was performed by clipping both the portal vein and the hepatic artery. The blood was collected 1, 3, and 5 minutes after the injection. The DNA degradation was examined by loading the blood samples on a 1% agarose gel for electrophoresis, which showed a lower clearance rate compared to that of liver uptake. In addition, using confocal microscopy and labeling both the DNA and endothelial cells, it was demonstrated that naked DNA is primarily taken up by the liver endothelial cells. The endothelial barrier could be overcome by manually massaging the liver (MML). Our TEM results clearly show that MML could enlarge the fenestrae, which increased uptake of DNA by hepatocytes and gene expression in the liver.

Acknowledgements: This work was supported by National Institutes of Health Grants DK065964 (to F. Liu) and A148851 (to L. Huang).

1077. Sequences from the Human Type 1α2 Procollagen Promoter Mediate Osteoblast-Specific Plasmid Nuclear Import

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Two major problems limiting the use of non-viral gene therapy include the low efficiency of gene transfer seen with non-viral vectors and limited methods for cell-specific targeting and expression. We and others have shown that translocation of plasmid DNA into the nucleus is an important rate-limiting step in gene transfer that affects transgene expression levels in non-dividing cells. Further, we have identified several DNA sequences that mediate the nuclear import of plasmids in non-dividing cells, thereby acting as DNA nuclear targeting sequences. These sequences contain multiple binding sites for transcription factors that allow for the formation of protein-DNA complexes in the cytoplasm that are actively transported into the nucleus of the non-dividing cell using nuclear localization signals present on the transcription factors, and hence the protein-DNA complex. We have previously identified both a universal sequence that acts in all cell types (the SV40 enhancer) and sequences that act in specific cell types based on the property that they bind cell-specific transcription factors (e.g., the SMGA promoter for use in smooth muscle cells). In order to identify a DNA nuclear targeting sequence that is restricted to osteoblast lineage cells, we tested a number of osteoblast-specific promoters for nuclear import activity, including those from osteocalcin, collagen, BSP and Runx2 genes. Although all of these promoters showed robust expression of reporter genes following transfection into dividing osteoblasts, when microinjected into the cytoplasm of human or rat osteoblast cell lines, only the human type Iα2 procollagen (hcolIα2) promoter had the ability to mediate nuclear import of the plasmid in the absence of cell division. This sequence did not support DNA nuclear import in chondrocytes, fibroblasts and marrow stromal cells, suggesting that it is osteoblast-specific. Site-directed mutagenesis of putative transcription factor binding sites indicated that multiple response elements were required for osteoblast specific nuclear targeting, including those for AP-1, AP-2, Nkx3, SRF, Krox20, and an E-box binding protein. Taken together, these results suggest that the hCol1α2 promoter contains a novel osteoblast-specific DNA nuclear targeting sequence that can be used to increase nonviral gene transfer and transgene expression uniquely in osteoblasts, thereby increasing the safety profile by specifically targeting osteoblasts in skeletal tissue for gene therapy. The research reported in this abstract was supported by the Dept of the Army Cooperative Agreement # (DAMD17-97-2-7016). The views and content of the information does not necessarily reflect the position or the policy of the government or NMTB, and that no official endorsement should be inferred.

