GENE TRANSFER, REPLACEMENT AND AUGMENTATION

Organizers: Inder M. Verma, Fred H. Gage and Richard C. Mulligan

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Poster Sessions

Model Systems

V 001 INDUCTION OF DORSO-ANTERIOR TISSUES IN XENOPUS. William C. Smith, Margaret E. Bolce, Richard M. Harland. Department of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology, 401 Barker Hall, University of California, Berkeley CA 94720

The formation of dorsal mesoderm is the critical event in the formation of the dorsoventral axis of vertebrates. In Xenopus, signals from the yolky vegetal hemisphere induce equatorial cells to become mesoderm. A special signal from the vegetal dorsalizing center (Nieuwkoop center) induces dorsal mesoderm. This dorsal mesoderm has "organizer" activity and can induce a complete body axis, including the nervous system. Activins and FGF have previously been implicated in the process of mesoderm induction, but neither can rescue a complete embryonic axis alone. We find that activin alone cannot induce notochord, but can induce other types of dorsal mesoderm with neural inducing activity. However the neural tissues induced are posterior to the midbrain and do not extend to anterior neural tissue. In order to identify other molecules that may take part in these inductions, we have assayed for mRNAs that can rescue dorsal structures in ventralized embryos. Ventralized embryos are injected with mRNAs from a cDNA expression library, and subsequently scored for dorsal structures. Sib-selection from the library has identified two separate dorsalizing RNAs. The first to be identified is Xwnt-8, from the Wnt family of signalling molecules (Smith and Harland, 1991). The second is predicted to encode a secreted polypeptide but has no apparent similarity to previously described peptide growth factors. Both these factors differ from FGF and activin in that they can rescue a complete axis, including anterior neural derivatives (e.g. eyes). Xwnt-8 is not expressed at the right time or place to be the endogenous inducer, but may be mimicking an endogenous inducer when introduced artificially by injection. In contrast the time and place of expression of the newer inducer is consistent with a role in axis formation in the normal embryo.

Smith W.C. and Harland, R.M. (1991) Injected Xwnt-8 RNA acts early in *Xenopus* embryos to promote formation of a vegetal dorsalizing center Cell 67, 753-765

V 002 THE DVR GENE FAMILY IN VERTEBRATE DEVELOPMENT, Brigid Hogan, Michael Jones, Nancy Wall, Christopher Wright, Karen Lyons and Manfred Blessing. Department of Cell Biology, Vanderbilt University Medical School, Nashville, TN 37232.

The DVR (Decapentaplegic-Vg-related) gene family is a subgroup of the larger TGF-β gene family. It constitutes at least 10 members in mammals and encodes secreted, dimeric molecules with a highly conserved C-terminal region held together by 7 di-sulphide bonds. These include the proteins originally named Bone Morphogenetic Proteins 2 through 7. We are particularly interested in the role of DVR-4 (BMP-4) and -6 (BMP-6, Vgr-1) in embryonic development. DVR-4 transcripts are first detected in the early somites stage mouse embryo, in the posterior, ventral mesoderm. Subsequently, transcripts are widely distributed in the embryo, particularly at sites of inductive tissue interactions. The initial localization raised the possibility that DVR-4 is involved in the specification of newly formed mesoderm and this has been tested using *Xenopus* embryos. Injection of RNA for human DVR-4 into oocytes blocks development at early gastrulation and no mesoderm is formed. However, animal caps dissected from injected embryos and cultured *in vitro* develop into vesicles containing ventral mesodermal cell types. The same animal caps transplanted into the blastocoel of host embryos induce the formation of secondary tails. These observations, combined with studies on gene expression and the effect of purified protein on animal caps, support the hypothesis that DVR-4 is involved in the specification of posterior/ventral mesoderm in the *Xenopus* embryo. Studies on DVR-4 function in the mammalian embryo will be discussed. DVR-6 transcripts are not detected in mouse embryos until a later stage than DVR-4, and are first seen in dorsal and ventral regions of the nervous system at around 9 days p.c.. A polyclonal rabbit antiserum specific for DVR-6 has been used to study the biosynthesis and localization of protein in the developing CNS and in a variety of other cell types.

V 003 MOLECULAR DISSECTION OF A *DROSOPHILA* MINICHROMOSOME, Gary H. Karpen[1] and Allan C. Spradling[2], [1] The Salk Institute, San Diego, CA, 92186 and [2] The Carnegie Institution of Washington, Baltimore, MD 21210.

Chromosome replication, segregation and transmission are the mechanisms used by organisms and cells to ensure that essential genetic traits are faithfully transmitted between generations. Errors in these processes can result in genomic abnormalities associated with a variety of human disorders. Meiotic and mitotic transmission is accomplished via interactions between a specific chromosomal region (the centromeric DNA) and the cellular "machines" responsible for moving chromosomes (kinetochores, centrioles, and spindle-associated microtubules). We are studying the molecular-genetics of chromosome transmission using a model eukaryotic system, the *Drosophila* minichromosome *Dp(1;f)1187*.

Higher eukaryotic centromeres are embedded in heterochromatin; molecular and genetic analyses of heterochromatin have been inhibited by the presence of large (up to Megabase-size (Mb)) arrays of highly repeated DNAs, or satellites. The presence of cloned, single-copy sequences adjacent to the *Dp(1;f)1187* heterochromatin provided a direct entry point that facilitated the structural analysis of the highly repeated DNA. Pulsed-field Southern analysis demonstrated that *Dp(1;f)1187* is approximately 1.3 Mb in length, including 1 Mb of heterochromatin. Restriction mapping in the vicinity of the euchromatin/heterochromatin junction revealed that the heterochromatin contains complex sequences in addition to the highly repeated satellite DNA. The molecular structure of *Dp1187* was used to demonstrate that heterochromatin can exert a surprisingly strong, negative position-effect on the polytenization of adjacent euchromatin. The molecular effect on DNA copy number was correlated with the phenotypic effect on euchromatic gene function, which led us to propose an alternative model of position-effect variegation based on the somatic elimination of heterochromatic sequences (Karpen and Spradling, Cell *63*, 97-107, 1990).

We used single P-element transposon insertion, and developed a new technique termed "local hopping," to further our analysis of the minichromosome. These transposons have provided entry points for mapping and cloning within the telomeric region, and have allowed us to characterize an extensive domain of subtelomeric heterochromatin (> 50 kb) that displays many of the structural and functional attributes of centromeric heterochromatin.

The structural information and the molecular-genetic tools generated by these studies have most recently been used to initiate the molecular identification, isolation and analysis of the chromosomal elements necessary for proper transmission of this minichromosome. The presence of the *rosy+* gene within the P element transposon has allowed us to generate deletion derivatives of *Dp(1;f)1187* that localize the *cis*-regulators of chromosome transmission, including the centromere, to a specific region of the heterochromatin. The practical applications of this research will be discussed; this includes the development of a minichromosome vector for genetic transformation (including "gene therapy") and for further analyses of chromosomal functions in *Drosophila* and other eukaryotes.

Cancer Genetics (Joint)

V 004 **REGULATING A "MASTER REGULATOR": CDK CONTROL OF THE RETINOBLASTOMA PROTEIN.**

Philip W. Hinds[1], Sibylle Mittnacht[1], Steven F. Dowdy[1], Andrew Arnold[2], Steven I. Reed[3], and Robert A. Weinberg[1], [1]Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142, [2]Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, and [3]The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037.

The retinoblastoma tumor suppressor protein (pRb) is thought to regulate passage through the cell cycle. In normal cells, pRb itself may be negatively regulated by phosphorylation, such that only the hypophosphorylated form of pRb is functional in blocking cell cycle progression. In some tumor cells this block is alleviated by mutational inactivation of pRb. Consistent with this idea, restoration of pRb expression in a variety of tumor cell lines leads to a loss of proliferative or tumorigenic capacity. In the human osteosarcoma cell line SAOS-2, this suppression of proliferation is accompanied by a marked phenotypic change, in that cells transiently transfected with pRb-encoding constructs become greatly enlarged. When co-transfected with a drug-resistance marker, these "flat cells" persist in culture without division for weeks. Tumor-derived and some in vitro pRb mutants are unable to elicit this phenotype, suggesting that the production of flat cells is a consequence of the tumor suppressor function of pRb. Wild-type pRb expressed in SAOS-2 cells is predominantly hypophosphorylated and associated with nuclear structures, consistent with the idea that this form of pRb is functional in suppression of proliferation. Indeed, the apparently total lack of phosphorylation of pRb upon transient expression in SAOS-2 cells suggests that the susceptibility of these cells to pRb may be due to levels of pRb that overwhelm the endogenous kinase system normally responsible for cyclically inactivating pRb. As many of the phosphate residues on pRb are thought to be added by a member(s) of the cdk family of kinases, the regulatory subunits of these kinases, known as cyclins, have been cotransfected with pRb into SAOS-2 cells in an attempt to provide the inactivating mechanism which is apparently normally insufficient in these cells. Two human cyclins, cyclin E and cyclin A, lead to a nearly complete loss of flat cell phenotype, and the pRb produced in the transiently transfected cells failed to associate with the nucleus due to hyperphosphorylation. In addition, two pRb mutants were identified which are resistant to this phenotypic "rescue" mediated by the cyclins, suggesting that cyclin overexpression directly inactivates pRb, rather than circumventing it. These results present evidence that the cyclin/cdk system is responsible for controlling the functional state of pRb, and thus the capacity for cell cycle progression.

V 005 TUMOR SUPPRESSOR GENES AND HEREDITARY TUMOR SYNDROMES IN THE HUMAN NERVOUS SYSTEM: TOWARD THE ISOLATION OF THE GENE CAUSING NEUROFIBROMATOSIS TYPE 2 (NF2), Bernd R. Seizinger[1,2], Nikolai Kley[1], Anil Menon[2], Terry Lerner[2], Mia MacCollin[2], James Trofatter[2], Jonathan Haines[2], James Gusella[2], Ronald Lekanne-Deprez[3], Ellen Zwarthoff[3], [1]Molecular Neuro-Oncology Laboratory, [2]Molecular Neurogenetics Laboratory, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, U.S.A., [3]Department of Pathology, Erasmus University, Rotterdam, The Netherlands.

Many human cancers are known to occur in two different forms: as sporadic tumors in the general population, and as hereditary tumors within families. Hereditary tumor syndromes offer unique model systems for isolating genes whose mutations lead to cancer. There is accumulating evidence that hereditary and sporadic tumors are caused by similar pathogenetic mechanisms affecting the same genes. The cloning and characterization of these genes may therefore have important implications for diagnosis and treatment not only of the relatively rare hereditary tumors, but also for their much more common sporadic counterparts.

Recombinant DNA technology has provided a powerful strategy for investigating inherited diseases in which the defective protein is unknown. This strategy has been termed "reversed genetics" because the first goal is not to find the defective protein, but rather to isolate the defective gene based on the determination of its chromosomal location in the human genome. However, most genes which have been cloned thus far using the "reversed genetics" approach, have been isolated with the additional aid of cytogenetically visible chromosomal aberrations, providing potential short-cuts to the defective genes. For example, the gene for neurofibromatosis type 1 (NF1) was recently identified by cloning the translocation breakpoints of two NF1 patients with constitutional translocations in the NF1 region on chromosome 17q11.2 (see first speaker).

NF is one of the most common inherited disorders affecting the human nervous system. There are two clinically and genetically distinct forms of NF: NF1 (von Recklinghausen NF) and a less frequent form, NF2 (bilateral acoustic NF). Although different cell types may be affected, the most common abnormalities in both NF forms are in the nervous system, in cells of neural crest origin. Acoustic neuromas, Schwann cell-derived tumors of the 8th cranial nerve, are the hallmark of NF2 and frequently lead to deafness. Furthermore, meningiomas and other neural-crest derived tumors such as spinal Schwannomas, are common in NF2 patients. We have previously shown that the gene causing NF2 maps to chromosome 22q. The region containing the NF2 gene is frequently deleted in tumors associated with NF2, in both their sporadic and hereditary forms, including acoustic neuromas, spinal Schwannomas, and meningiomas, suggesting that the NF2 gene belongs to the family of "tumor suppressor" genes, i.e., genes which normally confer growth suppression, and whose loss of function is associated with tumor formation.

The recent discovery of a meningioma tumor specimen with a translocation on chromosome 22 (Zwarthoff et al.) has provided a potential short-cut to the isolation of the NF2 gene. We have shown that the translocation breakpoint maps to the region on chromosome 22 identified as the NF2 region, based on tumor deletion and linkage studies. We have meanwhile identified flanking markers of the translocation breakpoint which recognize the translocation breakpoint on pulsed-field gel electrophoresis. These and other markers also appear to identify constitutional aberrations in patients with NF2 and multiple meningiomas. Thus, the search for transcripts in this region which are expressed in normal Schwann cells, but not in Schwann cell-derived tumors, may eventually lead to the identification of the NF2 gene and/or the or a meningioma "tumor suppressor" locus on chromosome 22.

Transplants

V 006 ANIMAL MODELS OF NORMAL AND LEUKEMIC HUMAN HEMATOPOIESIS BY TRANSPLANTATION INTO IMMUNE-DEFICIENT MICE. John E. Dick, Tsvee Lapidot, and Francoise Pflumio; Dept. of Genetics, Research Institute, Hospital for Sick Children and Dept. of Molecular and Medical Genetics, University of Toronto 555 University Ave. Toronto, Ontario Canada M5G 1X8

A complete understanding of the organization of the human hematopoietic stem cell hierarchy and of the molecular events regulating the stem cell developmental program has been hampered by the absence of suitable in vivo stem cell assays. Hematopoiesis is better understood in the mouse where single stem cells can rescue lethally irradiated or genetically deficient mice by repopulating all myeloid and lymphoid lineages. Perturbations in the normal stem cell program leading to neoplastic growth as the result of aberrant expression of key regulatory genes are also difficult to study because human leukemic cells are difficult to grow in culture. Recent advances in the transplantation of human cells into immune-deficient mice provides an opportunity to study human hematopoiesis -both normal and abnormal- in the context of a small animal model.

Our animal model was designed to reflect as close as possible the current approaches of bone marrow transplantation using IV injection of adult bone marrow into irradiated immune-deficient mice. We have evidence that the murine microenvironment can support human stem cells, but extensive differentiation does not occur probably because of the absence of human growth factors. Treatment of animals with human mast cell growth factor (MGF; kit ligand) and/or PIXY321 (GM-CSF/IL-3 fusion), starting immediately after the human bone marrow transplant or after waiting for 30 days, dramatically enhances the level of engraftment. There was extensive repopulation of the bone marrow with mature human cells of multiple myeloid, lymphoid, and erythroid lineages. Growth factor treated mice had elevated levels of CFU-GM, BFU-E, and multipotential CFU-GEMM progenitors compared to untreated mice that had low numbers of only CFU-GM. Inclusion of EPO in the growth factor cocktail resulted in the production of human RBC in the murine peripheral blood. The appearance of high levels of multiple lineages of human cells and progenitors in response to cytokines, in previously untreated transplanted mice, indicates that a very immature/stem cell can engraft mice. This system represents a novel in vivo method to detect human stem cells and to identify the growth factors that regulate their developmental program. A new system for transplanting human cells into newborn mice leading to engraftment of high levels of functional lymphoid cells will also be discussed.

Mice have also been engrafted with bone marrow from patients with genetic hematopoietic abnormalities including thalassemia and anemia providing a new approach to create animal models of human diseases. In addition, we have transplanted human bone marrow infected with retrovirus vectors into immune-deficient mice and recovered genetically manipulated progenitors up to 4 months later. The combination of an in vivo model with high efficiency gene transfer methods will provide an important preclinical system to test the feasibility of gene therapy.

In addition to normal cells, we have engrafted mice with cells obtained from patients with pre-B acute lymphoblastic leukemia (ALL) at different stages of their disease with the aim of establishing animal models of leukemia. All of the samples from patients at relapse grew rapidly and disseminated widely in the mice, while cells obtained from patients at diagnosis grew poorly if at all. This indicates that there is a correlation between growth in scid mice and clinical outcome. These results establish that the scid mouse is a powerful model to examine the biological characteristics of the growth of human leukemic cells. It will now be possible to evaluate, in vivo, various therapeutic strategies, including gene therapy, targeted against drug-resistant leukemic cells.

V 007 SURGICAL IMPLANTATION OF GENETICALLY-ENGINEERED CELLS INTO RODENTS USING A BIODEGRADABLE FRAMEWORK, Brian A. Naughton[1], Yifan Dai[2], Benson Sibanda[3], Raphael Scharfmann[2], Julia San Roman[3], and Inder M. Verma[2], [1]Hunter College School of Health Sciences, New York, NY 10010, [2]The Salk Institute, LaJolla, CA, [3]Advanced Tissue Sciences, Inc., LaJolla, CA.

The potential use of biodegradable meshes to deliver genetically altered cells was studied. A ß-galactosidase gene was inserted into fibroblasts derived from C57BL/6J mice, bone marrow stromal cells, and various populations of cells isolated after *in situ* perfusion of adult rat liver. The vector LNL-SLXßgal was generated by inserting a 3.1 kilobase-pair *Bam*HI fragment containing the complete coding sequence of the ß-galactosidase gene into the *Bgl* II site of plasmid LNL-SLX. The cells were infected in the presence of Polybrene while growing in monolayer culture. Selection using G418 was initiated 24 hr after infection. ß-galactosidase activity *in vitro* was indicated by the ability of cells to reduce 5-bromo-4-chloro-3-indolyl ß-D-galactoside (X-gal). ß-galactosidase-producing cells assume a blue color in the presence of this substrate. Monolayer cultures containing ≥ 80 % positive cells were treated with a collagenase-dispase solution and single cell suspensions were prepared. These cells were seeded at high density/low volume (2-5 x 10^6 cells/250 µL) onto 20 mm x 40 mm pieces of either Dexon polyglycolic (David + Geck, NJ) or Vicryl polyglactin 910 (Ethicon, Inc., NJ) biodegradable meshes or onto nylon filtration screens (Tetko, Inc., NJ) in Tissue Tek slide chambers. Cellular attachment was evident by 3-5 hrs after inoculation and an additional 3 mL of medium was added to the slide chamber. The meshes were transferred to 25 cm^2 flasks 8-10 hrs later and were grown until confluence was achieved. The maintenance of ß-galactosidase activity was verified using the X-gal method and 5 mm x 10 mm pieces of mesh were cut from the template for grafting. The following combinations of donor cells/recipients were studied: 1. embryonic C57BL/6J mouse fibroblasts into either nude mice or adult C57BL/6J mice. These grafts were either performed as "dermal replacements" or were implanted subcutaneously. 2. Long-Evans rat bone marrow stromal cells into Long-Evans rats. These grafts were either attached to the musculature at various locations on either side of the body wall or applied directly to organs. 3. Long-Evans rat acidophilic hepatic parenchymal cells or hepatic stromal cells into Long-Evans rats. These cells were either grafted onto the liver while attached to the biodegradable mesh or were enzymatically detached from nylon filtration screens and infused directly into the hepatic circulation, and 4. embryonic C57BL/6J fibroblasts growing on nylon screens were inserted into diffusion chambers with a pore size of 0.22 µm. The chambers were then filled with medium, capped, sealed, and implanted intraperitoneally into Long-Evans rats. Tissues containing the grafts were excised from the experimental animals after sacrifice and stained *in toto* with X-gal. After photographs were taken, the tissues were processed for histological sections. ß-galactosidase activity was found in all tissue grafts and at all time periods post-grafting which we studied. This included > 60 days for the nude mouse dermal replacement and subcutaneous grafts, > 30 days for the adult C57BL/6J mouse subcutaneous graft, and > 90 days for the subcutaneous and inner body wall musculature grafts in Long-Evans rats. Animals with longer-term grafts are currently being maintained for evaluation at a later date. These experiments also are being repeated using the gene for factor IX. The findings to date indicate that this method is efficacious for delivering genetically-altered cells.

V 008 RETROVIRUS-MEDIATED GENE TRANSDUCTION INTO CANINE HEMATOPOIETIC STEM CELLS, R. Storb, F.G. Schuening, K. Kawahara, R. To, W.R.A. Osborne, and A.D. Miller, Fred Hutchinson Cancer Research Center and the University of Washington School of Medicine, Seattle, Washington.

Amphotropic helper-free retrovirus vectors containing the bacterial neomycin phosphotransferase (neo) gene and the human adenosine deaminase (ADA) gene were used to transduce canine hematopoietic cells. Two methods of transduction were used. The first method used marrow that was cocultivated for 24 hours on vector-producing cells followed by incubation in a vector-containing long-term marrow culture system for four days. With the second method marrow was obtained at the time of the peripheral blood neutrophil nadir seven days after a single dose of cyclophosphamide (40 mg/kg IV) and cocultivated for 24 hours on vector-producing cells. In either case, autologous marrow was infused into dogs given an otherwise lethal dose of total body irradiation consisting of 920 cGy. Three of seven dogs so treated became long-term survivors, and their marrows showed intermittently at one to eight month intervals between 1 to 11% G418-resistant CFU-GM colonies for now over two years after transplantation. Concurrent polymerase chain reaction (PCR) analysis demonstrated the presence of both the neo and human ADA genes in marrow cells, peripheral blood granulocytes, and peripheral blood and lymph node lymphocytes. These findings provide evidence for gene transduction into pluripotent hematopoietic stem cells. Dilution experiments showed that the fractions of marrow, lymph node, and peripheral blood cells containing the transduced genes were 0.1 to 10%, consistent with the results of the CFU-GM colony studies. Repeated analyses of peripheral blood and marrow cells by starch gel electrophoresis to test for human ADA were negative. Peripheral blood samples of all dogs were free of helper virus, and no long-term side effects of the gene transduction were observed. To further investigate the observation of intermittent appearance of G418-resistant marrow-derived CFU-GM colonies, peripheral blood granulocytes were analyzed at weekly intervals for the presence of the neo gene, assuming that, because of their rapid turnover rate, granulocytes may best represent overall production by the marrow compartment. All three dogs showed neo-containing granulocytes intermittently at one to eight week intervals, suggesting that a small number of transduced long-term repopulating marrow cells gave rise to vector-containing differentiated cells on an intermittent basis, each time returning to a G_0 phase. Ongoing experiments are investigating by inverse PCR clonality of neo-containing granulocytes to determine whether they are derived from one or a few transduced stem cells and to estimate the time period during which transduced stem cells are producing differentiated cells before returning to a G_0 phase.

Delivery Vectors

V 009 INTRACELLULAR IMMUNIZATION AGAINST HIV USING RNA DECOYS, Eli Gilboa, Clay Smith, Seong-Wook Lee, Humilidad F. Gallardo, Bruce A. Sullenger, and Grace E. Ungers, Memorial Sloan-Kettering Cancer Center, New York.

We have previously shown that CEM SS cells which had been transduced with a retroviral vector expressing high levels of RNA transcripts encoding the HIV TAR sequence (TAR decoy) do not support HIV replication. To determine if this approach is applicable to clinical use, we have investigated whether 1) bulk populations of CD4+ T-lymphocytes could be efficiently transduced with retroviral vectors encoding TAR decoy templates, 2) whether transduction provides a survival advantage when challenged in vitro with a cytolytic strain of HIV, 3) whether TAR decoy retroviral vector transduction alters physiological properties of CD4+ T-cells, and 4) whether replication-competent helper virus is generated or IL-2 independent cells emerge in culture following transduction. Utilizing FACS analysis of peripheral blood CD4+ T-cells transduced with a lacZ-expressing retroviral vector or measuring survival in G418 following transduction with a neomycin phosphotransferase-expressing vector, transduction frequencies of 10-40% were achieved. Initial experiments utilizing normal human PBL enriched for CD4+ T-cells transduced with TAR decoy vector reproducibly demonstrated enhanced survival, and diminished gp120 and p24 production following challenge with HIV as compared to controls. No difference in growth kinetics of TAR decoy transduced CD4+ PBL was observed relative to controls. In addition, a CD4+ allospecific clonal cell line NBL-46 transduced with a TAR decoy vector demonstrated expression of TAR decoy RNA by RNA blot analysis while maintaining intact MLC proliferation and unaltered growth kinetics. No replication-competent helper virus was generated as measured by a sensitive amplification method, and no IL-2 independent CD4+ PBL subclones could be isolated following transduction and subsequent culturing for >8 weeks.

V 010 RECOMBINANT HERPES SIMPLEX VIRUS-MEDIATED GENE TRANSFER TO THE NERVOUS SYSTEM, J.C. Glorioso[1], W.F. Goins[1], L.R. Sternberg[1], M. Levine[2], D.J. Fink[3], [1]Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA, [2]Department of Human Genetics and [3]Department of Neurology, University of Michigan Medical School, Ann Arbor, MI

Herpes simplex virus (HSV) displays a natural propensity to establish lifelong latent infections within neurons characterized by the exclusive production of latency associated transcripts (LATs) in the complete absence of lytic gene expression. In order to exploit these natural features for gene transfer to CNS two fundamental problems must be overcome: (i) A mutant virus must be engineered which does not destroy brain tissue yet is capable of establishing latency in a substantial number of targeted neurons without the possibility of reactivation and (ii) a means of expressing the transferred gene of interest during latency must be devised which involves the identification of a latency active promoter/enhancer sequence and an appropriate genome location for insertion of the promoter-gene cassette.

To address the problem of viral neuropathogenesis, members of a significant panel of either replication defective or host-range mutants deficient for essential or accessory nonessential functions respectively were each tested as suitable vector backbones following stereotactic inoculation of rat hippocampus. The ability of these mutants to infect neuronal cells, replicate and spread to other brain regions was assessed using mutant virus recombinants carrying the *lacZ* reporter gene under transcriptional control of an HSV late gene promoter or the HCMV IE gene promoter. The effect of viral infection on neuronal cell viability was assessed using standard histopathologic procedures and electron microscopy. Latently infected cells were identified on the basis of *in situ* hybridization for the LAT RNAs from 2-8 days post-inoculation and long term (10 months) by PCR. The results of these studies identified a number of promising mutants potentially useful as gene transfer vector backbones. These include mutations deleted for essential functions such as the major immediate early gene ICP4 and its promoter inducer, VP16 (αTIF). Promising host-range mutants include deletions of the protein kinase gene US3, a VP16 modifier UL46, the viral ribonucleotide ribonucleotide reductase genes UL39 and UL40, and a partial deletion mutant of ICP4, which specifically blocks virus replication in neurons *in vivo* but not in standard cell cultures.

To address the second problem, studies were carried out to identify the natural promoters within the LAT region in order to exploit their latency active feature to drive foreign gene expression. In addition to a TATA box containing promoter reported by others, a second TATAless LAT promoter was uncovered. Both promoters were tested for their ability to express *lacZ* in the LAT region and several other ectopic loci during latency in both the peripheral and central nervous system. Both promoters were active long-term in the PNS but were weakly active in the CNS. Experiments are now in progress to modify the LAT promoters to enhance their activity and to test a variety of neuronal cell promoters for expression of *lacZ* in hippocampal neurons during latency.

Homologous Recombination (Joint)

V 011 EFFECTS OF LOSS-OF-FUNCTION MUTATIONS IN THE *PIM*-1 ONCOGENE *IN VITRO* AND *IN VIVO*, A. Berns, J. Domen, N. van der Lugt, M. van Lohuizen, H. te Riele, E. Robanus Maandag, C. Saris, P. Laird, A. Clark*, M. Hooper*, Division of Molecular Genetics of The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands; *Department of Pathology, University Medical School, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG, United Kingdom.

Transgenic mice overexpressing the *pim*-1 or c-*myc* proto-oncogene in their lymphoid compartment are predisposed to lymphomagenesis, although to a different extent. Infection of these mice with murine leukemia viruses results in the activation of other proto-oncogenes that collaborate with *pim*-1 or c-*myc* in lymphomagenesis. A number of additional genetic alterations contributing to tumor progression can be identified in this way. Recent findings with respect to some of these genes, such as *bmi*-1 and *pal*-1, will be discussed. In order to get insight into the normal function of these genes we have inactivated *pim*-1 and *bmi*-1 by homologous recombination in Embryonic stem cells. In doing so, we have analysed a number of factors that influence homologous recombination frequencies. We have studied in some detail mice lacking a functional *pim*-1 protein. They show no obvious phenotype. However, upon close examination of their hematopoietic compartment a number of effects were noted. Impaired growth factor responses were found both in mast cells and in lymphoid precursor cells. Implication for the function of *pim*-1 in signal transduction will be discussed.

V 012 TARGETED DISRUPTION OF THE MOUSE GLUCOCEREBROSIDASE GENE: DEVELOPMENT OF A MODEL OF GAUCHER DISEASE, Victor L.J. Tybulewicz[1], Michel L. Tremblay[2], Mary E. LaMarca[3], Barbara K. Stubblefield[3], Suzanne Winfield[3], Barbara Zablocka[3], Ellen Sidransky[3], Brian M. Martin[3], Sing-Ping Huang[2], Keith A. Mintze[1], Heiner Westphal[2], Richard C. Mulligan[1], and Edward I. Ginns[3], [1]Whitehead Inst for Biomed Res and Depart of Biology, MIT, Cambridge, MA 02142, and [2]Lab of Mam Genes and Dev, NICHD, Bethesda, MD 20892; and [3]Sec on Mol Neurogenetics, NSB, NIMH, Bethesda, MD 20892.

Gaucher disease, the most prevalent sphingolipidosis, results from the inherited deficiency of lysosomal glucocerebrosidase (GC). Despite its high frequency in humans, no animal model is available. A naturally occuring canine model of Gaucher disease was reported, but not propagated. Other attempts to create an animal model of Gaucher disease by inhibiting glucocerebrosidase in vivo have been inadequate. In order to produce mice having GC deficiency, we have disrupted the GC gene in mouse embryonic stem cells. The targeting plasmid for GC contained a neomycin gene inserted into exons 9 and 10 of GC, with an hsv-tk gene flanking the construct. This construct was introduced into ES cells by electroporation and the correct targeting event in doubly resistant clones was identified by Southern blot analysis. Four ES cell clones containing the targeted mutation in the GC gene in one allele were injected into blastocysts from C57BL/6 mice and transferred to FVB/N foster mice. One chimeric male transmitted the GC mutation to his progeny. A mouse strain carrying the mutation has been established. Mice homozygous for this "knockout" of the glucocerebrosidase gene have no detectable GC activity (<4% of control). These mice rapidly become cyanotic after birth, and have low weight, abnormal respiration, markedly decreased feeding and movement, and survive less than twenty-four hours. Histological analysis of affected mice shows massive infiltration of PAS positive lipid-laden cells in the liver, resembling Gaucher cells found in patients. Histological abnormalities, including PAS positive cells, are also present in the spleen, lung and bone. Although these mice have a rapidly progressive phenotype, they provide a model for the study of the pathogenesis of symptoms in patients with glucocerebrosidase deficiency. Glucocerebrosidase deficient mice with less severe phenotypes resembling the different types of Gaucher disease may be created either by introducing point mutations into the GC gene by homologous recombination, or by crossing transgenes carrying point mutations into the GC null mice described here. In this way we can obtain a model of Gaucher disease in which novel enzyme replacement, cellular transplantation and somatic gene transfer therapies can be evaluated.

