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Self-complementary AAV Vectors; Advances and Applications

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Numerous preclinical studies have demonstrated the efficacy of recombinant adeno-associated virus (rAAV) gene delivery vectors, and recent clinical trials have shown promising results. However, the efficiency of these vectors, in terms of the number of genome-containing particles required for transduction, is hindered by the need to convert the single-stranded DNA (ssDNA) genome into double-stranded DNA (dsDNA) prior to expression. This step can be entirely circumvented through the use of self-complementary vectors, which package an inverted repeat genome that can fold into dsDNA without the requirement for DNA synthesis or base-pairing between multiple vector genomes. The important trade-off for this efficiency is the loss of half the coding capacity of the vector, though small protein-coding genes (up to 55 kd), and any currently available RNA-based therapy, can be accommodated. The increases in efficiency gained with self-complementary AAV (scAAV) vectors have ranged from modest to stunning, depending on the tissue, cell type, and route of administration. Along with the construction and physical properties of self-complementary vectors, the basis of the varying responses in multiple tissues including liver, muscle, and central nervous system (CNS) will be explored in this review.

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THE PROBLEM WITH A SINGLE-STRANDED GENOME

Recombinant adeno-associated virus (rAAV) vectors have gained a respectable track record in preclinical gene-therapy studies and are likely to find a great many uses in clinical settings over the next few years. Transduction efficiencies generally range from 25 to several hundred vector genome-containing particles (VGP) per transducing unit, depending on the cell type. Initial investigations into the rate-limiting steps for transduction highlighted the importance of converting the single-strand DNA (ssDNA) vector genome into double-stranded DNA (dsDNA) prior to gene expression.[1,2] More recent studies have revealed additional bottlenecks, including transport to the nucleus and/or uncoating from the capsid.[3–5] There is also a transient period of vector genome instability after dsDNA conversion that leads to a significant loss of gene expression.[6] Losses at each of these steps are likely to contribute to the overall efficiency of the vector in terms of the dose of VGP required to achieve each transduction event. Regardless of these newly recognized hurdles, any rAAV genome that does reach the nucleus will still require the synthesis, or recruitment, of a complementary strand in order to achieve gene expression. This is the critical step that can be effectively bypassed through the use of self-complementary AAV (scAAV) vectors.

Like all parvoviruses, the AAV genome is packaged as a linear ssDNA molecule with palindromic inverted terminal repeat (ITR) sequences forming dsDNA hairpin structures at each end. These serve as replication origins during productive infection and as priming sites for host–cell DNA polymerase to begin synthesis of a complementary strand. During productive AAV replication, DNA polymerase delta, along with associated replication factors (replication factor C, proliferating cell nuclear antigen, and minichromosome maintenance complex), is the only DNA synthesis activity required, or normally used, for the exclusively leading-strand replication scheme.[7,8] It is currently unknown whether these are the only factors contributing to complementary strand synthesis in the context of rAAV vector transduction.

While the conventional replication scheme of AAV requires *de novo* synthesis of the complementary DNA strand, there is an alternative mechanism, involving the base pairing of complementary strands from two infecting viruses, which does not require DNA synthesis.[9] This interstrand base pairing, or strand annealing (SA), is possible because AAV, unlike many of the autonomous parvoviruses, packages either the plus or minus DNA strand with equal efficiency.[10] The likelihood of these two genomes forming dsDNA should increase with dose, following second order kinetics. However, host recombination factors probably play an important role in promoting SA, which makes it difficult to predict how efficiently this occurs in any particular cell type. There are other examples of second order interactions between rAAV genomes, in addition to SA, including end-to-end joining to form concatemers, and homologous recombination between overlapping regions of co-infected vectors.[9,11,12] Thus, it is highly likely that SA contributes to rAAV vector transduction when the multiplicity of infection is sufficient to promote base pairing.

The relative contributions of SA and DNA synthesis to AAV transduction have been investigated recently through the use of

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specialized ssAAV derivatives that can package only one polarity of the vector genome, thus precluding the SA pathway.[13,14] These single-polarity vectors transduced cells in many tissues, including liver, as efficiently as conventional ssAAV, suggesting that DNA synthesis is a major contributor to transduction under these conditions.

There are a number of host-cell treatments that increase the efficiency of dsDNA conversion including co-infection with adenovirus (Ad), DNA-damaging agents (ultraviolet or γ-irradiation, hydroxyurea), and specific inhibition of a host-cell factor that binds to the AAV 3′-ITR sequence.[1,2,15–18] Although revealing a great deal about the mechanisms behind barriers to dsDNA conversion, these treatments are unlikely to be useful for clinical gene therapy. γ-Irradiation leads directly to DNA double-strand breaks, and hydroxyurea and ultraviolet irradiation induce stalled replication forks which frequently convert to double strand breaks in dividing cells. Apart from being mutagenic, double strand breaks are targets for rAAV vector integration, and these treatments could exacerbate concerns over vector-related genotoxicity.[19–21] A possible exception, where the use of genotoxic agents to promote dsDNA conversion might be more readily accepted, would be in tumor cell–directed anticancer gene therapy, where the treated cells are likely to be cleared.[22]

scAAV VECTORS

The need for dsDNA conversion, either by SA or DNA synthesis, can be entirely circumvented by packaging both strands as a single molecule. This can be achieved by taking advantage of the tendency to produce dimeric inverted repeat genomes during the AAV replication cycle (**Figure 1**).[23] If these dimers are small enough, they can be packaged in the same manner as conventional AAV genomes, and the two halves of the ssDNA molecule can fold and base pair to form a dsDNA molecule of half the length. Although this further restricts the transgene carrying capacity of an already small viral vector, it offers a substantial premium in the efficiency, and speed of onset, of transgene expression because dsDNA conversion is independent of host-cell DNA synthesis and vector concentration.[24]

An scAAV vector, sometimes called dsAAV, can be made simply by reducing the vector construct size to ~2,500 base pair (bp) (2,200 bp unique transgene sequence plus two copies of the 145-bp ITR), such that the dimeric inverted repeat will be no larger than the normal AAV packaging capacity (~4,700 nucleotides). In this case, the vector product is a mixture, containing virions with self-complementary genomes and virions that have packaged either one or two, monomeric ssDNA molecules. The proportion of dimeric to monomeric genomes varies widely between preps, from ~5 to >50% dimer. It is not known whether two monomeric genomes contained within a single virion are necessarily complementary, or if so, whether their close proximity upon uncoating would favor SA. However, in a recent study in which scAAV vector was made by this method, and contained only 3% dimeric genomes, a 600-fold increase in therapeutic efficacy over ssAAV vector was reported.[25] This suggests the possibility that SA from two genomes contained within a single virion might have contributed to efficient transduction.

Although the two halves of a scAAV genome are complementary, it is unlikely that there is substantial base pairing while the DNA is contained within the virion shell. The structural properties of dsDNA are very different from those of ssDNA, particularly in terms of flexibility, and dsDNA viruses have specific mechanisms for condensing their genomes within the capsid. Parvoviruses would have no such mechanism, and much of the ssDNA within the parvovirus virion is situated with the bases in contact with amino acid residues of the inner capsid shell, and the phosphate backbone sequestered toward the center, which would preclude substantial base pairing.[26] Further, the packaging of AAV DNA is dependent on an active viral helicase function, suggesting that the DNA is unwound as it enters the capsid.[27] The greater likelihood is that the scAAV genome anneals rapidly after uncoating, in a pseudo first order reaction beginning with the ITR in the middle of the genome. This forms a dsDNA hairpin molecule, with a covalently closed ITR at one end and two open-ended ITRs at the other. The folded molecule would essentially mimic the structure of conventional AAV after dsDNA conversion by DNA synthesis (**Figure 1**, step 2) and would be permissive for active transcription.