1078. Silencing of Transgene Expression Following Plasmid Based Delivery to Murine Skeletal Muscle Is Dose Dependent

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Plasmid based gene delivery to skeletal muscle is an attractive method for the expression of therapeutic proteins. However, the level of transgene expression decreases over time and may be an obstacle to achieving sustained therapeutic levels of proteins. To investigate the mechanism responsible for loss of expression over time, a standard plasmid backbone containing the luciferase gene driven by the CMV promoter was injected into the skeletal muscle of the hind limb of C57Bl/6 mice at either a high dose (125μg) or a low dose (25μg) followed by electroporation. Transgene expression was monitored over time using in situ CCCD imaging. At the end of the study the quantity of transgene DNA and luciferase mRNA in the muscle was measured by quantitative PCR. At early time points (day 3 and day 7) luciferase activity in the high dose group was 2.5-fold higher than in the low dose group. However, luciferase activity in mice that received the high dose fell 230-fold between day 7 and day 42, while in mice that received the low dose of the same plasmid it fell only 4-fold. Vector DNA levels in the injected muscles at the end of the study (day 84) were not significantly different between the two doses indicating that the 27-fold lower luciferase activity in the high dose group compared to the low dose group at day 84 was not due to differences in vector DNA levels but rather to a difference in gene expression. Indeed, the luciferase mRNA to vector DNA ratio was 9-fold higher in the low dose group than in the high dose group at this time point. In the same study, a plasmid containing the identical CMV-luciferase expression cassette flanked by matrix attachment sites (MARs) and placed into a plasmid backbone from which all CpG dinucleotides had been removed, was tested at the same doses. At the low dose the CpG-depleted/MARs plasmid exhibited a similar luciferase expression profile to that of the standard plasmid at the same dose. However, in the high dose group the CpG-depleted/MARs plasmid partially prevented the decrease in luciferase expression over time that was observed with the standard plasmid at the high dose. At 28, 42 and 84 days after plasmid injection the CpG-depleted/MAR plasmid expressed 10-, 13- and 9-fold higher luciferase activity respectively than the standard plasmid. Vector DNA levels in the muscle at day 84 were only 2-fold higher in the CpG-depleted/MAR group than in the mice that received the standard plasmid. These results demonstrate that reducing the plasmid dose can result in a significant improvement in the duration of transgene expression, and that this effect was not due only to improved persistence of the plasmid DNA in the muscle. In addition, use of a CpG-free plasmid backbone plus MARs partially prevented the decline of transgene expression that occurred at the high plasmid dose, suggesting that the presence of CpG's and/or absence of MARs may contribute to loss of expression over time.

1079. Effect of WPRE on Transgene Expression Using Different Promoters in the Context of Hydrodynamically Delivered Plasmid Vectors

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The Woodchuck hepatitis virus (WHV) posttranscriptional regulatory element (WPRE) facilitates nucleocytoplasmic transport of RNA mediated by several alternative pathways that may be

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cooperative. This element has been included in many different gene therapy vectors including lentivirus, AAV, adenovirus etc., to stimulate heterologous cDNAs expression.

In this work we have analyzed the vitro and in vivo effect of WPRE element over transgene expression using two different promoters in the context of a non-viral vector, naked DNA: an ubiquitous promoter AFR with moderate transcriptional activity, and a strong liver specific promoter AAT-EnhALb. Two different versions of the WPRE element were included in the study: the conventional one, which include 60 aminoacids of theHBV X protein aminoterminal region (named as WPRE long), and a shorter version in which only 30 aminoacids remained (named as WPRE short). For the in vitro studies, HEPG2 cells were transfected with 7.5 x 10e11 copies of plasmid expressing luciferase, with or without WPRE elements, and a renilla-luciferase expressing plasmid, using lipofectamine. Enzyme activities were measured using the Dual-Luciferase Reporter Assay System (Promega). In vivo, 2.5 x 10e12 copies of each plasmid were administered using hydrodynamic injection via tail vein, thus, luciferase expression was mainly detected in the liver. Luciferase expression was analyzed using a cooled CCD camera at days 1, 7 and 21. 21 days after in vivo analysis, the mice were sacrificed and luciferase expression was measured in liver extracts. DNA and RNA were extracted from the livers, and plasmid copies and luciferase mRNA was quantified using quantitative PCR and RT-PCR.

Striking differences on WPRE activity were observed depending on the promoter, both in vitro and in vivo, 1 day after injection. WPRE elements significantly increased luciferase expression when gene expression was under control of AFR promoter while the expression was not significantly altered when the promoter driving luciferase expression was the liver specific AAT promoter. However, when luciferase expression was analyzed 7 or 21 days after hydrodynamic injection, WPRE element increased luciferase expression independently on which promoter was used. Moreover, significant differences were observed depending on the WPRE version. Short and long WPREs similarly increased the transgene expression from AFR promoter, while the increase was significantly higher when AAT promoter was combined with the WPRE long, than in with WPRE short. In vitro analysis of luciferase activity on liver extract corroborated the in vivo data.

In conclusion, the effect of modified versions of WPRE over transgene expression depends on the promoter and has to be analysed both in vivo and in vitro in order to get a correct picture of the interaction between the promoter and expression regulatory elements.