Positional Cloning & Major Diseases-I (Joint)

V 013 MOLECULAR GENETICS OF CYSTIC FIBROSIS AND NEUROFIBROMATOSIS, Mitchell Drumm, David Gutmann, Theresa Strong, Douglas Marchuk, Lisa Smit, Paula Gregory, James Koh, Lone Andersen, Tom Sferra, Susan Wilson-Gunn, Dan Wilkinson, Anna Mitchell, David Lawson, and Francis Collins, University of Michigan, Ann Arbor, Michigan, 48109-0650.

Positional cloning allows the identification of genes responsible for human disease without prior information on the normal function of the gene responsible for the disease. This strategy has recently yielded the genes for cystic fibrosis (CF) and neurofibromatosis (NF1). Research on these two diseases has now entered a different phase, which could be referred to as the "The Biology of Decoding". With the genes now in hand, investigations are now vigorously underway to deduce their normal function and to design better treatments for these two relatively common disorders.

Cystic fibrosis arises from mutations in the CFTR gene on chromosome 7; a consortium of laboratories working on this problem has identified more than 100 such mutations. Each of these mutations provides structure-function information about the CFTR protein. We are investigating mutant CFTR using a variety of expression systems, especially the Xenopus oocyte. Three days after RNA injection, wild type CFTR RNA leads to the production of a large chloride current which is cyclic AMP responsive. Surprisingly, the majority of other missense mutations (including ΔF508) have residual function in this assay, and can even be induced to generate near-wild type currents with high levels of phosphodiesterase inhibition. This result suggests a possible pharmacological approach to CF, which is being pursued by studying the same mutations in a variety of mammalian cells. In addition to these structure function analyses, a detailed investigation of the cis-acting sequences responsible for epithelial specificity of CFTR transcription is being pursued, and several potential regulatory sites have been identified by DNAse hypersensitivity, band shift analysis, footprinting, and expression analysis.

The full coding region of the NF1 gene has now been cloned and sequenced, and encodes a 2818 amino acid protein. In addition to the previously defined homology with GAP, other features of the gene are being actively studied. We have raised antisera against several domains of NF1, and using immunofluorescence demonstrated that the NF1 protein co-localizes with microtubules. This unexpected observation suggests that signal transduction mediated by NF1 may involve the cytoskeleton. A variety of mutations have also been identified in the NF1 gene which are associated with disease, and a vigorous search is being undertaken for evidence for mutation in this gene in tumors from patients who do not have NF1, though the large size of the gene makes such analysis fairly difficult.

V 014 MICRODELETIONS OF 22q11.2: A GENETIC ETIOLOGY FOR DiGEORGE SYNDROME, VELO-CARDIO-FACIAL (VCF) SYNDROME AND CHARGE ASSOCIATION. Beverly S. Emanuel[1], Deborah A. Driscoll[1], David Ledbetter[2], Fangrong Zhang[2] and Marcia L. Budarf[1], [1]The Children's Hospital of Philadelphia. Philadelphia, PA 19104 and [2]Baylor College of Medicine, Houston, TX.

DiGeorge Syndrome (DGS) is a developmental defect of the 3rd and 4th pharyngeal pouches. The disorder is characterized by aplasia or hypoplasia of the thymus and parathyroid glands and conotruncal cardiac malformations. Cytogenetic data suggests that the DGS critical region (DGCR) lies within 22q11.2. We have used dosage studies of probands and RFLP analysis of proband and parents to begin to define the DGS critical region in 14 patients. DNA Deletions have been detected in 5 DGS patients with visible interstitial deletions, del(22)(q11.21q11.23). Probands for whom cytogenetic studies were not unequivocally diagnostic of a deletion also have DNA deletions. In addition, microdeletions of 22q11 have been detected in 6 of 6 cytogenetically normal DGS probands. The minimal region of overlap for these fourteen patients includes loci D22S75 (N25), D22S66 (pH160b) and R32. Together these probes recognize a minimal region of 500 kb. The region is flanked proximally by locus D22S36(pH11) and distally, by BCRL2. By RFLP analysis in 5 families, 4 of 5 probands failed to inherit a maternal allele; the 5th proband failed to inherit a paternal allele. Based on these data and our observation of maternally and paternally inherited translocation-derived DGS probands, there does not appear to be a consistent parent of origin for DGS associated deletions of chromosome 22. Five additional DGS patients have been studied using at least one of the three commonly-deleted probes and they all have demonstrated 22q11.2 DNA deletions. Thus, 19/19 DGS patients show deletions of 22q11.2. YACs for probe N25 have been isolated and have been successfully utilized for detection of deletions by fluorescence *in situ* hybridization (FISH) techniques. Several other disorders, Velo-cardio-facial syndrome (VCF or Shprintzen) and the CHARGE association, are syndromes in which the the abnormalities which constitute the DiGeorge anomalad also play a significant role. We have detected N25 deletions in 5 patients with VCF syndrome; 2 by dosage analysis and 3 by FISH. Similarly, N25 deletions have been detected in 2 patients with the CHARGE association by dosage analysis. These data suggest a genetic etiology, deletion of 22q11.2, for all three disorders and support the combined use of cytogenetic and molecular analysis for the diagnosis of DGS, VCF and CHARGE association Progress using the YACs for analysis of the DGCR will be discussed.

V 015 POSITIONAL CLONING APPROACH TO POLYCYSTIC KIDNEY DISEASE, Stephen T. Reeders[1,2,3], Gregory G. Germino[1], Stefan Somlo[1], Debra Weinstat-Saslow[2], Marita Pohlschmidt4, and Anna-Maria Frischauf[4,1]Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06510; [2]Department of Genetics, Yale University School of Medicine, New Haven; [3]Howard Hughes Medical Institute, Yale, New Haven; [4]Imperial Cancer Research Fund Labs, London.

The most common form of autosomal dominant polycystic kidney disease (ADPKD) has been mapped to chromosome 16p13.3. The 500kb region in which the disease gene, PKD1, lies has been defined by flanking genetic markers. An overlapping set of ~40 genomic cosmid clones spanning the entire region has been isolated. Restriction mapping suggested that the region was packed with CpG islands. Genes corresponding to almost all of these islands have been identified by cDNA cloning.

No major rearrangements have been identified in the genes in the PKD1 region. Therefore a sequence-based approach is required to screen the ~20 genes from the region. The alternative strategies for mutational screening and the prioritization of PKD1 candidates based on likely function will be discussed.

Positional Cloning & Major Diseases-II (Joint)

V 016 THE LDL RECEPTOR SUPERGENE FAMILY, Joachim Herz[1], David Clouthier[2], Michael S. Brown[1], Joseph L. Goldstein[1], and Robert E. Hammer[2], [1] Departments of Molecular Genetics and Internal Medicine, [2] Howard Hughes Medical Institute and Department of Biochemistry, University of Texas, Southwestern Medical Center, Dallas TX 75235

Since the original cloning of the receptor for low density lipoproteins (LDL receptor), two other genes have been described whose primary structures are closely related to the LDL receptor. All members of this gene family are plasma membrane proteins and share the same basic structural components. Because of its structural resemblance to the LDL receptor and because it is predominantly expressed in the liver, the second member of this gene family, the LDL receptor-related protein (LRP), has been postulated to play a role in the removal of apolipoprotein E-rich lipoproteins from the circulation[1]. In addition to its ability to bind and internalize lipoproteins, LRP has recently been recognized to function as a receptor for the activated form of α_2-macroglobulin, an abundant plasma protease inhibitor[2,3]. The third member of the gene family, gp330, is predominantly found in the kidney. This protein was first described as the major antigen in an autoimmune disease in rats called Heymann's nephritis[4]. gp330 is found to cluster into clathrin-coated pits, and specific binding of plasminogen to this receptor has been recently reported[5]. Numerous mutations in the LDL receptor locus have been described that lead to a dominant monogenic disorder known as familial hypercholesterolemia (FH). To date no mutations that affect the function of LRP or of gp330 have been described. We report here the functional elimination of the gene for LRP by homologous recombination in mouse embryonic stem cells. The mutated gene is transmitted through the germline of male and female heterozygotes. However, homozygous deficiency of the LRP leads to early embryonic lethality.

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V 017 MOLECULAR ANALYSIS OF THE MILLER-DIEKER LISSENCEPHALY REGION (17P13.3), David H. Ledbetter[1], Susan A. Ledbetter[1], Akira Kuwano[1], Romeo Carrozzo[1] and William B. Dobyns[2], [1]Institute for Molecular Genetics, Baylor College of Medicine, Houston, TX 77030, [2]Departments of Neurology and Medical Genetics, Indiana University School of Medicine, Indianapolis, IN 46202.

Lissencephaly (agyria, pachygyria) is a severe brain malformation manifested by a smooth cerebral surface, resulting from an arrest of neuronal migration at 10-14 wk gestation. Type I, or classical, lissencephaly can occur either in association with Miller-Dieker syndrome (MDS) or as an isolated finding, termed "isolated lissencephaly sequence"(ILS). Approximately 90% of MDS patients (34/37) have cytogenetically visible or submicroscopic deletions of 17p13.3. Recently, we have shown that 20% of ILS patients (10/51) have submicroscopic deletions within the same region. The centromeric boundaries of MDS and ILS deletions are quite variable and overlap each other, while the ILS deletions do not extend as far in the telomeric direction as do most MDS deletions. Pulsed-field gel analysis and yeast artificial chromosome (YAC) cloning have established the size of the critical region as less than 350 kb. Using the St. Louis and CEPH total human YAC libraries, we have established a complete YAC contig of this interval. A NotI linking clone (LL132) mapping within this interval shows cross-hybridization to rodent DNA and has been used to screen two human fetal brain cDNA libraries (Stratagene, Clontech). A contig of 3.5 kb of cDNA, containing a canonical polyadenylation signal, has been assembled. Ubiquitous expression of this gene is suggested by RT-PCR experiments, which have been carried out using RNA samples from several fetal tissues. Cosmid clones from within the critical region are currently being utilized for detection of submicroscopic deletions and cryptic translocations by fluorescent in situ hybridization (FISH).

V 018 CYSTIC FIBROSIS: PROBING THE BASIC DEFECT, Lap-Chee Tsui[1,2], Danuta Markiewicz[1], Julian Zielenski[1], Ting-Chung Suen[1], Richard Rozmahel[1,2], Mark Dobbs[1], John Teem[1], Joseph Chien[1], Martha Glaves[1], Venus Lai[1], Mary Corey[3], Sasha Dho[4], Kevin Foskett[4], Peter Durie[3,5,6], and Johanna Rommens[1], Department of [1]Genetics and [3]Pediatrics, Divisions of [4]Cell Biology and [5]Gastroentorology, The Hospital for Sick Children, Toronto, Ontario M5G 1X8, and Departments of [2]Medical Genetics and [6]Pediatrics, University of Toronto, Toronto, Ontario M5S 1A8, Canada

Cystic fibrosis (CF) is a common autosomal recessive disorder in the Caucasian population. The mutated gene causing this disease was identified on the basis of its chromosomal localization by linkage analysis. As a basic defect in CF involved the regulation of a cAMP-inducible chloride permeability in the apical membrane of secretory epithelial cells, the encoded polypeptide was named *Cystic Fibrosis Transmembrane Conductance Regulator* (CFTR).

Expression of CFTR appears to be specific to the tissues that are affected in CF patients, e.g. lung, pancreas, intestine, liver, sweat gland and nasal epithelia. The primary sequence shows that this1480-amino-acid CFTR polypeptide contains 2 almost identical halves, each containing a membrane-spanning region followed by an ATP-binding domain. In order to delineate the basic defect in CF, four different lines of experiments are being pursued in our laboratory:

1. Identification of mutations in the CFTR gene. The major mutation causing CF is a 3-bp deletion (named ΔF508) removing a phenylalanine residue from the first ATP-binding domain of CFTR; it accounts for approximately 70% of the mutant chromosomes. To facilitate identification of the remaining mutations and to coordinate screening of population frequencies for each mutation, a worldwide consortium has been formed. Over 150 different mutations have been reported; most of them are rare and their frequencies vary among different ethnic groups. The data also support the predicted roles for the various domains of CFTR and provide a molecular basis for future structural analysis of the protein.
2. Correlation of genotype and phenotype. The concordance of pancreatic function among patients within the same family suggests that this clinical phenotype is predisposed by mutation in the CFTR gene. Two groups of mutant alleles are evident after examining over 500 CF patients in our Hospital. Patients carrying one or two *mild* mutant alleles are invariably pancreatic sufficient (PS) whereas patients with two *severe* mutations (such as ΔF508) are almost certainly pancreatic insufficient (PI). The *severe* alleles include nonsense, frame-shift, splicing mutations and missense mutations, some of which affecting the highly conserved residues within the ATP-binding domain. Besides the fact that all seven *mild* alleles identified are amino acid substitutions, the majority of them are located in the predicted transmembrane regions of CFTR. Possible correlation between genotype and other clinical symptoms are being further investigated.
3. Direct assay of CFTR function. Full-length CFTR cDNA clones have been generated and introduced into various cell types for functional analysis. Although the overall structure of CFTR resembles some of the prokaryotic and eukaryotic transport proteins, the results of numerous DNA transfection studies strongly suggest that CFTR itself functions as a small conductance chloride channel that is inducible by intracellular cAMP.
4. Model systems. In order to gain further insight into the pathophysiology of CFTR mutations *in vivo*, we have initiated attempts to create a mouse model for CF by trying to disrupt the mouse Cftr gene via homologous recombination in embryonic stem cells. Minigene constructs are also being generated to recreate the human mutations. In addition, we have inserted a segment of the first ATP-binding domain of the human CFTR into the corresponding region of the yeast STE6 protein but retained its transport activity for the *a*-mating factor. This activity can be destroyed by the introduction of the ΔF508 mutation into the CFTR segment and subsequently restored by second site mutations.

V 019 EXPRESSION OF NATURAL AND RECOMBINANT HUMAN DYSTROPHIN GENES. Ronald G. Worton, Henry J. Klamut, Lucine O. Bosnoyan, Christine Tennyson, Xiuyuan Hu and Peter N. Ray, Genetics Department and Research Institute, Hospital for Sick Children, and Department of Molecular and Medical Genetics, University of Toronto, 555 University Avenue, Toronto, Ontario, Canada, M5G 1X8.

Dystrophin is a high molecular weight cytoskeletal protein localized at the inner surface of the sarcolemmal membrane of skeletal muscle. Its function is not well understood, but its deficiency is responsible for the progressive degeneration of muscle in boys with Duchenne muscular dystrophy (DMD). Two outstanding problems in DMD research are to understand the function of dystrophin and to devise effective therapy for the disease. Genetic manipulation of the dystrophin molecule is one approach to the former and dystrophin replacement is a logical approach to therapy. Both types of study may benefit from the generation of recombinant dystrophin minigenes that can be genetically altered and expressed in cultured myogenic cells and in the skeletal muscle of affected boys. One potentially important aspect of such studies is the regulation of the dystrophin gene in the myogenic environment. To better understand dystrophin gene regulation we had previously isolated, sequenced and characterized the muscle-specific promoter of the dystrophin gene, defining a minimal promoter of 150 bp of upstream sequence. Within this we identified regions of homology to previously defined muscle-specific regulatory elements such as a CarG box and MEF-1 (MyoD) binding sites, and demonstrated tissue-specific and differentiation-specific expression. More recently studies with reporter gene constructs have revealed within the minimal promoter both positive and negative regulatory regions, and in intron 1 of the gene a muscle-specific enhancer. Other factors determining dystrophin level in muscle include efficiency of message elongation and stability of the message. The extreme size of the dystrophin gene (2300 kb) points to potential problems in completing transcripts and suggests a time to complete a transcript in excess of 20 hours. To investigate these factors, primer pairs that detect mature message at both the 5' and 3' end of the gene have been used to study message accumulation in human fetal muscle cells following fusion into multi-nucleated myotubes. The rate of accumulation of 3' sequences is considerably less than that for 5' sequences, suggesting premature termination or pausing during message elongation. Since, sequences in the 5' and 3' untranslated region (UTR) of several genes have been shown to affect message stability and translational efficiency. Sequences from the highly conserved 3' UTR of the dystrophin gene are being tested for their effect on expression of a growth hormone reporter gene. Finally, as a prelude to dystrophin functional studies, and to potential gene therapy, a functional dystrophin minigene has been constructed and expressed in myogenic cell culture. The gene is reduced in size by a 5.3 kb in-frame deletion that removes much of the central spectrin-like repeat domain from the protein, but dystrophin localizes properly in transfected myotubes. Additional constructs are being tested for membrane localization and for expression in transgenic *mdx* mice.

Gene Transfer/ Gene Therapy-I (Joint)

V 020 APPLICATIONS OF MURINE GENE TARGETING TO HUMAN GENETICS, Arthur L. Beaudet, Raymond W. Wilson, James E. Sligh, Christie M. Ballantyne, Wanda K. Lemna, Gerald Patejunas, E. Paul Hasty, Allan Bradley and William E. O'Brien, Howard Hughes Medical Institute and Institute for Molecular Genetics, Baylor College of Medicine, Houston, TX 77030.

We envision at least three applications of murine gene targeting for analysis of human genetic disease. First, mouse models of known human disorders can be generated with emphasis on studies of pathophysiology and therapy, particularly if the mouse phenotype is very similar to the human phenotype. If the mouse and human phenotypes are distinctly different, attempts to understand the basis of this difference still may shed light on pathophysiology and suggest therapeutic strategies. Second, mutations can be generated in genes where no mutants are yet identified in mammals. Analysis of the murine phenotype may allow for recognition of the molecular defect in homologous human disorders. Third, animals with mild phenotypes in heterozygotes or homozygotes can be used to analyze the role of candidate genes in polygenic disease processes. Following these rationales, gene targeting has been attempted in our laboratory for argininosuccinate synthetase (AS), cystic fibrosis (CF), CD18, and ICAM-1. Gene targeted clones were identified and chimeric mutant animals obtained for AS, CD18, and ICAM-1. Germline transmission of a hypomorphic allele was achieved for CD18, and homozygotes are fertile and demonstrate a mild phenotype. Homologous recombination was many orders of magnitude less frequent for the CF locus. Mutations in AS, CD18, and CF should provide mouse models for known human genetic disorders. Mutations in CD18 and ICAM-1 will be examined for polygenic effects on autoimmune processes, atherosclerosis, transplantation rejection, and other inflammatory processes.

V 021 Hepatic Gene Therapy in Animal Models. Savio L.C. Woo, Howard Hughes Medical Institute, Departments of Cell Biology and Molecular Genetics, Baylor College of Medicine, Houston, TX.

The liver is the major organ for metabolism and there are dozens of known metabolic disorders secondary to a variety of hepatic deficiencies in man. The development of technologies to deliver functional genes into hepatocytes in vivo would permit gene therapy for these disorders in the future. In our laboratory, two deficient animal models are being tested for the efficacy of gene therapy approaches for their phenotypic correction. Phenylketonuria (PKU) is an inborn error in amino acid metabolism that causes severe and permanent mental retardation in affected children. The genetic disorder is secondary to a deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH) which converts phenylalanine to tyrosine and constitutes the major metabolic pathway for the essential amino acid. We have previously reported the cloning of the human PAH cDNA and the use of it to express the human enzyme in heterologens mammalian cells by retroviral mediated gene transfer. More recently, we have also demonstrated that heterologous hepatocytes injected directly into the portal vein or the spleen of cogenic mice migrated to the liver, survived and continued to function as hepatocytes for the life of the recipients. Independently, a PAH-deficient mouse model has been created by the Laboratory of William Dove at the University of Wisconsin. Thus, we have initiated collaborative studies to attempt the correction of the deficient phenotype in the mutant mouse model. Virally transduced hepatocytes expressed high levels of human PAH, and these cells will be transplanted into deficient animals in order to determine if there will be long-term reconstitution of the enzyme activity in the liver. The second animal model is the Factor IX-deficient dog in the laboratory of Kenneth Brinkhous at the University of North Carolina in Chapel Hill. This is a severe bleeding animal model and the construction of recombinant retroviruses expressing human and canine Factor IX in fibroblasts and primary hepatocytes has previously been reported. We have recently established methods for the isolation of $3x10^9$ hepatocytes from a single liver lobe obtained from 5 kg dogs by partial hepatectomy, as well as autologous transplantation of these cells back into the same animal via injection into the portal vasculature. After retroviral transduction and transplantation, the cells survived for a minimum of 4 months in vivo. If successful, these technologies can be directly applied to the treatment of a variety of metabolic disorders in man in the future.

Gene Transfer/ Gene Therapy-I I (Joint)

V 022 HIGH EFFICIENCY GENE TRANSFER INTO HEMATOPOIETIC PRECURSORS, C. Thomas Caskey, Kohnoske Mitani and Annemarie Moseley, Institute for Molecular Genetics and Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX 77030

Adenosine deaminase (ADA) deficiency, a recessively inherited disease, accounts for 15% of severe combined immunodeficiency cases. Therapies include HLA-matched bone marrow transplantation, replacement with PEG-ADA and retroviral transduction of peripheral lymphocytes with the human ADA gene. While often associated with morbidity, transplantation with HLA-matched offers a potential cure, making ADA an ideal candidate for bone marrow gene therapy. Previously, we have demonstrated transduction of the hADA gene into bone marrow from normal donors and ADA deficient patients by means of co-cultivation with the virus-producing cell line, AM12st14, in the presence of rhIL3 and rhIL6. Infection efficiency, as determined by PCR detection of provirus integration into myeloid colonies grown in methylcellulose culture (CFU-GM), is 34% after 9 weeks in long-term culture. Since committed clonogenic precursors are not maintained in culture longer than 4-5 weeks, the data demonstrated that successful transduction of hematopoietic precursors was possible. Expression of the hADA gene in the CFU-GM was determined by RT-PCR and 50% of the CFU-GM which had integrated the provirus expressed the transduced gene. Microradioassay for adenosine deaminase activity correlated with mRNA expression. Subsequently we have focused on infection with supernatant from the AM12st14 cell line. We have achieved supernatant infection of normal bone marrow cells which were separated on Percoll gradient and were then maintained in culture for 2-3 weeks prior to infection. Supernatant was added every 12 hours in the presence of rhIL3 and rhIL6 for 72 hours. Proviral integration was present in 70% of the colonies at 7 weeks post-infection (*i.e.*, 10 weeks in LTC) and significant levels of transcript were seen. The presence of the stromal layer was essential for supernatant infection. We have expanded these studies to include the infection of a population of bone marrow cells enriched for more primitive hematopoietic precursors. Autologous stromal layers are initially developed from the low density Percoll gradient fraction. The remaining cells are cryopreserved until the stromal layers are nearly confluent, at which time the cells undergo positive selection for CD34+ cells using anti-CD34 coated flasks (Applied Immune Sciences). Infection efficiency is nearly equivalent to that seen with co-cultivation. The ability to infect the primitive hematopoietic progenitor containing population with retroviral supernatant greatly facilitates the clinical introduction of bone marrow gene therapy.

V 023 GRAFTING GENETICALLY MODIFIED CELLS TO THE BRAIN, Fred H. Gage, Department of Neurosciences, University of California, San Diego.

To assess the feasibility of using primary skin fibroblasts as a donor population for genetic modification and subsequent intracerebral grafting, we examined the structural and neurochemical characteristics of grafts of isogeneic primary fibroblasts over a period of six months. Following the implantation in adult rats from the same inbred strain, isologous grafts are stained immunohistochemically for fibronectin immunostaining which persists for at least six months. Immunostaining for laminin is intense within the grafts from one to eight weeks, but decreases by six months. Astrocytes respond dramatically to the implantation of primary fibroblasts although the intensity of immunostaining for glial fibrillary acidic protein diminishes between eight weeks and six months. The astrocytic border between the grafts and striatal neuropil remains intensely immunostained. Capillaries within the grafts stain immunohistochemically for glucose transporter as early as three weeks after implantation. At the ultrastructural level, grafts possess numerous fibroblasts and have an extracellular matrix filled with collagen. Reactive astrocytic processes filled with intermediate filaments are found throughout the grafts. Hypertrophied astrocytes and their processes also appear to form a continuous border between the grafts and the striatal neuropil. Grafts of primary fibroblasts also possess an extensive vasculature that is composed of capillaries with nonfenestrated endothelial cells; the occurrence of reactive astrocytic processes closely associated with or enveloping capillaries is variable. These results provide direct morphological and neurochemical evidence for the long-term survival of isologous fibroblasts after intracerebral implantation. We have transduced primary isologous fibroblasts with several different transgenes to assess the effectiveness of these genetically modified cells to delivery new genetic material to the brain, including Nerve Growth Factor (NGF), Tyrosine Hydroxylase, GABA Decarboxylase, and Choline Acetyltransferase. We will report on intracerebral grafts of primary skin fibroblasts genetically engineered to expressing several of these transgenes, including NGF which had been embedded within a collagen matrix prior to grafting. This later experiment was designed to assess the regenerative capacity of cholinergic neurons of the adult rat medial septum. The results reveal the following: first, NGF-producing grafts sustain a significant number of NGF receptor-immunoreactive septal neurons following axotomy. Second, NGF promotes the regeneration of septal axons, such that NGF-producing grafts possess large numbers of unmyelinated axons which use many a variety of substrates for growth. Grafts of control fibroblasts possess the same cellular and matrix substrates but contain only a very small population of axons. Advantages and disadvantages of these grafted genetically modified primary fibroblasts will also be discussed.

V 024 GENE THERAPY OF FAMILIAL HYPERCHOLESTEROLEMIA, Mariann Grossman and James M. Wilson, Departments of Internal Medicine and Biological Chemistry, and Howard Hughes Medical Institute, University of Michigan, Ann Arbor, Michigan.

Familial Hypercholesterolemia (FH) is an autosomal dominant disorder in humans caused by a defect in LDL receptor expression. Homozygous patients have severe hypercholesterolemia and premature coronary heart disease. We are developing two approaches for treating FH patients based on liver directed transfer of the LDL receptor gene. An animal model for FH called the WHHL rabbit has been used to demonstrate the feasibility and efficacy of our approaches. One strategy, involves the transplantation of autologous hepatocytes genetically corrected ex vivo with recombinant retroviruses. The feasibility and long-term efficacy of this approach has been demonstrated in the WHHL rabbit. Issues of safety have been addressed in experiments performed with dogs and baboons. Human trials should begin in 1992.

Another approach to gene therapy of FH is to construct a gene transfer substrate capable of targeting to hepatocytes when infused into the peripheral circulation. This substrate is formed by complexing a ligand to a hepatocyte specific receptor with a transfection-based, LDL receptor-expressing vector. When infused into the peripheral circulation of WHHL rabbits, the DNA/protein complex is targeted to hepatocytes and transiently expressed leading to a temporary improvement of hypercholesterolemia.

Model Systems; Cancer Genetics

V 100 INTRACELLULAR MOLECULAR ABLATION OF HIV TAT EXPRESSING CELLS, Hugh J.M. Brady, Colin Miles, Daniel Pennington and Elaine A. Dzierzak, Gene structure and Expression, National Institute for Medical Research, London U.K. NW7 1AA.

Human Immunodeficiency virus (HIV) has been identified as the etiological agent in human acquired immunodeficiency syndrome (AIDS). The molecular tools utilized in elucidating the nucleic acid structure and gene function of the virus suggest the potential for therapeutic strategies involving various molecular manipulations. We are exploiting such a strategy by an developing an intracellular molecular ablation system which specifically kills all cells in which the HIV-specific *trans*-acting factor, tat, is present.

To achieve, this have we generated molecular constructs using portions of the HIV genome (HIV-2 LTR) and a toxin-encoding gene (HSV1 thymidine kinase (TK)), in the context of retroviral vectors for subsequent delivery to the blood system. Since this system of intracellular molecular ablation requires that the HIV promoter is active only in the presence of tat, we sought to develop constructs which could be *trans*-activated efficiently but provide no basal transcriptional activity. We have made minimal HIV-2 promoter constructs incorporating base substitutions and deletions All constructs contain the TAR region of HIV-2.

We have tested for basal and tat-induced expression using the human growth hormone (hGH) as a reporter gene. Transient transfections of COS cells have shown that all the promoter sequences generated by PCR can be *trans*-activated but to varying degrees. Also, several of the constructs have the desired negligible basal expression of hGH both at the RNA and the protein level. We have also tested the constructs for transient expression in Jurkat cells.

From the constructs that have minimal or no basal expression and are sufficiently *trans*-activated in the presence of tat, we have inserted the TK gene and generated recombinant retroviruses. The results of infection, expression and Gancyclovir-mediated killing of human cell lines in the presence or absence of tat will be presented.

V 101 DIFFERENTIAL EXPRESSION OF DYSTROPHIN ISOFORMS IN STRAINS OF *MDX* MICE. Jeffrey S. Chamberlain, Gregory A. Cox, Andrea J. Maichele, Stephanie F. Phelps, Stephen D. Hauschka, and Verne M. Chapman. Dept of Human Genetics, University of Michigan Medical School, Ann Arbor, MI 48109, Dept. of Biochemistry, University of Washington, Seattle, WA, 98195, Molecular and Cellular Biology Dept., Roswell Park Cancer Institute, Buffalo, NY, 14224. Dystrophin is expressed predominantly as a 430kd protein in muscle and brain tissues. Antisera to the C-terminus also detects a 90kd protein transcribed from an internal promoter most active in brain, liver and testis. Northern analysis of dystrophin detects a mRNA of 14kb in muscle and brain, 4.9kb in a variety of tissues, and 6.5kb in liver and kidney. The 4.9kb transcript is most abundant in testis. PCR analysis of dystrophin mRNA reveals at least 10 isoforms are produced *via* alternative splicing near the C-terminus. The splicing pattern is similar in skeletal muscle and most non-muscle tissues, but a wide variety of spliced forms are differentially expressed in brain and heart. The original *mdx* strain does not express the muscle isoform of dystrophin, but transcription from the internal promoter in normal. In contrast, a mutation near the dystrophin C-terminus in a newer isolate of *mdx* mice eliminates expression of both the muscle and non-muscle transcripts. This new *mdx* strain displays a more severe phenotype and reproduces poorly, possible due to the lack of testis dystrophin. A permanent myoblast line from the original *mdx* mouse has been isolated and is being used to test dystrophin vectors. Expression vectors containing full-length, truncated, and separate isoforms of the dystrophin cDNA are being tested in the myogenic cultures and in transgenic mice for the ability to eliminate dystrophic symptoms. The newer *mdx* model and the *mdx* cell line should provide improved systems in which to test vectors for gene therapy of Duchenne muscular dystrophy.

