# 2ND MEETING OF
# THE AUSTRALASIAN GENE THERAPY SOCIETY

**April 27 to April 29, 2001**

**The Centenary Institute of Cancer Medicine and Cell Biology,**
**Royal Prince Alfred Hospital, Sydney, Australia**

The Australasian Gene Therapy Society (AGTS) held its second annual meeting on April 27-29, 2001, providing an opportunity for groups from all over Australia to present their latest contributions to 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.

AGTS held its inaugural meeting in Melbourne on February 22-23, 1999. It became an incorporated entity in New South Wales on October 17, 2000. The main aims of AGTS include:

- To facilitate the growth and development of the field of gene therapy in Australasia;
- To act as a resource of gene therapy protocols, reagents and scientific exchanges;
- To represent the goals of the Australasian gene therapy community before Government and the public;
- To disseminate information regarding the safe and ethical use of gene therapy methodologies.

The Organising Committee for the 2nd meeting of the society included:

Dr Ian Alexander, The Children's Hospital at Westmead, Sydney
Dr Gerald Both, CSIRO - Molecular Science, Sydney
Dr Panos Ioannou, The Murdoch Childrens Research Institute, Melbourne
Ms Margot Latham, The Children's Hospital at Westmead, Sydney
Dr John Rasko, Centenary Institute and Sydney Cancer Centre, Sydney
Dr Jason Smythe, Children's Medical Research Institute, Sydney
Dr Geoff Symonds, Johnson & Johnson Research Labs, Sydney
Prof Ron Trent, Royal Prince Alfred Hospital, Sydney

For further developments and other information please refer to the web page of AGTS at

**http://murdoch.rch.unimelb.edu.au/agts/**

# ORAL PRESENTATIONS

## *O1* DEVELOPMENT OF AN *IN VITRO* MODEL FOR THE TESTING OF NOVEL ANTI-LEUKEMIC AGENTS TARGETING THE RAS PATHWAY.

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Despite dramatic improvements in treatment, relapsed leukemia remains one of the most common causes of death from cancer both in children and adults. Mutations of the Nras gene have been identified amongst 30-40% of Acute Myeloid Leukemia (AML) cases, and activation of the ras pathway has often been found in Chronic Myeloid Leukemia (CML) lacking Nras mutations. The aim of this study was to develop an *in vitro* model for testing anti-ras agents using a dual-fluorescent reporter system for measuring ras and anti-ras expression at the single cell level by flow cytometry. Two retroviruses were engineered: one carrying oncogenic N-ras$^{m}$ and Green Fluorescent Protein (GFPtpz) and the other an anti-ras ribozyme (Rz) previously shown to effectively suppress N-ras expression[1]. In this study the Rz was fused to Red Fluorescent Protein (RFP). Target cells - NIH 3T3-based PG13 cells and leukemic K562 cells were first transduced with the Rz/RFP virus, and then with the Nras/GFPtpz virus. We demonstrated that Nras and Rz expression correlated with the relevant fluorescent reporter. Reduced GFPtpz fluorescence in cells expressing Rz/RFP was indicative of oncogene suppression. This suppressive effect was dependent on the relative level of ras/Rz expression, with the highest ras suppression seen at equimolar or greater than equimolar Rz to ras expression. The stoichiometry of this effect was determined by analysing sub-populations expressing different ratios of GFPtpz to RFP. Cell sub-populations expressing either Ras-GFPtpz alone or both Ras-GFPtpz and Rz-RFP, were futher sorted and RNA isolated to perform Northern blot analysis of ras and Rz expression. These data indicate that this dual fluorescent reporter system allows analysis of oncogene suppression and represents a useful model in which to analyse anti-leukemic genes. It can be used to analyse phenotype in NOD-SCID mice in collaboration with Alison Rice at the Childrens Cancer Institute Australia.

## *O2* A NOVEL B7-MEDIATED TRANS-COSTIMULATION STRATEGY FOR THE TREATMENT OF TUMOURS: *IN VITRO* AND *IN VIVO* ANALYSES.

Jason A. Smythe, Grant Logan, Christine Smyth, Afroditi Spinoulas, Irina Zaikina, Peter Rowe and Ian Alexander.

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Gene therapy has been proposed for a number of heritable and acquired human diseases including certain cancers and leukemia's, haematological defects, severe combined immunodeficiency (SCID), cystic fibrosis, and the acquired immunodeficiency syndrome, AIDS. We are developing a program within the Gene Therapy Unit focusing on the clinical application of an immune-augmentation gene therapy strategy for the treatment of paediatric cancer. Our initial strategy was based on genetically modifying human tumour cells to enhance their recognition and rejection by the immune system. By retroviral-mediated transfer of genes encoding the lymphocyte co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2) we aimed to stimulate tumour-specific cytotoxic T-lymphocytes effector cells, an immuno-therapeutic strategy that should be particularly applicable to the treatment of metastatic cancers. Although we were able to demonstrate that retrovirally transduced human tumour cell lines genetically modified to stably express CD80 and/or CD86 have enhanced immunogenicity as target cells in *in vitro* lymphoproliferation assays, when we attempted to translate these tumour cell line studies to primary paediatric tumour samples we encountered a number of problems with gene transfer efficiency, and ultimately were only able to successfully modify a small percentage of the primary tumour samples obtained.

In addressing this problem we developed a novel approach to the use of genetically modified non-neoplastic bystander cells (ie. primary fibroblasts) to augment anti-tumour immune responses. This strategy of using CD86-modified fibroblasts to provide costimulatory signals to effector T-lymphocytes (trans-costimulation) can be used to stimulate CD4 and CD8 T-lymphocytes in MHC class-II and MHC class-I restricted reactions *in vitro*, respectively. Not only do these results have significant implications for clinical implementation of this type of cancer immunotherapy by obviating problems associated with inefficient gene transfer to primary human paediatric tumour cells, but the observation that CD86 expressing bystander cells can provide costimulatory signals to T-lymphocytes also raise a number of fundamental questions about potential links between APC-mediated trans-costimulation *in vivo*, and the aetiology of autoimmune disease. We are currently evaluating the safety and efficacy of this CD86-mediated trans-costimulation strategy *in vivo* using a murine tumour model system, and the results of these studies will be discussed.

## *O3* DENDRITIC CELLS TRANSDUCED BY HIV-1 VECTOR ENCODING MURINE TRP-2 CONFER TUMOUR PROTECTION AND SUPPRESSION TO MICE CHALLENGED WITH B16 MELANOMA

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Dendritic cells (DCs) are the most potent professional antigen-presenting cells (APCs) with unique immunostimulatory properties, which play a vital role in primary immune response. Genetically engineering DCs does not require the information of the HLA haplotype of patient. Introducing genes into DCs will allow constitutive expression of the proteins that will prolong the presentation of the antigens. Additionally, multiple and unidentified epitopes encoded by the entire tumour-associated gene may enhance T cell activation. Vaccination of DCs modified to express tumour antigens has been reported to generate protective immunity. At present, retroviral vectors based on murine leukaemia virus (MLV) are the best-characterised viral vehicles used for cell gene transfer. However, limiting conditions for MLV vector to transduce DCs are the low transduction efficiency and the requirement of expensive transduction-enhancement reagent such as RectroNectin..

In this report, we have demonstrated HIV-1 based lentiviral vector mediated efficient transfer of genes to DCs. The transgene, *mTRP-2*, is a clinically relevant melanoma-associated antigen gene, encoding a tissue specific self-antigen naturally expressed by normal melanocytes, and recently found to be a tumour-rejection antigen for B16 melanoma. After gene transfer, the transduction efficiency, the proper processing of the transgene in DCs, in terms of RNA transcription activity and protein expression, was verified with RT-PCR and specific antibody respectively. Prophylactic treatment with the *mTRP-2* modified DCs (DC-HR'CmT2) in C57BL/6 mice resulted in protection from further tumour challenge. In the therapy model, DC-HR'CmT2 treated mice showed a significantly better survival rate. Also, our data indicated the presence $CD4^+$ and $CD8^+$ cells were essential for optimal tumour protection during both the priming and challenge phase.

## *O4* TARGETED GENE THERAPY FOR ANDROGEN-INDEPENDENT PROSTATE CANCER

Rosetta Martiniello-Wilks[1], Dale J.Voeks[1], Allison Dane[1], Linda J. Lockett[2], Dianna E. Brookes[2], Kylie Smith[2], Emma Bennetts[2], Gerald W. Both[2], Peter L. Molloy[2] and Pamela J. Russell[1].

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Prostate cancer (PCa) is the most common cancer in Western men but late stage androgen-independent disease is currently incurable. To target androgen-independent PCa, we have trialed two gene-directed enzyme/prodrug therapies (GDEPT). Replication incompetent adeno-viruses under the control of prostate promoters have been used to deliver 'toxin producing enzymes' to PCa grown in mice. Given intratumourally, we have used adenovirus delivering the Herpes Simplex Virus 'thymidine kinase' (TK) gene that converts the systemically given non-toxic prodrug ganciclovir to the toxin ganciclovir-triphosphate. Adenovirus delivering the *E. coli* 'purine nucleoside phosphorylase' (PNP) gene that converts prodrugs 6MPDR or fludarabine to their toxic metabolites 6-methylpurine or 2-fluoroadenine has also been used. Both GDEPT systems offer the added advantage that not all tumour cells require transduction to provide effective cell killing as the toxins produced can diffuse between cells, providing a bystander effect. Furthermore, the PNP metabolites offer a further advantage over the TK-GDEPT bystander effect, due to their highly toxic nature and their ability to freely cross cell membranes.

Specificity requires regulatory regions from a prostate expressed gene to drive enzyme expression. We have produced adenoviruses under the control of prostate specific antigen (PSA), rat probasin and the SV40 enhancer (SVPb) or the constitutive Rous Sarcoma Virus (RSV) promoters. Initial GDEPT studies were performed using replication defective human type 5 adenoviruses (Ad5) to deliver the TK or PNP to pre-established PC-3 tumours carried under the skin of nude mice followed by systemic prodrug treatment. The use of adenoviruses is particularly important in slow growing PCa cells as they can infect both dividing and non-dividing cells. Vectors Ad5-PSA-TK, Ad5-PSA-PNP, Ad5-SVPb-PNP were constructed and all produced a significant suppression in PC-3 tumour growth and increased mouse survival during GDEPT.

Currently, we are trialing a novel delivery vector, ovine adenovirus (OAdV). Importantly, unlike the pre-existing immunity to Ad5, humans are naive to OAdV. A new generation of OAdV vector carrying the PNP gene under the control of the SVPb promoter (OAdV218) was constructed. As this vector only transduced human PCa cells, a further OAdV carrying PNP under the RSV promoter (OAdV220) was also constructed for use in the aggressive murine RM-1 PCa mouse model. *In vitro* studies showed both OAdV218 and OAdV220 vectors delivered transgenes effectively to PC-3 cells. Furthermore, OAdV220 showed similar *in vitro* transduction in RM-1 cells. GDEPT with OAdV218 or OAdV220 followed by systemic fludarabine treatment in sc PC-3 tumour bearing nude mice showed a significant 82-85% suppression in tumour growth and increased mouse survival. A single intraprostatic injection of OAdV220 into pre-established aggressive RM-1 tumours followed by systemic treatment with fludarabine resulted in a 25-27% reduction in prostate volume at day 18. Improvements in vector dissemination to tumour cells *in vivo* and/or the involvement of the immune system may further improve efficacy.

## *O5* POXVECTORS FOR TUMOUR IMMUNOTHERAPY – STUDIES IN MICE AND HUMANS

Robinson BWS, Mukerjee S, Jackaman C, Lake R, Ramshaw I, Haenel T, Smith D, Musk A, Morey S, Van Hagen D, Himbeck R, Scott B, Harnett G, Stumbles P, Davidson J, Van der Bruggen I, Nelson D.

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Pox viruses have previously been used as vectors for tumour immunotherapy but only as vehicles for tumour antigens. It has been assumed that their strong antigenicity would prevent their being useful vectors for immunomodulatory molecules such as cytokines, where prolonged expression and multiple injections are required.

In our initial clinical trials using vaccinia virus (VV) with the transgene interleukin 2 (IL-2), delivered intratumorally in patients with mesothelioma, we noted prolonged transgene expression (analysed by PCR of sequential tumour biopsy samples) regardless of the level of the anti-VV antibody response at the time of study (1). This suggested that modification of the VV by insertion of the IL-2 gene into the thymidine kinase region of the virus, which rendered it non-cytopathic, reduced its immunogenicity so much that it would in fact be a useful virus for tumour immunotherapy. One of the problems encountered in the above study however was a failure to attain high levels of IL2 expression in tissues.

