CELL & GENE THERAPY INSIGHTS

CHALLENGES AND ADVANCES IN VIRAL VECTORS

SPOTLIGHT

EXPERT INSIGHT

Advances and challenges in the use of recombinant adeno-associated virus vectors for human gene therapy

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Recombinant vectors based on a non-pathogenic parvovirus, the adeno-associated virus (AAV), have taken center stage in the past decade. The well-established safety of AAV vectors in 162 Phase I/II clinical trials (and one recent Phase III trial) in humans to date, as well as their clinical efficacy in several human diseases, are now well documented. Despite these remarkable achievements, it is becoming increasingly clear that the full potential of AAV vectors composed of the naturally occurring capsids is unlikely to be realized. In this Expert Insight article, I will describe the advances that have been made, and the challenges that remain, in the optimal use of AAV vectors in human gene therapy applications. I will also attempt to provide additional avenues of research and development that could be pursued in order to further ensure both safety and efficacy of AAV vectors in targeting a wide variety of human diseases, both genetic and acquired, in the not-too-distant future.

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Adeno-associated virus 2 (AAV2), the most extensively studied prototype, is a small, naked icosahedral virus, which was first discovered in 1965 [1]. Approximately 90% of the human population is seropositive for AAV2 antibodies [2], implying that most humans have been exposed to the wild-type AAV2 (WT AAV2), yet there is no conclusive evidence that AAV2 infection leads to any known disease in humans, although there has been a recent report claiming that the WT AAV2 is the etiologic agent of hepatocellular carcinoma (HCC) [3], a claim that has been seriously questioned by us and others [4,5]. Recombinant vectors based on AAV2 (rAAV2), on the other hand, have been, or are currently being, used in a number of Phase I/II clinical trials, and thus far, no serious adverse events, much less cancer of any type have ever been observed

or reported [6]. Furthermore, the use of rAAV2 vectors has led to clinical efficacy in the potential gene therapy of at least three human diseases: Leber's congenital amaurosis (LCA) [7–10], aromatic L-amino acid decarboxylase deficiency (AADC) [11] and choroideremia [12]. In the past decade, at least 12 additional AAV serotype vectors, some derived from non-human primates, have also become available [13–21]. rAAV1 vectors have successfully been used in the gene therapy of lipoprotein lipase deficiency [22], and rAAV8 vectors have shown clinical efficacy in the potential gene therapy of hemophilia B [23,24].

Despite these remarkable achievements, I have argued that the first generation of rAAV vectors are unlikely to reach their full potential until we gain a better understanding of how rAAV vectors interact with the target cell, and have also posited that the WT AAV did not evolve to be used as a vector for the delivery of therapeutic genes [25]. A brief historical account follows.

VECTOR–HOST CELL INTERACTIONS: DISCOVERY OF THE CELLULAR RECEPTOR & CO-RECEPTORS FOR AAV2 & ITS IMPLICATIONS IN GENE THERAPY

AAV2 was discovered in 1965 [1], but for nearly three decades, it was generally assumed that infection by AAV2 was non-specific, because all cell types across the species barrier could be infected by AAV2. However, in 1996, we identified a human megakaryocytic leukemia cell line, MB-02, that could not be infected by the WT AAV2, or transduced by recombinant AAV2 vectors [26]. This observation prompted us to suggest that AAV2 infection of human cells is receptor-mediated. Indeed, Summerford and Samulski identified heparan sulfate proteoglycan (HSPG) as the first cellular receptor for AAV2 in 1998 [27]. The identification of HSPG as a cellular receptor provided an explanation as to why AAV2 infects all cell types across the species barrier since all cells express HSPG. Interestingly, we documented that MB-02, and a second human megakaryocytic leukemia cell line M07e, lack HSPG expression [26]. The discovery of the cellular receptor for AAV2 also provided the explanation as to why the very first Phase I clinical trial with AAV2 vectors for the potential gene therapy of cystic fibrosis, performed by Flotte and colleagues [28], did not show clinical efficacy since human airway epithelial cells express HSPG predominantly on the baso-lateral surface, rather than on the apical surface, and as a consequence, are not efficiently transduced by AAV2 vectors [29]. Thus, these observations further reinforced the value of basic science of AAV biology, with direct implications in the use of AAV vectors in human gene therapy.

Soon after the discovery of HSPG as the cellular receptor for AAV2, which is required for binding of AAV2 to the cell surface, we observed that HSPG alone was insufficient to mediate viral entry into cells. In 1999, we reported the identification of human fibroblast growth factor receptor 1 (FGFR1) as the first cellular co-receptor, which AAV2 utilizes to gain entry into cells [30]. In addition, Summerford *et al.* identified αVβ5 as yet another co-receptor for AAV2 [31].

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However, when Chen *et al.* isolated AAV sequences from various tissues, predominantly tonsils, from children, they observed that although 7% of these 'AAV2-like' sequences shared ~98% identity with the WT AAV2, they lacked the HSPG-binding site, and consequently, failed to bind to the cellular receptor [32]. These authors concluded that AAV2 either utilizes other putative cellular receptors as well *in vivo*, or the use of HSPG is a consequence of long-term propagation of AAV2 in tissue culture *in vitro*. Regardless, the use of rAAV2 vectors, from which the HSPG-binding domain has been deleted transduce murine brain and retinal tissues more extensively than their unmodified counterpart [33,34]. Furthermore, AAV2 has been shown to utilize at least four additional cellular co-receptors – hepatocyte growth factor receptor (HGFR) [35], α5β1 integrin [36]; laminin receptor (LamR) [37]; and CD9 [38] – in addition to FGFR1 [30] and αVβ5 [31], for viral entry. Thus, these studies have yielded a much clearer picture of AAV2–host cell interactions, none of which was available when the first clinical trial for the potential gene therapy of cystic fibrosis was pursued in 1996 [28].

In addition to AAV2, a number of additional AAV serotypes have since become available [13–21]. To date, at least 13 distinct AAV serotype vectors (AAV1 – AAV13) have been described, and it is highly likely that this number will continue to grow. The ten most commonly used AAV serotype vectors are depicted schematically in **Figure 1**. Although the precise mechanism of transduction by these AAV serotype vectors *in vivo* remains unknown, in general, it has been observed that AAV1 – AAV6 serotype vectors transduce tissue culture cells to various degrees of efficacy *in vitro*, and by and large, AAV7 – AAV10 serotype vectors transduce various tissues and organs efficiently in experimental animal models *in vivo*. There are only limited data on the transduction efficiency of AAV12 and AAV13 vectors.

Although it is clear that attachment to putative cell surface receptors is the initial step for successful transduction by each of the AAV serotype vectors, and the following 23 different glycan receptors have been identified: α2-3 and α2-6 N-linked sialic acid (SIA) for AAV1 [39,40]; HSPG for AAV2, AAV3 and AAV13 [21,27,41]; α2-3 O-linked and α2-3 N-linked SIAs for AAV4 and AAV5, respectively [42–44]; HSPG and α2-3 and α2-6 N-linked SIA for AAV6 [40,45,46]; and terminal N-linked galactose (GAL) of SIA for AAV9 [47,48]. The primary cellular receptors for AAV7, AAV8, AAV9, AAVrh10, AAV11, AAV12 and AAV13 serotypes have not yet been identified.

More recently, a trans-membrane protein, designated as an essential receptor for AAV2 infection (AAVR) was identified, which was shown to bind directly to AAV2, and was found to be a critical factor for infection by several AAV serotypes [49]. Thus, AAVR was reported to be a universal receptor for AAV infection, but what role, if any, AAVR plays in large animal models, and especially in humans, remains to be evaluated. It is clear, however, that binding to the primary cellular receptors is unlikely to be sufficient for AAV serotype vectors for gaining entry into cells, and most likely, additional cell surface as co-receptors are required. The following cellular co-receptors have been identified thus far: FGFR1 [30], αVβ5 [31]

FIGURE 1

The most commonly used recombinant AAV serotype vectors and their tissue-tropism.

Various murine tissues and organs that have been reported to be transduced efficiently with various AAV serotype vectors are indicated. AAV3 serotype vectors in particular, have been shown to transduce human hepatocytes well [60,63–65,100]. Similarly AAV6 serotype vectors transduce primary human hematopoietic stem/progenitor cells exceedingly well [141–143].

and α5β1 [36] integrins for AAV2; a putative integrin for AAV9 [50]; FGFR1 for AAV3 [51]; HGFR for AAV2 [35] and AAV3 [52]; platelet-derived growth factor receptor (PDGFR) for AAV5 [53]; epidermal growth factor receptor (EGFR) for AAV6 [54]; and laminin receptor (LamR) for AAV2, AAV3, AAV8 and AAV9 [37]. Based on these studies, the tissue-tropisms of AAV serotype vectors have been determined, which are also indicated in Figure 1. However, for the most part, a large body of our current knowledge of AAV vector tropism has been derived from studies with rodent models, which, in my opinion, are poor surrogates for humans [55]. Here, I will provide one specific example to corroborate my contention.

In 1997, we first reported the liver tropism of rAAV2 vectors, following intravenous administration, in a murine model *in vivo* [56], an observation that was subsequently replicated by other groups [57,58]. Based on those studies, a Phase I clinical trial for hemophilia B was carried out with rAAV2 vectors expressing the human clotting factor IX (h.FIX) [59]. Even though in

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pre-clinical studies with both hemophilic murine and canine models, rAAV2-F.IX vectors provided complete phenotypic correction of the disease for the entire lifespans of these animals, the predicted dose of these vectors in humans did not express therapeutic levels of F.IX in humans. Although the administration of a ten-fold higher vector dose did lead to expression of therapeutic levels of F.IX in one patient, it was short-lived due to the host immune response to AAV2 capsid proteins [59]. The lesson learned from this first liver-directed gene therapy trial was that AAV2 serotype vectors, although effective in mice and dogs, were not optimal for humans.