V 102 EXPRESSION OF THE HUMAN NEUTROPHIL ELASTASE GENE IN THE LUNGS OF TRANSGENIC MICE, Sami Damak[1]*, Anne M. Hill[1], Tania L. Smith[2], Peter M. George[2] and David W. Bullock[1] Dept. of Biochemistry and Microbiology[1], Lincoln University, Canterbury, and Clinical Biochemistry Dept.[2], Christchurch Hospital, Christchurch, New Zealand

Disruption of the protease anti-protease balance in the lung is believed to play a key role in the etiology of emphysema. In order to produce an animal model for emphysema, we have generated transgenic mice by microinjection of human neutrophil elastase (HNE) genomic DNA under the control of the rabbit uteroglobin promoter. A 4.3 kb cloned DNA fragment containing the complete HNE gene was ligated to 3.3 kb of 5'-flanking DNA containing the uteroglobin promoter region, which we showed previously could direct the expression of heterologous genes specifically to the lung of transgenic mice (De Mayo *et al.* Molec. Endocr. 1991, 5:311; De Mayo *et al.*, Am. J. Physiol., 1991, 261:L70). From 2082 injected zygotes, 87 pups were born, of which 11 were transgenic. Analysis of total RNA by Northern hybridization showed strong expression of HNE mRNA in the lung of transgenic mice in 7 out of 8 independent transgenic lines. Weak expression was also detected in the spleen in 2 lines. No HNE mRNA was detected in 10 other tissues. Immunohistochemistry using specific anti-HNE antibodies indicated the presence of HNE protein in the bronchial epithelium and the protein was detected by dot-blots of bronchoalveolar lavages, indicating that HNE protein is secreted. On Western blotting of lavage fluid, two bands of approximately 30 Kd and 80 Kd were detected with a biotinilated anti-HNE antibody. These bands correspond to free HNE and HNE-anti-protease complex, respectively. The presence of the complex suggests that HNE is properly processed in the bronchial epithelium and is secreted in an active form. Histological examination of mouse lungs for signs of emphysema is in progress.

(Supported in part by grant 91/107 from the Health Research Council of New Zealand.)

V 103 HUMAN DYSTROPHIN MINIGENE EXPRESSION CORRECTS THE MYOPATHIC PHENOTYPE IN TRANSGENIC *MDX* MICE, George Dickson, Kim E. Wells, Frank S. Walsh, Kay E. Davies* and Dominic J. Wells#. Department of Experimental Pathology, UMDS, Guy's Hospital, London SE1 9RT; *Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, OX3 9DU; #Unit of Molecular and Cellular Biology, The Royal Veterinary College, London NW1 0TU, U.K.

Duchenne and the less severe Becker form of muscular dystrophy (DMD,BMD) result from genetic deficiency in the level and/or activity of the protein dystrophin. The recent availability of cDNA based minigenes encoding recombinant dystrophin polypeptides has raised the possiblitiy of somatic gene transfer as a therapeutic approach to treat dystrophin deficiency. In this respect, the mdx mouse provides a useful model of DMD exhibiting features characteristic of both the early myopathic and later fibrotic phases of the human disease. Using a mutated human cDNA, compatible in size with virus-based somatic gene transfer vectors, the pathophysiological consequences of restoring dystrophin expression have been examined in transgenic mdx mice. Trangene expression was correlated with reduced serum creatine kinase levels and virtual elimination of the extensive skeletal myofibre necrosis and regeneration which is a major feature of the dystrophin deficient phenotype in young mdx mice. The cDNA construct which is based on a very mild BMD phenotype thus encodes a highly functional molecule whose reduced size renders it an attractive candidate for development as a therapeutic gene transfer reagent.

V 104 CELL SURFACE DIFFERENTIATION MARKERS OF SKELETAL MUSCLE CELLS USEFUL FOR MYOBLAST TRANSFER GENE THERAPY, Hannah Friedman Elson, Department of Molecular Cardiology, NHLBI, NIH, Bethesda, MD 20892

A promising approach to gene therapy is the formation of a stable source of recombinant gene product in skeletal muscle tissue (Salminen, Elson, Mickley, Fojo, & Gottesman (1991) *Human Gene Therapy* **2**, 15). For instance, a needed protein could theoretically be engineered to be secreted by muscle cells or bound to their outer cell surfaces by grafting it to the existing machinery of secreted or surface-bound proteins of skeletal muscle cells. Several cell surface differentiation markers have been identified for skeletal myoblasts and satellite cells. Developmental changes in the number and distribution of these markers have been documented. An overall survey of surface proteins, performed by an enzymatic radioiodination, revealed the major proteins exposed on the outer muscle cell surface. Specific proteins were identified. Enzymatic and immunological analysis demonstrated the presence of acetylcholinesterase on undifferentiated as well as mature cells. Acetylcholine receptors were revealed by binding the ligand α-bungarotoxin. Antibody studies indicated the differentiation-dependent presence of the receptor for extracellular fibronectin. Effects of the extracellular matrix molecule hyaluronic acid on myoblast differentiation suggested the presence of a specific receptor. Other researchers have identified several other markers. Surface markers present in the largest quantities would be the most useful for cell separation in preparation for myoblast implant therapy.

V 105 *Abstract Withdrawn*

V 106 DIRECT INJECTION OF PAPILLOMAVIRUS E2 GENE DNA REDUCES TUMOR XENOGRAFT FORMATION BY CERVICAL-DERIVED CELL LINES. Alejandro M. García-Carrancá and Ronit Ovseiovich Z. Department of Molecular Biology, Inst. Inv. Biomédicas, UNAM. Apdo. Postal 70-228, 04510 Mexico City, MEXICO.

Cervical neoplasias represent a major cause of dead among cancer patients in the developing world. Many of these neoplasias contain DNA from certain types of human papillomaviruses´ (HPVs´), including types 16 and 18. These HPVs´contain two oncogenes (E6 and E7) that are directly involved in cellular immortalization. The E2 gene product is a regulator that repress E6 and E7 transcription in HPV-16 and HPV-18. In malignant lesions viral DNA is found integrated into the cellular genome, usually with disruption of the E2 gene. There are several cell lines derived from cervical neoplasias (like HeLa and SiHa), that contain active HPV DNA integrated into their genome, with loss of the E2 gene. It has been reported that injections of pure preparations of DNA resulted in significant expression of the reporter gene.

To study the possible effect of injecting E2 gene DNA into tumors induced by cervical-derived cell lines, we developed a tumor xenograft assay using nude mice. Tumor formation was evaluated after subcutaneous injection of one million HeLa cells into 4 weeks male Balb/c-nu/nu mice. Injections of DNA preparations containing the bovine papillomavirus type 1 E2 gene (BPV-1 E2) were - started at different times. Some animals were - treated simultaneously with cisplatin. In all - cases, animals treated either with E2 alone or in combination with cisplatin developed tumors at a slower rate. This results indicate that papilloma virus E2 gene could be usefull for the treatment of cervical carcinomas.

Partially supported by CONACyT, The Miguel Alemán Foundation and CANIFARMA.

V 107 TARGETING ONCOGENES TO THE EPIDERMIS OF TRANSGENIC MICE: A MODEL FOR MULTISTAGE SKIN CARCINOGENESIS, Greenhalgh, D.A., Rothnagel, J.A., Longley, M.A., Bundman, D., Gagne, T., Quintanilla, B., and Roop, D.R., Departments of Cell Biology & Dermatology, Baylor College of Medicine, Houston, TX

The ability to target gene expression to specific tissues of transgenic mice provides a powerful model to study multistage carcinogenesis in the context of the whole animal. The epidermis is an attractive target tissue; not only is it a model for epithelial carcinogenesis in general but its accessibility allows easy detection of progressive pathological changes. We have developed a vector which specifically targets gene expression to the epidermis of transgenic mice and generated lines which express either v-ras^{Ha}, v-fos or the early region genes E6 and E7 of Human Papilloma Virus 18 (HPV 18). Transgenic mice expressing v-ras^{Ha} exhibit a massive epidermal hyperplasia at birth with up to a 20 fold thickening of the epidermis in 5 day old neonates and subsequently followed by a massive hyperkeratosis derived from keratinocyte terminal differentiation. With age, the phenotype diminishes and the histotype is consistently pre-neoplastic. Tumorigenesis is delayed, benign papillomas appear at 3-4 months and typically regress, suggesting that ras^{Ha} alone is insufficient to maintain even a benign phenotype. Transgenic mice expressing v-fos exhibit a very specific hyperplasia and hyperkeratosis of the ear. Again this is a non-neoplastic phenotype which initially appears in a wounded (tagged) ear at approximately 4 months and then becomes bilateral. Transgenic mice expressing HPV 18 E6/E7 initially failed to exhibit an obvious phenotype. However, rare lesions appear after a long latency (~9 months) that are typical of HPV induced benign papillomas. To date no mouse expressing a single targeted oncogene exhibits malignant conversion, all lesions being pre-neoplastic or benign. The availability of these strains will allow mating experiments to explore oncogene synergism and assessment of putative carcinogens/promoters. The pathological changes resulting from the study of these model systems may provide insights for therapeutic intervention.

V 108 In-vivo **Gene Transfer into Canine Myocardium.** Rüdiger von Harsdorf, Robert Schott, You-Tang Shen, Vijak Mahdavi, Stephen Vatner, Bernardo Nadal-Ginard. HHMI, Department of Cardiology, Children's Hospital and Harvard Medical School, Boston, MA

We have characterized the time course, dose-response curve, and histologic localization of reporter gene expression following direct myocardial injection of plasmid DNA into canine myocardium. Reporter constructs utilized the CAT, Luciferase and ß-galactosidase genes in front of the MSV or RSV promoters. Using these data we have further characterized in-vivo activity of the muscle specific ß-myosin heavy chain (ß-MHC) promoter, comparing a series of ß-MHC-CAT constructs, with co-injection of RSV-luciferase to control for transfection efficency. This model has enabled us to map the ß-MHC promoter in-vivo. We have also studied the effect of ischemia on the expression of reporter genes in the chronically instrumented dog. We have found that canine myocardium, well-characterized physiologically, is particularly useful for the study of pathophysiology and its effect on expression of directly injected reporter genes.

V 109 DIRECT INVOLVEMENT OF RETINOBLASTOMA GENE PRODUCT IN A NOVAL PROTEIN-DNA COMPLEX THAT REPRESSES NEU ONCOGENE EXPRESSION, **Mien-Chie Hung, Dihua Yu and Angabin Matin** Dept. of Tumor Biology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas 77030

The retinoblastoma susceptibility gene (RB) is a tumor suppressor gene involved in the etiology of many types of human cancers. However, the molecular mechanisms involved in tumor suppression by RB are largely unknown. The *neu* gene is a dominant transforming oncogene and both inactivation of the RB gene and overexpression of the *neu* gene are involved in human breast and lung cancers. Therefore, it is important to investigate the potential interactions between RB and *neu*. Here we report that RB suppresses *neu*-induced transformation by repressing transcription of the *neu* oncogene. The *cis*-acting element conferring RB-mediated repression was mapped to a recently identified enhancer in the *neu* promoter and RB protein was shown to be associated with a novel protein complex binding to the enhancer. The data demonstrate that RB can regulate *neu* gene expression as a transcriptional factor and reveal a new clue as to the molecular mechanisms of RB-mediated tumor suppression, namely, repression of a dominant oncogene expression at the transcriptional level.

V 110 **CHARACTERIZATION OF DEVELOPMENTALLY ACTIVE GENES IN MOUSE EMBRYOGENESIS BY GENE TRAPPING IN EMBRYONIC STEM CELLS,** **Mark D. Johnson and Kathleen A. Mahon, Laboratory of Mammalian Genes and Development, NICHD/NIH, Bethesda,MD 20892**

In order to gain a better understanding of the molecular biology of mammalian development, it is necessary to identify and analyze genes which determine the structure and functional design of the developing embryo. We are utilizing a strategy know as "Gene Trapping" (Gossler et al., SCIENCE 244:463-465, 1989) to identify, clone, and insertionally mutate genes which are transcriptionally active during mouse embryonic development. This strategy utilizes a gene trap construct containing a promoterless/enhancerless ß-galactosidase (*lacZ*) reporter gene. The *lacZ* reporter is only expressed when it integrates into a transcriptionally active cellular gene such that it can be activated by host *cis*-acting regulatory elements. Gene trap containing ES cell lines are screened by histochemical staining for interesting patterns of *lacZ* expression in culture and in ES cell generated chimeric embryos. Differential staining for *lacZ* reflects the developmental expression patterns of the host gene into which the construct has integrated. By its designed integration into a transcriptionally active gene, the gene trap construct serves as a molecular tag while simultaneously creating an insertional mutation within the host gene. A host gene-*lacZ* fusion transcript is produced, and therefore direct cloning of the interrupted host gene is simplified. We have established 2262 independent cell lines, of which 15 express *lacZ* in vitro. Analysis of ES cells and embryoid bodies differentiated in culture, and chimeric embryos generated from 2 of these 15 cell lines reveals interesting developmentally regulated patterns of *lacZ* expression. Both cell lines show loss of *lacZ* expression in the endoderm of embryoid bodies with restricted expression to the ectoderm. One cell line shows consistant *lacZ* expression predominantly in cardiac tissues of chimeric embryos suggesting integration of the construct within a gene locus active during cardiac development. Cloning of these genes with PCR techniques is ongoing. Breeding of chimeric mice bearing these mutated genes in their germline should lead to the generation of mouse mutants of these loci, allowing for study of their altered phenotypes.

V 111 RECOMBINANT HUMAN DYSTROPHIN EXPRESSION WITHIN DMD PATIENT MYOTUBES: SURFACE MEMBRANE LOCALIZATION AND IDENTIFICATION OF BINDING DOMAINS. Henry J. Klamut, Lucine O. Bosnoyan, Ronald G. Worton, and Peter N. Ray. Department of Genetics and Research Institute, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, M5G 1X8, Canada.

Mutations which alter the translational reading frame of the Duchenne muscular dystrophy (DMD) gene result in severe, progressive degeneration of muscle fibers. Although its function remains undefined, the 427 kDa protein product of the DMD gene (dystrophin) is believed to be a submembraneous cytoskeletal protein that acts to reinforce the surface membrane against contraction-induced stress. Becker muscular dystrophy (BMD) is a less severe, allelic form of the disease and results from mutations which maintain the translational reading frame but diminish the functional capacity of the protein. Histochemical analyses of patient muscle biopsies have demonstrated that dystrophin is absent from the surface membrane of DMD patient muscle fibers, while in BMD patient fibers dystrophin is present in discrete patches or can be distributed relatively homogeneously along the surface membrane. The domain responsible for surface membrane localization has therefore been attributed to unique sequences at the extreme carboxy-terminal end of the dystrophin molecule. In order to experimentally define the domain(s) responsible for dystrophin localization, a human DMD minigene has been prepared which, when expressed in DMD patient myotubes in vitro restores surface membrane antigenicity to several site-specific dystrophin antibodies. Histochemical analysis of patient myotubes expressing one of several DMD minigene constructs with defined in-frame deletions suggest that an intact carboxy-terminal domain is not an absolute requirement for surface membrane localization. Experiments aimed at precisely defining the sequences responsible for surface membrane localization are currently underway.

V 112 THE t(15;17) TRANSLOCATION OF ACUTE PROMYELOCYTIC LEUKEMIA GENERATES A FUNCTIONALLY ALTERED RETINOIC ACID RECEPTOR. Anne Dejean, Catherine Lavau, Christine Chomienne*, Agnès Marchio, Laurent Degos* and Hugues de Thé, UREG, Institut Pasteur and *Hôpital Saint-Louis, Paris, France.

A specific translocation t(15,17) has been reported in every patient with acute promyelocytic leukemia (APL). We have previously reported that, in an APL-derived cell-line (NB4), this translocation fused the retinoic acid receptor α (RARα) gene to a previously unknown locus, initially called *myl* and now renamed PML. Moreover, genomic alterations of either RARα or PML loci were demonstrated in most patients, suggesting that the fusion of these two genes might be a general situation in APL.

We now report the molecular cloning of the wild-type PML and hybrid PML-RARα transcripts. The PML gene product displays a C_3HC_4 motif found in several DNA-binding proteins and could encode a transcription factor. Two hybrid cDNAs, that differ by an alternatively spliced coding exon of PML, were isolated from the NB4 cell-line and shown to encode proteins containing most of the PML sequences fused to a large part of RARα, including its DNA- and hormone- binding domains. In transient expression assays, the hybrid protein exhibits altered *trans*-activating properties if compared to the wild-type RARα progenitor. These observations suggest that in APL, the t(15;17) translocation generates a retinoic acid receptor mutant that could contribute to leukaemogenesis through interference with promyelocytic differentiation. The analysis of the transforming properties of the PML-RARα hybrid are in progress.

V 113 PHENOTYPES OF TRANSGENIC MICE OVEREXPRESSING GLUTATHIONE PEROXIDASE. M.-E. Mirault, A. Tremblay, F. Pothier, P.F. Piguet*, M. Surette, P. Julien, S. Moorjani, J. Puymirat and P. Borgeat. CHUL Research Center, Laval University, Québec,PQ, Canada GIV 4G2, and Department of Pathology*, University of Geneva, CH-1211, Geneva, Switzerland.

The endogenous production of reactive oxygen intermediates (ROI) and fatty acid peroxides have been implicated in a large number of pathophysiological conditions, including cancer. Overexpression of glutathione peroxidases (GPx) has been shown to alter the responses and resistance of human cells to both endogenous and exogenous sources of ROI (Mirault et al., *J. Biol Chem.* **266**, 20752, 1991; Lavoie et al., ibid., in press). We are presently investigating the effects of overexpressing Se-GSHPx in an animal model. Four lines of transgenic mice containing human metallothionein-IIA promoter/GSHPx chimeric genes have been generated, which breed and develop apparently normally. However, several interesting anomalies have been observed in relation to inflammatory responses and fatty acid metabolism, including an unusual panniculitis. Alterations in the resistance of these mice to LPS, in arachidonic acid metabolism in macrophages, as well as in plasmatic levels of cholesterol and lipoprotein composition will be also presented and related to the distribution of GSHPx expression in all major tissues of these mice following induction of the transgene by Cd^{++} or LPS induction. These novel models could be of particular interest for studies of several (patho)physiological conditions involving the production and metabolism of hydrogen peroxide and/or fatty acid hydroperoxides.

V 114 SATURATION MUTAGENESIS AT CODONS 22 AND 31 AND *IN VIVO* EXPRESSION SCREENING FOR VARIANT METHOTREXATE-RESISTANT MURINE DIHYDROFOLATE REDUCTASES. Jill A. Morris, Anna Maria Platon, and R. Scott McIvor, Institute of Human Genetics, Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455.

Methotrexate-resistant dihydrofolate reductases (DHFR) have provided useful tools as dominant selectable markers for mammalian gene transfer and may be applicable to drug-resistance and selection *in vivo*. To investigate the variety of DHFR genotypes potentially conferring Mtx-resistance upon mammalian cells, we conducted saturation mutagenesis experiments at codons 22 and 31 of the murine DHFR coding sequence, positions at which amino acid substitutions have previously been observed which result in Mtx^r mammalian DHFRs. A PCR overlap extension protocol was used with degenerate primers at codons 22 and 31, generating complete DHFR transcription units regulated by the SV40 early promoter and a polyadenylation signal from the hepatitis B virus surface antigen gene. Sequence analysis verified the presence of all four nucleotides at each of the three nucleotide positions within codons 22 or 31 of the PCR-mutagenized products. These PCR-generated DHFR transcription units were transfected into DHFR-deficient Chinese hamster ovary cells, selecting in 0.1 μm methotrexate. Drug-resistant clones were isolated at a frequency of 1.7×10^{-4}, demonstrating that this procedure can be used for *in vivo* screening of new DHFR genotypes conferring drug-resistance function. These new Mtx-resistant DHFR variants are currently being characterized by nucleotide sequencing and enzyme inhibition studies and should be useful as selectable markers with heterogeneous pharmacological characteristics.

V 115 EVALUATION OF VASCULAR PROSTHESES SEEDED WITH GENETICALLY MODIFIED VASCULAR ENDOTHELIUM AND SMOOTH MUSCLE CELLS AS A MEANS FOR GENE THERAPY IN THE DOG. J.E. Sackman[1], M.B. Freeman[2] and C.D. Lothrop, Jr.[1], University of Tennessee College of Veterinary Medicine[1] and University Hospital[2], Knoxville, TN 37901

Canine endothelial cell(EC) cultures were established from normal donor blood vessels. Cell lines were transfected with retroviral vectors carrying the bacterial neomycin resistance gene. Cell clones were selected in G418. Resistant cell lines were verified by growth in G418 and PCR amplification of the Neo^R sequence. Resistant EC were seeded on Dacron grafts and implanted into donor dogs as aorticoabdominal bypass (2), femoral artery (2) or carotid artery (2) interposition grafts. Grafts were harvested at 250-360 days. All grafts were coated with an EC monolayer as visualized with light and scanning EM. The luminal surface of all grafts stained positive for vWF-Ag by immunoperoxidase verifying endothelial origin. DNA analysis and PCR amplification of the Neo^R sequence failed to demonstrate the Neo^R gene in any of the coated grafts. None of the cells cultured from the explants survived in G418. Preliminary results suggest that endogenous EC replaced seeded cells. Studies now are focused on the use of PTFE ($Gortex^R$) as a substrate for seeded vascular cells. PTFE's small internodal distance reduces endogenous ingrowth and displacement of seeded cells. PTFE grafts are seeded with EC and vascular smooth muscle(SM). Seeded SM may replace endogenous SM in graft healing. EC and SM are transfected with a retroviral vector containing either recombinant human or rat growth hormone gene (GH). High producing clones are selected in vitro by RIA assay for GH. Monitoring of recombinant protein activity in vivo is also done by RIA for GH, which is readily distinguished from endogenous canine GH. The recombinant secretory protein activity in EC and SM seeded on PTFE will be evaluated over a short-term (18 week) period to determine early cell fate. This model is felt to be potentially useful in the gene therapy of vascular and hematologic diseases.

V 116 DNA END-TO-END JOINING IN ILLEGITIMATE RECOMBINATION. Silke Thode, Antje Schäfer, Juliane Güldenpfennig, Walter Vielmetter and Petra Pfeiffer, Institute for Genetics, University of Cologne, Weyertal 121, D-5000 Köln 41, F.R.G.

Integration of foreign DNA into cellular genomes is a basic feature of gene therapy since it allows the maintenance of the foreign DNA in replicating cells. In higher eucaryotic cells random integration by illegitimate recombination processes is orders of magnitude more frequent than targeted integration by homologous recombination. Therefore, until now gene therapy cannot be applied to diseases that require the replacement of a defect cellular gene by its intact allele. Detailed knowledge of mechanisms that lead to random integration may help to optimize integration processes for many different applications of gene therapy.

Here, we investigate nonhomologous DNA end joining as the basic feature of illegitimate recombination in an *in vitro* system derived from eggs of *X. laevis*. As test substrates linearized plasmid DNA with two different restriction termini are employed that are recircularized during extract driven joining reactions. Sequence analyses of cloned junctions reveal that during joining processes noncomplementary protruding single strands (PSS) of the same polarity (5'PSS/5'PSS or 3'PSS/3'PSS) overlap by fortuitously matching basepairs (Pfeiffer & Vielmetter 1988, NAR 16:907). During joining processes of PSS to blunt ends the PSS sequences are conserved by fill-in DNA-synthesis. 3'PSS-ends that lack a primer to start DNA-synthesis utilize the 3'OH of the blunt ended partner without covalent connection between the 3'PSS template and the partner end (Thode et al. 1990, Cell 60:921). Both mechanism cannot be explained without the help of DNA-binding factors that align DNA-ends and stabilize overlap structures.

Further analyses of yet postulated alignment factors might lead to techniques that minimize illegitimate recombination in order to enhance targeted integration so that the spectrum of gene therapy could be extended to diseases that require targeted exchange of a defect gene.

V 117 **FUNCTION OF THE MEF-2 BINDING FACTOR IN MOUSE MYOGENESIS.** Richard A. Waldmann, Yie-Teh Yu, Roger E. Breitbart, Vijak Mahdavi and Bernardo Nadal-Ginard, Howard Hughes Medical Institute, Department of Cardiology, Children's Hospital, Harvard Medical School, Boston, Massachusetts 02115

The MEF-2 site is a highly conserved 10 base pair cis-acting regulatory element shown to be important to the function of a large number of muscle specific genes. It has been previously demonstrated that there is a muscle specific activity present in nuclear extracts that specifically binds to the MEF-2 site present in the MCK enhancer. Using this MEF-2 site as probe, we (Y.Y., B.N.G.) have cloned a cDNA from a human skeletal muscle expression library, which has the functional characteristics expected of the MEF-2 binding factor. Namely, it's *in vitro* transcribed and translated product binds to the MCK MEF-2 site, and can trans-activate a CAT reporter construct containing the MEF-2 site, when cotransfected into a non-muscle (HeLa) cell. A high level of CAT activity is seen when the reporter construct alone is transfected into muscle cells. Experiments are underway to disrupt the endogenous gene corresponding to our cDNA via homologous recombination in embryonic stem cells, with the aim of creating both a muscle cell line and ultimately a mouse line homozygous for the null allele. As a first step, mouse genomic clones have been isolated and mapped.

V 118 CANINE PYRUVATE KINASE DEFICIENCY DUE TO L GENE DEFECT. Katharine M. Whitney, Susan A. Goodman, Elizabeth M. Bailey, Tamio Noguchi, Takehito Tanaka, and Clinton D. Lothrop, Jr. Department of Environmental Practice, College of Veterinary Medicine, University of Tennessee, Knoxville, TN 37901.

Canine pyruvate kinase (PK) deficiency is an autosomal recessive disease of Basenji dogs. The enzyme deficiency results in chronic hemolytic anemia and persistent reticulocytosis similar to the disease in human beings. Erythrocytes from dogs homozygous for PK deficiency have an anomalous increase in PK activity. Characterization of erythrocyte PK activity in normal dogs and in Basenji dogs with PK deficiency by ion exchange chromatography, substrate kinetics, immunological reactivity and electrophoretic mobility suggests that the M_2-type PK isozyme is the major form of PK activity in erythrocytes of PK deficient dogs in contrast to normal dogs which have only R-type PK activity. The R-type monomer is readily detected by Western blot in erythrocyte lysates from normal dogs but is not detectable in lysates from PK deficient dogs using a heterologous antibody to rat L-type PK. The mRNAs for R- and M-type PK were present in reticulocytes from a PK deficient dog. Only the R-type mRNA was present in reticulocytes from normal dogs. Southern blots of DNA from normal and PK deficient dogs were identical. These results suggest that a mutation in the L gene is present in PK deficient dogs. The R-type PK cDNA from normal dogs and from PK deficient Basenjis is being cloned by the polymerase chain reaction method using primers based on conserved sequences from the human and rat cDNAs. Comparative sequencing is expected to reveal the precise molecular defect responsible for canine PK deficiency. The PK deficient dogs should be useful as a large animal model to evaluate gene therapy of blood diseases.

Transplants; Delivery Vectors

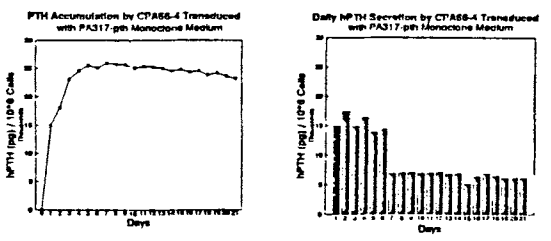
V 200 HIGH EFFICIENCY OF GENE TRANSFER TO MONKEY AIRWAY EPITHELIUM (MAE) BY RETROVIRAL INFECTION *IN VITRO*, Jean-Yves Bayle, Larry G. Johnson, John Olsen*, Judith St. George+ Ronald Swanstrom* and Richard C. Boucher. Department of Medicine, University of North Carolina Chapel Hill, *Lineberger Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599 +School of Veterinary Medicine University of Califorina, Davis, CA 95616.
Retrovirus have previously been shown to transfer foreign genes to monkey cell lines. As primates could be good models for gene therapy of airway diseases, we tested gene transfer efficiency in MAE cells in primary culture using a retroviral vector.
Methods: MAE cells were isolated from young rhesus monkey bronchus, plated on plastic dishes at 17.5 x 10^5 cells/cm^2 and fed with Ham's F12 media supplemented with 7 growth factors. The most efficient non-replication competent construct tested on MAE cells was the LPG4 virus. Containing a 55 Kd human interleukin 2 receptor cDNA (IL2R) as reporter gene, LPG4 was produced by a Gibbon ape leukemia virus based amphotropic packaging cell line. Each infection was performed for 2 hours at 37°C in a 5% CO_2 and humid atmosphere. The infection efficiency was determined on day 6, at least 48 hours after the last infection, using an IL2R immunocytochemistry assay by FACS. We studied effects of (1) the number and time of viral exposure (2) the viral dilution and (3) polybrene (PB) or protamine (PT) as adjuvant factors.
Results: (average of duplicates): Using 8μg/ml PB we found 77.2 ± 10.1% IL2R⊕ cells after multiple infection (once/day x 3 days) vs 11.4 ± 6.3% after single infection. Multiple infection on day 3 resulted in a higher efficiency than on day 1 with 81.6 ± 10.3% IL2R⊕ cells vs 29.3 ± 7.1 %. Dilution of virus reduced the gene transfer but we still obtained 8.4 ± 3.2% IL2R⊕ cells at 1/100 and 41.1 ± 4.8% at 1/10 vs 80.5 ± 13.4% without dilution after multiple infection. Furthermore after single infection we found 5.2 ± 1.6% IL2R⊕ cells with no adjuvant compared with either 5.8 ± 0.4%, 9.7 ± 1.0%, 14.4 ± 3.5%, 9.3 ± 5.3% and 23.0 ± 3.6% after respectively 1,2,4,6 and 8μg/ml PB or 16.4 ± 2.9% and 17.9 ± 2.2% after 10 and 20μg/ml PT.
Conclusion: A high efficiency of gene transfer was obtained in MAE cells by LPG4 infection even after decreasing of virus titer by dilution. Multiple infection increased the efficiency of gene transfer. PB was the best adjuvant factor with which it seemed a dose dependence effect but PT (20μg/ml) a pharmacological agent previously used in man, was as effective as 4μg/ml PB. These data suggest that an *in vivo* approach for gene transfer in monkey airways is warranted.