In order to improve the efficiency of VV as a vector we have therefore undertaken two additional approaches. Firstly, we have studied the capacity of dendritic cells (DCs) to act as vehicles for this vector. We analysed the capacity of VV-IL2 constructs to infect human DCs and found that they did so efficiently, in contrast to macrophages and T-cells. High levels of the transgene were produced, however to our surprise the vector preparation induced the DCs to release IL-4 rather than IL-12 ie a potentially dangerous 'tolerising' rather than 'immunostimulatory' pattern of cytokines. Secondly, we modelled the above in our murine mesothelioma model. In vivo studies of VV-IL-2 delivered intratumorally induced slowing or regression of tumor. Combination with VV-IL12 induced a synergistic effect.

We conclude that VV may be a good vector for tumour immunotherapy studies however use with DCs in humans requires careful exclusion of potential tolerising effects.

1. Mukherjee S, Haenel T, Himbeck R, Scott B, Ramshaw I, Lake RA, Harnett G, Phillips P, Morey S, Smith D, Davidson JA, Musk AW, Robinson BWS. Replication-restricted vaccinia as a cytokine gene therapy vector in cancer: persistent transgene expression despite antibody generation. Cancer Gene Therapy. 2000,7(5):663-670

## *O6* RETROVIRAL TRANSDUCTION OF HAEMOPOIETIC STEM CELLS TO CONFER CHEMORESISTANCE AND IN VIVO SELECTION

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Retroviral transduction of human haemopoietic stem cells to introduce drug-resistance genes has been shown to confer chemoresistance when measured in both in vitro and in vivo assays. In addition, the combination of a drug resistance gene with a second gene of interest (to correct for a genetic disease, for instance) may provide a selective advantage for engraftment and expansion of transduced cells within the bone marrow. Our aim is to develop a clinical protocol for retroviral transduction of human haemopoietic stem cells. We have chosen the sgene for the DNA repair enzyme Methyl-Guanine-Methyl-Transferase (MGMT) as our gene of interest, since function of the transgene in stem cells can both confer chemoresistance to a clinically relevant agent (BCNU) and act to provide a selection advantage for transduced cells in a transplant model.

Retroviral packaging lines for pseudotyping the MML-V based MFG-MGMT with both Gal-V and VSV-G proteins have been developed which can produce viral titres of 2.5 x $10^5$/ml (Gal-V) and 2 x $10^8$/ml (concentrated VSV-G). A Lenti-MFG-MGMT has also been produced using a transient triple transfection protocol resulting in a VSV-G pseudotyped virus. Transduction of CD34 selected peripheral blood stem cells or bone marrow following 2 or 3 days pre-stimulation with cytokines (IL-3, SCF, +/- IL-6, Flt3-L, TPO) in serum free medium has resulted in transduction efficiencies ranging between 3 – 30 % when measured by flow cytometry immediately following the transduction culture period. Short -term culture assays of transduced populations (CFU-GM colony assay) after exposure to BCNU show that expression of the MGMT protein can provide chemoresistance to BCNU. Analysis of transgene expression in CFU-GM colonies derived from Long Term-Culture Initiating Cell (LTC-IC) assays following transduction is currently being undertaken in order to determine the extent of transduction of more primitive CD34+ subpopulations. In addition, the NOD-SCID mouse model for analysis of the engraftment potential of human stem cells is being established as both a means of testing transduction efficiency in SCID Repopulating Cells and to provide a model for the use of MGMT as a selective marker for engraftment and expansion of gene modified stem cells.

Optimisation of transduction protocols using our vectors is our current focus, as we are working toward choosing producer clones to subject to regulatory testing for production of a Master Cell Bank and clinical grade vector stocks. Generation of pre-clinical data using the LTC-IC and NOD/Scid models will assist us in our longer-term

goal of establishing a clinical trial to provide chemoprotection for the stem cells of children with brain tumours being treated with BCNU.

## *O7* NOVEL APPROACHES TO GENETIC DISEASES FOR THE 21ST CENTURY

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The concept of gene therapy as a one-off procedure by which to treat otherwise incurable genetic diseases has attracted a lot of interest over the last twenty years. Yet despite intensive efforts by numerous groups all over the world, this objective has been much more difficult to achieve that it was ever anticipated.

The key obstacles that have bedevilled the field of gene therapy are the following:

Size/content of therapeutic insert: Most human genes are very large with complex regulatory elements that determine the developmental, tissue and locus specificity of gene expression. However, most therapeutic constructs use only the minimal coding and regulatory sequences and thus cannot achieve efficient gene expression under a variety of physiologically relevant conditions;

Method of delivery: A large variety of viruses are being investigated as vectors for gene delivery, because of their intrinsic properties to deliver their genomes efficiently to cells. However, most of these vectors have limited packaging capacity, thus forcing the use of "minigenes" which are devoid of most endogenous regulatory elements. Non-viral delivery has the potential to deliver genes with their regulatory sequences as intact functional units, but the efficiency of delivery is still much lower than that of viral vectors.

Safety: Although most viral constructs are largely devoid of viral sequences, efficient delivery is dependent on packaging into intact viral particles which may also incorporate a number of viral mRNAs that may be needed early in the infectious cycle. Proteins from the viral coat and after expression of packaged viral mRNAs may evoke a variety of host responses with undesirable effects. The random integration of therapeutic constructs and the recent demonstration of mobilisation of lentiviral constructs during HIV-1 infection also pose additional dangers that have not been adequately assessed.

The completion of the Human Genome Sequencing Project, the availability of most human genes as intact functional units in various high quality PAC/BAC libraries, and the recent development of techniques for the genetic engineering of PAC/BAC clones, are opening new avenues for the effective therapy of genetic diseases. While gene therapy by direct gene delivery using intact functional units, delivered either as episomes or as human artificial minichromosomes, is still some way off, the identification of agents for the targeted pharmacological modulation of the expression of specific genes is now possible through the creation of high throughput screening assays based on intact functional loci in physiologically relevant cell lines. It is anticipated that the development of such assays for each gene of interest will initially lead to the discovery of agents that can facilitate a better understanding of the regulatory pathways, while it is hoped that some of these agents will eventually prove to be clinically useful through the modulation of the expression of specific genes, so as to overcome or complement the effects of specific mutations.

## *O8* NEW STRATEGIES FOR THE TREATMENT OF DIABETIC RETINOPATHY

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Diabetic retinopathy is the major cause of blindness in people under the age of 65 in developed countries. The development of neovascularisation in the diabetic retina has been linked to the hypoxia-induced upregulation of vascular endothelial growth factor (VEGF) in ischaemic retinal cells. We selected VEGF as our target to develop therapies capable of preventing neovascularisation of the retina in a reliable manner for a prolonged period of times (decades). Recent developments in recombinant virus mediated gene delivery technologies suggest that the long term delivery and expression of transgenes in the retina is viable. Thus, we selected recombinant adenoassociated virus mediated gene delivery to facilitate the long-term expression of transgenes. One of the challenges of gene therapy is the delivery of transgenes into the diseased cells that are not always predisposed to transduction. In addition, the manipulation of cells in the macula is not desirable. To overcome these difficulties we proposed Secretion Gene Therapy (SGT). SGT is based on the expression of transgenes that are soluble and thus easily distributed throughout the diseased eye. We have suggested that the presence of the soluble fms-like tyrosine kinase (sflt) receptor in the diabetic eye may prevent the development of neovascularisation in the retina by trapping VEGF. We have demonstrated that recombinant virus produced sflt is capable of decreasing free VEGF levels in the eye. This decrease in free VEGF is accompanied by the reduction of VEGF induced new vessels.

## *O9* TOWARDS *IN VIVO* SELECTION FOR LIVER DIRECTED GENE THERAPY

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**Background:** It remains a challenge to achieve stable, high level expression of a therapeutic transgene in a significant proportion of hepatocytes *in vivo*. Clonal

expansion of gene-modified cells would provide a means to overcome inefficiencies of current delivery systems. This could be achieved by co-delivery of a therapeutic gene with a gene that provides a growth advantage when an exogenous selection pressure is applied. Increasing the ability of cells to produce glutathione (GSH), a natural cellular protectant, has the potential to provide cells with resistance to a number of exogenous agents including the hepatotoxin paracetamol. This resistance could be utilised in an *in vivo* selection strategy for gene therapy.

**Aim:** We aim to increase cells ability to produce GSH through overexpression of the heavy and light subunits of γ-glutamylcysteine synthetase (GCS) and show that such gene-modified cells have increased resistance to the *in vivo* toxicity of paracetamol.

**Methods:** A stable doubly transfected cell line (293HNClip) was generated in a two-step process, transfecting naïve 293 cells initially with the heavy subunit and then the light subunit of GCS, using neomycin and hygromycin resistance respectively. This cell line was characterised, measuring the steady state GSH levels by a colourimetric assay, and the GCS enzyme activity by HPLC. To further assess the ability of these cells to produce GSH, the GSH levels were measured before, during, and after exposure to diethylmaleate (DEM), an agent known to reversibly deplete GSH. The resistance of these cells to the cytotoxicity of NAPQI (the toxic metabolite paracetamol) was measured by an LDH release assay. To confirm the 293HNClip cells had a survival advantage, clonogenic growth assays and mixing assays were performed.

**Results:** The polyclonal cell line, 293HNClip, exhibited an enhanced capacity to synthesise GSH. The GSH content in the 293HNCLip cells was almost double that in the control cell line, (9.5 v's 5.9 nmols/$10^6$ cells ($p<0.0005$)) and the 293HNClip cells showed 1.5 times the enzyme activity of the control population ($p = 0.028$). On exposure to DEM the control cell showed a reduction in GSH levels by 1/3 after 2 hours of treatment, which returned to baseline by 4 hours. In contrast, the 293HNClip cells were able to maintain their GSH level despite DEM treatment. On withdrawal of DEM, the GSH level rose to well above baseline confirming these cells have an enhanced ability to synthesise GSH. On exposure to NAPQI at 125, 250 and 500 μM for 4 hours, the 293HNClip cells exhibited less LDH release than the control cells at all concentrations. The 293HNClip cells showed a greater survival after exposure to 5 – 15 μM NAPQI as demonstrated by clonogenic growth assay. After mixing the 293HNClip cells and control cells together and exposing the mixed population to a single dose of NAPQI, the proportion of 293HNClip cells in the population increased from 13% to over 30%, thus confirming that they have a growth advantage and can be selected *in vitro.*

**Conclusion:** Transfection of cells with the heavy and light subunits of GCS provides them with an in increased capacity to produce GSH and a survival advantage when exposed to the toxic metabolite of paracetamol. These results establish "proof of concept" for our selection strategy and provide a solid foundation upon which *in vivo* studies will be built.

## *O10* HUMAN ERYTHROPOIESIS IN A NOD/SCID MOUSE TRANSPLANTATION MODEL: A POSSIBLE *IN VIVO* XENOTRANSPLANTATION SYSTEM TO STUDY GENE THERAPY PROTOCOLS FOR THALASSAEMIA

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Approximately 300,000 severely affected patients with thalassaemia and various haemoglobinopathies are born each year, mainly among people of Mediterranean, Middle Eastern, African, South East Asian, Chinese, and Indian origin. The only curative treatment for thalassaemia is allogeneic bone marrow (BM) transplantation. The success of whole marrow transplantation for the correction of thalassaemia as well as other genetic disorders has focused attention on haematopoietic stem cells (HSC) derived from BM, mobilised peripheral blood (mPB) or human umbilical cord blood (UCB). HSC have the ability to divide, proliferate and differentiate into all mature blood cell lineages. Several research groups have established *in vivo* models for engrafting human HSC, based on the ability of human HSC to repopulate the BM and spleen of intravenously transplanted immunodeficient SCID or non-obese diabetic SCID (NOD/SCID) mice. Specific sorting for HSC is currently based on selection of CD34+ cells, a convenient positive selection marker to enrich HSC for stem cell transplantation.

The potential to genetically correct the entire haematopoietic system by modifying HSC has made the CD34+ cells a potential target for gene therapy. The development of human gene therapy protocols for erythropoietic disorders has been hampered by the lack of an appropriate *in vivo* animal model. In this study, we investigated whether NOD/SCID mice can be used as an *in vivo* model to evaluate human erythropoiesis following transplantation of CD34+ cells derived from UCB. We transplanted 4 x $10^4$ CD34+ cells into normal NOD/SCID mice or into NOD/SCID mice pre-treated with liposomes containing the drug dichloromethylene diphosphonate ($Cl_2$MDP-liposomes). $Cl_2$MDP-liposomes are used to selectively deplete the mononuclear phagocytic system and have previously been demonstrated to enhance engraftment levels of adoptively transferred cells in mice. The BM cells of all recipient mice, 8 weeks after the transplantation, contained various types of human haematopoietic cells, of both lymphoid and myeloid origin. The most abundant human cells in normal NOD/SCID mice and in mice pre-treated with $Cl_2$MDP-liposomes were found to be CD45+ (44±15%), CD19+ (23±10), CD33+ (16±8%) and CD34+ (5±3). Importantly, we only found human GlyA+/CD71+ (4±2%) erythroid

cells in the BM of NOD/SCID mice pre-treated with $Cl_2$MDP-liposomes.

