THE AUSTRALASIAN GENE THERAPY SOCIETY

3rd Meeting, April 30 – May 2nd, 2003

Queensland Institute of Medical Research, Brisbane

The Australasian Gene Therapy Society (AGTS) held its third biennial meeting on April 30 – May 2nd, 2003, providing an opportunity for groups across the Australasian region to present their latest contributions in gene therapy research. AGTS acknowledges and thanks the Journal of Gene Medicine for making the abstracts presented during the meeting available to the international research community.

The Organising Committee for the 3rd meeting of the society included:

Ming Wei, Brisbane
Ian Alexander, Sydney
John Rasko, Sydney
Gerald Both, Sydney
Panos Ioannou, Melbourne
Ann Simpson, Sydney
Steve Wilton, Perth
Don Anson, Adelaide
Kay Ellem, Brisbane
Malcolm West, Brisbane
John MacMillan, Brisbane
Ibrahim Diallo, Brisbane
Martin Lavin, Brisbane
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The meeting was sponsored by:

Murdoch Childrens Research Institute, Melbourne
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The Journal of Gene Medicine

Abstracts communicated by P Ioannou.

For further information, please refer to the web page of AGTS at http://www.agts.org.au

ORAL PRESENTATIONS

O1 Treatment of an Infant with Severe Combined Immunodeficiency (SCID)-X1 by Gene Therapy in Australia

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In April 2000 Alain Fischer and colleagues at L'Hopital Necker-Enfants Malades in Paris reported the successful treatment of two infants with the X-linked form of Severe Combined Immunodeficiency (SCID-X1) by gene therapy: a milestone in medical history. This condition is caused by mutations in the common γ chain of interleukin receptors 2, 4, 7, 9, 15 and 21. Affected infants commonly lack both T lymphocytes and Natural Killer cells (NK) in the peripheral blood (T^-NK^- phenotype), and have defective B cell function. IL-7 and IL-15 are known to provide survival and proliferative signals to T and NK cell progenitors, respectively, and the absence of functional heteromeric receptors for these cytokines is thought to underlie the failure of T and NK cell ontogeny. The molecular basis of B cell dysfunction is less well understood, but known to involve the absence of T lymphocyte help required for B cell maturation and class switching. Up to the recent adverse event-related suspension of SCID-X1 gene therapy trials worldwide, the French trial had been extended to a total of ten infants, including the Australian infant described here. Prior to initiation, the Australian arm of the trial was evaluated by the NHMRC Gene and Related Therapies Research Advisory Panel (GTRAP), the TGA under the CTX scheme, and the local Institutional Ethics Committee. The Australian infant is unique among those treated in that he had an atypical T^-NK^+ phenotype, the molecular basis of which (a novel 5'splice-site mutation in intron 3) is addressed in a separate abstract. The infant received 1.3 x 10^6 autologous γc-expressing (gene-modified) $CD34^+$ cells per kilogram at 9.5 months of age after *ex vivo* transduction according to the French protocol using amphotropic Mo-MLV-based retroviral vector supernatant supplied by Genopoietic (Lyon). Follow-up PCR, RT-PCR and FACS analyses have been performed on PBMC at 15 day intervals out to 90 days and monthly thereafter. Proviral γc mRNA was detected in PBMC from day 15 and proviral DNA from day 30. T cells first appeared at day 75. Concomitantly the infant began to gain weight, having previously failed to thrive, and cleared a chronic Rotavirus infection from the gut. Analysis of the T cell compartment revealed both CD4 and CD8 T cells, αβ and γδ subsets and increasing repertoire diversity as a function of time. At 11 months post-treatment, T cell counts remain below the normal range and a requirement for intravenous immunoglobulin therapy persists, albeit at reduced frequency. Studies are ongoing to map proviral integration sites (in collaboration with Christof von Kalle), look for evidence of persistent T cell production by TREC analysis, and explore the possibility that this infants atypical phenotype might be linked to the incomplete immunological reconstitution observed to date.

O2 Australian Patients in a Multi-centre Phase I/II Trial of AAV-Mediated Gene Transfer to the Liver for Severe Hemophilia B.

JEJ Rasko, K High, MA Kay, B. Glader, CS Manno, S Hutchinson, M Dake, M Razavi, R Kaye, VR Arruda, R Herzog, A McClelland, G Pearce P Rustagi, F Johnson, K Hoots, P Blatt, DGB Leonard, K Addya, B Konkle, A Chew, S Larsen, L Couto. Sydney Cancer Centre, Royal Prince Alfred Hospital and Centenary Institute, Sydney, Australia; Pediatrics and Genetics, Stanford University, Stanford, CA, USA; Children's Hospital of Philadelphia, Philadelphia, PA, USA; Radiology, Stanford University, Stanford, CA, USA; Avigen Inc, Alameda, CA, USA; University of Texas, Houston, TX, USA; Christiana Hospital, Christiana, DE, USA; Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA, USA

Gene therapy promises compelling advantages for the treatment of the X-linked bleeding disorder hemophilia, which is due to the absence of functional coagulation Factors VIII or IX. Successful pre-clinical studies of AAV-mediated gene transfer of the Factor IX (F.IX) gene in animal models have been reported as a foundation for initiation of trials in human subjects with severe hemophilia B (<1% normal F.IX). The first Phase I human trial of AAV-mediated F.IX gene transfer to skeletal muscle showed that vector administration at doses up to 1.8 x 10e12 vg/kg was safe with transduction rates comparable to animal models. Gene transfer and expression were demonstrated in all 8 subjects treated and persisted for at least 10 months post-injection. In animal studies, liver-directed gene transfer has a 10-50 fold dose advantage and reduced probability of forming inhibitors when compared to muscle delivery. With vector administration into the liver, preclinical efficacy studies have demonstrated a life-long correction of the bleeding diathesis in hemophilia B mice, and reconstitution of up to 14% of wildtype levels of canine F.IX in hemophilic dogs (average 5-8%). To date, one of the treated hemophilia B dogs followed for >4 years still maintains the initial level of F.IX following the administration of AAV-2. The current study design includes two subjects at a low dose (2 x 10e11 vg/kg), and up to four subjects each at a middle (1 x 10e12 vg/kg) and high dose (5 x 10e12 vg/kg). Subjects are infused with an AAV-2 vector containing a F.IX minigene and liver specific promoter with an intrahepatic artery injection. By comparison to the hemophilia B dog study, the high dose group in the clinical trial received a dose two-fold higher than the dose that resulted in 4 to 14% plasma F.IX levels in dogs. To ensure that minimal liver damage preexisted, three subjects with hepatitis C had liver biopsies prior to participation. To date, seven subjects have been enrolled in this trial and received AAV-2 vector, two from Australia. All four subjects in the two lower-dose cohorts tolerated the delivery procedure without incident, and the blood chemistries including the liver enzymes and blood counts remained normal after treatment. In all subjects, vector DNA was transiently detected in body fluids including the semen. Coagulation parameters including plasma F.IX levels remained unchanged in the low dose group. The highest F.IX levels to date (12%) were observed in the Australian patient enrolled in the highest dose cohort. However, one month following gene therapy asymptomatic hepatitis developed and F.IX levels declined towards baseline. We conclude that hepatic artery administration of recombinant AAV-F.IX to humans at doses up to 1 x 10e12 vg/kg was safe. Efforts must now be directed to explaining the mechanism of hepatitis induction using suitable models.

O3 FP253: A Prostate Targeted Gene-Directed Enzyme Prodrug Therapy For Androgen-Sensitive And Androgen-Independent Human Prostate Cancer

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Gene–directed enzyme prodrug therapy (GDEPT) based on the E.coli enzyme purine nucleoside phosphorylase (PNP) provides a novel strategy for treating tumours that have a low percentage of dividing cells, such as human prostate cancer. The PNP gene, controlled by a prostate-directed promoter, is delivered into the tumour by injection of a formulated ovine atadenovirus vector (FP253). Expressed PNP converts the systemically administered pro-drug, fludarabine phosphate to 2-fluoroadenine, a toxic metabolite that diffuses locally in a bystander effect that kills both dividing and non-dividing cells by inhibiting DNA, RNA and protein synthesis. FP253 showed highly selective PNP expression in prostate cancer cells compared with other cell lines *in vitro*. Following administration of FP253 to tumours in mice, PNP was expressed and remained active for over 7 days. Therapeutic efficacy in vivo was examined. A single intratumoural injection (on day 0) of 1-3 x 10^{10} particles of FP253 was followed by systemic treatment with fludarabine phosphate (at 75 mg/m^2/day) given intraperitoneally (ip) once daily on days 1-5 to nude mice bearing subcutaneous LNCaP-LN3 (androgen-sensitive) or PC3 (androgen-independent) human prostate cancer xenografts. Tumours in control mice received vehicle alone followed by saline or pro-drug ip, or FP253 followed by saline ip. GDEPT using FP253 and fludarabine was associated with a marked delay and a greater than 50% inhibition in tumour growth for both LN3 and PC3 tumours. Thus, FP253-GDEPT suppressed the growth of both androgen-sensitive and androgen-independent human prostate cancer xenografts in immune-deficient mice, suggesting that it should be appraised for use in the clinic.

O4 Gene-Directed Enzyme Prodrug Therapy (GDEPT) for Prostate Cancer in a Transgenic Mouse Model that Imitates the Development of Human Disease

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GDEPT most often uses a viral vector to deliver a non-mammalian gene (usually viral or bacterial) that encodes an enzyme capable of converting a non-toxic prodrug to a toxic metabolite in the tumour site. This approach restricts the systemic effects of cytotoxic agents. A further advantage of GDEPT is derived from a local bystander effect through which comprehensive cell killing is achieved without the need to express the gene in all cells. This is provided by the ability of the toxic metabolite to diffuse freely across cell membranes or via gap junctions, an important consideration given that *in vivo* gene transfer efficiencies are currently poor. GDEPT based on the *E.coli* enzyme purine nucleoside phosphorylase (PNP) provides a new approach for treating slow growing tumours like prostate cancer. Expressed PNP converts a systemically administered prodrug, fludarabine phosphate, to a toxic metabolite, 2-fluoroadenine. Infected and neighboring cells are killed by a local

bystander effect that results from the inhibition of DNA, RNA and protein synthesis.

These studies were carried out using the transgenic adenocarcinoma of the prostate (TRAMP) model that mimics human PCa development and progression. Control TRAMP mice were injected intraprostatically with vector vehicle and thereafter intraperitoneally with saline or fludarabine phosphate (~600mg/m^2/day) once daily for 5 consecutive days. Treated mice received a single intraprostatic injection containing 10^{10} particles of OAdV220, an ovine atadenovirus which expresses the PNP gene under the control of the Rous sarcoma virus promoter, followed by systemic fludarabine treatment. The weight of the genitourinary tract, seminal vesicles and the prostate as well as animal survival was monitored. Tumours were also analysed histologically. Preliminary studies showed that fludarabine alone caused no significant change in genitourinary tract weight in TRAMP mice. Animals injected with vector and prodrug showed a 36-47% reduction in GU tract weight and a 35-50% reduction in seminal vesicle weight in two separate experiments (ANOVA $p<0.001$). In particular, a reduction of 57% in prostate weight was observed (ANOVA $p=0.0007$). PNP-GDEPT mice also showed a survival advantage over control mice. Histological analysis suggested that the cancer progression was slowed in GDEPT treated animals.

Thus a single course of GDEPT based on ovine atadenovirus-delivered PNP and fludarabine produced a highly significant suppression of PCa progression in immune competent TRAMP mice.

O5 RNAi-mediated Gene Silencing for Sensitizing Tumor Cells to Radiation

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Radiation is the major therapy for cancer. However, toxicity associated with therapeutic doses often results in treatment failure. Mechanistically, radiation attacks cellular DNA, causing DNA damage and cell death. The attack is often counterbalanced by the cells' surveillance and DNA damage repair systems. The master controller of this defence system is a 370 kDa protein kinase, the product of the *ATM* gene. The aim of this study was to silence the *ATM* gene by the RNA-mediated RNAi, thus disabling the cells DNA damage repair system, and sensitising tumour cells to radiation.

Synthetic double stranded RNA against the sequence of a marker gene, the enhanced green fluorescent protein (EGFP) was prepared. Introduction of the dsRNA into tumor cell lines, i.e.: Spc-A1, A549 and HeLa cells inhibited 75-85% of the expression of the EGFP when an expression plasmid vector-encoding EGFP (pcDNA-EGFP) was co-transfected. Control sense and anti-sense constructs of synthetic RNA did not have the inhibitive effect at the concentration of 10 fold higher. These results demonstrated the ability of synthetic dsRNA to inhibit an episomal template (target). When the synthetic dsRNA was transfected into Spc-A1, A549 and HeLa cells transduced with a lentiviral vector-encoding EGFP, the inhibition was at 80-85%. Similarly, control constructs of sense and anti-sense did not have an inhibitive effect at 10 fold higher concentration. These results demonstrated the ability of synthetic dsRNA to inhibit a chromatin template.

However, synthetic dsRNAs are expensive to make and inefficient to transfer into tumor cells in vivo. To overcome the problem, we constructed viral vectors that can also direct the transcription of dsRNA. Transduction of Spc-A1, A549, B16 and U18 cells with the lentiviral vector demonstrated similar efficacy of the EGFP gene inhibition, at 85-95% (chromatin template), confirming that viral vectors were more efficient than synthetic dsRNA. When viral vectors-encoding dsRNA corresponding to the sequences of *ATM* were used to transduce Spc-A1, A549, B16 and U18 cells, RNAi effect on the cellular gene *ATM* was significant. Reduction of the ATM protein and mRNA was by 85-90% consistently. Most importantly, the ATM protein reduction was accompanied by sensitization to radiation by 8 fold. Since lentivectors are much less expensive than synthetic dsRNA and easier to handle, the approach represents a new strategy that has the potential for radiosensitizing tumor cells.

O6 rAAV mediated RPE65 gene therapy of mouse and dog models of Leber's Congenital Amaurosis

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To compare the response to recombinant adenoassociated virus (rAAV) mediated RPE65 gene expression in the Rpe65-/- mouse and Briard dog models of Leber's Congenital Amaurosis (LCA). The animals were subretinally injected with rAAV.RPE65 and at different time points subjected to behavioural studies, and electroretinography (ERG) recordings. Following euthanasia, the enucleated eyes were analysed by histology, immunohistochemistry and electron microscopy.

Following subretinal injection, rAAV.GFP and rAAV.RPE65 transgene expression was readily detectable by fluorescent microscopy and immunohistochemistry, respectively in both models and was maintained up to 18 months as shown in the mouse. ERGs showed that dark-adapted b-wave amplitudes to an average 25% of normal, and light adapted b-wave amplitudes to 20% of normal amplitudes in the mouse model and slightly more in the dog model and were not reduced in dogs during a 9-12 months follow-up period. No systemic side effects were observed in either model but 75% of rAAV.RPE65-injected dogs developed uveitis. Ultrastructurally a reversal of RPE lipid droplet accumulation at the rAAV.RPE65 injection site was observed in both animal models. In the mouse model there was a recovery of up to 50% of short wavelength cone opsin-positive cells. However, this functional recovery was not accompanied by a reduction of the degenerative process of photoreceptors in affected mice.

These observations have significant clinical implications as they suggest that gene therapy might provide a treatment that can prevent blindness or delay the onset of blindness in LCA suffers, potentially for decades.

