C57BL/6 and Balb/c mice were treated with (VSV)LV by intravenous injection. The content of viral RNA and vector provirus within the liver and spleen was monitored by real-time PCR. By 4 hours post-injection the majority of vector uptake and reverse transcription had occurred. RNase Protection Assay revealed that concomitant to vector entry there was a >10-fold induction of type I interferons (IFNs), TNFα, and the chemokine MIG in both the liver and spleen. This response was not due to contaminants in the vector preparation, as heat-inactivated and bald (envelope negative) LV particles did not result in innate activation. Cytokine expression following LV delivery was transient and largely subsided within 72 hours. However, clearance of vector genomes had already begun to occur by 24 hours. This effect may have been due to the antiviral activity of the IFNs, a possibility we will validate in IFN receptor knock-out mice.

We next set out to determine the factors involved in mediating the response to LV. Initially we sought to identify if particular DC subsets were involved. Indeed, in vitro studies revealed that plasmacytoid, but not myeloid DCs became activated to produce IFNs following exposure to LV. Similar findings were also seen with human DCs. Moreover, experiments with the bald LV suggested that endosome uptake was required to induce the cytokine response. Taken together, these results suggest a role for toll-like receptor (TLR)-7, a TLR that recognizes ssRNA and is found predominantly within the endosomes of plasmacytoid DCs, in LV-mediated innate immune activation. Studies are now underway to determine whether antagonists of TLR-7 can prevent the innate response to LV, and enable stable gene transfer in the absence of adaptive immunity.

80. Pre-Existing Cytotoxic T-Lymphocyte (CTL) Responses to the AAV CAP Protein Do Not Interfere with AAV Transgene Expression in Mice

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The use of adeno-associated viral (AAV) vectors as a delivery method for gene replacement strategies is currently being explored in several clinical indications. AAV vectors do not express any viral proteins suggesting that they represent a less immunogenic delivery system. However, recent data have suggested that administration of AAV vectors may actually result in the stimulation of T-cell responses that result in a loss of transgene expression and may limit the utility of these vectors. By using computer prediction algorithms, we identified several AAV peptides as putative MHC I binders. Immunization of mice with peptides predicted to have the highest binding affinities did not result in a detectable cytotoxic T lymphocyte (CTL) response. In addition, intravenous administration of increasing doses of AAV serotype 2 vector also failed to induce a measurable CTL response. In contrast, immunization with either a plasmid or an adenoviral vector encoding the full length AAV2 CAP protein, to mimic exposure to wild type AAV, generated a potent CTL response. A pre-existing CTL response is likely to be present in the human population and C57Bl/6 IgH6 knock-out (KO) mice were used to determine whether such CTLs could affect AAV transduction and subsequent transgene expression. When immunized with two doses of CAP plasmid, the IgH6 KO mice mounted a robust CTL response without the generation of AAV neutralizing antibodies. The mice were then injected with an AAV2 vector encoding the alpha-galactosidase (a-gal) protein. AAV transduction was unimpeded and the serum levels of a-gal protein as well as the longevity of transgene expression were comparable to those observed in mice pre-immunized with empty plasmid as a control. In contrast, wild-type C57Bl/6 mice pre-immunized with CAP plasmid, which developed both CTLs and neutralizing antibodies against AAV, did not show any evidence of a-gal expression after the injection of vector. Taken together, results of these experiments suggest that pre-existing AAV-specific CTLs may have little or no effect on AAV transduction or transgene expression and confirm that neutralizing antibodies significantly interfere with transduction by AAV vector.

I am an employee of Genzyme Corporation.

