compared to AAV vector transduction following other administration routes in mice. AAV serotype 2 (AAV2) was used to deliver a self-complementary CMV-GFP reporter cassette via intrastromal, intracameral, intravitreal, subretinal, or suprachoroidal injections. These injections were validated by fundoscopy and optical coherence tomography at the time of injection. Transduction was assessed six weeks later by fundoscopy and whole globes were evaluated for histology. Transduction of the stroma, ciliary body, retinal ganglion cells, outer retina, and retinal pigment epithelium could be seen in the various routes of delivery to the eye. In particular, the transduction of multiple retinal layers throughout the retina without the damage of retinal detachment and widespread distribution makes suprachoroidal injections a better delivery route than to subretinal injection.

545. Syngeneic AAV Pseudo-Vectors Potentiates Full Vector Transduction

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An excessive amount of empty capsids are generated during regular AAV vector production process. These pseudo-vectors often remain in final vectors used for animal studies or clinical trials. The potential effects of these pseudo-vectors on AAV transduction have been a major concern. In the current study, we systemically examined how the AAV pseudo-vectors interact with the full AAV vectors in the transduction processes. Three different types of AAV pseudo-vectors were used: syngeneic AAV pseudo-vector (sAAV, purified from the full AAV vector preparation with the same reporter gene), null AAV pseudo-vector (nAAV, produced by using AAV packaging plasmid and adenoviral helper plasmid), and allogeneic AAV pseudo-vector (aAAV, purified from the AAV vector preparation with a genome different to reporter gene). All three AAV2 pseudo-vectors exhibited inhibition effects on full AAV2 vectors particle transduction in a dose dependent manner *in vitro*. AAV2 pseudo-vectors not only inhibited full AAV2 vector transduction but also decreased gene expression from full AAV8 vectors. However, sAAV2 pseudo-vectors exhibited less inhibition effects on the transduction efficiency of full AAV2 or AAV8 vectors compared with nAAV2 or aAAV2 pseudo-vectors *in vitro*. In contrast, all three kinds of AAV8 pseudo-vectors did not show such inhibition effects on full AAV8 and AAV2 vectors *in vitro* or *in vivo*. Moreover, the sAAV8 pseudo-vector enhanced its full AAV8 vector transduction while nAAV8 and aAAV8 pseudo-vector did not have similar effects. Nine fold sAAV8 pseudo-vectors increased AAV8-TTR-hF8-X5 transduction by 2~5-fold in two different hemophilia A mouse models as measured the coagulation activity of factor VIII by aPTT assays and antigen levels by Elisa. Further characterization showed that sAAV8 and aAAV8 pseudo-vectors contain DNAs of varying sizes. In general, these DNAs still have AAV ITR sequences and partial vector genomes near ITRs. It is likely that such partial genome could anneal to the full AAV genomes and facilitate the second strand DNA synthesis thus increasing full AAV transduction.

