multiple sclerosis. Using AAV capsid DNA shuffling we created a library of novel AAV vectors. The primary recovered AAV capsid clone (Olig001) exhibited a novel >95% tropism for rat, striatal oligodendrocytes when a chicken beta actin (CBH) promoter drove GFP expression. Furthermore, when Olig001 is administered intravenously it is highly detargeted from peripheral tissues such as the liver. Surprisingly, Olig001 shares 99.3% homology (7 amino acid differences) with AAV8 within the VP3 coding region (aa204-738 using VP1 numbering); however, AAV8-CBH-GFP vectors are predominantly neurotropic. While AAV8 has some minimal tropism for oligodendrocytes, our in vitro studies using primary mixed glia cultures showed that Olig001 binds to oligodendrocytes with much higher affinity than AAV8, thus Olig001 exhibits a true gain-of-function. We sought to identify which amino acid(s) of Olig001 contribute to the oligodendrocyte-preferring tropism. Mutation of some or all of the four C-terminal amino acids of Olig001 (downstream of aa343 using VP1 numbering) back to AAV8 still produced an oligodendrocyte-preferring capsid. The N-terminal, mostly VP1/VP2-specific, portion of the capsid is a chimeric mixture of AAV1, 2, 6, and 9, and our results suggest that this region of the capsid is conferring the oligodendrocyte preferring phenotype. In summary, we report the generation of a novel AAV capsid with a tropism and biodistribution profile distinct from any naturally-occuring AAV capsids. Moreover, our results suggest that the N-terminus of the capsid specific to VP1/VP2 is the major contributor to this unique oligodendrocyte tropism.

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307. Rational Development of 12 Different AAV Serotypes as Scaffolds for Peptide Display

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Recombinant AAV vectors are some of the most attractive vehicles for therapeutic gene delivery available today, for many reasons. One is that AAV capsids are highly amenable to genetic engineering, permitting the creation and identification of vectors with novel properties including target cell specificities. One potent strategy is to insert a randomized peptide library into an exposed AAV2 capsid region, followed by iterative selection on desired cells to enrich single peptides binding to cell-surface receptors and hence mediating AAV transduction. However, several problems hamper the use of AAV2 as scaffold for peptide display: (i) neutralizing anti-AAV2 antibodies that are prevalent in the human population may interfere with transduction of AAV2 peptide mutants, and (ii) if not fully ablated, the AAV2 tropism for its genuine receptors will dampen retargeting efficiencies. Here, we aimed to overcome both problems by developing 11 other AAV serotypes as templates for peptide display, AAV1, 3-9, rh.10, po.1 and 12. We first engineered their capsid genes to contain unique restriction sites and then exploited these for insertion of 18 different oligonucleotides encoding peptides which were pre-selected in AAV2 on various cells. The resulting >200 AAV variants were subsequently produced as YFP-expressing vectors and screened in over 80 human or murine cells, including primary hepatocytes or keratinocytes, as well as T-cells and stem/iPS cells. Strikingly, many capsid-peptide combinations vastly outperformed the AAV2 counterparts, especially those based on AAV serotypes 1, 7-9 and rh.10, and those carrying 7mer peptides with an NxxRxxx motif (x = any amino acid). In addition, we found that disruption of residue R585 in AAV2 boosted the potency of several peptide display mutants by up to two orders of magnitude, whereas the same mutation had no effect in the chimeric capsid AAV-DJ, despite a common region surrounding R585. Another puzzling notion was that some serotypes were inactivated by peptide display, tempting us to model all 12 capsids after insertion of a prototype peptide. This revealed that in contrast to our prediction from linear sequence alignments, the exact position of the displayed peptide differed by up to four residues between the 12 capsids. Indeed, once adjusted accordingly, AAV3 and AAV6 were also markedly enhanced upon peptide insertion. Collectively, our data imply an enormous potential of alternative AAV serotypes as scaffolds for peptide display and provide a set of vital guidelines for their further development as gene therapy vectors. To aid in this process, we also present a protocol for AAV spotting and drying in 96/384-well plates which permits their long-term storage and shipping, and thus facilitates their evaluation in other labs.