This present study provides evidence for multilineage expansion in particular the erythroid lineage from human HSC transplanted into NOD/SCID mice only when pretreated with $Cl_2$MDP-liposomes. The NOD/SCID mice may be used as an *in vivo* model to evaluate the success of gene transfer protocols for the treatment of erythropoietic genetic disorders such as thalassaemia. We are currently investigating whether we can further increase the percentage of human erythroid cells in the BM, spleen and blood of NOD/SCID mice.

### *O11* TARGETED GENE CORRECTION IN BONE MARROW STEM CELLS: TOWARDS AN AUTOLOGOUS STRATEGY FOR GENE THERAPY IN THE *MDX* MOUSE

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Targeted genetic correction of mutations in cells has emerged as a potential strategy for treating many human conditions involving nonsense, missense and transcriptional splice junction mutations. We have used Single Stranded Short Fragment Homologous Replacement (ss-SFHR) to repair the exon 23 C to T *mdx* nonsense transition at the Xp21.1 *dys* locus in cultured myoblasts and then directly in *Tibialis Anterior* from male *mdx* mice. Whilst *mdx dys* locus repair was successful in 15 to 20% of cells in culture, the frequency of repair by direct intramuscular injection was lower, evident in 0.0005% and 0.1% of *dys* loci. Intramuscular gene repair needs to be significantly improved before *in vivo* SFHR can be considered a serious prospect for application in genetic conditions with primary muscle involvement. Systemically injected bone marrow stem cells from *wt* C57BL/10 *ScSn* mice have been shown capable of remodeling dystrophic *mdx* muscle. Six months following BM transplantation, we observed that sub-lethally irradiated as well as lethally irradiated *mdx* mice expressed *wt dys* loci (f~100% of cells) in their peripheral blood. On these bases, we have formulated a strategy for remodeling dystrophic tissue with systemically injected BMS cells that have been repaired by SFHR. In the *mdx* mouse, this procedure represents an isogeneic approach, possibly with low immune rejection of grafted cells. In humans, the possibility exists that such a strategy could be applied using autologous cells that have been genetically corrected to remodel dysfunctional tissue with functional autologous grafts. Such application of SFHR with autologous cell transplantation (SFHR-ACT) would potentially bypass the significant immune rejection issues that hinder current therapies for DMD.

### *O12* RESTORATION OF DYSTROPHIN EXPRESSION IN THE mdx MOUSE USING ANTISENSE OLIGONUCLEOTIDES IN A GENE "KNOCK-IN" APPROACH

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Duchenne muscular dystrophy (DMD) is a severe, muscle-wasting disease arising from mutations in the massive dystrophin gene that preclude the synthesis of a functional protein. Affected boys show signs of weakness between the ages of 3 to 5 years, become restricted to a wheelchair by the age of 12 years and 90% will die from cardiac or respiratory complications before their third decade. DMD dystrophin gene defects are typically nonsense or frame-shift mutations that lead to premature termination of translation. In-frame dystrophin gene mutations can result in a Becker muscular dystrophy (BMD), a milder, allelic form of DMD where the shortened dystrophin protein contributes to a milder phenotype. There is considerable variation in severity where some BMD patients become restricted to a wheelchair by the age of 15 years while others are almost asymtopmatic and have been diagnosed late in life (mid 60's). The very mild BMD patients clearly demonstrate that the intact or full-length dystrophin protein is not essential for near normal function.

Antisense oligonucleotides have been used to "knock-out" or suppress specific gene expression, either through the activation of a RNaseH activity or by blocking translation. We have used 2'-O-methyl antisense oligonucleotides (2'-O-Me AOs), which do not induce RnaseH activity, to "knock-in" a defective dystrophin gene so that a functional protein can be produced from a specifically modified transcript. These antisense oligonucleotides were directed to crucial splicing motifs in the dystrophin pre-mRNA, thereby blocking inclusion of specific exons in the mature dystrophin transcript. Such an approach would allow exons carrying nonsense mutations to be removed, or exons flanking a genomic deletion to be skipped to restore the reading frame.

The mdx mouse model of DMD has a nonsense mutation in exon 23 of the dystrophin gene which results in premature termination of translation. By targeting antisense oligonucleotides to the 5' splice site of intron 23 of the mouse dystrophin gene, it was possible to specifically induce skipping of exon 23 from the processed dystrophin mRNA. The induced skipping of dystrophin exon 23 removes the nonsense mutation without disrupting the reading frame so that a Becker-like protein could be produced. Delivery of 2'-O-Me AOs to the nucleus of cultured H-2K *mdx* cells was confirmed using an FITC-labelled oligo, and induced exon skipping was assessed by an RT-PCR assay that revealed amplification of a dystrophin transcript missing exon 23. Transfection

efficiency was improved by complexing 2'-O-Me AOs with commercially available liposomes. *In vivo* studies in the *mdx* mouse have shown synthesis and correct localisation of dystrophin and γ-sarcoglycan after repeated i.m. injection of a 2'-O-Me AO:liposome complex. This approach offers an alternative to gene replacement or correction, and future work will assess *in vivo* toxicity, persistence of the protein and further refine delivery.

### *O13* MOLECULAR CHANGES TO GLYCINE RECEPTORS BY GENE THERAPY, AND TRANSGENIC APPROACHES FOR THE STUDY OF SYNAPTIC NEUROTRANSMISSION

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Effects of mutations in the signal transduction domain of the glycine receptor (GlyR) have been studied in cell culture using electrophysiology. This method, however, does not mimic the *in vivo* situation where the receptor is a heteromeric pentamer activated by quantal releases of neurotransmitter. Our aim was to produce an *in vivo* system using mice with changes in the GlyR properties, for the study of synaptic events at the molecular level. Two approaches were used. Firstly, transgenic mice carrying one of two different GlyR human startle disease point mutations under the control of the rat neuron specific enolase promoter have been generated. This construct also contains the enhanced green fluorescent protein gene (EGFP). Three founder lines of each mutation are currently being bred to transgene homozygosity on a GlyR null background (oscillator), which is lethal. In these animals, gene expression has been demonstrated histologically by the presence of the EGFP. Secondly, microinjection of the transgenes (using a viral vector or naked DNA) into the tongue musculature has resulted in the retrograde transport of the constructs into the hypoglossal motor neurons where glycinergic current can easily be recorded. We are evaluating whether retrograde gene transfer results in an observable signalling change which is comparable with the transgenic mice; if so, many more mutations could be tested in a much smaller time frame. Concurrently, tongue injections are being used to trial fragments of the GlyR promoter for specificity and efficacy of gene expresssion. This promoter will then be used in gene therapy experiments to stimulate motor neuron regeneration.

### *O14* VIRAL VECTOR DIRECTED GENE THERAPY FOR THE TREATMENT OF DISEASES OF THE PERIPHERAL NERVOUS SYSTEM.

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Sensory neurons of the peripheral nervous system (PNS) are attractive targets for therapeutic gene transfer, being involved in the pathophysiology of a number of debilitating and incurable neurological conditions. A gene delivery system capable of efficient and stable genetic modification of these post-mitotic cells would, therefore, have significant therapeutic potential.

Virus vector systems are now available that can transduce neuronal cells with long-term transgene expression. We have, therefore, investigated the transduction efficiencies of both the recombinant adeno-associated virus (rAAV) and VSV-G pseudotyped lentivirus (HIV-1 based) vector systems in in vitro assays on newborn mouse, foetal human and foetal sheep dorsal root ganglia (DRG). In dissociated mouse cultures we achieved significant levels of reporter gene, enhanced green fluorescent protein (EGFP), expression using relatively low multiplicities of infection (MOI). Transgene expression was maintained for up to 28 days, the limit of our culture system. In addition, microinjection of both vectors into the interstitium of mouse DRG explants also achieved efficient transduction of neuronal cells over a 28-day period. Interestingly, our lentivirus vector stocks (pseudotyped with a VSV-G envelope) predominantly transduced neuronal cells, even at very high MOI. This phenomenon has previously been reported in lentivirus vector transduction of neurons in rat striatum and hippocampus (Blomer et al 1997, Naldini et al 1996). In contrast, the rAAV vector transduced all cell types in the DRG cultures (neurons, fibroblasts and Schwann cells). The same lentivirus vector stocks transduced equivalent numbers of all cell types in both dissociated human and sheep DRG cultures.

Since both vectors achieved efficient and sustained transduction of human sensory neurons in dissociated DRG cultures, they are both good candidate vectors for gene therapy to the human PNS. We are currently optimising vector design and delivery strategies, and investigating the mechanism of rodent-specific failure of VSV-G pseudotyped lentivirus vectors to transduce non-neuronal cells in dissociated DRG cultures.

### *O15* HIGH THROUGHPUT SCREENING OF RETROVIRUS PACKAGING CELL LINES

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Retroviral vectors have proven useful in a variety of gene transfer applications. A key feature of their utility is the availability of retrovirus packaging cells that allow production of retroviral vectors in the absence of helper virus. Although infectious retrovirus is produced within 48 hours following transfection of these packaging lines the infectious titre generated by these cells is on average low - necessitating identification of those rare clones that produce retrovirus at high titre. To date this has been accomplished by selecting clones from the transfected

population then testing each for its ability to produce virus. Titre assessment for vectors that encode fluorescent reporter genes is conveniently performed by flow cytometry. Due to the labour intensive nature of this type of analysis only a relatively small number of clones can be screened for each construct.

In order to identify high titre clones, we have developed a moderate/high throughput assay to screen retroviral packaging cell lines containing vectors which encode various fluorescent reporter genes. Target cells plated in 96 well plates were incubated in medium containing polybrene and virus from packaging cell line supernatant. 36-48 hours after transduction, plates were assayed on a Wallac 1420 Victor$^2$ multilabel counter to determine the percentage of cells expressing either the green (EGFP), yellow (EYFP), cyan (ECFP) or red (dsRed) fluorescent reporter gene. The relative fluorescent counts obtained for each packaging cell supernatant tested correlated with the level of target cell transduction by retroviral vectors encoding each specific autofluorescent reporter gene. With the exception of EGFP and EYFP which use the same filter set, detection is specific for one colour with cells exhibiting the highest signal above background at a density between $5x10^4$ and $7x10^4$ cell per well. Additionally we have achieved an accurate measure of cell density by assaying cells labelled with the nuclear dye DAPI on the multilabel counter. The specificity allows the option of multiplex assays where the percentage of cells expressing either flurochrome can be determined independent of the other. A cautious estimate is that a single operator will be able to screen approximately 2000 packaging cell lines in a day.

Optimising vector titre has important implications for clinical and research based gene therapy. The development of this high throughput assay greatly increases the chance of obtaining a high titre packaging cell line. This robust screening technology can be applied to any virus packaging system or indeed any fluorescent assay in which high-throughput selection is required.

## *O16* THE DEVELOPMENT OF NOVEL BOVINE LENTIVIRAL VECTORS FOR EFFICIENT GENE TRANSFER

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**Background.** The major stumbling blocks preventing the development of successful gene therapy protocols include inefficient gene transfer and transient gene expression. Recent reports from studies with Human Immunodeficiency Virus (HIV)-based vectors have shown efficient gene delivery and sustained gene expression in a variety of cell types in vivo, showing promises for lentivector-mediated gene therapy. However, it must be recognised that the HIV vectors are based on a human pathogen that causes Acquired Immune Deficiency Syndrome (AIDS). While the vector has been disabled of any pathogenic properties, it may be more prudent, practical and safer to use vectors derived from non-human retroviruses. In this report, we describe the development of a novel bovine lentivector based on a non-primate lentivirus, a Jembrana Disease Virus (JDV).

**Method.** Splicing PCR was used to amplify fragments from the JDV genome for the construction of a JDV transfer vector and a packaging construct. Disabled JDV viral particles pseudotyped with the vesicular stomatitis virus glycoprotein G (VSV-G) were produced by co-transfecting 293T cells with a JDV transfer vector, a JDV packaging vector and a VSV-G encoding plasmid. Viral titre was obtained by transducing cells with the supernatants harvested from co-transfection and by countning the number of cells expressing the transgene. PCR and Southern blot were used to detect transfer vector integration.

**Result.** Disabled JDV vectors could be efficiently packaged at approximately $1 \times 10^6$ transducing units per ml and used to transduce cells of different origins. Marker gene green fluorescent protein (GFP) was efficiently transferred and integration of the transgenes demonstrated in a range of primary and transformed cells. Several other genes were also being delivered, including suicide genes for cancer gene therapy.

**Conclusion.** This bovine lentiviral vector system may be applicable, if not more readily acceptable for human gene therapy.

## *O17* AN HIV-1 BASED GENE TRANSFER VECTOR WITH NOVEL SAFETY FEATURES

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Vectors derived from human immunodeficiency virus type 1 (HIV-1) appear an attractive option for many gene therapy applications due to their ability to stably transduce non-cycling cell populations. However, the pathogenic nature of HIV-1 has raised some considerable safety concerns because of the possibility of generating replication-competent lentivirus (RCL) through genetic recombination. To safeguard against the production of RCL, we have disassembled the genome of HIV-1 by isolating each of the primary transcriptional units encoding *trans* functions relevant for vector production (with the exception of Env, which has been substituted for by the vesicular stomatitis virus glycoprotein (VSV-G)). Each reading frame was isolated as a minimal or near minimal transcription unit and cloned into a separate expression construct. Together with a suitable HIV-1 derived vector construct, this panel of constructs was assayed for particle yield and for the transfer of the enhanced yellow fluorescent protein via transient expression from 293T cells.