MUTANT ITR CONSTRUCTS TO PROMOTE scAAV PRODUCTION

The generation of normal monomeric AAV genomes relies on the efficient resolution of the two ITRs in turn, with each round of DNA synthesis (**Figure 1**, steps 5m–6m). This reaction is mediated by the ssDNA endonuclease activity of the two larger isoforms of the AAV Rep protein.[28] Nicking the ITR at the terminal resolution site is followed by DNA elongation from the nick by host DNA polymerase (**Figure 1**, step 5m). Dimeric genomes are formed when Rep fails to nick the terminal resolution site before it is reached by the replication complex initiated at the other end (**Figure 1**, step 5d).

The yield of dimeric genomes in a scAAV prep can be increased dramatically by inhibiting resolution at one terminal repeat. This is readily accomplished by deleting the terminal resolution site sequence from one ITR, such that the Rep protein cannot generate the essential ssDNA nick.[29,30] The replication complex initiated at the other ITR then copies through the hairpin and back toward the initiating end (**Figure 1**, step 5d). Replication proceeds to the end of the template molecule, leaving a dsDNA inverted repeat with a wild-type ITR at each end and the mutated ITR in the middle (**Figure 1**, step 6d). This dimeric inverted repeat can then undergo normal rounds of replication from the two wild-type ITR ends (**Figure 1**, steps 7d–9d). Each displaced daughter strand comprises a ssDNA inverted repeat with a complete ITR at each end and a mutated ITR in the middle (**Figure 1**, step 9d). Packaging into the AAV capsid ensues from the 3′ end of the displaced strand. Production of scAAV from constructs with one mutated ITR typically yields >90% dimeric genomes.

Production and purification of scAAV vector from mutated ITR constructs is the same as conventional ssAAV. They can be purified by density gradient or by column chromotograpy. Quantification of VGP can be performed using either hybridization or reverse transcriptase PCR techniques. However, for either dot blot or Southern blot, it is important to apply the vector DNA to hybridization membranes under alkaline conditions to prevent reannealing of the complementary strands. Additionally, there have been scAAV constructs in which a spurious Rep-nicking

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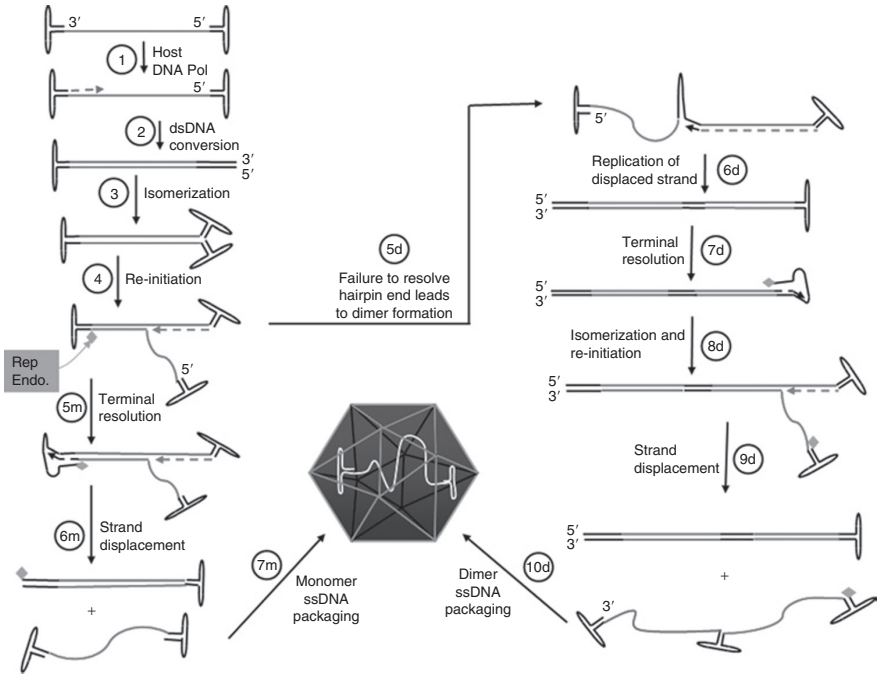

Figure 1 Adeno-associated virus (AAV) replication cycle and formation of dimeric inverted repeat (scAAV) genomes. Single-stranded virion DNA enters the host-cell nucleus and the 3′-inverted terminal repeat (ITR) acts as a primer for host DNA polymerase. (**1**) The 3′-ITR primer is elongated, displacing and replicating the ITR at the 5′ end. (**2**) The duplex ITR is re-folded into a double-hairpin configuration by host or viral DNA helicase, forming a new primer for DNA synthesis. (**3**) While the 3′-ITR is elongated and the complementary strand displaced, AAV Rep protein recognizes and binds to the ITR at the downstream end. (**4**) To generate complete monomeric genomes, Rep endonuclease nicks the terminal resolution site (*trs*) of the downstream ITR, initiating a second DNA replication complex, to copy the ITR before being reached by the complex initiated at the other end. (**5m**) The original replication complex displaces the daughter strand, including the newly synthesized ITR, and completes replication to the end of the genome, recreating the template for isomerization in step 3. (**6m**) The displaced single-stranded genome is packaged into the AAV capsid. (**7m**) Dimeric genomes are generated when Rep fails to nick the *trs* before being reached by the replication complex from the other end. (**5d**) Replication continues through the ITR, and the displaced strand, to generate a dimeric dsDNA template (**6d**) which can initiate a new round of DNA synthesis either by isomerizing the open end (as in step 4) or by terminal resolution of the hairpin end. (**7d**) Isomerization allows priming of DNA synthesis from the resolved end **(8d**), and replication of the dimeric template displaces a single-strand dimeric inverted repeat genome (**9d**), which can then be packaged into the AAV virion (**10d**). dsDNA; double-stranded DNA; ssDNA, single-stranded DNA.

site is close enough to the mutated ITR to allow terminal resolution and generation of monomer genomes. This can generally be dealt with by turning the transgene cassette around with respect to the mutant and wild-type terminal repeats. The yield of scAAV can also be affected by the choice of helper plasmid providing the AAV Rep proteins *in trans*.[31] The production of excess Rep may promote interactions with spurious nicking sites, again leading to the production of monomeric ssDNA genomes.

PACKAGING CAPACITY OF scAAV VECTORS

Clearly, packaging capacity is a limiting factor for scAAV vector applications. While ssAAV vectors generally deliver ~4.4 kb of unique transgene sequence, scAAV should be able to carry only half that, with a more substantial penalty in efficiency if this is exceeded because the unique sequence is doubled in the scAAV genome. Still, 2.2 kb is sufficient for a great number of useful applications, using relatively small transgenes with simple promoters, and for RNA-based therapies (short hairpin RNA and micro RNA, ribozymes).[32] With 500–1,000 bp for transcription elements (promoter, polyadenylation signal, intron), proteins of 40–55 kd can be encoded using scAAV vectors. Because space is at a premium in these vectors, careful optimization of transcriptional and post-transcriptional regulatory elements, as well as codon optimization, is likely to provide significant reward in overall levels of transgene expression.[33] Although it may not be possible to equal conventional ssAAV vectors in terms of transgene expression per transducing unit, scAAV can make up for that by transducing more cells at the same dose.

Two recent studies suggest that there may be greater latitude in packaging capacity than previously believed. Wu *et al.* have successfully packaged scAAV-2 constructs exceeding 3,300 bp and demonstrated dimeric inverted repeat genomes that were fully DNase resistant.[31] Further, these vectors yielded the expected increases in transduction efficiency over ssAAV when tested on cultured cells. Although it is not clear how this is happening, it may be a general feature of rAAV that the packaging capacity can be stretched beyond the previously described limits of 4.1–5.2 kb.[34,35] Allocca *et al.* have found that different serotypes of AAV

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have widely differing limits for the length of genome that can be packaged, ranging up to 8.9 kb in an AAV-5 capsid.[36] Although others had previously noted increased packaging capacities in AAV vectors, this was not serotype dependent.[37] Clearly, there is still more to be learned about the mechanisms and limitations of rAAV packaging, and a more complete understanding may open up significant new applications for scAAV vectors.

