboratory, John Curtin School of Medical Research, Australian National University, Canberra, ACT, Australia.*

Gene therapy has great potential for the treatment of debilitating human diseases. A major limitation however is the nuclear delivery of the DNA, where it is estimated that <1% of DNA taken up by a cell is actually expressed. This study investigates the use of engineered histones and reconstituted chromatin as an alternative to naked DNA

as a gene delivery vehicle, offering protection against nucleases through DNA compaction, and enhancement of nuclear uptake through the presence of nuclear localisation sequences (NLSs).
A series of engineered histones were generated and characterised. These included green fluorescent protein (GFP)-tagged histones to enable tracking of protein-DNA complexes, and histones with an additional, optimised NLS from the simian virus SV-40 large tumour T-antigen (T-ag), to confer recognition by the importin (IMP) components of the nuclear import machinery. These were characterised for IMP binding ability, nuclear localisation and DNA binding, with the most interesting characteristic being the ability to be taken up by intact cells in a non-endocytotic fashion.
The possibility of utilising chromatin for gene transfer was investigated by incorporating DNA encoding a dsRed reporter gene into chromatin utilising histone octamers containing the engineered histones. The engineered histones were able to condense and protect DNA, some to an even greater extent than octamers containing control histones, whilst remaining available for IMP recognition. Preliminary experiments suggest that the reconstituted chromatin can be taken up and expressed by intact cells.
Reconstituted chromatin appears to be a highly interesting prospect for use in gene transfer enhancement, allowing for the tracking of DNA-protein complexes to optimise gene delivery. A major advantage of the system will be the ability to deliver large DNA fragments, encoding multiple genes, enabling the treatment of complex diseases such as diabetes.

O12. Lentiviral Vectors for Gene Discovery: Application to the Molecular Basis of Regulatory T Cell Function

Elizabeth Melville,[1] Suzanne Bresatz,[1] Debbie Millard,[1] Bill Osborne,[2] Heddy Zola[1] and Simon Barry[1], [1]*Department of Paediatrics, Adelaide University, and Child Health Research Institute, Adelaide, Australia; and* [2]*University of Washington, Seattle, Washington, USA.*

The development of retroviral gene delivery vectors has generated a number of tools for gene discovery and validation, as well as for gene therapy. In particular the HIV1-based lentiviral vectors have shown promise as they have the ability to transduce a wide variety of cell types, and are not dependent on cell division for stable integration of their genome. In addition, the ability to pseudotype this virus with a range of envelope proteins has given broad tropism to these viruses. These vectors are multiply deleted to make them replication incompetent, and are produced in simple transient transfection protocols. Inclusion of cPPT and PRE elements into these vectors improves efficiency, and accessory molecules can enhance transduction efficiency further. Gene delivery *in vitro* and *in vivo* demonstrates sustained stable gene expression in a number of systems, including human immunomodulatory cells such as monocytes, dendritic cells and T cells. Data will be presented on the lifelong delivery of a cytokine to rats from a single intramuscular administration of lentivirus. In addition to stable gene expression these vectors can be used to deliver shRNAi cassettes into cells to allow targeted gene ablation. Data will be presented on constitutive ablation of p38alpha and the functional consequences, and on lentiviral tetracycline-regulated ablation of beta-catenin expression. These tools are being applied to the molecular identification of the genes required for the suppressor function of CD4+ CD25+ regulatory T cells, and data will be presented on the expression of the transcription factor Foxp3 using viral delivery systems, and application to chromatin immunoprecipitation. These discoveries may lead to therapeutic intervention in regulatory function, and may allow cell therapy approaches to treat autoimmune diseases such as type 1 diabetes and IBD. In addition, they may be applied to tolerance to transplant of either bone marrow or solid organs.

O13. Translation of the *gag* Gene Can Increase the Packaging Efficiency of Lentiviral Vectors

Don Anson,[1,2,3,4] Rachel Koldej[1,2] and Johnson Mak[5], [1]*Department of Genetic Medicine, Child, Youth and Women's Health Service, 72 King William Road, North Adelaide, SA 5006, Australia;* [2]*Department of Paediatrics, University of Adelaide, Australia;* [3]*Biotechnology Department, Flinders University, Australia;* [4]*School of Pharmacy & Medical Sciences, University of South Australia, Australia;* [5]*Macfarlane Burnet Institute for Medical Research, Melbourne, Australia.*

It has been shown that translation of the *gag* gene in *cis* increases the packaging efficiency of unspliced HIV-1 approximately 8-fold. In order to determine if this phenomenon could be used to construct more efficient lentiviral vectors we constructed a HIV-1 vector containing a full-length *gag* gene. A second version of this vector was made in which the *gag* gene initiation codon was mutated to a stop codon. An optimised vector, which contains 550 bp of an untranslatable *gag* reading frame, was used as a control. Apart from the *gag* sequence all the vectors were of identical construction and express enhanced yellow fluorescent protein as a reporter. Vectors were packaged and viral titres determined by FACScan analysis. The results of this analysis showed that with the vector containing the full length *gag* gene, mutation of the initiation codon resulted in a 6-fold decrease in titre. This suggests that, as with wild-type HIV-1, translation of the *gag* gene in *cis* leads to more efficient packaging of the vector genomic RNA. However, titres for the construct with the translatable *gag* gene were still lower than that of the optimised vector. Similar results were obtained when the vectors were packaged with the Gag and GagPol polyproteins provided in *trans* from separate constructs. The vector containing the translatable *gag* gene was also packaged with a construct that expresses only the HIV-1 GagPol polyprotein confirming expression of Gag from the vector. In conclusion, it appears that while there is a relatively large advantage for HIV-1 to have Gag translated in *cis*, this does not offer a facile means to make more efficient vectors. In addition, as we have noted before, there is a similar benefit in Gag and GagPol being translated from the same mRNA/helper plasmid.

O14. Intra-Articular Gene Therapy for Joint Disease in Mucopolysaccharidosis Type VI

S. Byers,[1] M. Rothe,[1] J. Lalic[1] and D. S. Anson[2], [1]*Matrix Biology Unit and* [2]*Gene Technology Unit, Department of Genetic Medicine, Women's and Children's Hospital, Nth. Adelaide, SA 5006, Australia.*

Mucopolysaccharidosis type VI (MPSVI) is an inherited metabolic disorder that manifests in childhood. The cardinal clinical feature is a severe, progressive skeletal dysplasia characterised by short stature, osteopenia and degenerative

joint disease. MPS VI arises from a deficiency in a lysosomal enzyme required for carbohydrate degradation. Therapy for MPS VI is thus a matter of replacing the missing enzyme, in all tissues displaying pathology, using recombinant protein or gene technologies. Several tissues are difficult to treat by systemic methods due to their avascular nature (cartilage/cornea) or the presence of a physical barrier (brain). Localised therapies have greater potential for benefit in these tissues. This study was undertaken to evaluate the efficacy of a lentiviral vector delivery system to infect different tissues in the joint (cartilage, synovium and ligament), after direct injection into the joint space. A lentiviral vector encoding the β-galactosidase gene was injected into the synovial cavity of the right knee joints of adult rats. The contralateral joint received carrier alone. After 1 week to 1 month the knee joints were removed, stained with X-gal and paraffin sections prepared. One week after administration of the vector, synovial cells stained strongly for β-gal throughout the synovial cavity. Strong staining persisted for 2 weeks and decreased thereafter but was still present at 8 weeks. Cells lining the outside of the cruciate ligament also stained strongly 1 week after injection. No staining was observed in cartilage cells at any time point. Work is underway to determine if cartilage cells are more amenable to transduction in a degenerative disease situation where the integrity of the cartilage matrix has been compromised. However, in a therapy setting the over-secretion of enzyme from synovial cells transduced with a lentiviral vector would act as a localised enzyme production factory and thus has potential to reduce MPS VI joint pathology.

O15. Methyl-Guanine-Methyl-Transferase (MGMT)-Expressing Transgenic Mouse for Developing Stem Cell Transplantation Models using *In Vivo* Selection

B. Kramer,[1] F. Lemckert,[1] I. Alexander,[2] P. Gunning[1] and G. McCowage[1], [1]*Oncology Research Unit, The Children's Hospital at Westmead,* [2]*Gene Therapy Research Unit, The Children's Hospital at Westmead, Children's Medical Research Institute and Discipline of Paediatrics and Child Health, University of Sydney, Australia.*

Methyl-guanine-methyl-transferase (MGMT) is a protein which repairs damage caused by exposure to the alkylating chemotherapy drug BCNU. Gene transfer of MGMT cDNA into haemotopoietic stem cells results in high levels of expression and confers drug resistance to gene-modified cells. One application for this gene transfer strategy is to provide chemoprotection to stem cells in patients being treated with alkylating drugs for brain tumours. Alternatively, MGMT expression in combination with a therapeutic gene(s) and *in vivo* drug selection could be used to treat genetic diseases. We aim to use the selective advantage conferred on MGMT-expressing haemotopoietic stem cells to develop therapeutic allogeneic transplant models and have generated a transgenic mouse line carrying the P140K mutant MGMT as a source of donor cells.

The transgenic line was derived from C57BL/6 ES cell clones transfected with the P140K mutant MGMT cDNA. BCNU resistant clones were injected into BALB/c blastocysts and chimaeric animals identified by coat colour. Transgenic progeny have been cross-bred to establish a homozygous line in which individual animals express MGMT at variable levels in their bone marrow and peripheral blood.

MGMT expression in bone marrow conferred chemoprotection against alkylating drugs *in vitro*, as has been demonstrated for virally transduced $MGMT^+$ bone marrow populations. Treatment over 2 months with three cycles of alkylating chemotherapy resulted in increasing proportions of MGMT-expressing cells in the bone marrow, peripheral blood and spleens of transgenic mice. This enrichment has been maintained for 6 months following the third cycle of chemotherapy. After one cycle, transgenic mice did not exhibit the transient fall in white cell count and haemoglobin seen with subsequent cycles in wildtype control animals. Chemotherapy also produced an increase in the proportion of Lin^- $Sca1^+$ stem cells expressing the transgene. This mouse line will be an ideal donor for developing strategies for *in vivo* selection of gene modified and MGMT-expressing stem cell populations.

O16. Evaluation of AAV-Mediated sFlt-1 Gene Therapy for Ocular Neovascularization

Chooi-May Lai,[1] Wei-Yong Shen,[1,2] Meliha Brankov,[1,2] Yvonne K. Y. Lai,[1,2] Shu-Yen Lee,[3] Ian Y. S. Yeo,[3] Ranjana Mathur,[3] Paul Pineda,[3] Amutha Barathi,[3] Chong-Lye Ang,[3] Ian J. Constable[1] and Elizabeth P. Rakoczy[1], [1]*Centre for Ophthalmology and Visual Science, The University of Western Australia, Australia,* [2]*Molecular Ophthalmology Department, Lions Eye Institute, 2 Verdun Street, Nedlands, WA 6009, Australia,* [3]*Singapore National Eye Centre, 11 Third Hospital Ave, Singapore.*

Vascular endothelial growth factor (VEGF) is one of the major mediators of retinal ischemia-associated neovascularization. The angiogenic actions of VEGF are mediated through its high affinity binding to two endothelium-specific receptor tyrosine kinases, Flt-1 and Flk-1/KDR. We have shown here that adeno-associated virus (AAV)-mediated expression of sFlt-1, a soluble form of the Flt-1 VEGF receptor, was maintained up to 8 and 17 months post-injection in trVEGF029 mice (transgenic mice generated through photoreceptor-specific upregulation of VEGF) and monkeys, respectively. The injection of AAV.sFlt-1 resulted in an increase in retinal function response as measured by electroretinography in trVEGF029 mice. It was also associated with the long-term (8 months) regression of neovascular vessels in 85% of trVEGF029 eyes. The majority of the treated trVEGF029 eyes (75%) retained high numbers of photoreceptors. In a laser photocoagulation-induced monkey model for choroidal neovascularization, AAV-mediated expression of sFlt-1 prevented the development of new vessels. There were no clinically or histologically detectable signs of toxicity present in either models following AAV.sFlt injection. These results suggest that AAV-mediated secretion gene therapy could be considered for treatment of retinal and choroidal neovascularization in chronic diseases.

O17. Liver Cell Transplantation for Crigler-Najjar Syndrome

Katrina Allen,[1,2,5] Daphne Cheah,[1] Winita Hardikar,[2] Paul Wright,[3] Mark Brooks,[4] Robert Jones[4] and Robert Williamson[1], [1]*Murdoch Children's Research Institute,* [2]*Department of Gastroenterology Royal Children's Hospital, Melbourne, Australia* [3]*Royal Melbourne Institute of Technology,* [4]*Victorian Liver Transplant Unit Austin Hospital,* [5]*University of Melbourne, Victoria, Australia.*

Background: Crigler-Najjar syndrome type 1 is a rare inherited inability to conjugate serum bilirubin, due to

malfunction of the hepatic enzyme bilirubin glucuronosyl transferase. The resulting high levels of unconjugated bilirubin can cause kernicterus and death. Standard treatment involves phototherapy followed by orthotopic liver transplantation.

Aims: The aim of this procedure was to increase hepatic bilirubin glucuronosyl transferase activity by infusing the native liver with ABO-matched hepatocytes from a cadaveric organ donor.

Methods: Fresh human hepatocytes were prepared from an ABO-matched organ by a standard two-step collagenase perfusion. Viability of cells, as determined by trypan blue exclusion, was 93% and an overnight plating efficiency of >90% was achieved. The 8-year-old female patient underwent plasmaphoresis prior to having a 5 French portal vein catheter placed radiologically. 140 mL of a 1×10^7/ml liver cell suspension was infused in over 60 min. Immunosuppression regime included tacrolimus, azathioprine and prednisolone.

Results: The procedure was simple and safe with no evidence of portal vein thrombosis or pulmonary embolism. The patient was discharged after 48 h for regular bilirubin monitoring. The cell transplant demonstrated function as evidenced by a significant reduction of the average serum bilirubin from 360 to 220 μmol/L while maintaining pre-transplant phototherapy times of 8 h per day. From 30 days post-transplant, the phototherapy time was reduced to 6 and then 4 h per day and was sustained for 1 month post-transplant. Over the next 2 months there was a gradual rise in phototherapy requirement to pre-transplant levels indicating a slow loss of graft function consistent with allograft rejection.

Conclusions: Liver cell transplantation is a safe and simple method for augmenting enzyme function in metabolic liver disease but, in this instance, was not sustained. A variety of method modifications are under consideration to improve graft survival for future cell transplants.

O18. Susceptibility of an Insulin-Secreting Liver Cell Line to the Toxic Effects of Cytokines Involved in the Autoimmune Destruction of Pancreatic Beta Cells

Janet Biady, Chang Tao, Bronwyn A. O'Brien and Ann M. Simpson, *Dept. Cell & Molecular Biology, University of Technology, Sydney, PO Box 123, Broadway, NSW 2007, Australia.*

Somatic gene therapy is one strategy being considered to correct patient blood glucose concentrations in type I diabetes. One of the mechanisms by which beta cells are killed by the autoimmune process is by the release of cytokines interleukin 1β (IL), tumour necrosis factor-α (TNF) and interferon-γ (IFN). The aim of the present study was to determine if an insulin-secreting liver cell line (Huh7ins) has resistance to these factors.

Cells were exposed to the cytokines for 12 days at the following concentrations of IFN (384 ng/mL), TNF (10 ng/mL) and IL (2000 pg/mL). Cell viability was measured by an MTT assay, chronic insulin secretion was sampled daily and measured by an insulin radioimmunoassay (RIA), and acute insulin secretion to 20 mM glucose was measured by static incubation experiments. Nitric oxide (NO) levels were determined by the Griess reaction. MIN-6, a glucose-responsive beta cell line, was used as a positive control.

The viability of MIN-6 cells was significantly ($P < 0.05$) affected by the triple cytokines from day 3 of culture. By comparison Huh7ins and the parent cells, Huh7 cells remained unaffected over the 12-day period. Chronic insulin secretion of the MIN-6 cells was significantly ($P < 0.05$) affected by the triple cytokines from day 1 of the experiment and by day 12 the cells became significantly ($P < 0.01$) less glucose-responsive than the untreated cells. In contrast, the chronic insulin secretion of Huh7ins cells were significantly ($P < 0.05$) affected from day 10; however, by day 12, the triple cytokines did not have a significant ($P > 0.05$) effect on glucose-responsiveness. Griess reaction showed that MIN-6 cells treated with the triple cytokine produced significantly ($P < 0.05$) more NO than the control cells. NO was not detected in treated or untreated Huh7 or Huh7ins cells. Therefore, our results indicate that Huh7ins cells are more resistant to the affects of autoimmune cytokines than pancreatic beta cells.