OLIGONUCLEOTIDE THERAPIES: NON-VIRAL TARGETS

81. Hippocampal Gene Knockdown of BACE1 by Lentiviral Gene Transfer of siRNA Reduces Amyloid Pathology and Improves Memory Performance in APP Transgenic Mice

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The neurodegenerative process in Alzheimer's disease (AD) is associated with increased β-secretase (BACE1) activity that results in progressive accumulation of amyloid precursor protein (APP) C-terminal fragments (CTFs) and amyloid-β (Aβ). Therefore, developing strategies to inactivate BACE1 might present an important approach toward the treatment of AD. To assess this possibility in vivo, APP transgenic mice that develop some aspects of AD neuropathology were treated with a lentiviral vector (LV) expressing small interfering RNA (siRNA) targeting BACE1. The LV-siBACE1 vector was expressed at high levels in HEK293T cells and reduced both BACE1 expression and production of Aβ1-42 in vitro. One-month post intra-hippocampal delivery of the LV-siBACE1 vector in APP transgenic mice, levels of BACE1 expression were significantly reduced at the site of the injection. This was accompanied by reduced deposition of Aβ, reduced intracellular CTFs and amelioration of the neurodegenerative alterations (dendritic / synaptic density) in the hippocampus as evaluated by confocal microscopy. Furthermore, the neuroprotective effects of LV-siBACE1 were associated with improved spatial learning and memory, tested by a Morris water maze. Our results suggest that lentiviral vector delivery of BACE1 siRNA can specifically reduce the cleavage rate of APP and neurodegeneration in vivo and indicates the potential therapeutic value of this approach for treating AD. We will discuss the possible outcome of long-term siRNA expression on the progression of AD in APP mice.

82. *Cftr* Gene Targeting in Murine ES Cells Mediated by the SFHR Technique

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Small Fragment Homologous Recombination (SFHR)-mediated targeting is a gene therapy strategy where a specific genomic locus is modified through a target exchange between a small DNA fragment (SDF) and genomic DNA. Here we demonstrate that SFHR can stably

introduce a 3-bp deletion (corresponding to ΔF508) within *Cftr* **(Cystic Fibrosis Transmembrane Conductance Regulator)** locus in the genome of mouse embryonic stem (ES) cells. SDFs (about 6.4 x10^5 molecules per cell) carrying the ΔF508 mutation were transfected by nucleofection protocol. About 12% of transcript corresponding to deleted allele was detected and about 60% of the electroporated cells no longer had measurable CFTR-dependent chloride efflux. The CFTR activity was also analyzed by measuring the chloride efflux by the fluorescence microscopy-coupled digital video imaging system in each ES cell colony, previously loaded with MQAE, a chloride sensitive dye. An average of 4-6 regions for each cell colony was analysed to verify the genotypic homogeneity of each colony. In fact all regions examined in each colony showed a similar significant chloride efflux after PKA activation. Moreover on twelve electroporated ES colonies analysed, eight were successfully mutated (Cl^- efflux not significantly different from zero) while four colonies showed Cl^-efflux CFTR-dependent not significantly different from the untreated ones.

After 15 passages, cells maintained their stem characteristics, verified by morphological and immunocytochemistry analyses, and also SDFs resulted to be stably integrated within their genome, as demonstrated by molecular test and chloride efflux measurement.

These data indicate that the SFHR technique can be used to target genomic sequences efficiently in ES cells. The produced cell lines, once differentiated, can be useful for elucidating gene function in specific tissue and for transplantation medicine in gene therapy protocols.

83. Cell-Type Specific Delivery of siRNAs with Aptamer-siRNA Chimeras

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Technologies that mediate targeted delivery of small interfering RNAs (siRNAs) are needed to improve the therapeutic efficacy and safety of siRNAs. Therefore, we have developed aptamer-siRNA chimeric RNAs capable of cell-type specific binding and delivery of functional siRNAs into cells. The aptamer portion of the chimeras mediates binding to PSMA, a cell-surface receptor overexpressed in prostate cancer cells and tumor vascular endothelium, while the siRNA portion targets the expression of survival genes. When applied to LNCaP cells, a prostate cancer cell line expressing PSMA, the chimeras are internalized and subsequently recognized and processed by Dicer, resulting in depletion of the siRNA target protein and cell death. In contrast, the chimeras do not bind or function in PC3 cells, a prostate cancer cell line that does not express PSMA. Thus, these studies demonstrate an approach for targeted delivery of siRNAs with a wide variety of potential applications including cancer therapeutics.