On the basis of subsequent studies with rAAV8 serotype vectors, which established the far superior efficacy of these vectors in murine hepatocytes, compared with rAAV2 vectors, rAAV8-F.IX vectors were used in a second Phase I clinical trial in patients with hemophilia B [23]. Although this landmark trial with rAAV8 vectors has been deemed highly successful [24], we raised the issue of whether AAV8 is really the optimal serotype for human hepatocytes. Nearly a decade ago, we identified the AAV3 serotype (which was largely ignored by the AAV community because it fails to transduce any cell/tissue/organ in mice) as the most efficient vector for transducing human hepatocytes, both malignant and primary [60].

We later discovered the basis of the selective tropism of AAV3, which was due to the use of HGFR as a co-receptor by AAV3 [52]. Although human and mouse HGFRs share 88% identity, there are four amino acids in the extracellular domain of human HGFR that AAV3 recognizes and binds to, which are different in the mouse HGFR. These studies were subsequently extended to include murine xenograft models to establish the remarkable specificity and efficacy of AAV3 vectors [61,62].

Interestingly, human and non-human primate HGFRs share 99% identity, and in our recent studies, we were able to achieve selective and high-efficiency transduction of NHP livers, both short-term (7 days) and long-term (91 days), following intravenous delivery of rAAV3 vectors, with no apparent toxicity at a relatively high dose of 1 x 10^{13} vgs/kg [63]. These studies were corroborated by Wang *et al.* [64], which further established the remarkable specificity, efficacy and safety of AAV3 vectors [63,64].

In our more recent studies with humanized mouse models, we have reported that rAAV3 vectors are approximately eight times more efficient than rAAV8 vectors, and approximately 82 times more efficient than rAAV5 vectors (the two serotypes that are currently being used in liver-directed gene therapy in humans), in transducing primary human hepatocytes [65]. Thus, my prediction is that, compared with rAAV8 and rAAV5 vectors that are currently being used (Table 1), rAAV3 vectors will prove to be far more efficacious in targeting human liver diseases in general, and gene therapy of hemophilia in particular.

THE WILD-TYPE VERSUS RECOMBINANT CAPSIDS: IMPLICATIONS IN HOST IMMUNE RESPONSE & GENE THERAPY

In 2001, I had emphasized not only the importance of gaining a better

understanding of underlying mechanism of AAV–host cell interactions, but also need to develop the more efficient, next generation of AAV vectors [66]. Whereas the former appeared to be relatively straightforward to achieve eventually, it was not readily apparent precisely how the latter could be accomplished. As it turned out, the first clue was available from our studies published in 2000 [67], in which we observed that only ~20% of the input rAAV2 vectors gain entry into the nucleus, whereas ~80% fail to escape the endosome in the cytoplasm. Duan *et al.* subsequently reported that AAV2 capsids become ubiquitinated in the cytoplasm, where they are targeted for degradation by the host cell proteasomal machinery [68].

In 2006, the results of the first Phase I clinical trial for the potential gene therapy of hemophilia B with the first generation of rAAV2 vectors were reported [59]. As described above, at low (8×10^{10} vgs/kg), and medium (4×10^{11} vgs/kg) vector doses, rAAV2 vectors failed to express F.IX in two patients. At the high dose (2×10^{12} vgs/kg), rAAV2 vectors did lead to expression of therapeutic levels of F.IX in one patient, but it was short-lived due to the host immune response to AAV2 capsid proteins. Following uptake of AAV2 vectors by dendritic cells, and then proteasomal degradation of capsid proteins, led to activation of AAV2 capsid-specific $CD8^+$ memory T cells, which in turn, led to the destruction of transduced hepatocytes and consequently, the loss of F.IX levels in this patient [69]. Thus, to a certain extent, these studies provided an explanation as to why a high dose of rAAV2 vectors induced a host immune response against the capsid proteins.

Since it appeared that the observed immune response correlated directly with the AAV2 vector dose, we pondered the following two questions: (i) Why is such a high vector dose needed to achieve therapeutic levels of F.IX? and (ii) What can be done to reduce the vector dose at least ten-fold, and yet achieve therapeutic levels of F.IX? As stated above, the answer to the first question came from our studies published in 2000, where we documented that ~80% of the input rAAV2 vectors fail to gain entry into the nucleus [67], as they are targeted for degradation by the host cell ubiquitination/proteasomal machinery [68]. The answer to the second question was predicated on our hypothesis that if we could circumvent the ubiquitination/proteasome pathway, it might be feasible to achieve more efficient nuclear transport of rAAV2 vectors, which, in essence, would allow for a reduction in the vector dosage. Serendipitously, we had previously observed that inhibition of the host cell EGFR protein tyrosine kinase (EGFR-PTK) resulted in a significant increase in the transduction efficiency of rAAV2 vectors [70]. Thus, we hypothesized that following infection, the AAV2 capsid protein becomes phosphorylated at surface-exposed tyrosine residues by EGFR-PTK, and that tyrosine phosphorylation leads to ubiquitination, followed by proteasomal degradation of rAAV2 vectors in the cytoplasm [71]. Indeed, we obtained experimental evidence to support this hypothesis, which we reported in 2007 [72]. These studies provided the impetus to mutagenize the surface-exposed tyrosine residues in the AAV2 capsid to circumvent this barrier.

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There are seven tyrosine (Y) residues in the AAV2 capsid that are surface-exposed (Y252, Y272, Y444, Y500, Y700, Y704 and Y730). Each of these Y residues was mutagenized to phenylalanine (F) residues to generate seven single-mutants (Y252F, Y272F, Y444F, Y500F, Y700F, Y704F and Y730F), the transduction efficiency of three of which (Y444F, Y500F and Y730F) was significantly higher than their WT counterpart. The Y730F single-mutant rAAV2 vector was the most efficient, the use of which resulted in the expression of therapeutic levels of hF.IX in three different strains of mice following intravenous or portal vein administration at ten-fold reduced vector doses [73].

In subsequent studies, seven double-mutants (Y252 + 730F; Y272 + 730F; Y444 + 730F; Y500 + 730F; Y700 + 730F; Y704 + 730F; and Y444 + 500F), one triple-mutant (Y444 + 500 + 730F), one quadruple-mutant (Y272 + 444 + 500 + 730F), two pentuple-mutants (Y272 + 444 + 500 + 704 + 730F and Y272 + 444 + 500 + 700 + 730F), one sextuple-mutant (Y252 + 272 + 444 + 500 + 704 + 730F), and one septuple-mutant (Y252 + 272 + 444 + 500 + 700 + 504 + 730F) were also generated, and the triple-mutant (Y444 + 500 + 730F) rAAV2 vector was found to the most efficient, and provided a long-term therapeutic and tolerogenic expression of hF.IX in hemophilia B mice [74]. Interestingly, the triple-mutant rAAV2 vector was also shown to minimize *in vivo* targeting of transduced hepatocytes by capsid-specific $CD8^+$ cells [75].

Although it appeared that the next-generation tyrosine triple-mutant rAAV2 vector, which circumvented the problems associated with the first-generation rAAV2 vectors, could potentially be used successfully in patients with hemophilia B, Nathwani *et al.* [23] reported that the use of rAAV8 serotype vectors, which had previously been shown to be far more efficient than rAAV2 serotype vectors in transducing murine hepatocytes [18,76,77], led to phenotypic correction of hemophilia B in two patients who received the highest vector dose, which appears to be sustained for more than 3 years [24], but with some diminution in hF.IX levels (Table 1). Despite these highly encouraging results, I would reiterate, as stated above, that rAAV8 vectors might not be the panacea, especially for patients with severe hemophilia B, since based on our studies with non-human primate and humanized mice models [63,65], rAAV8 vectors are approximately eight times less efficient than rAAV3 vectors. Table 1 also illustrates additional AAV serotypes and their variants that have been used, or are currently being used, in the potential gene therapy of both hemophilia B and hemophilia A. It should be noted, however, that most, if not all, of these vectors are composed of naturally occurring capsids, which are likely to induce host immune responses, especially when used at astronomically high doses in some instances. Thus, I was prompted in 2016 to also posit that the WT AAV did not evolve for the purposes of delivery of therapeutic genes [25]. In other words, rAAV vectors composed of naturally occurring capsid are unlikely to be optimal in human clinical trials.

In our quest to develop more efficient and potentially less immunogenic AAV vectors, we also extended our studies to include

TABLE 1

First generation of recombinant AAV serotype vectors used/being used for the potential gene therapy of hemophilia.

Investigators/ sponsors	Vector	Dose	Expression level	Total dose*	Ref.
Hemophilia B					
High/Kay	ssAAV2	8×10^{10} vgs/kg 4×10^{11} vgs/kg 2×10^{12} vgs/kg	0% 0% 11➔0%	5.6 trillion 28 trillion 140 trillion	[59]
Nathwani/ Davidoff	scAAV8	2×10^{11} vgs/kg 6×10^{11} vgs/kg 2×10^{12} vgs/kg	2% 2–4% 8–12% ➔~5%	14 trillion 42 trillion 140 trillion	[23,24]
Baxalta/Shire**	scAAV8	2×10^{11} vgs/kg 1×10^{12} vgs/kg 3×10^{12} vgs/kg	2–5% 20–25% 50➔?%	14 trillion 70 trillion 210 trillion	[113]
Spark Therapeutics***	Undis-closed	5×10^{11} vgs/kg	20–44%	35 trillion	[113]
uniQure	scAAV5	5×10^{12} vgs/kg	3–7%	350 trillion	[113]
Hemophilia A					
BioMarin	ssAAV5	2×10^{13} vgs/kg 6×10^{13} vgs/kg	2–5% 50–200%	1.4 quadrillion 4.2 quadrillion	[113]

*Based on an average patient's weight of 70 kg (estimated number of cells in a 70 kg 'reference man' = 3.0×10^{13} or 30 trillion) [139].
**This trial has now been stopped since the expression levels of F.IX were inconsistent among different patients, and in some patients, the level of expression decreased with time.
***One patient manifested an immune response to AAV capsid proteins 12-weeks post-vector administration, accompanied by a drop in F.IX activity level.

two additional amino acid residues in the AAV capsid that are surface-exposed, and can also be phosphorylated by cellular serine/threonine protein kinases. For example, in addition to seven tyrosine (Y) residues, the AAV2 capsid also contains 17 surface-exposed serine (S) and 15 surface-exposed threonine (T) residues, each of which has been mutagenized, and rAAV2 vectors containing various permutations and combinations thereof, have been generated [78], and a quadruple-mutant (Y444+500+730F+T491V) has been identified to be the most efficient rAAV2 vector to date, at least in the murine liver. In addition, since ubiquitination occurs on lysine (K) residues, all seven surface-exposed residues in the AAV2 capsid have also been mutagenized, and limited numbers of Y+S+T+K-mutant rAAV2 vectors have been generated [79]. Although there is circumstantial evidence that these modifications lead to reduced degradation of AAV vectors in the cytoplasm, and therefore improved intracellular trafficking to the nucleus, and consequently efficient transgene expression [73], it should be noted that Douar *et al.* [80] observed a lack of direct correlation between the fold increase in intracellular trafficking with the fold increase in transduction efficiency following treatment with inhibitors of cellular proteasome. Thus, it remains possible that additional mechanisms, such as induction of p53 expression, activation of stress kinases and induction heat-shock gene expression, postulated by Douar *et al.* [80], might also play a role.