O19. *In vivo* Delivery of the Human Insulin Gene Results in Long-Term Reversal of Streptozotocin-Induced Type 1 Diabetes in Rats

Binhai Ren,[1] Bronwyn O'Brien,[1] M. Anne Swan[2] and Ann M. Simpson[1], [1]*Dept. Cell & Molecular Biology, University of Technology, Sydney, PO Box 123, Broadway, NSW 2007, Australia.* [2]*Dept. Anatomy & Histology and Institute for Biomedical Research, University of Sydney, NSW 2006, Australia.*

Aims: One of the strategies being employed to replace the β-cells lost in type 1 diabetes is somatic gene therapy. The aim of this study was to reverse streptozotocin (STZ)-induced diabetes in Wistar rats.

Methods: The human proinsulin gene was cloned into a viral vector. Transduction efficiency was assessed by flow cytometry. We introduced the viral vector which expressed the insulin gene into male Wistar rats (250–300 g in body weight) via the portal circulation. Prior to vector delivery, diabetes was induced by the intraperitoneal injection of STZ (75 mg/kg body weight). Following delivery of the insulin gene or empty vector, rats were monitored for changes in body weight and blood glucose levels. An intravenous glucose tolerance test was performed after a 14 h fast in untreated (n = 3), empty vector-transduced (n = 4) and insulin vector-transduced (n = 6) rats. At various times (0, 2, 5, 10, 15, 30, 60, 120 min) after the infusion of glucose (0.5 g/kg body weight), blood samples were collected from the tail vein and assayed for glucose and insulin. Two months after vector infusion, liver, pancreas, kidney, spleen and lung were harvested. Expression of insulin was determined by RT-PCR, immunohistochemistry and transmission electron microscopy.

Results: The ideal amount of vector for successful delivery was 4×10^6 transduction units. Rats treated with the insulin-expressing vector, exhibited normalization of blood glucose levels within 48 h and normoglycaemia was maintained for over 12 months. Insulin was expressed only in the liver of insulin-transduced rats. Glucose tolerance tests revealed that insulin-transduced rats showed similar responses to normal rats. In contrast, diabetic rats that received the empty vector remained hyperglycaemic (blood glucose >24 mmol/L) and failed to increase body weight.

Conclusions: This study shows that hepatic insulin gene therapy can induce the long-term reversal of diabetes.

POSTER PRESENTATIONS

P1. Enhancing Transfection Efficiency of Short Interfering RNA Molecules for Telomerase Inhibition

Belinda Wigg, Catherine Durkin, Graham Flannery and Chee Kai Chan, *Department of Genetics, La Trobe University, Bundoora 3086, Melbourne, Australia.*

RNA interference (RNAi) has recently been recognised as an important tool for specific gene knockdown and gene therapy. The introduction of short interfering RNA (siRNA) molecules targeted against genes in mammalian cells has resulted in down regulation of the specific gene. There are however a number of problems still outstanding; one of which is transfection efficiency. Current gene delivery systems use a variety of viral and non-viral methods to achieve transfection, but both of these have their own individual advantages and disadvantages. To enhance delivery of siRNA, we have optimised and compared a number of non-viral transfection approaches used in conjunction with ultrasound and magnetofection. The siRNA used was designed to be targeted against telomerase in human cancer cell lines. Ultrasound has been shown to be effective in the creation of transient pores in the plasma membrane. The use of ultrasound-mediated delivery alongside siRNA molecules offers not only specific gene suppression due to the specificity of siRNA molecules, but also is a means of achieving a non-invasive organ specific gene targeting procedure. The siRNA was fluorescently labelled, and the enhancement of cellular uptake of the labelled siRNA, was examined using flow cytometry and confocal microscopy. The TRAPeze (telomerase repeat amplification protocol) assay was also utilised to determine the efficiency of telomerase inhibition as a result of the uptake of the siRNA. The double-stranded RNA used was shown to be able to produce a down regulation of telomerase activity in HL60, K562 as well as HeLa cells, as indicated by the TRAPeze assay. The optimisation of ultrasound to further enhance the uptake of siRNA in conjunction with lipofectamine, polyethylenimine and magnetofection in the different cell lines will be reported.

P2. Linker Histones (H1) and Dynein Association Motifs as Modules in Non-Viral Gene Therapy Constructs

Gregory W. Moseley, Michelle de Jesus, Daniela Roth, Kylie Wagstaff, Dominic Glover and David A. Jans, *Nuclear Signalling Laboratory, Dept. of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia.*

Virally based gene delivery constructs provide highly efficient gene transfer but shortcomings include immunogenicity, pathogenicity and the limited size of therapeutic DNA which can be incorporated into the viral genome. Non-viral approaches are not subject to these problems but, thus far, have proven inefficient in delivering therapeutic DNA to the nucleus.

Optimisation of non-viral DNA delivery includes the use of DNA-protein constructs incorporating DNA condensing proteins, nuclear localisation signals (NLS), cell-specific ligands and endosomal escape signals. The significance of the microtubule (MT) network in nuclear targeting, however, is only beginning to be appreciated. The dynein motor complex facilitates MT-dependent movement of cargo including signalling molecules and viruses toward the nucleus. A number of the molecular sites of cargo-dynein interactions have been defined, identifying motifs including K/R-x-S/T-QT and R/K-R/K-X-X-R/K-(xn)-V-S-K/H-T/S-X-V/T-T/S-N/Q-V for specific light chains of the dynein complex and alternative regions which interact with dynein indirectly. We are currently evaluating the potential for such regions, cloned from parathyroid hormone related protein (PTHrP), tumour suppressor p53, rabies P-protein and African swine fever virus p54 protein, in facilitating nuclear localisation of modular gene therapy constructs.

We also propose to forego previously used non-physiological methods of DNA condensation and deliver DNA as chromatin, produced *in vitro* using recombinant histones. This should deliver DNA in a native format, optimising gene expression. The linker histone, H1, which is peripherally associated with chromatin, will be used as a module with dynein association motifs, NLS and/or other modules as necessary. Here we show that GFP-fused H1, and fragments thereof, have functional nuclear localisation and DNA condensation properties. We also demonstrate, for the first time, that H1 has protein transduction capacity, being delivered from the extracellular space to the nucleus, and identify that the 98 residue C-terminal domain of H1 is necessary and sufficient for this activity.

P3. The Role of Microtubules in Nuclear Protein Import: Relevance to Gene Therapy

Daniela Martino Roth,[1,2] Greg Moseley,[1] Megan Waldhuber,[2] Colin Pouton[2] and David A. Jans[1], [1]*Nuclear Signalling Laboratory, Dept. of Biochemistry and Molecular Biology, Monash University, Clayton 3800, Melbourne, Australia;* [2]*Department of Pharmaceutical Biology and Pharmacology, Victorian College of Pharmacy, Monash University, Parkville 3052, Melbourne, Australia.*

Non-viral gene therapy holds great promise for the treatment of a number of human diseases. However, several physical barriers/limiting steps, such as slow trafficking through and degradation in the cytoplasm, hinder inefficient DNA delivery into the nucleus of target cells. Recently, the role of microtubules in cytoplasmic transport, facilitating nuclear import of viruses and certain nuclear proteins has become evident. Significantly, our *in vivo* studies of the cancer-related proteins: parathyroid hormone related protein (PTHrP) and the retinoblastoma protein (RB), both of which localise in the nucleus in conventional nuclear localisation signal (NLS)- and importin-dependent fashion, additionally implicate the cellular microtubule (MT) network as playing an integral role in nuclear import in transfected cells. We are presently examining the mechanistic basis of the dependence of nuclear import of PTHrP and RB on MT integrity, which we have not observed for other nuclear localizing proteins such as the simian virus SV40 large-tumor antigen, HIV-Rev protein, telomere binding factor 1 (TRF-1), sex determining protein (SRY), and the cAMP-response element binding protein (CREB). The results suggest that the ability to associate with MTs and/or be localised in the nucleus specifically through one importin-dependent pathway is not sufficient for MT-dependence of nuclear import. Our mapping of the MT association sequence (MTAS) within PTHrP by truncation analysis implicates PTHrP amino acids 38–108, which intriguingly also contains the PTHrP NLS. Since MTASs appear to facilitate NLS-dependent nuclear transport, they may have application in non-viral gene delivery by facilitating

the transport of large DNA molecules towards the nucleus of target cells; future work will test this directly.

P4. Evaluations of Novel Transfection Reagents Based on Lipopeptide Structure as Potential Gene Delivery Vehicles

Tarwadi, Jalal A. Jazayeri and Colin W. Pouton, *Department of Pharmaceutical Biology and Pharmacology, Victorian College of Pharmacy, Monash University, 381 Royal Parade, Parkville, VIC 3052, Australia.*

Delivering genes using non-viral vehicles is becoming more popular not only due to their safety compared to viral vehicles, but also several other advantages. These include compound stability, ease of chemical modification, low cost of production as bulk pharmaceuticals and potential to carry larger DNA fragments. However, low transfection efficiencies of non-viral delivery vehicles still remain a limiting step. Here we have designed a novel lipopeptide transfection reagent composed of alkyl chain (-R) and amino acid residues having a thiol group (-SH). In the presence of DNA template, the thiol group of the lipopeptide is stabilized by spontaneous oxidative reaction forming dimerized stable lipid/DNA particles. The relationship between lipopeptide structure, lipoplex formulation and transfection efficiency was studied by varying alkyl chain length and the number and fraction of amino acid residues present in the lipopeptide compound. The transfection study was performed using luciferase reporter gene. The optimal transfection efficiency in COS7 was achieved when the compound had a spacer arm of alkyl chain long enough to interact hydrophobically with DNA as well as having a number of positively charged lysine residues. The transfection efficiency of this compound is even significantly higher compared to the well-known transfection reagent *Polyethanolimine* (PEI) when it was complexed in Hepes glucose buffer. However, further biological evaluations of this polypeptide are still needed to improve the transfection efficiency *in vitro* and subsequently in *in vivo* experiments.

P5. Recombinant Suicidal *Listeria monocytogenes* for Oral Delivery of DNA Vaccines

C. Y. Kuo, J. A. Jazayeri and C. W Pouton, *Department of Pharmaceutical Biology, Victorian College of Pharmacy, Monash University, Parkville 3052, Australia.*

Listeria monocytogenes, a Gram-positive, food-borne, intracellular bacterial pathogen, is able to invade mammalian cells, escape from the endocytic vesicle, replicate within the cytoplasm and spread from cell to cell. As a result it has been investigated as a potential candidate in vaccine development. In this study, a mutant form of *L. monocytogenes,* rsΔ2, harboring the ply118 (cell wall hydrolysin gene), was constructed. The *ply*118 leads listeria to autolysis upon infection. *L. monocytogenes* rsΔ2 has shown normal growth rate in rich medium but its replication in the host cells is limited. The rsΔ2 was transformed with a shuttle vector carrying the luciferase reporter gene (p3LLuc). Upon infection into Caca-2 cells (human colon adenocarcinoma) the rsΔ2 showed higher luciferase activity compared to its parent strain, *L. monocytogenes* Δ2. In addition to Listeria-mediated delivery, a dual expression vector, pDuLX, containing the DNA-binding sequence of nuclear factor kappa B (NF-κB), promoter CMV and the promoter *hly*, was constructed. We demonstrated that the activity of the promoter *hly* carried on pDuLX is inducible and functional in nutrient-limited medium and in Caco-2 cells. NF-κB-assisted nuclear uptake of plasmid lead to an early expression of luciferase in a confluent monolayer of Caco-2 cells infected with *L. monocytogenes* rsΔ2/pDuLX. The Listeria-mediated delivery system will be easy and inexpensive to produce, and has the potential to lead to treatments for diseases such as malaria which affect large populations in developing countries.

P6. Chimeric IGF-IR Antisense Treatment Reduces Responses to Angiotensin II and Noradrenaline in Normotensive and Spontaneously Hypertensive Rats

T. T. Nguyen and P. J. White, *Department of Pharmaceutical Biology and Pharmacology, Victorian College of Pharmacy, Monash University, Parkville, Victoria 3052, Australia.*

We investigated the effects of a functional deficit in insulin-like growth factor-I (IGF-I) signalling in the rat cardiovascular system using IGF-I receptor antisense (AS) treatment. We designed 2′-sugar modified novel chimeric antisense oligonucleotides, with a view to allowing nuclease resistance and high affinity, allowing us to use far lower doses than those used by other groups. High doses of antisense used thus far in clinical trials have resulted in an unacceptable spectrum of class adverse effects.

IGF-I receptor (IGF-IR) AS treatments (5×0.5 nmol injection-1, 2 weeks) caused a rightward shift of the angiotensin II (AngII) dose-response (5.8-fold shift, $p < 0.001$, $n = 5–10$) The hypertensive responses to AngII gradually decreased over the 2-week treatment period, and were significantly reduced at day 14 compared to day 7 (3.83-fold rightward shift and the maximum response was decreased from 98.54 ± 2.0 mmHg to 85.18 ± 4.8 mmHg). There was a significant reduction in aortic medial thickness after 2 weeks IGF-IR AS treatment ($19.8 + 3.5\%$ reduction compared to vehicle treatment), whilst mismatch AS treatment had no effect in both Hooded Wistar and SHR.

IGF-IR and AT1R immunoreactivity was dramatically decreased in IGF-IR AS treated aortas. Western immunoblot analysis demonstrated a decrease in cardiac IGF-IR and AT1R to $70 \pm 7\%$ of vehicle-treated animals, $p < 0.01$, $n = 6–7$; and $69 \pm 8\%$, $p < 0.01$, $n = 3–4$, respectively, reflecting a tonic role of IGF-IR activation in the level of expression of AT1R.

Significant, specific *in vivo* antisense effects were observed using low ug doses, thus improving the therapeutic utility of these agents. The results of this study also suggest that an induced loss of IGF-IR has a predominantly depressor effect on vascular responses to vasoconstrictor agents, demonstrating the use of antisense knockdown in the characterisation of *in vivo* function of a target protein.

P7. Transcription Targeted Gene Therapy for Medullary Thyroid Carcinoma

Jan Shaw,[1] Marinella Messina,[1] Gerald Both,[3] Peter Molloy[3] and Bruce G Robinson[1,2], [1]*Kolling Institute of Medical Research, Royal North Shore Hospital,* [2]*University Of Sydney, Australia,* [3]*Molecular Science, CSIRO, North Ryde, NSW, Australia.*

Medullary thyroid carcinoma (MTC), a malignancy arising from C-cells of the thyroid, is primarily treated by surgery. Many patients develop metastatic disease that responds poorly to chemotherapy and radiotherapy. Targeted gene therapy may provide new strategies for treatment.

An adenoviral-mediated gene directed enzyme pro-drug (GDEPT) therapy is being developed to selectively target and induce killing in MTC cells. Our targeted gene therapy approach uses modifications of the promoter (T2) and splicing specificities (CT) of the calcitonin/calcitonin gene related peptide gene (CT/CGRP) to direct the expression of a pro-drug activating enzyme, *E. coli* purine nucleoside phosphorylase (PNP). We have introduced either a T2 driven PNP transgene (T2-PNP) or T2 driven, CT-specific splicing transgene (T2-CT/PNP) into recombinant replication defective adenovirus (AdV). *In vitro* we have demonstrated that while the modified promoter (T2) expressed sufficient PNP to inhibit the growth of TT cells (an MTC cell line) in the presence of pro-drug fludarabine, the combination of the modified promoter, plus the splicing feature, led to significantly greater cell-type specificity than either feature alone.

We have formulated a storage buffer for these Ad vectors which provides enhanced stability during multiple freeze thaw cycles (12), storage at RT (6 weeks), −20 °C and −70 °C.

In a TT xenograft nude mouse model we demonstrated that both targeted viruses produce PNP in subcutaneous tumours, with Ad.T2-PNP sustaining expression for 7 days. Ad.T2-CT/P showed lower expression by day 7 reflecting the *in vitro* data where PNP levels produced by Ad T2-CT/PNP were lower than those produced by Ad T2-PNP as measured by HPLC PNP assays.

We are continuing *in vivo* studies to examine the antitumour efficacy of these targeted viruses against MTC tumour cells in the presence of pro-drug, and to evaluate the toxicity and dissemination of systemically administered targeted adenovirus in immune competent mice.