APPLICATIONS AND PROPERTIES OF scAAV VECTORS

The potential applications of scAAV will depend on trade-offs between the ability to package the transgene and associated regulatory elements, and the gains in transduction efficiency realized in any particular therapeutic context. The relative efficiency of ssAAV vectors will rely more on the multiplicity of infection than scAAV, making the expected distribution of vector within the target tissue an important consideration. Because parameters such as the size of the tissue and the route of vector administration vary widely in different applications, direct comparisons between ssAAV and scAAV transduction, as well as therapeutic efficacy, are organized below by target tissue.

TRANSDUCTION IN LIVER

The kinetics of ssAAV and scAAV vectors are best exemplified in liver due to the relative ease of delivering a high dose homogeneously throughout the tissue. Using ssAAV serotype 2 vectors, transduction had been limited to ~5% of hepatocytes after intravenous injection of over 5×10^{10} VGP in mouse liver. While few hepatocytes expressed the transgene, most had taken up the viral DNA and these genomes could be rescued and expressed if the cells were subsequently co-infected with Ad.[2,38] This suggested that only a limited number of hepatocytes were competent for dsDNA conversion. Supporting this hypothesis, early experiments with scAAV demonstrated striking differences in the kinetics of gene expression from mouse liver after intravenous injection of ssAAV-2 versus scAAV-2 (2×10^{10} VGP) carrying an erythropoeitin reporter.[24] As expected, the scAAV vector initiated expression sooner and increased at a faster rate, ultimately sustaining significantly higher levels. Although scAAV commenced markedly sooner, there was still a slow increase in expression over a period of 5 weeks, similar to increases observed with ssAAV vectors, likely reflecting the slow transport of AAV-2 capsids to the nucleus. Thus, the expression kinetics represent the combined effects of inefficiencies at each step in processing the vector, with the scAAV effectively bypassing one important step. Using a GFP reporter, a dose of $2–5 \times 10^{10}$ VGP of scAAV-2 transduces the majority of hepatocytes after a single dose, suggesting that most of these cells are limited by second-strand synthesis.[29,30]

Because the packaging capacity of scAAV is small, there is a limited range of modifications that can be made to the transgene cassette to increase expression. However, careful optimization of these sequences can result in substantial improvements in tissue-wide expression, easily exceeding the overall levels reached by an ssAAV vector at the same dose. Wu *et al.* have optimized regulatory elements and codon usage for scAAV-FIX expression in liver, and compared its performance with a previously characterized ssAAV-FIX vector.[33] Expression levels per genome were compared at several doses by determining the vector genome copy number per cell and the overall Factor IX (FIX) expression level in the serum. While ssAAV vectors consistently produced two- to threefold more FIX per stable genome, the two- to fourfold increase in the number of available scAAV genomes in the liver allowed for an overall higher level of FIX in the serum. Further, the scAAV transgene expression was evenly distributed among hepatocytes throughout the liver, similar to the pattern of the endogenous gene, while expression from ssAAV was restricted to a relatively small number of cells producing large amounts of FIX product. It is not known whether there will be significant consequences to these differing patterns of gene expression, but it will be important to determine their effects on post-translational modification and potential transgene immune responses.

PSEUDOTYPED scAAV IN LIVER

The ability of ssAAV to transduce hepatocytes, or other specific cell types, is greatly improved through the use of alternate AAV serotypes, primarily by delivering more genomes to the nucleus through efficient intracellular trafficking and uncoating.[5,39,40] This is likely to promote dsDNA conversion by SA, particularly in cells that are not permissive for second-strand DNA synthesis. Using ssAAV-2/8 vectors (rAAV-2 ITR genome packaged in an AAV-8 capsid), transduction in mouse liver can approach 100% after a single tail vein or intraportal injection. This raises the question as to whether the benefits of a self-complementary vector also apply to AAV serotypes capable of highly efficient liver gene delivery.

Each of the known AAV serotypes is capable of packaging scAAV genomes with similar efficiency.[4] Using scAAV vectors packaged in serotypes 7 and 8, transgene expression (Factor IX) in the livers of nonhuman primates has been increased by as much as 1–2 orders of magnitude over ssAAV genomes packaged in the same vectors, largely by trafficking scAAV genomes more efficiently to the nucleus.[41–43] This demonstrates that the increased efficiency of specific AAV serotypes in target tissues also applies to scAAV vectors. Similarly, in a canine model of glucose-6-phosphatase deficiency, prolonged survival was achieved at a 600-fold lower dose of therapeutic scAAV than the comparable ssAAV vector when pseudotyped with a serotype 8 capsid.[25,44]

The relatively poor trafficking of AAV-2 vectors in some cell types, including liver and airway, can be improved through the use of proteasome or ubiquitin ligase inhibitors.[3] These improvements also apply to scAAV vectors, again, by effectively overcoming one of the earlier hurdles to AAV transduction.[45] Together, these studies support the idea that gains in vector transduction using a combination of scAAV and improved trafficking are likely to be synergistic. While the use of optimal serotype and ssAAV vectors in some tissues may allow near 100% transduction, as can be achieved in mouse liver with AAV-8, the same thing could be accomplished with scAAV-8 at a far lower dose. This would provide a significant advantage in safety as well as practicality for gene therapy.

MUSCLE TRANSDUCTION

Direct injection into muscle results in very high local concentrations of vector at the injection site, which facilitates transduction from ssAAV vectors, especially through the SA pathway. This can

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lead to ambiguity in comparisons between ssAAV and scAAV vectors in these tissues, particularly when using cell associated reporters (GFP, β-Gal, alkaline phosphatase) which are quantitative in terms of how many cells are transduced, but not in the number of expressing vector genomes. Even secreted reporters are likely to reach a saturation point when high doses of vector are used. More quantitative comparisons can be made in dose–response experiments, in which the minimal dose required to achieve comparable effects can be determined. In experiments using an erythropoietin reporter, ssAAV and scAAV vector doses of 5×10^9 VGP and above resulted in similar long-term levels of transgene expression, though the scAAV reached that level sooner.[29] This could lead to the interpretation that ssAAV transduction is slower but will eventually catch up through slow conversion to dsDNA in these tissues. However, at lower doses (1×10^9 and 5×10^8), the scAAV vector produced nearly the same increase in hematocrit as the higher dose, whereas the ssAAV vector had minimal effects. This suggests that scAAV transduces muscle cells more efficiently by approximately ~tenfold, and highlights the need for carefully designed dose–response experiments in evaluating transduction by direct injection into muscle, or other tissues where vector is concentrated at the injection site, to avoid saturation effects. This may have been a factor in a study of CEA antigen expression after injection of 10^{11} VGP of ssAAV or scAAV into mouse muscle.[46] While the expected faster rise in scAAV-CEA expression was observed (by immunohistochemical staining), the expression from the ssAAV vector reached the same level over a period of 3–8 weeks after injection.

In a similar time–course experiment using a single high dose (1×10^{11} particles) of GFP reporter vector, both scAAV and ssAAV tranduced all of the muscle cells in the region of the injection site.[30] Again, the scAAV expression commenced sooner and was visibly stronger than the ssAAV vector, with a 15-fold difference in expression level inferred from the camera exposure times required to produce equivalent signals.