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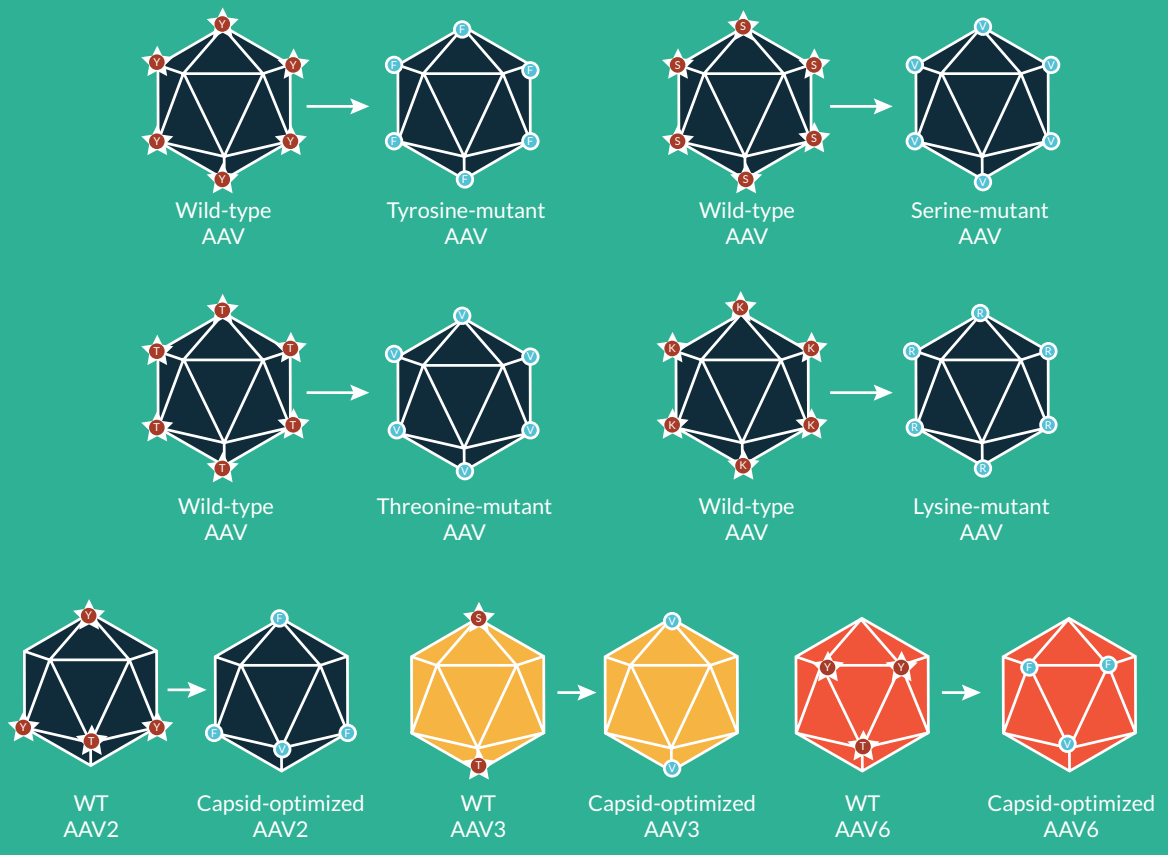
Interestingly, however, most, if not all of the surface-exposed Y, S, T and K residues are highly conserved among all ten commonly used AAV serotype vectors, and most of these residues have also been mutagenized in each of the ten AAV serotype vectors. Although further extensive studies would be needed to identify the most efficient combination of these mutations for a given serotype, cell or tissue type, and the host species, it has become abundantly clear that the use of the capsid-modified next generation of AAV vectors, as schematically illustrated in Figure 2, is likely to overcome some of the limitations associated with the first generation of AAV vectors. In this context, it is important to point out that three Phase I/II clinical trials with the tyrosine triple-mutant rAAV2 vectors have been initiated (Table 2), and that the initial results appear very promising in that two patients with Leber's hereditary optic neuropathy (LHON), who were administered a medium dose of the vector intravitreally, showed improvement in visual acuity at 90 days follow-up, without any loss of vision or any serious adverse events [81]. Thus, my

FIGURE 2

The capsid-modified next generation of recombinant AAV vectors.



Surface-exposed, specific tyrosine (Y), serine (S), and threonine (T) residues on AAV capsids can be phosphorylated, which is a signal for ubiquitination. Surface-exposed, specific lysine (K) residues on AAV capsids can be ubiquitinated, and subsequently degraded by the host cell proteasome machinery. Site-directed mutagenesis of these residues leads to the generation of AAV vectors that are more efficient at reduced vector doses, and consequently, less immunogenic. Specific examples of the most efficient rAAV2 [78], rAAV3 [62,63,65], and rAAV6 [141–143] serotype vectors generated thus far, are also depicted.

TABLE 2

Next generation of recombinant AAV2 serotype vectors being used in human gene therapy for retinal diseases.

Investigators/ sponsors	Vector	Dose*	ClinicalTrials.gov identifier
Leber's hereditary optic neuropathy			
Guy/National Eye Institute	Y444+500+730F-scAAV2	5 x 10^9 vgs/eye 2.5 x 10^{10} vgs/eye 1 x 10^{11} vgs/eye	NCT02161380
X-linked retinoschisis			
Applied Genetics Technologies	Y444+500+730F-AAV2	Not disclosed	NCT02416622
Achromatopsia			
Applied Genetics Technologies/ National Eye Institute	Y444+500+730F-AAV2	Not disclosed	NCT02599922

*Intravitreal delivery.

prediction is that the capsid-modified next generation of rAAV serotype vectors will prove to be far more efficacious than their WT counterparts in human gene therapy.

SINGLE-STRANDED VERSUS SELF-COMPLEMENTARY RECOMBINANT AAV GENOMES: IMPLICATIONS IN TRANSGENE EXPRESSION & GENE THERAPY

The genome of the WT AAV is a single-stranded DNA of 4,680 nucleotides [82], but single-stranded DNA of both [+] and [-] polarities are encapsidated into separate mature virions with equal frequency [83]. While advantageous for the WT AAV, which prefers to remain latent in host cells, the single-stranded nature of the genome in a rAAV vector is problematic, since single-stranded DNA is transcriptionally-inactive, and viral second strand-DNA synthesis is a rate-limiting step during rAAV vector-mediated transgene expression in tissue culture cell lines, as originally described by Fisher *et al.* [84] and Ferrari *et al.* [85] in 1996. However, since the AAV genome most likely exists as double-stranded circular episomes and concatemers, especially in post-mitotic cells and tissues, it appears unlikely that its single-stranded nature contributes to its latency. In 1997, we [56] and others [57] reported that following intravenous administration of rAAV2 vectors in a murine model *in vivo*, up to 95% of the mouse hepatocytes were transduced, but transgene expression occurred in ~5% of the hepatocytes [86].

However, the mechanism underlying the lack of viral second-strand DNA synthesis in ~95% of the hepatocytes remained unclear. There was robust debate among three groups of investigators, two groups favoring the viral second-strand DNA synthesis model [84,85] and the third group favoring the DNA strand-annealing model [87]. A preponderance of evidence suggested that the former was the predominant mechanism underlying rAAV vector-mediated transgene expression [88–95].

Using tissue culture cell lines as a model, we identified that a cellular protein, phosphorylated at tyrosine residues, binds specifically to the

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single-stranded sequence of 20 nucleotides, termed the D-sequence, within the AAV inverted terminal repeat (ITR) at the 3′-end of the viral genome, and that this phospho-protein strongly inhibits the viral second-strand DNA synthesis, resulting in impaired transgene expression [96]. We subsequently identified this cellular protein to be a 52 kDa protein that binds the immunosuppressant drug FK506, and hence the designation, FKBP52, a well-known cellular chaperone protein [97]. A number of strategies were developed to circumvent the barriers that hinder AAV second-strand DNA synthesis [90–94,98,99], the most significant of which was the generation of double-stranded, self-complimentary AAV (scAAV) vectors by McCarty *et al.* [88]. The use of scAAV vectors was shown to easily overcome the rate-limiting step of viral second-strand DNA synthesis, leading to early onset and robust transgene expression, both in tissue culture cell lines *in vitro*, and in murine models *in vivo* [89]. This observation was further validated by the successful clinical trial for hemophilia B by Nathwani *et al.* [23,24], who used scAAV8 vectors and achieved sustained levels of expression of hF.IX in ten patients. It is tempting to speculate that since AAV8 vectors transduce human hepatocytes less efficiently than mouse hepatocytes [63–65,100], the use of the scAAV vectors, rather than the AAV8 serotype, was largely responsible for the successful outcome.