O7 Gene Therapy For Gliomas Using Antisense ATM cDNA Lentivirus

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Gliomas are the commonest primary brain tumour of which death is due to failure to control the growth of the tumour. Effective therapies for malignant gliomas remain elusive and even

with the best therapeutic modalities currently available, a prognosis of less than a year, as for glioblastoma multiforme, is appalling. The ATM gene, which is mutated in the disease ataxia-telangiectasia (A-T), is implicated in response to radiation-induced DNA damage, leading to profound radiosensitivity. Taking advantage of the extreme radiosensitivity conferred by reduced levels of ATM, a method of reducing the levels of ATM within radioresistant glioma cells such as glioblastoma multiforme would be very beneficial, especially if it could transform the radioresistant tumour to radiosensitive. Concurrently, advances in science have demonstrated that lentivirus is the most effective method of delivering genes into cells. By producing safe non-replicating lentivirus, containing the ATM antisense knockout, malignant gliomas can be sensitised to radiotherapy. In conjunction with surgery, this strategy might provide an enhanced therapeutic intervention especially in the case of the glioblastoma multiforme where the tumour is untreatable.
AIM: To sensitise malignant glioma cells to radiation by decreasing/aborting the function of ATM in these cells using new lentivirus vector-mediated antisense gene transfer; hence enhancing radiotherapeutic efficiency: both in vitro and in vivo.
METHODS: Lentivirus, which contains ATM antisense cDNA, will be produced in high titre and malignant glioma cells will be infected in vitro and in vivo to confer radiosensitivity.
RESULTS: Antisense ATM cDNA has been successfully cloned into the lentiviral vector and lentiviruses expressing antisense ATM cDNA have been successfully developed. Glioblastoma multiforme cells infected with the knockout plasmid have demonstrated ATM protein reduction by more than 90% of normal levels and these tumour cells have 4-fold radiosensitisation ie. to the level of a homozygous A-T cell line.
CONCLUSION: Success in the animal model will provide an approach for the treatment of human brain tumours by gene transfer. This work will provide a novel strategy for the treatment of malignant gliomas and will improve survival in patients with these tumours.

O8 Optimisation of HIV-1 derived vector systems

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We have previously described a HIV-1 derived gene transfer vector system that utilises codon-optimised HIV-1 *gagpol* genes for expression of the HIV-1 Gag and GagPol polyproteins, either from a single transcriptional unit, or from separate transcriptional units (1). We have shown that this approach has clear advantages over using the native HIV-1 gene sequence. We have now further refined our vector system by producing codon-optimised reading frames for all HIV-1 proteins (with the exception of Env which is not used in our system). By developing more efficient expression vectors for these proteins, and by careful titration of the amount of each plasmid used in virus production, we show that viral titre can be significantly improved. In addition three different approaches to the expression of the Gag and GagPol polyproteins were compared for their efficiency and relative safety by analysis of viral titre and infectivity, and their predilection for inadvertent transfer of HIV-1 gene sequences to transduced cells, respectively. We have also developed HIV-1 based vectors by the systematic assessment of the effect of different elements from the HIV-1 genome on vector performance. This has allowed us to develop a vector that is both more efficient, and safer, than the widely used pHR' construct (2). Nonetheless, RNA analysis shows that further improvements to vector design are possible. We believe that by further developing our vector systems we will be able to produce a HIV-1 vector system that is safe for clinical application.

1. Fuller M, Anson DS. Helper plasmids for production of HIV-1 derived vectors. *Hum Gene Ther* 2001; 12: 2081-2093. 2. Naldini L, Blömer U, Gallay P, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 1996; 272: 263-267.

O9 Promoter Interference Mediated by the U3 Region of HIV-1-Derived Lentivirus Vectors: Effects on Transgene Expression

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In a previous study using an early-generation VSV-G pseudotyped lentivirus vector encoding EGFP under the transcriptional control of a human CMV immediate-early promoter we examined transduction efficiency in dissociated dorsal root ganglia (DRG) culture. In cultures of murine origin transgene expression was observed solely in the sensory neurons, with the stromal cell population failing to show evidence of transduction. In contrast, efficient and sustained transduction of both sensory neurons and the stromal cell population was observed in cultures of human origin. Given the widespread use of murine models in pre-clinical gene therapy studies we undertook, in the current study, to investigate the basis of this apparent neuron-specificity of lentivirus-mediated transduction in murine DRG cultures. The interspecies differences persisted at high multiplicities of infection, and irrespective of whether lentiviral vector stocks were packaged in the presence or absence of HIV-1 accessory proteins. Cell-type specificity of CMV promoter expression, tropism of the VSV-G envelope and blocks to molecular transduction were also precluded as possible mechanisms, thereby implicating transcriptional repression of the internal heterologous promoter. This recognised, but still poorly understood phenomenon, known as promoter interference, was first reported in the context of MoMLV-based retroviral vectors, but has not previously been observed in HIV-1-derived lentivirus vectors. In this study, the promoter interference effect was found to be mediated by *cis*-acting sequences upstream of the core promoter elements located in the U3 region of the proviral LTRs. Importantly, this effect has been inadvertently relieved in contemporary lentivirus vectors by a deletion in the U3 region, introduced to reduce the risk of insertional mutagenesis by ablating LTR promoter activity (SIN phenotype).

O10 Adenoviral Vector Mediated Flt3 Ligand Gene Therapy of Hepatocellular Carcinoma

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Objective: To investigate the *in vivo* antitumor effect of murine Flt3 ligand (mFL), mediated by recombinant adenoviral vector and the combinational effect with large-dose chemotherapy in a liver cancer model.

Methods: Recombinant adenoviral vector carrying mFL (AdmFL) was constructed using the bacterial homologous recombination system. After establishing a C57BL/6 murine hepatocellular carcinoma model (5×5 mm^2 in diameter) by subcutaneous inoculation of Hepa1-6 cells, we gave a single intratumor injection of $1x10^9$ cfu AdmFL or $1x10^9$ cfu empty vector and PBS, respectively. Four weeks after adenoviral injection, we re-challenged those animals that had complete primary tumor regression with parental Hepa1-6, or syngeneic EL4 cells. To examine the combinational effect of large-dose chemotherapy with mFL in the treatment of larger tumors (10×10

mm^2 in diameter), sublethal 5-FU chemotherapy, three times a week (lasting two weeks), was systemically administrated, with or without the simultaneous intratumor injection of $1x10^9$ cfu AdmFL (once a week, for three weeks). Tumor mass was measured and survival rate of each group recorded twice a week.

Results: Tumor transduction with adenovirus encoding mFL could induce significant tumor regression in 75% of mice at 18 days, the remaining mice had a much slower growth rate, both of which had significance compared with the control group. mFL could protect the tumor-free mice from the re-challenge of Hepa1-6 cells, but not EL4 cells, which showed that mFL could elicit specific long-lasting antitumor immunity. In the case of larger tumor burden, AdmFL had a synergistic action with large dose chemotherapy, with significant regression and improved survival rate, superior to either treatment alone.

Conclusions: Flt3 ligand intratumor gene transfer mediated by recombinant adenoviral vector had an antitumor activity *in vivo,* and the effect was synergized with chemotherapy. Our data shows the potential of clinical antitumor application of FL.

O11 Successful re-administration of adenoviral vectors by liposome-microsphere complexes.

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Adenoviruses are highly efficient in delivering therapeutic genes into a large variety of cell types. However, their use in clinical studies has been limited by the development of a humoral immune response to the vector by the host following initial exposure. Further treatment with adenovirus is rendered largely ineffective with large-scale production of neutralising antibody. In order for re-administration of adenovirus to be accomplished circumvention of the hosts immune system is necessary. This study describes the use of microspheres in conjunction with liposomes to protect adenovirus from neutralising antibody both *in vitro* an *in vivo* allowing re-administration of the virus. *In vitro* evaluation of the complexes shows the virus is significantly shielded from neutralising antibodies without a significant loss of infectivity. *In vivo* evaluation of the complexes shows that the viral shielding permits a comparable level of expression in animals pre-exposed to adenovirus to that of non-exposed animals and as such highlights the utility of this vector for re-administration of adenovirus or its use in patients with pre-exposure to adenovirus.

O12 Development of Intact Genomic loci from the Human Genome Project for Therapeutic Applications and Stem Cell Research

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The availability of most human genes as intact genomic loci in sequenced DNA fragments from the Human Genome Project has opened the way for their use in therapeutic applications for diseases arising from genetic or somatic mutations.

We have developed a series of eukaryotic BAC (EBAC) vectors to facilitate transfer of human BAC clones from bacteria back into human cells for functional studies. We have also developed the *GET Recombination* system for the precise engineering of BAC clones in bacteria, as well as novel counterselection strategies for the introduction of disease-causing mutations and other fine modifications in such clones.

We have used these technologies to generate humanised mouse models with normal genomic loci that depend entirely on human gene function for survival. Use of the modified human β-globin locus has allowed the first humanised mouse models for HbE/β-thalassaemia, as well as thalassaemia models for common splicing and deletion mutations in the human β-globin gene. These mouse models accurately recapitulate the molecular and haematological features of these mutations in patients and should prove invaluable for the preclinical evaluation of therapeutic strategies for thalassaemia based on gene therapy by gene supplementation, gene correction and the restoration of splicing specificity, or the pharmacological upregulation of foetal haemoglobin.

We have also used developed Genomic Reporter Assays (GRAs) by the in-frame insertion of EGFP into each of the human globin genes in the β-globin locus. The creation of stable cell lines in erythropoietic cells with these constructs is greatly facilitating studies on globin gene regulation and the development of strategies for the upregulation of foetal haemoglobin. Similarly, cellular and transgenic reporter assays based on the *FRDA* locus are greatly facilitating the evaluation of agents that may increase frataxin production as a means of therapy of Friedreich ataxia.

The direct delivery of genomic reporter constructs into embryonic and adult stem cells should greatly facilitate the development of therapeutic applications based on guiding stem cells to differentiate into specific cell types. Preliminary studies with our genomic reporter assays indicate that BAC DNA can be efficiently delivered by non-viral strategies into embryonic stem cells, but nuclear uptake is currently much less efficient than with the similar-sized genomes of herpes viruses. It is hoped that studies on the mechanism of nuclear uptake of herpes viral DNA will lead to non-viral strategies for facilitated nuclear uptake of genomic DNA as a potential approach for gene therapy.

O13 β-Thalassaemia mouse model containing the common IVS I-110 splicing mutation

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Thalassaemia is one of the most common genetic disorders affecting haemoglobin synthesis. In the case of β-thalassaemia, which is characterised by a reduction or absence of β-globin chain synthesis, free or unpaired α-globin chains aggregate and precipitate within red cells, resulting in haemolysis and severe anaemia. Over two hundred different types of mutations have been found to cause β-thalassemia, but the most common are splicing mutations. Many of these mutations activate cryptic splice sites without destroying the normal splice sites. Some mutations allow a significant level of normal splicing leading to thalassemia intermedia, while others reduce normal splicing to low or very low levels and lead to transfusion dependency in the homozygous form. The development of novel therapies for β-thalassaemia requires the availability of suitable animal models for preclinical studies.

In this study, we report the generation of a 'humanised' mouse model carrying the IVS I-110 splicing mutation in intron I of the β-globin gene in the context of the human β-globin locus. This mutation is one of the most common splicing mutations in the Eastern Mediterranean region and reduces normal splicing by ~90%. The 'humanised' mouse model carrying the IVS I-110 splicing mutation was created by crossing transgenic mice

carrying the human β-globin locus containing the IVS I-110 mutation, with heterozygous mouse β (^{mu}β)-globin KO mice ($^{mu}\beta^{+/-}$). The $^{mu}\beta^{+/-}$ mice are anaemic and display severe haematological abnormalities as measured by full blood examination (FBE). We show by HPLC that the 'humanised' mouse model carrying the IVS I-110 mutation ($^{mu}\beta^{+/-}$, $^{hu}\beta^{IVS\ I\text{-}110+/0}$) expresses ^{hu}β-globin chain at ~3% relative to ^{mu}α-globin chain, while $^{mu}\beta^{+/-}$ transgenic mice carrying the normal human β (^{hu}β)-globin locus express ^{hu}β-globin chain at 35%. We show that the low level of ^{hu}β-globin synthesis in the $^{mu}\beta^{+/-}$, $^{hu}\beta^{IVSI\text{-}110+/0}$ transgenic mice is the result of aberrantly spliced ^{hu}β-globin mRNA and that this 'humanised' IVS I-110 mouse model accurately recapitulates the degree of aberrant splicing that is found in patients with this mutation. The low level of ^{hu}β-globin chain synthesis from the IVS I-110 locus results in a small but significant improvement in the haematological values compared to the $^{mu}\beta^{+/-}$mice, whereas the normal human β-globin locus fully complemented the severe haematological abnormalities. We propose that the transgenic mice carrying the IVS I-110 ^{hu}β-globin locus on a KO background can serve as a platform for testing novel strategies for the restoration of normal splicing in erythropoietic cells.

O14 Development of Genomic Reporter Assays for Friedreich Ataxia.

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The expansion of a (GAA)n repeat in intron 1 of the *FRDA* gene results in reduced synthesis of frataxin by partial inhibition of transcription. The variation in severity of the disease with the length of the GAA expansion further indicates that a moderate increase in *FRDA* expression may have a significant impact on the development and progression of the disease. A possible approach to the therapy of Friedreich ataxia may therefore involve targeted pharmacological upregulation of *FRDA* expression. In order to facilitate studies on the regulation of frataxin expression and to develop targeted pharmacological approaches, we have developed a sensitive genomic reporter assay for frataxin expression from the intact *FRDA* locus. A fully sequenced bacterial artificial chromosome (BAC) clone containing exons 1-5b of the human *FRDA* gene and surrounding sequences (present on a 188 kb genomic fragment) was first shown to complement fully the embryonic lethality in homozygous knockout mice. Using the *GET Recombination* system, two in-frame fusions between the *FRDA* and *EGFP* (Enhanced Green Fluorescent Protein) genes were then constructed in this fragment. One fusion is within exon 2 of the *FRDA* gene. The other is at the end of exon 5a, producing the entire frataxin protein fused to EGFP. Both constructs were shown to drive the expression of EGFP from the regulatory elements of the *FRDA* locus, with the frataxin-EGFP fusion proteins targeted to the mitochondria. The exon 5a *FRDA-EGFP* fusion construct was subsequently used to establish stable cell lines of BHK21 (hamster) and HeLa, 293 and neuroblastoma cells (human). Stable cell lines were exposed to a number of test compounds and EGFP fluorescence was measured by flow cytometry. Hemin and butyric acid induced 30% and 19% increases in *FRDA* expression, respectively, in the BHK21 stable cell lines. However, there was little effect on expression of the HeLa stable cell lines. The iron chelators deferiprone and desferioxamine both elicited an almost two-fold increase in *FRDA* expression in the BHK21 stable cell lines, and approximately 50% induction in the HeLa stable cell lines. The antioxidant idebenone was shown to partially decrease *FRDA* expression. Interestingly, the combination of deferiprone and idebenone resulted in no overall modulation of *FRDA* gene expression, confirming that the two agents affected expression in opposite directions. These cell lines provide a robust assay to facilitate high throughput screening for pharmacological compounds that can modulate the expression of the *FRDA* gene in a clinically relevant manner.

O15 Neocentromere-Based Human Engineered Chromosomes for Gene Expression and Gene Therapy Studies

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Human engineered chromosomes (HECs) provide an alternative gene delivery tool for gene therapy. A HEC-based system has several useful features for gene therapy consideration, including episomal existence (minimising disruption of the host genome), very large DNA payload (allowing large genes/gene complexes to be delivered), and minimal risk of immunogenic reaction or infection.

A good HEC requires a functional centromere to confer mitotic stability. Normal human centromeres contain large tracks of functionally important repeated α-satellite DNA that can be difficult to manipulate and characterise. Our laboratory described the first case of human non-repetitive centromere found on a stable marker chromosome mardel(10). This centromere, which we termed neocentromere, forms epigenetically from normal euchromatic DNA found within the q25 band of chromosome 10, and is completely devoid of the heterochromatic α-satellite repeat DNA that typifies the normal human centromeres. Since then, over 60 cases of human neocentromeres arising from many different genomic sites have been described.