V 201 ANGIOGENIC ACTIVITY OF THE K-FGF/HST ONCOGENE IN NEURAL TRANSPLANTS, Oliver Brüstle[1], Adriano Aguzzi[2], Daniela Talarico[3], and Otmar D. Wiestler[1], [1]Institute of Neuropathology, University of Zürich, CH-8091 Zürich, Switzerland, [2]Research Institute of Molecular Pathology, A-1030 Vienna, Austria and [3]Department of Microbiology, NYU Medical Center, New York, N.Y. 10016, U.S.A.

Using retrovirus-mediated gene transfer into neural transplants, we have expressed the human K-*fgf/hst* oncogene in the central nervous system. Single cell suspensions of fetal rat brains were prepared at embryonic days 13 and 14, exposed to a retroviral vector encoding the K-*fgf* oncogene and stereotaxically implanted into the caudate-putamen of syngenic adult Fisher rats. Recipient animals were sacrificed at intervals of 6 to 16 months without evidence for neurological impairment. Mock-infected grafts showed the characteristic histopathological appearance of organotypically differentiated neural transplants. In contrast, grafts exposed to the K-*fgf* gene exhibited abundant capillary proliferation and capillary angiomas. By *in situ* hybridization analysis and immunohistochemistry, expression of K-*fgf* was detected in neural cells adjacent to vascular proliferations. Neurons and glia with abundant K-*fgf* transcripts were morphologically unaffected. In order to examine a transforming potential of the K-*fgf* gene in the nervous system, we combined retrovirus-mediated transfer of the K-*fgf* oncogene with a single transplacental exposure of the donor animals to the neurotropic carcinogen *N*-ethyl-*N*-nitrosourea (NEU). However, this combination of transforming agents did not result in tumor formation in the grafts. These results provide evidence for a powerful angiogenic effect of K-*fgf* on the developing brain *in vivo*.

V 202 A PROTOTYPE GENE THERAPY DELIVERY SYSTEM USING VASCULAR ENDOTHELIAL CELLS AND A PRE-PRO-PARATHYROID CONSTRUCT, Allan Callow, James A. Zwiebel*, Brendan A. Hayes, Eric Choi, Jeffrey Trachtenberg, Lynn Hu, Ruiqin Zhong, Sha-Ping Sun, Una S. Ryan, Department of Surgery, Washington University School of Medicine, St. Louis, MO 63110, *Georgetown University, Washington, D.C. 20007

Using a recombinant retrovirus encoding human pre-proparathyroid hormone we have generated an amphotropic packaging cell line by transinfection of the plasmid pMSV-hPTH-gpt into PA317 cells. Cells were selected with mycophenolic acid. Monoclonal and polyclonal PA 317 packaging cell lines were produced. Supernatants from both were used to infect bovine pulmonary artery ECs, human saphenous vein, rabbit and canine aorta and jugular vein ECs. Following selection of the transduced cells, immunoradiometric assay of the hPTH in the supernatant indicated that monoclonal cell lines tranduced with greater efficiency: bovine aorta: 35, 188, and 246 pg/ml from polyclonal versus 2741, 2695, and 3758 pg/ml for the monoclonal lines. Bovine aorta EC lines displayed less than 50% diminution in hPTH production over four months. Daily secretion showed an initial falloff over the first 14 days and stabilized thereafter. Biological activity was demonstrated by the stimulation of adenylate cyclase activity in ROS 17/2.8 cells by conditioned medium.



We have previously shown that retrovirally transduced cells can be grown on prosthetic vascular grafts, microcarriers and hollow fiber devices. Studies are underway to determine ease of creation, safety and efficiency of other methods of implantation.

V 203 INDUCTION OF PROTECTIVE IMMUNITY USING IL2- PRODUCING TUMOR CELLS

Federica Cavallo*, Antonio Soleti*, Alberto Gulino+, Mirella Giovarelli* and Guido Forni#, *Institute of Microbiology, University of Turin; +Department of Experimental Medicine, University of L'Aquila, #Center of Immunogenetics and Histocompatibility, CNR, Turin, Italy.

Lymphokines constitute communication code regulating the immune responses. Recombinant DNA technology has now made them unlimitedly available for pharmacological use in tumor immunology. As tumor-infiltrating lymphocytes and lymphocytes present in the lymph nodes draining neoplastic masses are unable to react against the tumor, we used lymphokines to restore their reactivity. To investigate the ability of locally released lymphokines to induce an immune recognition of tumors, we used expression vectors to introduce the cDNAs coding for several murine ILs into TS/A tumor cells, an apparently non-immunogenic murine adenocarinoma cell line. While the growth of TS/A cells engineered by gene transfer to produce IL-1ß or IL-4 is retarded but not totally inhibited, the tumorigenicity of TS/A clones producing high amounts of IL-2 (3,400-2,000 U/ml) is abrogated. The low-IL-2 producing clone (75 U/ml) is still able to form tumor in 40% of mice. More than 50% of mice that reject the high IL-2 producing clones are protected against a lethal challenge with the non-transfected TS/A parental cells. We observed that co-injection of high producing TS/A clones and parental cells results in efficient inhibition of tumor growth, even when highly tumorigenic doses of parental cells were used. Moreover, this protective effect was observed in few mice even if IL-2 secreting and parental TS/A cells were injected at the same time at different sites.

V 204 NOVEL RETROVIRAL VECTORS FOR CONSISTENT EXPRESSION AFTER TRANSDUCTION INTO HEMATOPOIETIC STEM CELLS, Pia M. Challita*, Carolyn Cook*, Leonard S. Sender*, Donald B. Kohn*, *Department of Microbiology, University of Southern California School of Medicine, Los Angeles, CA 90033, *Department of Pediatrics, University of Louisville School of Medicine, Louisville, KY 40292.

Successful gene therapy through bone marrow transplantation (BMT) requires gene transfer into a high percentage of hematopoietic stem cells (HSC), and persistent expression in the progeny of these cells. In the murine gene transfer/BMT model, gene expression of a variety of vectors has been achieved in long-term reconstituted animals. However, we have recently observed silencing of the MoMuLV long terminal repeat (LTR) expression in most of the secondary colony forming unit-spleen (CFU-S) when analyzed individually. There are at least two possible explanations for the inhibition of expression; first, repression through the presence of negative factors in the HSC, and second, inactivation by methylation of the vector LTR. In order to understand the mechanisms causing gene silencing, we designed a series of novel retroviral vectors to yield more consistent expression after HSC transduction. The reporter gene encoded by these vectors is the human glucocerebrosidase cDNA. These vectors are derivatives of the Myeloproliferative Sarcoma Virus (MPSV) and contain alterations in the transcriptional control elements such as deletion or substitution of negatively-acting cis-elements in the viral LTR, and insertion of a CpG-rich sequence, shown to prevent methylation in embryonic stem cells (ES). We are now studying the expression of the modified vectors in two different systems, ES cells and the murine gene transfer/BMT model. This analysis will allow us to determine, first, the relevance of the interaction between HSC negative factors and vector sequences in the LTR transcription, and second, the ability of the CpG-rich region to prevent methylation. Ultimately, we would like to generate a vector with persistent expression after HSC transduction, a feature that has important implications for effective gene therapy.

V 205 GENETIC ALTERATION OF DONOR CLASS I MHC ANTIGEN EXPRESSION IMPROVES KIDNEY TRANSPLANT FUNCTION. TM Coffman, RC Griffiths, S Geier, S Ibraham, F Sanfilippo, R Spurney, O Smithies and B Koller. Duke University Medical Center, Durham, NC and University of North Carolina, Chapel Hill, NC.

Class I MHC proteins are important determinants of alloimmune responses. To specifically examine the role of donor class I antigens in renal transplant rejection, we performed kidney transplants using donor kidneys from mice derived from embryonic stem cells in which the the $\beta_2$microglobulin (β_2M) gene had been disrupted by homologous recombination. Animals which are homozygous for the disrupted β_2M allele are grossly deficient in class I antigen expression. Kidneys from these H-2^b β_2M-/- mice, or H-2^b (C57/BLx129) F1 controls which express normal levels of class I, were transplanted into H-2^d (DBAxBALB/c)F1 recipients. Four weeks following transplantation, glomerular filtration rate (GFR) in the β_2M-/- group was significantly greater than that of the control allografts (5.22±1.05 vs 2.18±0.46 ml/min/kg; $p<0.025$) and was numerically less but not significantly different from that of syngeneic controls (8.17±1.97 ml/min/kg). Similarly, effective renal plasma flow (ERPF) was similar in the β_2M-/- grafts and syngeneic controls (15.90±3.34 and 19.85±5.14 ml/min/kg) and both were significantly greater than the control allografts (5.71±1.26: $p<0.025$). Mononuclear inflammatory cell infiltrates were present in both allograft groups but not in syngeneic controls and there were no differences in the pattern or severity if the interstitial and perivascular infiltrates in the allograft groups. Furthermore, immunoperoxidase staining revealed substantial and equivalent numbers of CD4+ and CD8+ lymphocytes in the β_2M-/- and control allografts. We observed no staining with anti-K^b in the β_2M-/- grafts confirming the lack of class I expression in the kidney and suggesting that expression of class I proteins in the kidney cannot be rescued by circulating β_2M derived from the recipient. IL-1β mRNA levels were reduced in the β_2M-/- group compared to control allografts. Thus, despite the development of histologic abnormalities that suggest ongoing rejection, kidney transplants from mice which lack normal class I proteins seem to be protected from immunologically mediated hemodynamic impairment.

V 206 *IN VIVO EVALUATION OF GENE TRANSFER TO PRIMATE CD34+ CELLS.* *R.E. Donahue, S.W. Kessler, D.M. Bodine, B.A. Agricola, E.R. Byrne, M.E. Metzger, K.T. McDonagh, J.D. Bacher, K.M. Zsebo, and A.W. Nienhuis. Clinical Hematology Branch, NHLBI, Bethesda, MD; Naval Medical Research Institute, Bethesda, MD; AMGEN,Inc. Thousand Oaks, CA.*

We have previously described a retroviral producer cell line (N263A2) that would permit, by co-cultivation, long-term gene transfer into unfractionated primate bone marrow mononuclear cells. This producer cell line produces high titer replication-defective and low titer replication-competent retroviral particles. To evaluate the contribution of CD34+ cells to hematopoietic reconstitution, bone marrow derived CD34+ cells from rhesus macaques treated with 50-70 mg/kg of 5-fluorouracil were isolated and marked with the neo^r gene. The immunoselected CD34+ cells were cultured for 4 days in medium conditioned by the neo^r retrovirus producer cell line, and combinations of growth factors. For 3 animals CD34+ cells were infected in suspension culture, and for 6 animals CD34+ cells were seeded onto pre-established autologous stroma. Following TBI(5Gyx2), the infected cells were reinfused, and peripheral blood cells were analyzed by the polymerase chain reaction(PCR). Reconstitution to granulocyte counts of greater than 1000/mm^3 occurred on average by day 20(16-25; n=9) compared to 14 and 16 days for animals receiving unmanipulated CD34+ cells, and 24 days for an animal receiving no cells. In 5 of the 9 animals, the neo^r gene was continuously present in 1-10% of the circulating leukocytes. The animal in which 10% of white blood cells contained the neo^r gene had its immunoselected CD34+ cells cultured on autologous stroma in the presence of 10ng/ml IL-3, 50ng/ml IL-6, 100ng/ml SCF, and 6ug/ml polybrene. The neo^r gene was detected in isolated circulating T-lymphocytes(98% CD2+), and granulocytes. Unfortunately the interpretation of our results has been complicated by the development of a chronic viremia from the murine amphotropic helper virus in these animals. So far, 3 of the 5 gene transfer animals developed thymic leukemias/lymphomas detected 180-234 days post-transplantation. This observation underscores the absolute requirement for the use of helper-free stocks for human gene therapy.

V 207 GENE TRANSFER TO JOINTS FOR ARTHRITIS THERAPY, Evans, C.H.[1,2], Bandara, G.[1], Robbins, P.D.[2], Mueller, G.[2], Georgescu, H.I.[1], and Glorioso, J.C.[2], Departments of Orthopaedic Surgery[1], Molecular Genetics and Biochemistry[2], University of Pittsburgh School of Medicine, Pittsburgh, PA 15261

To develop a novel, gene therapeutic approach to the treatment of arthritis, we are developing methods for introducing genes into the synovial lining of joints. Infection of rabbit synovial fibroblast cultures with retroviral vectors containing the lac Z and neo genes converts up to 10% of the cells to the lac Z$^+$ phenotype. Following selection in G418, essentially all cells become lac Z$^+$. Injection of these cells into recipient rabbit knees leads to colonization of the synovial lining by the transplanted cells, which remain in situ and express the lac Z$^+$, neo^r phenotype for at least three months. Retroviruses have also been used to transfer the human IRAP (Interleukin-1 receptor antagonist protein) gene into rabbit synoviocytes, which subsequently secrete up to 500ng IRAP/10^6cells into their culture medium. As these media inhibit the induction of metalloproteinases in chondrocytes treated with interleukin-1 (IL-1), the IRAP produced by infected synoviocytes has biological activity. Transplantation of these cells into the rabbit knee to block IL-1 in vivo is underway as a prelude to testing of the system in an animal model of arthritis.

V 208 CYTOKINE GENE THERAPY OF CANCER USING TRANSDUCED FIBROBLASTS, Habib Fakhrai, Ruth Gjerset, Daniel L. Shawler, Robert Naviaux, Ivor Royston, and Robert Sobol, San Diego Regional Cancer Center, The Salk Institute, and UC San Diego, San Diego, CA. 92121.

Tumor cells genetically modified to express cytokines such as interleukin 2 (IL-2) and interleukin 4 (IL-4) have been reported to induce immunity to both modified and unmodified tumor cells. However, application of this approach for human cancer therapy is limited by the need to culture the tumor cells for gene transfer, and by the possibility that implantation of live tumor cells may result in metastasis. We have developed a more practical approach where fibroblasts are genetically modified to secrete cytokines during co-immunization with irradiated tumor cells. We studied IL-2 expression levels in fibroblasts by constructing retroviral vectors with different promoters driving IL-2 expression. These vectors were designated as LXSN-IL2 (LTR promoter), and LNCX-IL2 (CMV promoter) and along with the double copy vector DC/TKIL-2 (TK promoter) were used to transduce different primary and established fibroblast cell lines. In all instances the level of IL-2 expression in fibroblast cell lines transduced with LXSN-IL2 and LNCX-IL2 exceeded the levels achieved with DC/TK-IL-2. The level of IL-2 expression achieved with DC/TK-IL-2 (4 ng/10^6 cells/ 24 hr) was comparable to those reported in the literature. IL-2 expression levels of up to 162 ng/10^6 cells/24 hr were achieved in a human embryonic fibroblast line using the LNCX-IL2 vector. In the murine CT26 colon carcinoma model, injection of 2 X 10^6 IL-2 expressing fibroblasts mixed with 10^5 live tumor cells resulted in either tumor rejection or a statistically significant ($p < 0.04$) decrease in the rate of tumor growth compared to tumor cells alone. In a different experiment, groups of Balb/c mice were immunized with 2.5 X 10^5 irradiated CT26 cells either alone or mixed with 2 X 10^6 transduced or unmodified fibroblasts, and challenged in the opposite flank one week later with 5 X 10^4 tumor cells. Animals co-injected with irradiated tumor cells and transduced fibroblasts were protected against subsequent tumor challenge, demonstrating the induction of systemic anti-tumor immunity. These results demonstrate the utility of transduced fibroblasts in cytokine gene therapy for cancer.

V 209 TUMOR REGRESSION WHEN A FRACTION OF THE TUMOR MASS CONTAINS THE HSV-TK GENE, Scott M. Freeman, Katharine A. Whartenby, David S. Koeplin, Frederick L. Moolten, Camille N. Abboud, and George N. Abraham. University of Rochester Medical Center, Rochester NY 14642 and Edith Nourse Rogers Memorial Hospital, Bedford, MA.

We have been investigating methods to kill tumor cells using the herpes simplex virus thymidine kinase (HSV-TK) gene as a negative selective marker. We have demonstrated that tumor cells expressing the HSV-TK are killed by the drug ganciclovir (GCV). We have further demonstrated that the toxic effects of GCV were not limited to HSV-TK positive cells, but also affected HSV-TK negative cells that were near HSV-TK positive cells. In a mixed population of tumor cells, as few as 50% HSV-TK positive cells eliminated the entire cell culture. This toxic effect of HSV-TK positive cells on HSV-TK negative cells may be a result of the mechanism of cell death of HSV-TK positive cells. In the process of cell death, apoptotic vesicles that may contain the HSV-TK enzyme or toxic ganciclovir metabolites are formed by the HSV-TK positive cells and may be transferred to the HSV-TK negative cells. This toxic effect was reproduced in an in vivo murine model by inoculating mixtures of HSV-TK positive and negative cells, either intraperitoneally or subcutaneously, as evidenced by tumor regression following GCV treatment. In addition, HSV-TK positive cells were inoculated into mice with HSV-TK negative intraperitoneal tumors and demonstrated prolonged animal survival. Evaluation of the results and their significance will be further discussed.

V 210 AGE AND PROMOTOR CHOICE INFLUENCE EXPRESSION OF DNA DIRECTLY INJECTED INTO SKELETAL MUSCLE

Dominic J. Wells and Geoffrey Goldspink. Unit of Veterinary Molecular and Cellular Biology, Department of Veterinary Basic Sciences, The Royal Veterinary College, London NW1 OTU, UK

Since the original report by Wolff et al. (1990) demonstrating the expression of reporter genes following the direct injection of plasmid DNA into rodent muscle, a number of studies have documented the same phenomenon in both skeletal and cardiac muscle of rodents and the skeletal muscle of fish. The technique has been proposed as a means of effecting somatic gene therapy, as the injected plasmids can exhibit prolonged expression, but it is hampered by variable results and the transfection of only a limited number of myofibers. We examined three possible causes of variation, namely the age and sex of the treated animal and the promoter used for treatment.

Variation in injected DNA expression due to age or sex was examined using pSV40CAT. Plasmid DNA was directly injected into the quadriceps muscle of C57/B10 mice followed 72 hours later by a standard CAT assay of extracts of the whole muscle. Age had a very major effect with the younger animals exhibiting greater plasmid expression, but there was no difference due to sex. A selection of different promoters coupled to the CAT gene produced a range of CAT expression.

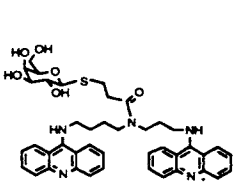
Therefore in this approach to somatic gene therapy the choice of promoter and the age of the patient may well be critical for the success of the treatment.

Wolff et al. (1990) Science 247:1465-1468

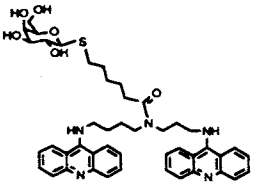
V 211 GALACTOSYLATED BIS-ACRIDINE: A POTENTIAL CARRIER FOR SPECIFIC DELIVERY OF GENES TO LIVER CELLS. Jean L. Haensler and Francis C. Szoka Jr., Department of Pharmaceutical Chemistry, School of Pharmacy, University of California San Francisco, CA 94143-0446.

One approach to the creation of gene transfer vectors is to construct the delivery system directly on the DNA. Methods to non-covalently attache molecules to DNA using a polylysine anchor have been devised but the stoichiometry of the resulting complexes is difficult to control.

We have attached galactose, a targeting ligand for liver cells, to a DNA bis-intercalator (spermidine bis-acridine) via a 3 or 6 carbon spacer arm, to create a better defined specific carrier for targeting of nucleic acids to liver cells.



Gal-3-bA



Gal-6-bA

The chemistry developed for the synthesis of Gal-3-bA and Gal-6-bA is versatile and can easely be adopted for other targeting carbohydrates. An ethidium bromide displacement assay was used to demonstrate that the galactosylated bis-acridines intercalate into DNA with affinity constants in the micromolar range. A gel retardation assay was used to demonstrate that a plasmid complexed with the galactosylated bis-acridines can interact with a soluble receptor for galactose (the lectin RCA-I from *Ricinus communis*), but as a result of an improved accessibility, the interaction is more effective with Gal-6-bA than with Gal-3-bA. This strategy can be used to attach multiple ligands to DNA and should permit the creation of multifunctional delivery vectors for gene therapy. The use of Gal-6-bA as a specific carrier for nucleic acids to liver cells *in vivo* is currently in progress. Partially supported by GM 25099.

V 212 TOWARDS GENE THERAPY FOR HEMOPHILIA A: TRANSPLANTATION OF FACTOR-VIII-SECRETING FIBROBLASTS INTO IMMUNE-DEFICIENT MICE Rob C. Hoeben[1], Frits Fallaux[1], Nico van Tilburg[2], Steve Cramer[1], Hans van Ormondt[1], Ernest Briët[2] and Alex J. van der Eb[1], Department of Medical Biochemistry[1], and Hematology[2], University of Leiden, P.O. Box 9503, 2300 RA Leiden, The Netherlands

Hemophilia A is caused by the lack of functional blood-clotting factor VIII. We have used retrovirus-mediated gene transfer to generate various fibroblast cell lines (rodent and human), that secrete significant amounts of a truncated, but fully functional (JBC 265:(1990) 7318-23), human factor-VIII protein (30 - 105 mU/10^6 cells per 24 h). To study whether transplantation of genetically modified fibroblasts is a feasible approach for gene therapy for hemophilia A, we implanted the factor-VIII-secreting cells into immune-deficient mice. Implantation of factor-VIII-secreting primary human skin fibroblasts ($2x10^7$ cells/mouse) resulted in long-term persistence of the transplanted cells. However, we were unable to detect any human factor VIII in plasma samples of the recipients (<20 mU/ml) by an ELISA specific for the human-factor-VIII light-chain. Cells recovered from the implants up to 2 months post-implantation still had the capacity to secrete factor VIII when regrown in tissue culture. This indicates that the absence of human factor VIII in the recipients' plasma is not due to (epigenetic) inactivation of the retroviral vector *in vivo*. In addition, we demonstrate that factor VIII is secreted as efficiently by density-arrested cell cultures (28 ± 0.8 mU/10^6 cells per 24 h) as by cultures of growing cells (31 ± 0.5 mU/10^6 cells per 24 h), suggesting that the apparent absence of human factor VIII is not due to the stationary state of the transplanted cells. In contrast, the absence of human factor VIII in the recipients can be explained by the rapid clearance of human factor VIII:CAg from the murine plasma. Upon intravenous injection of plasma-derived human factor VIII (40-50 mU/g) we noted a rapid disappearance of factor VIII ($t½ < 1$ h in mice vs. 12 h in humans). We can not, however, exclude the possibility that other mechanisms, too, thwart the detection of human factor VIII in murine plasma.

V 213 REGULATION OF EXPRESSION OF TYPE I COLLAGEN BY C-MOS PROTOONCOGENE IN TRANSGENIC MICE. Jaspal S. Khillan, Daniel C. Bertolette and Machiko Arita, Department of Biochemistry and Molecular Biology, Jefferson Institute of Molecular Medicine, Jefferson Medical Collegen, Thomas Jefferson University, Philadelphia, PA 19107

Earlier reports by Schmidt et al. (1985) have shown that v-mos oncogene product regulates the expression of CAT gene from the COL1A2 promoter in fibroblasts. In order to investigate the regulation of collagen genes by c-mos protooncogene we generated several transgenic lines with a fusion gene that contains -2.8 kb promoter region of human COL1A1 gene and the coding sequence of c-mos protooncogene. Several lines of transgenic mice were prepared. One line of transgenic mice developed abnormalities of the bone tissue including fractures of the long bones. Nodule-like outgrowths were observed at the bone-cartilage junctions of the ribs. These outgrowths were highly supplied with blood vessels. Tissue culture cell lines derived from the skin of transgenic animal expressed c-mos-specific RNA. Western blot analysis using antibodies specific for C-terminal domain of COL1A1 showed that the collagen synthesis in transgenic fibroblasts is down regulated as compared to the normal fibroblasts. Further studies are being carried out to investigate this regulation at the molecular level.

V 214 **MAST CELL GROWTH FACTOR (MGF) INCREASES RETROVIRAL VECTOR-MEDIATED GENE TRANSFER INTO HUMAN HEMATOPOIETIC PROGENITOR CELLS.** Donald B. Kohn, Jan A. Nolta, Gay M. Crooks, Robert W. Overell, and Douglas E. Williams. Childrens Hospital Los Angeles, U.S.C. School of Medicine, Los Angeles, CA 90027 and Immunex Research and Development Corporation, Seattle, WA 98101.

Retroviral vector-mediated gene transfer into human hematopoietic stem cells may permit gene therapy of numerous genetic diseases. Stimulation of marrow with hematopoietic growth factors (HGF) has been shown to increase the level of retroviral transduction. We have examined the effects of recombinant human Mast Cell Growth Factor (MGF), alone and in combination with other HGF, on the efficiency of gene transfer into human hematopoietic progenitor cells. MGF acts in concert with IL-3 and IL-6 to increase the percentage of $CD34^+$ progenitors transduced with a retroviral vector expressing the *neo* gene. The most potent combination of growth factors which we examined, IL-1/IL-3/IL-6/MGF, resulted in the conferral of G418-resistance on 45% of progenitors and long-term culture initiating cells. Extending the time of co-cultivation of the marrow cells with the vector-producing cells did not further increase gene transfer frequency, suggesting that the amount of available vector is not limiting. To analyze the effects of the HGF on gene transfer into more primitive hematopoietic progenitors, $CD34^+$ cells were isolated from marrow samples that were purged of committed progenitor cells by treatment with 4-hydroperoxycyclophosphamide (4-HC). Preculturing the $CD34^+$, 4-HC treated cells with the combination of four HGF (IL-1/IL-3/IL-6/MGF) permitted transduction of 20-28% of the progenitors which formed colonies after 30 days in culture. These results demonstrate that MGF in combination with other HGF enhances gene transduction of human hematopoietic progenitor cells.

V 215 THE INTESTINE AS A POSSIBLE SITE FOR GENE THERAPY, Chantal Lau, Humberto Soriano-Brücher, Tracy Hourigan, Milton Finegold, Fred D. Ledley and Susan J. Henning, Departments of Pediatrics, Pathology and Cell Biology, Baylor College of Medicine, Houston, Texas 77030

The intestinal epithelium is an attractive site for somatic gene therapy because of its ease of access via the luminal route. As the epithelium is continually proliferating, *in vivo* transduction using retroviral vectors should be feasible. Moreover, if the stem cell population can be transduced, long-term expression of retrovirally delivered genes should be possible. To assess the feasibility of such gene transfer we first used a rat intestinal epithelial cell line (IEC-6). Using the retrovirus Zen^+ ß-gal (from P. Soriano), we found that IEC-6 cells are transducible and give titers approximately 20% of those found with NIH3T3 cells. Incubation of Zen^+ ß-gal with intestinal washings indicated no deleterious effects on infectivity. The same retrovirus was then used in adult rats *in vivo*. Ligated 2-cm bowel segments were filled with virus-containing medium. Control segments similarly ligated were filled with medium only. The ligatures were removed 4 hrs later and the animals sacrificed 1–6 days after surgery. Frozen sections of the experimental segments were subjected to histochemical assay for ß-gal. Cells showing blue reaction product in the cytoplasm were scored as positive. Following blind scoring of the slides, significantly higher numbers of positive cells were found in the epithelium of experimental segments (exposed to retrovirus) than in control sections. Thus, it appears that the reporter gene transferred in this manner is expressed for at least 6 days following infection. These findings suggest that stem cells can be successfully transduced because complete replacement of the intestinal epithelium from progeny of stem cells is estimated to take 4–5 days, which is shorter than the time at which ß-gal was detected in the experimental segments.

V 216 AUTONOMOUS PARVOVIRUS VECTORS FOR GENE TRANSFER, Ian H. Maxwell[1], Françoise Maxwell[1], Solon L. Rhode III[2], Joe Corsini[3] and Jonathan O. Carlson[3]. [1]University of Colorado Health Sciences Center, Denver, CO 80262; [2]University of Nebraska Medical Center, Omaha, NE 68198; [3]Colorado State University, Fort Collins, CO 80523.

Parvoviruses are a widespread group of small DNA viruses containing a single strand genome of ≈ 5kb. They have potential as vectors since most of the genome can be replaced by foreign DNA, as previously demonstrated for the defective subgroup of AAV (adenoassociated viruses)(e.g. Samulski et al., J. Virol. 63 3822 (1989)). Here, we report that an autonomous parvovirus can also be used as a gene transfer vector. Recombinants based on the genome of the autonomous parvovirus, LuIII, were constructed by replacing the viral coding sequences in an infectious clone (pGLu883, constructed by Diffoot and Bates) by a luciferase reporter gene. When cells were transfected with these constructs, together with a plasmid supplying non-structural and capsid proteins, excision and replication of the recombinant genome occurred and luciferase-transducing virions accumulated in the culture medium. Transduction was specifically inhibited by antiserum to LuIII. Data will be presented on strand packaging and on transduction efficiency, assayed by in situ hybridization. We suggest that transducing autonomous parvoviruses will be useful in various gene transfer applications (including some types of gene therapy) where only transient expression is desired, since these viruses (in contrast to retroviruses and AAV) lack an efficient integration mechanism.