It is intriguing, therefore, that with a few exceptions, nearly all clinical trials reported thus far have been performed using ssAAV vectors, and yet clinical efficacy has been observed in the potential gene therapy of several human diseases, such as LCA, lipoprotein lipase deficiency, aromatic L-amino acid decarboxylase deficiency and choroideremia. If the expression cassettes of each of the therapeutic genes used in these trials were within the limited packaging capacity of ~2.5 kb for scAAV vectors, it is reasonable to suggest that the levels of the transgene expression would be significantly higher.

However, since it is unlikely that expression cassettes of all therapeutic genes can be encapsidated in scAAV vectors, it is clear that additional strategies to achieve higher levels of transgene expression from rAAV vectors containing single-stranded DNA genomes are warranted. Since, as stated above, we had observed that binding of FKBP52 to the D-sequence at the 3′-end in the AAV2-ITR strongly inhibits the viral second-strand DNA synthesis, and consequently, transgene expression, we hypothesized that deletion of the D-sequences from the viral genome would allow us to achieve that objective, but we observed that deletion of the D-sequences from the AAV genome resulted in failure of the viral progeny DNA strands to undergo genome encapsidation. Thus, we learned that the D-sequences are indispensable, as they serve as the 'packaging signal' for the AAV genome [101–103]. Interestingly, however, when only one of the two D-sequences was deleted from the AAV genome, successful encapsidation of the progeny viral DNA ensued, but depending upon which D-sequence was deleted, the resulting vectors contained either [+] or [-] polarity strands [104]. More interestingly, the transduction efficiency of these single-polarity ssAAV vectors was observed to be significantly higher than that of their unmodified counterpart in both established cell

lines *in vitro* and in murine hepatocytes *in vivo* [104].

Further detailed studies revealed that the D(-)-sequence at the 3′-end in the viral inverted terminal repeat contains the binding site for a cellular protein, FKBP52, phosphorylated forms of which bind to the proximal end of the D(-)-sequence, and strongly inhibit the viral second-strand DNA synthesis [96,97]. The D(+)-sequence at the 5′-end in the viral inverted terminal repeat contains the binding site for a cellular NF-κB repressing factor (NRF), which inhibits the viral transgene expression [105]. Whereas removal of both D-sequences is incompatible with vector genome encapsidation [102], removal of the D(+)-sequence leads to the generation of either the [+] or the [-] polarity ssAAV vectors, which mediate more efficient transgene expression due to the loss of the NRF binding site [104]. These one D-sequence–deleted genome-containing vectors are depicted schematically in Figure 3. Thus, the use of genome-modified rAAV vectors appear to partially overcome the limitation associated with the conventional ssAAV vectors, and expression cassettes of therapeutic genes of up to ~4.5 kb can easily be encapsidated to achieve improved transgene expression.

As expected, when the modified AAV genomes were encapsidated into the most efficient quadruple-mutant (Y444+500+730F+T491V) AAV2, or the double-mutant (S662V+T492V) AAV3 capsids, the resulting optimized vectors were documented to transduce cells and tissues significantly more efficiently at 20–30-fold further reduced vector doses [106]. These optimized AAV serotype vectors circumvent the problems associated with the first generation of AAV vectors. Thus, my prediction is that, in contrast to the enormously high vector doses that are currently being used, particularly for the potential gene therapy of hemophilia (Table 1), the optimized AAV serotype vectors, in addition to being far more efficacious, will also offer the potential advantages of being less immunogenic, and more cost -effective.

TRANSLATION INSIGHT

Gene therapy has had its ups and downs, but now there is little doubt that it is here to stay, and it is likely to cure a number of human diseases in the near future. Perhaps Dr Philippe Leboulch said it best in 2013 [107]: "The development of the field of gene therapy shares many similarities with the history of aviation. Each is based on deceptively simple principles: the introduction of a therapeutic gene into cells and the flow of air over an aircraft's wing. Each field was marred by shortcomings and adverse events early on. But in spite of naysayers lacking vision, both fields continued their quest, and now there is firm hope that gene therapy will soon do for medicine what airoplanes did for transportation."

As for rAAV serotype vectors and their successful use in a number of Phase I/II/III clinical trials, this sentiment has clearly been validated.

Commercially viable therapies

The AAV1 vector expressing the gene for lipoprotein lipase (LPL) was approved as a drug in Europe in 2012 [108]. It is designated as Alipogene tiparvovec, and marketed

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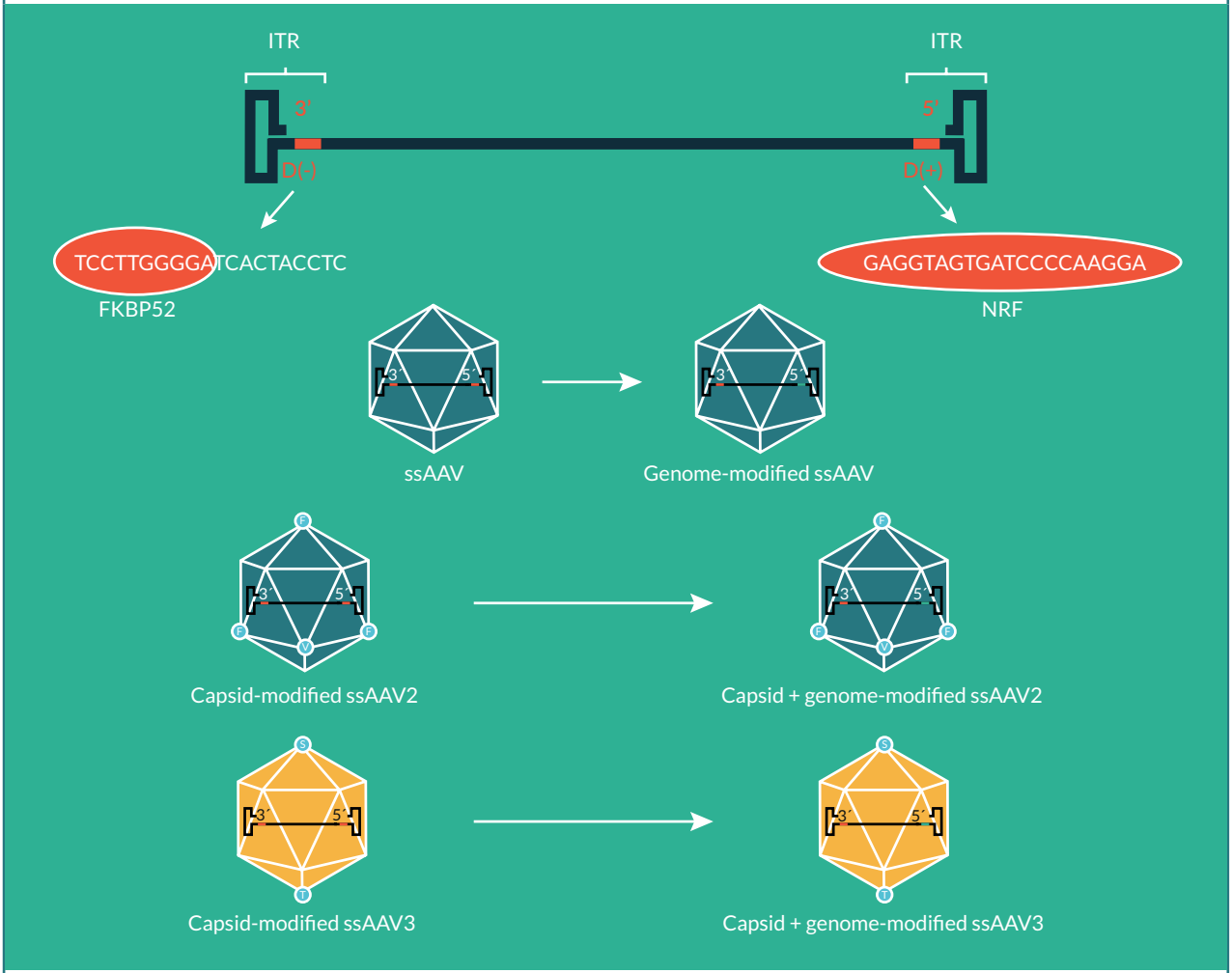
FIGURE 3

Genome-modified recombinant AAV vectors.



The D(-)-sequence at the 3′-end in the viral inverted terminal repeat contains the binding site for a cellular protein, FKBP52, phosphorylated forms of which strongly inhibit the viral second-strand DNA synthesis [96,97]. The D(+)-sequence at the 5′-end in the viral inverted terminal repeat contains the binding site for a cellular NF-κB repressing factor (NRF), which inhibits the viral transgene expression [105]. Whereas removal of both D-sequences is incompatible with vector genome encapsidation [102], removal of D(+)-sequence leads to the generation of either the [+] or the [-] polarity ssAAV vectors, which mediate more efficient transgene expression due to the loss of the NRF binding site [104]. Although the D(-)-sequence–deleted ssAAV genomes fail to package, encapsidation of D(+)-sequence–deleted viral genomes into capsid-modified vectors leads to the generation of optimized ssAAV2 and ssAAV3 serotype vectors that are far more efficacious at further reduced doses [106].

under the trade name Glybera®. Based on the recent successful Phase III trial for the potential gene therapy of LCA, it is likely that the AAV2 vector expressing retinal pigment epithelium-specific 65 kDa protein (RPE65), also known as retinoid isomerohydrolase, will soon be licensed as a drug in the USA. Licensing of various AAV serotype vectors and their variants, which have shown clinical efficacy in a number of gene therapy trials for hemophilia B and A [109], will soon follow as well. A number of additional clinical trials in which AAV vectors have already shown efficacy, such as hemophilia B with AAV8 vectors [23,24], aromatic amino acid decarboxylase deficiency and choroideremia with AAV2 vectors [12], and additional Phase I/II clinical trials are currently being pursued with AAV1 vectors for the potential

gene therapy of α1 anti-trypsin deficiency [110], AAV1 and AAV9 vectors for Pompe disease [111,112], and AAV8 and AAV5 vectors for hemophilia B and hemophilia A [113], respectively, and once their efficacy has been established, commercial viability will certainly be pursued. Thus far, AAV1, AAV2, AAV5, AAV8 and AAV9 serotype vectors have been, or are currently being used, in 162 Phase I/II clinical trials in humans to date [6,114], which will eventually lead to commercially viable therapies.