308. Inclusion of Heterologous ITRs in Dual AAV Vectors for Retinal Gene Therapy

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AAV cargo capacity limited to about 5 kb prevents their applications from treatment of inherited blinding conditions which require the transfer of genes with larger coding sequences. However, dual AAV vectors, each containing one of the two halves of a large gene expression cassette, are emerging as promising tools to overcome this limitation. However, dual AAV show lower levels of transgene expression than a single AAV vector and are associated with production of proteins shorter than expected from either the 5'- or 3'-half AAV. These limitations can be in part overcome by improving the productive tail-to-head AAV genome concatemerization by including heterologous inverted terminal repeats (ITRs) at both ends of each dual AAV vector genome.

We have generated dual AAV hybrid vectors with heterologous ITRs from AAV2 (ITR2) and AAV5 (ITR5) which are the most divergent among the various AAV serotypes. However, we found a significant reduction in the yields of AAV vectors with heterologous ITR2 and 5 when compared to those of vectors with homologous ITR2. In addition, the genome titer performed on ITR2 sequences results lower than that on a different part of the genome suggesting that ITRs might not be intact in AAV vectors with heterologous ITRs. Finally, the amount of full length protein relative to shorter was similar between vector with homologous and heterologous ITRs . We are currently testing dual AAV hybrid vectors with ITRs derived from other serotypes divergent from AAV2 which could overcome some of the limitations we have found using ITR5.

309. Optimization of Dual AAV Vectors for Gene Therapy of Inherited Retinal Diseases

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Retinal gene therapy with adeno-associated viral (AAV) vectors is safe and effective in humans. However, AAV limited cargo capacity

prevents its application to therapies of inherited retinal diseases (IRD) due to mutations in genes over 5 kb. Dual AAV vectors, each containing one of the two halves of a large gene expression cassette, are emerging as promising tools to overcome this limitation. Dual AAV trans-splicing and hybrid vectors transduce efficiently the mouse and pig retina and are effective in animal models of IRD. However, some of dual AAV limitations include lower levels of transgene expression compared to a single AAV vector and the production of proteins shorter than expected from either the 5'- or 3'-half AAV. Thus, further development of dual AAV vectors is required before their clinical translation. To increase dual AAV recombination we have exploited various regions of homology while to mediate the degradation of the proteins shorter than expected we have tested the ability of various degradation signals. We found that the levels of transgene expression achieved with the alternative regions of homology are similar to those achieved with dual AAV vectors carrying the AK region of homology we have previously shown to be effective. Notably, we have identified a degradation signal which mediates the degradation of proteins shorter than expected from dual AAV vectors. In conclusion, our study outlines optimized features of dual AAV vectors that improve their safety and efficacy. This represents a step towards the clinical translation of dual AAV for retinal gene therapy.