V 217 ADENO ASSOCIATED VIRUS: A VECTOR SYSTEM FOR HIGH EFFICIENCY GENE TRANSDUCTION INTO MAMMALIAN CELLS. Maureen McNally, Jane Lebkowski, Lisa Schain, Thomas Okarma. Applied Immune Sciences, Inc., Menlo Park, CA

Adeno Associated Virus is a dependent single stranded DNA parvovirus which can be easily engineered for efficient gene transduction. In the absence of adenovirus or herpesvirus, wild type AAV or rAAV vectors infect target cells and integrate their genomes by insertion of their molecular ends into host DNA. Recent evidence suggests that for certain human cells there are preferential sites for viral genome integration. We have constructed recombinant AAV vectors which are free of detectable wild type virus and which carry one or two of the following genes: the chloramphenicol acetyltransferase, the neomycin phosphotransferase or the apolipoprotein A1 gene. These recombinant AAV vectors infect numerous cell types including primary skin fibroblasts, and hematopoietic cell lines of diverse lineage, such as K562, NC37 and the CD34+ line KG1a. Stable transductants can be isolated with efficiencies of up to 10%. Cell lines transduced with rAAV vectors containing the neomycin phosphotransferase and apolipoprotein A-1 genes expressed both recombinant genes, although the expression levels of the two protein products were dependent upon the relative orientation of the two genes within the vector. Coinfection of two different recombinant viruses can be used to simultaneously introduce different sequences into a given cell with frequencies exceeding 50%. Current efforts to produce high titer rAAV stocks will be discussed.

V 218 G-CSF GENE TRANSFER INTO TUMOR CELLS INDUCES GRANULOCYTE-MEDIATED TUMOR INHIBITION.

Cecilia Melani, Mario P. Colombo, Luciano Lombardi, Antonella Stoppacciaro*, Giorgio Parmiani. Istituto Nazionale Tumori, Milano, and *Dipartimento di Biopatologia Umana, Università di Roma, Italy.

Several cytokines can exert antitumor activity when delivered at the site of tumor; we tested whether G-CSF transduction in a tumor cell line can interfere with its growth *in vivo* and analysed the host cells involved in the tumor inhibition. Retroviral mediated tranduction of G-CSF into the murine colon adenocarcinoma cell line C-26 resulted in the inhibition of its tumorigenicity *in vivo*, in both syngeneic BALB/c and nu/nu mice, while its injection in sublethally irradiated hosts gave rise to tumors which regressed after normalization of leukocyte count. Histological and immunocytochemical examination of tumors showed neutrophilic granulocytes as the major component of the tumor infiltrate in normal syngeneic mice, while T cells and macrophages were also involved in the tumor regression in sublethally irradiated hosts. When a mixture of infected and non infected C-26 cells was injected into mice, the resulting tumor was constituted only by the original C-26 cells, as shown by Southern blot analysis. To address the hypothesis that neutrophils recognize and selectively interfere with the growth of the G-CSF producing cells, a retroviral vector carrying the *E. Coli LacZ* gene was used to infect the the C-26 cells, and a mixture of G-CSF producing and b-gal positive/G-CSF non producing cells was injected into syngeneic mice. Treatment of tumor specimens with X-gal. allowed to recognize the two components of the tumor and to demonstrate, by electron microscopy and histological examination, that neutrophilic granulocytes were localized all around the G-CSF-positive/b-gal negative cells, with cell-cell contacts occurring only with these cells. These results indicate that G-CSF releasing at tumor site cause inhibition of C-26 tumor growth: this effect is mediated by neutrophilic granulocytes which are able to discriminate between G-CSF producing and non producing cells and to selectively interfere with their growth.

V 219 GENE TRANSFER INTO PRIMARY CULTURE PBL CAN PROTECT CELLS FROM HIV INFECTION

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Several protocols designed to treat patients with genetically engineered cells (gene therapy) are in progress. In viral infections (which can be thought of as acquired genetic diseases) it may be possible to apply the technology of gene therapy to deliver anti-viral agents directly to infected cells. We have developed several approaches that use retroviral vectors to genetically modify lymphocytes such that they now manifest anti-viral activity. It is within reason to consider that the appropriate genetic engineering of lymphocytes could be of therapeutic benefit to individuals suffering with life threatening viral infections (eg. HIV-1). Cells may be modified so that they either directly possess anti-viral activities, or that they can indirectly stimulate anti-viral activity. Different strategies for anti-HIV gene therapy approaches will be described. Retroviral vectors have been constructed that express one or more of the following gene products: sCD4 (or a sCD4 derivative), transdominant HIV rev mutants, a tat and rev regulated human α2-interferon, and an HIV inducible diphtheria toxin. Analysis of these AIDS gene therapy systems has demonstrated protection from HIV-1 infection in primary culture human lymphocytes.

V 220 OPTIMAL CONDITIONS FOR RETROVIRAL SUPERNATANT INFECTION OF CD34+ CELLS, Annemarie Moseley[1], Kohnoske Mitani[1,2], Albert Deisseroth[3] and C. Thomas Caskey[1,2]. [1]Institute for Molecular Genetics and [2]Howard Hughes Medical Institute, Baylor College of Medicine, [3]M.D. Anderson Cancer Center, Houston, TX 77030.

The CD34 surface antigen is a glycoprotein expressed on a population of bone marrow cells containing the primitive hematopoietic precursors. Autologous transplants have been successful with CD34 selected cells. Selection of these cells for bone marrow gene therapy would have multiple benefits: the cell volume which would need to be handled would be greatly decreased and transduction efficiency into the primitive target population could be enhanced. Bone marrow cells from normal donors are placed on a Percoll gradient, and cells are collected from the 40% / 60% interface. The low density cells are then placed on the anti-CD34 selection devices (Applied Immune Sciences). Using the My-1 clone anti-CD34 FITC (BD), the purity of the resulting population is 87-94% as analyzed by FACS analysis. The yield is approximately 0.5-1% of the Percoll fraction and when placed in methylcellulose culture, a 200-fold enhancement of colony-forming cells can be seen. The cells are placed into long-term culture under standard Dexter and Whitlock-Witte conditions with and without stromal support. Within 12-18 hours, retroviral supernatant containing the hADA gene is added, and then every 12 hours for 48-72 hours. Infection efficiency is determined by the percentage of positive colonies derived from methylcellulose culture after 5 weeks in long term culture as determined by PCR assay for proviral integration of the hADA gene. The cells require stromal support for infection (0% infection without stroma). Increased infection efficiency appears to occur with autologous stroma over allogeneic stroma, even when irradiation effects have been controlled for. While rhIL3 and rhIL6 (Genetics Institute) have been demonstrated to increase the infection efficiency in co-cultivation experiments, to date no significant difference over stroma alone has been detected in the infection of the CD34+ cells. Preliminary results suggest that the effects of the addition of stem cell factor (SCF; Immunex) may increase the early measurement of infection efficiency (30% over stroma only and rhIL3/rhIL6 groups), but that this enhancement may not be sustained. The significance of this result in an *in vivo* setting is unknown. Ongoing experiments address shorter infection periods as well as expression of the human ADA gene in myeloid colonies and B lymphocytes.

V 221 ANTISENSE P53 RNA REDUCES THE TUMOR SUPPRESSOR FUNCTION IN HUMAN LUNG CANCER CELL LINES CARRYING WILD TYPE OR MUTATED P53 GENE, Mukhopadhyay T., Cavender A., Roth J.A., Department of Thoracic Surgery, University of Texas M. D. Anderson Cancer Center, Houston, TX 77030.

Wild type p53 gene has been implicated in regulation and control of tumor development. Human lung cancer cell lines carrying either wild type or mutated p53 gene were transfected with a plasmid expression vector with a β-actin promoter driving wild type antisense p53 cDNA (ASp53). Stable transfectants were isolated after G418 selection and were grown separately. H226 human non small lung cancer cell (NSCLC) line has a wild type endogenous p53 gene and does not form tumors in nu/nu mice while H226 (ASp53) transfectants expressing antisense p53 RNA are highly tumorigenic although the growth rate in culture did not change significantly. Histochemical and western blot analysis indicated significant reduction in the p53 protein synthesis. Similarly, H322a cell line with a spontaneous mutation at 248 codon of the p53 gene show low tumorigenic potential, but H322(ASp53) clones readily formed tumors in nu/nu mice. Western blot analysis indicated reduced level of mutated p53 protein in these clones. These results indicate that specific p53 mutations still retain tumor suppressor function. This data presents direct evidence of tumor suppression function of wild type p53 gene.

V 222 GENETICALLY-MODIFIED ENDOTHELIAL CELLS ADMINISTERED INTRAVENOUSLY ARE INCORPORATED INTO SITES OF ACTIVE ANGIOGENESIS. John Ojeifo*, Reza Forough*, Thomas Maciag*, and James Zwiebel*. *Holland Laboratory, American Red Cross, Rockville, MD 20855, and *Lombardi Cancer Research Center and Department of Medicine (Hematology), Georgetown University, Washington, DC 20007.

A key step in somatic cell gene therapy is the implantation of genetically-modified cells. This process requires vascularization of the implant for the cells to survive and to maintain recombinant gene expression. To define the conditions for genetically-modified endothelial cell implantation, we first examined the capacity of acidic fibroblast growth factor-secreting mouse fibroblasts (aFGF/NIH3T3) to induce angiogenesis in nude mice. Animals which received non-irradiated cells developed rapidly growing, hemorrhagic tumors at the sites of implantation. These tumors led to the death of the mice within 2-3 weeks. Irradiation of the aFGF/NIH3T3 cells still allowed a brisk angiogenic response without causing the death of the animals. No angiogenic response was observed in control mice with implants of NIH 3T3 cells containing only the plasmid vector (pMEXNeo/NIH3T3). We then compared the survival of lacZ-expressing human umbilical vein endothelial cells (BAG-HUVEC) implanted subcutaneously (SC) with those injected intravenously (IV) into aFGF/NIH3T3 bearing nude mice. BAG-HUVEC appeared in the lungs within five minutes following tail vein injection into aFGF/NIH3T3-bearing nude mice. BAG-HUVEC were cleared from the lungs within three hours, but became noticeable fours days later at SC sites of aFGF/NIH3T3-induced angiogenesis. LacZ gene expression, demonstrated by X-gal staining, persisted for at least two weeks. In contrast, very few or no X-gal-stained cells were seen at the sites of the pMEX-Neo/NIH3T3 implant. Also, little X-gal staining was observed when BAG-HUVEC were innoculated SC into sites of aFGF/NIH3T3 implants. These results demonstrate that IV administered, genetically-labelled HUVEC can migrate into and survive within an angiogenic site in nude mice. Moreover, these findings suggest that IV injection may be more efficient than direct SC innoculation as a means of endothelial cell implantation.

V 223 AN SV40 PSEUDOVIRAL VECTOR FOR SOMATIC GENE THERAPY, Ariella Oppenheim, Nava Dalyot, Ziv Sandalon, and Ariella Gordon, Department of Hematology, Hebrew University-Hadassah Medical School, Jerusalem, Israel, 91120

An SV40 pseudoviral vector developed in our laboratory is very efficient in gene transfer into a variety of cells, including human hemopoietic cells. Plasmids carrying SV40 *ori* are encapsidated in COS cells, with helpers, as SV40 pseudovirions, and transmitted into the target cells by viral infection. The prokaryotic DNA is removed after propagation in bacteria and before encapsidation. The constructs include only 200 bp of SV40 DNA, with cloning capacity of over 5 kb. Thus plasmids carrying over 95% human DNA are efficiently transferred into human hemopoietic cells. A helper-free packaging cell-line, that will supply the viral T-antigen and the late proteins, is being constructed. We use CMT4 cells, which express T-antigen under control of metallothionein promoter. High expression of the late genes is achieved by co-amplification with the gene for multidrug resistance, MDR1. Electron microscopy revealed an abundance of virion-like particles, suggesting that the late proteins produced were self-assembled *in vivo*. Presently we investigate expression of the late genes from another inducible promoter. We anticipate that separate controls over the early and late helper functions will allow fine tuning of conditions for high-titer, helper-free pseudoviral stocks.

V 224 STUDIES OF THE TROPISM OF RETROVIRUSES TO SPECIFIC HUMAN TISSUES

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Retroviral vectors derived from murine leukemia virus (MuLV) have been an important method for transducing mammalian cells in vitro. Because of their broad tropism, vectors derived from amphotropic packaging cell lines are now being used experimentally to mark human cells with dominant selectable marker genes, and to introduce potentially therapeutic genes into target cells. While preliminary results of these studies are promising, the frequency at which human cells are transduced generally remains low (< 10%). In an effort to increase the frequency of in vitro transduction, we have been studying the interaction between the retrovirus and the cellular target by several approaches. A sensitive immunofluorescence based assay has been developed to determine whether a cellular target has surface receptors for a particular retrovirus. Using a rat monoclonal antibody against the envelope gp70 molecule which recognizes a shared epitope among the different MuLV's (ampho, eco and xeno), and a labelled secondary antibody, it has been possible to analyze the binding of these viruses to different cell types by FACS. We have been primarily interested in analyzing the binding to human peripheral blood lymphocytes and tumor infiltrating lymphocytes (TIL). The results obtained by this assay are envelope specific, and correlate with the known pattern of susceptibility to infection of human cells by MuLV's. In addition, we have found an inverse correlation between the length of time that the lymphocytes are cultured, and the percent of cells in that culture which remain receptor positive. These analyses have been limited to MuLV's because the antibody is specific and has a very low background. Reagents to other retroviral species (non-murine) are being tested to determine their suitability in this type of assay system. In an effort to study whether non-MuLV retroviruses target particular human cell types, we have used wild-type, primate retroviruses to infect cells in culture and look for productive infection by the presence of reverse transcriptase in the supernatants. In addition, vectors carrying a marker gene have been pseudotyped with various retroviruses to analyze cells that can be transduced, but do not permit replication of wild type viruses.

V 225 DEVELOPMENT OF METHODS TO ENSURE CONSTITUTIVE EXPRESSION OF AN INTERFERON GENE AS AN APPROACH TO SOMATIC CELL GENE THERAPY FOR HIV. I. Seif, E. Lauret, K. Macé*, I. Rivière, O. Cases, J. De Maeyer-Guignard, L. Gazzolo*, E. De Maeyer. URA 1343 - CNRS, Institut Curie, 91405 Orsay and *UMR 30, Faculté de Médecine Alexis Carrel, 69372 Lyon (FRANCE).

The aim of our experiments is to find ways of establishing stable antiviral expression (SAVE) in hemopoietic stem cell-dependent populations using interferon (IFN) genes, as a means of combating life-threatening viral infections with long incubation periods, for which no other therapeutic measures are available.

We have first shown that SAVE, directed against three nonretroviral RNA viruses, can be obtained in a significant percentage of murine BALB/c 3T3 cells transformed with an IFN-β expression plasmid under the control of the 0.6-kb XhoII-NruI promoter region of the murine H-2K^b MHC gene. In these cells, the low IFN-β synthesis was sufficient to induce a permanent antiviral state, without impeding cell survival and replication. Next, we have demonstrated the possibility of restricting HIV replication in human promonocytic cells (U 937) by establishing SAVE with the human IFN-β coding sequence placed under the control of the H-2K^b promoter. The presence of low amounts of construct-derived IFN-β mRNA was demonstrated by polymerase chain reaction amplification of cDNA, and the level of 2-5A synthetase, one of the major IFN-induced antiviral proteins, was shown to be constitutively increased. The antiviral state could be modulated by anti-IFN-β antibodies, in that the continuous presence of antibodies in the culture medium abolished the enhanced resistance to HIV-1 replication, whereas the withdrawal of the antiserum restored the antiviral state, indicating that it did indeed result from the constitutive synthesis of human IFN-β.

We are presently studying in the murine model ways of introducing the murine IFN-β construct into hemopoietic stem cells, using retroviral vectors. However, we have found that commonly used packaging cells, such as ψ2, produce very low retroviral titers, due to their high sensitivity to the autocrine interferon encoded by the vector. The presence of high-titered anti-IFN serum is unable to counter the antiviral effect of the thus produced IFN.

To study the effects of low-level constitutive interferon synthesis on hematopoiesis, immune function, and resistance to Friend Leukemia Virus, we have obtained mice transgenic for the H-2K^b IFN-β construct, in collaboration with C. Babinet of the Pasteur Institute.

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V 226 THE INDUCTION OF MYELOPROLIFERATIVE DISEASE AND LEUKEMIA BY THE ONCOGENES V-MYC AND V-ERB-B. Geoff Symonds, Lynn Bonham, Karen MacKenzie and Michelle Miller. Children's Medical Research Foundation, P.O. Box 61, Camperdown, N.S.W. 2050 Australia.

We have employed efficient recombinant retroviruses based on the genome of Moloney murine leukemia virus to introduce the MC29 v-myc and AEV v-erb-B oncogenes into immature bone marrow cells. Virus-producing φ2 cells were co-cultivated with post 5-fluorouracil bone marrow cells. These cells were then transplanted into lethally-irradiated recipient mice. All provirus-positive reconstituted animals were found to develop hematopoietic proliferative disorders. For v-myc, these were either myeloproliferative disease or leukemia (*myeloblastic, myelomonocytic or in one case* T lymphocytic) while v-erb-B induced an apparent erythroleukemia. Immortalised monocytes, myeloid progenitors and T lymphocytes were isolated from the mice repopulated with v-myc-containing progenitor cells reflecting the disease induced in the animals. However, no erythroid cell lines could be established from erythroleukemic mice; all cell lines derived from either spleen or bone marrow cultures had a pre-B lymphoid phenotype, indicating an in vivo transformed sub-population. The predominant target cell specificities for the two oncogenes thus appears to correlate with our previous in vitro findings of myelomonocytic (v-myc) (1,2) and erythroid/B lymphoid (v-erb-B) (3,4) transformation. This system provides a means by which to analyse leukemia development, in particular the target cell specificity, the sites of tumor development and the progression from a pre-leukemic disease to malignancy.

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V 227 THE LEVEL OF MHC CLASS I H-2D^k ANTIGENS CONTROLS THE METASTATIC PHENOTYPE OF MOUSE T LYMPHOMA CELLS. Thierry VandenDriessche and Patrick De Baetselier, Department of Cellular Immunology, Free University of Brussels, Paardenstraat 65, B-1640 Rode, Belgium.

In vivo inoculation of a low metastatic BW 5147 derived T-cell lymphoma variant into syngeneic mice, had led to the generation of a highly metastatic variant (VandenDriessche *et al.*, Inv. & Metastasis 10:65, 1990). Expression of membrane antigens was compared on the low and the highly tumorigenic variants. We found that the shift towards a more malignant phenotype is accompanied by an increase in H-2D^k antigen expression. This suggests that H-2D^k antigens may be involved in the control of the metastatic potential of BW T lymphoma cells. We have now corroborated this correlation by specifically altering the level of H-2D^k expression using different approaches: i) FACS sorting ii) IFN-γ treatment and iii) H-2D^k gene transfection. The highly metastatic BW variant which expressed a very high level of H-2D^k antigens was subjected to sequential FACS sortings to specifically enrich for cells which expressed only a low level of H-2D^k antigens (D^{low} variant) or a high level of H-2D^k antigens (D^{high} variant). The D^{low} cells were significantly less malignant as compared to the D^{high} population. The expression of H-2D^k antigens on these D^{low} cells could be induced by interferon-γ (IFN-γ) treatment which led to an increased malignancy. Moreover, a high level of H-2D^k antigen expression could also be obtained after transfection of a syngeneic H-2D^k gene into the D^{low} cells, which concomitantly became more malignant. These data demonstrate that a high level of H-2D^k antigen expression contributes to the acquisition of a malignant phenotype of mouse T lymphoma cells.

V 228 ANALYSIS OF TRANSFORMED CELLS AFTER TRANSDUCTION WITH RETROVIRAL VECTORS, Katharine A. Whartenby, James A. Zwiebel, Barbara K. Vonderhaar, Scott M. Freeman, and George N. Abraham. University of Rochester Medical Center, Rochester, NY 14642, Georgetown University Medical Center, and National Cancer Institute, NIH

We have been investigating mechanisms of malignant transformation of cells by transduction with retroviral vectors. Using NOG-8 murine mammary epithelial cells, 44 cell lines have been generated with 2 separate retroviral vectors, each containing an LTR-promoted gene for neomycin resistance and the gene for rat growth hormone (rGH) under the control of different promoters. 4 of 22 cell lines transduced with a retroviral vector containing rGH promoted by a prolactin inducible promoter were transformed in the presence of prolactin. Transformation was assessed by anchorage independent growth in soft agar. 7 of 22 cell lines transduced with a retroviral vector containing rGH under the control of a constitutive phosphoglyceraldehyde promoter were transformed. Cells were not transformed by growth in medium containing prolactin and growth hormone. Mechanisms of transformation by these vectors are being analyzed to determine the nucleotide sequences present within the vectors that are responsible for producing the transformed phenotype. Clonal populations of transformed cells have been isolated to facilitate this analysis. Cell lines are also being generated by transfection with the vectors. Southern and Northern analysis of these lines is currently underway.

V 229 NIH 3T3 CELLS TRANSDUCED WITH BASIC FGF STIMULATE MYELOPOIESIS IN VITRO, Robert Wieder[1], Sanjay Shirke[2], Efimia Kehagias[2], Ann A. Jakubowski[1], E. Lynette Wilson[3] and Janice L. Gabrilove[1], [1]Department of Medicine and [2]Laboratory of Leucocyte Biology, Memorial Sloan-Kettering Cancer Center, New York, NY 10021 and [3]Department of Cell Biology and Kaplan Cancer Center, New York University Medical Center, New York, NY 10016.

Exogenous human basic fibroblast growth factor (hbFGF) stimulates hematopoiesis in long term bone marrow cultures. NIH 3T3 cells are also able to support hematopoiesis in vitro. This study investigates the effects of constitutive bFGF production by NIH 3T3 cells on in vitro myelopoiesis. Retroviral vectors were constructed from N2 with a constitutive CMV or human ADA-promoted 1 kb hbFGF cDNA, and packaged in cell lines GP+E-86 and GP+envAM12. Titers were determined on NIH 3T3 cells. bFGF expression was assayed with a polyclonal rabbit anti-hbFGF antiserum using an ELISA. bFGF biologic activity was determined using a chromogenic plasminogen activation assay. NIH 3T3 cells were transduced, and G418-resistant clones producing low, intermediate and high levels of bFGF were selected. High bFGF levels correlated with high levels of vector-coded mRNA, phenotypic transformation, altered growth characteristics and growth in agar. CD34 antigen-enriched normal human bone marrow mononuclear cells were coincubated with gamma-irradiated bFGF producers for 24 hours, and cultured for 14 days in semisolid agar with 20 ng/ml rhGM-CSF. Low bFGF-producers stimulated CFU-GM (≥40 cells) but higher levels of bFGF suppressed colony formation. Cluster formation (<40 cells) was not significantly affected. In vivo effects of the transformed cells in mice are under investigation as a model for stromal transplantation as an adjunct for bone marrow transplantation and gene therapy.

V 230 Establishment of Intracellular Resistance to HSV-1 Production in Cells Transduced with an Adeno-associated Virus Based Antisense Vector, K.K. Wong, Jr. and Saswati Chatterjee, Divisions of Hematology and Pediatrics, City of Hope National Medical Center, Duarte, CA 91010-0269.

Herpes simplex virus (HSV) continues to represent a highly prevalent human pathogen, and the incidence of drug resistant strains appears to be increasing, especially in immunodeficient populations. Thus, novel approaches to control HSV replication are continually being sought. We have developed a eukaryotic viral vector based upon the nonpathogenic parvovirus AAV which confers resistance to G418, and expresses an RNA transcript complementary to the 5'-noncoding region and translational initiation site of the HSV-1 ICP4 transcript. The HSV ICP4 gene product has been shown to be the major viral transcriptional activator and to be requisite for viral replication. Twelve of twelve (100%) vector-transduced, clonally-derived murine L929 cell lines demonstrated minimal cytopathic effects following HSV-1 challenge (multiplicity of infection 0.1) in comparison to control cells. Antisense transcript expression was demonstrated by PCR. In addition, vector-transduced cells restricted HSV-1 production by a factor of 1000-10,000 (>99%), displayed reduced ICP4 expression, and exhibited increased cell viability (75% versus 0% at 4 days postinfection) in comparison to control cells following viral challenge. Furthermore, HSV-1 ICP4 antisense expressing cells also restricted replication of HSV-2, a genetically distinct but serotypically diverse herpesvirus, but were permissive to vaccinia virus replication. AAV-based vectors manifest a number of advantages for gene therapy including lack of toxicity, wide host range, high transduction frequencies, and possible site-specific genomic integration into human chromosomal DNA. Similar vectors may ultimately prove useful in the establishment of intracellular resistance to an assortment of human pathogens, in the control of aberrant gene expression, and in the replacement of dysfunctional genes.

V 231 CONSTRUCTION OF A RECOMBINANT ADENO-ASSOCIATED VIRUS (AAV) VECTOR CARRYING THE CYSTIC FIBROSIS cDNA. S. Zolotukhin*, J. Muzak*, R. J. Gregory+, A. E. Smith+, and N. Muzyczka* Department of Microbiology, SUNY Stony Brook Medical School*, Stony Brook, NY, 11794, and Genzyme+, Framingham, MA 01701

Recent experiments have demonstrated the usefulness of AAV vectors for transduction of foreign genes into mammalian cells. Here we report the construction of a new AAV vector which retains only the 145 bp terminal repeats. The vector was used to package the 4.6 kb cystic fibrosis transmembrane regulator (CFTR) cDNA. Two different constructs in which the CFTR gene is under the control of its own promoter or a herpes thymidine kinase promoter have been isolated and their levels of expression will be compared. New strategies for increasing the titers of recombinanat AAV vectors will also be discussed.

Homologous Recombination; Positional Cloning and Major Diseases

V 300 THE MURINE HOMEOBOX GENE, mNK-2, IS EXPRESSED IN NEWLY FORMED SOMITES,

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Drosophila homeobox genes encode DNA binding proteins that play important roles in regulating embryonic pattern formation. Like the Drosophila genes, murine homeobox genes are expressed in a stage and tissue specific manner and appear to perform analogous developmental functions. A new murine homeobox sequence was isolated from mouse genomic DNA by PCR technology using degenerate oligonucleotide primers specific for conserved regions of the Drosophila engrailed homeobox. This unique sequence showed only 35% homology to the Drosophila engrailed homeobox, and was designated mNK-2 because it was most similar (56% homology) to the Drosophila NK-2 homeobox (Kim and Nirenberg PNAS 86:7716, 1989). Genomic and cDNA clones containing mNK-2 were isolated to study expression of this gene in the mouse. A 2KB mNK-2 specific transcript was detected in both adult and embryonic tissues. In adults, expression was restricted to brain and kidney. During embryogenesis, expression was most abundant at 9.5 days with decreasing expression in later stages of development. In situ localization of mNK-2 transcripts in 8 to 10.5 day embryos showed hybridization restricted to the most terminal somites. Expression is extinguished as the somite differentiates; first in the dermomyotome, then the sclerotome. mNK-2 is the first gene shown to be exclusively expressed in newly formed somites, suggesting that mNK-2 may play a regulatory role in the segmentation and differentiation of somitic mesoderm in the mammalian embryo.

V 301 TARGETED MUTATION OF THE PLATELET-DERIVED GROWTH FACTOR A CHAIN GENE IN EMBRYONAL STEM CELLS.

Christer Betsholtz, Håkan Hedstrand, Samuel Gebre-Medhin, Hans Boström, Fredrik Rorsman, Per Leveen, Catherine Hazzelin, Helen Abud & John K. Heath. Departments of Pathology, University of Uppsala, Sweden, and Biochemistry, University of Oxford, UK.

Platelet-derived growth factor (PDGF) occur in three dimeric isoforms (AA, AB or BB) of two polypetide chains, A and B. The B chain gene is the cellular homolog (*SIS*) to v-sis, the transforming gene of simian sarcoma virus. The PDGF isoforms bind to two different receptor molecules, α and β, which upon ligand binding dimerize to active receptor complexes. The A chain has only affinity for the α-receptor subunit whereas the B chain bind to both subunits. The subunits appear to have in part common and in part distinct signalling pathways. The cellular responses to PDGF therefore depend both on the PDGF isoforms present as well as the particular cell's repertoire of PDGF receptor subunits. These parameters are variable and the genes for the individual chains and receptor subunits have been shown to be expressed independently by a variety of cell types. The physiological functions of PDGF, however, are not fully understood. Roles in early embryogenesis, development of the central nervous system and in tissue repair in the adult organism have been postulated as well as functions in a number of pathological conditions (e.g. neoplasia, atherosclerosis, fibrosis). Informative germ line mutations of the PDGF genes have not been identified, however, the α-receptor subunit appears to be deleted in the mouse patch mutation. Whether additional genes are affected in the patch mutation is not known. In order to increase our understanding of the (early) physiological functions of PDGF, we are trying to introduce germ line mutations in the PDGF genes by homologous recombination in mouse embryonal stem (ES) cells. So far, we have been able to target the PDGF A chain gene in HPRT-negative ES-cells with a construct lacking the 4th exon and carrying neomycin and HPRT genes as positively and negatively selectable markers, respectively. Chimeras carrying this mutation have been generated and are currently bred.

V 302 CLONING OF HUMAN TYPE VII COLLAGEN AND LINKAGE TO DOMINANT DYSTROPHIC EPIDERMOLYSIS BULLOSA.

Angela M. Christiano, Linda C. Chung-Honet, M. Gabriela Parente, Markku Ryynänen, Robert Knowlton, Mon-Li Chu, and Jouni Uitto. Jefferson Medical College, Philadelphia, PA 19107.

Dystrophic EB is associated with abnormalities in the anchoring fibrils, morphologically recognizable attachment structures, which provide stability to the association of the cutaneous basement membrane and the underlying dermis. Since type VII collagen is the major component of the anchoring fibrils, we tested for genetic linkage of dominant dystrophic EB (DDEB) and the type VII collagen gene (COL7A1).

We have previously reported the characterization of a 1.9 kb human type VII collagen cDNA (K-131) isolated from a keratinocyte cDNA library (PNAS 88:6931, 1991). This cDNA was used to search for RFLPs using 15 different restriction endonucleases and revealed a PvuII polymorphism in COL7A1. We have recently isolated several overlapping cDNA clones from WISH, keratinocyte and fibroblast cDNA libraries which span ~8 kb of the entire type VII collagen mRNA, estimated to be ~9 kb. Inheritance of this RFLP, detected by PvuII restriction enzyme digestion or PCR amplification, was followed in four Finnish DDEB families. Close genetic linkage of COL7A1 and DDEB was demonstrated, with a maximum LOD score of 15.64 at a recombination fraction of zero. COL7A1 was mapped to chromosome 3p by somatic cell hybrid analysis and by chromosomal in situ hybridization. The localization of DDEB locus was confirmed by the linkage of the disease to D3S30 and D3S32, two RFLPs on chromosome 3p21. Complete characterization of the type VII Collagen mRNA and gene structure will provide critical information about the normal biology of type VII collagen, and will facilitate our understanding of the pathogenetic importance of mutations in patients with DDEB. Furthermore, this will facilitate the introduction of type VII collagen normal sequences into transplantable human keratinocytes via retroviral insertion, potentially reversing their defective expression in the skin of patients with DDEB.