In a recent study directed at transduction of heart muscle, Andino *et al.* compared ssAAV and scAAV GFP vectors pseudotyped with the AAV-1 capsid.[47] In this case, a dose of 1.85×10^{11} vector particles was injected into the cardiac chamber of 4-day-old mice, rather than directly into the tissue, with the result that clear differences were observed in the numbers of cells transduced at each time point.

Thus far, the weight of evidence suggests that scAAV will transduce muscle cells more efficiently than ssAAV vector by a factor of at least 10- to 15-fold. While ssAAV may transduce a similar number of cells at a high dose, this does not necessarily equate to an equivalent level of transgene expression, which may, or may not be important for particular applications. Again, the ability to transduce a similar number of cells using a lower dose of scAAV offers practical, as well as safety advantages in a therapeutic setting. This will be particularly relevant to systemic vascular delivery strategies to muscle cells, where vector will not be so highly localized.[48,49]

CNS

The adult central nervous system (CNS) is largely composed of postmitotic cells and there have been numerous reports of long-term expression from rAAV vectors.[50] As in many tissues, expression from conventional rAAV-2 increases to a maximum over a period of ~5 weeks and then remains stable. A comparison between scAAV and ssAAV at this time point reveals a significant difference in the numbers of cells transduced by either direct injection into the thalamus or injection into the dorsal third ventricle.[29] As is characteristic of AAV-2 with a cytomegalovirus promoter, primarily neurons were transduced in the direct parenchymal injection (2×10^8 particles), and both vectors showed detectable transduction to a radius of ~3.5 mm from the injection point. However, within that radius there were marked differences in the number of cells expressing GFP and the intensity of fluorescence. The spread of vector particles in the injection bolus depends on interactions between virus capsid and cellular receptors (heparan sulfate for AAV-2) and would not be influenced by the genome content. It is therefore unlikely that a widespread distribution of transduction in parenchymal tissue can be achieved solely through the use of scAAV vectors. Therefore, the primary advantage of scAAV in direct injections will be in achieving a greater saturation of transduced cells within a limited area.

A much broader distribution of CNS transduction can be achieved by injecting vector into the ventricular space, due to diffusion and bulk flow in the cerebral spinal fluid. Because of this wider distribution, fewer cells will be multiply infected, and transduction by SA is less likely. While ssAAV achieves minimal transduction at a dose of 5×10^8 VGP, the scAAV-2 transduces a large number of ependymal cells lining the ventricles, even in remote regions of the brain, as well as parenchymal cells close to the ventricular periphery. In this context, the benefits of the scAAV vector are clear and can be generalized to other applications where vector can be widely disseminated.

Self-complementary vectors have also been useful in biodistribution studies aimed at delivery to CNS tissues.[51] The ability to detect transduction, even at low levels of delivery, has allowed the optimization of vector serotype and injection protocol so that significant delivery could be achieved. Once optimized, the procedures were applied to ssAAV vectors carrying a therapeutic transgene, in this case for the treatment of a lysosomal storage disease, which was too large to be accommodated as an scAAV vector.[52] Though the ssAAV would be more limited in transduction, the distribution in tissue and cell types remains the same, and a significant therapeutic effect was observed.

An alternate method for delivery to the CNS is retrograde transport of vector from peripheral axons infected by intramuscular injection.[53–55] Gene delivery to these cells has important applications for the treatment of diseases including amyotrophic lateral sclerosis and spinal muscular atrophy. While spinal motor neurons can be transduced with ssAAV vectors, the efficiency is low. However, significantly higher transduction rates have been achieved recently using scAAV vectors after either intramuscular injection or direct injection into the sciatic nerve.[56] Among serotypes 1–6, scAAV-1 pseudotyped vectors yielded the highest number of transduced cells by either injection route (4.1 and 7.5% of target motor neurons for intramuscular and intrasciatic injection, respectively). In a direct comparison between this scAAV-1 vector and the correlate ssAAV-1 after intrasciatic injection, the scAAV transduced eightfold more neurons at a tenfold lower dose. The presence of DNA from both vectors in the CNS was confirmed by

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PCR assay, supporting the interpretation that dsDNA conversion is rate-limiting in neurons.

TRANSDUCTION IN THE EYE

The retina is an attractive application for AAV gene therapy for a number of reasons including relatively easy access with minimal exposure to other tissues, small volume and low dose requirements, post-mitotic target cells, and a variety of monogenic and complex disorders amenable to treatment.[57–59] Two recent clinical trials for Leber Congenital Amaurosis using AAV vectors show promising results.[60,61]

scAAV vectors have been compared to ssAAV in retina and other ocular tissues in a number of studies. Yang *et al.* infected mouse retina with AAV serotype 2 and 5 vectors of full length or half length (capable of generating scAAV) and evaluated gene expression.[62] In a direct comparison of AAV-2 long and short vectors, the short vectors transduced 2,500-fold and 370-fold more retinal cells per particle than ssAAV at 5 and 15 weeks after infection, respectively. Interestingly, the short vectors also maintained 50-fold more genome copies in retinal cells at 14 weeks after infection, suggesting the loss of ssAAV genomes that were not converted to dsDNA in this tissue.

Yokoi *et al.* compared scAAV and ssAA-GFP serotype 2 vectors by subretinal and intravitreous injections in mice.[63] Subretinal doses as low as 10^7 particles of scAAV yielded rapid expression in retinal pigment epithelial cells and strong expression in photoreceptor cells at 28 days after infection. The same dose of ssAAV yielded strong expression in retinal pigment epithelium but little expression in photoreceptor cells. A tenfold higher dose yielded uniform transduction of photoreceptors and substantial transduction in the inner retina using scAAV vector. The ssAAV showed significantly less transduction of retinal pigment epithelium, and transduction of inner retina cells was not detectable until 28 days after infection. When the effects of subretinal injection were quantified by area of fluorescence, the scAAV transduction was higher by 4-, 2.2-, and 1.9-fold at 7, 14, and 28 days after infection, respectively. Similarly, at the lowest intravitreous injection dose, scAAV transduced widespread ganglion cells, and some cells in the inner nuclear layer, while no expression was detected from the ssAAV at this dose until 28 days after infection, when a small number of GFP-positive ganglion cells appeared. These differences were less pronounced at higher doses. This study revealed potentially important differences in the ability to target specific cell types accessible by subretinal injection, with both vectors effectively transducing retinal pigment epithelial cells, but the scAAV more effective in photoreceptors.

A more extensive comparison of vectors packaged in serotypes 2, 5, and 8, in subretinal, intravitreous, and intracameral injections corroborated the earlier results.[64] In all three serotypes, the scAAV yielded faster onset and higher stable levels of gene expression, with differences of 1.2-fold, 2.2-fold, and 2.3-fold for serotypes 5, 2, and 8, respectively. Serotype 8 capsids yielded the most efficient transduction in all cell types and, in contrast to AAV-2, allowed transduction of photoreceptors with ssAAV vector. This again suggests that the ability to transduce any given cell type with ssAAV is highly dependent on the number of vector genomes that can be delivered to the nucleus, by increasing either the probability of SA or second-strand DNA synthesis.