In order to both optimise the expression of GagPol and make expression Rev/RRE independent, a series of

reading frames that were codon optimised for high level expression in mammalian cells was created. Subsequently, the Gag and GagPol polyproteins were expressed from two separate constructs allowing removal of the translational frameshift signal in the *gagpol* gene. This resulted in a similar amount of viral particles but with a lower titre compared to that achieved when GagPol was expressed from one plasmid. The predicted safety afforded by this approach was demonstrated by the measurement of transfer of functional *gag* and *gagpol* sequences to recipient cells.

Several HIV-1 based gene transfer vectors have been developed by careful evaluation of the viral *cis* sequence elements on vector function. Apart from the minimal sequence elements required for effective vector performance, other sequence elements were shown to alter the efficiency of virus production. The design of HIV-1 vectors will almost invariably involve compromises (usually conflicting) of safety and efficiency. The ultimate analysis of safety will depend as much on the systems used to provide packaging functions and detection of replication competent virus, as it does on the vector design.

## *O18* A LENTIVIRAL GENE TRANSFER VECTOR FOR AIRWAY EPITHELIUM

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Modified lentiviruses are attractive gene transfer vectors as they can stably transduce dividing as well as terminally differentiated cells. We have constructed a lentiviral vector encoding the LacZ cDNA (LV-LacZ) based on the HIV type 1 that has been reconstructed to remove all viral proteins necessary for replication.

**Methods:** 20ul of LV-LacZ (1 x $10^4$ LacZ-forming-units) were instilled into the right nostril of C57Bl/6 mice one hour after pretreatment with 4ul of 1% LPC (lysophosphatidylcholine) or PDOC (polidocanol) - both known to injure epithelium - or after control pretreatment (PBS). Gene transfer was measured after 7 days via X-gal staining.

**Results**: No transduced cells were observed in any control groups [plasmid LacZ, carrier solution or after PBS pretreatment (0.0(0) cells)]. LPC pretreatment resulted in significantly higher numbers of transduced cells (11(0.6) cells, n=3,.v. PBS group, $p<0.05$, ANOVA) as did PDOC (4(0.6) cells, n=3, .v. PBS group, $p<0.05$). Transduction occurred in ciliated, non-ciliated, basal, and sub-epithelial cells.

**Discussion:** Our finding that an injurious dose of LPC or PDOC was required for transduction of intact airway is consistent with the only other published work in intact airway (Johnson et.al, Gene Therapy 2000: 7, 568). This vector appears to transduce most cell types in respiratory epithelium, including the basal cells that are thought to be a primary airway stem-cell group. We are now examining methods to optimise vector titre and pretreatment techniques, for inclusion in protocols employing an LV-CFTR vector designed for gene therapy trials in CF mouse airways.

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## *O19* NON-HUMAN ADENOVIRAL VECTORS

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The characterisation and development of non-human adenoviruses as vectors lags significantly behind that of human adenoviruses, understandably so, as the prototype human viruses have been studied at the molecular level for many years. Recently however, the number of characterised non-human adenoviruses has increased substantially. Ovine, bovine, avian and frog adenoviruses that do not fit into the known taxonomic groups have been identified and sequenced and the ovine isolate has been adapted as a gene delivery vector. Canine, porcine, bovine and avian isolates in the mast- and aviadenoviruses have also been completely characterised and are being used in the same way. Exploitation of these vectors for gene delivery depends on the target cells they infect and their behaviour in those cells. Non-human adenoviruses have a lytic cycle in their natural host but often replicate abortively in heterologous cells, although the molecular basis for this is not well understood. Many genes carried by these viruses pose new questions whose answers will ultimately lead to new insights into virus/cell interactions. For example, the clear differences between avi- and mastadenoviruses in their ability to transform cells compared with ovine adenovirus indicates a fundamental difference in the interaction with cell cycle control proteins by the latter virus. The challenges are to understand the biology of the new adenoviruses, and having engineered them as gene delivery systems, to identify the best areas for their application. Issues relating to biosafety, vector production and scale up will also be discussed.

## *O20* FUNCTIONAL DELIVERY OF LARGE GENOMIC DNA TO HUMAN CELLS BY AN EPSTEIN-BARR VIRUS VECTOR

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Persistent expression of a transgene at therapeutic levels is required for successful gene therapy. Most of the currently used vector and virus systems have a small capacity and thus usually employ a cDNA copy of the transgene under control of a heterologous promoter. These are prone to vector loss and promiscuous expression or transcriptional silencing. The delivery of genomic DNA would enable genes to be transferred as complete loci, including regulatory sequences, introns and native promoter elements. Our program has been to implement technology (episomal systems, transfection methods, viral packaging) to use genomic DNA for functional gene expression.

We developed large-insert vectors incorporating elements of the bacterial artificial chromosome (BAC) cloning system and the episomal maintenance mechansims of Epstein-Barr virus (EBV). We have demonstrated that constructs of up to 242 kb can be delivered to cultured cells with relatively high efficiency (10%) by a nonviral gene therapy vector, LID (Lipid-Integrin-DNA). When LID vectors were used to deliver a large genomic locus (NBS1 [Nijmegen Breakage Syndrome], encoding nibrin) as a 143 kb construct to primary NBS cells, at least 57% of cells expressing GFP also expressed functional nibrin (White et al., submitted).

We have used this system to stably correct a human genetic deficiency in vitro (Wade-Martins et al., 2000) When a 115 kb genomic fragment spanning the complete human hypoxanthine phosphoribosyltransferase (HPRT) locus was introduced into HPRT-deficient human cells, the transgene was both maintained as a low-copy episome and expressed stably for six months in rapidly dividing cell cultures.

Recently we have shown that with appropriate EBV elements in the vector and a suitable helper packaging cell line, our vector, carrying up to 150 kb human genomic DNA, can be packaged as infectious virus. Cell-free, purified viral particles have allowed introduction of the genomic DNA vector by infection into EBV-negative B-cells followed by recircularisation of the linear viral genome, re-establishment of the vector as a stable episome and stable, long-term expression of the transduced transgene (White et al., manuscript in preparation).

Wade-Martins, R., White, R.E., Kimura, H., Cook, P.R. and M.R. James. Stable correction of a genetic deficiency in human cells by an episome carrying a 115 kb genomic transgene. Nat.Biotech. 18:1311-1314 (2000).

## *O21* GENE THERAPY FOR TYPE I DIABETES - REGULATED SECRETION OF INSULIN FROM A GENETICALLY MODIFIED HUMAN CELL LINE

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Type I diabetes mellitus (IDDM) is caused by severe insulin deficiency secondary to the autoimmune destruction of pancreatic beta cells. Somatic gene therapy is one strategy being considered to correct patient blood glucose concentrations. A previous study has shown that insertion of human insulin cDNA ($pC_2$) and the glucose transporter GLUT2 into the human hepatoma cell line Hep G2 resulted in synthesis, storage and regulated secretion of insulin to glucose by the Hep G2ins/g cells. The present study is examining if regulated secretion of insulin to the physiological stimulus glucose can be engineered in another human hepatoma cell line - Huh7. Human insulin cDNA under the control of the constitutive mammalian expression vector RcCMV was introduced into Huh7 cells by electroporation (Huh7ins). Insulin was measured by radioimmunoassay. The levels of human insulin constitutively secreted into the culture supernatant from 5 stably selected clones of Huh7ins cells differed 1.8-fold: $0.16 \pm 0.3$ to $0.3 \pm 0.1$ pmoles insulin/$10^6$ cells/ day, (n=10). Examination of acid ethanol extracts of these cells indicated that insulin was also stored by these cells in varying amounts: $4.75 \pm 0.5$ to $6.93 \pm 0.8$ pmoles/ $10^6$ cells (n=5). Storage of insulin was further confirmed by immunohistochemical analysis and immunoelectron microscopy, the latter of which revealed that insulin was stored in discrete secretory granules. Three-fold stimulation of insulin secretion $0.14 \pm .02$ pmoles/ $10^6$ cells over basal levels ($.05 \pm 0.03$) was detected when 20 mM glucose was applied as a stimulus to Huh7ins cells for one hour. The immediacy of the response was confirmed by perifusing the cells with 20 mM glucose and collecting 2 minute samples.

To further confirm that the secretion of insulin from Huh7ins cells was regulated two further experiments were carried out. Firstly, calcium was removed from the stimulation medium. This abolished the response of the cells to glucose, as was the case with MIN6 cells (insulinoma cell line), used as a positive control. Secondly, Brefeldin A (BFA)(10 μg/ ml), an agent that blocks movement of proteins into the Golgi apparatus, and therefore constitutive release of insulin, was added at the same time as 20 mM glucose. BFA inhibited 30% of the glucose-stimulated insulin secretion and it can therefore be concluded that 70% of the insulin secretion of Huh7ins cells is regulated.

These results offer hope that genetically engineered liver cells may have some clinical benefit in Type I diabetes.

## *O22* ß CELL CHARACTERISTICS OF INSULIN-PRODUCING HUMAN LIVER CELL LINES

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An alternative approach to the treatment of type 1 diabetes is the use of genetically altered liver cells to synthesize, store and secrete insulin. To achieve this goal we previously transfected two human liver cell lines, HEP G2

and HUH7, with human insulin cDNA under the control of the cytomegalovirus promoter, and in the case of HEP G2 cells, which has no GLUT2, also with GLUT2 cDNA. Both cell lines synthesized (pro)insulin, stored it in granules in the cytoplasm, and secreted it in a tightly controlled manner in response to glucose. The HUH7-ins but not the HEP G2ins/g cells cleaved proinsulin to diarginyl insulin. When the HUH7-ins cells were transplanted into diabetic immunoincompetent mice, they continued to synthesize, process, store and secrete diarginyl insulin in a rapid regulated manner in response to glucose. As a result blood glucose levels of the mice were normalized.

What is intriguing about these data is that liver cells can be made to store and secrete insulin in a rapidly regulated manner. These are features of pancreatic ß cells rather than liver cells. To determine in what way the transformed cell lines were similar to ß cells, we examined protein extracts of them for ß cell transcription factors, PDX-1 (mol wt 46 kD), NeuroD (55 kD) and NKx6.1 (43 kD). These factors are necessary for development of the ß but not the liver cell. Western blots were performed with antibodies to these factors. The ß cells used as controls were from mouse, MIN-6 and NIT-1, and rat, RIN 10468. The parent liver cell lines, HEP G2 and HUH7, also were examined. We found both the transformed and parent human liver cell lines contained the transcription factors, NeuroD and NKX6.1, but not PDX-1. Extracts of HUH7-ins cells that normalized blood glucose levels when transplanted into diabetic mice gave similar results. As expected, the beta cells tested positive for all three transcription factors.

To further test the similarities between the transformed human liver cell lines and pancreatic ß cell, we analysed protein extracts of them for proinsulin converting enzymes 1 and 2. These enzymes are found in ß but not liver cells and cleave proinsulin to diarginyl insulin. We found both enzymes to be present in the HUH7-ins and parent HUH7 cells but not the HEP G2ins/g cells.

These results show that human liver cell lines possess a number of pancreatic endocrine features that reflect the common endodermal ancestry of liver and pancreas, perhaps due to ontological regression of the neoplastic liver cells from which the lines are derived. Introduction of the insulin gene under the control of the CMV promoter induces changes in these cells to make them akin to pancreatic ß cells. Our results support the view that liver cells can be induced to become substitute pancreatic ß cells and be a therapy for the treatment of type 1 diabetes.

## *O23* GOOD MANUFACTURING PRACTICE (GMP) AND GENE THERAPY TRIALS

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Investigators manufacturing (and administering) genetically modified cells for *ex vivo* gene therapy have always been required to comply with the *Therapeutic Goods Act 1989.* Thus all clinical trials (and individual treatments) to date have come under the scrutiny of the Therapeutics Goods Administration (TGA). In addition, investigators now wishing to undertake human clinical trials with genetically modified cellular therapeutics will also have to comply with the recent changes to the *Therapeutic Goods Act 1989* and the recently assented *Gene Technology Act 2000.*

The *Gene Technology Act 2000* provides mandatory conditions for the genetic manipulation of material including material intended for therapeutic purposes. These provisions detail the nature and extent of containment and the licensing requirements to cover the potential risk of unintended release of genetically modified organisms (GMOs). Additionally, the recent changes to the *Therapeutic Goods Act 1989* have emphasised the requirements for compliance with the code of Good Manufacturing Practice (GMP). These requirements are apparent in both Part 3 (relating to drug safety) and Part 4 (relating to licensing of manufacturers) of the Act, and give the TGA more regulatory oversight of clinical trials by increasing their powers to compel clinical investigators to supply detailed manufacturing information.