310. Development of Generation X Recombinant AAV Vectors for Human Gene Therapy

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We have previously reported the development of capsid-modified next generation (NextGen) AAV serotype vectors that transduce cells and tissues more efficiently at reduced vector doses (*Proc. Natl. Acad. Sci., USA*, 105: 7827-7832, 2008). More recently, we have also described the development of genome-modified generation X (GenX) AAV vectors that also transduce cells and tissues more efficiently (*J. Virol*, 89: 952-961, 2015). The recombinant AAV genome contains inverted terminal repeats (ITRs) of 145 nucleotides at both ends. In these studies, a 20-nucleotide sequence, termed the D-sequence, was replaced with a substitute sequence, which led to enhanced transgene expression in human cell lines *in vitro* an in murine hepatocytes *in vivo* (*J. Virol*., 89: 952-961, 2015). In our present studies, we observed that a sequence, GGTTCCT, at the end of the D-sequence, shares partial homology to the consensus glucocorticoid receptor-binding element (GRE) site, 5'-GGTACANNNTGTT/CCT-3'. The TGTTCT half-site is an essential core element, which has been reported to be sufficient to relay glucocorticoid signaling. In electrophoretic mobility-shift assays (EMSAs), we documented that purified GR protein could specifically bind to double-stranded D-sequence oligonucleotides, suggesting that the D-sequence potentially functions as a ½ GRE site. Based on these results, we hypothesized that replacement of the D-sequence with a full GRE binding-site in the ITR might further increase transgene expression from these GenX AAV genomes. To this end, recombinant AAV vectors were generated in which the D-sequence was replaced with a fully functional GRE site. Insertion of a full GRE binding-site in the ITR significantly increased the transgene expression from these GenX AAV genomes following encapsidation in the wild-type (WT) AAV2 capsid vectors in human cell lines *in vitro*, and the extent of the transgene expression was further increased by dexamethasone-treatment. When $1x10^{10}$ vgs of recombinant AAV2 vectors containing the Gaussia luciferase (Gluc) reporter gene in the unmodified AAV genome, or those containing the GRE sequence, were administered via tail-vein into C57BL6/J mice, the transduction efficiency of the AAV-GRE vectors was ~8-fold higher in murine hepatocytes *in vivo* up to 9-weeks post-vector administration. More interestingly, when AAV-GRE genomes containing the enhanced green fluorescence protein (EGFP) reporter gene were encapsidated in the optimal NextGen AAV capsid-modified quadruple-mutant (Y444F+Y500F+Y730F+T491V) AAV2 vectors, the transduction efficiency of these vectors was further increased by ~8-fold in murine hepatocytes *in vivo* at a dose as low as $5x10^{8}$ vgs/mouse. Taken together, the availability of these novel GenX AAV vectors containing the D-sequence substitution, and the fully functional GRE site insertion, to achieve high-efficiency transgene expression, has implications in the use of these vectors in human gene therapy.

311. AAV8 Vexosome Vectors Enhance Cell Transduction In Vitro and Outperform Conventional AAV8 Vectors in Liver-Transduction In Vivo in the Presence of Anti-AAV Neutralizing Antibodies

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Adeno-associated virus vectors based on serotype 8 (AAV8) have demonstrated superior efficiency of transduction of hepatocytes in vivo in animal models and in humans. Recently, we described extracellular vesicle (exosomes) -associated AAV vectors termed vexosomes (vAAV). We tested vAAV1, 2, and 9 and found them to enhance transduction and antibody evasion capabilities compared to conventional AAV vectors.

With the goal of developing an enhanced vector for liver gene therapy, we sought to characterize the in vitro and in vivo transduction, biodistribution, and immunogenicity profile of vAAV8 vectors compared to conventional AAV8 vectors purified from the same preparation.

Several cell lines were transduced with conventional AAV8 or vAAV8 vectors expressing luciferase, at multiplicity of infection (MOI) ranging from 250 to 25000, and luciferase expression was measured after an overnight incubation. In HeLa and HEK293 cells, levels of luciferase up to 1 log higher were observed with vAAV8 compared with AAV8 vectors, with detectable levels of luciferase at MOIs as low as 250. In hepatocyte cell lines, the magnitude of enhancement was about 3-fold, with lower max relative light unit signal measured compared to the other cell lines. Notably, although at low levels, transduction with vAAV8 vectors was also observed in a Jurkat T cell line, in which no transduction was observed with conventional AAV8 vectors.

We then evaluated the efficiency of liver targeting in vivo with this vector system using the secreted factor IX (F.IX) transgene. Conventional or vAAV8 vectors expressing human F.IX under the control of a liver-specific promoter were administered i.v. to C57BL/6J male mice at $5x10^{10}$ vg/mouse. In naïve animals, no difference in F.IX transgene expression levels was observed between the two vector types; similarly, vector genome were found at similar levels in liver, spleen, muscle, lungs, heart, and kidneys. In these animals, i.v. vector administration also resulted in identical levels of anti-AAV antibody formation. We next evaluated the effect of vAAV8 on liver transduction in the presence of antibodies. Animals were passively immunized with IVIg intraperitoneally (0.5, 5.0, and 15 mg/mouse) followed by administration of vector ($5x10^{10}$ vg) intravenously 24 hours later. At a neutralizing antibody titer of ~1:10 (0.5mg IVIg), vAAV8 vector-injected mice had levels of expression identical to those in naïve animals. At higher titers (>1:100, 5.0 mg), low levels of residual expression of F.IX were measure with vAAV8