The fact that several Big Pharma companies, such as Baxter, Bayer, Biogen, BioMarin, Bristol-Myers Squibb, GlaxoSmithKline, Novartis, Pfizer, Sanofi and Shire, among others, have invested well over $3 billion since 2014 [115], bodes well for the commercial viability of AAV vector-mediated gene therapy in humans.

Challenges

Despite the remarkable progress that has been made in the use of rAAV vectors for human gene therapy, and the future prospects that appear very promising, several challenges also remain. One of the major challenges is pre-existing antibodies to AAV. A significant proportion of humans are sero-positive for one or more of the AAV serotypes, and studies have documented that anti-AAV antibody titers as low as 1:10 are sufficient to neutralize systemically administered rAAV vectors [116,117]. Cross-reactivity of these pre-existing antibodies against one AAV serotype to many other AAV serotypes is also a significant barrier [118].

The second challenge is the inability of the currently available rAAV vectors to selectively target a given cell type, tissue or organ following systemic administration. Highly regulated transgene expression restricted to a given cell type, tissue or organ also remains a desirable goal.

The third challenge with the currently available rAAV serotype vectors is the lack of standardization of vector titers and potency, although reference standards for at least two serotypes (AAV2 and AAV8) are now available [119,120]. However, reproducibility among different production methods also remains a challenge.

The final challenge is the inherently limited packaging capacity of ~5 kb for the conventional ssAAV vectors and ~2.5 kb for scAAV vectors. Although there was a lone report [121] claiming that AAV genomes of up to 8.9 kb could be packaged in rAAV5 serotype vectors, at least three independent groups failed to reproduce those results [122–124]. Several groups have reported the use of dual vectors to achieve the delivery and expression of oversized genes [125–128].

Research requirements

The value of basic science research on rAAV vector biology cannot be overstated. As I have emphasized previously [55], it was entirely due to the sustained efforts of a very few investigators who continued to pursue basic science research on AAV for nearly three decades despite the complete lack of interest of the scientific community at large, that was instrumental in the development of rAAV vectors. In my opinion, detailed molecular studies on every aspect of the AAV lifecycle – attachment and entry, intracellular trafficking, nuclear transport, viral uncoating, second-strand DNA synthesis, and transgene expression – must continue to be pursued.

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In this context, it is important to reiterate that, as stated above, and illustrated in Figure 3, although removal of the D(+)-sequence from the inverted terminal repeat (ITR) at the 5′-end leads to generation of either the [+] or the [-] polarity ssAAV vectors, which mediate more efficient transgene expression due to loss of the NRF binding site [104], it has thus far not been possible to generate ssAAV vectors that lack the D(-)-sequence at the 3′-end in the ITR since ssAAV genomes lacking the D(-)-sequence fail to undergo encapsidation [103]. Thus, the development of additional strategies are warranted to generate ssAAV genomes that lack the FKBP52-binding site, and yet can be packaged, such that efficient viral second-strand DNA synthesis can ensue, leading to robust transgene expression.

Unless and until the astronomically high vector doses that are currently being used in human clinical trials [Table 1], which clearly trigger the host immune response, can be reduced to achieve clinical efficacy, detailed studies on the intricacies of the AAV vector immunology must also be pursued.

For the most part, rAAV vector genomes have been shown to remain episomal for extended time periods lasting years and decades, but thus far, those studies have been carried out with post-mitotic cells, tissues and organs. There is clearly a need to develop rAAV vectors that can also stably transduce actively dividing cells. This would necessitate that rAAV vector genomes undergo integration into the host cell chromosomal DNA. In order to circumvent the possibility of insertional mutagenesis due to random integration, efforts must also be made to achieve site-specific integration of rAAV vector genomes, akin to what has been observed with the WT AAV [129,130].

It has become increasingly clear that despite the extensive use of mouse and rat models in biomedical research in general, for the most part, these rodent models are poor surrogates for humans, as well as poor predictors for evaluating the efficacy of rAAV serotype vectors for human diseases [55]. Thus, at the very least, the use of non-human primates, or humanized mouse models, should be considered as more reliable model systems. In addition, individual difference among humans may also significantly influence the reproducibility of AAV vector-mediated gene therapy, as was illustrated by differences in transduction efficiency of rAAV3 and rAAV8 vectors in hepatocytes from various donors [63–65,100]. In this context, it is also important to note that despite the limited lifespan of primary hepatocytes, sustained transgene expression mediated by rAAV vectors lasting decades, warrants further mechanistic studies.

Manufacturing needs

There are currently two common rAAV vector production protocols that are being used: human embryonic kidney cell line, 293 (HEK293) and plasmid triple-transfections; and insect cell line, sf9, and baculovirus vector infections. Although both systems have their advantages as well as disadvantages, additional systems, including suspension cultures, would need to be refined to achieve scalable production of various rAAV serotype vectors to meet the ever-growing need as additional clinical trials for a wide variety of human diseases are contemplated and pursued.

As pointed out above, there is an urgent need to develop standardized protocols not only to produce high-quality rAAV serotype vectors, but to also determine their titers and potency accurately, such that vectors produced and clinical trials performed at various geographical locations can be compared consistently and in meaningful ways. During the process of vector packaging, a large amount of empty capsids are produced. A better understanding of the underlying mechanism of vector assembly might also lead to strategies that significantly increase vector production. It should also be noted that thus far, all recombinant AAV vectors are generated using AAV2-ITRs and AAV2-Rep proteins, regardless of the AAV serotype capsid, which is not optimal. We have suggested the use of the homologous ITRs and Rep proteins, specific for each serotype, which, at least for rAAV3 serotype vectors, appears to significantly improve the titers as well as the potency [131].

The cost associated with large-scale rAAV vector production, especially for clinical grade vectors, is not insignificant. With the steadily shrinking research support from the National Institutes of Health, it is becoming prohibitively expensive for academic investigators to pursue clinical trials. Thankfully, as mentioned above, the Big Pharma companies have stepped in and begun to fill this void. The establishment of additional Clinical Manufacturing Organizations (CMOs) would also go a long way to meet this critical need.

As stated above, although the very first rAAV vector as a drug, Glybera®, was approved in Europe, nearly 64% of all gene therapy clinical trials have been, or are currently being performed in the USA, and ~20% in Europe [6]. Yet, a large population of patients in dire need of life-altering and life-saving treatments lives in the third world. Thus, all efforts should be made to make gene therapy not only cost-effective, but also available to eligible patients worldwide.

Regulatory framework

The US Food and Drug Administration (FDA) has recently begun to grant expedited review and approval, termed Breakthrough Therapy, for specific gene therapy trials, among Priority Review, Accelerated Approval and Fast Track, to facilitate such treatments as rapidly as possible, which is highly commendable.

However, the current emphasis, both by academia and Big Pharma, is still on targeting the orphan diseases. Now that the safety of rAAV vectors has been well established in 162 Phase I/II clinical trials, and one Phase III clinical trial, one would hope that the regulatory agencies, including the FDA, would consider granting approval for diseases where the rates of incidence is higher, and the life expectancy is shorter.

These regulatory agencies should also consider granting approval for gene therapy clinical trials with rAAV vectors for diseases where the end of life is imminent.

Although at least two Phase I clinical trials with modified AAV vectors were approved by the FDA, one contained five amino acid substitutions from AAV1 to AAV2 (AAV2.5) [132], and the second in which three surface-exposed tyrosine residues were mutagenized and replaced with phenylalanine residues (AAV2-Y444+500+730F) [81], it remains to be seen what regulatory hurdles, if any, additional AAV variants, both rationally

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designed and shuffled, might face in the future.

Next steps

In addition to pursuing various research avenues outlined above, the quest for the isolation of novel AAV serotypes from other species, both vertebrates and non-vertebrates, and their development as vectors must also continue. Although several approaches, including directed evolution [133], DNA shuffling [134], rational design [73,78,135–137], dual vectors [124–127] and chemical modifications [138] are currently being used, further optimization of not only the capsid, but the vector genomes should be pursued as well [104].

Finally, efforts towards the development of site-specific integrating vectors; tissue- and organ-specific vectors; vectors capable of escaping pre-existing antibodies; and vectors capable of repeat administrations, should also continue, in order to realize the full potential of these remarkable biological entities that were once considered a 'biological oddity' [139].

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FINANCIAL & COMPETING INTERESTS DISCLOSURE

The author holds several issued patents on recombinant AAV vectors that have been licensed to various gene therapy companies.

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REFERENCES

1. Atchison RW, Casto BC, Hammon WM. Adenovirus-associated defective virus particles. *Science* 1965; 149(3685): 754–6.
2. Blacklow NR, Hoggan MD, Sereno MS *et al.* A seroepidemiologic study of adenovirus-associated virus infection in infants and children. *Am. J. Epidemiol.* 1971; 94(4): 359–66.
3. Nault JC, Datta S, Imbeaud S *et al.* Recurrent AAV2-related insertional mutagenesis in human hepatocellular carcinomas. *Nat. Genet.* 2015; 47(10): 1187–93.
4. Berns KI, Byrne BJ, Flotte TR *et al.* Adeno-associated virus type 2 and hepatocellular carcinoma? *Hum. Gene Ther.* 2015; 26(12): 779–81.
5. Buning H, Schmidt M. Adeno-associated vector toxicity-To be or not to be? *Mol. Ther.* 2015; 23(11): 1673–5.
6. Ginn SL, Alexander IE, Edelstein ML, Abedi MR, Wixon J. Gene therapy clinical trials worldwide to 2012 – an update. *J. Gene Med.* 2013; 15(2): 65–77.
7. Bainbridge JW, Smith AJ, Barker SS *et al.* Effect of gene therapy on visual function in Leber's congenital amaurosis. *N. Engl. J. Med.* 2008; 358(21): 2231–9.
8. Maguire AM, Simonelli F, Pierce EA *et al.* Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N. Engl. J. Med.* 2008; 358(21): 2240–8.
9. Cideciyan AV, Aleman TS, Boye SL *et al.* Human gene therapy for RPE65 isomerase deficiency activates the retinoid cycle of vision but with slow rod kinetics. *Proc. Natl Acad. Sci. USA* 2008; 105(39): 15112–7.
10. Hauswirth WW, Aleman TS, Kaushal S *et al.* Treatment of Leber congenital

amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: Short-term results of a phase I trial. *Hum. Gene Ther.* 2008; 19(10): 979–90.