Current data indicate that neocentromeres are structurally and functionally indistinguishable from their normal counterparts, making them suitable for use in HEC construction. We have previously described the construction of a series of neocentromere-based HECs in the form of 0.7- to 2-Mb minichromosomes using a process of targeted truncation of the proximal p- and q-chromosome arms flanking the mardel(10) neocentromere. These minichromosomes show full mitotic stability and centromere function and, unlike other repetitive DNA-based HECs, are small in size and fully manipulable and characterisable by sequencing.

An essential requirement of a HEC-based gene therapy system is to be able to demonstrate that gene expression is permissible within the vector. We have begun to define the structural domains of our neocentromere and relate these to the transcriptional competency of endogenous genes found within and flanking these domains. The study is particularly important as recent data in yeast and Drosophila suggest a domain-like centromere structure with a modified chromatin core surrounded by flanking regions of heterochromatin that are traditionally associated with gene silencing. Using the mardel(10) neocentromere, we have defined a region of increased chromosome scaffold attachment that overlaps two other distinct and non-overlapping domains of constitutive centromere proteins and a third domain of heterochromatin protein binding. Transcriptional competency is normal throughout the scaffold-enriched region and within a major constitutive centromere protein-associated domain. Treatment with a low dose of histone deacetylase inhibitor Trichostatin A causes a reduction in the expanse of the scaffold region, a shift in the positions of constitutive centromere protein binding, and the dissociation of heterochromatin proteins, but has no direct effect on gene expression. These results highlight the permissibility of transcription within the constitutively modified chromatin of our

neocentromere, and provide a sound basis for the further evaluation of the expression properties of inserted therapeutic genes in our neocentromere-based HECs.

O16 Targeted Corrective Gene Conversion (TCGC) and Human Disease

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Point mutation, deletion, insertion and triplet contraction and expansion are all classes of mutation involved in human disease. Targeted Corrective Gene Conversion (TCGC) has an obvious potential for treating human conditions involving nonsense, missense and transcriptional splice junction mutations, provided that therapeutic strategies can be delivered effectively to relevant tissue(s). Non-point mutations affect encoded protein function either by disruption of functionally critical peptide sequence or by disruption of codon reading frame. TCGC has some potential in restoring small deletions and insertions to normal sequence at the chromosomal level, and in the case of frame shift, TCGC may be used to restore codon reading frame by either inserting or deleting a single nucleotide at or just downstream of the deletion interface. We have used a TCGC method, Short Fragment Homologous Replacement (SFHR) to correct the exon 23 C3815T nonsense mutation at the Xp21.1 dystrophin locus (*dys*) of the *mdx* mouse, and to restore the *dys* codon reading frame in lymphoblast culture derived from a boy with Duchenne muscular dystrophy (DMD). In these experiments, correction frequencies of the *mdx* mutation have been improved in myogenic precursors to better than 20% of cells in culture. Correction of the *mdx* mutation has been achieved *in vivo*, although to far lower frequencies (<1% of cells). TCGC clearly has a widespread applicability and in our hands has demonstrated utility for restoration of codon reading frame and point mutations at the chromosomal level. We are currently developing a number of additional TCGC applications, including strategies to "loop out" triplet expansions or to "loop in" triplet contractions which will cover a wider variety of mutations involved in human disease

O17 Redirecting Dystrophin pre-mRNA Splicing Using Antisense Oligonucleotides: An Overview.

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We are developing an alternative therapy for Duchenne muscular dystrophy (DMD) using antisense oligonucleotides (AO) to displace factors involved in the normal splicing of the dystrophin exons during pre-mRNA processing. Masking selected motifs involved in splicing prevents normal splicesome assembly so that specific exons are omitted from the mature mRNA along with the flanking introns. In this manner, an exon containing a nonsense mutation could be by-passed as long as the reading frame was not disrupted. The most common type of mutations in the dystrophin gene are genomic deletions of one or more exons which disrupt the reading frame. Specific removal of one or more exons flanking the genomic deletions could restore the reading frame.

The dystrophin gene should be a most amenable target for this type of AO approach as it has been well established that the complete dystrophin protein is not required for near normal activity. A milder allelic version of DMD, Becker Muscular Dystrophy, arises from in-frame deletions in the dystrophin gene, which allows synthesis of a shorter but still functional protein. Furthermore, dystrophin-positive revertant fibres, which arise from some naturally occurring exon skipping mechanism, have been detected in dystrophic tissue of many DMD patients and animal models of the disease.

The *mdx* mouse model of muscular dystrophy carries a nonsense mutation in exon 23 of the dystrophin gene. AO-induced removal of this exon will by-pass the premature termination codon without disrupting the reading frame so that a dystrophin protein missing only 71 amino acids can be produced. Refinement of AO design and targeting has resulted in high levels of induced exon 23 skipping in the induced dystrophin mRNA, thereby restoring dystrophin synthesis in transfected cultured cells. *In vivo* administration through intramuscular injection of AOs into the tibialis anterior of *mdx* mice has resulted in strong and sustained dystrophin expression with correct sub-sarcolemmal localization. AO treated muscle shows significant improvement in muscle strength over untreated dystrophic mdx muscle after as few as a two injections of 1 ug AO:liposome complex.

Other AO chemistries (morpholino and peptide nucleic acids) can also be used to displace splicing factors. These have uncharged backbones and, although very resistant to nuclease degradation, have been limited by poor cellular/nuclear uptake. We have annealed sense-strand oligonucleotide leashes to the uncharged AO to allow complex formation with cationic liposomes. Although the PNA:leash:liposome complex was unable to induce any exon skipping, the morpholino AO:leash:liposome complex induced exon skipping at concentrations more than three orders of magnitude lower than other groups have demonstrated antisense effects with uncomplexed morpholino AOs in cultured cells.

O18 Morpholino antisense oligonucleotides for inducing exon skipping

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The *mdx* mouse model of muscular dystrophy arose due to a nonsense mutation in exon 23 of the dystrophin gene. We have previously demonstrated 2' O-methyl phosphorothioate antisense oligonucleotides (AOs) induced the removal of exon 23 from the mRNA during processing of the primary gene transcript, producing an in-frame transcript which results in expression of dystrophin in *mdx* muscle. Refinement of AO design has allowed efficient exon skipping to be induced in *mdx* mouse muscle cultures at nanomolar concentrations.

Another chemistry that is fast gaining recognition as a potential therapeutic oligonucleotide is the morpholino AO which have been reported to overcome some of the limitations faced when using phosphodiester (PO) and the phosphorothioate (PS) AOs. The backbone modifications of the morpholino structural type produce an AO that is neutral and has greater biological stability. Splicing intervention by morpholino AOs has been successfully applied *in vitro* to the β-globin gene pre-mRNA to correct aberrant splicing when delivered in the micromolar concentration.

We present data showing that dystrophin exon skipping in *mdx* muscle cells may be induced by morpholino AOs at nanomolar concentrations when annealed to a 'leash' or sense oligonucleotide and then complexed with a cationic liposome. We have investigated a number of leash designs and chemistries, including mixed backbone oligonucleotides, and their ability to influence the ability of a 25-mer morpholino AO to induce specific exon skipping. Typically, each leash has a central phosphodiester core, which contains between 15-25 complimentary bases that anneal to the morpholino AO. Flanking

the core region are 5 to 10 nucleotides on a PS backbone which generate overhangs at the 5' or 3' terminus of the morpholino AO. Varying the length and design of the leashes has enabled the morpholino AO to induce targeted pre-mRNA skipping and protein synthesis at concentrations several magnitudes below those required by uncomplexed morpholino AO, presumably due to improved delivery. The superior stability of the morpholino chemistry is expected to provide efficient and prolonged exon skipping and is currently under investigation *in vivo*.

O19 Oligonucleotide Gene Therapy for Choroidal Neovascularisation Enhanced by Dendrimer Mediated Delivery

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Purpose: To determine if lipid-lysine dendrimers are a viable option for the delivery of oligonucleotides for use in gene therapy.

Methods: D407 cells were transfected with nine different dendrimers complexed with an oligonucleotide (ODN-1) proven to possess an anti vascular endothelial growth factor (VEGF) effect. The efficacy of the dendrimers to deliver ODN-1 to the target site was determined by calculating the levels of VEGF protein and mRNA expression under hypoxic conditions at 24 and 48 hours post transfection using ELISA and RT-PCR respectively, and comparing this to results obtained using a commercially available transfecting agent. The two most effective dendrimer complexes were subsequently injected into the vitreous of rat eyes and later laser photocoagulated to induce choroidal neovascularisation (CNV). The extent of CNV was determined using fluorescein angiography.

Results: *In vitro* data indicated that all of the dendrimer / ODN-1 complexes resulted in a 40% to 60% decrease in the production of both VEGF protein and mRNA in the first 24 hour period. However, after 48 hours, several of the dendrimers were unable to maintain a reduction in the expression of VEGF indicating poor DNA protection qualities. Both the transfecting and protective ability seemed to be related to the length and number of lipidic amino acids (Laa's) associated with each dendrimer. It was found that dendrimer 4, which possessed two C14 Laa's and eight free amino groups, achieved the second highest transfection efficacy of 89% and in addition maintained the greatest reduction in VEGF expression for the 24 and 48 hour time periods (48% - 50% respectively). *In vivo*, eyes that were treated with dendrimer 4 showed a 70% lower rate of CNV compared to that of eyes treated with dendrimer minus the oligonucleotide for up to 3 months post injection / lasering.

Conclusion: We have shown that synthetic lipophilic charged dendrimers can be used for gene delivery both *in vivo* and *in vitro*, resulting in a therapeutic outcome and will be a valuable tool in gene therapy.

O20 Inhibiting neovascular signals from multiple cell targets in the eye by secretion gene therapy

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Ocular neovascularisation is a severe and major complication of many ocular conditions. This uncontrolled growth of blood vessels and its associated vascular hyperpermeability are the most common pathologic causes of vision loss in developed countries. Although many complex interactions and multiple contributing factors could be involved, vascular endothelial growth factor (VEGF) has emerged as the main mediator of neovascularisation in the eye. The recent developments in virus-mediated gene therapy in the retina suggest that inhibition of ocular neovascularisation by anti-angiogenic gene therapy is possible. However, a difficulty with using cell-specific gene therapy is that several types of cells in the retina have been identified as potential contributors to VEGF upregulation. Some of these target cells are difficult to access, transduce or undesirable to disturb especially within the macula, which is responsible for central vision. Therefore, to achieve stable ocular anti-angiogenic therapy, we explored the use of virus-mediated secretion gene therapy. We generated a recombinant adenovirus vector (Ad-CMV.sflt) and an adeno-associated virus vector (AAV-CMV.sflt) encoding the soluble receptor for VEGF, sFlt-1. The efficacies of these vectors were tested using the cautery-induced corneal neovascularisation model and the laser photocoagulation-induced choroidal neovascularisation model. Delivery of Ad-CMV.gfp and AAV-CMV.gfp, which carried the green fluorescence protein (gfp) reporter gene, into the anterior chamber of non-pigmented RCS-rdy$^+$ rats indicated that viral transduction occurred in the trabecular meshwork, corneal endothelium or iris. When injected with Ad-CMV.sflt and AAV-CMV.sflt, the relative neovascular score in the cornea was reduced by at least 37% and 35%, respectively. This indicated that secretion of sFlt-1 from the transduced cells could suppress VEGF upregulation and neovascularisation at the limbus and corneal stroma. Similarly in the choroidal neovascularisation model, subretinal delivery of AAV-CMV.sflt near the equator of pigmented RCS-rdy$^+$ eyes suppressed new vessels at the laser lesions around the optic nerve by 19%. These results show that secretion of sFlt-1 can inhibit the neovascular processes that occur at distinct and distant cellular areas. Hence, virus-mediated secretion gene therapy is a feasible approach to overcome the complexity of multiple cellular sources of angiogenic factors that lead to ocular neovascularisation.

O21 Insulin trafficking in a glucose-responsive human liver cell line-Huh7-EGFPins

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An alternative approach to the treatment of type I diabetes is the creation of an 'artificial beta cell' to synthesise, store and secrete insulin in response to metabolic stimuli. It has been previously shown that the stable transfection of the full length insulin cDNA into the human liver cell line HuH7 resulted in synthesis, storage and regulated release of insulin to the physiological stimulus glucose. Proteins such as insulin can be easily fused to the green fluorescent protein (GFP) and be visualized directly within living cells. To reveal the mechanisms underlying glucose stimulated insulin secretion, HuH7 cells were stably transfected with insulin fused to enhanced GFP (EGFP). As a positive control the glucose-responsive pancreatic beta cell line MIN 6 was also transfected with the insulin-EGFP construct. Using the resulting cell lines HuH7-EGFPins and MIN6-EGFPins, we were able to visualize the subcellular localization and trafficking of insulin by confocal and live cell imaging. Insulin storage following acid/ ethanol extraction and acute responsiveness to glucose following 1 hour incubations were analysed by radioimmunoassay.

Huh7ins-EGFP cells stored 3.9 ± 0.9 pmoles/ 10^6 cells (n=4). Confocal microscopy revealed the intracellular expression of EGFP-ins as punctate fluorescence. Upon glucose (5-20 mM) stimulation, the response was imaged as time-dependent loss of fluorescence in the cytoplasm, as insulin granules were released by exocytosis. Response to the ATP-sensitive potassium channel (K_{ATP}) activator diazoxide (150 μM) and the blocker glibenclamide (20 μM), confirmed the presence of K_{ATP} channels involved in the regulated release of insulin. Cells exposed to diazoxide, accumulated brighter cytoplasmic fluorescence. Exposure to glibenclamide resulted in loss of fluorescence. Exposure to 20 mM glucose resulted in an increase in insulin secretion from 0.08 ± 0.02 to 0.24 ± 0.03 and glibenclamide from 0.06 ± 0.01 to 0.26 ± 0.03 pmoles insulin/ 10^6 HuH7-EGFPins cells. Diazoxide inhibited glucose-stimulated insulin release. Similar results were seen in the positive control MIN 6-EGFP cells.

Our results show that glucose-responsive insulin secretion in HuH7-EGFPins cells is comparable to the mechanism of secretion in the glucose-responsive pancreatic beta-cell line (MIN6) and indicates that the cells respond to glucose via a similar mechanism.

O22 The Conversion of Electrically Inert Cells into Cells Capable of Electrical Excitation and Intercellular Coupling by Gene Transfer: Toward Gene Therapy of Heart Block

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Congenital, surgically-induced or acquired block in cardiac atrioventricular (AV) conduction via the AV node is a long-term clinical problem for both paediatric and adult patient populations. The only therapy currently available for significant heart block is the insertion of a permanent pacemaker device. The insertion and maintenance of these devices, particularly in the long-term, is associated with the potential for complications and morbidity.

In this research study we sought to reproduce, by gene transfer, the properties required for efficient intercellular electrical conduction. In the context of cardiac conduction these properties include the possession of an excitable cell membrane capable of generating an action potential and the capacity for gap junctional intercellular communication (GJIC) to transmit electrical current between cells.

Replication incompetent, VSV-G pseudotyped, third-generation lentiviral vectors were produced by transient transfection into permissive HEK293 cells. Vector was purified by filtration and concentrated by centrifugation. High-titre (>10^8 TU/mL) vectors encoding MyoD (myogenic determination factor) and connexin43-GFP fusion (gap junctional protein) were generated. Titre was assigned by assessing transgene expression in HEK293 cells, devoid of MyoD and connexin43. In addition to these transgenes, the expression cassette also contained a deletion in the U3 region of the 3'LTR and the transduction enhancing central polypurine tract and Woodchuck virus post-transcriptional regulatory element.