The regulations for human tissues have also been modified and extended (primary through the new *Code of GMP for 'Human Blood and Tissues'*, which has superseded the two previous Codes) to include various levels of manipulation; minimal to very highly manipulated products. The extent of regulation required will depend on the 'level' of manipulation of the cellular product; gene therapy is considered as "Very High Level Manipulation" and thus will require processing in a TGA-licensed facility with the need for Technical Master Files and CTX/CTN submissions. In summary, highly manipulated product that involves gene therapy must be manufactured in a facility licensed under Part 4 of the Act.

It is anticipated that as part of TGA's policy of risk management the initial focus will be on TGA auditing/licensing of blood collection centres (having commenced in late 2000), cord blood and tissue banks, followed by fresh blood products in hospitals. Consequently, it is very likely that those intending to process highly-manipulated blood/tissue (i.e. purging, ex vivo expansion and gene therapy) will be required to undergo audit/licensing by the TGA in the not-too-distant future.

In establishing GMP compliant facilities/operations there is a risk of 'collateral damage' to other areas. Namely, the stifling of research activities or overwhelming associated service units within the institute/hospital. In conjunction with the Australian Red Cross Blood Service we have established a model system which facilitates the development of appropriate quality systems using expertise already available within the Blood Service whilst shielding the rest of the hospital from the impact of GMP compliance. The acquisition of our Part 4 licence is initially for manufacture of highly modified cell therapeutics followed by the manufacture of genetically modified cellular therapeutics. We will further detail the principles used to establish this process.

# POSTER PRESENTATIONS

## *P1* CRYPTIC SPLICING INVOLVING THE SPLICE SITE MUTATION IN THE CANINE MODEL OF DUCHENNE MUSCULAR DYSTROPHY

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Golden retriever muscular dystrophy (GRMD) arises from a mutation in the acceptor splice site of intron 6 of the dystrophin gene. Consequential skipping of exon 7 disrupts the mRNA reading frame and causes premature termination of translation. We are using this animal model to evaluate treatments for Duchenne Muscular Dystrophy, including gene repair induced by chimeric oligonucleotides.

After injection of GRMD muscle with a chimeric oligonucleotide to repair the lesion, immunostaining revealed a modest increase in the number of dystrophin-positive fibres at the injection sites. This result was supported by RT-PCR studies which detected low levels of dystrophin transcripts containing exon 7. Since this exon should have been omitted due to the GRMD mutation, the inclusion of exon 7 suggested low levels of splice site conversion may have occurred as a result of the chimeric oligonucleotide. However, DNA sequencing of these apparently normal dystrophin transcripts revealed that the first 5 bases of exon 7 were missing.

This transcript had arisen from the selection of the first AG in exon 7 as an alternate splice acceptor site. This phenomenon of exon 7 inclusion in GRMD dystrophin transcripts has not been previously reported in dystrophic canine muscle and has the potential to compromise the interpretation of gene correction studies. This transcript is out of frame and cannot be responsible for the origin of dystrophin-positive fibres. It will be important to be aware of this phenomenon with respect to further gene correction studies in the canine model.

## *P2* GENE THERAPY TRIALS IN THE OVINE MODEL OF McARDLE'S DISEASE

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McArdle's disease is an autosomal recessive metabolic myopathy characterised by a deficiency in muscle glycogen phosphorylase and results in exercise intolerance, rhabdomyolysis and recurring myoglobinuria. Sustained disabling weakness may also occur late in the course of the disease. At present, no satisfactory treatment is available for this disorder. There are three mammalian isoenzymes of glycogen phosphorylase (muscle, brain and liver forms) which are encoded by different loci. The brain and muscle isoforms are expressed in foetal muscle, whereas the muscle isoform is expressed during the late phases of muscle differentiation and is the only isoform present in adult skeletal muscle. McArdle's disease is caused by genetic defects in the muscle-specific isoform resulting in either the absence or decreased levels of muscle glycogen phosphorylase, with minimal or no detectable enzymatic activity.

A sheep model of McArdle's disease, characterised by diminished exercise tolerance and impaired glycogen degradation has been described. Studies revealed the absence of glycogen phosphorylase cytochemical activity in the muscle fibres. A splice site mutation in the myophosphorylase gene results in the loss of eight bases at the 5' end of exon 20 of the transcript. This deletion disrupts the reading frame, thereby removing the last 31 residues of the myophosphorylase.

We report results from trials in which a first generation adenovirus, expressing the (a) human muscle phosphorylase gene driven by the Rous Sarcoma virus promoter (ADV myophos) or (b) Lac Z reporter gene with a cytomegalovirus promoter (ADV Lac Z) was injected into the semitendinosus muscle of affected lambs at sites with or without prior injection of notexin. Ten days after injection of ADVmyophos alone, there was an approximately 10-fold increase in glycogen phosphorylase activity when compared to levels from uninjected muscle. Similar results were seen at 30 days, with a slight decrease at 60 days. Combining notexin and ADV myophos resulted in 40 times the glycogen phosphorylase activity at 10days, however, at 30 and 60 days results were similar to those obtained for ADV myophos alone. When either notexin or ADV LacZ alone were injected, average glycogen phosphorylase activity was comparable to that from ADV myophos only sites. Phosphorylase expression was not seen in uninjected muscle but was evident at sites injected with notexin. This is likely to be due to upregulation of another ovine phosphorylase isoform and is currently being investigated.

### *P3* HIGH RESOLUTION PCR-BASED METHODS FOR DETECTION OF GENE CORRECTION AT THE *DYS* LOCUS IN THE *MDX* MOUSE MODEL OF DUCHENNE MUSCULAR DYSTROPHY

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The *mdx* mouse model of muscular dystrophy harbours a point mutation in exon 23, resulting in a nonsense mutation. Our laboratory is currently using Single Fragment Homologous Replacement (SFHR) to convert the *mdx* point mutation to wild type. To assess the success of *mdx* gene conversion to wild type using SFHR a number of PCR based methods were developed. DNA and RNA transcripts were both assessed for the presence of the wild type allele in treated *mdx* cells.

Analysis of DNA was carried out using Allele specific PCR (AS-PCR) and RFLP analysis of PCR products. AS-PCR could detect low levels (0.001% to 0.05%) of wild type:*mdx* sequence, but despite the sensitivity of this method the quantitative capacity is limited. However RFLP of $^{32}$P labelled PCR products allowed quantification of wild type levels down to 2% wild type.

Application of these techniques was extended to RNA analysis where cDNA copies of dystrophin transcripts yielded similar results. RT-PCR was used to generate both wt and *mdx* dystrophin cDNA, AS-PCR was then applied to a titration of wt:*mdx* cDNA. This technique showed that wt transcripts could be detected at a frequency of 1/10,000 (0.01%) and above, similar to the sensitivity of this method on DNA.

These combined methods allow high resolution screening and quantification of gene correction at both the DNA and RNA level. With the advent of gene correction methods, the efficiency of locus conversion as well as the expression of the converted gene must be efficiently and accurately characterised to establish the relationship between gene repair and expression.

### *P4* GENE THERAPY FOR HYPERTENSION: THE RATIONALE AND APPROACHES

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Primary hypertension affects up to 25% of the population. It is also one of the most important risk factors for renal failure and cardiovascular diseases. Pharmacological intervention has been relatively successful in normalizing the elevation in blood pressure associated with hypertension. However, exogenous or synthetic drugs only produce a temporary relief in blood pressure, and undesirable side effects such as sexual dysfunction, coughing, and lethargy diminish patient compliance. Somatic gene therapy may potentially provide a safe and long-lasting tool to control blood pressure without side effects.

Indeed, success in laboratory experiments suggests that gene transfer can reverse hypertension, either by inserting extra copies of genes associated with ameliorating effects or inhibiting genes associated with exacerbating effects. The antisense (AS) oligonucleotide (ODN) approach has been shown to be **e**ffective in control of blood pressure when targeted to mRNA for renin, angiotensinogen, ACE, and $AT_1$-R in 3 different animal models of hypertension. Generally, a single dose of AS ODN reduced 20 to 30 mmHg of BP within 3 to 9 hours and lasted for up to 7 days. The effect could be maintained by repeated administration. Recently, a retrovirus (LNSV) encoding an antisense $AT_1$-R injected into the newborn spontaneously hypertensive rat (SHR) has been shown to prevent the development of hypertension in adults. An adeno-associated virus (AAV) encoding antisense DNA to AGT or AT1-R demonstrated reduction and slow development of hypertension, also only with a single dose administration. Double transgenic mice (human renin and AGT) with high angiotensin II have high blood pressure, however, when these animals were treated with AAV-AT1-R-AS, a normalization of blood pressure for over six months was followed. Injection of plasmid containing atrial natriuretic peptide (ANP), or endothelial nitric oxide synthase, or human tissue kallikrein gene in SHR also effectively reduced blood pressure and a single injection reduced BP for up to 10 weeks. Therefore, all these experiments suggest the feasibility of using gene therapy as an alternative method for the control of hypertension.

The recent development of lentiviral vectors that transfer genes to dividing as well as nondividing cells efficiently in vivo and integrate into the genomic DNA of the host cell has shown considerable promises for human gene therapy. Such vectors may also be useful in the clinical therapy of hypertension in vivo.

### *P5* AMPLIFICATION OF SUICIDE CELL DEATH BY CO-TRANSFER OF HSV THYMIDINE KINASE AND VP22

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**Background** The inefficiency of Herpes Simplex Virus thymidine kinase (TK) gene transfer and toxicity of ganciclovir (GCV) at high concentrations *in vivo* limits the use of this strategy for suicide cell death in clinical settings. To overcome the problem, we have sought evidence of amplification of cytotoxicity by cotransfer of the TK gene fused with the gene encoding HSV-1

structural protein VP22, which has a remarkable ability for intercellular trafficking.

**Methods** The expression of the fusion proteins from the chimeric VP22-TK or VP22-EGFP genes was shown by Western blot and VP22 promoted TK or EGFP intercellular trafficking by an indirect immunofluorescent assay. The cytotoxicity was demonstrated by a colorimetric cell proliferation assay followed by an assessment of the bystander effect on admixtures of transfected with nontransfected naive cells.

**Results** The expression of the VP22 fusion proteins and their spread to varying numbers of bystander cells was observed in up to 30 cells, confirming VP22-assisted intercellular trafficking of the fusion proteins. This VP22 promoted TK spreading resulted in killing by 2.5 μg/mL of GCV of virtually all cells in cultures that had been transfected at an efficiency of only 27.5%. In contrast, less than 80% of cells were killed when transfected with "tk alone" at the same efficiency. The cell killing effect was exponentially dependent on GCV concentration in cells transfected with "tk alone" at GCV concentrations between 0.25 and 0.5 μg/mL, but not those transfected with VP22-TK, probably due to the continuously variable, high sensitivity of about 50% of cells. Even at low concentration of GCV (0.2 μg/mL), the enhancement of cell killing by VP22 was four fold higher in cells transfected with VP22-TK than in cells transfected with "tk alone".

**Conclusions** VP22 enhanced intercellular trafficking of TK and amplified the TK/GCV killing effect, especially in the lower range of GCV concentrations. This offers a new approach to enhance the effectiveness of suicide cell death in gene therapy of cancers as well as some cardiovascular diseases.

### *P6* PRODUCTION OF TRANSGENIC MOUSE MODELS FOR β-THALASSAEMIA AND HAEMOGLOBINOPATHIES AS MODELS FOR GENE THERAPY

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β-thalassaemia is the most common, single gene disorder of hemoglobin synthesis. Over the past decade, various attempts at viral and non-viral based therapies have been conducted to determine effective and safe therapies for patients. Despite the identification of numerous regulatory elements in the β-globin locus during this period, there is still no gene therapy vector that can confer stable, tissue-specific and physiologically responsive long-term expression of the globin gene. Furthermore, there are no accurate transgenic animal models for specific thalassaemia mutations in the context of the intact β-globin locus. Our group is aiming to produce transgenic mice for the normal human β-globin locus and for the most common thalassaemia mutations. Such models may be used not only for the evaluation of various gene therapy approaches but also for the evaluation of pharmacological approaches aimed at overcoming the thalassaemia defect by the stimulation of the production of other β-like globin chains, or the suppression of specific types of mutations.

