The AAV Vector Toolkit: Poised at the Clinical Crossroads

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The discovery of naturally occurring adeno-associated virus (AAV) isolates in different animal species and the generation of engineered AAV strains using molecular genetics tools have yielded a versatile AAV vector toolkit. Promising results in preclinical animal models of human disease spurred the much awaited transition toward clinical application, and early successes in phase I/II clinical trials for a broad spectrum of genetic diseases have recently been reported. As the gene therapy community forges ahead with cautious optimism, both preclinical and clinical studies using first generation AAV vectors have highlighted potential challenges. These include cross-species variation in vector tissue tropism and gene transfer efficiency, pre-existing humoral immunity to AAV capsids and vector dose-dependent toxicity in patients. A battery of second generation AAV vectors, engineered through rational and combinatorial approaches to address the aforementioned concerns, are now available. This review will provide an overview of preclinical studies with the ever-expanding AAV vector portfolio in large animal models and an update on new lead AAV vector candidates poised for clinical translation.

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FOREWORD

The biology of adeno-associated virus (AAV) has been extensively studied since the discovery of the first adeno-associated satellite virus in the 1960s.[1] The potential application of recombinant AAV as vectors for gene transfer was realized over the next three decades. A vast majority of these seminal studies were carried out with AAV serotype 2, the most prevalent strain found in the human population. As a result, receptor usage, infectious pathways, tissue tropism, antigenicity, immune profile, and persistence of AAV2 vectors in a wide range of animal models are now well known. Also, several phase I clinical trials for gene therapy of inherited and acquired diseases using first generation AAV2 vectors have been completed or are currently in progress.[2] For instance, AAV2 vectors have been evaluated for gene transfer within the liver in the treatment of hemophilia B, in the lung for treatment of cystic fibrosis, in the brain for treatment of Parkinson's, Batten's, and Canavan's disease; within the joints for patients with rheumatoid arthritis; and in the eye for treatment of Leber's congenital amaurosis and age-related macular degeneration.[2] Perhaps the most striking example of successful gene therapy in a clinical setting is the phase I trial of Leber's congenital amaurosis.[3–9] Persistent improvement in vision of affected patients has been reported in over 40 patients treated with AAV2 vectors delivering a corrective version of the *RPE65* gene. However, other clinical trials reporting partial success, and in some cases none at all, have still been instrumental in highlighting critical challenges including pre-existing humoral immunity, vector dose-dependent toxicity and significant cross-species differences in the nature of the immune response to AAV2 vectors and transgene products.

Over the most recent decade of this bench-to-bedside transition of AAV2 vectors, several other serotypes and novel AAV strains have been isolated.[10–13] Comprehensive efforts to unravel the biology of such new AAV isolates have established key differences in AAV capsid structure, their antigenic diversity and varying tissue tropisms demonstrated in preclinical animal models. A summary of current knowledge pertaining to the biology and capsid structure of different AAV serotypes and pertinent literature is outlined in **Table 1**. These features have enabled the rapid transition of different AAV serotypes into promising lead vector candidates currently being evaluated in several phase I clinical trials. For instance, muscle-tropic AAV1, originally isolated as a contaminant in adenovirus stocks, is being evaluated for intramuscular gene delivery in α-1 antitrypsin deficiency, lipoprotein lipase deficiency, Pompe's disease, limb girdle muscular dystrophy, and cardiac failure. The closely related AAV6 strain is also being evaluated for therapeutic gene transfer in patients with heart failure. Another nonhuman primate isolate, AAV8, has demonstrated promising early stage results in a clinical trial for gene therapy

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Table 1 Biology of naturally occurring AAV strains and isolates

AAV clades and clones	Representative members	Primary (glycan) receptor	Secondary receptor (Coreceptor)	References
A	AAV1	α2,3/α2,6 N-linked sialic acid	— (Not known)	119
	AAV6	α2,3/α2,6 N-linked sialic acid/heparan sulfate	Epidermal growth factor receptor (EGFR)	119–121
B	AAV2	Heparan Sulfate	Fibroblast/hepatocyte growth factor receptor (FGFR/HGFR) Laminin receptor (LR) Integrin αVβ5/α5β1	122–128
C	AAV2-AAV3 hybrid	—	—	60
D	AAV7	—	—	129
E	AAV8	—	LR	124,130
	AAV10	—	—	131
	AAVrh.10	—	—	60
F	AAV9/AAVhu.14	Galactose	LR	124,132
Clones	AAV3a/3b	Heparan sulfate	HGFR/LR	124,133,134
	AAV4	α2,3 O-linked sialic acid	—	135,136
	AAV5	α2,3 N-linked sialic acid	Platelet-derived growth factor receptor (PDGFR)	137–139
	AAV11	—	—	131
	AAVrh32.33	—	—	30
	AAV12	—	—	79

Abbreviation: AAV, adeno-associated virus.

of hemophilia B. Importantly, however, the lack of expression in one subject enrolled in the latter trial reiterated the challenge of pre-existing neutralizing antibodies (NAbs) against different AAV capsids in the human population.