Carrier Development and Therapeutic Evaluation

1080. Receptor-Targeting Smart Vectors for Efficient Gene Transfer to Tumours

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We seek to develop novel synthetic vector formulations that can be administered systemically to target therapeutic genes to tumours. Cationic nonviral vectors are cleared rapidly from the circulation by the reticuloendothelial system as a result of binding to plasma proteins and vector aggregation. Circulation times may be extended by shielding the vectors with polyethylene glycol (PEG) moieties. However, PEGylation often leads to greatly reduced transfection efficiency due to excessive vector stability. The aim of this project was to develop novel formulations of PEGylated vectors which may be administered intravenously (i. v.), and persist in the circulation and target tumour-associated receptors. The vector has been further modified to disassemble within the cell in response to the intracellular environment to achieve high transfection efficiency. Such virus-like vector formulations are often referred to as "smart vectors".

The vector developed is a lipopolyplex-class formulation based on an integrin-targeting peptide with an oligolysine nucleic acid-binding element, and a cationic lipid that enhances integrin-targeted transfection by promoting endosomal release. New cleavable peptides (CP) were designed containing CP motifs located between the DNA-binding and the integrin-targeting domains. Peptide cleavage should enhance transfection by promoting the trafficking of DNA to the cytoplasm and so to the nucleus. Cleavable PEGylated lipids (PEG-CL) were also synthesised for improved *in vivo* stability in blood, enhancing intracellular complex dissociation and endosomal escape.

The cleavage studies showed PEG-CL were efficiently hydrolysed at acid pH using TLC, and CP were cleaved in the presence of endosomal proteases using HPLC. The size of lipopolyplexes comprising PEG-CL in physiological salt solution was 394±14 nm by light scattering methods, and stable for at least one hour, which was considerably smaller ($p<0.05$) than complexes made of unPEGylated lipids (1388±198 nm after one hour). The smaller particles generated with PEG-CL were less efficient in *in vitro* transfections unless a centrifugation (1500 rpm, 5 min) was performed to promote their sedimentation and cell contact. After centrifugation, in murine neuroblastoma cell line (Neuro2A), transfection efficiency with lipopolyplexes containing PEG-CL, CP and a plasmid encoding the luciferase gene was 2 times more efficient than with Lipofectin and non-CP ($p<0.05$). The transfection efficiency of PEG-CL/CP lipopolyplexes using a plasmid encoding the GFP protein, was about 40% in Neuro2A and mouse fibroblast (AJ3.1) cells, whereas only 20% of these cells were transfected using Lipofectin and non-CP ($p<0.05$). Finally, in a murine neuroblastoma model, i. v. administration of these integrin-targeted PEG-CL/CP lipopolyplexes showed a higher level of luciferase expression in tumour (52142±7121 RLU/mg) than in lung (2347±577 RLU/mg) or in liver (1198±58 RLU/mg) ($p<0.05$).

In conclusion, we have developed a novel, targeted, synthetic, smart vector formulation that offers exciting prospects for tumour-specific therapeutic gene transfer.

1081. Targeted Nanocapsules for Liver Cell-Type Delivery of Plasmids *In Vivo*

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In contrast to the efficient delivery afforded by viral vectors, the use of nonviral vectors for gene therapy has been hindered by the lack of adequate *in vivo* delivery systems. Although hydrodynamic push has been used extensively for hepatic plasmid delivery, this method delivers DNA nonselectively to liver cell types. One approach for increasing delivery to a specific cell type is by targeting receptor(s) that are either unique or highly expressed by that cell. Hepatocytes (heps) express aisaloglycoprotein receptors (ASGPr), and liver sinusoidal endothelial cells (LSECs) express hyaluronan receptors (HAr) in high abundance providing ideal targets for ligand-mediated receptor uptake. The **aim** of this study was to determine if liver cell type specific delivery of DNA could be achieved *in vivo* using asialoorosomucoid (ASOR) targeting to the hep ASGPr and HA for the LSEC HAr. Using a novel dispersion atomization method (*Mol Cell Biochem* 274:77, 2005) that forms sub 50 nm capsules with the receptor ligand noncovalently bound to the capsule coating, a red fluorescent protein (DsRed2) reporter plasmid was encapsulated using either ASOR for hep or HA for LSEC uptake. Eight week (wk) old C57/BL6 mice received 100 $_{\mu}$g of the encapsulated

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