V 303 TARGETED DISRUPTION OF THE MAP-1b GENE

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MAP-1b is a member of the high molecular weight MAPs (Microtuble Associated Proteins) and a major component of the neuronal cytoskeleton. It is expressed in axons and dendrites of neurons and in glia. MAP-1b is a polyprotein and can be detected in phosphorylated and unphosphorylated forms. Because the expression and phosphorylation of MAP-1b is prominent in growing axons, a role in neurogenesis has been suggested.
In order to investigate the role of MAP-1b in the neuronal development, a replacment vector was constructed and the MAP-1b gene was disrupted in embryonic stem cells. After positive-negative selection and PCR screening two targeted cell lines were isolated and verified by southern blot analysis. Positve-negative selection using gancyclovir gave an 3-4 fold enrichment and the average targeting frequency was 1 in 1480 G418 resistant colonies. We are currently injecting these cell lines into blastocysts in order to obtain chimeric animals.

V 304 TARGETED INACTIVATION OF BOTH ALLELES OF A HUMAN IFN-INDUCIBLE GENE, Jane E.Itzhaki and Andrew C.G.Porter, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK.

A genetic analysis of autosomal genes in mammalian somatic cells requires the targeted mutagenesis of both alleles. We have created null mutants for the interferon(IFN)-inducible gene 6-16 in human fibrosarcoma cells, by two consecutive targeting events. In the first we used a screen based upon the secretion of human growth hormone(hGH) which became IFN-dependent following homologous recombination of an insertion-type targeting plasmid with the 6-16 gene. In addition to a promoterless hGH gene, the plasmid carried a constitutively expressed *neo* gene which allowed pools of G418-resistant colonies to be screened. Alteration of a 6-16 allele in the selected clones was confirmed by Southern analysis. We have used such clones for a second round of targeting involving a replacement-type construct that carried a silenced *gpt* gene within the first intron of a promoterless fragment of the 6-16 gene. Homologous recombination between this construct and the 6-16 gene was expected to place the *gpt* gene under the control of the 6-16 promoter such that *gpt* expression would become IFN-dependent. Clones were therefore selected in mycophenolic acid (MPA) and IFN and tested for their dependence on IFN for MPA-resistance. PCR and Southern analyses of IFN-dependent clones showed targeting of *gpt* either to the previously altered 6-16 allele, or to the wild-type 6-16 allele. In the latter case, complete functional disruption of both 6-16 alleles was confirmed by RNA analysis. Phenotypic analysis of such null mutants is underway.

V 305 TWO TYPES OF DELETIONS OBSERVED IN RETROVIRUSES CONTAINING HUMAN PNP GENOMIC SEQUENCES. Jon J. Jonsson, Andrea Converse and R. Scott McIvor, Institute of Human Genetics, Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455.

T-cell immunodeficiency associated with purine nucleoside phosphorylase (PNP) deficiency is considered a prototype disease for gene therapy. We demonstrated previously that inclusion of the 2.9 kb first intron was essential for efficient expression of the human PNP gene in murine fibroblasts. More recently we have identified an enhancer in the first intron which in conjunction with the PNP promoter or a heterologous promoter increases CAT expression 3 fold in fibroblasts. We are interested in evaluating the effect of this enhancer (currently localized to a 444 bp sequence) on PNP expression after retroviral-mediated gene transfer. We thus constructed retrovectors containing a methotrexate-resistant DHFR gene transcribed from the MoMLV-LTR and various PNP minigenes downstream in the reverse orientation. A vector containing a 547 bp PNP promoter, PNP cDNA and 1.2 kb of 3' flanking sequences (MoDC4i0R) underwent two deletions in the PNP gene after shuttle packaging into PA317 cells. Sequence analysis of PCR products amplified from the transduced provirus indicated a 398 bp deletion in the PNP coding sequence due to fortuitous splice donor and acceptor signals in the PNP complementary strand. In addition, there was an 1100 bp 3' flanking sequence deletion starting 109 bp downstream of the PNP poly(A) signal, presumably due to misalignment during reverse transcription. PNP vectors containing the full length first intron underwent more extensive deletions. We also constructed a PNP minigene containing the 547 bp promoter, an 855 bp abbreviated first intron and a PNP poly(A) signal with an 117 bp 3' flanking sequence recovered from transduced MoDC4i0R provirus by PCR. This 2980 bp PNP minigene was expressed efficiently after transfection into fibroblasts both alone and when inserted in the reverse orientation downstream of the neo gene in a LN type retroviral vector. However after packaging and transduction of this vector into PA317 cells, only low levels of human PNP expression were observed in heterogeneous cultures. Proviruses in infected PA317 clones contained deletions extending between the 5' UTR and 3' end of the PNP gene (RT misalignment) as well as involving the coding sequence, abbreviated intron 1 and the PNP 5' UTR (fortuitous SD and SA sites). We concluded that human PNP genomic sequences, when transduced in the reverse orientation in retrovirus vectors, frequently undergo various deletions by at least two different mechanisms necessitating an alternative approach to PNP genomic vector design.

V 306 TARGETED DISRUPTION OF GLUT4 IN EMBRYONIC STEM CELLS, E.B. Katz, K. Hatton, R. DePinho, and M. J. Charron, Departments of Biochemistry , and Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, N.Y. 10461.

The regulation of blood glucose is thought to be partially controlled by a family of facilitated glucose transport proteins (GT's). Two of these proteins, GLUT1 and GLUT4, are expressed in tissues where glucose uptake is stimulated by insulin (i.e., adipose cells and muscle). GLUT4 is thought to be the major GT in these tissues where glucose transport greatly increases after the tissue is exposed to insulin. Insulin stimulates glucose transport in these cells primarily by eliciting the translocation of an intracellular pool of GT's to the plasma membrane.

We and others have shown that GLUT4 is down-regulated in insulin-sensitive tissues in diabetes mellitus. In order to test the hypothesis that GLUT4 is essential for normal glucose homeostasis in vivo, we have replaced by homologous recombination the endogenous GLUT4 gene in embryonic stem (ES) cells with a disrupted GLUT4 gene contained in a "knockout" plasmid. The plasmid consisted of a 6kb fragment containing the promoter and most of the GLUT4 gene with the neomycin resistance gene,driven by the PGK promoter, inserted into a unique SacI site in the 10th exon of the gene. The HSV thymidine kinase gene (gancyclovir sensitivity), also driven by the PGK promoter, was put into a SalI site in the plasmid backbone. The "knockout" plasmid was linearized and electroporated into ES cells. Clones containing the targeted integration of GLUT4 were obtained using "positive-negative" selection. Disruption of the endogenous gene was confirmed by Southern analysis. These ES cells will be used to generate mice which contain the targeted mutation so that the biological consequences of the GLUT4 disruption may be studied.

V 307 TARGETING THE MITOCHONDRIAL UNCOUPLING PROTEIN GENE, Charles P. Lerner, Anders Jacobsson*, Leslie Kozak, and Jeffrey D. Saffer, The Jackson Laboratory, Bar Harbor, ME 04609, *The Wenner Gren Institute, University of Stockholm, S-10691 Stockholm, Sweden

The mitochondrial uncoupling protein, UCP, is a brown adipose tissue-specific nuclear gene product that uncouples respiration from ATP synthesis to increase heat production. The UCP gene, located on mouse Chromosome 8, consists of six exons, each of which codes an α-helical transmembrane domain. UCP expression is induced 30-fold in a cold environment and likely plays an important role in non-shivering thermogenesis. The regulation of energy utilization by UCP may also contribute to obesity. Elucidation of the roles played by UCP may be aided by the availability of a mouse mutant lacking this gene product.

V 308 FLP RECOMBINASE EXPRESSION IN MOUSE EMBRYONIC STEM CELLS: DEVELOPMENT OF A DELIVERY SYSTEM FOR GENE TARGETING, Dale L. Ludwig* and James R. Stringer, Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati, Cincinnati, OH 45267-0524. The endogenous 2μm plasmid of *Saccharomyces cerevisiae* encodes a site-specific recombinase, FLP, which recognizes a minimum DNA substrate of no more than 28 base pairs. We sought to utilize FLP as a means to deliver genes site-specifically into the chromosomes of ES (embryonic stem) cells, in an effort to generate a readily manipulable system for the analysis of general homologous recombination. We constructed an FLP gene expression plasmid, utilizing the polyoma TK promoter, for transient expression of the recombinase, and several substrate plasmids for the detection of FLP-mediated site-specific recombination in transfected cells. Cultured ES cells were co-transfected, via lipofection, with the FLP plasmid and two substrate plasmids containing FRTs (FLP recognition targets). FLP-mediated site-specific recombination was detected by colorometric staining of cells for transient expression of β-galactosidase from recombined substrate plasmids. Additionally, several ES cell lines were generated which contain stable integrants of an FRT containing plasmid for targeting of general recombination substrates to unique sites within the chromosomes. The placement of recombination substrates to specific loci would allow for the precise measurement of the rates of intrachromosomal genetic recombination in correcting various genetic mutations at homologous chromosomal locations.

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V 309 THE DIPHTHERIA TOXIN A-CHAIN GENE CAN BE USED IN A POSITIVE-NEGATIVE SELECTION SYSTEM TO TARGET GENES NOT EXPRESSED IN MOUSE EMBRYONIC STEM CELLS, James W. McCarrick*, Jane R Parnes#, Davor Solter@ and Barbara B. Knowles*, *The Wistar Institute, Philadelphia, PA 19104; #Stanford University Medical Center, Stanford, CA; Max-Planck Institute, Freiburg, Germany.

The generation of mice of a desired genotype is now possible through gene targeting in embryonic stem cells. Isolation of successfully targeted cells is greatly facilitated by the positive-negative selection (PNS) system when the gene is transcriptionally silent in ES cells. Targeting vectors which contain toxin genes which are directly toxic to the recipient cell have the advantage in that they eliminate the need for exposure of cells to additional drugs, however, it is conceivable that transient expression of the toxin gene could lead to unacceptable nonspecific toxicity. We are interested in the targeted ablation of the mouse CD4 gene, a gene which is critical to normal T cell development and function and not normally expressed in ES cells. We find that an expression cassette which contains the diphtheria toxin A-chain (DT-A) gene under control of a fairly strong enhancer/promoter, and with independent polyadenylation signals: 1) exhibits only low levels of nonspecific toxicity, and 2) acts as an effective negative selection element in a PNS vector, yielding 10- to 25-fold enrichment for targeted clones (of which about 1 of 10 are appropriately targeted). Such targeted clones can contribute extensively to chimeras. Further applications of this construct are being investigated.

V 310 DIRECT DETECTION OF ENZYMATIC ACTIVITY OF RECOMBINANT HUMAN PROTEIN C IN THE MILK OF TRANSGENIC ANIMALS, Rekha K. Paleyanda, Da-Wei Zhang, William Velander, William N. Drohan and Henryk Lubon, Plasma Derivatives Laboratory, American Red Cross, Rockville, MD 20855.

The anticoagulant property of human plasma Protein C (hPC) plays an important role in maintaining hemostasis. Activated PC is being investigated as an anticoagulant therapeutic in certain disease states and in hetero- and homozygous deficiencies of hPC. Tissue culture systems have proved to be unable to yield sufficient rhPC to meet clinical needs. We are therefore exploring the production of rhPC using transgenic animals. Using the regulatory elements of the murine Whey Acidic Protein (WAP) gene to target tissue-specific expression, we have expressed functional rhPC in the mammary gland of transgenic pigs and achieved secretion into the milk at levels between 50-200 Ug/ml. The same construct yielded transgenic mice expressing about 3 Ug/ml rhPC in their milk. However, accurate enzymatic assay of rhPC in pig milk which contains more whey protein than ruminant milks has posed some problems. We have developed assays for the direct enzymatic detection of rhPC activity in whey. This involves SDS-PAGE of total whey proteins under nonreducing conditions and the electrophoretic transfer of protein onto nitrocellulose. Direct activation of the membrane-bound rhPC and hPC by Protac, a snake venom-derived hPC activator, followed by proteolytic cleavage of the chromogenic substrate S-2366 was achieved. The strong signals obtained on these blots correspond in molecular weight to the nonreduced forms of rhPC and hPC molecules. No amidolytic activity was detected in the whey of a nontransgenic control pig. This assay allows evaluation of amidolytic activity of rhPC in the milk of transgenic pigs throughout the course of lactation. This method can have significant application in detecting enzymatic activity of various heterogenous forms of rhPC, as well as in evaluating the rhPC product obtained after purification from milk.

V 311 GENERATION OF APOLIPOPROTEIN-E-DEFICIENT MICE BY GENE TARGETING IN EMBRYONIC STEM (ES) CELLS, *Jorge A. Piedrahita, Sunny H. Zhang, John R. Hagaman, and Nobuyo Maeda, Department of Pathology, University of North Carolina, Chapel Hill, N.C. 27599-7525.

Apolipoprotein E (apo-E) is a constituent of very low density lipoproteins synthesized by the liver, of a subclass of high density lipoproteins involved in cholesterol transport among cells and is the major protein constituent of chylomicrons remnants. In addition to being involved in the uptake of chylomicron remnants by the liver, apo-E mediates high affinity binding to the low density lipoprotein (LDL) receptor and is thus responsible for the cellular uptake of apo-E-containing lipoprotein particles. In humans, a mutant form of apo-E that is defective in binding to the LDL receptor is associated with familial type III hyperlipoproteinemia, a disease characterized by elevated levels of plasma cholesterol and premature coronary heart disease. To better understand the relationship between apo-E and atherogenesis, as well as to further elucidate the functions of the apo-E, we have inactivated the mouse apo-E locus by homologous recombination and transferred the desired modification through the germ line.

Two different targeting constructs were used. Construct pJPB69 contained an incomplete apo-E gene disrupted by a copy of the neomycin gene (neo). Construct pNMC109 contained, in addition to the apo-E gene disrupted by a copy of neo, a copy of the thymidine kinase gene at the 3' end. ES colonies targeted after electroporation with construct pJPB69, were identified by the polymerase chain reaction (PCR) followed by genomic Southerns. Of 648 G418-resistant colonies analyzed, 9 were positive by PCR of which 5 were confirmed targeted by Southern analysis. When construct pNMC109 was used, of 177 G418- and ganciclovir-resistant colonies analyzed 39 contained a disrupted apo-E gene. Results from four electroporation with plasmid pNMC109 indicated that colonies composed of slow growing ES cells were preferentially targeted when compared to colonies composed of fast growing ES cells. Blastocyst injection experiments utilizing a targeted ES cell line generated chimeras transmitting the disrupted apo-E trough the germ line. Mice heterozygous for the disrupted apo-E grow and reproduce normally. We are now in the process of analyzing the lipoprotein profiles of mice produced from brother-sister matings of animals heterozygous for the apo-E deficiency.

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V 312 TARGETED MUTATIONS OF THE MOUSE POMC GENE BY HOMOLOGOUS RECOMBINATION IN EMBRYONIC STEM CELLS

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Proopiomelanocortin (POMC) generates a diverse family of peptides that differ widely in their biological properties and may function as hormones and mitogens (ACTH and α-MSH) or neurotransmitters (β-endorphin). Because the physiological role of the individual peptides in development and the unique contribution of β-endorphin to the total opioid activity of the brain have been difficult to determine by available techniques, we decided to disrupt the entire POMC gene or selectively mutate β-endorphin coding sequences by homologous recombination in embryonic stem (ES) cells. The construction POMCXX3 contains a 9.8 kb fragment of murine POMC genomic DNA subcloned in Bluescript SK(+/-) (Stratagene, CA). 0.5 kb of coding sequences were deleted at the 3' end of the third exon avoiding the production of any POMC active peptides and replaced by a neomycin resistance gene (*neo*). To screen for homologous recombination events using the positive and negative selection method, a herpes simplex virus thymidine kinase gene (*tk*) was inserted at the 5' end of the mouse POMC gene fragment. Both *neo* and *tk* cassettes are under the transcriptional control of the phosphoglycerate kinase-1 promoter which is highly active in ES cells. The construction POMCX*4 is similar to the one described above but instead of having the 0.5 kb deletion, a single base was inserted in the codon for the N-terminal tyrosine of β-endorphin converting it to a termination codon (TAC to TAA). AB1 ES cells are grown on γ irradiated SNL76/7 mouse fibroblasts on gelatinized plates. Typical electroporations are being performed with 2 x 10^7 ES cells in 0.8 ml of Hepes buffered saline with 25 μg of linearized plasmid at 330 μF and 280 V in a BRL cell porator. ES cell survival is 50 to 60%. After 48 h cells receive 300 μg/ml G418 and 2 μM gancyclovir for 10 to 12 additional days. DNA from pools of 10 double resistant colonies is extracted and analyzed by PCR to identify targeted genomic integrations. One of the pools of ES colonies electroporated with POMCXX3 showed a band of the expected size that hybridized to an internal 1 kb fragment present at the 3' end of the region of homology. We are in the process of obtaining additional recombinant ES colonies to generate chimeric mice by blastocyst injections.

V 313 GENE TARGETING At A MAMMALIAN PLASMID,

Keiko Sakagami*, Hiroshi Yoshikura+, Ichizo Kobayashi*. *: Dept. Molecular Biology, Institute of Medical Science, Univ. Tokyo, Shiroganedai, Tokyo 108. +: Dept. Bacteriology, Medical School, Univ. Tokyo, Tokyo 113, Japan.

The mechanism of homologous recombination in gene targeting has been difficult to analyze because it involves the complex chromosomes. We have developed a model system employing a mammalian plasmid, BPV-1, as a target. BPV-1 (Bovine papillomavirus type 1) replicates as a multi-copy, double-stranded, circular plasmid, taking a chromatin-like structure, in nuclei of cultured mouse cells. A recipient cell line harboring a plasmid consisting of BPV, an *E. coli* plasmid and one deletion allele of *neo* gene was first established by morphological transformation. Transfer of donor DNA carrying another *neo* deletion allele to this resulted in many Neo+ (G418R) colonies. Extrachromosomal DNA from these cells were recovered in *E. coli* for analysis. We found *neo+* plasmid molecules expected from precise correction by homologous recombination. We also found *neo+* plasmids which carry a non-homologous joint as well as a homologous joint. We are systematically varying the structure of both the recipient DNA and the donor DNA to elucidate factors affecting efficiency and outcome of gene targeting.

V 314 Pathophysiological effects of mice rendered IL-2 deficient by gene targeting

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Interleukin-2 deficient mice were generated by targeted recombinbation in murine embryonic stem cells. In mitogen stimulated T-cells of the mutant animals neither IL-2 m-RNA nor IL-2 Protein activity could be detected. Analysis at 4 days and 4 weeks of age showed a normal distribution of $CD4^+8^+$, $CD\ 4^-8^+$ and $CD\ 4^+8^-$ cells in the thymus. The distribution of γδ T-cells was also unaffected. However, proliferation of mitogen stimulated thymus, spleen and lymph node cells were reduced. There was no alloreactivity of $CD8^+$ T-cells of the IL2 -/- mice in an Chromium release assay. Also the $CD4^+$ cells of these mice are not able to overcome the negative signal for IgM secretion of B-cells after massive B-cell receptor crosslinkage. All these effects could be restored by addition of IL-2.

The effects of the IL-2 deficiceny on B-cells were much more pronounced and manifested in dramatic changes in Ig-Isotype distribution.

Currently we are investigating the pathological effects in older IL-2-/- mice. A progress report will be presented.

V 315 DELETION AND REPLACEMENT OF THE IGH INTRON ENHANCER BY HOMOLOGOUS RECOMBINATION VIA THE HIT & RUN PROCEDURE,

Matthias Serwe, Betina Marquardt and Fred Sablitzky, Max-Delbrück-Laboratorium in der Max-Planck-Gesellschaft, Carl-von-Linné-Weg 10, 5000 Köln 30, FRG

Enhancers have been defined as cis-regulatory elements which control the transcription of nearby genes. In the case of the intron enhancer (Eμ) of the Ig heavy chain (H) locus many sequence motifs have been defined which are bound by regulatory proteins, some of which are identified as transcription factors. However, it appears that the intron enhancer could fulfil additional functions different from the transcriptional control. For example, experiments by P. Ferrier et al. (EMBO J. **9**, 117 [1990]) suggested that Eμ act as a cis-regulatory element in the control of variable gene segment (V[D]J) recombination. To elucidate the functions of the IgH intron enhancer during B cell development *in vivo* we want to establish mice which either lack the intron enhancer or carry heterologous enhancer within the IgH locus. Using the "Hit & Run" procedure developed by Hasty et al. (Nature **350**, 243 [1991]) our laboratory has established targeted ES cell lines and is currently generating chimeric animals.

V 316 CLONING AND CHROMOSOMAL ASSIGNMENT OF CUTANEOUS BASEMENT MEMBRANE ZONE GENES EXPRESSED IN HUMAN EPIDERMAL KERATINOCYTES: CANDIDATE GENES FOR EPIDERMOLYSIS BULLOSA. Jouni Uitto (1), Angela M. Christiano (1), Kehua Li (1), George Giudice (2), Mon-Li Chu (1) and Robert Knowlton (1). (1) Thomas Jefferson University, Philadelphia, PA; (2) University of Wisconsin, Milwaukee, WI.

The cutaneous basement membrane zone (BMZ) consists of a large number of collagenous and non-collagneous macromolecules which are necessary for stable association of the epidermis and dermis. Some of them serve as candidate genes in epidermolysis bullosa, a group of heritable diseases manifestated as fragility and easy blistering of the skin and mucous membranes. We have isolated complementary DNAs corresponding to human type VII collagen (COL7A1), 230-kD bullous pemphigoid antigen (BPAG1) and 180-kD bullous pemphigoid antigen (BPAG2) sequences from keratinocyte λgtll expression libraries by immunoscreening and probe hybridizations. The identity of the clones was confirmed by comparison with protein sequences and by selection of antibodies which bind to epitopes expressed in fusion proteins. The corresponding genes were mapped by chromosomal in situ hybridizations and Southern analyses of DNA from human x rodent hybrid cells. COL7A1 was mapped to 3p21, and the BPAG1 and BPAG2 loci were on 6p11-12 and 10q24-25, respectively. This information indicates that the BMZ genes are widely dispersed within the human genome. Further characterization of these genes will allow their insertion into transplantable human keratinocytes, potentially correcting their deficient expression in the skin of patients with EB. Furthermore, isolation of mouse homologs of these genes will allow inactivation of the endogenous genes through targeting by homologous recombination, potentially leading to development of animal models for different forms of EB.

V 317 ANTIBODY EXPRESSION FROM TARGETED AND NON-TARGETED IMMUNOGLOBULIN GENES IN TRANSGENIC MICE, Reitha Weeks, Vicki Mizuno, Harald Haugen, Chris Clegg and Kim Folger, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA 98121

Antibody-transgene expression in mice generated from embryonic stem cells and in traditional transgenic mice is being compared. The transgene's effect on allelic exclusion and antibody repertoire, and its affinity maturation after immunization will be addressed. The specific antibody being studied is L6, an antibody to a human tumor associated antigen. The functionally rearranged heavy chain VDJ region for L6 was joined to germline IgM sequences in a vector containing Neo and HSV-Tk.

The contribution of heavy and light chains to the L6 antibody affinity has been analyzed by expressing the L6 heavy chain construct in cell lines with various light chains.

Traditional transgenic mouse lines, with different copy numbers of the L6 IgM construct, have been established. Serum levels of L6-like antibodies have been measured before, during, and after immunization protocols with L6 antigen. Hybridomas from various transgenic mice are being characterized for L6 affinity and somatic mutation.

The L6 heavy chain construct was introduced into the mouse heavy chain locus in embryonic stem cells using homologous recombination. The targeted event was detected by Southern band analysis at a frequency of ~1/35. Chimeric mice derived from the recombined stem cells are being examined. Heterozygous transgenic mouse lines will be examined for antibody-transgene expression in serum and in hybridomas and compared to results from traditional transgenic mice.

Positional Cloning and Major Diseases

V 400 EXPERIMENTAL MANIPULATION OF HUMAN HEPATOCYTES FOR HEPATIC GENE THERAPY: TRANSDUCTION WITH AMPHOTROPIC AND XENOTROPIC VECTORS AND HEPATOCELLULAR TRANSPLANTATION IN SCID MICE. R. Mark Adams, Humberto Soriano, Fred D. Ledley, Departments of Cell Biology and Pediatrics, Baylor College of Medicine, Houston, TX 77030.

Studies in experimental animals have suggested the feasibility of performing hepatic gene therapy by harvesting hepatocytes, transducing primary cells ex situ with retroviral vectors, and transplanting these cells into the liver via the spleen or portal vasculature. We describe studies aimed at assessing the suitability of these methods for human applications. Human hepatocytes were harvested from organs preserved in Belzer (UW) solution and were cultivated in a serum-free, tyrosine-free, hormone-defined media. These cells exhibited characteristic hepatocyte morphology and expressed liver-specific functions including phenylalanine hydroxylase, alpha$_1$-antitrypsin, and glutamine synthase. Transduction with the LNL6 vector resulted in stable incorporation of the provirus into 10^{-2} of the cells, a frequency >10 fold less than identical transductions in primary murine hepatocytes. Consistently higher transduction efficiencies was observed in human hepatocytes (>10^{-1}) using a xenotropic N2 vector. To demonstrate the feasibility of transplanting human hepatocytes, $2x10^6$ cells were labeled with a florescent dye (DiI) and transplanted into the spleen of immunodeficient (SCID) mice or immunocompetent C57Bl/6 mice. Fifteen days later, human hepatocytes were identified engrafted into the hepatic parenchyma, exhibiting a normal histological appearance, and in the spleen. No hepatocellular engraftment was identified in the immunocompetent mice. These studies demonstrate the feasibility of transducing primary human hepatocytes for hepatic gene therapy and obtaining engraftment of these cells in the liver after hepatocellular transplantation. These data, together with experiments in baboon which demonstrate that 5% of the liver can be constituted from a hepatocellular graft, suggest that the efficiency of transduction may not yet be adequate for gene therapy. Studies with xenotropic vectors suggest that alternative envelope determinants in the recombinant vector may enhance transduction efficiency.

V 401 TWO MURINE MODELS FOR THERAPEUTIC GENE TRANSFER: AN MHC CLASS I GENE AND THE MULTI DRUG RESISTANCE (MDR) GENE. David M. Bodine Ph.D., B.P. Sorrentino M.D., M. Sykes M.D*., D.A. Sachs M.D.*, and A.W. Nienhuis M.D., NIH and *Massachusetts General Hospital

We have constructed a retrovirus vector containing the murine major histocompatibility complex class gene (H-2 K^b). Bone marrow cells from B10.AKM mice (homozygous for the H-2 K^k allele) were infected with the H-2 K^b virus or a control N2 virus. Six weeks post bone marrow transplantation, both the N2 control and H-2 K^b animals received skin grafts from B10.MBR (homozygous H-2 K^b) mice and or B10.BR (homozygous for a different mismatched class I gene). In the N2 control group the both grafts both were rejected in an average of 30 days. In the animals reconstituted with cells containing the H-2 K^b virus, the negative control B10.BR skin grafts were rejected in 30 days, while the B10.MBR grafts survived an average of 110 days. These results indicate that transduction of a specific MHC class I gene prolongs specific allogeneic graft survival, which may improve organ transplantation from mismatched donors.

In a second series of experiments, a retrovirus vector containing the human Multi Drug Resistance (MDR) gene was introduced into mouse bone marrow stem cells. Treatment of the MDR animals and a group N2 control animals with the cytotoxic drug Taxol lead to a 40% reduction in the white blood cell count (WBC) in both groups. Following hematopoietic recovery at day 13, it was shown that the average copy number of the MDR provirus in peripheral blood cells had increased from 19.7% to nearly 62.5%, while the copy number of the N2 virus remained unchanged. Upon re-treatment with Taxol, the WBC of the MDR animals was unchanged, while the WBC of the N2 group decreased. These results demonstrate in vivo selection of the transduced MDR gene and suggest that introduction of the MDR gene to the bone marrow of patients undergoing chemotherapy for the treatment of non hematopoietic malignancies may be advantageous.

V 402 CORRECTION OF DYSTROPHIC MUSCLE BY RECOMBINANT DYSTROPHIN EXPRESSION IN MDX TRANSGENIC MOUSE. C. Thomas Caskey[1,2], Cheng Chi Lee[1], Françoise Pons[3], Pamela G. Jones[1,2], Roger D. Bies[1] and Jean J. Leger[3]. [1]Institute for Molecular Genetics and [2]Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX 77030, [3]INSERM, U300, Faculté de Pharmacie, Avenue Charles Flahaut, 34100 Montpellier, France.

Duchenne muscular dystrophy (DMD) is a common and lethal X-linked progressive myopathy. The gene locus for DMD and its allelic form, Becker muscular dystrophy, encodes for a large cytoskeletal membrane protein, dystrophin. The mouse model for DMD is the *mdx* mouse, which has a stop codon mutation in its mRNA transcript and is dystrophin deficient. *Mdx* mice have similar biochemical and histological defects compared with patients with DMD, although they do not display severe progressive myopathy. We have recently described the cloning of a full-length cDNA for dystrophin and its expression at COS cell membranes. To better study the biological effect of rescuing dystrophic muscle with recombinant dystrophin, we have developed a model system in the transgenic mouse. A vector based on the muscle creatine kinase (MCK) promoter and two enhancers was developed to drive the dystrophin cDNA in *mdx* transgenic mice. The correction of the *mdx* mouse was achieved by expression of a single copy recombinant dystrophin transgene. Cardiac and skeletal muscle expression of recombinant dystrophin was achieved using this MCK promoter. Immunostaining with anti-dystrophin antibodies shows that the recombinant dystrophin is localized to muscle fiber membrane consistent with the known localization of native dystrophin but there is variability in the level of recombinant dystrophin expression. Histopathological benefit based on the lower of fiber necrosis, centralized nuclei and general uniformity of the transgene muscle fibers is also observed. Our studies indicate that muscle fibers expressing recombinant dystrophin will progressively predominate over the degenerative dystrophin-deficient fibers, suggesting a therapeutic value for dystrophin gene replacement for Duchenne muscular dystrophy patients. This transgenic mouse would provide the basis for the development of strategies and the assessment of therapeutic effects for Duchenne muscular dystrophy gene therapy.