P8. Fibroblasts Modulate Cardiomyocyte Excitability: Insights from Fibroblasts with Genetically Altered Connexin43 Expression

Eddy Kizana,[1,2,3] Samantha L. Ginn,[3] Christine Smyth,[3] Stuart P. Thomas,[1] David G. Allen,[2] David L. Ross,[1] and Ian E. Alexander[3,4], [1]*Department of Cardiology, Westmead Hospital, Westmead NSW, Australia,* [2]*Institute for Biomedical Research and Department of Physiology, University of Sydney, NSW, Australia,* [3]*Gene Therapy Research Unit of The Children's Hospital at Westmead and Children's Medical Research Institute, Westmead NSW, Australia and* [4]*Department of Pediatrics and Child Health, University of Sydney, NSW, Australia.*

Heterologous gap junction-mediated coupling of fibroblasts and cardiomyocytes within the heart is likely to have important electrophysiological consequences in both normal and pathophysiological states. While a conductive function for fibroblasts in this context has been reported, the current study provides the first evidence that fibroblasts can modulate cardiomyocyte excitability in a connexin43-dependent manner. In a co-culture system neonatal rat cardiomyocytes were grown on monolayers of mouse fibroblasts in which the fibroblasts had genetically altered connexin43 expression. Intrinsic beat frequencies of the cardiomyocytes were counted. Cardiomyocytes grown on wild-type fibroblasts expressing native levels of connexin43 (Cx43) beat significantly slower than cells grown on fibroblasts devoid of this molecule (germline knockout) or with dominant-negative functional suppression. Expression of Cx43 in fibroblasts from Cx43 knockout mice reduced beat frequency back to that observed in co-culture with wild-type fibroblasts. In conclusion, fibroblasts couple to cardiomyocytes and modulate their excitability by a Cx43-dependent mechanism. This electrophysiological effect of fibroblasts on cardiomyocytes may be important in the context of stem cell-based strategies for cardiac repair where donor cells with the capacity for cell membrane excitability are usually grafted into fibrotic myocardium. In this setting, donor cell electrophysiology may be altered by coupling with scar tissue-resident fibroblasts.

P9. Liver-Targeted Gene Delivery using Adeno-Associated Virus Vectors in the Treatment of Urea Cycle Disorders

S. C. Cunningham,[1] A. P. Dane,[1] J. A. Curtin[1] and I. E. Alexander[1,2], [1]*Gene Therapy Research Unit, The Children's Hospital at Westmead and Children's Medical Research Institute, Westmead, NSW 2145, Australia.* [2]*Discipline of Paediatrics and Child Health, University of Sydney, NSW 2000, Australia.*

Adeno-associated virus (AAV) is a promising vector for gene therapy due to its capacity to confer long-term transgene expression *in vivo* and theoretical safety profile. AAV8 pseudo-serotyped vectors have been shown to be highly efficient in the transduction of muscle and non-muscle tissues following systemic administration, in particular the liver. We are interested in developing treatments for urea cycle disorders, in particular ornithine transcarbamylase (OTC) deficiency. In its common severe neonatal form, liver transplantation is the only option for long-term survival. However, this treatment has its own shortcomings. The gene delivery system investigated in this project has the potential to provide a safer and more effective alternative to liver transplantation.

We have constructed an AAV vector (type 2 genome) containing a liver-specific promoter (two copies of the apolipoprotein E enhancer upstream of the hAAT promoter) driving expression of a reporter gene EGFP, and encapsidated this with the AAV8 capsid. Vector was produced in 293 cells by triple plasmid transfection, and purified by cesium chloride gradient centrifugation. This vector successfully transduces mouse, rat and human hepatoma cells *in vitro*, and mouse hepatocytes *in vivo*. The *in vivo* experiments were carried out in adult mice. Vector was inoculated by portal vein injection, and the dose was 8.95×10^5 TU (5×10^{10} vector genomes as established by real-time PCR). The mice were harvested at 1, 3 and 6 weeks after injection. At each time point, liver was sectioned and analysed for EGFP expression. Experiments are currently being carried out with this vector to establish the optimal age and route of delivery into mice.

Based on these results, AAV8 pseudo-serotyped vectors have been constructed expressing the mouse and human cDNAs for OTC. These vectors are currently being function-tested in OTC-deficient cell lines, and will subsequently be evaluated in a mouse model of OTC deficiency.

P10. Investigation of a Virus Vector-Mediated Approach for the Treatment of the Neurological Component of the Friedreich Ataxia Disease

A. Spinoulas,[1] J. Fleming,[1] M. Zheng,[1] S. C. Cunningham,[1] S. L. Ginn,[1] P. B. Rowe[1] and I. E. Alexander[1,2], [1]*Gene Therapy Research Unit, The Children's Hospital at Westmead and Children's Medical Research Institute, Westmead, Sydney, NSW 2145.* [2]*Discipline of Paediatrics and Child Health, University of Sydney, NSW 2000, Australia.*

The sensory neuropathy Friedreich ataxia (FRDA) is a progressive neurodegenerative disease associated with the loss of large sensory neurons from the dorsal root ganglia (DRG). Unfortunately, current treatments are inadequate and we have, therefore, chosen to investigate a gene therapy approach for the treatment of the neurological component of this debilitating disease.

In a previous study we were able to show efficient and sustained gene delivery to sensory neurons *in vitro* using adeno-associated virus (AAV) and lentivirus (LV) vectors. In this study both virus vectors encoding human frataxin cDNA were assessed for both frataxin protein expression and function in primary FRDA patient fibroblasts. Antibody staining in transduced FRDA cells was shown to be consistent with correct sub-cellular localisation of frataxin to the mitochondrial compartment. In addition, frataxin mRNA levels revealed frataxin expression in vector-treated FRDA cells remained at or above physiological levels. Transduced fibroblasts consistently exhibited resistance to BSO-induced oxidant stress, with LVFrat-transduced patient fibroblasts demonstrating a significant 2.9-fold increase in cell viability ($p = 0.031$) versus untransduced patient cells. Furthermore, transduced cells demonstrated a small reduction in mitochondrial iron levels ($p = 0.078$, not significant) below those detected in naïve FRDA patient cells. Therefore, despite the inherent difficulties experienced in working with primary fibroblasts, both vectors were able to achieve appropriately targeted frataxin expression and evidence of phenotype reversal.

In addition to studies using patient fibroblasts, we have established neuronal cell lines that over-express frataxin protein. Over-expression of transgene in these cells did not appear to be associated with any changes in cell viability or resistance to oxidative stress. Studies are now underway to characterise frataxin-deficient sensory neurons in culture.

P11. Functional Role of Interleukin-15 in the Development of NK Cells in SCID-X1 with an NK+ Phenotype

Christine M. Smyth,[1] Grant J. Logan,[1] Samantha L. Ginn,[1] Peter B. Rowe,[1] and Ian E. Alexander[1,2], [1]*Gene Therapy Research Unit, Children's Medical Research Institute and The Children's Hospital at Westmead, Locked Bag 23 Wentworthville NSW 2145, Australia,* [2]*Discipline of Paediatrics and Child Health, University of Sydney, Australia.*

X-linked severe combined immunodeficiency (SCID-X1) results from mutations in the gene encoding the common gamma chain (γc) of the interleukin receptors 2, 4, 7, 9, 15 and 21. Characteristically, patients lack T and NK cells and have near normal numbers of functionally defective B cells (T-NK-B+). However, NK cells are detected in a minority of SCID-X1 patients (T-NK+B+ phenotype), and the reasons for this remain incompletely understood.

We have identified a SCID-X1 infant with an NK+ phenotype with a putative splice-site mutation in the gene encoding γc. This was confirmed by RT-PCR analyses, on mRNA derived from both patient PBMC and EBV-transformed bone marrow cells, which demonstrated two aberrantly spliced γc mRNA species and trace levels of correctly spliced message. However, no cell-surface γc protein was detected by FACS on these populations.

We hypothesized that low but undetectable amounts of cell surface γc in this patient were sufficient for IL-15 receptor-mediated signaling (enabling NK cell development) but insufficient for IL-7 receptor-mediated signaling thereby preventing T-cell development. To test this hypothesis, we measured STAT5 phosphorylation by immunofluorescence in EBV-transformed patient B cells after stimulation with either IL-15 or IL-7.

Interestingly, the patient cells were able to phosphorylate STAT5 in response to IL-15 at levels similar to control cells expressing wild-type γc suggesting that undetectable γc was expressed at the surface of patient cells. To determine whether IL-7 stimulation could also transduce a signal through γc, patient and control cells were genetically modified to express IL-7Rα. After modification, IL-7 stimulation induced phosphorylation of STAT5 in control cells but was unable to transduce a similar signal in patient cells.

These results support the conclusion that expression of limiting amounts of cell-surface γc preferentially impairs signaling via the IL-7 receptor relative to the IL-15 receptor, and provide a mechanistic explanation for the NK+ phenotype observed in the infant described.

P12. A Versatile Adenoviral Vector Platform

Charles G. Bailey,[1] Cynthia Ng[1] and John E. J. Rasko[1,2], [1]*Gene and Stem Cell Therapy, Centenary Institute of Cancer Medicine and Cell Biology.* [2]*Sydney Cancer Centre, Royal Prince Alfred Hospital, Missenden Rd, Camperdown, 2050, Sydney, Australia.*

Adenoviral vectors based on adenovirus serotype 5 (Ad5) are widely used for *in vitro* and *in vivo* research applications. They offer broad tropism, can be produced at high titres, and can package more genetic information than many other viral vector systems. A number of methods for generating recombinant adenoviral vectors exist, including use of homologous recombination in *E. coli*, an *in vitro* ligation method and site-specific recombination using Cre-LoxP or lambda bacteriophage-derived recombination (Gateway® Technology). All commercially available systems offer increased efficiencies in the construction time of adenoviral vectors by improving the cloning strategy or using a universal cloning technology. However, all systems lack versatility in design, functionality and the ability to express multiple transgenes.

We have developed an adenoviral vector platform incorporating Gateway® Technology. This technology enables whole expression cassettes to be shuttled into our adenoviral vectors without using conventional cloning techniques. This platform offers the following advantages: the vector pseudotype can be altered; any reporter gene can be used in combination with the gene of interest; and different promoters can be incorporated. Different cell populations or tissues can be targeted using different fibre genes to alter the vector pseudotype. Any marker or autofluorescent reporter gene can be added allowing for adenoviral vectors with different reporters to be used in co-transduction experiments and then analysed by

two- or three-colour FACS. A range of promoters for constitutive, inducible or tissue-specific expression can be easily incorporated. We have also increased the transgene packaging capacity up to 10 kb allowing the expression of large cDNAs or multiple transgenes from the same virus. To date we have generated numerous Ad5- and Ad35-pseudotyped multi-transgene vectors incorporating green or orange fluorescent proteins at titres up to 10^{10} pfu/mL. These have been successfully used to transduce human and mouse cells including those of haemopoietic origin using the Ad35 vector.

P13. Specific Serotypes of Adeno-Associated Virus Vectors Facilitate Gene Transfer in a Non-Human Primate Model of Mesenchymal Stem Cell-Directed Gene Therapy

Keefe Chng,[1] Stephen Larsen,[1] Sally Thomson,[2] Annemarie Hennessy[2] and John Rasko[1,3], [1]*Gene Therapy Research Unit, Centenary Institute,* [2]*Renal Dept, RPAH,* [3]*Sydney Cancer Centre, RPAH, Sydney, NSW, Australia.*

Mesenchymal stem cells (MSCs) are multi-lineage potential cells that can be isolated from the bone marrow. The capacity of these cells to differentiate in vitro and in vivo into multiple mesenchymal lineages including adipogenic, osteogenic, chondrogenic, and myogenic phenotypes have been described using a variety of animal models. These properties coupled with their accessibility from a bone marrow aspirate, their relative ease of culture in vitro, and their reported long-term engraftment in vivo, has contributed to the growing interest in developing these cells as vehicles for gene therapy. Our aim is to establish a non-human primate model of MSC-directed gene therapy. To accomplish this we have isolated and characterized bone-marrow-derived MSCs from baboons (*Papio hamadrayas*) using surface phenotypic markers, and by in vitro clonal assays of osteogenic and adipogenic differentiation. To define the transduction profile of our baboon MSCs we are investigating the use of different serotypes of the adeno-asociated virus (AAV) as vectors to transduce these cells with the LacZ reporter transgene. AAV vectors based on serotypes 2 and 5 were found to transduce baboon MSCs at a much higher efficiency, with a 100-fold and 10-fold improvement, respectively, compared to serotype 1. Preliminary results also show that human MSCs have a similar pattern of transduction efficiency using the above serotypes, albeit the level of transduction was found to be lower than that achieved on baboon MSCs.

To test the feasibility of our model in vivo, we have initiated a cardiotoxin-induced muscle injury and regeneration NODSCID mouse model to test whether transduced baboon MSCs can be delivered into damaged muscle and engraft in vivo to contribute to ongoing muscle repair. We are currently establishing methods to detect injected transduced baboon cells in mouse tissue by LacZ expression, immunohistochemistry, and species-specific PCR.

P14. Mesenchymal Stem Cells as Gene Delivery Vehicles for Cancer Gene Therapy

Rosetta Martiniello-Wilks,[1,2] Keefe Chng,[2] Stephen R. Larsen[2] and John E. J. Rasko[1,2], [1]*Cell and Molecular Therapy Unit, Sydney Cancer Centre, Royal Prince Alfred Hospital;* [2]*Gene Therapy Research Unit, Centenary Institute of Cancer Medicine & Cell Biology and the University of Sydney, Locked Bag No 6, Newtown, NSW 2042, Australia.*

We are exploring a therapeutic strategy utilising bone marrow mesenchymal stem cells (MSC) as gene delivery vehicles for the targeted delivery and local production of biological agents that kill tumours. MSC are highly proliferative, capable of self renewal by proliferation without differentiation and respond to tissue injury signals that contribute to the maintenance and regeneration of connective tissue. MSC are also multipotent and can differentiate under controlled conditions into cell types including chondrocytes, adipocytes, osteocytes and fibroblasts that form mesenchymal tissues, cartilage, fat, bone, and stroma, respectively. Two studies have shown that MSC can deliver genes encoding therapeutic proteins to melanoma and breast carcinoma during tumour stroma establishment. Due to their ability to self-renew, their ability to evade attack by the immune system and their potential to differentiate into mesenchymal tissues, MSC have attracted attention as cellular vehicles for therapeutic gene transfer.

Mouse bone marrow MSC with the greatest fibroblast colony forming cell (CFU-F) potential show a Lin$^-$ Sca-1^{bright}CD45$^-$ CD31$^-$ phenotype. We have sorted Sca-1^{bright}CD45$^-$ (MSC) or Sca-1^{bright}CD45$^+$ (control) adherent bone marrow cells isolated from the femur and tibia of C57BL/6 mice. As a further control, unsorted adherent bone marrow cells (mBM) were cultured along with these subpopulations in alpha MEM + 20% FCS for the first four passages followed by alpha MEM + 20% FCS + 10 ng/mL basic fibroblast growth factor. When subjected to tri-lineage differentiation media, only Sca-1^{bright}CD45$^-$ cells showed osteogenesis, adipogenesis and chondrogenesis. When subjected to various eGFP gene transfer methodologies, the Sca-1^{bright}CD45$^-$ cells showed lentivirus>nucleofection>adeno-associated virus serotype 2>ecotropic retrovirus eGFP transfer. The ability of gene marked MSC to home to and engraft cancer in the prostate and lungs of C57BL/6 mice will now be explored.

P15. Combination of Cytosine Deaminase with Uracil Phosphoribosyl Transferase Leads to Local and Distant Bystander Effects Against RM1 Prostate Cancer in C57BL/6 Mice

Aparajita Khatri,[1,2] Pamela J. Russell,[1,2] Bing Zhang,[3] Eboney Doherty,[1,2] Kim Ow,[1] Jane Chapman,[1,2] and Rosetta Martiniello-Wilks[4], [1]*Prince of Wales Hospital, Randwick, Australia,* [2]*University Of New South Wales, Sydney, Australia,* [3]*University of Queensland, Brisbane, Australia,* [4]*Centenary Institute of Cancer Medicine & Cell Biology, Sydney, Australia.*

We are evaluating the therapeutic potential of gene directed enzyme prodrug therapy (GDEPT) using cytosine deaminase (CD) in combination with uracil phosphoribosyl transferase (UPRT) for treating prostate cancer (PCa). CDUPRT converts 5 fluorocytosine (5FC) to freely diffusible metabolites, including 5-fluorouracil (5FU), that disrupt the metabolic pathways for both DNA and RNA synthesis, resulting in the killing of both dividing and non-dividing cells. This is especially relevant to slow growing PCa.