A study of gene delivery to the trabecular meshwork was somewhat more revealing mechanistically.[65] These cells were not permissive to ssAAV-2 transduction, or to an AAV vector engineered to display an integrin-binding motif, even though the trabecular meshwork cells are rich in integrin, suggesting that receptor binding and internalization were not rate limiting.[66] However, they were highly permissive for scAAV vectors, and transduction with ssAAV was increased >20-fold by co-infection with Ad, which promotes second-strand DNA synthesis.[1,2] Gene array analysis of ssAAV-GFP infected and uninfected trabecular meshwork cells showed minimal changes in gene expression, but those that were changed generally correlated with reduced cell cycling and DNA synthesis, as had been previously observed in normal fibroblasts infected with AAV vectors.[67] In contrast, the Ad co-infected trabecular meshwork cells had a much larger number of upregulated genes, suggesting that downregulation of DNA replication was responsible for the low rates of transduction with ssAAV vectors. While these observations were consistent with second-strand DNA synthesis being the rate-limiting step for ssAAV transduction, one caveat remains in that co-infection with Ad also increases the intracellular translocation of AAV-2 to the nucleus, which might also increase the number of genomes available for SA.[68]

The eye, as mentioned above, has unique attributes that affect the efficacy of gene delivery with AAV vectors. Most important for this discussion is the ability to isolate high concentrations of vector in the immediate vicinity of the target cells, which facilitates high multiplicity infection. While in every situation examined thus far, scAAV has been more efficient in transducing the various cell types of the eye, therapeutically useful levels of transduction could be achieved with ssAAV at higher doses, or using different serotypes. Therefore, additional factors, such as the ability to include large, cell type–specific promoters, may outweigh the transduction efficiency gained from scAAV even when the transgene-coding region is small enough to be accommodated.[69]

TRANSDUCTION IN BONE MARROW

There are a great many reasons to develop gene delivery to bone marrow–derived cells including reconstitution of the immune system, antigen presentation, and correction of hemoglobinopathies. While this is generally the realm of efficiently integrating gamma retrovirus and lentivirus vectors, which can exploit stem cell expansion, AAV vectors have also been shown to stably integrate in bone marrow long term–regenerating cell populations.[70–72] However, the efficiency of AAV infection is limited by a number of factors including variable expression of cellular receptors, intracellular trafficking, nuclear uncoating, and conversion to dsDNA, such that vector particle:cell ratios exceeding 10^4 per cell are typically required for effective transduction.[73,74] This level can be increased using scAAV vectors and optimized serotypes, and stable integration of the scAAV genome can be demonstrated in serial bone marrow transplantation in mice.[75–77] However, it did not appear that the integration frequency was significantly higher than ssAAV, which typically ranges ~0.1–0.5% of infecting genomes.[78] Analysis of the integration sites suggests that there was no preference for highly transcribed regions, as had been noted

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for ssAAV integration in hepatocytes, which may provide a safer alternative to retrovirus vectors.[79,80]

Bone marrow–derived dendritic cells (DCs) have applications in genetic immunotherapy for cancers and chronic infections because of their ability to express and present antigens to induce T-cell responses. The ability to express antigens from AAV vectors in DC would be advantageous because they would not express viral proteins, which can otherwise dominate the immune response. However, transduction of DC with AAV has been difficult and variable, though AAV-5 vectors have recently shown promise. Because only the major immunoreactive epitopes of the antigen need to be expressed in DC, scAAV would be an attractive option for these applications, and two recent studies have demonstrated greater efficacy with scAAV vectors in various serotypes. Aldrich *et al.* specifically tested bone marrow–derived DC with serotypes 1 through 6 and found maximimal transduction with AAV-6 vectors.[81] A comparison of ssAAV-6 and scAAV-6 vectors expressing CEA antigen showed an ~threefold increase in transduction after a single infection (7% versus 20%) and after multiple infections (12% versus 35%). The DC transduced with scAAV were functional and induced a CEA-specific immune response upon injection into naive mice. In the second study, Veron *et al.* tested ssAAV and scAAV serotypes 1 and 2 on plasmacytoid DC, myeloid conventional DC, or langerhans cells.[82] After a single exposure, transduction efficiencies were four- to tenfold greater in the various types of progenitor and differentiated DCs using the scAAV vectors. This difference was more pronounced for committed DC transduction. While the ability to transduce DCs for the purpose of immunotherapy is promising, it also suggests the possibility that scAAV gene therapy may carry the risk of inducing an immune response to the transgene through DC antigen presentation unless a cell type–specific promoter is used.

ANTICANCER THERAPIES

Another application where speed and efficiency of vector expression offers a compelling premium is in cancer gene therapy, targeting the cancer cells themselves rather than Ag-presenting cells. There are numerous reports describing the use of rAAV for the expression of cytokines and signaling molecules in tumor tissues, to stimulate an immune response or apoptosis, or antiangiogenisis factors to limit tumor growth. Most of these genes are small and are easily accommodated in scAAV vectors. The relative efficiency of ssAAV and scAAV, pseudotyped into different AAV capsids, has been tested in several tumor cell lines.[45,83,84] As expected, transduction was significantly greater with scAAV, with improvements ranging from three- to eightfold. As noted above, synergistic effects between scAAV and proteosome inhibitor treatment were observed in tumor cells that were highly resistant to AAV transduction. In one study, the efficacy of an scAAV-5 vector expressing CD40L was demonstrated in an *in vivo* xenograft model.[84] After injection of vector into an established tumor, a 66% inhibition of tumor growth was observed.

CELLS NOT RESPONDING TO scAAV

There have been at least two reports to date of cell types that do not appear to show improved transduction with scAAV vectors. Polarized airway epithelial cells are generally resistant to transduction from the apical surface with ssAAV-2 vectors, although this is improved dramatically by treatment with proteosome inhibitors. Transduction with ssAAV-2/5 is marginally higher and also increased by proteosome inhibitors. Ding *et al.* used scAAV-2 and 2/5 vectors to determine whether the barrier to transduction was at the level of dsDNA conversion.[85] After careful characterization of the scAAV vector genome content and demonstration of the expected improved transduction in HeLa cells, they did not see any improvement in the polarized airway cultures. This suggested that dsDNA conversion was not a barrier to transduction in this airway cell model.

In a second study, primary B-cell chronic lymphocytic leukemia (B-CLL) cells could be transduced with ssAAV only after co-cultivation with HeLa cells expressing CD40L as a stimulatory molecule.[86] A different stimulatory treatment, CpG oligodeoxynucletide, did not increase ssAAV transduction alone, but did have a synergistic effect with CD40L feeder cells. The use of a carefully characterized scAAV vector did not eliminate the requirement for CD40L stimulation to get efficient transduction, nor did it increase the level of transduction of stimulated cells compared to ssAAV vector. Analysis of ssAAV-transduced B-CLL cells stimulated with CD40L alone, or in combination with CpG oligodeoxynucletide, revealed very high vector genome copy numbers (2,480 and 4,080/cell, respectively). Although there is as yet no definitive explanation for the inability of scAAV to improve on transduction in these cell types, we would speculate that when a relatively small population of cells takes up a large number of vector genomes, dsDNA conversion, either by SA or DNA synthesis, is likely to be very efficient.

FATE OF scAAV GENOMES

There has been a great deal of effort made toward understanding what happens to the AAV vector genome once it enters the nucleus, particularly in terms of the potential for genotoxicity through integration in the chromosome.[21] It is clear at this point that the vast majority of vector genomes persist as episomes, either circular monomers or circularized concatemers.[78] This generally appears to be the fate of scAAV genomes as well, although subtle differences in processing by host DNA repair pathways are likely to emerge because the scAAV is not recognized by the host as ssDNA, unlike the ssAAV genome. The circularization of scAAV has been characterized in a panel of DNA repair–deficient cell lines and found to be highly dependent on several factors related to homologous recombination including ATM, NBS1, MRE11, WRN, and BLM, but not DNA-PK_{CS} or ATR.[87] The ATM protein generally responds to DNA double-strand breaks, whereas ATR responds to DNA damage containing significant ssDNA regions. When injected into muscle *in vivo*, circularization of scAAV DNA proceeds over the course of 5–6 weeks in normal mice, and is significantly diminished in ATM and DNA-PK_{CS}, but not NBS1-deficient mice. The different requirements for DNA-PK_{CS} and NBS1 in cultured cells and muscle probably relates to differing cell-cycle status.