546. Development of MFP-Inducible System for AAV5 Gene Therapy of Chronic Diseases in the Liver

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Introduction: Gene therapy offers long term solutions for chronic diseases, whereby the transgene is continuously expressed upon single vector administration. However in some cases it would be desirable to tightly regulate or switch off transgene expression. Methods: We are investigating regulated gene expression based on the mifepristone (MFP)-inducible GeneSwitch system. The GeneSwitch protein comprises yeast Gal4 DNA-binding domain, a human p65 activation domain and a MFP controlled domain derived from the human progesterone receptor. The classical GeneSwitch system consists of two expression cassettes on two separate vectors; one containing the GeneSwitch sequence and one containing the transgene. We compared this two-vector system to a single-vector system, where the two cassettes were put into one vector for efficacy *in vitro* and *in vivo*. Results: We show inducible expression of EPO, IGF and GNDF obtained *in vitro* upon addition of MFP to cells transfected with plasmids containing GeneSwitch and the gene expression cassette. The kinetics of EPO mRNA and protein expression followed a dose dependent fashion in the range of 0.1 to 10 nM MFP and reached a plateau at higher MFP concentrations. Surprisingly, the GeneSwitch protein expression decreased 48h after MFP induction. The indicibility of the single versus the two-vector system of GeneSwitch-EPO was compared. Both systems were equally inducible based on total amount of EPO produced in the presence of MFP and related to background expression in the absence of MFP. *In vivo* proof of concept was obtained for EPO in the liver. EPO is characterized by clear expression kinetics in plasma and raises blood hematocrit, hence provides a reliable in-life read-out for gene inducibility. Mice were injected with different doses of AAV5-AAT-GeneSwitch-EPO and gene expression was induced in two separate rounds at 4 and 8 weeks p.i. EPO plasma levels increased approximately 2-logs in the single or two-vector system-injected mice, compared to un-induced groups. Moreover in the absence of MFP background expression of EPO was lower in the single-vector system and hematocrit levels were unaffected. Measurements of MFP in tissue matrices and in plasma by mass spectrometry show the presence of MFP in plasma and liver, validating applicability of the GeneSwitch system in the liver. Conclusion: Overall, our data indicate that transgene expression can be repeatedly regulated in the liver using the GeneSwitch system and provides us with a novel AAV5 vector for further development.

547. AAV Preparations Contain Contamination from DNA Sequences in Production Plasmids Directly Outside of the ITRs

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Despite having the best safety profile of any current clinical viral vector, it is known that AAV preps contain contaminating sequences that are packaged alongside the expression cassette at a low rate. These sequences can originate from production plasmid DNA, or chromosomal DNA from producer cell lines. It has been reported

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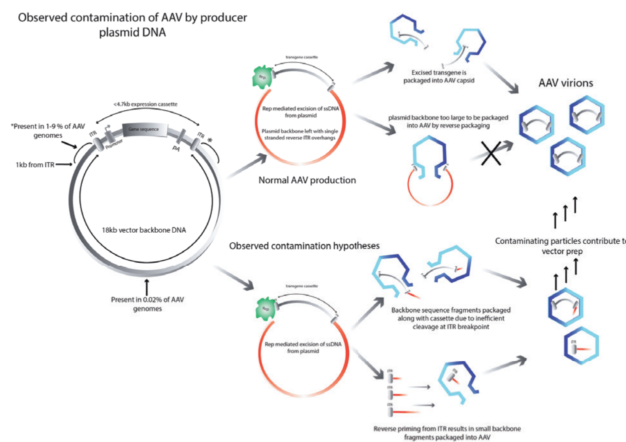
that sequences from the expression cassette producer plasmid are more likely to be packaged into AAV than DNA from other producer plasmids, or chromosomal DNA. We hypothesised that in the expression cassette plasmid, backbone sequences directly flanking the ITRs might be packaged into AAV at a higher rate than sequences further away from either ITR. We first confirmed the presence of these sequences via PCR amplification of non-expression cassette DNA flanking the ITR sequence up to 1.5kb in length in an AAV prep. Sequential qPCR assays showed that plasmid sequences at a range of distances up to 2kb from the ITR make up between 1% and 9% of AAV particles. Most significantly, there was an observable decreasing trend in contaminant titer as distance from the ITR increased. Contaminant sequences closer to the ITRs (within 1kb) are detected at a 100 fold greater rate than distal plasmid DNA (9kb from ITRs on the same plasmid). The disparity in the levels of ITR adjacent DNA sequences, compared to sequences 9kb from either ITR, suggest that the origin of this DNA is from within AAV particles rather than residual plasmid DNA remaining after purification procedures. ITR adjacent contamination is present at both a TRS mutated ITR (required for self-complementary vectors) and non TRS mutated ITRs. Contaminating plasmid sequences were present when the transgene was half of the packaging capacity (2.3kb FIX prep) and at the full capacity (5kb FVIII prep) at comparable levels, suggesting that increasing the transgene size with stuffer DNA to create a full genome will not solve this issue. Previous studies have concluded that increasing the size of the backbone with stuffer DNA reduces the level of plasmid backbone contamination, as the two ITRs are then not in range of each other to facilitate reverse packaging. However, the total plasmid size in our studies was >20kb. Therefore, the ITRs should not be in range for this to occur. We hypothesise that these ITR adjacent sequences are either a product of read-through from the expression cassette into flanking sequences, due to inefficient cleavage at the ITR breakpoint, or from reverse priming mediated by only 1 functional ITR. In the current expression cassette plasmids examined, the Kan^r gene and bacterial f1origin of replication are within the range of the flanking sequences that could be packaged. With current, unsolved clinical challenges for AAV, including transaminitis post high dose infection, it is clear that clinical AAV vectors should be designed to contain as little contamination as possible. We conclude that newly designed AAV production plasmids should contain significant lengths of stuffer DNA flanking each ITR (at least 2kb) to ensure that bacterial sequences are not packaged into AAV preps. Further research into vector design is required to eliminate this source of non-functional DNA from AAV produced for the clinic.