Gene transfer of MyoD to electrically inert human dermal fibroblasts (HDF) resulted in the efficient conversion of these cells into the skeletal muscle phenotype as evidenced by morphological changes of myotube formation and the expression of muscle-specific proteins on immunocytochemistry. Moreover, the appearance of calcium transients during confocal microscopy, following loading with the fluorescent indicator Fluo-3 and extracellular electrode stimulation, confirmed the presence of an excitable cell membrane in these MyoD-converted myotubes. Consistent with known biology, adjacent myotubes did not display synchronised calcium transients indicating the absence of intercellular electrical coupling. The additional transfer of the connexin43-GFP gene resulted in the dense accumulation of this protein at the interface between contiguous myotubes. Stimulation of adjacent MyoD-converted myotubes, also expressing connexin43-GFP, resulted in the synchronisation of calcium transients indicating the presence of GJIC between these excitable cell types.

These data demonstrate that electrically inert cells can be rendered excitable and capable of electrical coupling following gene transfer. Ultimately, this technology may have an application in the treatment of heart block.

O23 Lentivirus and adeno-associated virus vector-mediated gene therapy for the treatment of sensory neurons of the PNS.

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There are a number of acquired and inherited neurological conditions that affect the sensory neurons of the PNS, including the sensory neuropathies, neuropathic pain syndromes and the spasticity associated with upper motor neuron damage. At present there are no established therapies available for the amelioration or cure of these debilitating diseases, gene therapy, therefore, offers an attractive long-lasting therapy for these conditions.

Sensory neuropathy Friedreich ataxia (FRDA) is an inherited neurodegenerative disease associated with a progressive loss of dorsal root ganglia (DRG) sensory neurons. The disease has been well characterised and is caused by mutations in the frataxin gene. Since a viable mouse model for FRDA has recently been developed, which recapitulates many of the clinical and biochemical features of the human disease, we have chosen to use FRDA as a model disease to study virus vector-mediated gene delivery to the PNS.

In a previous study, we demonstrated that recombinant adeno-associated virus (rAAV) and recombinant lentivirus (rLV) vectors encoding the reporter gene EGFP exhibited efficient and sustained transduction of dissociated newborn mouse and fetal human DRG cultures. Excitingly, at a low multiplicity of infection, rAAVEGFP (MOI 1) and rLVEGFP (MOI 10) transduced 68% and 97% of dissociated mouse neurons respectively. We have now constructed a rAAV and a rLV vector encoding human frataxin cDNA under the control of the immediate early cytomegalovirus promoter. Initial experiments, using both vectors, have demonstrated increased frataxin protein expression in transduced human cervical carcinoma (HeLa) cells, which have negligible endogenous frataxin expression. Transgene expression also appeared to co-localise with a mitochondrial marker dye, suggesting that the frataxin protein had been transported to the mitochondrial compartment. Similarly, FRDA patient fibroblast cell lines (kindly provided by Dr Martin Delatycki) demonstrated increased expression of frataxin protein in transduced compared to untransduced patient cells. Since FRDA patient cell lines have previously been reported to exhibit sensitivity to oxidative stress and an increased concentration in mitochondrial iron, we are currently evaluating the ability of the vectors to reverse the mutant phenotype of the transduced cells.

Once functionality of both vectors has been established by phenotype correction, we will pursue virus vector-mediated gene delivery to DRG cultures from the mouse model of FRDA *in vitro*, before embarking on *in vivo* studies. To date both rAAV and lentivirus vector, continue to be promising gene delivery vectors for use in gene therapy applications involving the PNS.

O24 Immortalized Human Adult Liver Cells Express ß-Cell Markers

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People with type I diabetes are missing insulin producing (ß) cells. Replacing these missing cells is the ideal approach to treat this form of diabetes. Until now, the focus has been on isolating insulin producing cells from the pancreas of humans or pigs. Both have proven to be problematic to apply therapeutically.

We and others are developing a novel source of insulin producing cells, by genetically modifying liver cells to produce insulin. Two human tumour liver cell lines, HEP G2 and HUH7, have been genetically modified to carry the insulin ± GLUT 2 genes. Both cell lines have been shown to synthesize, store and secrete (pro)insulin in a glucose responsive manner (Simpson et al, *Gene Therapy* 1997; 4:1202-15; Tuch et al, *Gene Therapy* 2003, in press). Interestingly, both the parent and transfected cell lines express the ß cell transcription factors, NeuroD and Nkx6.1 as well as ß cell markers, prohormone convertase 1 and 2.

In this study, a non-tumourigenic immortalized human liver cell line was obtained to determine if lines of this nature express ß cell transcription factors and other markers. The advantage of these cell lines over HEP G2 and HUH7 is that they can be used more readily for therapeutic application. Immortalized human liver cells were produced by retroviral transduction with a retrovirus expressing Simian virus 40 (SV40) large T antigen and herpes simplex virus-thymidine kinase (Kobayashi et al, *Hum Cell* 2000;13:7-13). We analysed twenty-eight of these immortalized liver cell clones by RT-PCR. Initial studies show that seventeen of the clones expressed NeuroD, Neurogenin3 and GLUT 2; a further seven clones expressed NeuroD and Neurogenin 3 alone. None of the analysed clones was positive for prohormone convertase 2 mRNA. These data indicate that when a human adult liver cell is immortalized, there is an alteration of the make up of the cell, with expression of some ß cell characteristics.

POSTER PRESENTATIONS

P1 Mutational and NK Analyses in an Infant with Severe Combined Immunodeficiency (SCID-X1) Prior to Gene Therapy.

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The X-linked form of SCID (SCID-X1) is characterised by defective development of both T and natural killer (NK) lymphocyte subsets, which are typically absent in the peripheral blood of affected infants. Although B cells are present, they fail to undergo class switching and antibody production. SCID-X1 is caused by mutations in the gene encoding the common γ chain (γc), a component of interleukin receptors 2, 4, 7, 9, 15 and 21. Failure of T and NK cell ontogeny is thought to result from loss of signalling through the receptors for interleukin 7 and 15, respectively. In 2000 a French group headed by Alain Fischer reported the successful treatment of two infants with SCID-X1 by gene therapy: a milestone in medical history (Cavazzana-Calvo *et al.*, 2000). To date 10 infants have received gene therapy as part of the French trial. Of these, the Australian infant reported here is the only one to have an NK^+ phenotype, an incidence consistent with that reported in the literature (12%).

Here we describe a mutation that leads to this atypical SCID-X1 phenotype. CFSE-labelled NK cells from this patient were incapable of proliferating in response to IL-2, but retained cytotoxic activity when assayed on the NK-sensitive K562 cell line. DNA sequence analysis revealed a novel mutation not previously reported (http://www.nhgri.nih.gov/DIR/LGT/SCID). This mutation was an A to C substitution at the third base of intron 3 (nucleotide 1130 of GenBank L19546) and appeared to be a putative splice-site mutation. To confirm aberrant splicing of this intron, RT-PCR was performed on RNA from peripheral blood, with PCR primers spanning this region. In contrast to normal control samples containing a single γc mRNA species, three separate mRNA species were identified. The major species was found to contain the whole of intron 3 thereby confirming that the nucleotide substitution observed did indeed result in splice-site dysfunction. Based on the DNA sequence, protein translated from this product would be predicted to have a premature stop codon upstream of the transmembrane domain. Of the two minor species, one was found to contain part of intron 3 indicating the use of a cryptic splice-site, and the other to be correctly spliced wild-type message. Translation of the former species would be predicted to produce a γc protein containing a 20 amino acid insertion in the extracellular domain. We hypothesise that the limiting amount of "normal" γc transcript observed in this patient is sufficient to allow signal transduction through the IL-15 receptor and NK ontogeny, but insufficient to allow signal transduction through the IL-7 receptor necessary for T cell development.

Cavazzana-Calvo,M., Hacein-Bey,S., de Saint,B., Gross,F., Yvon,E., Nusbaum,P., Selz,F., Hue,C., Certain,S., Casanova,J.L., Bousso,P., Deist,F.L., and Fischer,A. (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* 288, 669-672.

P2 Differential Retroviral Transduction of Primitive Hematopoietic Progenitors Rather Than Gene Silencing Accounts for Variation in Transgene Expression.

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Hematopoietic progenitor cell (HPC) gene transfer remains an attractive approach to treat genetic diseases and cancer. Despite improved retroviral transduction, in general long-term persistence of only a relatively small number of transduced and contributing HPC has been observed. We have sought to analyse human cord blood CD34+ cell transduction using two day prestimulation with early acting cytokines, two days RetroNectin facilitated transduction and two further days culture. While 50-80% of CD34+ cells were consistently transduced, long-term persistent GFP expression was seen only in approximately 20% of engrafted human cells in NOD/SCID mice. *In vitro* analysis of transgene expression revealed rapid decline in the proportion of GFP^+ cells in the first 5-7 days following retroviral transduction of CD34+ cells. Transgene silencing during cell maturation was excluded because bright GFP fluorescence was clearly seen in both spontaneously differentiated myeloid cells and cells induced to myeloid differentiation by over-expression of mutant Ras (*N-Ras*^{m}). The decline in the proportion of GFP+ cells correlated with rapid myeloid differentiation. In this case, over-expression of *N-ras*^{m}, shown to induce hyperproliferation and premature myeloid differentiation, accelerated this decline in GFP-positive cell number. In addition, the proportion of transduced cells was lower in the clonogenic progeny of earlier, more primitive progenitors

compared to more mature progenitor cells. Conversely, over-expression of *myc* or stromal support both shown to inhibit cell differentiation, delayed the decline in GFP+ cell number. Our results indicate differential transduction of the CD34+ cell population: CFSE staining of CD34+ cells prior to retroviral infection, demonstrated that cells with the greatest number of cell divisions exhibited the highest level of retroviral transduction. Thus the retrovirus targets more mature subsets of hematopoietic progenitors and the percentage of vector-transduced cells declines as more primitive cells take over. Based on these results, we suggest that rapid differentiation and apoptosis of the more mature progenitor cells and low transduction of earlier primitive progenitor cells accounts for the rapid decline in transgene expression.

P3 *In vivo* insulin delivery to diabetic rats using lentiviral vectors

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PURPOSE: Type 1 diabetes mellitus is caused by severe insulin deficiency secondary to the autoimmune destruction of the β-cells of the pancreas. Patients need periodic insulin injections to prevent ketoacidosis and chronic complications, which can be fatal. The goal of this study is to test whether prevention of acute complications in severe diabetic rat is possible with insulin gene therapy using lentiviral vector.

MATERIALS and METHODS: A recombinant lentiviral plasmid containing human mutated proinsulin cDNA which had a furin endopeptidase-cleavage site and a lentiviral plasmid containing the green fluorescent protein (GFP) gene were constructed. The resulting plasmids, pRRL-cPPT-CMV-InABC-PRE-SIN or pRRL-cPPT-CMV-GFP-PRE-SIN, were transfected into 293T cells with packaging plasmids to produce recombinant lentivirus. Virus titre was determined by FACS analysis of GFP expressing cells after lentiviral vector transduction and measurement of p24gag protein in the supernatant of 293T cells. The viruses were concentrated by high-speed centrifugation. Diabetes mellitus (DM) was induced by the intraperitoneal administration of streptozotocin (STZ) in Fisher 344 inbred rats. Five days later, concentrated recombinant lentiviruses were injected into the skeletal muscle of the groin (treatment group). Blood glucose and weight were measured serially and survival observed.

RESULTS: In the presence of sodium butyrate, virus titre was $1.5x10^8$ infection unit/ml, which was much higher than that of $2.5x10^7$ IU/ml without sodium butyrate. Insulin production from the Hela cells infected with recombinant lentivirus was 20.5 μIU/ml in 48 hours, 160.5 μIU/ml in 120 hours and 184.5 μIU/ml in 168 hours. Before administration of recombinant lentiviral vector (5 days after DM induction with STZ), random glucose concentrations of the treatment group (n = 5) and control group (n = 5) were 421.7±46.83 mg/dl and 422.7±46.01 mg/dl, respectively. The 1-, 2- and 4-week glucose levels of the treatment group were 303.7±61.45 mg/dl, 292.6±54.52 mg/dl and 370.3±78.65 mg/dl respectively and of the control group were 487.63±5.90 mg/dl, 497.3±2.05 mg/dl and 652.5±38.54 mg/dl respectively. Before lentiviral vector administration, body weights of the treatment group (n = 5) and control group (n = 5) were 115±5.0 g and 116±1.5 g, respectively. The 1-, 2- and 4-week weights after lentivirus administration were 117±6.6 g, 122±10.7 g and 125±12.4 g respectively and of the control group were 103±5.7 g, 98±2.0 g and 91±1.4 g respectively. The overall survival time of the treatment group was 63±16.1 days, which was significantly longer than that of 34±21.6 days in the control group.

CONCLUSION: The intramuscular administration of recombinant lentivirus containing the mutant proinsulin gene could prevent acute complication of DM rats, leading to prolongation of survival time. This suggests that *in vivo* delivery of insulin by gene therapy could become an effective treatment modality for insulin-dependent diabetes mellitus of man in the future.

P4 Suppression of HBV by Trans-acting HDV Ribozyme in HepG2.2.15 Cells

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Objective: To explore whether the trans-acting HDV (Hepatitis Delta Virus) ribozyme has the ability to suppress HBV (Hepatitis B Virus) in vivo.

Methods: To select potential HDV ribozyme target sites within the HBV genome an HBV full sequence was cloned from the genomic DNA of HepG2.2.15 cells. The clones were sequenced and aligned with 202 HBV full sequences of various genotypes retrieved from the GenBank database, to identify potential target sites conserved in most of the genotypes (homology >90%). Some sites located in the C region were examined by RNase H hydrolysis with oligonucleotides to find out the sites which were accessible for the ribozyme. To search the cleavage activity of trans-acting HDV ribozyme in vitro, the cDNAs of both HDV ribozyme and the fragment of HBV were chemically synthesised and transcripted in vitro with labeled α-^{32}P UTP through T7 RNA polymerase. Under the appropriate reaction conditions two transcripts, ribozyme and substrate, were mixed and incubated at 37^{o}C. Cleavage products were assayed by denatured PAGE and autoradiography. Research in vivo: the cDNA of HDV ribozyme was inserted into the vectors pcDNA3.0 and ptV which include the hMCV and $tRNA^{Val}$ promoters respectively. Recombinant pcDHRz1 and ptVRz1 DNA were transfected into HepG2.2.15 cells by Lipofectomine^{TM}2000 (Invitrogene). A pcDNA3.0 control as well as medium without transfected DNA were also included. The HBeAg and HBsAg of the culture medium were detected by ELISA assay after 48 hours in culture.

Results: In the full extent of HBV genome thirty-two conserved sites which include more than 18nt were identified. There were seven sites located in the C region but only one site (2174-2191, GenBank accession number: U95551) was accessible by 18mer-oligonucleotide. The RNA fragment transcripted in vitro including this site could be cleaved by the HDV ribozyme which targeted the site. The cleavage rate was 56%, Km was 0.58μM and Kcat 0.60 Min^{-1}. After transfecting into HepG2.2.15 cells for 48 hours the average OD_{450} value of HBeAg in mediums of three groups, pcDHRz1, ptVRz1 and the pcDNA3.0 control, were 0.289, 0.286 and 0.398 respectively and there was a significant difference ($p < 0.05$) between two transfected groups and the control respectively. There was no significant difference in the levels of HBsAg between them.

Conclusion: The ability of trans-acting HDV ribozyme cleaving the HBV mRNA fragment in vitro and specially suppressing the expression of HBeAg in vivo showed it was a new potential antisense regent for HBV gene therapy.

P5 Gene Delivery of Bone Morphogenetic Proteins and Type 2 Receptors Using Adenoviral Vectors.