In mouse liver, a high dose of scAAV leads to the early formation of monomeric circles and linear concatemers at 1 day after injection, with the concatemers converting to concatemeric circles by 42 days after injection.[42] Although these transformations

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do require host–cell DNA recombination and DNA synthesis factors, they are not essential for efficient vector gene expression, which can occur from linear molecules in the absence of specific DNA repair factors.[87,88] Several of the studies discussed above have noted the relative stability, or persistence, of the scAAV genome, and this may be a consequence of the instability of ssAAV during the single-stranded phase.[46] However, Wang *et al* have recently characterized a transient period of instability of AAV vector genomes in mouse liver and found that it applies to scAAV as well. Further, a significant loss of ssAAV genomes occurred after dsDNA conversion. This suggests that there may be multiple pathways for degradation of conventional ssAAV genomes, some acting during the single-strand phase and some on the dsDNA genomes after conversion. Because scAAV and ssAAV present a biologically different substrate to the cell upon infection, it will be important to fully characterize any differences in processing between the two.

FUTURE DIRECTIONS

Numerous studies have documented the increased transduction efficiency of scAAV relative to the cognate ssAAV vectors in rodent, canine, and nonhuman primate animal models. The greatest differences are observed in applications where the vector is widely disseminated, such as systemic delivery strategies to liver or other tissues. Smaller, though still significant, differences are observed with local injection of vector into parenchymal tissues or constrained spaces, such as the eye. The likely explanation for why scAAV improves systemic delivery more than local delivery strategies is that high copy numbers of ssAAV genomes favor conversion to dsDNA by SA and increase the probability of dsDNA conversion by DNA synthesis. Therefore, the size and accessibility of the therapeutic target will be an important consideration when evaluating the potential benefits of using scAAV and weighing them against the reduced capacity for carrying transcriptional and post-transcriptional regulatory elements. While the size of scAAV vectors is limiting, the recent observation of larger genomes being packaged in AAV-5 raises the exciting possibility of greatly expanding the potential applications for these vectors.

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REFERENCES

1. Ferrari, FK, Samulski, T, Shenk, T and Samulski, RJ (1996). Second-strand synthesis is a rate limiting step for efficient transduction by recombinant adeno-associated virus vectors. *J Virol* **70**: 3227–3234.
2. Fisher, KJ, Gao, GP, Weitzman, MD, DeMatteo, R, Burda, JF and Wilson, JM (1996). Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. *J Virol* **70**: 520–532.
3. Duan, D, Yue, Y, Yan, Z, Yang, J and Engelhardt, JF (2000). Endosomal processing limits gene transfer to polarized airway epithelia by adeno-associated virus. *J Clin Invest* **105**: 1573–1587.
4. Sipo, I, Fechner, H, Pinkert, S, Suckau, L, Wang, X, Weger, S *et al.* (2007). Differential internalization and nuclear uncoating of self-complementary adeno-associated virus pseudotype vectors as determinants of cardiac cell transduction. *Gene Ther* **14**: 1319–1329.
5. Thomas, CE, Storm, TA, Huang, Z and Kay, MA (2004). Rapid uncoating of vector genomes is the key to efficient liver transduction with pseudotyped adeno-associated virus vectors. *J Virol* **78**: 3110–3122.
6. Wang, J, Xie, J, Lu, H, Chen, L, Hauck, B, Samulski, RJ *et al.* (2007). Existence of transient functional double-stranded DNA intermediates during recombinant AAV transduction. *Proc Natl Acad Sci USA* **104**: 13104–13109.
7. Nash, K, Chen, W, McDonald, WF, Zhou, X and Muzyczka, N (2007). Purification of host cell enzymes involved in adeno-associated virus DNA replication. *J Virol* **81**: 5777–5787.
8. Nash, K, Chen, W and Muzyczka, N (2008). Complete *in vitro* reconstitution of adeno-associated virus DNA replication requires the minichromosome maintenance complex proteins. *J Virol* **82**: 1458–1464.
9. Nakai, H, Storm, TA and Kay, MA (2000). Recruitment of single-stranded recombinant adeno-associated virus vector genomes and intermolecular recombination are responsible for stable transduction of liver *in vivo*. *J Virol* **74**: 9451–9463.
10. Berns, KI (1990). Parvovirus replication. *Microbiol Rev* **54**: 316–329.
11. Duan, D, Yue, Y and Engelhardt, JF (2001). Expanding AAV packaging capacity with trans-splicing or overlapping vectors: a quantitative comparison. *Mol Ther* **4**: 383–391.
12. Halbert, CL, Allen, JM and Miller, AD (2002). Efficient mouse airway transduction following recombination between AAV vectors carrying parts of a larger gene. *Nat Biotechnol* **20**: 697–701.
13. Zhong, L, Zhou, X, Li, Y, Qing, K, Xiao, X, Samulski, RJ *et al.* (2008). Single-polarity recombinant adeno-associated virus 2 vector-mediated transgene expression *in vitro* and *in vivo*: mechanism of transduction. *Mol Ther* **16**: 290–295.
14. Zhou, X, Zeng, X, Fan, Z, Li, C, McCown, T, Samulski, RJ *et al.* (2008). Adeno-associated virus of a single-polarity DNA genome is capable of transduction *in vivo*. *Mol Ther* **16**: 494–499.
15. Alexander, IE, Russell, DW and Miller, AD (1994). DNA-damaging agents greatly increase the transduction of nondividing cells by adeno-associated virus vectors. *J Virol* **68**: 8282–8287.
16. Alexander, IE, Russell, DW, Spence, AM and Miller, AD (1996). Effects of gamma irradiation on the transduction of dividing and nondividing cells in brain and muscle of rats by adeno-associated virus vectors. *Hum Gene Ther* **7**: 841–850.
17. Zhao, W, Wu, J, Zhong, L and Srivastava, A (2007). Adeno-associated virus 2-mediated gene transfer: role of a cellular serine/threonine protein phosphatase in augmenting transduction efficiency. *Gene Ther* **14**: 545–550.
18. Zhong, L, Chen, L, Li, Y, Qing, K, Weigel-Kelley, KA, Chan, RJ *et al.* (2004). Self-complementary adeno-associated virus 2 (AAV)-T cell protein tyrosine phosphatase vectors as helper viruses to improve transduction efficiency of conventional single-stranded AAV vectors *in vitro* and *in vivo*. *Mol Ther* **10**: 950–957.
19. Kay, MA and Nakai, H (2003). Looking into the safety of AAV vectors. *Nature* **424**: 251.
20. Miller, DG, Petek, LM and Russell, DW (2004). Adeno-associated virus vectors integrate at chromosome breakage sites. *Nat Genet* **36**: 767–773.
21. Russell, DW (2007). AAV vectors, insertional mutagenesis and cancer. *Mol Ther* **15**: 1740–1743.
22. Peng, D, Qian, C, Sun, Y, Barajas, MA and Prieto, J (2000). Transduction of hepatocellular carcinoma (HCC) using recombinant adeno-associated virus (rAAV): *in vitro* and *in vivo* effects of genotoxic agents. *J Hepatol* **32**: 975–985.
23. Carter, BJ, Khoury, G and Rose, JA (1972). Adenovirus-associated virus multiplication. IX. Extent of transcription of the viral genome *in vivo*. *J Virol* **10**: 1118–1125.
24. McCarty, DM, Monahan, PE and Samulski, RJ (2001). Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. *Gene Ther* **8**: 1248–1254.
25. Koeberl, DD, Pinto, C, Sun, B, Li, S, Kozink, DM, Benjamin, DK Jr. *et al.* (2008). AAV vector-mediated reversal of hypoglycemia in canine and murine glycogen storage disease type Ia. *Mol Ther* **16**: 665–672.
26. Chapman, MS and Rossmann, MG (1995). Single-stranded DNA-protein interactions in canine parvovirus. *Structure* **3**: 151–162.
27. King, JA, Dubielzig, R, Grimm, D and Kleinschmidt, JA (2001). DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. *EMBO J* **20**: 3282–3291.
28. Im, D-S and Muzyczka, N (1990). The AAV origin binding protein Rep68 is an ATP-dependent site-specific endonuclease with DNA helicase activity. *Cell* **61**: 447–457.
29. McCarty, DM, Fu, H, Monahan, PE, Toulson, CE, Naik, P and Samulski, RJ (2003). Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction *in vivo*. *Gene Ther* **10**: 2112–2118.
30. Wang, Z, Ma, HI, Li, J, Sun, L, Zhang, J and Xiao, X (2003). Rapid and highly efficient transduction by double-stranded adeno-associated virus vectors *in vitro* and *in vivo*. *Gene Ther* **10**: 2105–2111.
31. Wu, J, Zhao, W, Zhong, L, Han, Z, Li, B, Ma, W *et al.* (2007). Self-complementary recombinant adeno-associated viral vectors: packaging capacity and the role of rep proteins in vector purity. *Hum Gene Ther* **18**: 171–182.
32. Xu, D, McCarty, D, Fernandes, A, Fisher, M, Samulski, RJ and Juliano, RL (2005). Delivery of MDR1 small interfering RNA by self-complementary recombinant adeno-associated virus vector. *Mol Ther* **11**: 523–530.
33. Wu, Z, Sun, J, Zhang, T, Yin, C, Yin, F, Van Dyke, T *et al.* (2008). Optimization of self-complementary AAV vectors for liver-directed expression results in sustained correction of hemophilia B at low vector dose. *Mol Ther* **16**: 280–289.
34. Dong, JY, Fan, PD and Frizzell, RA (1996). Quantitative analysis of the packaging capacity of recombinant adeno-associated virus. *Hum Gene Ther* **7**: 2101–2112.
35. Hermonat, PL, Quirk, JG, Bishop, BM and Han, L (1997). The packaging capacity of adeno-associated virus (AAV) and the potential for wild-type-plus AAV gene therapy vectors. *FEBS Lett* **407**: 78–84.
36. Allocca, M, Doria, M, Petrillo, M, Colella, P, Garcia-Hoyos, M, Gibbs, D *et al.* (2008). Serotype-dependent packaging of large genes in adeno-associated viral vectors results in effective gene delivery in mice. *J Clin Invest* **118**: 1955–1964.
37. Grieger, JC and Samulski, RJ (2005). Packaging capacity of adeno-associated virus serotypes: impact of larger genomes on infectivity and postentry steps. *J Virol* **79**: 9933–9944.
38. Miao, CH, Nakai, H, Thompson, AR, Storm, TA, Chiu, W, Snyder, RO *et al.* (2000). Nonrandom transduction of recombinant adeno-associated virus vectors in mouse hepatocytes *in vivo*: cell cycling does not influence hepatocyte transduction. *J Virol* **74**: 3793–3803.