Andogen-independent mouse RM1 cells were stably transformed with plasmids containing GFP/CDUPRT, GFP or GFP/LacZ genes (controls). CDUPRT expression in cell lysates from RM1-GFP/CDUPRT cells/tumors was confirmed by estimation of enzymic conversion of its substrate, 5FC, into 5FU using HPLC. Treatment of C57BL/6 mice bearing intraprostatic RM1-GFP/CDUPRT tumors with 5FC resulted in complete regression of the tumors. Further, intraprostatic implantations with mixtures of RM1-GFP/CDUPRT and RM1-GFP cells in different proportions in C57BL/6 mice

resulted in a 'local bystander effect', even though only 20% of the cells were expressing the transgene. To determine if there was any distant bystander effect, pseudometastases in the lungs were established and the lung colony counts at necroscopy (day 19) indicated the presence of a 'distant bystander effect'. Indeed, the pseudometastases were absent in ~50% of mice in the RM1-GFP/CDUPRT+5FC group compared with the control groups. This is the first demonstration of a distant bystander effect using CDUPRT-GDEPT. Furthermore, immunohistochemical evaluation of the GDEPT showed an increase in immune cell infiltration by $CD4^+$ T cells, macrophages and natural killer cells. There was increased tumor necrosis and apoptosis and a decrease in tumor vascularity after GDEPT. We conclude that CDUPRT-GDEPT significantly suppressed the aggressive growth of RM1 prostate tumors via mechanisms involving necrosis and apoptosis, accompanied by strong infiltration of immune cells in the prostate tumors. The latter may be associated with the decrease in lung pseudometastases.

P16. Promise of BLCA-38 as a Targeting Antibody for Tissue Specific Gene Delivery to Prostate Cancer

Tim Ho,[1,2] Ann Cunningham,[1,2] Leif Lindholm,[3] Yong Li,[2,4] Pamela J. Russell[1,2] and Aparajita Khatri[1,2], [1]*Oncology Research Centre, Prince of Wales Hospital, Randwick, Australia,* [2]*University of New South Wales, Australia,* [3]*Department of Medical Microbiology & Immunology, University of Göteborg, Sweden,* [4]*Cancer Care Centre, St. George Hospital, Kogarah, Australia.*

BLCA-38 is a novel murine IgG1 monoclonal antibody (MAb), which specifically binds various epithelial cancers including prostate cancer (PCa), significantly, without cross-reactivity with normal tissue. The promise of BLCA-38 as a targeting antibody for treatment of PCa is evident from the fact that it binds to both PCa cell lines and human PCa biopsies. Its successful use for targeting immunotoxins to cancer cells suggests that BLCA-38 MAb may have the potential to serve as a tool for targeted gene delivery to PCa using transductionally modified gene transfer vectors. In this study, we explored the potential of BLCA-38 mediated transductional targeting of a tropism-modified adenoviral vector. Specifically, an Ad5-C2C2 virus, developed by Dr. Leif Lindholm's• laboratory, is used. It expresses the green fluorescent protein (GFP) and contains fibre with a C2 domain (an Fc-binding oligopeptide derived from protein G of *Streptococcus*), thus allowing it to conjugate with different targeting antibodies. The specific binding of the virus to BLCA-38 MAb was confirmed via dot blot assays. Immunostaining studies showed that BLCA-38 antigen is expressed in both androgen-independent (PC3, DU145) and androgen-sensitive (LN3) PCa cell lines. Infection of the cell lines with Ad5-C2C2-GFP-BLCA-38 conjugate was monitored by GPF expression using UV microscopy and flow cytometry. BLCA-38 antigen negative murine PCa Tramp C1, irrelevant antibody/Ad5-C2C2-GFP and Ad5-GFP/BLCA-38 conjugates served as the negative controls. The antibody conjugated Ad5-C2C2-GFP virus showed a significant increase in infection rates compared to Ad5-GFP. Up to 40-, 30- and 85-fold increases were observed for LN3, DU145 and PC3 cells, respectively. In conclusion, BLCA-38 conjugated Ad5-C2C2 shows antigen-specific cell targeting *in vitro*. Currently, the efficiency and specificity of the system is being evaluated in three-dimensional PCa spheroid models in collaboration with Dr. Yong Li. This will be followed by *in vivo* evaluation of its specificity and efficiency.

P17. Gene Therapy to Induce Immunological Tolerance for Autoimmune Disease

Frank Alderuccio, Judith Field, Zeyad Nasa, Warren Clements, Kim Murphy, Mark Biondo, James Chan, and Ban-Hock Toh, *Department of Immunology, Monash University, Commercial Road, Prahran, Victoria 3181, Australia.*

Autoimmune diseases such as type 1 diabetes and multiple sclerosis pose a significant health burden on our society. They affect approximately 6% of the population and are the third largest cause of disease burden after heart disease and cancer. Despite much effort, there is no known cure, with treatment restricted to toxic immunosuppressive regimes or replacement therapy. Autoimmune diseases are a consequence of the adaptive immune response and characterised by T and B cell responses to defined autoantigens. The failure of complete T cell tolerance to self-antigens in the thymus, together with genetic predisposition, renders certain individuals prone to developing autoimmune disease. We hypothesize that gene therapy is a viable strategy that can be used not only to prevent but also to cure autoimmunity. Our approach is focused on directing autoimmune disease-specific autoantigens for ectopic expression in the thymus to induce tolerance. Our hypothesis stems from experimental studies we have performed with the mouse model of experimental autoimmune gastritis (EAG) and more recently with the NOD model for type 1 diabetes. We have demonstrated that ectopic expression of disease-initiating autoantigen in MHC class II-positive cells, including those in the thymus, can render mice resistant to autoimmune disease induction. Furthermore, we find that tolerance can be transferred to naïve mice via the bone marrow compartment. These findings reinforce the importance of bone-marrow-derived antigen-presenting cells in establishing concrete thymic tolerance, and forms the cornerstone of our studies. Gene therapy tools offer a powerful approach that can be used to direct autoantigen expression to bone-marrow-derived cells, such as dendritic cells, and induce thymic tolerance.

P18. Ectopic Expression of Neuro D in Insulin Secreting Liver Cells

Mathiyalagan Appavoo and Bernard E. Tuch, *Diabetes Transplant Unit, Prince of Wales Hospital and University of New South Wales, Sydney, NSW 2031, Australia.*

The ideal therapy for type 1 diabetes is to replace the destroyed insulin-producing cells. However, transplantation of a whole pancreas or islets from human cadaveric donors, although successful, is limited by the scarcity of human donors. Gene therapy is a potential strategy to overcome this shortfall. A gene-therapy-based treatment of type 1 diabetes requires the development of surrogate β-cells that can synthesize, store and secrete insulin in response to glucose.

It has been demonstrated that rat hepatoma cells, FAO, stably transfected with the insulin gene, FAOins, synthesize and release insulin constitutively. NeuroD, a transcription factor, is a key regulator of insulin gene transcription and required for pancreatic islet morphogenesis. We hypothesized that NeuroD plays a role in regulated secretion of insulin.

Accordingly, we transfected human NeuroD cDNA into FAOins. Clonal selection (FAOinsNd) was carried out using antibiotic pressure (hygromycin). FAOinsNd cells were analyzed for key β-cell factors and their ability to secrete insulin in response to calcium or glucose. Vector-derived NeuroD gene expression was confirmed by RT-PCR. Both, FAOinsNd and FAOins expressed the genes for β-cell factors Pdx1, NeuroD, Pax4, Pax6, Isl1, HB9, MafA, Nkx6.1, Nkx2.2, chromogranin A, chromogranin B, prohormone convertase 1, prohormone convertase 2, synaptotagmin 1 and SNAP25, with only FAOinsNd cells expressing Ngn3. Both lines expressed β-cell proteins, GLUT2 glucose transporter, glucokinase, NeuroD and chromogranin A. The FAOinsNd cells secreted insulin in response to calcium, exhibiting a seven-fold increase (64.2 ± 3.9 ng/10^6 cells/hr) from basal (8.8 ± 0.3 ng/10^6 cells/hr), but not to glucose. In contrast, the FAOins cells were unresponsive to both calcium and glucose. Intracellular insulin content was increased three-fold ($P < 0.0001$) in FAOinsNd compared to FAOins cells.
Our results indicate that ectopic expression of NeuroD markedly enhanced intracellular insulin content and induced calcium responsiveness in FAOinsNd cells. However, the expression of NeuroD alone was not sufficient to render these liver cells glucose responsive.

P19. Glucose-Stimulated Insulin Secretion from an Engineered Human Liver Cell Line Is Regulated by the L-Type Calcium Channel

Edwin Ch'ng,[1] Chang Tao,[1] Donald K. Martin,[2] Mark Lutherborrow,[3] Bernard E. Tuch,[3] and Ann M. Simpson[1], [1]*Dept. Cell & Molecular Biology &* [2]*Dept. Health Sciences, University of Technology, Sydney, PO Box 123, Broadway, NSW, 2007, Australia.* [3]*Diabetes Transplant Unit, Prince of Wales Hospital, High St., Randwick, NSW, 2031, Australia.*

Introduction: One approach to the treatment of type 1 diabetes is the creation of an 'artificial beta cell'. The stable transfection of insulin cDNA into the human liver cell line HuH7 resulted in synthesis, storage and regulated release of insulin to glucose stimulus (Huh7ins cells). Glucose responsiveness of Huh7ins cells is regulated by ATP-sensitive potassium (K_{ATP}) channels. The aim of the present study was to determine if Huh7ins cells respond to glucose via activation of voltage-gated calcium channels, as is the case with pancreatic beta cells.
Methods: The effect of 20 mM glucose and the calcium blockers verapamil (10 μM), ryanodine (10 μM), thapsigargin (1 μM), oleic acid (20 μM) and the activator Bay K8644 (1 μM) on acute insulin secretion was assessed. Insulin secretion was measured by radioimmunoassay. The glucose-responsive beta cell line MIN-6 was used as a positive control. Primers were designed to the K_{ATP} subunits, Kir 6.2 and SUR 2A, and the L-type calcium channel (α1D subunit) of beta cells. RT-PCRs were performed on cDNA from Huh7, Huh7ins, and human beta cells.
Results: Exposure to glucose for 1 h increased insulin secretion from 0.15 ± 0.01 to 0.52 ± 0.05, Bay K8644 from 0.19 ± 0.02 to 0.46 ± 0.04 and thapsigargin from 0.09 ± 0.01 to 0.18 ± 0.01 pmol insulin/10^6 Huh7ins cells ($n = 6$). Verapamil inhibited glucose-stimulated insulin release. Similar results were obtained for MIN 6 cells. PCR analysis revealed the presence of the K_{ATP} channel subunits Kir 6.2 and SUR 2A and the L-type calcium channel in Huh7ins cells.
Conclusions: Huh7ins cells respond to glucose by the same mechanism as β cells. This provides useful information for the future engineering of insulin-secreting liver cells and suggests that the transplantation of such cells may provide a cure for type I diabetes.

P20. Anti-Tumor Study of Suicide Gene Therapy with an Ovarian-Specific Promoter for Ovarian Cancer and Polyethylenimine Mediated Transfection

Kong Bei-Hua,[1] Jin Ping[1] and Xu Yu-Hong[2], [1]*Department of Gynecology and Obstetrics, Qilu Hospital of Shandong University, Jinan, Shandong 250012, China;* [2]*School of Pharmacy of Shanghai Jiao Tong University, Shanghai 200030, China.*

Objective: The improvement of transfection efficiency and design of targeted gene transfer vector are two critical issues in the field of gene therapy. Many approaches have been developed to solve these problems. Polycationic polyethylenimine (PEI), as a new kind of gene vector, has provided a potential solution. This study aimed to investigate the anti-tumor and targeted delivery effects of HSV-TK suicide gene therapy utilising an ovarian-specific promoter (OSP1) for human ovarian carcinoma by PEI-mediated transfection.
Methods: (1) Human ovarian carcinoma SKOV3 cells were transfected by pGL3-Luc plasmid mediated with PEI, DOTAP liposome and naked DNA, respectively. The transfection efficiency was measured by RLU (relative luciferase unit). (2) The pOSP1-HSVtk plasmids containing an ovarian-specific promoter were transfected using PEI into the human ovarian carcinoma cell SKOV3, human lung carcinoma cell NCI-H460 and human heptocellular carcinoma cell HepG2. The cytotoxicities resulted from GCV were evaluated using the MTT assay. The concentrations of GCV in SKOV3 cells were detected by high performance liquid chromatography (HPLC). The apoptosis of three kinds of carcinoma cells were observed by flow cytometry (FCM) and the TdT-mediated dUTP nick end labeling (TUNEL) technique. (3) A SKOV3 ovarian cancer xenograft model was established in BALB/C nude mice. The xenografts were transfected with pOSP1-HSVtk using PEI as the transfection agent. The expressed TK activities were estimated by monitoring the GCV concentration change using a HPLC assay. The weight of the nude mice, the tumor volumes and tumor weights were all recorded to calculate the tumor inhibition rates by weight and by volume. Histopathological analysis and TUNEL method were also performed.
Results: (1) The PEI-transfected group had the highest luciferase activity (187.35 ± 6.48) compared with other two groups (45.74 ± 5.98, 0.03 ± 0.00) with significant statistical difference ($P < 0.01$); (2) GCV was shown to be toxic only in SKOV3 cells, not in NCI-H460 and HepG2 cells. The concentrations of GCV in SKOV3 cells detected by HPLC gradually decreased with increase in time. FCM showed the apoptotic rate of cells significantly increased in SKOV3 exposed to GCV for 24, 48 and 72 h ($8.42 \pm 0.76\%$, $18.50 \pm 1.78\%$, $34.80 \pm 3.46\%$). The substantial cell apoptosis in SKOV3 was confirmed by TUNEL. (3) Compared with the control group, GCV concentrations in xenografts transfected pOSP1-HSVtk were shown to be significantly lower by HPLC ($0.05 > P > 0.01$). Besides, the tumor volume and the tumor weight were also significantly decreased ($P < 0.01$). The tumor volume inhibition rate and

the tumor weight inhibition rate were estimated to be 63.66% and 58.98%, respectively. Histological examination revealed heavy haemorrhage and necrosis in the tumor tissues, and TUNEL confirmed substantial cell apoptosis in the treated group.

Conclusion: The suicide gene therapy system using an ovarian-specific promoter by PEI-mediated transfection has a significant killing activity for human ovarian cancer, which indicated its potential to improve the efficiency and the targeting in the field of gene therapy.

P21. Anti-Tumor Study of Double Suicide Gene Therapy with an hTERT Promoter for Ovarian Cancer and Polyethylenimine Mediated Transfection *in vivo*

Jin Ping,[1] Kong Bei-Hua[1] and Xu Yu-Hong[2], [1]*Department of Gynecology and Obstetrics, Qilu Hospital of Shandong University, Jinan, Shandong 250012, China;* [2]*School of Pharmacy of Shanghai Jiao Tong University, Shanghai 200030, China.*

Objective: To investigate the anti-tumor effects of double suicide gene therapy adopting a human telomerase reverse transcriptase (hTERT) promoter and polyethylenimine (PEI)-mediated transfection for human ovarian carcinoma by several methods and further to evaluate the dose-effect, the period-effect relationship.

Methods: SKOV3 cells were implanted intraperitoneally to BALB/C nude mice and the ovarian cancer survival model was established. Diverse doses (5, 30, and 50 μg) of pOSP1-HSVtk were given by intraperitoneal injection using PEI as the transfection agent. Diverse treatment times were started by intraperitoneal injection of pOSP1-HSVtk plasmid on the 1st, 10th and 20th day after tumor implantation. The two kinds of plasmid (PEI/pBTdel-279-TK, PEI/pBTdel-279-CD-TK) treatment were also observed. Then 5-fluorocytosine (5-FC) and ganciclovir (GCV) were injected into the cavity of the peritoneum. The survival period and the survival prolong rate were calculated.

Results: The average survival period of the treated group to which pBTdel-279-CD-TK was injected on the 1st day after tumor implantation was longer than other two groups ($P < 0.05$). There were no significant differences between the 10th day treated group and the 20th day treated group ($P > 0.05$). The average survival period of the pBTdel-279-CD-TK group was prior to the pBTdel-279-TK group ($P < 0.05$), and the survival prolongation rate of these were 73.41% and 146.44%, respectively. The dose of pBTdel-279-CD-TK plasmid had no relation to the average survival period ($P > 0.05$).