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Primary pulmonary hypertension (PPH) is a fatal disease characterised by abnormal proliferation of pulmonary vascular endothelial and smooth muscle cells. Recent evidence indicates

defects in the bone morphogenetic protein receptor type 2 (BMPR2) pathway in this disease. These mutations are predicted to lead to a downregulation in BMPR2 signalling, a pathway which involves a SMAD-protein signal cascade. Thus, gene replacement therapy using a normal BMPR2 gene could potentially be useful in this disease. Previously, we developed a technique to achieved selective gene expression in pulmonary vascular endothelium using adenoviral (Ad) vectors, based on transductional targeting and transcriptional control using the endothelial-specific flt-1 promoter. Hypothesis: A targeted Ad vector system could be developed to deliver functional BMPR2 receptors and BMPs to pulmonary vascular endothelium. Aims: In this phase of the study we sought to develop Ad vectors containing the BMPR2 gene, then evaluate gene delivery in vitro using immunohistochemistry and a functional assay using a SMAD-sensitive reporter construct (p3GC2wt-Lux) whereby increased SMAD levels drive the expression of the luciferase reporter gene. Methods: The cDNAs for the human BMPR2 receptor, incorporating a c-terminal myc tag (for ease of later immunohistochemical detection), or for the BMPR2 ligands (BMP-2 or BMP-7) were cloned into Ad vectors by standard techniques. Cells in culture were infected with AdBMPR2-myc then stained with an anti-myc antibody, with signal detected with immunofluorescence. Evidence of receptor function was sought by transfecting cells first with p3GC2wt-Lux, then co-infecting with AdBMPR2 and AdBMP-2. Results: Positive fluorescent signal was detected in AdBMPR2-myc infected cells in a distribution consistent with membrane localisation. Analysis of luciferase activity in cells infected with BMPR2-myc/BMP-2 Ads revealed 10-fold greater luciferase activity then cells transfected with p3GC2wt-Lux alone or with control virus. Conclusion: Functional BMPR2 and BMPs can be delivered by Ad vectors, at least in vitro. In vivo studies are currently under way. Supported by: NHMRC, NIH.

P6 Variations in the Patterns of Transgene Expression Mediated by Recombinant Adeno-Associated Virus Type 2 in the Retina.

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OBJECTIVES. This study aimed to evaluate the impacts of different preparations of recombinant adeno-associated virus type 2 (rAAV2) on the patterns of transgene expression in the retina, and to examine whether the impurity of rAAV2 and local micro-environmental changes affect the patterns of rAAV2-mediated transgene expression.

METHODS. A rAAV2 carrying the enhanced green fluorescent protein (GFP) gene driven by a cytomegalovirus (CMV) promoter was produced by either co-infecting the 293 cell line with E1-defective adenovirus and purified by one step $CsCl_2$ density gradient centrifugation (rAAV2-$CsCl_2$), or by transfecting with an adenoviral helper plasmid and purified by iodixanol density gradient centrifugation followed by heparin column chromatography (rAAV2-heparin). The protein contents in rAAV2-$CsCl_2$ and rAAV2-heparin were assessed by SDS-PAGE. The impacts of different virus preparations on the patterns of transgene expression were evaluated following subretinal injection in adult rats. Factors that may impact the patterns of transgene expression have been examined. These included the effects of cellular proteins extracted from 293 cells, contaminant proteins in rAAV2-$CsCl_2$, inactivation of the helper adenovirus, cell stress induced by DNA-damaging agents and laser photocoagulation to change the profiles of local retinal gene expression.

RESULTS. SDS-PAGE confirmed the impurity of rAAV2-$CsCl_2$. rAAV2-$CsCl_2$ predominantly transduced RPE and with less efficiency in PR. In contrast, rAAV2-heparin predominantly transduced PR but with much less efficiency in RPE. Supplement of cellular proteins extracted from 293 cells into rAAV2-heparin, heat-treatment of rAAV2-$CsCl_2$ to re-inactivate any possible helper adenovirus contaminants and introduction of cell genotoxic stress did not change the patterns of gene transduction. However, supplement of a small amount of rAAV2-$CsCl_2$ into rAAV2-heparin, and retinal laser photocoagulation prior to subretinal injection of rAAV2-$CsCl_2$ significantly changed the cell target specificity.

CONCLUSIONS. This study revealed the differences in retinal cell target specificity between rAAV2-heparin and rAAV2-$CsCl_2$ following subretinal injection. Further identification of the factors affecting the retinal cell target specificity will undoubtedly contribute to the development of new gene therapy approaches to PR- and RPE-specific retinal diseases.

P7 A Quick and Convenient Molecular Method for Titration of Lentiviral Vector Directly from Culture Supernatant

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At present, there are several methods available for titration of lentiviral vectors, including functional and molecular titration. However, each of them has advantages and disadvantages, but both are time-consuming. In addition functional titration is difficult for vectors that do not contain a marker gene.

We report the development of a real-time quantitative RT-PCR (RQ-RTPCR) method for the titration of lentiviral vector directly from viral harvest of cell culture supernatant. Primers for the amplification of the U5 region of the lentiviral long-terminal repeats (LTR) were synthesised. Direct harvests of lentiviral vectors or concentrates were treated with DNase to rid it of any plasmid contamination and used for real time RQ-RTPCR. PCR amplifications were performed in the ABI Prism 7700 sequence analyser. A standard curve was generated using the lentiviral plasmid. Vector titre was expressed as number of vector molecules, which was calculated by comparing the threshold cycle (Ct) values of the sample with that of the plasmid standard curve. When compared with existing functional as well as molecular based assays, including maker gene expression, RNA titration by RQ-RTPCR and DNA titration by RQ-PCR using isolated DNA and RNA was found to be quicker, more sensitive and convenient because there was no transduction, gene expression, or DNA or RNA isolation required. Furthermore, the method is applicable to all lentiviral vectors regardless of the transgene.

P8 Recombinant Adeno-Associated Virus and Lentivirus Vector-Mediated Gene Transfer to Cultured Proximal Airway Epithelia from Fetal Sheep

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Cystic fibrosis (CF) is the most common lethal genetic disease in Caucasian populations. Mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) cause abnormal transepithelial salt and fluid regulation predominantly affecting the lung and, to a lesser extent, the gut. Repeated lung infections and the cumulative damage caused commonly result in respiratory failure at an early age. Since the identification of the CFTR gene, cystic fibrosis has been an

attractive target for gene therapy. Unfortunately, successful gene replacement therapy of CF by targeting well-differentiated adult airway epithelium has proven elusive using currently available gene transfer technologies and delivery strategies. *In utero* gene transfer to the developing airway epithelium offers several theoretical advantages, including the possibility of transducing self-renewing basal cells inaccessible in adult epithelium following luminal vector delivery. In this study, we therefore investigated transduction of cultured proximal airway epithelia from fetal sheep using recombinant Adeno-associated virus (rAAV) type 2 and third generation HIV-1-derived lentivirus vectors (VSGg pseudotyped). Fetal sheep provide an excellent large animal model, approximating the size and developmental time-table of the human fetus, with the added advantage of being exceedingly tolerant of fetal manipulation without miscarriage. Six pregnant ewes were euthanized at 92 to 134 days gestation. The fetus was removed via caesarean section and the lungs and trachea harvested under aseptic conditions. Intact epithelial explants from both the trachea and main bronchi were placed in culture media specifically formulated for epithelial cells. After 2 days in culture, epithelial cells (confirmed by immunohistochemistry) could be seen growing out from the explant. Lentivirus or rAVV 2 were added at 7 and 13 days after culture initiation and gene transfer followed out to 35 days. In all instances, the epithelial explants were visibly ciliated, while epithelial cells growing out onto the plate appeared less well differentiated, lacking cilia and evidence of polarisation. Both rAAV 2 and HIV-based lentivirus vectors encoding enhanced green fluorescent protein (eGFP) stably transduced these less well differentiated cells at all gestation ages examined, however transduction of ciliated cells within intact explants was consistently highly inefficient. Lentivirus-mediated transduction was markedly more efficient than rAAV-mediated transduction at equivalent multiplicities of infection. These pilot data highlight the impact of differentiation state on epithelial transduction, and indicate a need to study more immature epithelium earlier in gestation and also more distal epithelium at the gestational ages investigated in the current study.

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P9 Development of a Model of Autologous Haemopoietic Stem Cell Transplantation For Gene Transfer using a Less Toxic Preparative Regimen

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Gene-modified haemopoietic stem cells (HSC) lacking a selective survival advantage require that some form of conditioning of the recipient be performed to achieve engraftment. Several groups have examined the use of nonmyeloablative conditioning with a view to avoiding the toxicity of myeloablation. Although toxicity has been low in nonmyeloablative protocols, the level of gene marking has generally been below levels of therapeutic utility (0.01% to 15%). To further explore the use of nonmyeloablative conditioning we are developing a baboon (Papio hamadryas) model of HSC gene transfer using 600cGy of megavoltage x-rays. This dose approximates the median lethal dose in Rhesus macaques and results in 3 weeks of profound pancytopenia in the absence of HSC rescue (Wagemaker et al. Stem Cells 1998;16:375). To optimise the mobilisation of peripheral blood HSC, we are exploring different cytokine combinations. In our model, we have found that even large doses of G-CSF alone (100mcg/kg/d) did not result in significant CD34+ cell mobilisation. In contrast, the combination of G-CSF + SCF (50mcg/kg/day) resulted in a significantly higher number (11-28 CD34+ cells/uL (n=3) vs 27-64 CD34+ cells/uL (n=3) in the peripheral blood on day 5 respectively). Leucapheresis was performed using the Cobe Spectra Apheresis System on baboons under general anaesthesia. The leucapheresis products contained 0.4-0.6 x 10e6/kg (G-CSF) vs. 4.2-13 x 10e6/kg (G-CSF + SCF) total CD34e6 cells. To assess safety and feasibility, we have performed one autologous transplant using non-marked cells. Peripheral blood mononuclear cells containing 4.2 x 10e6/kg CD34+ cells were harvested on day 5 of G-CSF + SCF mobilisation in a 21kg male animal. The leucapheresis product was frozen and stored in liquid nitrogen. At 48 hours after total body irradiation, the animal received an infusion of the autologous cells. The animal tolerated the procedure well, although an infection occurred during white cell recovery that resolved with antibiotic treatment. Transient pancytopenia was observed with a neutrophil nadir of 0.6 x 10e9/L on day 8, platelet nadir of 18 x 10e9/L on day 10 and recovery of all cell counts by day 12 post-irradiation. In establishing a nonmyeloablative primate model of autologous haemopoietic stem cell transplantation, we have examined several variables including mobilisation regimens, radiation dosage and safety. A recent report suggests that G-CSF + SCF-mobilised cells are more efficiently transduced than G-CSF-alone or G-CSF + Flt3L-mobilised cells (Hematti et al, Blood, 2002 online). Based on this report and our results showing more efficient mobilisation using G-CSF + SCF, we conclude that this combination may be optimal in the context of haemopoietic stem cell gene transfer protocols.

P10 FRL 19, an embryonic liver stem cell line particularly sensitive to lentivirus vector-mediated gene transfer

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Gene transfer is a promising novel approach to the treatment of a variety of disorders including infectious, genetic, neurological and cardiovascular disorders and cancer. The efficiency of gene transfer could be defined as the ability to reach all target cells with the therapeutic gene. To achieve high efficiency gene transfer, a high titre gene transfer vector is important. The number of active vector particles (vector titre) can be determined by transduction of a marker gene into cell lines such as 293, 293T or NIH 3T3 (functional titre). However, the sensitivity of these cells to the vector often influences the calculation of vector titre.

In this study, we compared the sensitivity of these cell lines to lentiviral vector-mediated gene transfer, and showed that conventional cell lines are less sensitive, thus, underestimating vector titre. A new embryonic stem cell line, FRL 19, is sensitive to lentiviral vector transduction. It reached a transduction efficiency of 96.7% when 400 μl of viral vector was used while the transduction efficiency was only 87.9% for 293T cells, 77.1% for NIH 3T3 cells and 63.9% for 293 cells. Lentiviral vector-mediated gene expression in all tested cell lines was consistent and stable, and real-time PCR study further confirmed that. Our data demonstrated that FRL 19 is more sensitive to lentiviral vector-mediated gene transfer than conventional cell lines tested, indicating FRL 19 is a useful cell line for determination of lentiviral vector titre. Furthermore, culture of FRL 19 is relatively easier than conventional cell lines. As lentiviral vector has emerged as the vector of choice for *in vitro* and *in vivo* gene therapy studies, FRL19 should become the most suitable tool for lentiviral vector titration.

P11 Insertional Oncogenesis and Insertional Virogenesis – Two Aspects of the Same Coin?

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Gene therapy holds great promise for the effective therapy of many genetic diseases. While it is still hoped that it may be possible in the future to achieve gene therapy by the correction of genetic defects directly in genomic DNA, most gene therapy research involves the construction, delivery and expression of synthetic minigenes after their random integration into the genome. The efficient mechanisms of integration of retroviral and lentiviral DNA into the genome of host cells has therefore made these vectors the systems of choice for the development of many gene therapy constructs, despite the clearly perceived risks of insertional mutagenesis. The interruption or the modification of the activity of genes essential for DNA synthesis, repair and cell division by insertional mutagenesis may lead to cell death, but it may also lead sometimes to dysregulation of cell division and oncogenesis. The recent description of two adverse events in a French gene therapy trial because of insertional oncogenesis indicates that the risks of insertional mutagenesis have been significantly underestimated. However, given the benefits that some patients have already derived from this gene therapy strategy, it is important that a careful risk-benefit analysis is carried out.

Human cells are often hosts to a variety of viruses and it is therefore essential to consider the potential implications of insertional integration of gene therapy constructs into the genomes of such viruses. The herpes and pox viruses are the most interesting in this respect, since they may present targets comparable in size to the human genome in their life cycles, while they may readily package and transmit intact retroviral and gene therapy constructs after their integration into their genomes. Evidence will be reviewed in this presentation for the spontaneous insertional integration of partial and complete copies of retroviral genomes into the genomes of herpes and fowlpox viruses in natural isolates of these viruses and after co-infection in cell culture. Such integration events may lead on the one hand to novel herpes and poxviruses with altered virulence and pathogenicity properties, while on the other hand they may also facilitate the transmission of retroviruses and lentiviruses through the normal mechanisms of transmission of herpes and poxviruses. It is therefore considered essential that the risks of insertional integration of retroviral and lentiviral constructs into the genomes of human herpes and poxviruses are carefully assessed in experimental systems and that surveillance systems are put in place for the early detection of such hybrid viruses in the course of gene therapy studies.

The targeted integration of therapeutic constructs into specific sites in the human genome should reduce the risks from both insertional oncogenesis and insertional virogenesis.

P12 Flt3 Ligand Gene Therapy Greatly Enhanced Tumor Regression and Expanded the number of Dendritic Cells and Natural Killer Cells.

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Flt3 ligand is not only an important early hematopoiesis factor, but also an immune enhancing factor. Recent studies by others showed that it could prevent the apoptosis of primitive hematopoiesis cells by modulating the balance of the Bcl-2 family of proteins. Therefore, we conducted a series of experiments to explore whether the application of an adenovirus vector carrying the Flt3 ligand gene (Ad-FL) can enhance tumor regression and prolong the survival of colon cancer-bearing mice when used in combination with 5-Fu. The dynamic changes of peripheral mononuclear cells showed that Ad-FL can protect the mice from 5-Fu caused hematopoiesis injuries and promote the recovery of hematopoiesis. Natural killer cells and dendritic cells were also greatly expanded, which play an important role in non-specific and specific anti-tumor activities, respectively. Mice from the combinatory therapy group survived longer than those from control groups. In conclusion, Flt3 ligand gene therapy can improve the therapeutic effect of chemotherapy by protecting the host from hematopoiesis injures, promoting immune recovery and may provide a novel way for cancer therapy.

P13 Over-Expression of TIMP 3 Causes Apoptosis in Lung Cancer Cells.