11. Hwu L, Muramatsu S, Tseng SH et al. Gene therapy for aromatic L-amino acid decarboxylase deficiency. *Sci. Transl. Med.* 2012; 4(134):134ra61.
12. MacLaren RE, Groppe M, Barnard AR *et al.* Retinal gene therapy in patients with choroideremia: Initial findings from a phase 1/2 clinical trial. *Lancet* 2014; 383(9923): 1129–37.
13. Xiao W, Chirmule N, Berta SC, McCullough B, Gao G, Wilson JM. Gene therapy vectors based on adeno-associated virus type 1. *J. Virol.* 1999; 73(5): 3994–4003.
14. Muramatsu S, Mizukami H, Young NS, Brown KE. Nucleotide sequencing and generation of an infectious clone of adeno-associated virus 3. *Virology* 1996; 221(1): 208–17.
15. Chiorini JA, Yang L, Liu Y, Safer B, Kotin RM. Cloning of adeno-associated virus type 4 (AAV4) and generation of recombinant AAV4 particles. *J. Virol.* 1997; 71(9): 6823–33.
16. Bantel-Schaal U, Delius H, Schmidt R, zur Hausen H. Human adeno-associated virus type 5 is only distantly related to other known primate helper-dependent parvoviruses. *J. Virol.* 1999; 73(2): 939–47.
17. Rutledge EA, Halbert CL, Russell DW. Infectious clones and vectors derived from adeno-associated virus *(AAV) serotypes other than AAV type 2. J. Virol.* 1998; 72(1): 309–19.
18. Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J, Wilson JM. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc. Natl Acad. Sci. USA* 2002; 99(18): 11854–9.
19. Mori S, Wang L, Takeuchi T, Kanda T. Two novel adeno-associated viruses from cynomolgus monkey: Pseudotyping characterization of capsid protein. *Virology* 2004; 330(2): 375–83.
20. Schmidt M, Voutetakis A, Afione S, Zheng C, Mandikian D, Chiorini JA. Adeno-associated virus type 12 (AAV12): A novel aav serotype with sialic acid- and heparan sulfate proteoglycan-independent transduction activity. *J. Virol.* 2008; 82(3): 1399–1406.
21. Schmidt M, Govindasamy L, Afione S, Kaludov N, Agbandje-McKenna M, Chiorini JA. Molecular characterization of the heparin-dependent transduction domain on the capsid of a novel adeno-associated virus isolate, AAV(VR-942). *J. Virol.* 2008; 82(17): 8911–6.
22. Gaudet D, Methot J, Dery S *et al.* Efficacy and long-term safety of alipogene tiparvovec (AAV-lpls447x) gene therapy for lipoprotein lipase deficiency: An open-label trial. *Gene Ther.* 2013; 20(4): 361–9.
23. Nathwani AC, Tuddenham EG, Rangarajan S *et al.* Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N. Engl. J. Med.* 2011; 365(25): 2357–65.
24. Nathwani AC, Reiss UM, Tuddenham EG *et al.* Long-term safety and efficacy of factor ix gene therapy in hemophilia B. *N. Engl. J. Med.* 2014; 371(21): 1994–2004.
25. Srivastava A. Adeno-associated virus: The naturally occurring virus versus the recombinant vector. *Hum. Gene Ther.* 2016; 27(1): 1–6.
26. Ponnazhagan S, Wang XS, Woody MJ. Differential expression in human cells from the p6 promoter of human parvovirus B19 following plasmid transfection and recombinant adeno-associated virus 2 (AAV) infection: Human megakaryocytic leukaemia cells are non-permissive for aav infection. *J. Gen. Virol.* 1996; 77 (Pt 6): 1111–22.
27. Summerford C, Samulski RJ. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J. Virol.* 1998; 72(2): 1438–445.
28. Flotte T, Carter B, Conrad C. A phase I study of an adeno-associated virus-CFTR gene vector in adult cf patients with mild lung disease. *Hum. Gene Ther.* 1996; 7(9): 1145–59.
29. Duan D, Yue Y, Yan Z, McCray PB, Jr., Engelhardt JF. Polarity influences the efficiency of recombinant adeno-associated virus infection in differentiated airway epithelia. *Hum. Gene Ther.* 1998; 9(18): 2761–76.
30. Qing K, Mah C, Hansen J, Zhou S, Dwarki V, Srivastava A. Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. *Nat. Med.* 1999; 5(1): 71–7.
31. Summerford C, Bartlett JS, Samulski RJ. αVβ5 integrin: A co-receptor for adeno-associated virus type 2 infection. *Nat. Med.* 1999; 5(1): 78–82.
32. Chen CL, Jensen RL, Schnepp BC *et al.* Molecular characterization of adeno-associated viruses infecting children. *J. Virol.* 2005; 79(23): 14781–92.
33. Muzyczka N, Warrington KH, Jr. Custom adeno-associated virus capsids: The next generation of recombinant vectors with novel tropism. *Hum. Gene Ther.* 2005; 16(4): 408–16.
34. Boye SL, Bennett A, Scalabrino ML *et al.* The impact of heparan sulfate binding on transduction of retina by rAAV vectors. *J. Virol.* 2016; 90(8): 4215–31.
35. Kashiwakura Y, Tamayose K, Iwabuchi K *et al.* Hepatocyte growth factor

 DOI: 10.18609/cgti.2016.061

receptor is a coreceptor for adeno-associated virus type 2 infection. *J. Virol.* 2005; 79(1): 609–14.

36. Asokan A, Hamra JB, Govindasamy L, Agbandje-McKenna M, Samulski RJ. Adeno-associated virus type 2 contains an integrin α5β1 binding domain essential for viral cell entry. *J. Virol.* 2006; 80(18): 8961–9.
37. Akache B, Grimm D, Pandey K, Yant SR, Xu H, Kay MA. The 37/67-kilodalton laminin receptor is a receptor for adeno-associated virus serotypes 8, 2, 3, and 9. *J. Virol.* 2006; 80(19): 9831–6.
38. Kurzeder C, Koppold B, Sauer G, Pabst S, Kreienberg R, Deissler H. CD9 promotes adeno-associated virus type 2 infection of mammary carcinoma cells with low cell surface expression of heparan sulphate proteoglycans. *Int. J. Mol. Med.* 2007; 19(2): 325–33.
39. Chen S, Kapturczak M, Loiler SA *et al.* Efficient transduction of vascular endothelial cells with recombinant adeno-associated virus serotype 1 and 5 vectors. *Hum. Gene Ther.* 2005; 16(2): 235–47.
40. Wu Z, Miller E, Agbandje-McKenna M, Samulski RJ. Alpha2,3 and alpha2,6 N-linked sialic acids facilitate efficient binding and transduction by adeno-associated virus types 1 and 6. *J. Virol.* 2006; 80(18): 9093–103.
41. Handa A, Muramatsu S, Qiu J, Mizukami H, Brown KE. Adeno-associated virus (AAV)-3-based vectors transduce haematopoietic cells not susceptible to transduction with AAV-2-based vectors. *J. Gen Virol.* 2000; 81(Pt 8): 2077–84.
42. Kaludov N, Handelman B, Chiorini JA. Scalable purification of adeno-associated virus type 2, 4, or 5 using ion-exchange chromatography. *Hum. Gene Ther.* 2002; 13(10): 1235–43.
43. Walters RW, Yi SM, Keshavjee S *et al.* Binding of adeno-associated virus type 5 to 2,3-linked sialic acid is required for gene transfer. *J. Biol. Chem.* 2001; 276(23): 20610–6.
44. Walters RW, Pilewski JM, Chiorini JA, Zabner J. Secreted and transmembrane mucins inhibit gene transfer with AAV4 more efficiently than AAV5. *J. Biol. Chem.* 2002; 277(26): 23709–13.
45. Seiler MP, Miller AD, Zabner J, Halbert CL. Adeno-associated virus types 5 and 6 use distinct receptors for cell entry. *Hum. Gene Ther.* 2006; 17(1): 10–9.
46. Wu Z, Asokan A, Samulski RJ. Adeno-associated virus serotypes: Vector toolkit for human gene therapy. *Mol. Ther.* 2006; 14(3): 316–27.
47. Shen S, Bryant KD, Brown SM, Randell SH, Asokan A. Terminal N-linked galactose is the primary receptor for adeno-associated virus 9. *J. Biol. Chem.* 2011; 286(15): 13532–40.
48. Bell CL, Gurda BL, Van Vliet K, Agbandje-McKenna M, Wilson JM. Identification of the galactose binding domain of the adeno-associated virus serotype 9 capsid. *J. Virol.* 2012; 86(13): 7326–33.
49. Pillay S, Meyer NL, Puschnik AS *et al.* An essential receptor for adeno-associated virus infection. *Nature* 2016; 530(7588): 108–12.
50. Shen S, Berry GE, Castellanos Rivera RM *et al.* Functional analysis of the putative integrin recognition motif on adeno-associated virus 9. *J. Biol. Chem.* 2015; 290(3): 1496–504.
51. Blackburn SD, Steadman RA, Johnson FB. Attachment of adeno-associated virus type 3h to fibroblast growth factor receptor 1. *Arch. Virol.* 2006; 151(3): 617–23.
52. Ling C, Lu Y, Kalsi JK *et al.* Human hepatocyte growth factor receptor is a cellular coreceptor for adeno-associated virus serotype 3. *Hum. Gene Ther.* 2010; 21(12): 1741–7.
53. Di Pasquale G, Davidson BL, Stein CS *et al.* Identification of PDGFR as a receptor for AAV5 transduction. *Nat. Med.* 2003; 9(10): 1306–12.
54. Weller ML, Amornphimoltham P, Schmidt M, Wilson PA, Gutkind JS, Chiorini JA. Epidermal growth factor receptor is a co-receptor for adeno-associated virus serotype 6. *Nat. Med.* 2010; 16(6): 662–4.
55. Srivastava A. *In vivo* tissue-tropism of adeno-associated viral vectors. *Curr. Opin. Virol.* 2016; 21: 75–80.
56. Ponnazhagan S, Mukherjee P, Yoder MC *et al.* Adeno-associated virus 2-mediated gene transfer *in vivo*: Organ-tropism and expression of transduced sequences in mice. *Gene* 1997; 190(1): 203–10.
57. Snyder RO, Miao CH, Patijn GA *et al.* Persistent and therapeutic concentrations of human factor ix in mice after hepatic gene transfer of recombinant AAV vectors. *Nat. Genet.* 1997; 16(3): 270–6.
58. Su H, Lu R, Chang JC, Kan YW. Tissue-specific expression of herpes simplex virus thymidine kinase gene delivered by adeno-associated virus inhibits the growth of human hepatocellular carcinoma in athymic mice. *Proc. Natl Acad. Sci. USA* 1997; 94(25): 13891–6.
59. Manno CS, Pierce GF, Arruda VR *et al.* Successful transduction of liver in hemophilia by AAV-factor IX and limitations imposed by the host immune response. *Nat. Med.* 2006; 12(3): 342–7.
60. Glushakova LG, Lisankie MJ, Eruslanov EB *et al.* AAV3-mediated transfer and expression of the pyruvate dehydrogenase E1 alpha subunit gene causes metabolic remodeling