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© The American Society of Gene Therapy

39. Rutledge, EA, Halbert, CL and Russell, DW (1998). Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. *J Virol* **72**: 309–319.
40. Gao, GP, Alvira, MR, Wang, L, Calcedo, R, Johnston, J and Wilson, JM (2002). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc Natl Acad Sci USA* **99**: 11854–11859.
41. Gao, GP, Lu, Y, Sun, X, Johnston, J, Calcedo, R, Grant, R *et al.* (2006). High-level transgene expression in nonhuman primate liver with novel adeno-associated virus serotypes containing self-complementary genomes. *J Virol* **80**: 6192–6194.
42. Nathwani, AC, Gray, JT, Ng, CY, Zhou, J, Spence, Y, Waddington, SN *et al.* (2006). Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. *Blood* **107**: 2653–2661.
43. Nathwani, AC, Gray, JT, McIntosh, J, Ng, CY, Zhou, J, Spence, Y *et al.* (2007). Safe and efficient transduction of the liver after peripheral vein infusion of self-complementary AAV vector results in stable therapeutic expression of human FIX in nonhuman primates. *Blood* **109**: 1414–1421.
44. Koeberl, DD, Sun, BD, Damodaran, TV, Brown, T, Millington, DS, Benjamin, DK Jr. *et al.* (2006). Early, sustained efficacy of adeno-associated virus vector-mediated gene therapy in glycogen storage disease type Ia. *Gene Ther* **13**: 1281–1289.
45. Hacker, UT, Wingenfeld, L, Kofler, DM, Schuhmann, NK, Lutz, S, Herold, T *et al.* (2005). Adeno-associated virus serotypes 1 to 5 mediated tumor cell directed gene transfer and improvement of transduction efficiency. *J Gene Med* **7**: 1429–1438.
46. Ren, C, Kumar, S, Shaw, DR and Ponnazhagan, S (2005). Genomic stability of self-complementary adeno-associated virus 2 during early stages of transduction in mouse muscle *in vivo*. *Hum Gene Ther* **16**: 1047–1057.
47. Andino, LM, Conlon, TJ, Porvasnik, SL, Boye, SL, Hauswirth, WW and Lewin, AS (2007). Rapid, widespread transduction of the murine myocardium using self-complementary Adeno-associated virus. *Genet Vaccines Ther* **5**: 13.
48. Blankinship, MJ, Gregorevic, P, Allen, JM, Harper, SQ, Harper, H, Halbert, CL *et al.* (2004). Efficient transduction of skeletal muscle using vectors based on adeno-associated virus serotype 6. *Mol Ther* **10**: 671–678.
49. Wang, Z, Zhu, T, Qiao, C, Zhou, L, Wang, B, Zhang, J *et al.* (2005). Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. *Nat Biotechnol* **23**: 321–328.
50. McCown, TJ (2005). Adeno-associated virus (AAV) vectors in the CNS. *Curr Gene Ther* **5**: 333–338.
51. Fu, H, Muenzer, J, Samulski, RJ, Breese, G, Sifford, J, Zeng, X *et al.* (2003). Self-complementary adeno-associated virus serotype 2 vector: global distribution and broad dispersion of AAV-mediated transgene expression in mouse brain. *Mol Ther* **8**: 911–917.
52. Fu, H, Kang, L, Jennings, JS, Moy, SS, Perez, A, Dirosario, J *et al.* (2007). Significantly increased lifespan and improved behavioral performances by rAAV gene delivery in adult mucopolysaccharidosis IIIB mice. *Gene Ther* **14**: 1065–1077.
53. Foust, KD, Poirier, A, Pacak, CA, Mandel, RJ and Flotte, TR (2008). Neonatal intraperitoneal or intravenous injections of recombinant adeno-associated virus type 8 transduce dorsal root Ganglia and lower motor neurons. *Hum Gene Ther* **19**: 61–70.
54. Kaspar, BK, Llado, J, Sherkat, N, Rothstein, JD and Gage, FH (2003). Retrograde viral delivery of IGF-1 prolongs survival in a mouse ALS model. *Science* **301**: 839–842.
55. Boulis, NM, Willmarth, NE, Song, DK, Feldman, EL and Imperiale, MJ (2003). Intraneural colchicine inhibition of adenoviral and adeno-associated viral vector remote spinal cord gene delivery. *Neurosurgery* **52**: 381–387; discussion 387.
56. Hollis, ER 2nd, Kadoya, K, Hirsch, M, Samulski, RJ and Tuszynski, MH (2008). Efficient retrograde neuronal transduction utilizing self-complementary AAV1. *Mol Ther* **16**: 296–301.
57. Alexander, JJ and Hauswirth, WW (2008). Adeno-associated viral vectors and the retina. *Adv Exp Med Biol* **613**: 121–128.
58. Buch, PK, Bainbridge, JW and Ali, RR (2008). AAV-mediated gene therapy for retinal disorders: from mouse to man. *Gene Ther* **15**: 849–857.
59. Surace, EM and Auricchio, A (2008). Versatility of AAV vectors for retinal gene transfer. *Vision Res* **48**: 353–359.
60. Bainbridge, JW, Smith, AJ, Barker, SS, Robbie, S, Henderson, R, Balaggan, K *et al.* (2008). Effect of Gene Therapy on Visual Function in Leber's Congenital Amaurosis. *N Engl J Med* **358**: 2231–2239.
61. Maguire, AM, Simonelli, F, Pierce, EA, Pugh, EN Jr., Mingozzi, F, Bennicelli, J *et al.* (2008). Safety and Efficacy of Gene Transfer for Leber's Congenital Amaurosis. *N Engl J Med* **358**: 2240–2248.
62. Yang, GS, Schmidt, M, Yan, Z, Lindbloom, JD, Harding, TC, Donahue, BA *et al.* (2002). Virus-mediated transduction of murine retina with adeno-associated virus: effects of viral capsid and genome size. *J Virol* **76**: 7651–7660.
63. Yokoi, K, Kachi, S, Zhang, HS, Gregory, PD, Spratt, SK, Samulski, RJ *et al.* (2007). Ocular gene transfer with self-complementary AAV vectors. *Invest Ophthalmol Vis Sci* **48**: 3324–3328.
64. Natkunarajah, M, Trittibach, P, McIntosh, J, Duran, Y, Barker, SE, Smith, AJ *et al.* (2008). Assessment of ocular transduction using single-stranded and self-complementary recombinant adeno-associated virus serotype 2/8. *Gene Ther* **15**: 463–467.