Conclusion: The suicide gene therapy system using a human telomerase reverse transcriptase (hTERT) promoter by PEI-mediated transfection had a significant killing activity on human ovarian cancer in vivo and in particular it had remarkable therapeutic effects in the early period of the tumor development.

P22. Knockdown of Epidermal Growth Factor Receptor by RNAi Inhibits A549 Lung Adenocarcinoma Cell Growth

Li Bai, Chun-xue Bai, Rong Zhu, and Lei Gao, *Department of Pulmonary Disease of Zhong Shan Hospital, Fudan University, Shanghai 200032, P.R. China.*

Objective: Overexpression of epidermal growth factor receptor (EGFR) plays an important role in the formation and proliferation of various cancer types including lung cancer. The importance of the EGFR axis in tumorigenesis and tumor progression makes it an attractive target for the development of anticancer therapies. RNAi provide a new and reliable way to investigate gene function and has the potential for gene therapy. The aim of the study was to examine the anti-tumor effects of a decrease in the protein level of EGFR by RNAi in A549 cells.

Methods: A plasmid-based polymerase promoter system for siRNA targeting EGFR was used to reduce the expression of EGFR in A549 cells. The mRNA and protein level of EGFR were measured by real-time PCR and flow cytometry, respectively. We assessed the effects of EGFR silencing on tumor growth by cell growth curve and colony formation assay.

Results: Plasmids expressing siRNA targeting EGFR reduced the protein level of EGFR by up to 80%. Furthermore, real-time PCR showed that EGFR mRNA was effectively degraded by over 90%. Cell growth and colony formation were inhibited by 67.8% and 59.4%, respectively.

Conclusion: A plasmid-based system by decreasing EGFR expression by RNAi against EGFR could inhibit tumor growth significantly, which provides another available therapeutic tool in the treatment of non-small-cell lung carcinoma overexpressing EGFR.

P23. Silencing of the AT1-Receptor Gene by Vector-Mediated RNA Interference for the Development of a Genetic Therapy for Hypertension

Jian Xu, Bing Zhang, Tao Yang, Glenda Gobe, Ming Q. Wei, and Malcolm J. West, *Department of Medicine, University of Queensland, The Prince Charles Hospital, Brisbane, Australia.*

Objective: Hypertension is a worldwide health problem. The renin-angiotensin system (RAS) is considered to be the major regulator of blood pressure, and the key factor in most essential hypertension. Interruption of the components of the RAS has been shown to reduce blood pressure in various animal models. In this study, we aim to test the inhibitory effect of vector-mediated RNAi on the expression of angiotensin II type 1 receptor (AT1-R).

Methods: Plasmid vectors encoding dsRNA were constructed by placing synthetic, short and complimentary double-stranded DNA either specific for AT1-R RNA or an unrelated sequence under the control of a polymerse-III H1-RNA promoter (PIII). Various cell lines were transfected with the plasmids. The levels of the AT1-R expression before and after the transfection on these cell lines were monitored by immuno-fluorescent assay using fluorescent microscopy and flow cytometry.

Results: Transfection of a vascular smooth muscle cell line and an epithelial cell line with the plasmid encoding dsRNA specific for AT1-R showed a significant inhibitory effect on the expression of AT1-R. When compared with non-transfected cells, or cells transfected with plasmid encoding unrelated dsRNA, the inhibitory effect was about 80% for the VSMC line and 85% for the epithelial cell line at 24 h post-transfection. At 48 h, the effects were the most obvious at about 90% for the VSMC line and about 95% for the epithelial cell line. Non-specific control of cells without addition of the specific antibody always stained negative for AT1-R. A dose effect of the transfection and a Western blot experiment to examine the reduction of the total AT1-R protein are underway.

Conclusion: Our results showed plasmid-mediated RNAi can specifically inhibit the expression of the AT1-R in a VSMC cell line and epithelial cell line, suggesting vector-delivered RNAi to AT1-R may be developed as a therapy to diminish the vasoconstricting effect of rennin-angiotensin, thus developing a potential viable therapy for high blood pressure.

P24. A Multi-Modality Strategy for the Treatment of Ovarian Cancer

Ping Zhang,[1] Bei-hua Kong[1] and Ming Q. Wei[2], [1]*Department of Obstetrics and Gynecology, Qilu Hospital, Shandong University, Jinan 250012, P. R. China;* [2]*Gene Therapy Unit, Department of Medicine, University of Queensland, Prince Charles Hospital, Australia.*

Objective: To investigate the in vitro killing effect of a multi-modality strategy involving a suicide gene and a chemokine gene transfer in combination with the use of macrophage.
Methods: The herpes simplex virus thymidine kinase (TK) and the monocyte chemoattractant protein 1 (MCP-1) cDNA fragment were separated by an internal ribosome re-entry site (IRES) and cloned into a retroviral vector based on pLXSN. This vector was then packaged in PA317 and used to transduce the ovarian cancer cell line, NuTu-19. RT-PCR and ELISA were used to confirm the expression of HSV-TK and MCP-1. Cells transduced with the backbone vector packaged virus were used as a control. The MTT method was used to investigate the tumoricidal effects. Flow cytometry was used to analyse the apoptotic effect and the expression of CD25 or CD44v6 on the trasduced cells. SEM was used to examine the morphology of cancer cells after GCV treatment.
Results: The retroviral vector encoding TK, and both TK and MCP-1, were successfully constructed and packaged. Transduction of NuTu-19 with HSV-TK or TK-MCP-1 vectors resulted in significant cell killing after treatment with GCV. Bystander effect was seen in cells transduced with the vector encoding TK. However, cells transduced with vectors encoding TK-MCP-1 showed a higher expression rate of CD25 (38.82%) than in cells transduced with vectors encoding HSV-TK (9.50%). Similarly, the expression of CD44v6 in cells transduced with HSV-TK or TK-MCP-1 vectors was significantly lower than control NuTu-19 cells. Most significantly, addition of macrophage (co-culture) caused a significant greater degree of apoptotic ratio (13.48%), S-phase block (52.92%) in cells transduced with TK-MCP-1 vector than those transduced with TK vector (apoptotic 9.50%, S-phase block 28.31%). This compared with no obvious apoptotic effect and only S-phase block ratio of 28% for macrophage alone. These results suggest a significant synergistic effect on the growth inhibition of the NuTu-19 cells ($P < 0.05$) when chemokine (MCP-1) gene transfer and macrophage were used.
Conclusion: Combined transfer of suicide gene and chemokine gene inducing nonspecific and specific antitumor immunity leads to a significant enhancement of the efficacy of suicide gene therapy. This strategy, when further combined with macrophage, could be developed as a modality of anti-ovarian cancer therapy.

P25. Induction of Specific Cytotoxic T Lymphocytes (CTL) Against Ovarian Cancer by MUC1/Y Gene-Modified Dendritic Cells

Lan Qi,[1] Bei-hua Kong,[1] Mei-xiang Yang,[2] Xun Qu,[2] and Ming Q. Wei[3], [1]*Department of Obstetrics and Gynecology, Qilu Hospital, Shandong University, Jinan 250012, P.R. China;* [2]*Basic Medical Science Institute, Qilu Hospital, Shandong University, Jinan 250012, P. R. China;* [3]*Gene Therapy Unit, Department of Medicine, University of Queensland, Prince Charles Hospital, Australia.*

Objective: Dendritic cells (DCs) are potent inducers of cytotoxic T lymphocytes (CTL) when pulsed with antigenic peptides or tumor lysates, but the effects are transient. In this report, we used plasmid-mediated gene transfer to DCs to examine whether plasmid DNA encoding a fused human MUC1/Y gene has the ability to elicit antigen-specific CTL responses to ovarian cells, hoping to develop a long-lasting therapy for ovarian cancer.
Methods: The eukaryotic expression vector pEGFP-N1/MUC1/Y encoding a fusion gene of MUC1/Y and the enhanced green florescent protein (EGFP) was constructed. DCs were generated from human peripheral blood, allowed to differentiate and then transfected with pEGFP-N1/MUC1/Y or backbone plasmid pEGFP-N1 (as control group) using Lipofectamine 2000. These DCs were 6 days old at the time of transfection. Then, 18–48 h after gene transfer, transfected DCs were confirmed of EGFP expression using fluorescence microscopy and MUC1/Y gene expression by RT-PCR. Subsequently, transfected DCs were co-cultured with autogeneic T cells to induce CTL activity for 5 days which were then used to target an ovarian cancer cell line, A2780 (MUC1/Y^+). The cytotoxic activity of the induced CTL to A2780 was detected using an MTT assay.
Results: DC precursors were isolated from the peripheral blood and induced to differentiate and mature using medium containing GM-CSF/IL-4/TNF-α for 6 days. After transfection with pEGFP-N1/MUC1/Y or pEGFP-N1, the transfected DCs had high level expression of the transgenes, i.e.: green fluorescence in the cytoplasm but not in the control group. Similarly, MUC1/Y mRNA was detected in pEGFP-N1/MUC1/Y transfected cells, but not in pEGFP-N1 transfected cells. Allogeneic T cell proliferation induced by DC-MUC1/Y was obviously higher in pEGFP-N1/MUC1/Y-transfected cells than in pEGFP-N1-transfected cells or the nontransfected DC group ($P < 0.05$). The cytotoxicity of CTL to the A2780 cell line was higher in pEGFP-N1/MUC1/Y-transfected cells than that of DC-pEGFP-N1-transfected cells.
Conclusion: We have successfully transfected the MUC1/Y gene into peripheral blood isolated DCs. Our results showed that as a cell transmembrane molecular, the MUC1/Y protein can enhance the ability of DCs to stimulate allogeneic T cell proliferation and induce potent cytotoxicity of CTL to ovarian cells that are MUC1/Y positive, suggesting the possibility of further development as a potential therapy for ovarian cancer.

P26. A New Eukaryotic Expression Vector: Co-expression of Enhanced Green Florescent Protein (EGFP) and the MUC1/Y

Lan Qi,[1] Bei-hua Kong,[1] Xun Qu,[2] Mei-xiang Yang,[2] and Ming Q. Wei[3], [1]*Department of Obstetrics and Gynecology, Qilu Hospital, Shandong University, Jinan 250012, P.R. China.* [2]*Basic Medical Science Institute, Qilu Hospital, Shandong University, Jinan 250012, P.R. China.* [3]*Gene Therapy Unit, Department of Medicine, University of Queensland, Prince Charles Hospital, Australia.*

Objective: MUC1/Y is a dominant antigen on the ovarian cancer cells. In this study, we aim to develop a eukaryotic expression vector to deliver and express MUC1/Y.
Methods: MUC1/Y cDNA was amplified by RT-PCR from an ovarian cancer cell line and cloned into pMD 18-T vector for sequence analysis. The correct sequence was then subcloned into the pEGFP-N1 eukaryotic expression vector as a fusion gene resulting in pEGFP-N1/MUC1/Y. The newly constructed vector was transfected into COS7 cells and then observed under a fluorescence microscope for positive clones. Forty-eight hours after transfection, COS7 cells were collected and MUC1/Y expression was detected by RT-PCR.
Results: The full sequence of the MUC1/Y cDNA was cloned and was successfully fused with the N-terminal of the EGFP gene in the pEGFP-N1 eukaryotic expression vector. Eighteen hours after transfection, green fluorescence was seen on the cell membranes in pEGFP-N1/MUC1/Y-transfected cells. After 48 h, MUC1/Y expression was detected by RT-PCR that showed high level of the MUC1/Y mRNA in pEGFP-N1/MUC1/Y-transfected cells.
Conclusion: We have successfully constructed the pEGFP-N1/MUC1/Y eukaryotic expression vector. Fusion of MUC1/Y with the EGFP gene does not influence the character of the MUC1/Y protein being secreted on the cell membrane. This experiment made it possible to further investigate the function of the MUC1/Y gene and to develop a nucleic acid vaccine based on this dominant tumour antigen gene.

P27. Combination of Angiostatin Gene Therapy with Chemotherapy for the Treatment of Human Ovarian Carcinoma in a Xenograft Mouse Model

Ping Sun,[1] Beihua Kong,[1] Dianning Dong,[1] and Ming Q. Wei[2], [1]*Department of Obstetrics and Gynecology, Qilu Hospital, Shandong University, Jinan 250012, P.R. China.* [2]*Gene Therapy Unit, Department of Medicine, University of Queensland, Prince Charles Hospital, Brisbane, Australia.*

Objective: To examine the combinational effects of angiostatin gene therapy with chemotherapy for the treatment of human ovarian carcinoma in a xenograft nude mouse model.
Methods: The xenograft nude mouse model was established by intraperitoneal injection of approximately 1×10^7 human ovarian epithelial carcinoma cells (SKOV3). Seven days after the injection, mice were randomly divided into four groups ($n = 8$), and injected in group 1 with a plasmid vector encoding angiostatin (pAng) + cisplatin; group 2 with plasmid pAng alone; group 3 with cisplatin alone; and group 4 with backbone plasmid, respectively. In group 1, where the combinational treatment was applied, angiostatin plasmid, pAng, was injected first followed by cisplatin 24 h later. Animals were sacrificed 1 week later and tumor samples were collected for analysis of the expression of angiostatin, and vascular endothelial growth factor (VEGF) with immunohistochemistry and Western blot. The microvessel density (MVD) in the tumor was also determined with immunohistochemistry and tumor cell viability (cell apoptosis) with TUNEL staining. A T test was used for statistical analyses.
Results: Significant inhibition of the tumor growth and the ascites formation was observed in groups 1–3. However, the inhibitive effect in group 1, the combination of angiostatin gene therapy with cisplatin, was the most significant. In this group the MVD was the lowest, but the apoptotic index (AI) was the highest.
Conclusion: Angiostatin gene therapy can inhibit the growth of ovarian cancer in nude mice by reducing angiogenesis. When combined with chemotherapy, it can lead to better inhibitive effects on ovarian cancer, including inhibition of angiogenesis and ascites formation and induction of cell apoptosis.

P28. Utilising the RCAS-TVA System to Investigate Gene Function in a Bovine Mammary Epithelial Cell Culture Model

Peta Phillips, Peter Wynn and Paul Sheehy, *CRC for Innovative Dairy Products and Centre for Advanced Technologies in Animal Genetics and Reproduction, Faculty of Veterinary Science, The University of Sydney, 425 Werombi Rd, Camden 2570, Australia.*

The RCAS-TVA system is a retroviral gene transfer system comprising of RCAS vectors derived from an avian sarcoma/leucosis virus, and TVA, an avian specific receptor for this virus. An immortalised line of bovine mammary epithelial (MAC-T) cells was transfected and selected to stably express TVA allowing for infection and hence transfer of genes from RCAS viral particle. RCASBP(A), a standard RCAS vector, has been modified to incorporate a Gateway cassette for ease of gene insertion. Two reporter genes, luciferase (Promega) and DsRedExpress (BD Biosciences, Clontec), have been recombined into Gateway competent RCASBP(A) vectors. A line of immortalised chicken embryonic fibroblasts (DF-1) which express native TVA will be transfected with these vectors to produce viral particle. TVA expressing MAC-T cells will then be infected with RCASBP(A)*luc* and RCASBP(A)DsRedExpress viral particle. This will allow for visual analysis as well as a quantitative measurement of gene transfer and expression. Evaluation methods include real-time PCR, luciferase assays and immunohistological staining.
Future work includes further adaptation of the system to incorporate a tissue-specific promoter as well as a gene regulation system for the expression of TVA. Other RCAS vectors will also be utilised, with some allowing for larger gene inserts or for infection of mammalian cells not expressing TVA.
The major application of this system is as an alternative method to study gene function and interaction in a bovine mammary epithelial cell culture model.

P29. Targeting the IRF2 Transcription Factor to Inhibit Leukaemic Cell Growth

S. Shen,[1] Passioura,[2] A. Choo,[1] G. Symonds[2] and A. Dolnikov[1], [1]*Children's Cancer Institute Australia for Medical Research, Randwick, NSW, Australia.* [2]*School of Physiology and Pharmacology, University of New South Wales, Randwick, NSW, Australia.*

IRF1 and its functional antagonist IRF2, originally discovered as transcription factors that play a role in the regulation of the interferon-β gene, have been recognized to be involved in the regulation of normal haematopoiesis and leukaemogenesis. The role of these two factors in the control of cell growth has led to the definition of IRF1 as a tumour suppressor gene and of IRF2 as an oncogene. We have shown that IRF1-mediated growth suppression can be induced by the activation of N-Ras signaling in certain human leukaemiac cells lines: K562 and U937, but not in TF-1 cells. In addition, the activation of the cdk inhibitor $p21^{WAF1}$ regulated by IRF1 was triggered in K562 and U937 (class 1 cell lines) but not in TF-1 cells (class 11 cell line). In contrast to other cell lines, TF-1 cells exhibited abnormally high levels of both IRF1 and IRF2 expression, the latter apparently acting to abrogate IRF1 tumour suppression in these cells. A high-throughput cDNA library screen in one class 1 cell line (U937) identified IRF2 as specifically involved in the abrogation of N-Ras-induced growth suppression in class 1 cells. We have shown that IRF2 acts to repress IRF1 function through the repression of cdk inhibitor $p21^{WAF}$ critical for cell growth control. These results indicate that the balance between IRF1 and IRF2 expression is a major determining factor in leukaemic cell growth control. We have hypothesized that IRF2 suppression may be a specific and effective means to inhibit leukaemic cell growth.