V 403 TRANSDUCTION OF INTRACELLULAR RESISTANCE TO HIV BY AN ADENO-ASSOCIATED VIRUS-BASED ANTISENSE VECTOR Saswati Chatterjee and K.K. Wong Jr., Divisions of Pediatrics and Hematology, City of Hope National Medical Center, Duarte, CA 91010

We explored the possibility of conferring perpetual resistance to human immunodeficiency virus (HIV) replication by specific genetic alteration of individual cells. A recombinant adeno-associated virus (AAV) based vector was designed to encode both G418-resistance and the constitutive production of a dominant negative antisense RNA complementary to the HIV TAR sequence as well as the polyadenylation signal, regions common to all HIV transcripts. Additionally, the interaction of TAR with Tat is critical for efficient viral transcription and replication. 293-based G418-resistant clonal cell lines that constitutively expressed the antisense RNA, specifically and significantly inhibited (>90% in several clones) HIV LTR-directed gene expression, synthesis of all species of HIV transcripts and viral replication following transfection of an infectious proviral clone. Replication of the related pathogenic lentivirus simian immunodeficiency virus (SIV) was also inhibited in these clonal cell lines, albeit to a lesser extent. Significantly, encapsidated stocks of the antisense vector transduced stable intracellular resistance to HIV-1 infection in $CD4^+$ human lymphocyte lines without evidence of toxicity. Following virus challenge, the accumulation of HIV-specific mRNA, virus replication as well as the production of infectious HIV was significantly reduced (>90%) in lymphocyte lines transduced with the HIV antisense vector as compared to controls. Because of the properties of site-specific integration, lack of recombination with the target retrovirus, HIV, lack of pathogenicity and high transduction frequencies of AAV vectors, we conclude that the system described here for the permanent induction intracellular resistance to HIV may be the basis of a promising form of antiretroviral gene therapy.

V 404 Expression of Cytokine Genes Using Retroviral Mediated Gene Transfer. Yawen L. Chiang[1], Marlene Hammer[1], Pearl Chen[1], Michael Christy[1], Daniel Kuebbing[1], Paul Tolstoshev[1], and W. French Anderson[2]. 1 - Genetic Therapy, Inc., Gaithersburg, MD. 2 - Molecular Hematology Branch, National Institues of Health, Bethesda, MD.

To evaluate the utility of augmenting adoptive immunotherapy approaches to cancer therapy by the use of somatic cell gene transfer, retroviral vectors capable of transferring and expressing the gene for human Tumor Necrosis Factor (TNF), human Interleukin-2 (IL-2) and human Interferon α_2 (IFNα2) were constructed. These vectors are designed to be used in studies to augment immunotherapy procedures such as TIL (Tumor Infiltrating Lymphocytes) cell therapy for malignant melanoma by using lymphokine modified TIL cells to locally deliver these agents. Clinically certifiable retroviral vector supernatants were generated, and used to show that human TIL cells can be transduced with cytokine-containing retroviral vector, and produce biologically active product. We have demonstrated a G418 selection method for transduced TIL cells for higher cytokine expression. Additionally, vectors containing the genes for human IL-1β, IL-4 and others have been constucted and expression of these genes will be evaluated. The cytokine vectors generated for this study are also being used to introduce genes into tumor cells in animal models, to explore this potential therapeutic approach in stimulating immune responses.

V 405 LYMPHOCYTE GENE THERAPY FOR ADENOSINE DEAMINASE DEFICIENCY. Kenneth W. Culver, A. Dusty Miller, W. French Anderson and R. Michael Blaese. National Institutes of Health, Bethesda, Md 20892 and the Fred Hutchinson Cancer Center, Seattle, Wa 98104

Deficiency of adenosine deaminase (ADA) results in severe combined immunodeficiency. T-lymphocytes in these patients fail to mature and function due to high intracellular concentrations of 2'-deoxyadenosine and deoxyATP. Matched bone marrow transplantation (BMT) is the treatment of choice. Unfortunately, not all children with ADA deficiency have a matched marrow donor and may not be a candidate for the ablative therapy that may be required for a mismatched marrow transplant. As a result, children have been treated with PEG-ADA (polyethylene glycol-conjugated to bovine ADA) as enzyme replacement therapy. The weekly injection of PEG-ADA has resulted in variable, partial immunologic and clinical improvement. The fact that engraftment of donor T-lymphocytes following BMT can correct the immunodeficiency in humans suggests that the transplantation of a large number of autologous ADA gene-corrected T-lymphocytes may further enhance or replace the effect of PEG-ADA treatment. The LASN retroviral vector [LTR--hADA--SV40--NeoR--LTR] was utilized for these studies. LASN has reliably produced hADA and has remained free of replication competent retrovirus. In 1990, we began infusions of OKT3 monoclonal antibody stimulated, rIL-2 grown (1000U/ml) LASN gene-corrected T-cells in a 4 year old girl. She received 8 infusions in the first year (9 x10^10 total cells) which has resulted in a substantial increase in her number of circulating T-cells (571/µl pre-gene therapy vs. a mean of 2108µl with gene therapy infusions every 6-8 weeks). The ADA activity in recovered peripheral blood T-cells has increased >10 fold. The increase in T-cells numbers and ADA activity has been associated with the development of positive delayed type hypersensitivity reactions to Candida, Diphtheria and Tetanus, regrowth of tonsils and a decreased number of infectious illnesses. These findings suggest that therapy with OKT3 monoclonal antibody stimulated and rIL-2 grown, LASN gene-corrected T-cells provides recontitution of immune functions not attained with PEG-ADA alone.

V 406 RETROVIRAL-MEDIATED TRANSFER OF A DYSTROPHIN MINIGENE INTO *MDX* MOUSE MUSCLE *IN VITRO* AND *IN VIVO*. M.G. Dunckley, D.J. Wells*, K.E. Davies#, F.S. Walsh and G. Dickson. Department of Experimental Pathology, UMDS, Guy's Hospital, London SE1 9RT, U.K. - *Unit of Molecular and Cellular Biology, The Royal Veterinary College, London NW1 0TU, U.K. - #Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, OX3 9DU, U.K.

Duchenne muscular dystrophy (DMD) is a severe recessive, X-linked myopathy caused by a lack of functional dystrophin, a 427 Kd cytoskeletal protein normally most abundant in skeletal muscle. In-frame deletions within the dystrophin gene result in expression of truncated semi-functional proteins and the consequently milder phenotype of Becker muscular dystrophy (BMD). Retroviral vectors have been widely used for stable and efficient gene transfer into eukaryotic cells *in vitro* and *in vivo*, but the 10-12 Kb packaging limit of retroviruses precludes incorporation of the 14 Kb normal human dystrophin cDNA. Nevertheless, we have demonstrated expression of a 6.3 Kb BMD human dystrophin cDNA at the sarcolemma of differentiated myotubes following retroviral-mediated transduction of cultured myoblasts from the dystrophin-deficient *mdx* mouse. In addition, the recombinant retrovirus was injected into quadriceps muscles of *mdx* mice *in vivo*. One to three weeks later, immunostaining of cryostat sections of these muscles with anti-dystrophin antisera revealed numerous positively-labelled myofibres around the injection site relative to contralateral muscles injected with control preparations. Further functional and biochemical analyses of recombinant dystrophin expression after retroviral-mediated transfer of dystrophin minigenes *in vitro* and *in vivo* may lead to an evaluation of this technique as a potential human gene therapy for DMD.

V 407 EVALUATION OF IN SITU RETROVIRAL-MEDIATED GENE TRANSFER FOR THE TREATMENT OF LIVER GENETIC DISEASES. Nicolas Ferry°+, Sophie Branchereau*+, Didier Houssin*, Olivier Danos+ and Jean-Michel Heard+. (+) Laboratoire Rétrovirus et Transfert Génétique, Institut Pasteur, 75015 Paris France. (°) INSERM U75, CHU Necker, 75015 Paris France (*) Laboratoire de Recherche Chirurgicale, UER Cochin-Port Royal, 75014 Paris France.

We have shown that stable genetic modification of hepatocytes could be obtained in vivo by perfusing the regenerating liver of rats with a helper-free amphotropic retroviral vector (Ferry et al, Proc. Natl. Acad. Sci. 1991, 88: 8377). These studies were performed with a reporter gene encoding a ß-galactosidase with nuclear localisation whose activity was revealed on histological sections. Animals were analysed 3 to 15 weeks after gene transfer and up to 5% of the hepatocytes in the liver parenchyme displayed ß-galactosidase activity in their nuclei. The stability of transgene expression has now been documented in animals analysed 4, 9 and 12 months after surgery. All animals remained healthy and the proportion of ß-galactosidase positive hepatocytes in their livers was comparable to that observed in short-term animals.

The efficacy of our gene transfer method in restoring a normal phenotype in an animal model of hepatic genetic disease is now being tested. Gunn rats lack bilirubin UDPGT activity and accumulate high levels of unconjugated bilirubin in their serum. We have produced a high titer amphotropic retroviral vector carrying the rat bilirubin UDPGT cDNA. This vector was perfused in the liver of Gunn rats. The presence and activity of the transgene is currently monitored by: a) PCR analysis of the liver DNA; b) measure of the bilirubin UDPGT activity in liver peroxysomes and c) detection of conjugated bilirubin in the bile. Phenotype reversal will be followed up by measuring the serum bilirubin levels over time after gene transfer.

As a next step toward the use of gene transfer in the therapy of human liver genetic disease, we have documented the ability of our vectors to deliver genes into primary cultures of human hepatocytes and the perfusion procedure is being adapted to larger mammals (pigs and dogs).

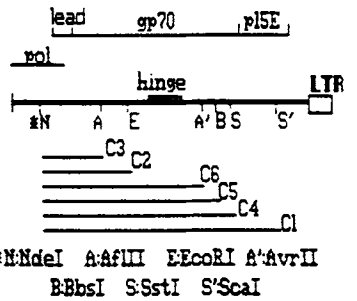
V 408 RETROVIRAL MEDIATED GENE TRANSFER OF HUMAN ORNITHINE TRANSCARBAMYLASE INTO PRIMARY HEPATOCYTES OF *SPF* AND *SPF-ASH* MICE, Markus Grompe*[1], Stephen N. Jones[1,2], Hermela Loulseged[1] and C. Thomas Caskey[1,2], [1]Institute for Molecular Genetics and [2]Howard Hughes Medical Institute, Baylor College of Medicine, One Baylor Plaza, Houston TX 77030

The sparse fur (*spf*) and the sparse fur/abnormal skin and hair (*spf-ash*) mice are 2 murine models of the human X-linked disorder ornithine transcarbamylase (OTC) deficiency. A defective recombinant retrovirus, ΔN2OTC was utilized to infect primary hepatocytes derived from these mutant animals. Infection of the primary cultures was highly efficient, with an average proviral copy number of 0.5–2 per cell in the population of infected hepatocytes. Northern analysis and slot blots of total RNA isolated from infected cells showed levels of human OTC mRNA to be equivalent to that present in normal human liver. Enzymatic assays demonstrated that a partial biochemical correction of the defect was achieved. After retroviral transduction, the hepatocytes were trypsinized and replated for long-term culture. Viability after replating exceeded 90%, indicating that the infected cells might be useful for transplantation. The successful *in vitro* correction of OTC deficiency by this vector suggests that it will also be useful in somatic gene therapy experiments.

V 409 Study of Host Range Determinants of Murine Retroviruses Using Chimeric Viral Envelope. Lei Han[1], Yawen L. Chiang[1] and W.French Anderson[2] 1 - Genetic Therapy, Inc., Gaithersburg, MD, 2 -Molecular Hematology Branch, NIH, Bethesda, MD.

Host-range for different viruses is determined by the glycosylated envelope protein, specifically by the extracellular portion of the viral envelope protein (SU). Amphotropic virus will infect both mink and mouse cells, while xenotropic virus will infect only mink cells. In order to study the host range determinant of amphotropic virus, chimeric envelopes having a xenotropic backbone and different pieces of amphotropic virus envelope protein were constructed (see the diagram below).

Constructs containing C1 and C2 generated live viruses having amphotropic host range specificity. A construct containing C3 failed to produce infectious viral particles. These results imply, as expected, that host range determinants reside in the N-terminal portion of the molecule. The C3 substitution may alter the conformation of the xenotropic envelope protein, preventing production of infectious virions. Other constructs, C4, C5 and C6, are being designed and evaluated. Studies of chimeric envelopes such as these will allow us to understand virus-cell interactions, and will be useful in the development of future gene transfer technology.



V 410 TRANSFER AND EXPRESSION OF THE GENE FOR ADENOSINE DEAMINASE IN HUMAN HEMOPOIETIC PROGENTORS AND PROTOCOL FOR TREATMENT OF HUMAN PATIENTS.

Peter M. Hoogerbrugge[1,2], Victor W. v. Beusechem[1], Jaak M.J.J. Vossen[2], Dinko Valerio[1]. [1]Dept. of Gene Therapy, TNO-Inst. for Applied Radiobiology and Immunology, Rijswijk, [2]Dept. of Pediatrics, Univ. Hosp. Leiden, The Netherlands.

Long-term in vivo expression of a newly introduced human adenosine deaminase (ADA) gene has been observed in mice (v. Beusechem et al., J. Exp. Med. 1990,172,729) and rhesus monkeys (V. Beusechem et al., this meeting) following transplantation of bone marrow co-cultured with the recombinant ADA-retrovirus-producing cell line POC-1. Moreover, the hypersensitivity of ADA-SCID lymphocytes to dAdo could be restored (Braakman et al., Eur. J. Immunol., in press). The recombinant virus produced by POC-1 is encoded LgAL(ΔMo+PyF101). In the present study, normal human bone marrow is co-cultured with the POC-1 cell line for 3 days. Unseparated buffy-coat cells and density-gradient enriched bone marrow cells, prior to as well as after T-cell depletion were seeded onto 70% confluent, irradiated POC-1 cells in the presence of rec. hIL-3. Following co-cultivation, *in-vitro* colony formation in the presence of GM-CSF and hIL-3 was determined. Hemopoietic progenitor cells overexpressing ADA were selected by adding xylofuranosyl-adenine (Xyl-A) and deoxycoformycin (dCF) to the medium. The presence of Xyl-A/dCF resistant colonies in co-cultured marrow revealed successful gene transfer. PCR analysis of individual colonies for the presence of the provirus is in progress.

Based on the data obtained in the rhesus monkey experiments (V. Beusechem et al., this meeting), a clinical protocol aimed at the treatment of patients with ADA-deficient SCID has been submitted to the authorities. In this protocol, the patients hemopoietic stem cells will be genetically corrected by co-cultivation with POC-1 cells and subsequently reinfused. Details of the protocol, which has been approved by the Dutch government, will be presented.

V 411 ADENO-ASSOCIATED VIRUS-MEDIATED TRANSDUCTION OF ORNITHINE TRANSCARBAMYLASE ACTIVITY INTO PRIMARY HEPATOCYTES DERIVED FROM *spf* MICE. Stephen N. Jones, Markus Grompe, Manal Morsy, Richard J. Samulski*, and C. Thomas Caskey. Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX 77030, and *Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260.

Ornithine transcarbamylase (OTC) deficiency is the most common and severe urea cycle defect in man. The often-untreatable clinical manifestations of OTC deficiency make this disorder an excellent candidate for gene therapy. To this end, we have explored the use of adeno-associated virus (AAV) vectors for OTC gene transfer. The use of AAV parvovirus-based vectors for gene therapy offers several potential advantages over the more commonly used retroviral vector systems. AAV in non-pathogenic to humans and animals, has a broad host range and tissue tropism, and exhibits site-specific integration into the human genome.

An AAV vector which contains human OTC cDNA and the neomycin resistance gene has been created and used to generate recombinant AAV particles. The resulting virus is capable of stably transducing OTC cDNA into primary cultures of hepatocytes derived from OTC-deficient mice (sparse fur mutation). Northern analysis and OTC enzyme assays indicate an increase in OTC production and activity in the infected hepatocytes, suggesting that AAV may be a useful vector system for hepatic gene therapy.

V 412 A CANINE MODEL FOR HEPATIC GENE THERAPY. M.A. Kay, S. Rothenberg, P. Baley, T.J. Liu, M. Finegold, G. Darlington, W. Pokorny, and S.L.C. Woo. Howard Hughes Medical Institute, Departments of Molecular Genetics, Cell Biology, Pathology and Surgery. Baylor College of Medicine, Houston, TX 77030.

This laboratory has focused efforts on developing methods for autologous hepatocellular transplantation after genetic reconstitution with retroviral vectors. We have recently shown that at least 1 x 10^9 hepatocytes or 5% of the liver mass of a 5 Kg dog can be transplanted via the portal vasculature. Hepatocytes have been transduced with a retroviral vector containing the human alpha-1-antitrypsin (hAAT) cDNA under the transcriptional control of the intermediate-early CMV promoter-enhancer. About 25% of the hepatocytes are transduced and 50 to 75 ug/2 x 10^6 hepatocytes/48 hours of hAAT is synthesized in vitro. In two animals, the transduced hepatocytes were transplanted autologously. Both transplants lead to significant serum concentrations of hAAT for one month (maximum 4.6 ug/ml). Semi-quantitative PCR analysis demonstrated that the transduced hepatocytes remained viable in the liver parenchyma for at least 4.5 months after transplantation. This strongly suggests that CMV promoter-enhancer is inactivated in hepatocytes in vivo. To resolve this issue, retroviral vectors containing the hAAT cDNA under the transcriptional control of the albumin promoter-enhancer and MoMLV LTR have been contructed and their abilitity for long-term expression of hAAT in transplanted animals is being investigated.

V 413 ISOLATION AND INFECTION OF RAT SKELETAL MUSCLE SATELLITE CELLS FOR RETROVIRAL-MEDIATED GENE TRANSFER. C.R Kirby, A. Sheldon, and F.W. Booth. Department of Physiology and Cell Biology, University of Texas Medical School, P.O. Box 20708, Houston, TX 77225.

Gene transfer into adult skeletal muscle holds promise for both therapeutic and experimental applications. One approach entails insertion of foreign gene(s) into muscle precursor cells (satellite cells or SC) and subsequent SC implantion into adult skeletal muscle. Herein we report preliminary results on the isolation, infection, and implantation of rat SC into adult skeletal muscle. SC from inbred-Lewis rats (350-400 g) were harvested by incubating ground skeletal muscle in 1.25 mg pronase/ml PBS. Multiple PBS washes and centrifugations yielded a crude preparation which was partially purified by centrifugation on a 20%/60% Percoll density gradient. SC (3 x 10^3 cells/gram muscle wet weight) were cultured on Basement Membrane Matrigel coated plates. *In vitro* SC doubling times averaged 18 hours. After 7-10 d in culture, 90% of these cells fused to form myotubes. SC were infected *in vitro* with a foreign gene, the ß-galactosidase reporter gene driven by the 5' LTR promoter, by the replication-incompetent *Moloney murine leukemia virus*. Satellite cells (4 x 10^3 cells/cm^2) were cultured for 72 hrs with 4 µg/ml polybrene and retrovirus-containing media (1x10^3 cfu/ml). *In vitro* staining for ß-galactosidase activity revealed a transfection efficiency of 30%. Cells were removed from culture with 200 µg/ml trypsin, washed with PBS, and then injected into rat tibialis anterior muscles treated previously (24 hrs.) with bupivacaine. Bupivacaine, a local anesthetic, produces an environment suitable for SC proliferation and fusion in adult skeletal muscle. *In vivo* expression of the reporter gene is currently under evaluation by immuno-histochemical techniques.

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V 414 GENE TRANSFER INTO CANINE BONE MARROW CELLS, Clinton D. Lothrop, Jr., Zuhair S. Al-Lebban, Glenn P. Niemeyer, Leslie W. Mishu, J.B. Jones, Joey R. Smith, Martin A. Eglitis, and W. French Anderson. Department of Environmental Practice, College of Veterinary Medicine, University of Tennessee, Knoxville, TN 37901.

A Moloney murine leukemia virus based retroviral vector with the neomycin resistance gene (neo^R) was used to characterize gene transfer into canine hematopoietic cells. The neo^R gene was present in a small percentage of bone marrow cells following retroviral infection of bone marrow and autologous bone marrow transplantation in 2 dogs based on G418-resistant CFU-GM and DNA analysis. The neo^R gene was not detected in either dog after 100 days. The effect of hydrocortisone and 5-flurouracil (5FU) pretreatment on gene transfer into in vitro CFU-GM was investigated in an attempt to improve the efficiency of gene transfer into hematopoietic progenitor cells. The % G418-resistant CFU-GM (mean ± SEM) was significantly ($P<0.05$) increased with bone marrow incubated with 10^{-5}M hydrocortisone prior to (7.1 ± 2.0) or during (6.1 ± 1.1) retroviral infection compared to cells not treated with hydrocortisone (2.0 ± 0.4). The % G418 resistant CFU-GM was also significantly ($P<0.05$) increased 120 hours following treatment with 5FU (7.0 ± 1.2) compared to dogs not treated with 5FU (2.0 ± 0.4). The percent of G418-resistant CFU-GM was improved the most when bone marrow cells from 5FU treated dogs were incubated with 10^{-5} M hydrocortisone prior to (15.9 ± 4.2) or during retroviral infection (18.2 ± 5.5). Bone marrow was obtained from 4 dogs treated with 5FU, infected with the neo^R vector in the presence of hydrocortisone and used for autologous transplantation. Two dogs were reconstituted but the % G418-resistant CFU-GM was not increased compared to dogs not treated with 5FU and hydrocortisone. These results suggest that an exogenous gene can be transiently expressed in canine bone marrow cells after retroviral transduction and bone marrow transplantation. Gene transfer protocols will have to be further optimized to obtain stable expression of an exogenous gene in a majority of bone marrow cells for gene therapy.

V 415 LONG-TERM EXPRESSION OF HUMAN ADENOSINE DEAMINASE IN VASCULAR SMOOTH MUSCLE CELLS OF RATS: A MODEL FOR GENE THERAPY.
Carmel M. Lynch[1], Monika M. Clowes[2], William R.A. Osborne[2], Alexander W. Clowes[2], and A. Dusty Miller[1].
[1]Program in Molecular Medicine, Fred Hutchinson Cancer Research Center, Seattle, WA 98104
[2]Department of Surgery University of Washington, Seattle, WA 98195

Many somatic cell types have been examined as targets for gene therapy, but difficulties in obtaining efficient gene transfer and persistent gene expression in animal models has limited the application of these techniques to humans. We examined vascular smooth muscle cells as a potential target for human gene therapy.

Vascular smooth muscle cells were isolated from an inbred strain of rats and transduced in culture with retroviral vectors expressing E.coli β-galactosidase (β-gal) or human adenosine deaminase (hADA). The transduced cells were introduced into the distal left common carotid artery of rats following removal of the endothelium by intraluminal passage of a balloon catheter. The carotids were examined at various time intervals after transplantation. Incubation with the X-gal chromagen revealed blue-staining of carotids seeded with β-gal transduced cells and of ballooned and sham-seeded control carotids at a lower intensity. Thus, histochemical analysis of β-gal activity was not a definitive assay for gene transfer in this system.

Human ADA was used to unambiguously document gene transfer, as it is readily distinguishable from endogenous rat ADA. Carotids seeded with ADA-transduced cells produced hADA at a level comparable to that of endogenous rat ADA over 6 months of observation post-transplantation. Within a month a transplantation the arteries had reached a stable state, smooth muscle cell proliferation had subsided and the vessels were completely re-endothelialised. No further increase in the size of the neo intima was observed. Long-term persistence of ADA expression in the stable vessel wall indicates that vascular smooth muscle cells are a suitable target tissue for gene therapy.

V 416 TRANSFER OF THE ADA GENE INTO HUMAN ADA-DEFICIENT T-LYMPHOCYTES RECONSTITUTES SPECIFIC IMMUNE FUNCTIONS. F. Mavilio, G. Ferrari, S. Rossini, and C. Bordignon. *Istituto Scientifico San Raffaele, Milano, Italy.*
Deficiency of the enzyme adenosine deaminase (ADA) results in severe combined immunodeficiency (SCID), the first genetic disorder considered for human somatic cell gene therapy. We have developed a pre-clinical in vivo model for correction of ADA-deficient SCID by retroviral vector-mediated gene transfer. Peripheral blood lymphocytes obtained from a patient affected by ADA deficiency were infected with a retroviral vector containing two copies of a human ADA minigene (DCA), and injected into BNX immunodeficient mice. Long term survival of human cells was demonstrated in recipient animals. 6 to 10 weeks after injection human T cells were cloned from spleens of treated animals by stimulation with PHA and IL-2, and analyzed for T-cell markers, expression of ADA activity, integration of retroviral sequences, and TCR rearrangement. All clonable T-lymphocytes obtained from the spleen of recipient animals showed high levels of vector-derived ADA enzyme and showed predominantly a CD4+phenotype. Analysis of retroviral integrations and TCR-β gene rearrangement demonstrated the presence of a variety of different clones in the mouse spleens. Combined analysis of vector integration and TCR rearrangement provided evidence that a circulating progenitor T-cell preceding TCR rearrangement was infected by the retroviral vector. Human T-cells were also cloned from the spleen of immunodeficient mice boosted with tetanus toxoid (tt), by in vitro stimulation with tt in the presence of autologous PBLs as antigen-presenting cells, and showed tt-specific proliferative response. Our results demonstrate that restoration of enzyme activity in human ADA-deficient peripheral blood T-cells by retroviral-mediated ADA gene transfer allows survival *in vivo* and reconstitution of specific immune functions, and that gene transfer can be successfully achieved in immature, progenitor lymphoid cells.This implies that gene transfer in PBL could be successful not only in maintaining a preexistent immune repertoire, but also in protecting progenitor cells capable of developing further immune specificity. These are fundamental pre-requisites for utilization of genetically engineered PBLs for somatic cell gene therapy of ADA deficiency.

V 417 COMPARISON OF ARG22 AND TRP31 MURINE DIHYDROFOLATE REDUCTASES IN RENDERING ERYTHROID COLONY FORMATION METHOTREXATE-RESISTANT AFTER RETROVIRAL MEDIATED GENE TRANSFER, R. Scott McIvor, Kirstin M. Thompson, and Laura R. Bean. Institute of Human Genetics, Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455.
Variant, methotrexate-resistant dihydrofolate reductases (DHFR) have proven to be useful as dominant selectable markers and may also be useful for induction of drug-resistance *in vivo*. DHFR variants containing a leucine to arginine substitution at codon 22 (arg22) or a phenylalanine to tryptophan substitution at position 31 (trp31), while both conferring methotrexate-resistance on mammalian cells, differ with respect to methotrexate sensitivity and catalytic activity of the enzyme. To compare the relative effects of expressing these two DHFR's in hematopoietic cells, we constructed recombinant retroviruses transducing either the arg22 or trp31 variants under transcriptional regulation of either the Moloney leukemia virus LTR or the spleen focus-forming virus (SFFV, anemia strain) LTR. Murine bone marrow cells were exposed to recombinant DHFR virus in a 2-day co-cultivation and then subjected to a 6-day *ex vivo* preselection protocol to enrich for transduced cells expressing the integrated DHFR provirus. Hematopoietic colony-formation *in vitro* was then assessed in the presence of 50 nM Mtx, a concentration which completely inhibited background (non-transduced) colony-formation. The appearance of drug-resistant colonies, primarily erythroid in character, was dependant on preselection for a 6-day period, and was not observed when preselection was omitted or limited to 3 days. Trp31 DHFR virus containing the SFFV LTR (MoTSF) provided the highest frequency of drug-resistant colonies (5%) compared to the other DHFR viruses, consistent with the increased catalytic activity of this DHFR variant and transcriptional activity of the SFFV LTR in erythroid cells. Transduction of marrow cells with this trp31 DHFR retrovirus might thus be utilized to render hematopoietic cells resistant to methotrexate *in vivo*, alleviating hematopoietic toxicity for the purpose of improved chemotherapy using methotrexate, a possibility we are currently investigating in murine marrow transplant experiments.

V 418 DEVELOPMENT OF DEFECTIVE VIRAL VECTORS FOR GENE TRANSFER INTO HUMAN HEMATOPOIETIC PRECURSORS, Kohnoske Mitani[1], Frank L. Graham[2] and C. Thomas Caskey[1], [1]Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX 77030 and [2]Department of Biology, McMaster University, Ontario L8S 4K1, Canada

Adenosine deaminase (ADA) deficiency is a rare autosomal recessive immunodeficiency. We have developed methods of ADA gene transfer into human hematopoietic precursors which utilize defective viral vectors (retrovirus and adenovirus). Initially, mononuclear bone marrow cells from normal allogeneic donors were infected by co-cultivation with the amphotropic virus producing cell line in the presence of IL-3 and IL-6. Gene transfer efficiency into hematopoietic progenitors was measured by a PCR based method capable of detecting retroviral insertion and ADA expression in individual colonies. The infection efficiency of clonogenic progenitors was 50-90% with 50-100% of provirus-positive colonies expressing transcript. Using myeloid long term culture (LTC) supported on irradiated NIH/3T3 cells. ADA transcripts were detected in 10-60% of total colonies at 1 to 5 weeks post-infection. The expression was 20% at 6 weeks. The transduced ADA activity was analyzed by a microradioassay and ADA activity was 3.1 and 2.0 fold higher than controls at week 5 and 9, respectively. Since clonogenic cells are not maintained in LTC for longer than 4 to 5 weeks, gene transfer into primitive hematopoietic progenitors related to the pluripotent stem cells has been achieved.

Since retroviral infection of non-replicating cells is inefficient, we are exploring the potential of adenovirus vectors for ADA gene delivery. The adenovirus transfer vector pXCX2, which has a deletion of approximately 2.8kb of E1 DNA and a cloning site for insertion of foreign DNA was used. An expression cassette of human ADA, which utilized a SV40 enhancer and a promoter of human polypeptide chain elongation factor 1, was cloned into pXCX2. The cassette has been shown to have a high level of ADA expression in vitro. By in vivo recombination, a defective recombinant adenovirus, whose replication is dependent on the helper cell line, 293 cells, was produced. The utility of the defective ADA-adenovirus for delivery and expression in hematopoietic precursors is being pursued.

V 419 TRANSDUCED GENE EXPRESSION IN MULTIPOTENTIAL HEMATOPOIETIC CELL LINES, Deborah K. Moore, Yong Ki Shin, Stella Lau, N. Ramesh, William Osborne, Department of Pediatrics, University of Washington, Seattle, WA 98195

Adenosine deaminase (ADA) deficiency and purine nucleoside phosphorylase (PNP) deficiency are autosomal recessive diseases associated with severe combined immunodeficiency which is fatal if untreated. Gene transfer of a normally functioning ADA or PNP gene into the patient's somatic cells, in particular self renewing hematopoietic stem cells, is a potential treatment.