84. RNA Silencing: Role of Interferon System Components in Non-Specific Effects Induced by Chemically Synthesized Small Interfering RNAs, Enzymatically Synthesized Small Interfering RNAs and Short Hairpin RNAs

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In mammalian cells, long double-stranded RNAs (dsRNAs) activate interferon (IFN) signaling as well as interferon inducible RNA-dependent enzymes that affect translational activity including the protein kinase (PKR) and 2',5'-oligoadenylate synthetases (2',5'-OAS). DsRNA also is the mediator of RNA interference (RNAi), a widely used gene silencing strategy with utility both as a basic research tool and potential therapeutic. RNAi mediated by short dsRNA molecules (~20-bp) was originally thought to circumvent the IFN response to achieve specific target silencing without triggering potentially problematic non-specific effects. However, reports suggest that some small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) produced from transfected plasmids can induce cytokine signaling and cause other non-specific effects. We therefore undertook a further combined genetic and biochemical examination of the involvement of IFN system components in RNAi to directly address the potential involvement of IFN-regulated gene products in non-specific responses to siRNAs. We examined the contribution of IFN signaling, PKR and RNase L to both on-target and non-specific gene silencing as well as induction of cytokines induced by three RNAi strategies. We show, through the use of cell lines genetically deficient in signaling and action components of the IFN system, that chemically synthesized siRNAs, T7 RNA polymerase-synthesized siRNAs, and shRNAs differentially activate IFN signaling and non-specific gene silencing. ShRNAs with up to 50 nucleotides complementary to a target gene mediated specific silencing and did not trigger non-specific gene silencing. While the plasmids that delivered the shRNA constructs induced IFN-β in a wild type cell line sensitive to CpG DNA, there was no differential induction of IFN-β caused by the shRNAs in this cell type or other cell types not sensitive to CpG DNA. By contrast, both 20-bp and 50-bp T7 synthesized siRNAs caused significant non-specific gene silencing and induced higher levels of IFN-β in wild type cells than chemically synthesized siRNAs. The IFN-β induction capacity of the T7 synthesized siRNAs varied between preparations and could be reduced by gel purification of the siRNA. Chemically synthesized siRNAs caused non-specific gene silencing only at extremely high concentration and did not induce significant IFN-β production in any cell type tested. Non-specific siRNA-mediated silencing was independent of PKR and RNase L, and only partially dependent upon IFN receptor-mediated signaling. We also found a relationship between the non-specific gene silencing and the induction of apoptosis by the different RNAi mediators in different cell lines.

85. Von Willebrand Factor (VWF) Gene Targeting by Double-Strand Break Enhanced Homologous Recombination

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Gene targeting by replacing the aberrant nucleotide sequence in the defective gene with the corresponding functional or "normal" sequence offers an **idealized** solution for treating genetic disorders. However therapeutic gene targeting has yet to be realized. Major advances have been made in gene targeting by utilizing site-directed DNA double strand break enhanced homologous recombination (DSB-GT). Most recently Urnov et al (2005) demonstrated DSB-GT efficiencies approaching 20% in a gene, IL2Rγ, whose dysfunction can lead to human X-linked severe combined immune deficiency (X-SCID). This remarkable validation of DSB-GT technology utilized four fingered zinc-finger nucleases (ZFN) that recognized a unique endogenous sequence at a very high affinity.

In this report we employ zinc-finger nucleases (ZFN) for DSB-GT at the Von Willebrand Factor (VWF) locus for the purposes of demonstrating its potential for correcting mutations associated with the severe bleeding disorder, Von Willebrand Disease (VWD) in