The creation of accurate transgenic mouse models for specific thalassaemia mutations requires the prior insertion of each of the mutations into the intact human β-globin locus. We have developed *GET Recombination*, an inducible homologous recombination system based on the simultaneous induction of the *RecE* pathway and the inhibition of the *recBCD* nuclease for the targeted modification of PACs and BACs in the recombination deficient (recA-) *E. coli* DH10B strain in which most PAC and BAC libraries are made. However, the insertion of disease-causing mutations, polymorphisms and other modifications which do not include any selectable markers and which do not leave behind any operational sequences requires a two-stage homologous recombination process. In the first stage, a cassette consisting of a counterselection marker and an antibiotic resistance gene is inserted in the region of interest. Recombinants are isolated by the use of the antibiotic selectable marker. In the second stage, the counterselection cassette is replaced by *GET Recombination* between its flanking regions and the two ends of a genomic DNA fragment which contains the desired modification, without the inclusion of any operational sequences. True recombinants are identified by the selection for the cells that have lost the counterselection marker. Recently we have used the *GET recombination* system with a tetracycline counterselection cassette to introduce the IVS I-110 (G→A) splicing mutation, the most common Mediterranean mutation, into the β-globin gene in a 200kb BAC clone. Here we demonstrate the further use of the *GET Recombination* system to introduce three mutations causing thalassaemia, Hb E (G→A), 4bp deletion (-TCTT) and IVS I-5 (G→C), which are very common in South-East Asia. Work is in progress to establish transgenic mice with the modified genomic fragments carrying these mutations. It is hoped to breed these transgenic mice with the β- and α-globin knockout mice to produce accurate transgenic models for each of these mutations and commonly occuring combinations.

### *P7* DEVELOPMENT OF AN EFFICIENT METHOD FOR THE DETECTION OF EMBRYONIC AND FOETAL HAEMOGLOBIN INDUCERS FOR THE TREATMENT OF B-THALASSAEMIA

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β-thalassaemia is one of the most common inherited genetic disorders and is characterised by a reduction or absence of β-globin chain synthesis. Free or unpaired α-globin chains then aggregate and precipitate within red cells, resulting in hemolysis and severe anaemia. However, individuals with hereditary persistence of foetal haemoglobin (HPFH), a genetic condition that results in maintenance of a high level of γ-globin chain synthesis in adult life, are able to compensate for the imbalance between the α-globin and β-globin chains. There is also experimental evidence that increased production of ε-globin chains may compensate for the imbalance between α– and β–globin chains. The subject of current interest is whether pharmacological or genetic agents can be used to upregulate ε-globin or γ-globin synthesis in individuals with β-thalassaemia. A number of compounds including sodium phenylbutyrate and isobutyramide have been identified as compounds that can increase foetal haemoglobin synthesis, but drug toxicity, low specificity and efficacy limit their use.

High throughput screeninjg for haemoglobin inducers has been hampered by the lack of *in vitro* and *in vivo* techniques. In this study we describe the development of a physiologically relevant assay system which can be used to screen for potential inducers of embryonic and foetal haemoglobin. Using *GET Recombination*, an inducible homologous recombination system developed in our group, we were able to create EGFP reporter constructs cloned within the human β-globin locus as a 205kb BAC. The EGFP cassette was inserted at the start of each of the ε–, $^{A}$γ–, $^{G}$γ–, δ–, and β-globin genes. Expression of EGFP thus reflects globin chain synthesis, and therefore the constructs can be used to evaluate globin gene expression in erythroid cells. We transiently transfected EGFP-modified β-globin BAC into the erythroleukemia cell lines K562 and K562-expressing EBNA1 (KEB). We found that the transfection efficiency of K562 cells with EGFP modified BACs was very low (<0.1%), whereas the transfection efficiency of KEB cells was significantly higher (10%). In this study, we report the transfection efficiency and responsiveness of the EGFP-modified ε–, $^{A}$γ–, $^{G}$γ–, δ–, and β-globin constructs in KEB cells following transient transfection, and haemin induction. We demonstrate that EGFP expression from the EGFP-modified ε–, $^{A}$γ–, and $^{G}$γ-globin constructs is significantly enhanced with haemin induction, resulting in a 50% increase in the number of transfected KEB cells, which were expressing high levels of EGFP. EGFP expression of EGFP-modified δ– and β-globin constructs following transfection and haemin induction resulted in only low levels of EGFP expression. We are currently evaluating KEB clones stably transfected with EGFP-modified ε–, $^{A}$γ–, $^{G}$γ–, δ–, and β-globin BACs.

Our results indicate that the use of an EGFP reporter gene under the regulatory elements of each of the globin genes may provide a simple assay system to detect and evaluate compounds that can increase ε-, and γ-globin gene expression under physiologically relevant conditions.

## *P8* DELIVERY OF EBV-BASED GLOBIN BACS INTO ERYTHROLEUKEMIC CELLS

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One of the major limitations to successful gene therapy is the inability of current vectors to provide a continuous and regulated level of gene expression. A possible solution to this problem is the use of replicating episomal vectors. Episomal vectors are ideal because they have the capacity to carry large human genomic inserts (100-300kb). These inserts are large enough to allow the recovery of most genes together with their long-range control elements, which are essential for regulated gene expression in a tissue-specific manner. The best-studied episomal system is based on Epstein-Barr virus (EBV) replication. EBV-based vectors contain an origin of plasmid replication (*ori-P*) and the gene for the EBV nuclear antigen-1 (EBNA-1). EBNA-1 is the only EBV viral protein encoded by the episome and it plays an essential role in extrachromosomal replication, episomal maintenance and nuclear localization. However, delivery of large EBV-based BACs (EBACs) remains a challenge. Compounding the problem is the fact that certain cell types are resistant to transfection. This is particularly the case with erythroleukemic cell lines, such as K562.

This study investigated the factors limiting the transfection efficiency and nuclear localization of 200kb EBACs in K562 cells. A K562 cell line constitutively expressing the EBNA-1 protein (KEB cells) was also created. All the EBV-based vectors used contained the enhanced green fluorescent protein (EGFP), as a reporter gene. Transfection efficiency was determined by flow cytometry. The transfection efficiency of KEB cells was significantly higher (10%) than the transfection efficiency of K562 cells (<0.1%). Cellular localization of EBACs was determined by rhodamine labelled DNA (R-DNA). Analysis by confocal microscopy showed that both cell types, transfected with R-DNA, accumulated equal amounts of R-DNA/liposome complexes in the cytoplasm. Nuclear localisation was determined by fluorescent *in situ* hybridisation (FISH). Importantly, FISH confirmed that episomes were only present in the nucleus of KEB cells. These results clearly demonstrate that vector uptake into the cytoplasm by K562 and KEB cells does not correlate with transfection efficiency. Moreover, cells that constitutively express EBNA-1 showed significantly higher transfection efficiency, which was associated with nuclear localisation of DNA. It is hypothesised that in the cytoplasm EBNA-1 binds to *ori-P* DNA sequences to facilitate the nuclear entry of EBACs. This suggests that EBNA-1 negative cells cannot mediate nuclear import of EBACs effectively; hence the majority of EBAC DNA remains in the cytoplasm.

KEB cells have since been used to generate stable cells-containing functional genomic inserts. Furthermore, other cell types have also been created which constitutively express EBNA-1. These cells also show up to a 100-fold increase in transfection efficiency. This suggests that monitoring of gene expression of large EBACs in a variety of cell types may now be possible, by simply expressing the EBNA-1 protein in these cells. Currently, investigations are under way to determine the extent to which the DNA sequence contributes to transfection. It is hoped that a better understanding of the cellular mechanisms by which EBACs can be transferred into the nucleus will help to develop improved gene therapy vectors.

### *P9* DEVELOPMENT OF PHYSIOLOGICALLY RELEVANT MODELS FOR FRIEDREICH ATAXIA

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Friedreich's ataxia (FRDA) is the most common inherited ataxia. 98% of FRDA chromosomes harbour an abnormal trinucleotide repeat sequence (TRS) within the first intron of the FRDA gene. Unlike other TRS related diseases, the repeat sequences occur within an intron rather than an exon. The GAA repeat expansion causes FRDA by reducing frataxin mRNA levels, resulting in insufficient frataxin, a nuclear encoded mitochondrial protein. This leads to the progressive neurodegeneration and cardiomyopathy characteristic of FRDA. Since most FRDA patients have intact *frataxin* coding sequences, understanding the reasons for the reduction of the transcription levels caused by the GAA repeats will provide a basis for the targeting of therapeutic intervention. A number of current models show that frataxin homologues have similar functions in different organisms, which may be related to mitochondrial iron transport. Human frataxin has also been shown to be able to complement many of the phenotypes of the yeast mutant. However the pathogenesis of human FRDA is unclear, and the regulation of the gene remains unknown.

Our aim is to make accurate mouse models harbouring the entire human genomic locus carrying the GAA expansion from patient DNA. This is necessary for proper evaluation of therapeutic approaches under physiologically relevant conditions. A fully sequenced bacterial artificial chromosome clone, containing exons 1-5b of the frataxin gene and adjacent flanking sequences (pFraBAC) has been chosen for this study. The genomic fragment (187kb) was isolated from pFraBAC and used to generate transgenic mice by pronuclear microinjection. Three founder lines have been established. Characterisation of these transgenic mice has been carried out by PCR for end sequences to confirm the integrity of the genomic fragment, as well as by Southern blot to determine copy number and by FISH to determine chromosomal localisation relative to the endogenous locus. Work is in progress to breed these mice with mice carrying a knockout deletion mutation in the mouse frataxin locus, which results in embryonic lethality in the homozygous form. It is hoped that these studies will demonstrate that the human frataxin locus, as present in our pFraBAC clone, contains all necessary sequences to complement the mouse knockout mutation.

Two-stage *GET Recombination*, an inducible homologous recombination system that has been developed in our laboratory is being used to insert the GAA expansion, cloned directly from patient DNA, into pFraBAC. In the first stage a counterselection cassette is being inserted into intron 1 of the FRDA locus, while in the second stage a fragment carrying the GAA expansion will be used to replace precisely the counterselection cassette (pFraBAC::GAA). The GAA construct will be used to generate an accurate transgenic mouse model of FRDA. In addition, insertion of a reporter gene downstream of the GAA construct will be used to establish a high throughput screening assay system for agents that may enhance transcription through the expansion region. Any promising compounds will be tested in the transgenic mouse model.

### *P10* DEVELOPMENT OF EGFP REPORTER CONSTRUCTS IN THE FRIEDREICH ATAXIA LOCUS BY HOMOLOGOUS RECOMBINATION.

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Friedreich Ataxia (FRDA), an autosomal recessive neurodegenerative disease, is the most common inherited ataxia. Expansion of a GAA trinucleotide repeat within the first intron of the *FRDA* gene is found in 98% of FRDA patients. The GAA expansion is postulated to form a triplex DNA structure which impedes the transcription machinery. Reduction in the level of *FRDA* mRNA results in a severe reduction of frataxin protein in FRDA patients. The exact physiological function of the mitochondrial-located frataxin is unknown but it is likely to be involved in iron homeostasis. Lack of frataxin results in oxidative stress mostly affecting large neurons and heart muscle. While gene therapy approaches may eventually prove amenable for treating FRDA and related diseases, there are still numerous obstacles to overcome before such procedures become feasible. Therefore, our group is aiming to develop assays for the evaluation of pharmacological approaches to FRDA treatment, by utilising the increasing knowledge of the structure, regulation and function of the *FRDA* locus.

Our objective is to design reporter constructs to monitor *FRDA* expression in the context of the entire *FRDA* gene locus and its flanking regions in relevant cell lines. Two types of investigation are then possible. Firstly, a construct containing a reporter within the normal *FRDA* locus will permit detailed analysis of endogenous regulatory elements controlling *FRDA* expression. To date, there is no information available on the position of the promoter or

any other *cis*-acting regulatory elements of the *FRDA* locus. Secondly, a construct containing a reporter within an FRDA locus harbouring a GAA expansion will be used to screen for compounds able to upregulate *FRDA* expression, either by affecting regulatory factors or by reversing the inhibitory effect of the triplex structure.

A fully sequenced bacterial artificial chromosome (BAC) clone containing the entire *FRDA* locus and surrounding sequences was used to create fusions with the reporter, EGFP. Two in-frame fusions between the *FRDA* and EGFP genes were created. One is within exon 2 of the FRDA gene forming a fusion containing a truncated N-terminal portion of frataxin, and another is within exon 5a immediately following the final codon in the coding sequence, to form a fusion containing the entire frataxin protein fused to EGFP. Insertion was carried out using the *GET* homologous recombination system developed in our laboratory. Cassettes consisting of the EGFP gene linked to a kanamycin/neomycin resistance determinant were amplified by PCR using primers carrying targeting sequences corresponding to the site where insertion of the cassette was to take place on the *FRDA* locus. Insertion of each cassette into the correct targeted position was confirmed by short and long range PCR analysis using primers within the *FRDA* locus and the cassette, by DNA sequencing of the junctions, and by Southern blot analysis using various probes specific for each of the targeted *FRDA* gene exons and for the cassettes. Recombinant clones were also 'fingerprinted' by restriction endonuclease digestion and shown to contain no unwanted rearrangements. It is anticipated that expression studies with the modified frataxin clones, as well with clones carrying various deletions in the flanking regions will lead to a delineation of the regulatory elements determining the tissue and developmental specificity of frataxin expression.