and apoptosis of human liver cancer cells. *Mol. Genet. Metab.* 2009; 98(3): 289–99.

61. Cheng B, Ling C, Dai Y *et al.* Development of optimized AAV3 serotype vectors: Mechanism of high-efficiency transduction of human liver cancer cells. *Gene Ther.* 2012; 19(4): 375–84.
62. Ling C, Wang Y, Zhang Y *et al.* Selective *in vivo* targeting of human liver tumors by optimized AAV3 vectors in a murine xenograft model. *Hum. Gene Ther.* 2014; 25(12): 1023–34.
63. Li S, Ling C, Zhong L *et al.* Efficient and targeted transduction of nonhuman primate liver with systemically delivered optimized AAV3B vectors. *Mol. Ther.* 2015; 23(12): 1867–76.
64. Wang L, Bell P, Somanathan S *et al.* Comparative study of liver gene transfer with AAV vectors based on natural and engineered AAV capsids. *Mol. Ther.* 2015; 23(12): 1877–87.
65. Vercauteren K, Hoffman BE, Zolotukhin I *et al.* Superior *in vivo* transduction of human hepatocytes using engineered AAV3 capsid. *Mol. Ther.* 2016; 24(6): 1042–9.
66. Srivastava A. Gene therapy with viral vectors: The hope, the problems, and the solution. *J. Hematother. Stem Cell Res.* 2001; 10(2): 321–2.
67. Hansen J, Qing K, Kwon HJ, Mah C, Srivastava A. Impaired intracellular trafficking of adeno-associated virus type 2 vectors limits efficient transduction of murine fibroblasts. *J. Virol.* 2000; 74(2): 992–6.
68. Duan D, Yue Y, Yan Z, Yang J, Engelhardt JF. Endosomal processing limits gene transfer to polarized airway epithelia by adeno-associated virus. *J. Clin. Invest.* 2000; 105(11): 1573–87.
69. Mingozzi F, Maus MV, Hui DJ *et al.* CD8(+) t-cell responses to adeno-associated virus capsid in humans. *Nat. Med.* 2007; 13(4): 419–22.
70. Mah C, Qing K, Khuntirat B *et al.* Adeno-associated virus type 2-mediated gene transfer: Role of epidermal growth factor receptor protein tyrosine kinase in transgene expression. *J. Virol.* 1998; 72(12): 9835–43.
71. Zhong L, Li B, Jayandharan G *et al.* Tyrosine-phosphorylation of AAV2 vectors and its consequences on viral intracellular trafficking and transgene expression. *Virology* 2008; 381(2): 194–202.
72. Zhong L, Zhao W, Wu J *et al.* A dual role of egfr protein tyrosine kinase signaling in ubiquitination of AAV2 capsids and viral second-strand DNA synthesis. *Mol. Ther.* 2007; 15(7): 1323–30.
73. Zhong L, Li B, Mah CS *et al.* Next generation of adeno-associated virus 2 vectors: Point mutations in tyrosines lead to high-efficiency transduction at lower doses. *Proc. Natl Acad. Sci. USA* 2008; 105(22): 7827–32.
74. Markusic DM, Herzog RW, Aslanidi GV *et al.* High-efficiency transduction and correction of murine hemophilia Bb using AAV2 vectors devoid of multiple surface-exposed tyrosines. *Mol . Ther.* 2010; 18(12): 2048–56.
75. Martino AT, Basner-Tschakarjan E, Markusic DM *et al.* Engineered AAV-vector minimizes *in vivo* targeting of transduced hepatocytes by capsid-specific CD8+ T cells. *Blood* 2013; 121(12): 2224–33.
76. Sarkar R, Tetreault R, Gao G *et al.* Total correction of hemophilia a mice with canine FVIII using an AAV8 serotype. *Blood* 2004; 103(4): 1253–60.
77. Thomas CE, Storm TA, Huang Z, Kay MA. Rapid uncoating of vector genomes is the key to efficient liver transduction with pseudotyped adeno-associated virus vectors. *J. Virol.* 2004; 78(6): 3110–22.
78. Aslanidi GV, Rivers AE, Ortiz L *et al.* Optimization of the capsid of recombinant adeno-associated virus 2 (AAV2) vectors: The final threshold? *PLoS One* 2013; 8(3): e59142.
79. Li B, Ma W, Ling C *et al.* Site-directed mutagenesis of surface-exposed lysine residues leads to improved transduction by AAV2, but not AAV8, vectors in murine hepatocytes *in vivo*. *Hum. Gene Ther. Methods* 2015; 26(6): 211–20.
80. Douar AM, Poulard K, Stockholm D, Danos O. Intracellular trafficking of adeno-associated virus vectors: Routing to the late endosomal compartment and proteasome degradation. *J. Virol.* 2001; 75(4): 1824–33.
81. Feuer WJ, Schiffman JC, Davis JL *et al.* Gene therapy for leber hereditary optic neuropathy: Initial results. *Ophthalmology* 2016; 123(3): 558–70.
82. Srivastava A, Lusby EW, Berns KI. Nucleotide sequence and organization of the adeno-associated virus 2 genome. *J. Virol.* 1983; 45(2): 555–64.
83. Berns KI, Adler S. Separation of two types of adeno-associated virus particles containing complementary polynucleotide chains. *J. Virol.* 1972; 9(2): 394–6.
84. Fisher KJ, Gao GP, Weitzman MD, DeMatteo R, Burda JF, Wilson JM. Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. *J. Virol.* 1996; 70(1): 520–32.
85. Ferrari FK, Samulski T, Shenk T, Samulski RJ. Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. *J. Virol.* 1996; 70(5): 3227–34.
86. Miao CH, Nakai H, Thompson AR *et al.* Nonrandom transduction of recombinant adeno-associated virus vectors in mouse hepatocytes *in vivo*:

 DOI: 10.18609/cgti.2016.061

Cell cycling does not influence hepatocyte transduction. *J. Virol.* 2000; 74(8): 3793–803.