P30. An *In Vivo* Assay for Retrovirally Transduced Human Haematopoietic Stem/Progenitor Cells (HSC/HPC) using Nonobese Diabetic/Severe Combined Immunodeficiency (NOD/SCID) Mice

S. Shen, G. Symonds and A. Dolnikov, *Children's Cancer Institute Australia for Medical Research, Randwick, NSW, Australia.*

A unique retroviral transduction model to enforce expression of exogenous genes in human HSC/HPC has been established. A gene transfer protocol designed specifically for the HSC/HPC has permitted testing exogenous gene expression in this compartment. Various genes such as marker genes (*GFP*), oncogenes ($N\text{-}ras^m$ and *v-myc*) and therapeutic genes (retrovirally delivered dsRNA) are tested. Using this model we have shown that retrovirally transduced human HSC/HPC efficiently engraft and repopulate bone marrow of the sub-lethally irradiated host mice. Of the order of 12% chimerism was observed for the *GFP* and $N\text{-}ras^m$ transgenes. These retrovirally transduced cells efficiently reconstituted both lymphoid and myeloid lineages. Activation of ras signaling significantly increased engraftment of human CD45+ cells through the activation of an autocrine/paracrine mechanism. Increased expression of SDF-1, VEGF, metalloproteinases and VLA5, known to increase human CD45+ cell engraftment, was induced by N-ras. In addition, the new glycogen synthase kinase 3(GSK) inhibitor 6-bromoindirubin-3′-oxime (BIO), recently shown to activate the Wnt pathway in embryonic stem cells, has been tested. We have shown that BIO activates the Wnt signaling and induces nuclear accumulation of ß-catenin in human HPC. In addition, BIO-treated human HPC showed increased *ex vivo* cell viability and cell cycling. BIO appears to represent a new promising candidate compound that can potentially be used to expand human HSC/HPC *ex vivo* for stem/progenitor cell gene therapy.

P31. *In vivo* Airway Conditioning for Improved Gene Delivery: Mucosubstance Release and Lentiviral Gene Transfer

P. Cmielewski,[1] D. W. Parsons,[1,3] A. Stocker[1,4] and D. S. Anson[2,3], [1]*Dept of Pulmonary Medicine,* [2]*Genetic Medicine; Children, Youth and Women's Health Service, North Adelaide;* [3]*Dept of Paediatrics,* [4]*Dept of Physiology; University of Adelaide, Adelaide, South Australia.*

To establish an effective gene therapy for cystic fibrosis lung disease, we have used airway conditioning with lysophosphatidylcholine (LPC) with lentiviral vectors to produce long-lasting gene expression in intact nasal airways of normal and cystic fibrosis mice. We have examined the relationship between LPC dose on mucosubstance release and lentiviral-mediated gene transfer in nasal respiratory epithelium.

Methods: A single dose of LPC (0.03–2% w/v) was instilled as a 4 µl bolus into one nostril of C57Bl/6 mice. One hour later mice were killed and airway sections were examined for injury and mucosubstance presence. In other studies lentivirus (LVLacZ) vector (20 µl bolus, 6×10^8 tu/ml) was instilled 1 h after LPC delivery to examine the dose-dependence of LPC on gene expression analysed at 7 days post-treatment.

Results: Increasing concentrations of LPC resulted in a dose-dependent release of mucosubstances from goblet cells anteriorly, but not towards the nasopharynx. At the highest doses (1%, 2%) areas of exfoliation were also noted anteriorly. Compared to control (PBS), LPC at 0.1–2% displayed significant increase in mucosubstance release (ANOVA, $p < 0.05$, $n = 8$). Doses of LPC at 0.1–2% also significantly increased the efficiency of gene transfer with the LVLacZ vector (ANOVA, $p < 0.05$, $n = 8$). Gene transfer was localised to respiratory and transitional epithelium on the treated side of the nose and was similar anteriorly and in the nasopharynx.

Conclusions: The release of luminal mucosubstances does not appear to be the primary factor influencing the efficiency of gene transfer.

P32. The Optimisation of Methods for The Purification and Concentration of Human Immunodeficiency Virus Type-1 Derived Gene Transfer Vectors

Rachel Koldej[1,2] and Donald S. Anson[1,2,3,4], [1]*Gene Technology Unit, Department of Genetic Medicine, Children, Youth and Women's Health Service, Adelaide, South Australia.* [2]*Department of Paediatrics, University of Adelaide, Adelaide, South Australia.* [3]*Department of Biotechnology, Flinders University, Adelaide, South Australia.* [4]*School of Pharmacy & Medical Sciences, University of South Australia, Adelaide, South Australia.*

Human immunodeficiency type-1 derived gene transfer vectors are proving to be extremely promising tools for gene therapy. However, their relatively low titres mean that they require substantial concentration before use. In addition, the final preparation needs to be as

pure as possible. Therefore, effective methods to remove contaminants need to be developed. To facilitate processing, virus was collected in serum-free medium then clarified by low-speed centrifugation and 0.45 μm filtration. As an initial purification step, the virus was concentrated using a 750 kDa Mw cut-off hollow fibre system. Ultrafiltration allowed the clarified cell culture supernatant to be concentrated 20-fold in 2–3 h with virus recoveries of approximately 50% and a >10-fold reduction in total protein.

Ultracentrifugation was then used to pellet the virus allowing it to be resuspended in a small volume (approximately 1000th of the starting volume) of the buffer of choice. Recovery of virus was again about 50% and total protein was reduced a further 10-fold.

The combination of ultrafiltration and ultracentrifugation allowed us to develop a method that results in the rapid concentration and purification of large volumes of virus supernatant with overall recoveries of infectious virus of >25% and a 50-fold increase in the number of infectious units/mg of total protein. The approach is rapid, having an overall processing time from collection of viral supernatant to resuspension of the final virus preparation of <8 h. However, SDS-PAGE analysis suggests the material is still somewhat impure.

We are currently optimising different parameters in the ultrafiltration and ultracentrifugation steps with the aim of raising the overall recovery to closer to 50%. In addition, we are also exploring the option of using an anion-exchange chromatography step to increase the purity of the final virus preparation.

P33. A Cre Recombinase System for Exchange of Therapeutic Genes on Human Artificial Chromosomes

Margaret Shaw, Richard Saffery and K. H. Andy Choo[1,2], [1]*Chromosome Research, Murdoch Children's Research Institute, Royal Children's Hospital, Flemington Road, Parkville, Australia.* [2]*Department of Paediatrics, University of Melbourne, Parkville, Australia.*

Human artifical chromosomes (HACs) provide an attractive alternative to viral vectors for gene therapy due to their autonomous replication and human origin. We have developed a number of small, mitotically stable HACs by truncation of a neocentromere-based chromosome 10 derivative. In order for HACs to be a useful therapeutic tool for a range of genetic disorders, there needs to be an easy method for exchange of different therapeutic genes carried on the HAC.

We have developed an efficient system for gene exchange using the site-specific Cre recombinase. By co-transfection of a plasmid containing the lox-flanked gene with in vitro transcribed Cre mRNA, clones with the desired gene targeted to the HAC can be generated at relatively high frequency (~4% of antibiotic-resistant clones).

P34. Analysis of Chimeric Mice Containing Human Neocentromere-Based Chromosomes

Lee H. Wong, Richard Saffery, Julie Quach, Melissa Anderson, Elizabeth Earle, Kerry Fowler and K. H. Andy Choo, *Murdoch Children's Research Institute, Royal Children's Hospital, Flemington Road, Parkville, Victoria 3052, Australia.*

Human neocentromeres are functional centromeres that are devoid of the typical human centromeric α-satellite DNA. We have transferred a 60-Mb chromosome 10-derived neocentric marker chromosome, mardel(10), and its truncated 3.5-Mb derivative, NC-MiC1, into mouse embryonic stem (ES) cells and have demonstrated a relatively high structural and mitotic stability of the transchromosomes in a heterologous genetic background. We have also produced chimeric mice carrying mardel(10) or NC-MiC1. Both transchromosomes were detected as intact episomal entities in a variety of adult chimeric mouse tissues, including haemopoietic stem cells. Genes residing on these transchromosomes were expressed in the different tissues tested. Meiotic transmission of both transchromosomes in the chimeric mice was evident from the detection of DNA from these chromosomes in sperm samples. In particular, germline transmission of NC-MiC1 was demonstrated in the F1 embryos of the chimeric mice. Variable [low in mardel(10)- or NC-MiC1-containing ES cells and chimeric mouse tissues and relatively high in NC-MiC1-containing F1 embryos] levels of missegregation of these transchromosomes were detected, suggesting that they are not optimally predisposed to full mitotic regulation in the mouse background, in particular during early embryogenesis. These results provide promising data in support of the potential use of neocentromere-based human marker chromosomes and minichromosomes as a tool for the study of centromere, neocentromere, and chromosome biology, and for gene therapy studies in a mouse model system. They also highlight the need to further understand and overcome the factors that are responsible for the definable rates of instability of these transchromosomes in a mouse model.

P35. A Comprehensive Mutation Screening Strategy of the Dystrophin Gene of DMD Patients Preceding Antisense Oligoribonucleotide Clinical Trials

M. Gibbs, S. Bouwer, C. Johnson, S. Fletcher and S. Wilton, *EMML, LNND, QEII Medical Centre, Perth, Western Australia.*

Duchenne muscular dystrophy (DMD), a fatal X-linked recessive disorder, is caused by mutations in the dystrophin gene. Identifying the exact genomic boundaries of the causative mutations in the dystrophin gene is a prerequisite to a potential customized antisense oligonucleotide (AO) therapy for these patients. Induced exon skipping using AOs to redirect processing of the pre-mRNA aims to restore the reading frame to encode a shorter, but functional protein. This approach aims to mimic the type of mutations found in Becker muscular dystrophy, a milder allelic disorder caused by in-frame mutations in the dystrophin gene. We present a high-resolution mutation screening method that will detect all major genomic deletions across all 79 exons of the dystrophin gene. Multiplex PCR encompassing all exons will be utilized as the first line of screening to allow the detection of regions showing duplication and deletion, comprising approximately 70% of disease-causing mutations in DMD. The regions flanking the mutation will be sequenced to detect any polymorphic sequences which could hinder AO efficiency. For the remaining patients with single nucleotide changes a full sequence analysis would be carried out on cDNA products representing the coding region. Fine mutation mapping will facilitate genetic counseling for other at-risk family members.

P36. Induction of Revertant Fibres in the *mdx* Mouse using Antisense Oligonucleotides

A. M. Fall, K. Honeyman, S. J. Errington, S. Fletcher and S. D. Wilton, *Experimental Molecular Medicine Group, Centre for Neuromuscular and Neurological Disorders, University of Western Australia, QEII Medical Centre, Nedlands, WA 6009.*

Duchenne muscular dystrophy (DMD) is a fatal genetic disorder caused by dystrophin mutations that preclude synthesis of a functional protein. The *mdx* mouse model has a nonsense mutation in exon 23. Removal of this single defective exon induces an in-frame mRNA transcript that encodes a shortened but still functional dystrophin protein.
Despite the primary dystrophin gene lesion, at least 50% of DMD patients, *mdx* mice and a canine model of DMD have rare dystrophin-positive or 'revertant fibres' which arise from some naturally occurring exon-skipping event. Immunostaining studies have shown that the majority of revertant fibres miss multiple exons flanking the DMD mutation. These revertant fibres could provide a template for more functional dystrophin design, rather than skipping of single exons.
We aim to emulate these naturally occurring revertant fibres using either bi-functional or combination antisense oligonucleotides (AO) to induce multiple exon skipping. We have developed AO cocktails that consistently induce removal of exons 19–25 and 21–25, two revertant transcripts that have been detected in untreated dystrophic mouse muscle. We are investigating whether these 'induced revertant' transcripts generate a more functional dystrophin protein than the minimal exon 23 skip to by-pass the nonsense mutation.

P37. Antisense Oligonucleotides and Exon Skipping: Does Size Matter?

Penelope L. Harding, Susan Fletcher and Stephen D. Wilton, *Experimental Molecular Medicine Group, Centre for Neuromuscular and Neurological Disorders, University of Western Australia, Nedlands, Perth 6009, Australia.*

Duchenne muscular dystrophy (DMD) is an X-linked genetic disorder that arises from mutations in the dystrophin gene. The disease is characterised by severe and progressive wasting of skeletal and cardiac muscles.
The *mdx* mouse model of muscular dystrophy has a nonsense mutation in exon 23 of the dystrophin gene. Our group has previously shown that both 2′-O-methyl antisense oligonucleotides (AOs) and morpholino AOs can induce skipping of exon 23 in H2k-*mdx* cells. These AOs redirect splicing by masking the normal donor splice site to induce exon 23 skipping, causing an in-frame transcript to be produced which can then be translated into a shorter but functional dystrophin protein.
We have compared 2′-O-methyl AOs with morpholino AOs, to determine which chemistry is the most efficient at inducing exon skipping and presumably which chemistry should be used in human clinical trials. Two different sequences targeting the intron 23 donor splice site, each prepared as 2′-O-methyl or morpholino AOs, were evaluated to determine if the length of the AO influences efficiency of exon skipping. Both morpholino and 2′-O-methyl AOs were used to transfect H2k-*mdx* cells across a range of concentrations. Persistence of corrected in-frame transcript was also used as a measure to determine the most efficient AO for inducing exon skipping.
We found the longer AO for each chemistry was more efficient than the corresponding shorter compound. The morpholino AOs, when delivered as a leash-lipoplex, were more efficient at inducing sustained exon skipping than the corresponding sequences prepared as 2′-O-methyl AOs *in vitro*. Both chemistries are now being evaluated *in vivo* for safety and efficacy.

P38. Redirecting Splicing to Restore Dystrophin Expression in a Canine Model of Muscular Dystrophy

G. McClorey, S. Fletcher and S. Wilton, *Experimental and Molecular Medicine Group, Centre for Neurological and Neuromuscular Disorders, University of Western Australia, Nedlands, WA 6009.*

Duchenne muscular dystrophy (DMD) is an X-linked fatal muscle-wasting disease. It manifests itself through mutations in the dystrophin gene that cause a non-functional protein to be produced. A canine model of this disease, the Golden Retriever model of muscular dystrophy (GRMD), has been used to examine the use of antisense oligonucleotides (AOs) to alter splicing of the dystrophin pre-mRNA to restore protein synthesis.
The GRMD animal has a splicing mutation in intron 6 of the dystrophin gene that causes exon 7 to be excluded from the mRNA, resulting in a frame-shift that produces a truncated protein. Low dose AOs were directed to block splice site recognition sequences and splicing enhancer elements of the surrounding exons 6 and 8. Subsequent removal of these exons resulted in the restoration of the GRMD mRNA reading frame that should allow translation of a near full-length protein. One of the major challenges that have arisen though has been to induce sufficient correction at the RNA level that detectable levels of dystrophin protein are produced. Preliminary *in vivo* experiments indicate that improvement in design and/or delivery will be necessary for this approach to be successful in the GRMD model.
Ultimately it is hoped that this AO-mediated exon skipping approach will be used to treat DMD patients by producing a semi-functional protein similar to that observed in the milder Becker muscular dystrophy.