Against the backdrop of ongoing clinical trials, the aforementioned AAV serotypes and several other AAV isolates covering a broad tissue tropism range have now been extensively characterized in preclinical animal models. In addition, an arsenal of engineered AAV strains have now been generated with clinically relevant challenges in mind. In the following review, we will update progress in the ever-expanding AAV vector toolkit, highlight recent preclinical studies in large animal models and provide specific examples of naturally occurring as well as engineered strains poised for clinical translation. While we have made every effort to highlight recent contributions to the AAV vector repertoire, it should be noted that the current version of the AAV portfolio was made possible by contributions worldwide, some of which might have been omitted due to the scope of the current review.

NATURAL AAV SEROTYPES: ON BOTH SIDES OF THE CLINICAL FENCE

Comprehensive evaluations of the tissue tropisms and biodistribution of AAV serotypes 1 through 9 in mouse models have been published to date. In addition, several AAV serotypes are now being evaluated in clinical trials as outlined earlier, and their performance in the clinic has been reviewed elsewhere.[2] More recently, collaborative efforts to establish the preclinical transduction profile of different AAV serotypes in large animal models such as primates, pigs, and dogs have increased. These studies have provided valuable insight into cross-species differences in transduction efficiency and immune response to AAV vectors. We highlight key findings from recent preclinical studies evaluating different AAV serotypes in large animal models below (summarized in **Table 2**).

Liver gene transfer: AAV8

Several studies have validated preferential liver transduction in nonhuman primates and dog models by AAV8 vectors. While the absolute transgene expression levels in primate and dog liver have been shown to be lower than mouse models, promising therapeutic indices have been achieved in large animals.[14,15] For instance, intravenous administration of self-complementary AAV8 vectors in the nonhuman primate liver can mediate expression levels of factor IX sufficient for phenotypic correction in hemophilia patients[16–18] and sustained correction of disease in a canine model of hemophilia.[19–21] Effects of transient immunosuppression on AAV8 liver transduction in nonhuman primates and the ability of proteasomal inhibitors to enhance AAV8 liver transduction in canine models have also been evaluated.[20,22] Moreover, a recent study evaluating intrauterine gene transfer with AAV8 vectors in rhesus macaques yielded robust liver-specific expression of factor IX in injected offspring for up to 2 years.[23] In another recent study, AAV8-mediated hepatic gene transfer in neonatal rhesus macaques was found to be less stable when compared to adolescent animals. Although infant monkeys displayed transgene expression in nearly 98% of hepatocytes within 1 week of administration, a significant loss of transgene expression was observed ~1 month postinjection.[24]

Taken together with the successful completion of St Jude's phase I clinical trial in hemophilia patients,[25] the aforementioned studies corroborate the choice of AAV8 vectors as the lead candidate for factor IX gene transfer in the human liver. These studies also provide a roadmap for evaluating AAV8 vectors in clinical liver gene transfer protocols for treatment of conditions such as

Table 2 Summary of current and emerging AAV vectors suitable for specific clinical applications

Organs	Disease targets	AAV serotypes and isolates	Emerging vector candidates	References
Liver	Hemophilia, α-1 antitrypsin deficiency, ornithine transcarbamylase deficiency	AAV8	AAV2 (Y→F), AAV7, AAV-HSC15/17	84, 18, 140, 82
Heart	Congenital heart failure, cardiomyopathies	AAV1, AAV6, AAV9	AAVM41, AAV2i8, AAV9.45	112, 96, 113
Skeletal Muscle	Muscular dystrophies, α-1 antitrypsin deficiency, lipoprotein lipase deficiency, lysosomal storage disorders	AAV1, AAV6, AAV9	AAV7, AAV2.5, AAV6 (Y445F/Y731F), AAV2i8, AAV9.45	141, 92, 86, 96, 113
Lung	Cystic fibrosis, α-1 antitrypsin deficiency	AAV5	AAV6.2, AAV2.5T, AAV-HAE1/2	93, 110, 111
CNS	Parkinson's, Alzheimer's, Batten's, Canavan's, epilepsy, amyotrophic lateral sclerosis, spinal muscular atrophy, Rett syndrome, lysosomal storage disorders	Intracranial: AAV1, AAV5, AAV8 Systemic: AAV9	For systemic use: AAVrh.10, AAV Clone 32/83	61, 62, 116
Eye	Leber's congenital amaurosis, macular degeneration	AAV4, AAV8	AAVShH10, AAV2 (Y→F), AAV8(Y733F)	114, 84, 89

Abbreviation: AAV, adeno-associated virus.

hemophilia B, α-1 antitrypsin deficiency, phenylketonuria and lysosomal storage disorders to name a few.