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New options are needed for the treatment of advanced lung cancer. Tumour invasion and spread involves a complex interplay between tumour, stromal and inflammatory cells with the extracellular matrix (ECM). The matrix metalloproteinases (MMPs) are a family of enzymes involved in ECM maintenance and tissue inhibitors of matrix metalloproteinases (TIMPs) are important factors in the regulation of the activity of MMPs. Gene delivery of TIMP 1 and 2 has been shown to inhibit tumour growth in animal models. There is evidence that TIMP 3 may also be therapeutic in this way, and in contrast to TIMP 1 and 2, may directly induce apoptosis in tumour cells, although this has not yet been evaluated in lung cancer. Hypothesis: Overexpression of TIMP 3 delivered by adenoviral (Ad) vectors to lung cancer cells causes apoptosis and increased cell death. Aims: To compare the effect of TIMPs 1, 2 and 3 gene delivery on lung cancer cell lines. Methods: A549, 1299, H1466 and 522 cells were plated at a known concentration, then infected with Ad vectors carrying the genes for TIMP 1, 2 and 3. Following infection, the cells were examined for changes consistent with apoptosis and cell death. Analysis by flow cytometry using Propidium Iodide and Anexin V staining was used to establish the presence of apoptosis. Cell counts were used to evaluate any reduction in cell number. Results: For all lines maximal reduction in cell number was seen with TIMP 3 overexpression and this was dose dependent. Flow cytometry confirmed the induction of apoptosis by TIMP 3 (eg for A549 cells 32.5 ± 6.3% at 72 hours vs 2.2 ± 2.1% for uninfected cells). Rates of apoptosis with TIMP 1 and 2 infected cells were similar to controls. Conclusion: TIMP 3 induces a reduction in cell numbers and causes increased apoptosis, which was not seen with either TIMP 1 or 2, in lung cancer cell lines. Further study using animal models of lung cancer is thus warranted and is currently being pursued.

P14 Promise of the MYCN Mouse Model of Spontaneous Neuroblastoma as a Pre-Clinical Tool for Investigating Novel Cancer Immunotherapy Strategies

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While localised neuroblastoma (NB) can be cured in the majority of children by conventional therapy, the prognosis for

disseminated NB remains dismal. Clearly, the development of novel therapies is urgently needed, a process that would be greatly facilitated by the availability of well characterised and clinically relevant animal models. Recently, a transgenic mouse model has been developed in which the proto-oncogene, MYCN, is over-expressed in the neuroectoderm. These mice develop spontaneous neuroblastoma and accurately model many of the biological, technical and logistic challenges inherent in the treatment of human disease. The primary aim of this study is to characterise this unique model as a preclinical tool for evaluation of immuno-therapeutic strategies for treatment of paediatric NB. We have established a MYCN mouse colony and successfully optimised a quick and sensitive quantitative PCR-based assay for determination of zygosity using *real time PCR* technology. We find that the tumour incidence in homozygous and hemizygous animals is 100% and 30%, respectively. Tumour latency studies show that the age of onset of the clinical disease is 5-6 weeks in homozygous animals, and 6-12 weeks in hemizygous animals. Microscopic evidence of disease in consistently present and multifocal by 17 days in homozygous animals, but most commonly appears later and is unifocal in hemizygous animals. Importantly, while not syngeneic, phenotyping revealed that these mice are homozygous at the MHC gene loci. Gene-expression profiling studies performed on five independently arising tumours from separate animals using micro-array technology showed that more than 200 genes are consistently more highly expressed in tumours than in central nervous system tissue (used as a surrogate for neurectoderm). Interestingly, a subset of these genes are known to be over-expressed in human NB (e.g. Tyrosine Hydroxylase (TH), survivin and alpha-prothymosin), and a further subset (including MYCN) have established potential as targets for immunotherapy. These observations provide good preliminary evidence that the neuroblastomas arising in these mice are viable and clinically relevant targets for immunotherapy. Based on current data, we hypothesise that a polyvalent cellular vaccine (e.g. gene-modified dendritic cells) will induce tumour-specific protective and therapeutic immunity in this animal model. Our current efforts are geared towards demonstrating tumour immunogenicity *in vitro* and *in vivo***.** We then plan to evaluate the immuno-therapeutic potential of a polyvalent DC-based cellular vaccine in this model for the induction of tumour-specific immunity.

P15 The Role of ATM Gene in Cancer and Radiosensitivity

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Ataxia-telangiectasia (AT) is a complex and autosomal recessive disorder, characterized by cerebella ataxia and telangiectasia. Major hallmark features in AT are predisposition to develop a range of lymphoid malignancies and a profound hypersensitivity to the cytotoxic effects of ionizing radiation which involved in chromosomal instability, defective repairing in apoptosis and DNA damage. The gene defective in AT is a member of the phosphatidylinositol 3-kinase family that include DNA-dependent protein kinase (DNA-PK), ATM, Rad3 related protein and ATR. Studies reveal that ATM gene has been regarded as a central controller of cellular response to DNA damage, and several cell cycle checkpoints activated by different targets at different stages of the cell cycle. ATM is rapidly activated as a pre-existing protein in response to radiation induced double strand breaks in DNA damage. Once activated ATM rapidly phosphorylates a series of substrates important in DNA damage recognition and cell cycle control.

DNA damage leads to genetic instability, which in turn may enhance cancer development rate, the deficiencies in signalling and repair of DNA damage are fundamental to the etiology especially in human cancers. The relative risk for developing some particular tumor types is several hundred-fold higher in AT as compared to the normal population. AT heterozygotes also exhibit a measurable risk for developing breast cancer. ATM may play a role as a tumor suppressor in T-prolymphocytic leukemia, which associated highly with loss or mutation in ATM locus.

A novel strategy to increase the radiosensitivity of human tumor based on abrogating the function of ATM using new lentivirus vector-mediated antisense gene transfer to enhance radiotherapeutic efficiency, the development in molecular biology provides new possibilities for the biological enhancement of radiotherapy. Retro-viruses were the viral vehicle of choice but adenoviruses now seem more promising due to their ability to transfect resting and proliferation cells and the relative stability of the transfected genes. Attenuated ATM protein expression in human glioblastoma cell by expressing antisense RNA to functional domain of ATM gene followed by increased constitutive expression of *p53* and *p21*, demonstrated radioresistant DNA synthesis, and increased radiosensitivity and may provide a new way for the cancer therapy.

P16 Treatment of Ovarian Cancer Cells with the Transfer of *E.coli* cd/HSV-1 tk Fusion Gene Using the Human Telomerase Reverse Transcriptase Gene Promoter

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BACKGROUND: Genetic prodrug activation (GPAT) has great potential as a gene therapy strategy for cancer. The most frequently used GPAT involves the HSV-1 TK and *E. coli CD* gene. We are interested in the treatment of ovarian cancer using this approach and developing a tumor-specific approach to restrict the treatment to tumor cells. Previous research has indicated that activation of telomerase is tightly regulated at the transcriptional level of the human telomerase reverse transcriptase (hTERT). Because the vast majority of ovarian cancers have telomerase activity, we hypothesise that the hTERT promoter may be telomerase responsive. **OBJECTIVE:** To investigate the *in vitro* effect of an hTERT promoter-driven vector system encoding *E. coli* cd/HSV-1 tk fusion gene in 3AO ovarian cancer cells. **METHODS:** An expression vector (pBTdel 279-cd/tk) encoding cd/tk fusion gene (pWZLneoCDgly TK) under the hTERT promoter was constructed, and then transfected into 3AO ovarian cancer cells by cationic liposome. Following the transfection, 5-FC and GCV were added, and MTT and flow cytometry methods were applied to examine cell survival and antitumor effects. **RESULTS:** Expression vector pBTdel279-cd/tk induced apoptosis in hTERT-positive ovarian cancer cells, but not in hTERT- negative normal ovarian epithelial cells and fibroblasts. In addition, pBTdel 279-cd/tk leads to greater cell death in 3AO than pBTdel 279-tk does. Flow cytometry suggests that there is a greater degree of S-phase block in the pBTdel 279-cd/tk transfected 3AO than in pBTdel 279-tk transfected 3AO. None of these effects is seen following transfection with a control vector that does not encode cd or tk gene. **CONCLUSIONS:** The telomerase-specific transfer of the cd/tk fusion gene under the hTERT promoter is a novel targeting approach for the treatment of ovarian cancer and may lead to an effective and specific gene therapy.

P17 Cationic Liposome-mediated Transfer of Caspase-3 together with *E. coli* cd/HSV-1 tk Fusion Gene Induces Significant Apoptosis in 3AO Ovarian Cells

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BACKGROUND: Genetic prodrug activation therapy (GAPT) has great potential as a gene therapy strategy for cancer, but clinical trials are disappointing when HSV-1 tk or *E. coli* cd were used. Caspase-3 plays a critical role as an executioner of apoptosis. **OBJECTIVE:** To investigate the in vitro effect of caspase-3 on cell survival when combined with *E. coli* cd/HSV-1 tk fusion gene after cationic liposome facilitated gene transfer to 3AO ovarian cancer cells. **METHODS:** Expression vector encoding caspase-3 (pcDNA3-casp3) and expression vector consisting of cd and tk fusion gene under the hTERT promoter (pBTdel 279-cd-/tk) were constructed, and then were transfected into 3AO and normal control cell lines by cationic liposome. Following the treatment of the transfected cells with 5-FC and GCV, MTT and flow cytometry were applied to observe their antitumor effects and Western blot was used to observe caspase-3 and its activation. **RESULTS:** 5-FC and GCV treatment lead to greater cell death in pro-caspase-3-expressing clones of 3AO than in control clones of 3AO, as well as more rapid cleavage of PARP. Flow cytometry suggests that there is a greater degree of S-phase block in the pro-caspase-3-expressing clones than in control clones of 3AO following treatment with cd/5-FC and tk/GCV. None of these effects is observed in normal ovarian epithelial cells and fibroblasts, or following transfection with a control vector that dose not encode cd/tk. **CONCLUSIONS:** Co-expression of pro-caspase-3 may lead to a significant enhancement of the efficacy of cd/tk fusion gene therapy, thus providing a new strategy for anticancer gene therapy.

P18 Inhibition of Androgen-Independent Prostate Tumour Growth and Lung Metastases Following Gene-Directed Enzyme Prodrug Therapy

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Gene-directed enzyme prodrug therapy (GDEPT) based on the *E.coli* enzyme purine nucleoside phosphorylase (PNP) provides a new approach to the treatment of slow growing tumours like prostate cancer. The PNP gene is delivered directly into the tumour by injection of an ovine atadenovirus vector (OAdV) that is not neutralised by human antisera that readily neutralize human adenoviruses. Expressed PNP converts a systemically administered prodrug, fludarabine phosphate, to a toxic metabolite, 2-fluoroadenine, that kills infected and nearby cells in a bystander effect by inhibiting DNA, RNA and protein synthesis. We previously constructed OAdV220 containing the Rous sarcoma virus (RSV) promoter to drive PNP expression and, with fludarabine, demonstrated suppression of primary prostate tumour growth. The present study was conducted to determine if a distant effect could be induced against experimental lung metastases after injection of OAdV220 into orthotopic prostate tumours in immune competent mice. **Methods:** RM-1 cells were implanted intra-prostatically (iprost) in male C57BL/6 mice on day 0 to produce a primary prostate tumour. On day 6, the mice were injected intravenously with RM-1 cells via the tail vein to establish lung pseudo-metastases. Mice (10 per group) in treatment group received a single iprost injection of 10^{10} particles of OAdV220 on day 4, while control mice received an iprost injection of vector dilution buffer. All mice received an intraperitoneal (ip) injection of fludarabine daily (600mg/m^2/day) for 5 consecutive days from day 5. Mice were sacrificed on day 18; prostate tumours and lungs were harvested. In a separate experiment, 3 mice from each group were sacrificed on days 7, 12 and 18; prostate tumours and lungs were harvested for histologic analysis. **Results:** OAdV220 and fludarabine treatment (GDEPT) significantly inhibited prostate tumour growth and formation of lung metastases, showing a 42% reduction in prostate tumour weight ($P < 0.05$), a 47% reduction in prostate tumour volume ($P < 0.05$) and a 68% reduction in lung colony number ($P < 0.001$) when compared with the control group. Analysis by TUNEL assay showed a two fold increase in apoptotic cells in GDEPT- treated prostate tumours on day 18, but no difference in apoptosis in lung tissue was found between treatment and control groups. Proliferating activity in both prostate tumours and metastatic lung colonies, as evaluated by proliferating cell nuclear antigen (PCNA) immunostaining, was significantly suppressed after GDEPT treatment. **Conclusion:** Ovine atadenovirus-delivered GDEPT based on PNP and fludarabine can induce a systemic antitumour response and inhibit the growth of prostate cancer pseudo-metastases in lung in immune competent mice. Antitumour efficacy is associated with an increase in apoptosis in prostate tumours and suppression of tumour cell proliferation.

P19 Application of a new transgene expression system for gene therapy in the retinal pigment epithelium

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The construction of vectors targeting gene expression to specific cell type or tissue in an organism is one of most significant challenges in the field of gene therapy. In most viral delivery systems, cell/tissue-specific promoters and/or related *cis*-activator elements (enhancers) are used to ensure therapeutic gene expression level in defined regions. However, gene expression in specific sites is limited or hampered by the poor transcriptional activity of cell-/tissue- specific promoters and the lack of related *cis*-acting positive regulatory elements. Here, we present a new transgene expression system mediated by a chimeric transcriptional activator for the enhancement of transgene transcriptional activity in retinal pigment epithelial (RPE) cells and its potential application for gene therapy targeted at the retinal pigment epithelium.

To achieve a high level of reporter gene expression in RPE cells, we used the chimeric transcriptional activator GAL4-VP16 DNA and its' DNA binding sites. To ensure specificity, we chose a human or mouse RPE 65 promoter (R65P), which regulates the expression of the major protein specific to the vertebrate RPE, to drive a reporter gene (luciferase) and GAL4-VP16. We prepared plasmid DNA pGV and pLuc series, where GAL4-VP16 is controlled by R65P, and the reporter gene is controlled by different promoters, respectively. We also constructed single plasmid constructs in which GAL4-VP16 and the reporter genes are within the same plasmid DNA.

The results of transient cotransfection of pGV and pLuc DNA constructs showed that in RPE cells the enhancement of reporter gene expression is up to 13-14 fold when the human RPE65 promoter drives the reporter and GAL4-VP16 genes in the system. There was no significant transgene expression observed in other human cells. The constructs containing the mouse counterpart indicated high species-specificity in the human RPE

cells. The levels of the reporter gene expression remained low in most of the single DNA constructs except for one plasmid, where GAL4-VP16 and the reporter genes were regulated by the human RPE65 promoter. This single construct retained high level of specificity and increased the activity of the RPE65 promoter over 4 fold in RPE cells.

Our study showed that the gene expression system can dramatically increase or amplify the transcriptional activity of weak and cell-specific promoters. The system can be used any cell type or tissue if related cell-/tissues- specific promoters are available. Most interestingly, our presentation offers a considerable step forward to cell-specific gene therapy vehicles.

P20 The silencing effect of RNA Interference in 293T Cells

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RNA interference (RNAi) is a process where double-stranded RNA (dsRNA) directs sequence-specific degradation of messenger RNA (mRNA) in animal and plant cells. In several model systems, RNAi had been developed into a useful tool for the investigation of gene function. In order to study the effectiveness of RNAi in mammalian cells, we introduced chemically synthesized 21-nucleotide short double stranded RNA (dsRNA) duplexes corresponding to EGFP into 293T-EGFP cells. 293T-EGFP is a cloned cell line transduced with a lentiviral vector encoding EGFP. It thus expresses EGFP constitutively. Three methods were used for transfection, including TransIT-TKO, Oligofectamine™ and Lipofectamine™2000. Our results showed that EGFP expression was significantly and specifically inhibited by the introduction of the dsRNA, but not by unrelated dsRNA. Furthermore, dsRNA was effective at 10nM, a very low concentration. Amongst the three different transfection reagents, lipofectamine™2000 demonstrated the highest transfection efficiency with a 48 h exposure. Although TransIT-TKO and Oligofectamine™ displayed similar trends, they had lower transfection efficiency. The silencing effects were 79.58% for Lipofectamine™2000, 61.72% for TransIT-TKO and 50% for Oligofectamine™ respectively. However, TransIT-TKO and Oligofectamine™ reagents were somewhat toxic to the 293T cells. Therefore, we concluded that the Lipofectamine™2000 was the best method for the transfection of dsRNA for RNAi. RNAi pathway is active in mammalian embryo cells and may be developed into a potential tool for gene therapy.