P39. *In vitro* Modification of Tissue-Specific Alternative Splicing of the Mouse TPM3 Gene with Antisense Oligonucleotides

Clint Johnson, Steve Wilton and Sue Fletcher, *Experimental Molecular Medicine Group, Centre for Neuromuscular and Neurological Disorders, QEII Medical Centre, Perth, Western Australia.*

Alternative splicing is one of the principle mechanisms to increase protein diversity in higher eukaryotes. From a single pre-mRNA, multiple mRNAs can be generated by selective exon inclusion, exclusion or, in some instances, exon extension or shortening. This mechanism dramatically increases the coding capacity of a single gene. Numerous genes undergo tissue-specific and/or developmentally-regulated splicing, an example of which is the tropomyosin 3 (TPM3) gene. Antisense oligonucleotides (AOs) were applied to cultured cells to investigate modifying tissue-specific splicing of the TPM3 gene transcript. It was found that AOs targeting mutually exclusive exons 6a or 6b were able to partially redirect splicing from one tissue-specific exon to the other. AOs annealing to exonic sequences showed the highest efficacy which suggests that these sequences contain exonic splicing enhancer (ESEs) elements controlling the

splicing process. With both single and multiple transfections (blocking both exons 6a and 6b) exclusion of both exons from the same transcript could not be induced, suggesting some mechanism to ensure inclusion of either mutually exclusive exon. Conversely, a single AO blocking constitutively spliced exon 7 was able to induce exclusion of this exon. These results are contrary to the general model of exon selection, where constitutive spliced exons are strongly selected for by the splicing machinery, whereas alternatively spliced exons are supposedly weakly selected. AO-induced modification of tissue-specific splicing patterns indicates that this approach could potentially to be used therapeutically to alter isoform expression in a variety of conditions.

P40. Adult Muscle Derived Stem Cells Contribute to Myo-Regenerative Capacity in the *mdx* Mouse

Magdalena Kita,[1] Kym Lowes,[1] Anita Quigley,[1] Andrew J. Kornberg,[1,2] and Robert Kapsa[1], [1]*National Muscular Dystrophy Research Centre, St Vincent's Hospital, Melbourne, Australia.* [2]*Department of Neurology, Royal Children's Hospital, Melbourne, Australia.*

Duchenne muscular dystrophy (DMD) is a degenerative muscle disorder, which results from a lack of dystrophin protein. This lack of functional protein is due to a number of mutations in the dystrophin gene. Because of this genotype, therapy may potentially be achieved through the use of targeted corrective gene conversion (TCGC) on autologous cells *ex vivo*, followed by transplantation of the corrected cells into the affected tissue. In order to succeed, this activity requires the use of cells with high level capacity for remodelling muscle. Myoblast transfer therapy in humans has so far shown limited success, thus the utilisation of stem cells emerged as an attractive alternative.

We have used the *Rosa(26)* mouse to expand and characterize a population of muscle-derived cells, which show potential in facilitating regeneration of dystrophic muscle. By culturing these cells in suspension using defined, serum-free media conditions, we have enriched a population of cells that co-express stem cell (Sca-1, CD34, Oct-4), muscle satellite cell (pax-7, m-cadherin) myogenic (desmin, MyoD) and neurogenic (nestin, β-III-tubulin) factors. These cells can be propagated in culture for an extended period of time while retaining this expression pattern, which may characterise cells with wider applicability to dystrophic muscle remodelling than just replacement of degenerated muscle fibres.

We are currently investigating this aspect using *in vivo* transplantation into *mdx* (a model of DMD) mouse muscle. At 1 week post-transplant into a myotoxin damaged muscle, regions of dystrophin-positive immature myofibres were evident, while, at 5 weeks post-transplant, mature fibres were localised in proximity to myotoxin damaged areas.

These results suggest that we have isolated a population of cells from mouse muscle with *in vitro* characteristics of multipotent stem cells. These may represent a pool of adult stem cells residing in muscle, which under the dystrophic regenerative stimulus will re-establish functional, non-dystrophic muscle following cell transplantation.

P41. Adipose and Skin-Derived Mesenchymal Stem Cells as Potential Vehicles for Gene Delivery

Kym Lowes,[1] Magdalena Kita,[1] Anita Quigley,[1] Mark Cook,[2] Andrew Kornberg[3] and Robert Kapsa[1], [1]*National Muscular Dystrophy Research Centre, Howard Florey Institute, Parkville 3052, Melbourne, Australia.* [2]*Centre for Clinical Neurosciences and Neurological Research, St Vincent's Hospital, Fitzroy 3065, Melbourne, Australia.* [3]*Department of Neurology, Royal Children's Hospital, Parkville 3052, Melbourne, Australia.*

Mesenchymal stem cells (MSCs) can be isolated from many tissues, have multilineage differentiation potential, and hence show promise as a source of cells for cell replacement therapeutic strategies. Ideally, cells for tissue engineering would come from a source that is easily accessible and highly renewable. Accordingly, we have isolated populations of putative MSCs from mouse adipose tissue and skin that we have termed mAdSs and mDmSs, respectively. Unlike MSCs that are typically selected for based on their adherence to tissue culture plastic, mAdSs and mDmSs are selected using a highly defined, serum-free growth medium and are propagated as non-adherent cell clusters/spheres. Cell surface marker analysis has revealed that mAdSs and mDmSs are Sca-1$^+$, CD45$^-$, CD34$^-$, CD31$^-$ and c-kit$^-$. Currently, we are investigating the potential of mAdSs and mDmSs to remodel dystrophic muscle in the *mdx* mouse model of Duchenne muscular dystrophy (DMD). The pathological basis of DMD involves mutation of the *dys* gene and subsequent absence of the protein dystrophin, which results in progressive muscle deterioration. Five weeks after transplantation of ROSA 26 mAdSs and mDmSs into preconditioned gastrocnemuis muscle, up to 119 β-galactosidase$^+$/dystrophin$^+$ myofibres were observed although there was wide variation in the success of cell engraftment. This suggests that mAdSs and mDmSs could be used, in conjunction with targeted gene conversion methodologies, to correct the *dys* mutation, for delivery of wild-type *dys* in an autologous manner. We also observed that a large number of engrafted cells that remained in the injection site expressed β-galatosidase 5 weeks post-transplantation. This suggests that although the cells did not contribute to new myofibre formation they were still viable. This ability to survive the engraftment procedure suggests that mAdSs and mDmSs may be ideal vehicles to deliver genes into muscle for therapeutic applications.

P42. Plasmid Based Gene Delivery of the Survival Motor Neuron Gene

Anita Quigley,[1,2] Andrew Kornberg[1,3] and Robert Kapsa[1,2], [1]*National Muscular Dystrophy Research Centre, St. Vincent's Hospital, Fitzroy, Victoria 3065, Australia.* [2]*Howard Florey Institute, Melbourne University, Parkville, Victoria 3052, Australia.* [3]*Neurology, The Royal Children's Hospital, Parkville, Victoria 3052, Australia.*

Spinal muscular atrophy (SMA) is a hereditary motor neuron disease and the leading cause of infant mortality with an incidence of 1/10 000. SMA is caused by homozygous mutations of the survival motor neuron (SMN) gene. In normal individuals this gene exists in duplicate (SMN1 and SMN2) due to a 500-kp inverted repeat on 5q13 in humans; however, a polymorphism at the start of exon 7 in SMN2 promotes the exclusion of exon 7 from the majority of final transcript, resulting in production of SMN

protein with reduced function. SMA patients with mutations or deletions of SMN1 are reliant on intact copies of SMN2 for the production of full-length transcript, resulting in a deficit of fully functional SMN protein. This can be visualized by loss of nuclear GEMS (complexes formed between SMN, Geminins and other proteins).

We are currently developing non-viral-based gene delivery methods for expression of the SMN gene in SMA patient cell lines. Full-length SMN cDNA was cloned into pDEST47 (Invitrogen) and patient cell lines transfected with pDEST47-SMN complexes using Metafectine or Lipofectine plus. Treated cell lines were analyzed by immunohistochemistry for the presence of SMN protein and the increased formation of GEM complexes. An increased number of GEMS was observed in the nucleus and cytoplasm in a number of treated patient cell lines. We are currently analyzing treated cell lines for the restoration of other proteins associated with functional GEMS such as p80-coilin.

P43. Establishing Effective Correction of the Dystrophin Mutation in the *mdx* Mouse Model of Duchenne Muscular Dystrophy

Marian Todaro, Magda Kita, Anita Quigley, Kym Lowes, Rosetta Marotta, Mark Cook, Andrew J. Kornberg and Robert Kapsa, *National Muscular Dystrophy Research Centre and Department of Clinical Neurosciences, St.Vincent's Hospital and The Howard Florey Institute, Melbourne, Australia.*

Targeted correction of mutations in the genome holds great promise for the repair/treatment of disease-causing mutations either on their own applied directly to the affected tissue, or in combination with other techniques such as stem cell transplantation. Various DNA or RNA/DNA based corrective nucleic acid (CNA) molecules such as chimeraplasts, single-stranded oligonucleotides, triplex forming oligonucleotides and SFHR have been used to change specific loci. Significant variation in the success of these techniques from laboratory to laboratory can be attributed to factors such as differences between application protocols, loci and/or target cell/tissue type. However, a number of recent publications have highlighted the potential of PCR-based locus detection methods to generate artifactually over-estimated positive evidence of mutation correction. This potential for artifact universally affects all of the mutation correction methods in use today, and usually arises from the presence of the corrective molecule. It is important to establish however that in all instances, such artifact may be eradicated from results by relatively simple but effective controls/steps to provide accurate information regarding the effectiveness of protocols in promoting mutated locus correction. This study reports the potential sources/conditions by which such artifact may be generated by the SFHR process and presents the steps that prevent these from contaminating genuine data.

P44. Heterogeneity in Clonal Populations of Muscle Derived Stem Cells

R. M. I. Kapsa, M. Kita, A. F. Quigley, K. N. Lowes, M. J. Cook and A. J. Kornberg, *National Muscular Dystrophy Research Centre, Howard Florey Institute and St Vincent's Hospital, Fitzroy, VIC, Australia.*

Duchenne muscular dystrophy (DMD) is a myodegenerative disorder affecting 1 in every 3500 live-born males, in which the functional absence of a dystrophin is caused by frame-shift or nonsense mutations of the 2.5 Mb DMD gene located at Xp21.1. Restoration of functional dystrophin in dystrophic *mdx* mouse muscle (nonsense mutation) has been achieved to varying levels of success by cell transplantation, viral and non-viral gene therapies. Our strategy for dystrophin restoration in dystrophic muscle involves targeted corrective gene conversion (TCGC) in mature, non-dividing muscle fibres, combined with ex vivo TCGC of mutations in cell types that display optimal myoremodelling capacity, followed by transplantation of corrected cells into the dystrophic muscle. This forms the basis of an autologous cell transplantation protocol that may be developed in the *mdx* mouse model for hereditary neuromuscular disorders. One of the issues of autologous transplantation in muscular dystrophy is a depleted pool of myogenic precursor cells in dystrophic muscle. We investigated clonal populations of myogenic stem cells to determine whether single muscle derived stem cells can give rise to populations of muscle precursors capable of reconstituting muscle. Myoblasts were derived from C57BL10 mice and isolated using the pre-plate (pp) method. PP6 cells were extensively passaged before a single cell was isolated and expanded to a clonal population in myogenic media. This clonal population was fractionated based on adherence and size, and grown under specific media conditions. RT-PCR analyses showed the expression of myogenic markers such as myf-5, myf-6, pax-7 and myo-D in all populations similar to the parental culture; however, there was marked variation in the expression of the stem cell marker Sca-1 (0–30%). Similarly, all clonal cell populations were negative for CD45, CD34, CD31 and c-kit by FACS analysis. This study demonstrates that single clones isolated from a population of MDSCs are capable of reconstituting heterogeneous MDSC populations.

P45. Development of an HPFH-Specific Assay for HbF Inducers

Preeyachan Lourthai,[1,2,3] Jim Vadolas,[1] Hady Wardan,[1] Pranee Winichagoon,[2] Jisnuson Svasti,[3] Suthat Fucharoen[2] and Panos Ioannou[1] (jim.vadolas@mcri.edu.au), [1]*CAGT Research Group, Murdoch Children's Research Institute, Royal Children's Hospital, Flemington Road, Melbourne, VIC 3052, Australia.* [2]*The Thalassemia Research Center, Institute of Science and Technology for Research and Development, Mahidol University, Bangkok, Thailand.* [3]*Department of Biochemistry, Mahidol University, Bangkok, Thailand.*

Augmentation of fetal hemoglobin (HbF) synthesis can reduce the severity of β-thalassemia and β-globin chain hemoglobinopathies by improving the imbalance between α- and non-α-globin chains. This is supported by observations that β-thalassaemia patients with hereditary persistence of fetal hemoglobin (HPFH) are transfusion independent. A number of pharmacological agents have also been found to induce HbF through various mechanisms. However, all known HbF inducers have low efficacy and specificity and may result in long-term toxicity.

We have previously described the development of episomal and stable genomic reporter assays (GRA) based on a bacterial artificial chromosome (EBAC) containing the human β-globin locus with an EGFP reporter cassette replacing in-frame the $^{G}\gamma$- and $^{A}\gamma$-globin genes (pEBAC/148β::$\Delta^{G}\gamma^{A}\gamma$ EGFP). Human erythroleukemia K562 cell lines carrying this construct in episomal format or stably integrated have

been shown to respond to a range of known HbF inducers. High-throughput screening of a large chemical library, FDA-approved drugs and traditional medicines is currently underway. Despite the high information content, sensitivity and specificity of this assay, many agents affecting general chromatin structure and other common pathways of gene expression may be expected to have a significant impact on HbF expression.

In this study, we investigate the induction potential of several DNA-binding agents on γ-globin gene expression using the cellular GRA described above. We also describe the introduction of the $-175(T\rightarrow C)$ HPFH mutation into the pEBAC/148β::$\Delta^{G}\gamma^{A}\gamma$ EGFP construct and compare level of EGFP expression from this construct with the parent construct. We anticipate that the availability of this cellular GRA will greatly facilitate the identification and evaluation of agents that specifically mimic the effect of the HPFH mutation on HbF expression.

P46. siRNA-Mediated Knockdown of EGFP Expression in Human Erythroid Cells: Application for β-Thalassaemia

Hsiao Phin Voon, Jim Vadolas, Hady Wardan, Sara Howden, Faten Zaibak, Novita Puspasari and Panos Ioannou, *Cell and Gene Therapy Research Group, The Murdoch Children's Research Institute, Royal Children's Hospital, Flemington Road, Parkville 3052, Melbourne, Australia.*

β-Thalassaemia is a recessively inherited haemoglobinopathy which arises due to mutations in the β-globin genes resulting in reduced β-chain synthesis. Much of the pathology of this disease is caused by excess α-chains forming toxic insoluble precipitates within erythroid progenitor cells resulting in cell death. Decreased α-chain expression and globin chain imbalance leads to milder symptoms, exemplified in individuals who co-inherit α-thalassaemia and β-thalassaemia. Therefore, downregulating α-globin gene expression in β-thalassaemia individuals could potentially improve phenotypic outcomes.

The discovery of RNA interference in mammalian cells in response to small interfering RNA (siRNA) effectors has simplified the process of reducing gene expression and allowed development of novel therapeutic strategies involving gene knockdowns. Numerous investigations have demonstrated promising results utilising siRNA in *in vitro* and *in vivo* models. These have yielded largely positive results and are encouraging towards other studies, such as the reduction of α-globin expression as a therapy for β-thalassaemia.

In this investigation, we have performed preliminary experiments to assess the feasibility of a siRNA-mediated reduction of highly expressed genes, such as α-globin, in erythroid cells. siRNA molecules were introduced into the human erythroleukemic cell line K562 by electroporation and directed against transient, episomal and stably integrated constructs expressing increasing amounts of the enhanced green fluorescent protein (EGFP) reporter. Decreasing knockdown effects with increasing amounts of EGFP expression were noted. To further investigate this phenomenon, increasing amounts of EGFP mRNA (1, 5 and 10 μg) were electroporated with constant amounts of siRNA oligonucleotides (5 μg). A dose relationship was confirmed, with increased target mRNA leading to lower knockdown effects.

Our results show that higher doses of siRNA molecules are required to knockdown highly expressed genes. Nonetheless, we were able to demonstrate siRNA-mediated knockdown of highly expressed EGFP in a stable integration system. These initial results indicate siRNA-mediated knockdown of α-globin in erythroid cells is theoretically possible and justifies further investigation.