65. Borras, T, Xue, W, Choi, VW, Bartlett, JS, Li, G, Samulski, RJ *et al.* (2006). Mechanisms of AAV transduction in glaucoma-associated human trabecular meshwork cells. *J Gene Med* **8**: 589–602.
66. Shi, W and Bartlett, JS (2003). RGD inclusion in VP3 provides adeno-associated virus type 2 (AAV2)-based vectors with a heparan sulfate-independent cell entry mechanism. *Mol Ther* **7**: 515–525.
67. Stilwell, JL and Samulski, RJ (2004). Role of viral vectors and virion shells in cellular gene expression. *Mol Ther* **9**: 337–346.
68. Xiao, W, Warrington, KH Jr., Hearing, P, Hughes, J and Muzyczka, N (2002). Adenovirus-facilitated nuclear translocation of adeno-associated virus type 2. *J Virol* **76**: 11505–11517.
69. Komaromy, AM, Alexander, JJ, Cooper, AE, Chiodo, VA, Acland, GM, Hauswirth, WW *et al.* (2008). Targeting gene expression to cones with human cone opsin promoters in recombinant AAV. *Gene Ther* **15**: 1049–1055.
70. Chatterjee, S, Li, W, Wong, CA, Fisher-Adams, G, Lu, D, Guha, M *et al.* (1999). Transduction of primitive human marrow and cord blood-derived hematopoietic progenitor cells with adeno-associated virus vectors. *Blood* **93**: 1882–1894.
71. Fisher-Adams, G, Wong, KK Jr., Podsakoff, G, Forman, SJ and Chatterjee, S (1996). Integration of adeno-associated virus vectors in CD34+ human hematopoietic progenitor cells after transduction. *Blood* **88**: 492–504.
72. Ponnazhagan, S, Yoder, MC and Srivastava, A (1997). Adeno-associated virus type 2-mediated transduction of murine hematopoietic cells with long-term repopulating ability and sustained expression of a human globin gene *in vivo*. *J Virol* **71**: 3098–3104.
73. Ponnazhagan, S, Mukherjee, P, Wang, XS, Qing, K, Kube, DM, Mah, C *et al.* (1997). Adeno-associated virus type 2-mediated transduction in primary human bone marrow-derived CD34+ hematopoietic progenitor cells: donor variation and correlation of transgene expression with cellular differentiation. *J Virol* **71**: 8262–8267.
74. Srivastava, A (2005). Hematopoietic stem cell transduction by recombinant adeno-associated virus vectors: problems and solutions. *Hum Gene Ther* **16**: 792–798.
75. Zhong, L, Li, W, Li, Y, Zhao, W, Wu, J, Li, B *et al.* (2006). Evaluation of primitive murine hematopoietic stem and progenitor cell transduction *in vitro* and *in vivo* by recombinant adeno-associated virus vector serotypes 1 through 5. *Hum Gene Ther* **17**: 321–333.
76. Han, Z, Zhong, L, Maina, N, Hu, Z, Li, X, Chouthai, NS *et al.* (2008). Stable integration of recombinant adeno-associated virus vector genomes after transduction of murine hematopoietic stem cells. *Hum Gene Ther* **19**: 267–278.
77. Maina, N, Han, Z, Li, X, Hu, Z, Zhong, L, Bischof, D *et al.* (2008). Recombinant self-complementary adeno-associated virus serotype vector-mediated hematopoietic stem cell transduction and lineage-restricted, long-term transgene expression in a murine serial bone marrow transplantation model. *Hum Gene Ther* **19**: 376–383.
78. McCarty, DM, Young, SM Jr. and Samulski, RJ (2004). Integration of adeno-associated virus (AAV) and recombinant AAV vectors. *Annu Rev Genet* **38**: 819–845.
79. Nakai, H, Montini, E, Fuess, S, Storm, TA, Grompe, M and Kay, MA (2003). AAV serotype 2 vectors preferentially integrate into active genes in mice. *Nat Genet* **34**: 297–302.
80. Russell, DW (2003). AAV loves an active genome. *Nat Genet* **34**: 241–242.
81. Aldrich, WA, Ren, C, White, AF, Zhou, SZ, Kumar, S, Jenkins, CB *et al.* (2006). Enhanced transduction of mouse bone marrow-derived dendritic cells by repetitive infection with self-complementary adeno-associated virus 6 combined with immunostimulatory ligands. *Gene Ther* **13**: 29–39.
82. Veron, P, Allo, V, Riviere, C, Bernard, J, Douar, AM and Masurier, C (2007). Major subsets of human dendritic cells are efficiently transduced by self-complementary adeno-associated virus vectors 1 and 2. *J Virol* **81**: 5385–5394.
83. Lee, HS, Shin, OK, Kim, SJ, Lee, WI, Jeong, S, Park, K *et al.* (2007). Efficient gene expression by self-complementary adeno-associated virus serotype 2 and 5 in various human cancer cells. *Oncol Rep* **18**: 611–616.
84. Wu, JQ, Zhao, WH, Li, Y, Zhu, B and Yin, KS (2007). Adeno-associated virus mediated gene transfer into lung cancer cells promoting CD40 ligand-based immunotherapy. *Virology* **368**: 309–316.
85. Ding, W, Yan, Z, Zak, R, Saavedra, M, Rodman, DM and Engelhardt, JF (2003). Second-strand genome conversion of adeno-associated virus type 2 (AAV-2) and AAV-5 is not rate limiting following apical infection of polarized human airway epithelia. *J Virol* **77**: 7361–7366.
86. Theiss, HD, Kofler, DM, Buning, H, Aldenhoff, AL, Kaess, B, Decker, T *et al.* (2003). Enhancement of gene transfer with recombinant adeno-associated virus (rAAV) vectors into primary B-cell chronic lymphocytic leukemia cells by CpG-oligodeoxynucleotides. *Exp Hematol* **31**: 1223–1229.
87. Choi, VW, McCarty, DM and Samulski, RJ (2006). Host cell DNA repair pathways in adeno-associated viral genome processing. *J Virol* **80**: 10346–10356.
88. Choi, VW, Samulski, RJ and McCarty, DM (2005). Effects of adeno-associated virus DNA hairpin structure on recombination. *J Virol* **79**: 6801–6807.

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