### *P11* GENE THERAPY FOR INBORN ERRORS OF METABOLISM: PAST, PRESENT AND FUTURE.

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Inborn errors of metabolism are a group of individually rare single gene disorders in which the disease mechanism characteristically results from absent or reduced enzyme activity. This leads to either a deficiency of substrate or accumulation of intermediate metabolites, which may be toxic or disrupt cell function through storage. Many of these disorders have devastating clinical effects and treatment where feasible requires intensive lifelong dietary and pharmacological management. Despite such treatment modalities, morbidity and mortality frequently remain high. Whilst there has been progress in the molecular and biochemical understanding of these disorders, development of improved conventional treatments has been slow. It is postulated that a small percentage of enzyme activity correction would be sufficient for significant improvements in clinical status. Gene and Cell therapies aimed at replacing enzyme function would provide the ultimate therapy for these disorders.

Development of such approaches had been limited by genes not cloned and a lack of adequate animal models. These issues are being addressed with the development of a number of animal models. We are currently developing both a transgenic and knockout mouse model for Methylmalonic Aciduria (MMA). We plan to cross the knockout with strains carrying the normal human MMA locus looking for correction. Correction will also be attempted by ex vivo transfer of the normal human locus into the mouse hepatocytes. It is anticipated results from this model would be applicable to other organic acidaemias.

Here we summarise the current state of cell and gene therapy for inborn errors of metabolism and discuss prospects for the future.

### *P12* *IN VITRO* AND *IN VIVO* DOWNREGULATION OF MRP1 BY ANTISENSE OLIGONUCLEOTIDES: A POTENTIAL ROLE IN NEUROBLASTOMA THERAPY

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Neuroblastoma is the most common solid tumour of childhood. It is thought to arise from the anomalous arrest of multipotential embryonal cells of neural crest origin during differentiation. In otherwise good prognosis patients, the presence of multiple copies of the N-myc oncogene in tumour biopsies, is sufficiently predictive of poor outcome to warrant the intensification of first line therapy. Relapse and drug resistance in neuroblastoma cells with amplification of the N-myc oncogene presents a *fait accompli*. This may in part be due to the reported correlation of MRP1 (multidrug resistance protein -1) expression with the amplification of N-myc.

In this study, MRP1 antisense oligonucleotides (ASO) (ISIS 7597) were used in a NOD-SCID mouse-human xenograft model of neuroblastoma to down-regulate MRP1. The MRP1 ASO reduced protein levels of MRP1 to an average of 40% of the nil treated controls (p=0.007). There was significant chemosensitisation to single agent chemotherapy: VP16 (etoposide) at doses between 1mcg/mL and 10mcg/mL, which was not seen with control oligonucleotides. In comparison, MDR1-ASO (multidrug resistance gene 1) produced significant chemosensitisation only at 10mcg/mL of VP16. The downregulation of MRP1 was also associated with an increase in tumour cell death (49% increase in apoptosis index p=0.0012) and a reduction in cell turnover (40% reduction in mitotic index p=0.004) which was not seen with any other oligonucleotide. This work is the first to concurrently explore the effects of downregulation of MRP1 and *MDR1*

in neuroblastoma cell lines. The potential involvement of MRP1 in the regulation of apoptosis and cell cycle progression, presents a fresh avenue for investigation of the biological consequences of MRP1 expression which occurs in many tumour cell types. It also provides rationale for the use of gene-therapy adjuvants such as antisense oligonucleotides, in the treatment of those malignancies.

### *P13* CONSTRUCTION OF NEOCENTROMERE BASED HUMAN MINICHROMOSOMES WITH POTENTIAL THERAPEUTIC APPLICATIONS

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We have constructed a series of minichromosomess from a mardel(10) marker chromosome containing a previously characterized human neocentromere. These minichromosomes range in size from ~0.7-1.8 Mb and contain single-copy intact genomic DNA from the 10q25 region. Two of these neocentromere-based minichromosomes (NC-MiCs) appear circular while one is linear. All demonstrate high stability in both structure and mitotic transmission in the absence of drug selection. Presence of a functional neocentromere is shown by binding a host of key centromere-associated proteins. These NC-MiCs represent the first examples human minichromosomes lacking centromeric repeats making them readily amenable to full sequence characterisation. Current studies are aimed at incorporation of potential therapeutic genes into the NC-MiCs followed by transfer into various cell types to test the feasibility of NC-MiCs as an *ex vivo* gene delivery system.

### *P14* DENDRITIC CELLS, BUT NOT MACROPHAGES, EXHIBIT A CYTOPLASMIC FOCAL CONCENTRATION OF THE CD86 COSTIMULATORY PROTEIN.

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Immune gene therapy strategies for cancer are designed to boost the immune system to eliminate the target tumour cell. These strategies have included the translocation of cytokines or costimulatory molecules (like CD80 or CD86), using retroviral gene transfer, to target cells. *In vivo*, cell mediated immunity relies on the ability of dendritic cells (DCs) to provide the costimulatory signal (CD80, CD86) and interact with tumour antigen-specific lymphocytes in the lymph nodes. The (tumour) antigen-specific signal in combination with the costimulatory signal from dendritic cells signal the cytotoxic T lymphocytes to expand and kill the tumour cells.

In attempting to test the strategy of enhancing tumour immunogenicity by transduction of cells to express CD80 or CD86 we made an interesting observation. We observed an unexpected focal concentration of the CD86 protein in the cytoplasm of the CD86 transduced (CEM) cells. Extending these studies to the analysis of endogenous CD86 gene expression in normal blood mononuclear cells (PBMCs) a similar intracellular focal concentration of CD86 was observed in monocytes (which constitutively express CD86). The intracellular concentration of CD86 in monocytes was not due to storage within the Golgi apparatus, and relied on intact microtubules to retain structural integrity.

We present further studies which examined the cellular localisation of CD80 and CD86 costimulatory molecules in human monocyte derived antigen presenting cells - macrophages (after culture in media) and DCs (after culture in IL4 and GM-CSF supplemented media). Unlike macrophages, dendritic cells (like monocytes) constitutively express the CD86 costimulatory molecule. Moreover, in a subset of DCs (and in DCs after further maturation with TNFα) there was a prominent focal concentration of CD86 in the cytoplasm which was never observed in macrophages. CD80 was not expressed on macrophages and at very low levels on a small proportion of DCs. Furthermore, there was no evidence of an intracellular focal concentration of CD80 protein in either antigen presenting cell. Drug and immuno-electron microscopy studies confirm that the focal concentration of cytoplasmic CD86 in DCs was not Golgi associated.

The evidence we present in macrophages (which are primarily cells which present antigen) and DCs (antigen presenters but also capable of initiating immune responses) supports our previous findings in monocytes that the presence of an intracellular reservoir of CD86 protein reflects the importance of this costimulatory molecule in those cells which are important in initiating lymphocyte mediated immune response.

### *P15* SUSCEPTIBILITY OF INSULIN-SECRETING HEPATOCYTES TO TOXICITY OF AUTOIMMUNE CYTOKINES.

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The liver has been suggested as a suitable target organ for reversing Type 1 diabetes by gene therapy; however whether insulin-secreting hepatocytes are resistant to the autoimmune process which kills pancreatic β cells is unknown. One of the mechanisms by which β cells are

killed is by the release of the cytokines interleukin-1β (IL), tumour necrosis factor (TNF) and interferon-γ (IFN) by immune cells. To test the effect of these cytokines, they were added individually and in combination for 14 days to a genetically modified human hepatocyte cell line (HEP G2ins/g). This cell line has the ability to synthesise, store and secrete insulin in a regulated fashion to a glucose stimulus and has receptors for the cytokines. The cytokine concentrations used were 10-1000U/ml for human TNF and IFN and 20-2000pg/ml for IL.

The cells were resistant to the cytokines during the first 6 days as determined by cell viability (propidium iodide fluorescence assay) and mitochondrial function (MTT assay). However by day 10, IL 1000pg/ml and the triple cytokine combination (1000U/ml TNF and IFN, and 2000pg/ml IL) were toxic to the cells with the triple combination being the most toxic. Acute insulin secretion in response to glucose, daily insulin secretion, insulin content and insulin mRNA measurements were not adversely affected up to day 6 but detrimental effects were observed from day 10. Addition of like cytokines to the parent cell line HEP G2, the rat hepatoma cell line H4-II-E, rat primary liver cells and human fetal liver cells showed the same results as with the transfected cells, namely lack of adverse effects up to day 6 but toxicity from day 10. In contrast, cells of the beta cell line NIT-1 were mostly destroyed during 3 days of culture with the cytokines.

These results offer hope that liver cells *in-vivo* which are made to produce insulin by gene therapy are less likely to be destroyed by cytokines released during autoimmune destruction.

### *P16* RETROVIRAL TRANSDUCTION OF BONE MARROW STEM CELLS TO DELIVER HUMAN (PRO)INSULIN

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Pancreatic β cells are destroyed by an autoimmune process in type I diabetes. A potential therapeutic approach to this disease is to manipulate somatic cells to function as β cells. Therefore the possibility of using the bone marrow stem cells as an alternative source for β cell replacement, and more specifically, the haematopoietic stem cells and the non-haematopoietic mesenchymal stem cells, was investigated. Two retroviral constructs were designed and used, that contained the cDNA of (1) human insulin, and (2) the human insulin and glucose transporter 2 (Glut2). Both haematopoietic progenitor cell lines K562 (CD34-) and Tf-1 (CD34+) representing the haematopoietic component of the bone marrow, and the primary human mesenchymal stem cells, were transduced with both constructs. Insulin synthesis, secretion and storage were assessed. All transduced cells tested positively for the insulin gene by RT-PCR. (Pro)insulin was secreted from all 3 cell types transduced with the human insulin gene alone at 120-220 μU per $10^6$ cells per day. Comparable levels of (pro)insulin were also secreted from cells transduced with the insulin and Glut-2 genes. Radioimmunoassay of acid-ethanol cell lysate and immunohistchemical staining for insulin were negative for all transduced cells, indicating lack of insulin storage. In conclusion, we have demonstrated that, by insertion of the human insulin gene, bone marrow stem cells of both haematopoietic and non-haematopoietic lineage have the capacity to synthesise and secrete insulin, but lack the ability to store this hormone.

### *P17* DELIVERY OF A SOLUBLE VEGF RECEPTOR USING RECOMBINANT AAV AS A THERAPY AGAINST OCULAR NEOVASCULARIZATION

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Vascular endothelial growth factor (VEGF) is believed to be the major mediator involved in the growth of new blood vessels. There has been evidence of upregulated VEGF expression in proliferative diseases of the eye including diabetic retinopathy and age-related macular degeneration. We have constructed a recombinant adeno-associated virus (rAAV) encoding a soluble VEGF receptor, sFLT-1 and determined its ability to inhibit proliferation of vascular endothelial cells in a rat corneal neovascularization model.

Vectors AAV-CMVsflt and control AAV-CMVgfp were injected intracamerally into the anterior chamber of RCS-$rdy^+$, $p^-$ rats. The central corneas of injected and non-injected eyes were cauterized using silver nitrate applicator sticks at 3 weeks post-injection and the corneas were examined several days after cautery.

Slit-lamp examination of AAV-CMVsflt-injected eyes indicated no new blood vessels had formed after cautery. However, uninjected and AAV-CMVgfp-injected control eyes showed extensive neovascularization extending from the limbus to the cautery lesion. This was confirmed by histologic examination of paraffin-embedded eye sections, which revealed presence of numerous blood vessels in uninjected and AAV-CMVgfp-injected eyes, but not in AAV-CMVsflt-injected eyes.

These results indicate that AAV-CMVsflt can inhibit VEGF-induced corneal neovascularization. We have previously demonstrated the ability by rAAV vectors to mediate long-term transgene expression in the retina. Therefore, AAV-CMVsflt may also provide a potential long-term treatment against retinal and subretinal neovascularization.

### *P18* ADENOVIRUS-MEDIATED SFLT-1 INHIBITION OF EXPERIMENTAL CORNEAL ANGIOGENESIS.

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Pathological angiogenesis, or the production of new capillary vessels from pre-existing vasculature, within the eye is a serious event that often leads to blindness. Upregulation of vascular endothelial growth factor (VEGF) has been linked to neovascularization in the eye, suggesting that it could be a suitable target to inhibit angiogenic changes. This work investigated whether the presence of a proven anti-angiogenic factor, the soluble variant of the VEGF receptor sflt-1, in the anterior chamber is sufficient to inhibit new vessel formation in the cornea in an animal model of corneal neovascularization. A recombinant adenovirus vector that can mediate efficient *in vivo* gene transfer and expression into ocular cells was selected as a delivery agent. We have shown that following the injection of Ad.βgal into the anterior chamber of normal and cauterized rat eyes, corneal endothelial cells and cells of the trabecular meshwork were efficiently transduced and that βgal expression was maintained up to 10 days post-injection. Cauterization significantly increased the amount of immunoreactive VEGF in vehicle- or Ad.null-injected animals (t-test, $p \leq 0.001$ and $p \leq 0.001$, respectively). However, when cauterization was combined with Ad.sflt injection there was no statistically significant increase in the amount of immunoreactive VEGF ($p \geq 0.12$). The injection of Ad.sflt into the anterior chamber slowed down or inhibited VEGF-induced angiogenic changes. Following cauterization, 100% of uninjected and vehicle-injected and 82% of Ad.null-injected animals developed moderate to severe corneal angiogenesis in contrast to 18% of Ad.sflt-injected animals. These *in vivo* results suggest that the transient presence of anti-angiogenic agents in the anterior chamber can be successfully used to inhibit the development of corneal angiogenesis.