P21 New Method to Suppress Gene Expression in Lung Cancer Cells

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Purpose: RNA interference (RNAi) is a new technology for gene silencing. Can RNAi be used in gene suppression in malignant lung tumors? Using the enhanced green fluorescent protein (EGFP) gene as a reporter, we have demonstrated RNAi effects in human embryo and lung cancer cells in vitro. **Methods:** Plasmid pIRB containing two opposite CMV promoters was constructed by gene recombination. Plasmid pIRBEGFP was made from pIRB by inserting short EGFP fragments into the sequence between the CMV promoters. pIRBEGFP could express sense and antisense mRNA strands of EGFP fragment simultaneously. pHR'EGFP is an EGFP expression plasmid. pIRBEGFP and pHR'EGFP were co-transfected into the human embryo kidney cell line 293T and lung adenocarcinoma cell line SPC-A1. The expression of EGFP was monitored by Flow Cytometry. **Results:** (1) The percentage of EGFP positive cells and mean fluorescent intensity in 293T cells transduced with pHR'EGFP and pIRBEGFP decreased significantly when compared with pHR'EGFP alone (by 50%, $P < 0.01$). (2) The RNAi effect of pIRBEGFP on 293T cells showed dose-effect relation. (3) When the lung cancer cell line SPC-A1 was transfected with pHR'EGFP and pIRBEGFP, the EGFP expression also declined by 50% compared with the control group transfected with pHR'EGFP. **Conclusion:** The constructed new plasmid was effective in both human embryonic and lung cancer cells for suppression of EGFP marker gene. It may be useful for tumor gene suppression in lung cancer cells for human gene therapy purposes in the future.

P22 Implications for involvement of the ENO1/ MBP-1 gene in neuroblastoma tumorigenesis.

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Neuroblastoma is the most common solid tumour of childhood. It is a tumour of the postganglionic sympathetic nervous system and common genetic features of this tumour are amplification of the oncogene MYCN and deletions of part of chromosome arm 1p. Both these features are strongly associated with aggressive tumour and bad outcome for the patient.
A smallest region of overlap of deletion has earlier been determined in primary neuroblastomas to an approximately 2 Mbp region in 1p36.2-3. It is likely that this region contains one or more tumour suppressor genes important in neuroblastoma tumourigenesis.

The ENO1-gene is located within this region on chromosome 1 and one alternative translated product of the ENO1-gene known as MBP-1, acts as a negative regulator of the *c-myc* oncogene. This makes ENO1/MBP-1 a good candidate for being a neuroblastoma tumour suppressor gene.

Here we have been able to show, by transfection studies, that introduction of ENO1 into 1p-deleted neuroblastoma cell lines (SKNAS & IMR32) causes reduction of cell growth and induces apoptosis. This effect could however also be seen when the gene was transfected into non-neuroblastoma cells (293-cells), indicating that overexpression of ENO1 has a general growth reduction effect.

We can also shown by RT-PCR studies of primary neuroblastoma tumours, that the expression of the MBP1-part of ENO1 is absent or very reduced in 70 % (12/17) of stage 4 neuroblastomas, independently of 1p-deletion status, compared to low stage (stage 1, 2 ,4S) neuroblastomas and ganglioneuromas, where expression could be detected in all 9 cases tested.

However after mutation screening, by sequencing and/or DHPLC, of all exonic sequences and the promoter region of ENO1 in 50 primary neuroblastomas of all different stages, no mutations had been detected. This suggests that a different mechanism alters the expression of the MBP-1 part of the ENO1 gene, such as splicing errors or some other mechanism for early termination of the transcript.

P23 Multiple exon skipping in the dystrophin gene – a more effective therapy?

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Duchenne muscular dystrophy (DMD) is an X-linked recessive muscle wasting disorder characterised by the absence of the protein dystrophin. Rare dystrophin-positive revertant fibres exist in DMD muscle, arising from a naturally occurring phenomenon where the splicing machinery has been re-directed to exclude the DMD mutation. Furthermore, protein and mRNA transcript studies have indicated that revertant fibres in the mouse model of muscular dystrophy involved skipping of at least 5 exons. The most common revertant fibres were missing more than 20 exons. Alternatively processed dystrophin gene transcripts missing only exon 23, the minimum rearrangement to by-pass the mutation, have never been detected in untreated mdx muscle. In-situ hybridisation studies indicated that there were no gross genomic rearrangements and suggested that the revertant exon skipping arose through alternative splicing.

Antisense oligonucleotides have been used to re-direct dystrophin pre-mRNA processing by blocking sequences crucial to pre-mRNA splicing, thereby inducing skipping of target exons. We wished to determine if AO targeting of multiple exons in the dystrophin gene transcript was possible and if we could induce a revertant-like transcript. Based on the observation that revertant fibres increase with age, there may be some selective advantage to dystrophin missing the protein encoded by several exons. The revertant dystrophins may be more biologically functional than a protein missing only 71 amino acids encoded by exon 23.

Antisense oligonucleotides were directed at motifs involved in pre-mRNA splicing of dystrophin exons 19-25. Cultured *mdx* mouse myotubes were transfected with either single antisense oligonucleotides or combinations. RT-PCR studies were undertaken to determine both specificity and sensitivity of induced multiple-exon skipping. Singly, each antisense oligonucleotide was able to induce the skipping of the target dystrophin exon at which it was directed. Selected combinations of antisense oligonucleotides were able to induce the removal of the targeted exons. No other regions of the dystrophin message were altered in the final transcript as a consequence of these antisense oligonucleotide treatments. The ability to manipulate the outcome of the final message provides real potential for a genetic therapy, as these modifications may emulate naturally occurring processes within the dystrophic muscles of Duchenne muscular dystrophy patients.

P24 Inducing dystrophin pre-mRNA exon skipping as a potential genetic therapy: target selection for improved antisense oligonucleotide activity.

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Antisense oligonucleotides (AOs) have emerged as a potential genetic therapy for a number of inherited diseases including Duchenne muscular dystrophy (DMD). DMD is a multi-system disorder whose primary feature is progressive and fatal muscle wasting caused by a lack of functional dystrophin, a large mechano-sensory protein normally localised to the sarcolemma of muscle fibres. We have previously demonstrated that a 25-mer 2'-*O*-methyl phosphorothioate AO targeted against the 5' (donor) splice site of intron 23 was able to induce exclusion of exon 23 from the mature dystrophin mRNA transcript in the *mdx* mouse model of DMD, both *in vitro* and *in vivo*. Exclusion of exon 23 removed the *mdx* mouse nonsense mutation whilst maintaining the mRNA reading frame, thereby enabling synthesis of a slightly shorter, but still functional, dystrophin protein. Our more recent results have indicated that refining AO design could improve their activity markedly, even if the target site was only subtly shifted. By contrast, we have never observed exon 23 skipping when using several different AOs targeting the 3' (acceptor) splice site of intron 22, suggesting that the donor splice site may be the more amenable target for AO-induced skipping of exon 23. However, this trend was not maintained when we used AOs to induce specific removal of exon 19. In this study, we found that exonic splicing enhancer (ESE) motifs were marginally better targets than consensus splice sites and that exon 19 skipping could be induced by AOs targeting the acceptor splice site.

In order to improve our understanding of which target sites and sequence motifs might be most amenable to AO activity, we wished to compare target site sensitivity and induction parameters for a large range of exons in multiple species. Here we report the AO designs and effectiveness for a broad range of targets. Included are comparisons of different splicing motifs in several exons in both transfected mouse and human cultured cells. Relative AO activity, and thus target site sensitivity, was determined by the minimum effective dose of AO required to induce specific skipping of the target exon as demonstrated by RT-PCR assays. AOs were delivered to cultured cells as complexes with the cationic liposome Lipofectin. We also investigated the possibility of non-specific exon skipping in different regions of the dystrophin transcript and several other mRNAs, including spectrin and the homologous gene utrophin, both of which share similarities with dystrophin. The broader aim of these studies was to provide information that might improve the rational design of AOs designed to interfere with the splicing process and to assess the relative specificity, and thus safety, of AOs.

P25 Transient Suppression of the Myostatin Gene Transcript – A Comparison of Different Antisense Nucleic Acid Mechanisms.

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Antisense nucleic acids function on the basis of Watson-Crick hybridisation with a target sequence and induce changes in the flow of information from gene sequence to protein. There are various outcomes from these interactions, depending upon the mechanism through which a particular antisense molecule induces its effect. These may include the targeted degradation of RNA products, redirection of natural splicing mechanisms, the induced correction of genomic mutations (gene correction) or the inhibition of translational events. We have investigated three different mechanisms of antisense action and compared their ability to induce transient suppression of the products of the *myostatin* gene. This gene product is known to be a negative regulator of muscle growth that inhibits myoblast proliferation and may potentially be a target with which to investigate antisense effects in muscle. In this study, we compare three different antisense strategies that are known to invoke fundamentally different mechanisms. These are: 1) the phosphorothioate deoxyoligoribonucleotides (PS-ODN) known to activate the ubiquitous RNAseH enzyme and induce targeted destruction of DNA-RNA hybrid molecules 2) the 2'-O-methyl oligoribonucleotides (2OMeAO) that redirect nuclear splicing events to exclude exons from the mRNA transcript 3) A dsRNA molecule with 2'ACE chemistry that is reported to induce a potent silencing pathway in plant and mammalian systems. Transient suppression of the *myostatin* product could result in hyperplasia and/or hypertrophy of muscle and this may hold potential as a treatment for patients with muscle wasting conditions.

P26 Apoptosis and expression of Bcl-2 and Bax gene in mice viral myocarditis

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Objective: To assess the role of apoptosis in viral myocarditis (VM) and if it is modulated by the Bcl-2 and Bax genes.

Methods: One hundred and twenty five Balb/c mice were included in the experiment. Twenty mice in each experimental group inoculated with 10^9 $TCID_{50}$ CVB_3 in 0.12 ml and five mice in each control group inoculated with saline were sacrificed on 7, 10, 14, 21, 28 days post-inoculation (p.i.) respectively. Light microscopic, electron microscopic and terminal transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assays quantified inflammation, necrosis, and apoptosis in myocardium. Presence in myocardium of apoptosis-related proteins Bcl-2 and Bax were determined by immunohistochemistry.

Results: 1. The incidence of VM in the mice inoculated with CVB_3 was 86%. Apoptotic myocytes, endothelial cells and infiltrating cells were detected in myocardium of CVB_3-injected mice by electron microscope and TUNEL. TUNEL-positive myocytes increased significantly from 7 to 14 days, then reduced after 21 to 28 days p.i. ($p<0.05$). The positive rate of mice with TUNEL-positive cells was higher in mice exhibiting moderate to severe histopathology than that in mice exhibiting mild histopathology ($p<0.01$). No apoptotic cells and TUNEL-positive cells were detected in myocardium of saline-injected controls.
2. There was no Bcl-2 immunoreactivity in myocardium from control mice; however, the dynamic changes of Bcl-2 expression levels in experimental groups showed significant positive correlation with the changes of myocardial histopathologic scores ($r=0.93$, $p<0.01$). The amount of Bax protein increased prominently in the myocardium from experimental groups compared with the control group ($p<0.05$). With apoptosis in experimental groups increasing significantly from 7 to 14 days p.i., both expression of Bcl-2 and Bax increased remarkably.

Conclusion: 1. Myocyte apoptosis does exist in Balb/c mice with acute VM and may be one of the mechanisms causing myocyte damage in VM. 2. Bcl-2 and Bax genes were involved in the regulation of apoptosis of myocytes and infiltrating lymphocytes of mice with VM. Over expression of Bax might promote apoptosis in myocytes, while Bcl-2 can protect myocytes.

P27 Myogenic Differentiation of Adipose-Derived Cells: A Potential Cell Source for Autologous Cell Transplantation in Duchenne Muscular Dystrophy.

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Duchenne Muscular Dystrophy (DMD) is an X-linked recessive disorder that affects 1 in 3500 live male births. The pathological basis of DMD involves mutation of the dystrophin gene (*dys*), which results in progressive muscle deterioration until death in the early 20's. Previous data have shown that the *dys* mutation in the *mdx* mouse (a mouse model of DMD) can be corrected *in vitro* in up to 20% of myoblasts using short-fragment homologous recombination. Myoblasts transplanted into the muscle of the *mdx* mouse have been shown to remodel disease-damaged muscle fibres suggesting that cell therapy is a potential way to deliver corrected *dys* gene loci to dystrophic muscle in an autologous manner. However, in individuals with DMD the progressive degeneration of muscle results in a deletion of specific muscle-derived stem cells for transplantation due to the considerable strain already imparted on the cell-mediated regenerative capacity. Therefore, an alternative cell source for autologous treatment of dystrophic muscle would be beneficial. We have isolated a novel population of cells from juvenile mouse and human adipose tissue that formed spheres of round, non-adherent cells *in vitro*. These adipose-derived spheres were differentiated in *mdx* myoblast conditioned medium. After 3 weeks, cells with myoblast morphology that expressed the myogenic marker desmin were observed in both mouse and human cultures. Isolation and expansion of cells with myogenic potential from easily accessible (and potentially autologous) tissue source such as fat may have significant therapeutic applications for the treatment of degenerative neuromuscular conditions.

P28 Short Fragment Homologous Replacement strategies for frame shift and splice junction mutations involved in neuromuscular disorders

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The muscular dystrophies are a heterogeneous group of disorders that affect 1/1000 individuals. Duchenne Muscular Dystrophy (DMD) is a progressive muscle wasting disease affecting 1/3500 males resulting from codon reading frame disrupting mutations of the dystrophin gene. These include point mutations (35%) and deletions (65%), which in turn result in an absence of functional dystrophin protein. We have successfully corrected the point mutation in the *mdx* mouse model of DMD in 20% of cultured *mdx* myoblasts using a technique called short fragment homologous replacement and developed strategies for the conversion of frame shift mutations (deletions and deletion/insertions) in lymphoblast cell lines from patients with DMD. These strategies involve modification of the patient sequence by insertion or removal of nucleotides to restore the reading frame of dystrophin. This template is used for the preparation of transfection fragments used for targeted corrective gene conversion. This modified sequence restores the reading frame of the dystrophin protein, potentially resulting in a less severe "Becker-like" dystrophin protein in successfully converted cells. We have also developed strategies for the conversion of the centromeric copy of the survival motor neurone (SMN) gene involved in the pathogenesis of spinal muscular atrophy (SMA). This strategy involves targeted "de-activation" of a splice site that is responsible for the removal of exon 7 from the centromeric SMN transcript. Targeted modification of the polymorphism responsible for alternative splicing of the SMN gene potentially results in production of full-length functional SMN protein in treated cell lines. These strategies form a basis for ex-vivo SFHR-mediated TCGC in two different neuromuscular disorders, DMD and SMA.

P29 Microarray Analysis of a β Cell Surrogate: The Insulin Producing Liver Cell Line HEP G2ins/g

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As a potential cure for Type I diabetes, regulated hepatic insulin production is being considered and investigated. The hallmark of endocrine cells is the possession of storage granules

that allow the rapid, regulated secretion of hormones, such as insulin. Without these storage granules minute by minute regulation of blood glucose levels is not possible. The biogenesis of such storage granules in endocrine cells is at this stage unknown.