This study was designed to investigate gene expression in transduced cells to determine if expression is altered with cell differentiation. The human promyelocytic leukemia cell line HL-60 can be induced to differentiate into cells with macrophage-like characteristics by the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) or into cells with granulocyte-like characteristics with retinoic acid (RA). The human monocytic leukemia cell line THP-1 can also be induced to differentiate into monocytes or macrophage-like cells by TPA or RA.

These cell lines were infected with retroviral vectors containing neomycin phosphotransferase (Neo) as a selectable marker and human ADA or PNP cDNA under the control of the Moloney murine leukemia promoter (LASNL, LPNSNL) or the immediate early promoter of human cytomegalovirus (LNCAL, LNCPNL). Cells were then induced to differentiate.

Infected cells induced with TPA or RA expressed vector encoded ADA or PNP activity ranging from 5 to 100 μmol of enzyme converted per hour per mg of protein, up to 20 times the level of expression before induction, indicating that hematopoietic differentiation did not result in loss of transduced gene expression. Vector expression was highest in HL60 cells infected with LNCAL and induced to differentiate to macrophage-like cells by TPA, which resulted in ADA levels of 100.0 μmol/hr/mg compared to 4.9 μmol/hr/mg in infected uninduced cells. Increased vector expression in terminally differentiated hematopoietic cells may be beneficial to stem cell directed gene therapy.

Northern blots, prepared from total RNA from each infected cell line were hybridized with ADA or PNP and Neo probes. RNA levels were in agreement with enzyme assays.

V 420 EXPRESSION OF ANTI-HIV COMPOUNDS DIRECTED FROM THE HIV-LTR: A POTENTIAL GENE THERAPY FOR HIV INFECTED INDIVIDUALS. J. Mosca, P. Perera, Z. Yu, D. Ritchey, L. d'Arcy, D. Burke, Henry M. Jackson Foundation for the Advancement of Military Medicine, Rockville, MD and Walter Reed Army Institute of Research, Rockville, MD.

Our objective is to construct inducible retroviral vectors which express compounds that inhibit HIV expression and replication in response to HIV infection. The strategy was to DNA transfect hybrid anti HIV genes in the presence and absence of HIV infection. Expression of the hybrid gene product was monitored at both the RNA and when possible the protein level. HIV expression was monitored by RNA analysis and p24/RT production.

Previous work showed that alpha 2 interferon production regulated by the HIV-LTR in permanently transfected cell lines render the cells resistant to HIV infection (PNAS 86:4958 '89). To extend and confine antiviral production to HIV infection, we made a series of constructions where the HIV-LTR regulated production of antisense RNA, ribozymes, tat/gag transdominant and protease inhibitor peptides, in addition to alpha 2 interferons missing the signal sequence to prevent extracellular transport from infected cells. We purposely constructed each hybrid to be regulated by a kB deleted HIV-LTR, imposing HIV-encoded tatIII inducibility but unresponsiveness to heterologous stimulation. In transient assays cotransfected with the HIV-LTR antiHIV hybrid construction and HIV provirus the antisense construction inhibited 53 to 84%, the transdominant 30 to 79%, the ribozymes 21 to 53%, the protease inhibitor 51%, and the various alpha 2 interferon contructions 7 to 60%. Inhibition was measured by a decrease in S1 nuclease resistent RNA transcripts initiated from the HIV-LTR. Quantitation was obtained by phosphoimager analysis using cotransfection of vector sequences with the HIV provirus as a control.

It is our aim to determine the most promising hybrid construction to inhibit HIV expression and replication and to insert that into a retroviral vector. The insertion into retroviral vectors of tatIII inducible hybrid genes expressing anti-HIV compounds could provide future therapeutic and/or prophylactic usefullness towards HIV infection in humans.

V 421 MURINE SKIN FIBROBLASTS AND BONE MARROW CELLS AS TARGETS FOR IN VIVO TRANSFER AND EXPRESSION OF THE HUMAN ß-GLUCURONIDASE cDNA. Philippe Moullier, Valérie Maréchal, Jean-Michel Heard and Olivier Danos. Laboratoire Rétrovirus et Transfert Génétique, Institut Pasteur, Paris 75015, France.

The ß-glucuronidase lysosomal enzyme deficiency is a human inherited disease for which cDNA and animal models are available. Peripheral uptake of injected ß-glucuronidase via the mannose-6-phosphate receptor has been demonstrated and used in enzyme replacement therapies. This property offers great potentials for phenotypic correction through somatic gene therapy.

Gene transfer to skin fibroblasts: We used an ecotropic recombinant retrovirus vector, carrying the human ß-glucuronidase cDNA to infect primary culture of skin fibroblasts from C3H/He mice. Cells actively secreting the human enzyme were implanted into organoids which had been previously installed in the peritoneal cavity of C3H/He recipients (2 to 5.10^6 cells per recipient). The organoids were obtained with extra cellular matrix-coated polytetrafluoroethylene fibers (Gore-Tex). After 35 to 70 days, most of the cells present in the organoid, synthetize and secrete human ß-glucuronidase. Active human enzyme was characterized after immunoprecipitation on liver and spleen extracts from the recipient animals. Our data demonstrate the capacity of organoid structures to provide a viable environment for genetically modified fibroblasts and to supply distant organs with exogenous enzyme.

Gene transfer to hematopoietic stem cells: Bone marrow cells from 5-fluoro-uracil treated C3H/He mice were infected with the ß-glucuronidase retroviral vector and used to reconstitute the hematopoietic system of lethally irradiated recipients. Ninety days after bone marrow transplantation, Southern blot analysis demonstrated the presence of the human cDNA in all hematopoietic lineage.

Data will be presented in both somatic gene transfer systems, with emphasis on the long term survival of genetically modified cells and the systemic uptake of the transgene product.

V 422 ISOLATION AND EXPRESSION OF A MURINE PURINE NUCLEOSIDE PHOSPHORYLASE cDNA, David M. Nelson, Mark D. Foresman, Bridgette J. Ronnei, and R. Scott McIvor. Institute of Human Genetics, Department of Genetics and Cell Biology, Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455.

Purine nucleoside phosphorylase is an enzyme which functions in the salvage pathway to recycle purine bases. Human PNP deficiency, inherited in an autosomal recessive manner, leads to development of a fatal T-cell immunodeficiency, a candidate condition for human gene therapy. To isolate murine purine nucleoside phosphorylase (PNP) cDNA sequences, a murine BALB/c liver cDNA library was screened for recombinants hybridizing to a human PNP cDNA probe. The largest clone recovered was sequenced, revealing an 867 nucleotide open reading frame encoding 289 amino acids with 84% residue identity to human PNP. All human PNP residues recently implicated in crystallographic studies to be of structural and functional importance were identical in the murine sequence with the exception of a Cys at position 142 instead of a Ser. The 5' end of the murine message was found to contain 78 bp of untranslated sequence as determined by rapid amplification of cDNA ends (RACE) technology. The base at position -3 relative the the translational initiation site was observed to contain a "T" in the murine and a "G" in the human sequence. This observation was suprising in that this position has been identified as important for translational fidelity with a "G" leading to high and a "T" leading to low levels of translation. Comparison of the murine and human 3' UTR's revealed regions of up to 25 bp identity in a background less than 60% homologous. The murine PNP cDNA sequence was used to probe northern blots of mouse NIH 3T3 cell RNA, identifying a 1.6 kb murine PNP message. Biological function for this cloned sequence was confirmed by transfection of human 293 cells with a construct expressing the murine PNP coding sequence from the Moloney murine leukemia virus LTR. Isoelectric focusing followed by histochemical staining for PNP revealed the presence of activity which co-focused with murine PNP in the transfected human cells. The availability of the murine PNP cDNA will facilitate cloning of the murine PNP gene for the purpose of creating a murine model of PNP-deficiency by homologous interruption. In addition, successful expression of murine PNP from a retroviral construct suggests studies using murine PNP as a biochemical marker to optimize transduction and PNP expression in normal human hematopoeitic cells.

V 423 EFFICIENT TRANSFER AND EXPRESSION OF THE HUMAN GLUCOCEREBROSIDASE GENE IN MURINE HEMATOPOIETIC STEM CELLS, Toya Ohashi, Sallie Boggs, Paul Robbins, Al Bahnson, Juan Li, and John A. Barranger, Departments of Human Genetics, Molecular Genetics and Biochemistry, and Radiation Oncology, University of Pittsburgh, Pittsburgh, PA, 15261

Expression of the human glucocerebrosidase (GC) gene in cells derived from transduced bone marrow has been problematic. Although transduction of bone marrow previously has been accomplished efficiently, expression of the GC gene in the progeny of the marrow on a long term basis has been disappointing. The cDNA for GC was placed in the retroviral vector, MFG, developed by Richard Mulligan and tested in the mouse model of bone marrow transplantation. This vector (MFG-GC) efficiently transduces stem cells. In a protocol utilizing IL-3, IL-6, and stem cell factor (modified from Bodine, Exp. Hematol. 19:206, 1991), the MFG-GC vector is capable of transducing stem cells with an efficiency approaching 100%. This was judged from the copy number in tissues of animals whose bone marrow had been reconstituted by syngeneic donor marrow infected by co-cultivation with the MFG-GC producer. The recipient animals were studied at between 4 and 5 months post-transplantation. Macrophages cultured from the bone marrow of long-termed reconstituted mice have an average copy number of the human gene of 1/cell. In addition, CFU-S_{12} in secondary bone marrow transplanted mice carry the human gene in 28/28 foci. This provides further evidence of highly efficient stem cell transduction. Expression of the GC gene is robust in CFU-S_{12}, hematopoietic and non-hematopoietic tissues of long term reconstituted mice (>4 mos), and macrophages. All show increases of enzymatic activity several fold above the endogenous mouse background activity. Furthermore, assays for helper virus in viral supernatants and animal blood are negative. The studies to be presented demonstrate the possibility of targeting the human GC gene to bone marrow and the potential of the MFG-GC vector to correct the genetic deficiency of GC in macrophages on a long term basis.

V 424 SKELETAL MUSCLE AND GENE THERAPY: LONG-TERM EXPRESSION OF HUMAN FACTOR IX IN RATS. **Theo D. Palmer, Stephen J. Tapscott and A. Dusty Miller. Program in Molecular Medicine, Fred Hutchinson Cancer Research Center, Seattle, WA, 98104.**

Many somatic tissues have been proposed as suitable targets for gene therapy but several likely candidates show only transient expression of retrovirally transduced genes following transplant. We have tested several primary tissues and have found that myocytes from smooth muscle and skeletal muscle continue to express transduced genes for several months following transplant. The large volume of accessible skeletal muscle make this tissue especially promising for gene therapy. In preliminary experiments, normal human skeletal myoblasts were retrovirally transduced with a human clotting factor IX gene. The transduced cells produced several micrograms of properly modified factor IX per 10^6 cells per day and, when transplanted into immune deficient rats, continued to produce factor IX for more than three months. In addition to using the more common retroviral vectors, several muscle specific vector systems are being developed. Muscle specific enhancer-promoter combinations are being used to augment expression of therapeutic genes in muscle cells as well as for expression of selectable markers in a muscle specific manner. Muscle specific selection vectors will be useful in heterogeneous primary cultures where they provide transfer of a therapeutic gene and enrichment for myoblasts following selection.

V 425 CONTROL ELEMENTS OF THE HYPERPROLIFERATION-ASSOCIATED KERATIN K6 GENE. Angel Ramírez, Miguel Vidal, Ana Bravo[1], José L. Jorcano, Department of Molecular and Cellular Biology, CIEMAT, Madrid and [1]School of Veterinary, University of León, León (Spain).

Keratins are the constituents of the cytoskeleton of intermediate filaments of epithelial cells. They form a complex and conserved multigene family which encodes more than twenty proteins. On the basis of biochemical and functional properties, they can be divided in two subfamilies: type I or acidic and type II or basic. Keratin filaments are heteropolimers containing equimolar amounts of basic and acidic polypeptides. Keratins are expressed in a tissue-specific and developmentally controlled manner so that specific pairs of keratins are characteristic of the different types of epithelial cells.

The expression pattern of keratins is also influenced by other stimuli. Thus, under hyperproliferative conditions of the epidermis, a different set of keratins are synthesized. For instance, the pair formed by keratins K6 and K16 is induced by hyperproliferative stimuli such as wound healing, neoplasia and topical treatments with agents like TPA or retinoids. These two keratins are also found constitutively in other stratified epithelia.

To investigate the control elements of the keratin K6 gene we have used the bovine KIV$^-$ gene (BKIV$^-$, the bovine counterpart of the murine K6 gene) to generate transgenic mice. Constructs containing genomic fragments of BKIV$^-$ fused to the E.coli lac Z gene were introduced in the germ line of mice, and the different transgenic lines were analyzed both for constitutive and hyperproliferation- induced expression. Our results show that a 9 Kb fragment of 5' flanking and promoter sequences directs the tissue-specific expression of the reporter gene in a manner resembling to the mouse K6 gene. A 2.4 Kb fragment is inducible by retinoic acid treatment, although its constitutive expression is much weaker than that of larger 5' flanking fragments.

V 426 SELECTIVE CD3γ DEFICIENCY IN MAN

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The molecular basis of a familial T-cell receptor (TCR)/CD3 expression defect was determined. Previous biochemical evidence suggested that the CD3γ component was missing or abnormal in two affected siblings. We therefore amplified and sequenced or analyzed by dot blotting complementary DNA and/or genomic DNA specific for CD3γ in 16 family members from three generations. Two independent point mutations were identified in the CD3γ genes which had segregated to the two affected individuals: a paternally-inherited A-to-G transition which changed the initiator ATG (methionine) codon to GTG (valine), and a maternally-inherited G-to-C transversion in the last nucleotide of intron 2 which abolished normal splicing and displaced cleavage to a cryptic splice site within exon 3, causing the deletion of 17 nucleotides in the messenger RNA and, as a consequence, a reading frameshift. Both mutations gave rise to abnormal transcripts coding for severely truncated, if any, CD3γ proteins which hindered, but did not completely abolish, the normal association and surface expression of the TCR/CD3 complex. Disparate clinical phenotypes were associated to this human CD3γ deficiency: the proband died with severe combined immunodeficiency features when three years old, whereas his sibling remains healthy at ten years of age. We believe this may be a candidate disease for human gene therapy.

V 427 RETROVIRAL GENE TRANSFER, BONE MARROW TRANSPLANTATION AND ENZYME REPLACEMENT THERAPIES FOR MURINE MPS VII, Mark Sands, Jane Barker, John Wolfe[1] and Edward Birkenmeier, The Jackson Laboratory, 600 Main St., Bar Harbor, ME 04609, [1]University of Pennsylvania, School of Veterinary Medicine, 3800 Spruce St., Philadelphia, PA 19104.

Murine mucopolysaccharidosis type VII (MPS VII) is a lysosomal storage disease caused by the absence of β-glucuronidase. The genetic defect is inherited as an autosomal recessive and maps to the distal end of chromosome 5 near the β-glucuronidase structural gene. Murine MPS VII closely resembles the corresponding human disease and provides a useful model to evaluate therapeutic approaches such as bone marrow transplantation (BMT), enzyme replacement and retroviral gene transfer.

Adult mice with extensive lysosomal storage disease given BMT from normal syngeneic donors had an extended life span. Liver, spleen, cornea and glomerular mesangeal cells showed essentially complete correction. Storage was partially corrected in meningeal and perivascular cells in the brain and in renal tubular epithelial cells. Skeletal deformities can be dramatically lessened if either BMT or enzyme replacement therapy is initiated at birth. Although neuronal storage in the CNS responded poorly to BMT, enzyme replacement results in detectable levels of enzyme in the brain. Detailed pathology of the CNS is in progress.

The response of affected animals to BMT suggests that transfer and expression of functional β-glucuronidase cDNAs into mutant hematopoietic stem cells may be an effective form of therapy for murine MPS VII. Efficient infection of gradient enriched hematopoietic stem cells was achieved with recombinant retroviruses containing the rat β-glucuronidase cDNA driven by the Herpes TK promoter. Long term reconstitution and expression of the retroviral gene was observed in lethally irradiated mutant mice following transplantation. Histochemical staining for active enzyme demonstrated the presence of positive cells in the spleen and bone marrow. Liver and spleen contained 2-5% of normal enzyme levels and showed a marked decrease in lysosomal storage.

V 428 **CROSS-CORRECTION OF MPSVII HUMAN, MOUSE AND DOG FIBROBLASTS BY MPSVII CELLS CONTAINING RETROVIRAL CONSTRUCTS FOR ß-GLUCURONIDASE**

RM Taylor and JH Wolfe, Dept of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, 19104-6051.

Deficiencies of lysosomal enzyme activity can be corrected by co-cultivation of defective and normal cells. Cells take up enzyme via mannose-6-phosphate receptors. Mouse, human and dog fibroblasts deficient in ß-glucuronidase (GUS-B) activity have been successfully corrected with retroviral constructs containing human or rat GUS-B cDNA driven by the TK promotor. In this study we examined the ability of these corrected cells to cross-correct co-cultivated deficient cells. GUS-B deficient human, mouse and dog fibroblasts were plated as targets in the lower wells of Transwell plates with the corrected, enzyme donor cells separated in the upper wells. Three different retroviral constructs were used; NTKBH (human GUS-B cDNA), NTKBGEO (rat GUS-B cDNA) and N2 (control, not containing GUS-B sequence).The GUS-B activity was measured in recipient cells and culture media. α-galactosidase was examined as a reference enzyme. The cells corrected with NTKBGEO produced 3 to 5 fold more GUS-B than those corrected with NTKBH. The N2 construct did not correct the enzyme deficiency. The corrected cells released significant amounts of enzyme into the culture media, proportional to the cell enzyme activity. The enzyme was efficiently taken up by co-cultivated deficient cells, regardless of the species of donor or recipient cells. The GUS-B activity in cross-corrected cells declined over 7 days when the cells were replated separately, compared to the α-galactosidase activity which continued to increase.These replated cells were susceptible to G418 demonstrating they had not been contaminated by retrovirally corrected cells. These studies provide encouraging in-vitro support for the future prospects of retrovirally-mediated gene therapy in inherited lysosomal storage disease. It is clear that corrected MPSVII cells are an effective source of stable GUS-B which is able to correct the enzyme deficiency in other cells, without any requirement for cell-to-cell contact. The higher levels of GUS-B activity produced by the NTKBGEO construct may be useful in increasing enzyme production by deficient cells.

V 429 PHENOTYPIC CORRECTION OF CHRONIC GRANULOMATOUS DISEASE *IN VITRO* BY RETROVIRUS-MEDIATED GENE TRANSFER INTO B-LYMPHOCYTES. Adrian Thrasher and Colin Casimir, Department of Medicine, Rayne Institute, University College London, London WC1E 6JJ. U.K.

Chronic Granulomatous Disease (CGD) is an inherited condition characterised by an extreme susceptibility to serious and recurrent infections. This is due to to the failure of an NADPH-dependent oxidase located in the membranes of phagocytic leukocytes (predominantly neutrophils). Though most commonly an X-linked condition, about one third of cases are due to an autosomal recessive disease, caused by deficiency of a 47kDa cytoplasmic protein (p47-*phox*). B-lymphocytes immortalised by Epstein-Barr virus (EBV) express oxidase function, albeit at a reduced rate compared to neutrophils. These B-cell lines, when produced from p47-(*phox*) deficient CGD patients, both fail to produce superoxide and lack the p47-(*phox*) protein. Using a retroviral vector containing p47-(*phox*) cDNA sequences, we have succesfully restored oxidase function to such a B-cell line, established from a patient with no p47-(*phox*) expression. Investigaton of the cells following gene transfer by Western blot and PCR analysis demonstrated the presence of p47-(*phox*) protein and transgene-specific RNA, respectively.

V 430 FACTORS AFFECTING THE TRANSDUCTION OF PLURIPOTENT HEMOPOIETIC STEM CELLS: LONG TERM EXPRESSION OF A HUMAN ADENOSINE DEAMINASE GENE IN MICE.

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An amphotropic retroviral vector, LgAL(ΔMo+PyF101) containing a human adenosine deaminase (ADA) cDNA has been used to optimize procedures for the lasting genetic modification of the hemopoietic system. The variables tested included: addition of hemopoietic growth factors (IL-1α, IL-3 and SCF) to co-cultivations, adherence of hemopoietic cells and 5-FU pre-treatment. Cells that generate CFU-S in the bone marrow of lethally irradiated recipients (MRA CFU-S) were used to determine the yield of marrow regenerating cells following co-cultivation and the frequency of retroviral gene expression. Transduction efficiencies in the progeny of pluripotent hemopoietic stem cells (PHSC) were measured by quantifying the fraction of transduced cells in the bone marrow, spleen and thymus of long term reconstituted mice (6 months post transplantation).
We observed that approximately 80% of the MRA CFU-S, adhered to the fibroblastoid virus producing cells. Thus for optimal regeneration of a hemopoietic system, adherent cells should be included in the transplant. The presence of IL-1α and IL-3 during co-cultivation reproducibly resulted in the highest frequency of PHSC transduction. Human ADA activity was detected in circulating white blood cells following long term repopulation of the hemopoietic system proving that the LgAL(ΔMo+PyF101) vector was capable of directing sustained expression of hADA. The frequency of hADA expression was quantified in transduced MRA-CFU-S derived spleen colonies. Approximately one out of three transduced MRA-CFU-S derived spleen colonies expressed hADA.
The observed efficiencies of gene transfer and expression should be sufficient for the development of bone marrow gene therapy protocols for ADA—SCID, since healthy T-cells have a selective repopulation advantage in ADA deficient patients.

V 431 LONG-TERM MULTI-LINEAGE EXPRESSION OF HUMAN ADENOSINE DEAMINASE IN THE HEMOPOIETIC SYSTEM OF NON-HUMAN PRIMATES FOLLOWING AUTOLOGOUS TRANSPLANTATION OF GENETICALLY MODIFIED BONE MARROW CELLS, Victor W. van Beusechem, Leonie C.M. Kaptein, Julia A.K. Bart-Baumeister and Dinko Valerio, Dept. of Gene Therapy, TNO-Institute of Applied Radiobiology and Immunology, 2280 HV Rijswijk, The Netherlands.
Congenital deficiency of adenosine deaminase (ADA) is considered as one of the prime candidates for bone marrow gene therapy. As a preclinical test for such a procedure, eight rhesus monkeys were autologously transplanted with bone marrow cells (BMC) that were genetically modified by co-cultivation with the human ADA (hADA)-virus producing cell line POC-1 (Van Beusechem *et al.*, J. Exp. Med. 172(1990):729), in the presence of the recombinant hemopoietic growth factors (HGF) human IL-1α and/or rhesus monkey IL-3 (RhIL-3). Following transplantation, all monkeys were shown to express the functional hADA enzyme in their erythrocytes. The frequency of provirus-containing cells in the peripheral blood was determined using a semi-quantitative PCR analysis. When RhIL-3 was the solely added HGF during co-cultivation all monkeys carried the provirus in their mononuclear cells (PBMC) and granulocytes, with equal frequencies (approximately 0.1%) and for the duration of the experiment (up to more than a year). Moreover, in all PBMC samples tested hADA expression could be detected. At various times post transplantation monkeys were sacrificed for PCR-analysis of their hemopoietic tissues. Genetic modification could be demonstrated in BMC of various densities, in thymus, in spleen and in lymph nodes.
More recently two modifications of the procedure were tested. Firstly, we co-cultured purified $CD34^+CD11b^-$ progenitor cells with POC-1 cells. Three months post autologous transplantation of these BMC, $CD34^+CD11b^-$ cells capable of *in vitro* colony formation could be retrieved from the bone marrow and shown to carry and express the introduced sequences. Long-term analysis of these animals is currently being undertaken. Secondly, two monkeys have received BMC co-cultured with a new virus-producing cell line exhibiting a 2 log higher virus-titer than POC-1. The effects of the increased multiplicity of infection on the efficiency of hADA gene transfer into BMC will be presented.

V 432 RETROVIRAL VECTORS WITH LIVER SPECIFIC CONTROL REGIONS: HEPATOMA SPECIFIC EXPRESSION, Dwayne A. Wolf and John Papaconstantinou, Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX 77550
Albumin and α-fetoprotein (AFP) are major proteins produced in the fetal liver. AFP is also expressed in fetal yolk sac and gut. After birth, the AFP gene is repressed but albumin is expressed at a high level throughout adulthood. The postnatal repression of AFP is promoter-dependent and the AFP enhancers can direct expression from the albumin promoter in fetal or adult liver. In order to confer liver specificity on retroviral vectors, we constructed a series of vectors containing the 308-bp mouse albumin promoter linked to the marker gene *lacZ*. In one design, albumin promoter-*lacZ* was 5′ of an internal SV40 promoter-*neo* expression cassette. This virus, due to expression from the LTR in fibroblasts, and inherent instability, gave equally high levels of expression in NIH-3T3 fibroblasts or BWIC3 cells (a mouse hepatoma line derived from BWTG3). However, replacement of the viral enhancer with a portion of the AFP enhancer, the minimal enhancer region I or MER-I, decreased expression from the LTR in fibroblasts but permitted expression from the albumin promoter specifically in BWIC3 cells. Reversing the orientation of MER-I abolished this activity. A different vector, containing the albumin promoter downstream of a promoterless *neo* gene, in which the latter is driven from a wild type LTR, demonstrated a high degree (10-fold) of specificity of expression of β-gal in BWIC3 relative to NIH-3T3 cells. The stability of this provirus and maintenance of expression from the internal albumin promoter was assessed by growing infected BWIC3 *in vivo* as a tumor in nude mice for two weeks. Expression from the proviral insert was maintained during passage as a tumor. Moreover, the provirus was maintained, and expression of both the *neo* and *lacZ* genes were continued even after re-establishment of the tumor cells in culture.

V 433 OVER EXPRESSION OF β-GLUCURONIDASE FROM A DOUBLE-COPY RETROVIRAL VECTOR IN MPS VII CELLS. J.H. Wolfe, R.M. Taylor, and M.J. Parente. School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce St., Philadelphia PA 19104.

Retroviral vectors were constructed to transfer and express a cDNA encoding the human lysosomal hydrolase β-glucuronidase (GUSB) under control of the human GUSB promoter (minigene). Expression of the transferred minigene was evaluated in a GUSB-negative cell line established from a mouse with the lysosomal storage disease mucopolysaccharidosis (MPS) type VII. Vectors which were constructed from the N2 retroviral vector to transfer single copies of the minigene (N2HβH vectors) corrected the deficiency by expressing normal levels of GUSB acvtivity in the MPS VII cells. The levels of GUSB expression were increased to several times greater than normal levels by inserting the minigene into a double-copy vector (DCHβH vectors), which places one copy of the minigene transcriptional unit upstream of the retroviral transcriptional unit in the integrated provirus in the target cell DNA. The level of GUSB activity in vector-corrected cells was found to be dependent on the phase of cell growth in a manner similar to that seen in normal control cells. The levels of specific enzymatic activity of both GUSB and a control lysosomal enzyme, α-galactosidase A (GLA), were higher in stationary cells from confluent cultures than in sub-confluent dividing cells. When the levels of GUSB and GLA activities were compared, their ratio was similar at all phases of cell growth, with the levels of GUSB expression from the DCHβH vectors being several-fold higher than from the single copy vectors. To determine if this effect was controlled by the GUSB promoter, a vector was constructed using the TK promoter to drive the human GUSB cDNA (NTKβH vectors). The levels of GUSB in cells corrected with these vectors exhibited the same cell-density-dependent pattern as when the GUSB promoter was used indicating that the density-dependent variation in enzymatic activity is not a function of the GUSB promoter. GUSB was also released from the DCHβH-infected cells at higher levels than from the corrected cells expressing normal amounts of GUSB. The ability of the DCHβH vectors to express and export very high levels of GUSB even in dividing cells may be useful for correcting GUSB-deficiency in vivo since the current strategy is to use a limited number of vector-corrected cells as a source of enzyme for the multple tissues affected in this disease.

V 434 RETROVIRAL MEDIATED GENE CORRECTION OF MUCOPOLYSACCHARIDOSIS TYPE I FIBROBLASTS.

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Three retroviral constructs containing a full length human a-L-iduronidase cDNA were made. The first, pLIdSN, is designed so that expression of the IDUA cDNA is from the 5' viral LTR. The second, pLNCId is designed to express the IDUA cDNA from the cytomegalovirus immediate early promoter, while in the third, pLNTId, the CMV promoter is replaced by a promoter fragment of the mouse CD45 (T200) gene. All vectors transduce resistance to G418 (neomycin). High titre virus producing cell lines for these constructs were made by infection of the amphotropic packaging cell line PA317 after transient expression in, and virus rescue from, the ecotropic packaging cell line ψ CRE. The high titre virus producing cell lines were assayed for absence of helper virus, synthesis of human IDUA and for integrity of proviral structure. Suitable lines were used as a source of virus to infect two different MPS I skin fibroblast cultures. All three of the recombinant viruses corrected the enzymatic defect in MPS I fibroblasts. This resulted in correction of the storage phenotype of these cells, as assayed by accumulation of ^{35}S labelled glycoaminoglycans within the cell, to differing degrees, depending on the level of IDUA synthesized.

Late Abstract

USE of ADENOVIRUS as a GENE TRANSFER VEHICLE *in vivo*. Leslie D. Stratford-Perricaudet, P. Briand, & M. Perricaudet. Institut Gustave Roussy, Villejuif, FRANCE and ICGM, Paris, FRANCE.

Neonatal and adult mice were IV-injected with a recombinant adenovirus harboring the LacZ gene. Gene transfer was assessed by histochemical staining for β-galactosidase activity in various tissues. The extent of blue staining reveals that a large percentage of cells within different tissues are infected. Organs from control animals did not express β-galactosidase activity. At 15 or 21 days post-injection (p.i.) positive nuclear staining was observed in many organs such as lung, liver, intestine, heart, and skeletal muscle. Because of the exciting implications of efficient gene transfer into myocytes, we sought to characterize transduction to these cells in particular.

Examination of the intact heart, in addition to skeletal muscles from the experimental animals, reveals the impressive efficiency of gene transfer after only a single injection of the recombinant adenovirus. Because the intravenous route was used, the viral vector is not concentrated in any one area of the muscle tissue, and dispersion is favored.

Expression of the transferred gene in both cardiac and skeletal muscle was remarkably stable since β-galactosidase - positive nuclei were observed as late as 10 months p.i. of the recombinant adenovirus.

The present report has serious implications for the treatment of muscular disorders, heart diseases included.