548. Recombinant AAV3 Serotype Vectors Generated by Using AAV3 ITRs and AAV3 Rep Proteins Efficiently Transduce Human Liver Cancer Cell Lines *In Vitro* and Human Liver Tumors in a Murine Xenograft Model *In Vivo*

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Our initial observation of the selective tropism of AAV3 serotype vectors for human liver cancer cell lines and primary human hepatocytes (*Mol Genet Metabol*., 98: 289-299, 2009; *Hum Gene Ther*., 21: 1741-1747, 2010; *Gene Ther*., 19: 375-84, 2012), led to renewed interest in this serotype, since AAV3 vectors and their variants have recently proven to be extremely efficient in targeting human and non-human primate hepatocytes *in vitro* as well as *in vivo* (*Nature*, 506: 382-386, 2014; *Hum Gene Ther*., 25: 1023-1034, 2014; *Mol Ther*., 23: 1867-1876, 2015; *Mol Ther*., 23: 1877-1887, 2015). Our previous studies also documented that the combined use of AAV3 ITRs, AAV3 Rep proteins, and AAV3 capsids led to the production of recombinant AAV3 vectors with higher titers than those produced using AAV2 ITRs, AAV2 Rep proteins, and AAV3 capsids. We also observed that the transduction efficiency of Rep3/ITR3 AAV3 vectors was ~4-fold higher than that of Rep2/ITR2 AAV3 vectors in a human hepatocellular carcinoma (HCC) cell line, Huh7, under identical conditions. In the present studies, we extended these observations to include capsid-optimized AAV3 vectors in which two surface-exposed residues (Serine 663 and Threonine 492) were mutated to generate a S663V+T492V double-mutant vector to examine whether the transduction efficiency of the Rep3/ITR3-S663V+T492V-AAV3 vectors could be further augmented. To this end, two human HCC cell lines, Huh7 and LH86, were transduced with WT-AAV3 and S663V+T492V-AAV3 vectors generated with ITR2/Rep2 and ITR3/Rep3, respectively, under identical conditions. Consistent with our previously published studies, the transduction efficiency of the S663V+T492V-AAV3 vectors was ~10-fold higher than its WT counterpart, and interestingly, there was a further 2-fold increase in the transduction efficiency of both vectors generated with ITR3/Rep3, compared with those generated with ITR2/Rep2 (Fig. 1A). We next evaluated the transduction efficiency of these vectors in a murine xenograft model bearing human liver tumors. AAV3-EGFP-Neo vectors generated with either ITR2/Rep2, or with ITR3/Rep3, were injected intra-tumorally at a dose of $1x10^{11}$ vgs/tumor. Forty-eight hrs post-vector administrations, transgene expression was determined in each tumor by Western blot assays. These results indicated that AAV3 vectors generated with ITR3/Rep3 transduced human liver tumors *in vivo* ~2-fold more efficiently than those generated with ITR2/Rep2 (Fig. 1B). It is anticipated that the combined use of ITR3/Rep3, and S663V+T492V-AAV3 capsids will further increase the transduction efficiency of these vectors. Taken together, our data suggest the transduction efficiency of AAV3 vectors can be significantly improved both by using homologous Rep proteins and ITRs as well as by capsid-optimization. The use of these modified AAV3 vectors should prove useful in liver-directed human gene therapy.

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