P47. Chromatin-Binding Domains within the Amino-Terminus of Epstein-Barr Nuclear Antigen-1 Mediate the Nuclear Uptake of Epstein-Barr Virus-Based Vectors

Sara Howden, Jim Vadolas, Hady Wardan and Panos Ioannou (s.howden@pgrad.unimelb.edu.au), *Murdoch Children's Research Institute, Royal Children's Hospital, Melbourne, VIC 3052, Australia.*

Epstein-Barr virus (EBV)-based vectors permit the transfer and long-term expression of therapeutic transgenes in human cells, providing a potential vehicle for the treatment or correction of many inherited and acquired diseases. EBV-based vectors encode the Epstein-Barr nuclear antigen-1 (EBNA-1) protein and carry the origin of replication (*ori*P) derived from human EBV, which act jointly to effectively replicate and retain the plasmid in the nucleus of dividing cells. We and others have reported dramatic increases in the nuclear uptake and subsequent expression of EBV-based vectors following their nonviral delivery into human cell lines that constitutively express EBNA-1. The strong binding affinity between EBNA-1 and *ori*P, as well as the presence of a nuclear localisation signal (NLS) in the central region of the EBNA-1 protein, have previously been implicated in the nuclear transport of EBV plasmids. However, the molecular mechanism by which this process occurs has never been directly observed. Although our experiments confirm that EBNA-1 can only facilitate the nuclear uptake of plasmids that carry *ori*P sequences, we also demonstrate that a derivative of EBNA-1 comprising just the NLS and DNA-binding domain (DBD) of the protein has no effect on the transfection of *ori*P plasmids. Moreover, we show that a version of EBNA-1 devoid of a functional NLS, and therefore unable to be actively transported into the nucleus, enhances the transfection of a 21 kb EBV-based plasmid almost 10-fold. A similar increase in transfection efficiency was observed following the delivery of plasmid DNA in cells expressing wildtype EBNA-1. We propose that regions within the amino terminus of EBNA-1 which are known to bind cellular chromosomes, and not the NLS, mediate the nuclear uptake of EBV plasmids upon their entry into cells. Further experiments involving the construction and expression of a fusion protein comprising the chromatin-binding domain and DBD of EBNA-1 are being conducted to confirm this hypothesis.

P48. Targeted Gene Correction of a Mouse Model of β^0-Thalassemia

Faten Zaibak, Duangporn Jamsai, Jim Vadolas, Hady Wardan and Panayiotis A Ioannou (faten.zaibak@mcri.edu.au), *The Murdoch Children's Research Institute, Royal Children's Hospital, Flemington Road Parkville, Melbourne, Victoria 3052, Australia.*

Ex vivo gene correction of hematopoietic stem cells (HSC) is a promising option for the treatment of hematological disorders. It enables the patient's own HSC to be corrected and then retransplanted. Here we describe the development of a mouse model for the evaluation of targeted gene correction strategies in β-thalassaemia. This model was then

used to determine the repair efficiency of single-stranded oligonucleotides (ssODN) at the β-globin locus.
A humanized mouse model of β^0-thalassemia was created by microinjection of a bacterial artificial chromosome carrying the entire human globin locus with a common β-thalassemia deletion in codons 41–42 (-TTCT) of the β-globin gene. This mutation causes a frame shift, abolishing human β-globin expression. Transgenic mice with this deletion were crossed with β-globin knockout mice to produce mice carrying the transgene on a heterozygous knockout background (DH). DH mice exhibit a β-thalassaemia intermedia phenotype, while transgenic mice on a homozygous knockout background are embryonic lethal. As DH mice have enhanced extramedullary erythropoiesis, fetal liver, spleen and adult bone marrow cells are rich sources of HSC for gene correction studies.
BM cells from these mice were electroporated with sense or antisense ssODN (70mers). Forty-eight hours after delivery, genomic DNA was extracted and the repair efficiency determined by allele-specific PCR. Samples were treated with S1 nuclease to reduce the risk of PCR artefacts. Gene correction efficiency with ssODN was below the level of detection (<0.1%).
We conclude that the 4 bp mouse model is an excellent model to test gene correction strategies and that the endogenous level of correction of BM cells at the β-globin locus is low and must be improved to become a feasible gene therapy option.

P49. Eliminating PCR Artefact in Gene Correction Protocols

Faten Zaibak and Panayiotis A Ioannou (faten.zaibak@mcri.edu.au), *The Murdoch Children's Research Institute, Royal Children's Hospital, Flemington Road, Parkville, Melbourne, Victoria 3052, Australia.*

Targeted gene correction with oligodeoxynucleotides (ODN) has many advantages over other gene therapy strategies. Firstly, it can directly correct a disease-causing mutation, thus preserving the genes regulatory and splicing machinery. It can be used to treat both recessive and dominant mutations. In addition, gene correction is permanent and only requires a single treatment, unlike antisense and RNAi approaches. However, gene correction with ODN is poor, thus detection relies on PCR-based strategies that are subject to artefact due to carryover of the ODN into the PCR reaction. Here we describe an optimised protocol for detecting gene correction using S1 nuclease.
To minimise the possibility of ODN carryover into the PCR reaction, genomic DNA from ODN-treated cells was digested with S1 nuclease (a ssDNA exonuclease). To verify that the S1 nuclease was completely removed and did not inhibit the PCR, by degrading the primers, the mouse APRT gene was used as a positive control. In all cases, the PCR yield did not vary significantly between treated and untreated samples. The ability of S1 nuclease treatment to eliminate PCR artefact was then established by spiking the PCR reaction with ODN at various concentrations. As little as 100 pg of ODN resulted in a band and S1 nuclease treatment significantly removed this PCR artefact.
The S1 protocol was then tested in gene correction experiments. A 70 nt ODN was electroporated into HSC carrying a 4 bp deletion (codons 41–42) in the β-globin locus. Forty-eight hours after electroporation, genomic DNA was extracted and S1 nuclease treated. In the absence of S1 nuclease treatment gene correction efficiency was >1%. However, when samples were digested with S1 nuclease, <0.1% correction was observed. This suggests that, in the absence of S1 nuclease treatment, the initially high correction efficiency was due to the ODN serving as a primer and template during the PCR amplification. Thus, S1 nuclease treatment is essential to eliminate PCR artefacts.

P50. High Throughput Screening using a Genomic Reporter Assay for Friedreich Ataxia Therapy

Lingli Li, Joseph P. Sarsero, Sanne van den Hengel and Panos A. Ioannou (joe.sarsero@mcri.edu.au), *Cell and Gene Therapy Research Group, Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, Victoria 3052, Australia.*

Friedreich ataxia (FA) is characterised by neurodegeneration and cardiomyopathy induced by a GAA trinucleotide expansion in the first intron of the *FRDA* gene that causes reduced synthesis of frataxin. As the coding sequence is unaltered and there is a correlation between expansion length, the amount of residual frataxin produced and the severity of disease, pharmacological upregulation of *FRDA* expression may restore frataxin to therapeutic levels in patients. To facilitate screening of compounds that modulate *FRDA* expression in a physiologically relevant manner, we have established a genomic reporter assay (GRA) consisting of stable human cell lines containing an *FRDA-EGFP* fusion construct. Following exposure to test compounds, EGFP levels are measured by flow cytometry. The highest level of induction has been observed with the anti-cancer drug cisplatin, which elicits an almost 2.5-fold increase in *FRDA* expression. In contrast, antioxidants have shown a significant, time-dependent decrease in *FRDA-EGFP* expression.
We have also optimised the assay for use in a high-throughput screening format by measuring EGFP levels fluorometrically. Cell seeding and growth conditions have been defined for a 96-well format. Due to the low level of expression of the *FRDA-EGFP* genomic reporter, the use of optical grade plates is essential to maximise the signal-to-noise ratio. Correction for variations in cell number following exposure to compounds is achieved by performing a fluorescent cell viability assay. The assay was evaluated using cisplatin as a control test compound. EGFP levels and cell viability were measured following exposure to the drug for 72 h. A 1.6-fold level of induction by cisplatin was consistently obtained. The reproducibility of the assay was assessed by whole plate analysis, which demonstrated that well-to-well variation was not significant. Any compound that increases *FRDA* expression by more than 2.5-3-fold should be detectable in this assay. An increase in frataxin levels by several-fold in FA patients could be therapeutic.

P51. *FRDA-EGFP* Genomic Reporter Transgenic Mice

Joseph P. Sarsero,[1] Timothy P. Holloway,[1] Lingli Li,[1] Samuel McLenachan,[1] Kerry J. Fowler,[2] Ivan Bertoncello,[3] Lucille Voullaire,[1] Sophie Gazeas,[2] and Panos A. Ioannou[1] (joe.sarsero@mcri.edu.au), [1]*Cell and Gene Therapy Research Group,* [2]*Disease Model Unit, Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, Victoria 3052, Australia.* [3]*Stem Cell Research Laboratory, Peter MacCallum Cancer Centre, East Melbourne, Victoria 3002, Australia.*

Friedreich ataxia is an autosomal, recessive disorder caused by a GAA trinucleotide expansion in the first intron of the

Friedreich ataxia gene (*FRDA*) that causes reduced synthesis of frataxin, a mitochondrial protein likely to be involved in biosynthesis of iron-sulfur clusters. Frataxin insufficiency leads to increased oxidative stress, progressive loss of large sensory neurons and hypertrophic cardiomyopathy. To elucidate the mechanisms regulating *FRDA* expression and to develop an *in vivo* assay for agents that might upregulate *FRDA* expression in a therapeutically relevant manner we have generated transgenic mice with a BAC genomic reporter construct consisting of an in-frame fusion between the *FRDA* and *EGFP* genes. Production of full-length frataxin-EGFP fusion protein was demonstrated by immunoblotting. EGFP expression was observed as early as day E3.5 of development. Most tissues of adult transgenic mice were fluorescent. The level of *FRDA-EGFP* expression in peripheral blood, bone marrow and in cells obtained from enzymatically disaggregated tissues was quantitated by flow cytometry. There was a two-fold increase in EGFP expression in mice homozygous for the transgene when compared to hemizygous mice. *FRDA-EGFP* transgenic mice were mated with mice heterozygous for a knockout mutation of the murine *Frda* gene, to generate mice homozygous for the *Frda* knockout mutation and hemizygous or homozygous for the human transgene. The *FRDA-EGFP* transgene was able to rescue the embryonic lethality of the homozygous *Frda* KO mice with no signs of any abnormality as determined by behavioural and histological tests. The *FRDA-EGFP* transgene thus appears to produce a fully active bifunctional hybrid protein. These transgenic mice are a valuable tool for the examination of spatial and temporal aspects of *FRDA* gene expression, and for the preclinical evaluation of pharmacological inducers of *FRDA* expression in a whole animal model. In addition, tissues from these mice should also be valuable for stem cell transplantation studies.

P52. Gene Regulation Studies of the Friedreich Ataxia Locus using Genomic Reporter Assays

Novita Puspasari, Joseph P. Sarsero and Panos A. Ioannou (joe.sarsero@mcri.edu.au), *Cell and Gene Therapy Research Group, Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, Victoria 3052, Australia.*

The neurodegeneration and cardiomyopathy in Friedreich ataxia (FA) is caused by a trinucleotide repeat expansion in the first intron of the *FRDA* gene resulting in insufficiency of frataxin protein. As the coding sequence is unaltered, pharmacological upregulation of *FRDA* gene expression may restore cellular frataxin to therapeutic levels in patients. Understanding the mechanisms regulating *FRDA* expression should enable a rational approach for the pharmacological restoration of gene expression and the therapy of FA. There is currently no information about the position of any long-range, *cis*-acting regulatory sequences that control *FRDA* expression. We have utilised data from the sequence assemblies of the human and other mammalian genomes to perform cross-species comparative genomics analysis and have identified a number of conserved, non-coding regions upstream of the *FRDA* gene. The role of these sequences will be evaluated by their deletion in the context of an *FRDA-EGFP* genomic reporter construct. Genomic reporters, in which a reporter fusion is made to a gene in the context of its entire genomic locus on a BAC vector, preserve the normal location and spacing of many long-range regulatory elements and facilitate the recapitulation of normal gene expression patterns. To facilitate functional studies with the genomic reporter in human cells we have developed a dual-reporter enhanced BAC (EBAC) vector. The EBAC construct contains eukaryotic selection markers and is capable of episomal maintenance. It harbours both an *FRDA-EGFP* genomic reporter and the gene encoding the DsRed-Express fluorescent protein. The EGFP/DsRed-Express ratio will provide a sensitive and specific assay for detecting the effects of deletions of regulatory regions of the *FRDA* gene while concurrently allowing correction for differing transfection efficiencies. A combination of bioinformatic analysis to identify conserved non-coding regions of DNA, in conjunction with their experimental targeted deletion in the context of a genomic reporter, can be used to identify regulatory elements in any gene of interest.

P53. Development of Genomic Reporter Assays and Upregulation of Gene Expression as a Potential Therapeutic Strategy for Methylmalonic Aciduria

Mahmoud S. Khaniani,[1,3] Heidi Peters,[1] Jean-Francois Benoist,[2] Leonie Wood,[1] Lingli Li,[1] Hady Wardan,[1] Jim Vadolas[1] and Panos A. Ioannou[1,3] (heidi.peters@mcri.edu.au), [1]*Cell and Gene Therapy Research Group, Murdoch Children's Research Institute, Royal Children's Hospital, Victoria 3052, Australia;* [2]*Service de Biochimie-Hormonologie, Hôpital Robert Debré, 75019 Paris, France;* [3]*Department of Paediatrics, University of Melbourne, Australia.*

Methylmalonic aciduria (MMA) is an autosomal recessive disorder resulting from a functional deficiency of methylmalonyl-CoA mutase (MCM) enzyme. A significant number of mutations leading to MMA as well as other genetic diseases do not abolish completely normal gene expression. MMA patients with low level of residual activity of the normal MCM enzyme are referred to as *MUT*$^{-}$. It is postulated that increasing expression of the MCM enzyme gene in this group could potentially be therapeutic for such individuals. Lower levels of induction may still be beneficial clinically in decreasing disease progression and severity.

We have developed a sensitive genomic reporter to facilitate the development of a physiologically relevant assay for potential inducers of MCM expression. Using homologous recombination an *EGFP* (enhanced green fluorescent protein) cassette was introduced at the last codon of exon 13 of the *MUT* gene in a 172 kb fully sequenced BAC clone containing the intact *MUT* locus and extensive flanking sequences. The linearised 172 kb genomic fragment from the modified clone was transfected into HeLa cells by using Lipofectamine 2000 and single positive clones were isolated. Based on the FACS data, single clones were identified which showed a single uniform peak of *EGFP*, but different levels of median peak fluorescence. One of the cell lines with a robust level of basal EGFP expression was selected for screening of various compounds. Cisplatin and propionic acid induced two-fold and three-fold increases in *MUT* expression, respectively, while a number of other compounds, including gamma-hydroxybutyric acid and butyric acid, had little effect on *MUT* gene expression.

These stable cell lines provide a robust assay to facilitate high-throughput screening for pharmacological compounds that could modulate the expression of the *MUT* gene in a clinically relevant manner.

P54. Gentamicin-Induced Readthrough of a Premature Stop Codon in a 'Humanised' Mouse Model of Methylmalonic Aciduria (MMA)

Leonie R. Wood, Heidi Peters, Natasha J. Hamilton and Panos A. Ioannou (heidi.peters@mcri.edu.au), *Cell and Gene Therapy Research Group, Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, Victoria 3052, Australia.*

Methylmalonic aciduria (MMA) is a human autosomal recessive disorder of organic acid metabolism. It results from a functional defect in the activity of methylmalonyl-CoA mutase (MCM). Several mutations have been identified in the MUT locus of patients with the disease, including some stop codon mutations. A knockout mouse model of MMA has been previously generated with homozygous MCM knockout mice exhibiting neonatal lethality, with disease characteristics similar to the human condition. In order to generate a 'humanised' mouse model of MMA, a stop mutation involving a single base change in exon 6, identified from an MMA patient, was introduced using two-step homologous recombination onto a BAC vector containing the entire human MUT locus. Transgenic mice have been produced using this modified BAC. Breeding of transgenic mice with heterozygous MCM knockout mice was subsequently used to produce 'humanised' mice containing the modified human mutase gene on a homozygous knockout background. These mice exhibit the key phenotypic and biochemical features of MMA.

Aminoglycoside antibiotics, such as gentamicin, function by specific binding of rRNA, disturbing codon-anticodon recognition and causing translational readthrough of stop codons. Primary fibroblast cell lines have been established from the 'humanised' mouse model of MMA in order to investigate the potential of gentamicin as a treatment for MMA. These cells were cultured in the presence of 0–800 μg/mL gentamicin over a period of 4 days. RNA extracted from the cells was analysed for human MCM mRNA expression by real time RT-PCR. Results show a 1.5- to 2-fold increase in human MCM gene expression in two separate fibroblast cell lines over this concentration range of gentamicin. In addition, [^{14}C]-labelled propionate incorporation was measured in these cells, in order to indirectly assess MCM activity. Preliminary results indicate an increase in propionate incorporation with gentamicin treatment.