Previously we reported the creation of the liver cell line HEP G2ins/g (Simpson AM et al, *Gene Therapy*, 1997; 4, p1202-1215). Human cDNA for both insulin and the glucose transporter GLUT2 were stably transfected into the parental cell line HEP G2. This cell line was then able to synthesise, store and secrete proinsulin in a regulated manner with glucose-controlled secretion. Importantly, the HEP G2ins/g cells possessed dense-core storage granules similar to those found in β cells.

By comparing the mRNA expression in this cell line with that in the parent cell line using microarray technology, it was hoped that the genes involved in the biogenesis of storage granules would become evident.

Data obtained using a human 6K cDNA array and a human 19K oligonucleotide array has showed changes of expression of more than 2-fold in 192 (70 down-regulated, 122 up-regulated) and 434 (215 up-regulated, 219 down-regulated) genes or ESTs respectfully in HEP G2ins/g cells.

As expected the expression of insulin mRNA in the HEP G2ins/g cell line was up-regulated greater than 40 fold. 5 histone/chromatin related genes showed an expressional increase in HEP G2ins/g, which may involve a chromatin re-arrangement related event. Such chromatin rearrangement events may allow the access of transcription factors to genes involved in the formation of storage granules. Collectively, many genes associated with the cytoskeleton were up-regulated, which may be due to the movement of granules from the cytoplasm to the cell membrane for proinsulin exocytosis.

Pancreatic transcription factors and proteins whose expression was not detected by microarray include PDX-1 (confirmed by PCR), PC1, PC2 (confirmed by PCR), Pax4, and Isl-1, whilst NeuroD (confirmed by PCR) was present in both cell lines though showed no change. The pancreatic enriched transcription factor Pax6 was up-regulated in the insulin producing HEP G2ins/g cells (confirmed by PCR). Pax6 is critical in the development of the endocrine pancreas, and is essential for hormone (insulin) gene expression. Pax6 also influences the expression of the pancreatic transcription factor PDX-1, which is involved in the expression of many β cell specific genes.

In conclusion, expression of the insulin and GLUT2 cDNA in the HEP G2 cell line affected the expression of a considerable number of genes. This transgenic expression did not induce the expression of many pancreatic transcription factors, although an increase in the expression of Pax6 was observed. It is not known if Pax6 has any influence in secretory granule biogenesis. Future studies involving anti-sense Pax6 may elucidate the effect of Pax6 expression on secretory granule biogenesis in HEP G2ins/g cells.

P30 Supercoiled Plasmid DNA : a Generic Purification Process from Lab to Large-scale Production

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In the last decade, the administration of therapeutic genes to patients has become a reality in many clinical trials. Non-viral vectors are often preferred to minimize the risk for transfer of pathogens. This increases the demand for highly purified plasmids in relatively large amounts for use in gene therapy and plasmid-based vaccines.

Stringent demands from regulatory authorities dictate that nearly homogeneous preparations for supercoiled DNA should be used for clinical applications. The production of plasmid DNA includes several steps from fermentation to the final product. The scalability of column chromatography approaches to purification issues is an obvious advantage in this perspective. This poster describes a three-step chromatograhy process for the selective purification of supercoiled plasmid DNA from a clarified bacterial cell lysate, which is specially designed for easy transfer to GMP manufacturing.

Step 1 : Group separation by preparative size exclusion chromatography for RNA removal

Step 2 : Thiophilic aromatic chromatography for selective capture of supercoiled plasmid DNA

Step 3 : Anion exchange chromatography for sample concentration and endotoxin removal

The resulting plasmid DNA preparation is of excellent quality and purity and meets stringent requirements for clinical use. The purification process outlined above has several benefits including the fact that it is a completely scalable (generic) process. The purification of plasmids can easily be scaled up from lab to GMP compliant production scale if other requirements such as clean facilities are met.

The process does not include any precipitation steps and no additives like detergents and alcohols are required. It completely removes RNA without the use of RNase and it contains no ultrafiltration steps that may shear the DNA. The extremely high selectivity of thiophilic aromatic chromatography (PlasmidSelect) for plasmid DNA isoforms enables purification of plasmids in their native supercoiled form.

P31 Potential use of cathepsin D proximal promoter in gene therapy targeting retinal pigment epithelium cells

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In view of the importance of retinal pigment epithelium (RPE) in maintaining the health and integrity of the retina, any RPE abnormalities could lead to a damaging cascade of degenerations. Recently, recombinant adeno-associated virus (AAV) mediated gene therapy has been shown to have a potential for the treatment of ocular diseases affecting the RPE layer. A limitation with the current approach however is the lack of RPE-specific gene expression following virus-mediated gene delivery. Therefore, our aim is to develop a RPE-specific promoter targeting RPE cells and to functionally test such a promoter in the context of an AAV vector.

A 368 (-1 to –367) fragment of cathepsin D (CatD) promoter was cloned upstream of a reporter gene encoding the enhanced green fluorescent protein (GFP). This construct was transiently transfected into RPE cell lines (D407 and RPE51) and control kidney epithelium cells (HEK-293). The cassette containing the CatD promoter and GFP gene was then cloned into a rAAV vector (rAAV.CD.gfp). Two microlitres of rAAV.CD.gfp particles ($3.38x10^8$ transduction unit (t.u)/mL) were injected subretinally into non-pigmented RCS/rdy^+ rats. At 8 and 12 weeks post-injection, the RPE layer was separated from the neuroretina layers, flatmounted and examined for GFP expression by fluorescence microscopy. As control, rAAV.CMV.gfp was also injected subretinally.

In vitro analysis showed that the 368bp CatD proximal promoter is relatively specific for the RPE cells. Flow cytometry analysis results showed that there was stronger GFP signal intensity in cultured RPE cells than in HEK-293 cells. In addition,

there were no signs of cytotoxicity observed during the study. Clinical ophthalmic examination and colour fundus photography of the experimental animals indicated a normal retinal morphology. Fluorescence microscopical analysis of the separated RPE and neuroretina flatmounts of rAAV.CD.gfp-injected animals demonstrated GFP expression in the RPE layer with no or low level of expression in the photoreceptor cells. In contrast, injection with rAAV.CMV.gfp resulted in strong GFP expression in both the RPE and photoreceptor layers.

In conclusion, this study shows the transcriptional activity of 368bp-proximal CatD promoter in the RPE cells. Therefore, it has the potential for rAAV-mediated gene therapy targeting the RPE cells.

P32 Development of a Novel Bacterial Artificial Chromosome Cloning System for Functional Studies

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Bacterial artificial chromosomes (BAC) cloning systems currently in use generate high quality genomic libraries for gene mapping, identification and sequencing. However, the most commonly used BAC cloning systems do not facilitate functional studies in eukaryotic cells. To overcome this limitation, we have developed pEBAC190G, a new BAC vector that combines the features of the first generation PAC/BAC vectors with eukaryotic elements that facilitate the transfection, episomal maintenance and functional analysis of large genomic fragments in eukaryotic cells. A number of different cloning strategies may be used to retrofit genomic fragments from existing libraries into the new vector. The system was tested by the retrofitting of a 170kb *Not*I genomic fragment carrying the human *mma* locus from the RPCI-11 BAC library into the *Not*I site of pEBAC190G. Clones from any eukaryotic genomic library harboured in this vector can be transferred from bacteria directly to eukaryotic cells for functional analysis. The new vector should greatly facilitate the experimental characterisation of genes and loci for which no function has been assigned, speed up the identification of various regulatory elements on known genes and facilitate the use of intact genomic loci for therapeutic applications.

P33 Mouse Models for Methylmalonic Aciduria (MMA) and their Potential Application for Cell and Gene Therapies.

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MMA is an inherited disorder of organic acid metabolism, in which despite conventional treatment to modify disease and allow survival, high mortality and morbidity remain. Alternative forms of therapy are needed to improve outlook. Correction of the enzyme defect with gene/cell therapy approaches would be ideal. As there is currently no MMA animal model, our objective is to develop accurate disease models to enable evaluation of such therapeutic approaches.

We have produced transgenic mice for **1)** the normal human mutase locus, **2)** the human mutase locus carrying a point mutation identified from our patient molecular studies, and **3)** a knockout of the mouse mutase locus. A fully sequenced bacterial artificial chromosome (BAC) clone of 170 kb, containing the intact human mutase locus and significant flanking sequences was used to generate transgenic mice by pronuclear microinjection. Four founder lines have been established and characterised. All lines express the human transgene in blood, liver, kidney and brain. The level of expression correlates with the type of tissue and the number of copies of the transgene. Two-stage *GET Recombination,* was used to introduce a single base change point mutation into the identified human BAC. Transgenic mice have been generated by microinjection.

We have also produced mice that have a targeted deletion of exon 3 of the mutase locus. Homozygous knockout mice recapitulate the key biochemical changes that are characteristic of the human disease and display early neonatal lethality. The availability of this knockout mouse model should greatly facilitate studies on the pathophysiology of the disease and the development of novel cell and gene therapy approaches. The homozygous knockout mice are also being bred with our transgenic mouse models to look for functional complementation with the human *MMA* locus and to allow evaluation of therapeutic approaches under physiologically relevant conditions.

P34 Development of Accurate Transgenic Mouse Models of Friedreich Ataxia

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Two new types of transgenic mouse models of Friedreich ataxia have been developed. Each utilises a fully sequenced 188 kb genomic DNA fragment containing exons 1-5b of the human *FRDA* gene obtained from a bacterial artificial chromosome clone. Three independent transgenic mouse lines carrying the intact genomic fragment have been established. Fluorescent *in situ* hybridisation showed that each line had a single site of integration of the transgene. A competitive PCR assay was used to estimate the transgene copy number in all three lines. RT-PCR analysis demonstrated that human *FRDA* mRNA is expressed in all the lines. The h*FRDA*/m*Frda* RNA ratio between the three lines in different tissues demonstrated that the pattern of expression is similar, confirming that the 188 kb fragment confers position-independent expression of the *FRDA* locus. However, there are major differences in this ratio in different tissues. The human/mouse mRNA ratio is highest in the brain and lowest in the blood, while heart, liver and skeletal muscle show similar levels of expression. These studies indicate that the human *FRDA* locus in this genomic fragment may contain a number of regulatory elements that confer tissue specificity in gene expression. Homozygous *Frda* knockout mice, which normally die *in utero*, can be rescued by the presence of this particular *FRDA* genomic fragment, in hemizygous or homozygous form. To date, this is the smallest human genomic fragment shown to complement the embryonic lethal phenotype of the *Frda* knockout. The availability of the complete sequence of this genomic fragment should facilitate the targeted modification of the *FRDA* gene by the introduction of a GAA trinucleotide expansion, or known point mutations, for the generation of accurate transgenic mouse models of Friedreich ataxia. To facilitate studies of *FRDA* gene expression *in vivo*, another two transgenic lines have been established with a genomic reporter construct consisting of an in-frame fusion of *Enhanced Green Fluorescent Protein (EGFP)* at the end of exon 5a of the *FRDA* gene. Both lines exhibit whole-organ green fluorescence confirming the expression of the fusion protein in these mice. Differential levels of *FRDA-EGFP* expression in these mice can be analysed by flow cytometry after enzymatic dissociation of different tissues, while subcellular patterns of *FRDA-EGFP* expression can be examined by cryosectioning of various tissues. These *FRDA-EGFP* genomic reporter mice should allow examination of spatial and temporal aspects of *FRDA* gene expression, and the evaluation of pharmacological agents on *FRDA* expression in the context of a whole animal model.

P35 Development of Genomic Reporter Assays for Pharmacologically Mediated Induction of Fetal Hemoglobin in Human Erythroid Cells.

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Reactivation of foetal haemoglobin genes has been proposed as a potential therapeutic procedure for patients with β-thalassemia, sickle cell disease and other haemoglobinopathies. Despite a number of clinical trials investigating the potential of HbF-inducing chemical agents, such as 5-azacytidine, butyrate and hydroxyurea, there are continued concerns with the long-term use of such agents. Many of these drugs have low efficacy, specificity, and associated cytotoxicity while some are potentially carcinogenic. Therefore, there is an urgent need to identify new pharmacological agents that can induce HbF with greater efficacy and less toxicity.

In vitro model systems based on small plasmid constructs, incorporating minimal regulatory elements of the β globin gene have been used in studies to study the mechanisms of Hb-F regulation, and reactivation by pharmacological agents. Such systems, however, cannot recapitulate the requirements for induction of fetal hemoglobin in adult patients. In this study we report the development of Genomic Reporter Assays (GRAs) using the modified human erythroleukemic cell line K562-EBNA-1, stably expressing the enhanced green fluorescent protein (EGFP) gene placed under the control of the Gγ promoter of the human β globin locus and retaining all of the upstream regulatory elements. The cell lines maintained a uniform level of basal EGFP expression and profile throughout a continuous culturing period extending over 230 days. In our GRA haemin proved to be a potent inducer of EGFP expression resulting in a dose dependent increase reaching a maximum of 764±145%, mirroring the induction levels of the endogenous globin genes. Protein analysis by SDS-PAGE showed EGFP levels following hemin induction constituted 5-10% of total cellular protein. Hydroxyurea and butyric acid resulted in lower induction levels (75±10% and 82±29% respectively) but were noted to have an additive effect (159±72%), suggesting independent mechanisms of action. Conversely, short chain fatty acid derivatives (α-methylhydrocinnamic acid and 2,2-dimethylbutyric acid) failed to induce EGFP expression.

This genomic reporter assay based on the K562-EBNA-1 cell line containing the EGFP-modified human β globin locus can be potentially used for high-throughput screening to identify and evaluate new compounds with the capacity to upregulate the expression of fetal hemoglobin under physiologically relevant conditions. The technology used in the development of this assay could also be implemented in an approach to produce therapeutic proteins *in vitro* and *in vivo* for diseases amenable to protein replacement therapy.

P36 Enhancing homologous recombination - implications for gene therapy

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Gene correction has the potential to repair an endogenous mutation in its chromosomal context via homologous recombination (HR). It also avoids safety concerns associated with random integration. However, progress has been limited due to the poor efficiency of HR in eukaryotic cells and the incomplete understanding of the factors involved.

Gene correction efficiency in prokaryotic cells was determined using the prokaryotic/eukaryotic shuttle vector, pZamp+4. This vector expresses a mutant zeocin resistance gene that has been inactivated by a 4-bp insertion. A very low rate of spontaneous reversion of this mutation was observed in recA- cells which was enhanced about 1000-fold with oligonucleotides in RecE (an exonuclease) and RecT (a homologous recombination pairing protein) expressing cells, indicating the potential of the recombination proteins RecE and RecT to enhance the efficiency of homologous recombination in eukaryotic cells.

The RecE / RecT system and other recombination proteins are currently being adapted for eukaryotic expression using *in vitro* transcription and electroporation of mRNA. Electroporation of Enhanced Green Fluorescent Protein (EGFP) mRNA revealed a transfection efficiency of >80% in human erythroleukaemia (KEB) cells, that the levels of protein expression are similar from cell to cell and that the level of expression can be easily controlled by varying the amount of mRNA added. Gene correction in human erythroleukaemia (KEB) cells is being evaluated using the original pZamp+4 plasmid, as well as pEBAC190GZamp+4, a modified eukaryotic BAC vector allowing episomal maintenance of the mutant zeocin gene and direct visualisation of cells carrying the episome by EGFP expression. Stable cell lines have also been created with pEBAC190GZamp+4 to evaluate gene correction directly at the chromosomal level.

In order to evaluate the efficiency of gene correction in primary cells, we have also developed a humanised mouse model for β-thalassaemia carrying a common 4 bp deletion in the human β-globin gene on a mouse knockout background. The model accurately recapitulates the thalassaemic features typical of this mutation in patients. HPLC and human β-globin immunostaining studies indicate that correction of this mutation in haemopoietic /erythropoietic cell precursors with an efficiency of 1% or higher should be readily detectable.

We hope that the use of mRNA delivery as a means to express bacterial and eukaryotic recombination proteins in human cell lines and primary cells will facilitate the enhancement of gene correction in eukaryotic cells to a clinically useful level.

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