### *P19* EVALUATION OF THE EFFICIENCY AND INTRAOCULAR SAFETY OF RECOMBINANT ADENOVIRUS-MEDIATED GENE DELIVERY IN THE RETINA

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This study aimed to re-evaluate the longevity, particularly, the intraocular safety of recombinant adenovirus (rAd) mediated gene delivery following subretinal injection, and to prolong transgene expression through combination of two synergistic immunosuppressants. A rAd vector carrying green fluorescent protein (GFP) gene was delivered subretinally in the rat eye. GFP expression was monitored in real time by fundus fluorescent photography. Intraocular safety was examined by observation of changes of retinal pigmentation, cell infiltration in virus contacted area, immunophenotyping for $CD4^+$ and $CD8^+$ cytotoxic T lymphocytes, $CD68^+$ macrophages, histology and dark-adapted electroretinography (ERG). Two synergistic immunosuppressants, cyclosporin A (CsA) and rapamycin (RAPA) were used alone or in combination to prolong transgene expression by temporary immunosuppression. GFP expression peaked on day 4, dramatically decreased on day 10 and was not detectable on day 14. The decreased GFP expression was coincident with cell infiltration in virus contacted area. Immunostaining revealed that the infiltrating cells were $CD4^+$ and $CD8^+$ cytotoxic T lymphocytes and $CD68^+$ macrophages. Clumped retinal pigmentation and decreased b-wave of dark-adapted ERG were observed at 3~4 weeks post injection. Histology confirmed rAd-induced retinal degeneration. Transient immunosuppression by CsA and RAPA, either alone or in combination, improved transgene expression, with the combination the most efficient, extending transgene expression from 10 days to at least 10 weeks (6-fold). The combined immunosuppression attenuated but did not retard the rAd-induced retinal damage. Our studies demonstrate that rAd-mediated transgene expression following subretinal delivery is short-term and toxic to the retina. Combination of CsA and RAPA may act as an immunosuppressive adjunct to prolong rAd-mediated gene transfer in the eye. The intraocular safety of rAd following subretinal delivery should be carefully considered before clinical application.

### *P20* EVALUATION OF PERSISTENCE, BIODISTRIBUTION AND GENE EXPRESSION OF AN OVINE ADENOVIRUS (OADV) IN MOUSE TISSUE BY TAQMAN REAL TIME PCR.

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Gene therapy is a rapidly developing area, which provides the promise of a viable option for the treatment of localized or regional cancers. Human adenovirus (hAd) mediated gene transfer is one such method being investigated and has become a widely used tool for use in gene therapy. However its successful use in clinical trials is hampered by the pre-existing immunity encountered in human tissues due to the human origin of the virus. We have developed a modified ovine adenovirus containing an E. coli purine nucleoside phosphorylase (PNP) gene

cassette for use in tissue targeted pro-drug gene therapy, which offers the advantage of not encountering pre-existing immunity in human tissues. We are currently investigating the use of this virus in the treatment of prostate cancer.

An important component in the evaluation of any method of gene therapy is the determination of the delivery vector's persistence, bio-distribution and expression in a broad range of tissue types in an animal model system. To meet this end we have developed a sensitive Taqman real time assay using the Applied Biosystems 7700 sequence detection system to measure both the presence and expression of the PNP gene cassette in C57BL/6 mice following intravenous or intraprostatic administration of OAdV.

As any nucleic acid isolation method produces variable yields and as real time PCR is extremely sensitive, it is also vital that adequate internal DNA and mRNA controls are included so that results can be related back to an equal amount of starting material or cell number. To address this need we have identified and designed primer/probe sets to the mouse Parathyroid hormone-like peptide (Pthlh) genomic sequence and the Hypoxanthine phospho-ribosyltransferase (HPRT) mRNA sequence as DNA and RNA controls, respectively. Where possible, regions of identical homology between the mouse and human sequences have been chosen in order to add versatility to the system.

## *P21* VIRAL TRANSDUCTION OF HUMAN PROSTATE TISSUE

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Aims: In prostate cancer gene therapy, the ability of delivery vehicles to infect a wide range of human prostate cancer cells is critical. We are developing gene therapy strategies for advanced prostate cancer using replication-incompetent human type-5 adenovirus (Ad5) or an ovine adenovirus (OAdV). Although such viruses can infect some human prostate cancer cell lines, these may represent only a very specialised subgroup of prostate cancers. To examine viral infectivity of human prostate tissue, samples were obtained from patients undergoing radical prostatectomy (RP) or transurethral resection of the prostate (TURP) and placed into culture for subsequent Ad5 or OAV transduction.

Methods: Recombinant OAdV and Ad5 viruses were constructed bearing green fluorescent protein (GFP) as a reporter under the transcriptional control of the CMV and elongation factor-1 promoters, respectively. RP and TURP tissues were placed into culture and transduced with varying concentrations of Ad5GFP or OAVGFPin the presence or absence of several cationic lipid formulations. Reporter expression and tissue viability were assessed by fluorescence microscopy.

Results: Both Ad5 and OAV in the presence and absence of lipid were able to transduce human prostate tissue representing a range of prostate samples from BPH to high-grade adenocarcinoma as evidenced by surface fluorescence of the reporter gene product.

Conclusions: OAdV and Ad5 may provide for suitable gene delivery to a broad spectrum of prostate cancers. Significantly, RP and TURP culture may provide a system for the evaluation of human gene therapeutics in a diversity of relevant primary tissue prior to clinical trial procedures.

## *P22* SAFETY FEATURES OF AN OVINE ADENOVIRUS VECTOR

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Ovine adenovirus (OAdV) is a gene delivery vector that is derived from a genus of adenoviruses that is distinct from all known human adenoviruses. OAdV lacks an obvious E1A gene and has several distinct genes and capsid proteins. It is necessary to assess the safety of OAdV, particularly with respect to its ability to transform cells and to interact with or complement the replication of other adenoviruses, prior to its application in the clinic. Transforming ability was examined in primary rat cells using human AdV5 and OAdV623, a recombinant that carries an expression cassette inserted at a non-essential site in the complete OAdV genome. Whereas AdV5 E1A/B sequences produced transformed colonies within 14-21 days, no such colonies were observed with OAdV623 after 30 days.

In addition, human cell lines that can be infected by both OAdV623 and human AdV5 were transduced singly or with both viruses and the development of cpe was monitored. Virus was passaged three times and at each step viral DNA was extracted and analysed to determine whether replication had occurred. AdV5 replicated efficiently in the three cell types examined but no aberrant genome fragments were detected in the presence of the OAdV623 genome. Moreover, OAdV623 did not replicate in human cells, even in the presence of replication competent AdV5.

The data indicate that OAdV is an abortively replicating gene delivery vector that exhibits desirable safety features in the systems that have been examined.

### *P23* GENERATION OF A TETRACYCLINE-REPRESSIBLE RETROVIRAL VECTOR FOR *IN VIVO* USE

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The ability to regulate gene expression *in vivo* would have widespread benefit in the field of gene therapy, particularly for genes whose over-expression or sustained expression is detrimental. To date, reproducible regulation *in vivo* without selection has proved difficult to achieve. Variable regulation has predominantly been due to neighbouring enhancer effects on the vector minimal promoter causing high levels of basal transcription. Such enhancer effects also preclude construction of single vectors carrying both a gene of interest under control of a regulatable minimal promoter and the transactivator protein driven by a strong promoter/enhancer. This problem may be overcome, in theory, by the use of "insulator" or "boundary" elements to isolate the minimal promoter from neighbouring effects.

An alternative approach to gene transactivation is that of repression by steric hindrance. We are attempting to generate a retroviral vector in which the LTR promoter is repressible by tetracycline. To do this, we have introduced tandem tetracycline operator (tetO) sites between the TATA box and transcription start site of the mouse stem cell virus (MSCV) LTR. When the LTR(tetO) modified promoter is used to drive the luciferase gene in a plasmid context following transient transfection of a HEK293 cell line stably over-expressing the tetR, addition of tetracycline results in 500-750 fold derepression of luciferase gene expression. Hence, the LTR can be effectively repressed by the tetR in the absence of tetracycline. We have introduced a tetR cassette into a modified repressible MSCV retroviral construct, in which the LTR(tetO) drives an IRES-linked GFP gene, to generate a single regulatable vector system. This vector is currently being tested for its ability to regulate expression in response to tetracycline. Such a vector could be of significant benefit for basic and applied research *in vitro* and *in vivo*.

### *P24* HIGH EFFICIENCY OF MURINE STEM CELL RETROVIRAL VECTOR (MSCV) IN CONFERRING STABLE TRANSGENE EXPRESSION IN THE PROGENY OF PRIMITIVE HEMATOPOIETIC STEM CELLS (PHSC) *IN VITRO* AND *IN VIVO*

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A major goal of gene therapy is the rescue of deficient gene function by an exogenous normal functional gene through an efficient delivery system. Among all the gene delivery systems currently being tested, retroviruses are still the choice vehicles to introduce new genetic materials into primitive hematopoietic stem cells (PHSC) that are rare in normal bone marrow or peripheral blood after treatment with cytokine (e.g. G-CSF).

One of the major obstacles in using Moloney Leukemia virus-based (MuLV) retroviral vectors as robust vehicles for gene therapy is their relative inconsistency in conferring stable long-term transgene expression *in vivo*. In a number of studies, MuLV-LTR driven transgene expression declined as a function of time after transplantation. Others reported a high frequency of gene silencing events during *in vivo* differentiation of the 'transduced' PHSC.

Mouse Stem Cell virus-based (MSCV) retroviral vectors have recently been developed and are now widely used. However the use of MSCV based retroviral vector did not always show significant advantages over the MuLV based vector when comparing results from individual groups. Complicating the interpretation of these results is the lack of information on the clonal make up of the engrafted donor derived cells. So the observation of decline in the number of transgene-expressing cells may be due to (i) retroviral gene repression or (ii) clonal succession between expressing and non-expressing clones or (iii) a combination of both scenarios. Here we report preliminary results on our systematic clonal analysis on the efficacy of MSCV retroviral vector in sustaining stable transgene expression in the progeny of the transduced PHSC using eGFP as the reporter gene.

Our data showed that MSCV retroviral vector conferred stable multi-lineage transgene expression from the transduced PHSC both *in vitro* and *in vivo*. The decline in circulating peripheral blood cells is due to the decline of donor-derived cells rather than transcription repression of the 'retrovirally-transduced' gene. In addition, our studies suggest the presence of a novel short-term 'erythropoietic' repopulating cell subset. These preliminary observations support the notion that MSCV retroviral vector is an ideal basic vector system for genetic manipulation of the PHSC.

## *P25* CONCENTRATION OF LARGE VOLUMES OF HIGH TITRE LENTIVIRAL VECTORS BY POLY-$_L$-LYSINE TREATMENT

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**Introduction** Lentiviral vectors pseudotyped with Vesicular Stomatitis Virus glycoprotein (VSV-G) are emerging as the vectors of choice for *in vitro* and *in vivo* gene therapy studies. However, current method for harvesting lentivectors relies upon ultracentrifugation at 50,000 g for 2 hours. At this ultra-high speed, rotors in use generally have small volume capacity. Therefore, preparations of large volumes of high titre vectors are not only time consuming but laborious to perform. The aim of this study was to develop a new method for harvesting high titre, large volumes of lentiviral vectors.

**Methods** Viral vector supernatant harvests from vector-producing cell (VPC) were pre-treated with various amount of poly-$_L$-lysine (PLL), and concentrated by low speed centrifugation. The method was then directly compared with ultracentrifugation. Electron microscopic analysis in combination with immunogold-labelling was carried out to elucidate the mechanism of precipitation by low speed centrifugation.

**Results** Optimal conditions were established when 0.005% of PLL (w/v) was added to vector supernatant harvests, and followed by incubation for 30 minutes and centrifugation at 10,000 g for 2 hours. Direct comparison with ultracentrifugation demonstrated that the method consistently produced larger volumes (6 mL) of high titre viral vector titre at $1 \times 10^8$ transduction unit (TU)/mL from as much as 3,000 mL of supernatant in one round of concentration. Electron microscopic analysis showed that PLL/viral vector formed small aggregates, which probably resulted in easy precipitation at low speed concentration, a speed does not usually precipitate viral particles efficiently.

**Conclusions** Treatment of viral vector supernatant with PLL followed by a low speed centrifugation provided a new method for harvesting large volumes of high titre lentivectors. The method should expedite animal experiments or human gene therapy trials, in which large amount of lentiviral vectors are a prerequisite.