87. Nakai H, Storm TA, Kay MA. Recruitment of single-stranded recombinant adeno-associated virus vector genomes and intermolecular recombination are responsible for stable transduction of liver *in vivo*. *J. Virol.* 2000; 74(20): 9451–63.
88. McCarty DM, Monahan PE, Samulski RJ. Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. *Gene Ther.* 2001; 8(16): 1248–54.
89. Wang Z, Ma HI, Li J, Sun L, Zhang J, Xiao X. Rapid and highly efficient transduction by double-stranded adeno-associated virus vectors *in vitro* and *in vivo*. *Gene Ther.* 2003; 10(26): 2105–11.
90. Zhong L, Chen L, Li Y *et al.* 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*. M*ol. Ther.* 2004; 10(5): 950–7.
91. Zhong L, Qing K, Si Y, Chen L, Tan M, Srivastava A. Heat-shock treatment-mediated increase in transduction by recombinant adeno-associated virus 2 vectors is independent of the cellular heat-shock protein 90. *J. Biol. Chem.* 2004; 279(13): 12714–23.
92. Qing K, Li W, Zhong L *et al.* Adeno-associated virus type 2-mediated gene transfer: Role of cellular T-cell protein tyrosine phosphatase in transgene expression in established cell lines *in vitro* and transgenic mice *in vivo*. *J. Virol.* 2003; 77(4): 2741–6.
93. Zhong L, Li W, Yang Z. Improved transduction of primary murine hepatocytes by recombinant adeno-associated virus 2 vectors *in vivo*. *Gene Ther*. 2004; 11(14): 1165–9.
94. Zhao W, Wu J, Zhong L, Srivastava A. Adeno-associated virus 2-mediated gene transfer: Role of a cellular serine/threonine protein phosphatase in augmenting transduction efficiency. *Gene Ther.* 2007; 14(6): 545–50.
95. Zhong L, Zhou X, Li Y *et al.* Single-polarity recombinant adeno-associated virus 2 vector-mediated transgene expression *in vitro* and *in vivo*: Mechanism of transduction. *Mol. Ther.* 2008; 16(2): 290–5.
96. Qing K, Wang XS, Kube DM, Ponnazhagan S, Bajpai A, Srivastava A. Role of tyrosine phosphorylation of a cellular protein in adeno-associated virus 2-mediated transgene expression. *Proc. Natl Acad. Sci. USA* 1997; 94(20): 10879–84.
97. Qing K, Hansen J, Weigel-Kelley KA, Tan M, Zhou S, Srivastava A. Adeno-associated virus type 2-mediated gene transfer: Role of cellular FKBP52 protein in transgene expression. *J. Virol.* 2001; 75(19): 8968–76.
98. Abmayr S, Gregorevic P, Allen JM, Chamberlain JS. Phenotypic improvement of dystrophic muscles by rAAV/microdystrophin vectors is augmented by IGF1 codelivery. *Mol. Ther.* 2005; 12(3): 441–50.
99. Ma W, Li B, Ling C, Jayandharan GR, Srivastava A, Byrne BJ. A simple method to increase the transduction efficiency of single-stranded adeno-associated virus vectors *in vitro* and *in vivo*. *Hum. Gene Ther.* 2011; 22(5): 633–40.
100. Lisowski L, Dane AP, Chu K *et al.* Selection and evaluation of clinically relevant AAV variants in a xenograft liver model. *Nature* 2014; 506(7488): 382–6.
101. Wang XS, Ponnazhagan S, Srivastava A. Rescue and replication signals of the adeno-associated virus 2 genome. *J. Mol. Biol.* 1995; 250(5): 573–80.
102. Wang XS, Ponnazhagan S, Srivastava A. Rescue and replication of adeno-associated virus type 2 as well as vector DNA sequences from recombinant plasmids containing deletions in the viral inverted terminal repeats: Selective encapsidation of viral genomes in progeny virions. *J. Virol.* 1996; 70(3): 1668–77.
103. Ling C, Wang Y, Lu Y *et al.* The adeno-associated virus genome packaging puzzle. *J. Mol. Genet. Med.* 2015; 9(3).
104. Ling C, Wang Y, Lu Y *et al.* Enhanced transgene expression from recombinant single-stranded D-sequence-substituted adeno-associated virus vectors in human cell lines *in vitro* and in murine hepatocytes *in vivo*. *J. Virol.* 2015; 89(2): 952–61.
105. Jayandharan GR, Aslanidi G, Martino AT *et al.* Activation of the NF-kappaB pathway by adeno-associated virus (AAV) vectors and its implications in immune response and gene therapy. *Proc. Natl Acad. Sci. USA* 2011; 108(9): 3743–8.
106. Ling C, Li B, Ma W, Srivastava A. Development of optimized AAV serotype vectors for high-efficiency transduction at further reduced doses. *Hum. Gene Ther. Methods* 2016; 27(4): 143–9.
107. Leboulch P. Gene therapy: Primed for take-off. *Nature* 2013; 500(7462): 280–2.
108. Yla-Herttuala S. Endgame: Glybera finally recommended for approval as the first gene therapy drug in the European union. *Mol. Ther.* 2012; 20(10): 1831–2.
109. Dolgin E. Early clinical data raise the bar for hemophilia gene therapies.

Nat. Biotechnol. 2016; 34(10): 999–1001.

110. Flotte TR, Trapnell BC, Humphries M *et al.* Phase 2 clinical trial of a recombinant adeno-associated viral vector expressing alpha1-antitrypsin: Interim results. *Hum. Gene Ther.* 2011; 22(10): 1239–47.
111. Byrne PI, Collins S, Mah CC *et al.* Phase I/II trial of diaphragm delivery of recombinant adeno-associated virus acid alpha-glucosidase (rAAV1-CMV-GAA) gene vector in patients with pompe disease. *Hum. Gene Ther. Clin. Dev.* 2014; 25(3): 134–163.
112. Corti M, Cleaver B, Clement N *et al.* Evaluation of readministration of a recombinant adeno-associated virus vector expressing acid alpha-glucosidase in Pompe dise*ase: Preclinical to clinical planning. Hum. Gene Ther. Clin. Dev.* 2015; 26(3): 185–93.
113. Herzog RW. A cure for hemophilia: The promise becomes a reality. *Mol. Ther.* 2016; 24(9): 1503–4.
114. Gene Therapy Clinical Trials. http://www.wiley.com/legacy/wileychi/genmed/clinical
115. Sheridan C. Biogen and UPENN join forces to commercialize gene therapies. *Nat. Biotechnol.* 2016; 34(8): 791–3.
116. Calcedo R, Vandenberghe LH, Gao G, Lin J, Wilson JM. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. *J. Infect. Dis.* 2009; 199(3): 381–90.
117. Calcedo R, Morizono H, Wang L. Adeno-associated virus antibody profiles in newborns, children, and adolescents. *Clin. Vaccine Immunol.* 2011; 18(9): 1586–8.
118. Boutin S, Monteilhet V, Veron P *et al.* Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: Implications for gene therapy using aav vectors. *Hum. Gene Ther.* 2010; 21(6): 704–12.
119. Lock M, McGorray S, Auricchio A *et al.* Characterization of a recombinant adeno-associated virus type 2 reference standard material. *Hum. Gene Ther.* 2010; 21(10): 1273–85.
120. Ayuso E, Blouin V, Lock M *et al.* Manufacturing and characterization of a recombinant adeno-associated virus type 8 reference standard material. *Hum. Gene Ther.* 2014; 25(11): 977–87.
121. Allocca M, Doria M, Petrillo M *et al.* Serotype-dependent packaging of large genes in adeno-associated viral vectors results in effective gene delivery in mice. *J. Clin. Invest.* 2008; 118(5): 1955–64.
122. Lai Y, Yue Y, Duan D. Evidence for the failure of adeno-associated virus serotype 5 to package a viral genome > or = 8.2 kb. *Mol. Ther.* 2010; 18(1): 75–9.
123. Dong B, Nakai H, Xiao W. Characterization of genome integrity for oversized recombinant AAV vector. *Mol. Ther.* 2010; 18(1): 87–92.
124. Wu Z, Yang H, Colosi P. Effect of genome size on AAV vector packaging. *Mol. Ther.* 2010; 18(1): 80–6.
125. Duan D, Yue Y, Yan Z, Engelhardt JF. A new dual-vector approach to enhance recombinant adeno-associated virus-mediated gene expression through intermolecular cis activation. *Nat. Med.* 2000; 6(5): 595–8.
126. Nakai H, Storm TA, Kay MA. Increasing the size of rAAV-mediated expression cassettes *in vivo* by intermolecular joining of two complementary vectors. *Nat. Biotechnol.* 2000; 18(5): 527–32.
127. Sun L, Li J, Xiao X. Overcoming adeno-associated virus vector size limitation through viral DNA heterodimerization. *Nat. Med.* 2000; 6(5): 599–602.
128. Maina N, Zhong L, Li X *et al.* Optimization of recombinant adeno-associated viral vectors for human beta-globin gene transfer and transgene expression. *Hum. Gene Ther.* 2008; 19(4): 365–75.
129. Kotin RM, Siniscalco M, Samulski RJ *et al.* Site-specific integration by adeno-associated virus. *Proc. Natl Acad. Sci. USA* 1990; 87(6): 2211–5.
130. Samulski RJ, Zhu X, Xiao X *et al.* Targeted integration of adeno-associated virus (AAV) into human chromosome 19. *EMBO J.* 1991; 10(12): 3941–50.
131. Ling C, Yin Z, Li J, Zhang D, Aslanidi G, Srivastava A. Strategies to generate high-titer, high-potency recombinant AAV3 serotype vectors. *Mol. Ther. Methods Clin. Dev.* 2016; 3: 16029.
132. Bowles DE, McPhee SW, Li C *et al.* Phase 1 gene therapy for Duchenne muscular dystrophy using a translational optimized AAV vector. *Mol. Ther.* 2012; 20(2): 443–55.
133. Maheshri N, Koerber JT, Kaspar BK, Schaffer DV. Directed evolution of adeno-associated virus yields enhanced gene delivery vectors. *Nat. Biotechnol.* 2006; 24(2): 198–204.
134. Koerber JT, Jang JH, Schaffer DV. DNA shuffling of adeno-associated virus yields functionally diverse viral progeny. *Mol. Ther.* 2008; 16(10): 1703–9.
135. Lochrie MA, Tatsuno GP, Christie B *et al.* Mutations on the external surfaces of adeno-associated virus type 2 capsids that affect transduction and neutralization. *J. Virol.* 2006; 80(2): 821–34.
136. Gabriel N, Hareendran S, Sen D *et al.* Bioengineering of AAV2 capsid at specific serine, threonine, or lysine residues improves its transduction

 DOI: 10.18609/cgti.2016.061

efficiency *in vitro* and *in vivo*. *Hum. Gene Ther. Methods* 2013; 24(2): 80–93.

137. Sen D, Gadkari RA, Sudha G *et al.* Targeted modifications in adeno-associated virus serotype 8 capsid improves its hepatic gene transfer efficiency *in vivo*. *Hum. Gene Ther. Methods* 2013; 24(2): 104–16.
138. Kelemen RE, Mukherjee R, Cao X, Erickson SB, Zheng Y, Chatterjee A. A precise chemical strategy to alter the receptor specificity of the adeno-associated virus. *Angew Chem. Int. Ed. Engl.* 2016; 55(36): 10645–9.
139. Berns KI. My life with adeno-associated virus: A long time spent studying a short genome. *DNA Cell Biol.* 2013; 32(7): 342–7.
140. Sender R, Fuchs S, Milo R. Revised estimates for the number of human and bacteria cells in the body. *PLoS Biol.* 2016; 14(8): e1002533.
141. Song L, Kauss MA, Kopin E *et al.* Optimizing the transduction efficiency of capsid-modified AAV6 serotype vectors in primary human hematopoietic stem cells *in vitro* and in a xenograft mouse model *in vivo*. *Cytotherapy* 2013; 15(8): 986–98.
142. Song L, Li X, Jayandharan GR *et al.* High-efficiency transduction of primary human hematopoietic stem cells and erythroid lineage-restricted expression by optimized AAV6 serotype vectors *in vitro* and in a murine xenograft model *in vivo*. *PLoS One* 2013; 8(3): e58757.
143. Ling C, Bhukhai K, Yin Z *et al.* High-efficiency transduction of primary human hematopoietic stem/progenitor cells by AAV6 vectors: Strategies for overcoming donor-variation and implications in genome editing. *Sci. Rep*. 2016; 6: 35495.

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