A common observation in clinical and preclinical studies is that pre-existing low levels of NAbs profoundly impact gene transfer efficiency. A recent mechanistic study demonstrated that AAV8 capsid-specific NAb diminished liver deposition of genomes and increased genome distribution to the spleen.[26,27] Three separate studies have established the prevalence of NAbs to AAV8 and other serotypes in the human population. The NAb prevalence for AAV8 was noted to be lowest amongst other serotypes in case of pediatric hemophilia patients (22.6%),[28] healthy human subjects (19%)[29] and a worldwide epidemiology study.[30] Despite this relatively favorable antigenic profile for AAV8 in humans, development of strategies to evade NAbs would make a diverse patient cohort eligible for enrollment in clinical trials. Within this framework, generating Nab-escape AAV8 variants and reengineering antigenic domains on the AAV8 capsid[31] are likely to yield next generation vector candidates for liver gene transfer. Furthermore, the availability of the AAV8 crystal structure[32] should facilitate engineering new lab-derived AAV strains.

Cardiac and musculoskeletal gene transfer: AAV1, AAV6, and AAV9

Intracoronary delivery of AAV1 vectors carrying the *SERCA2a* gene has been shown to prevent cardiac dysfunction, improve ventricular remodeling and vascular reactivity in a porcine model of heart failure.[33,34] These studies provided the foundation for the current phase I/II clinical trial of gene therapy for cardiac failure with AAV1/SERCA2a vectors.[35] In addition to cardiac muscle, regional intravenous delivery of AAV1 vectors to the hind limb of nonhuman primates has also been reported to mediate robust transgene expression similar to AAV8 in different skeletal muscle groups without any signs of immunotoxicity.[36–38]

Similar to AAV1, coronary infusion of the closely related serotype AAV6 mediates efficient and long-term myocardial gene expression.[39] Effective cardiac delivery of shRNA encoding vector genomes by AAV6 has also been demonstrated in canine models.[40] Furthermore, a recent report utilizing a novel technique for myocardial gene delivery demonstrates robust cardiac gene transfer using AAV6 vectors in a sheep model.[41] Likewise, high transduction efficiencies have also been demonstrated in skeletal muscle of nonhuman primates and a canine muscular dystrophy model following intramuscular administration of AAV1 and AAV6 vectors, respectively.[42,43] An open label, dose-escalation study in hemophilia patients involving intramuscular administration of AAV2 vectors encoding factor IX revealed the need for higher transduction efficiency in muscle to attain a sustained therapeutic effect. More recently, phase I and II clinical trials for gene therapy of α-1 antitrypsin deficiency involving intramuscular administration of AAV1 vectors have been successfully completed thereby supporting the safety and feasibility of this approach.[44,45] Similar application of AAV1 vectors for correction of lipoprotein lipase deficiency through intramuscular administration in human subjects has also been demonstrated.[46]

Early studies with AAV9 vectors in infant rhesus macaques have demonstrated preferential cardiac transduction following intravenous administration in nonhuman primates similar to mouse models.[47] These studies have now been corroborated by a preclinical study demonstrating efficient cardiac-specific gene transfer following intracoronary administration of AAV9 vectors in a porcine model of post-ischemic heart failure.[48] However, another study in adult human primates comparing different AAV vectors has shown that AAV9 vectors mediate less efficient cardiac gene transfer than AAV6 in adult nonhuman primates.[49] That said, an interesting related observation is that in contrast to liver studies, cardiac gene transfer efficiency of different AAV serotypes generally appears to display a lesser extent of cross-species variation. In addition to delivery to the heart, widespread skeletal muscle expression of human mini-dystrophin in a neonatal golden retriever muscular dystrophy model following intravenous administration of AAV9 vectors has been reported.[50] Taken together, preclinical studies in large animal models strongly argue for the evaluation of AAV serotypes 1, 6, and 9 as lead vector candidates in gene therapy of cardiac and musculoskeletal disease.

CNS gene transfer: AAV5 and AAV9

In the past 5 years, several studies evaluating the transduction efficiency of different AAV serotypes in the primate brain have been reported against the backdrop of phase I clinical trials using AAV2 vectors for gene therapy of Canavan's, Parkinson's, and Batten's disease. For instance, convection-enhanced delivery,[51,52] a dose–response study and long-term assessment have demonstrated

clinical improvement in Parkinsonian monkeys with AAV2 vectors.[53–55] Convection-enhanced delivery of AAV1 vectors in the nonhuman primate brain revealed transduction of oligodendrocytes and astrocytes in addition to the neuronal population.[56] In another recent study in adult cynomolgus monkeys involving striatal injection of AAV1, AAV5 and AAV8, AAV5 and AAV1 were superior to AAV8 in transducing neurons in the nonhuman primate striatum.[57] Such efficient intracerebral gene transfer using AAV5 vectors following intracerebral injection in nonhuman primates has now been corroborated in other preclinical studies.[58,59] Another emerging candidate for central nervous system (CNS) gene delivery is AAVrh.10, a rhesus macaque isolate belonging to clade E (AAV8 family[60,61]). This AAV isolate has shown promising results in the rat brain and found suitable for therapeutic CNS gene transfer in a mouse model of Batten disease.[62]

Recent studies have also focused on cross-species comparison of the ability of AAV9 vectors to traverse the blood–brain barrier in mouse models and nonhuman primates following intravenous administration.[63–65] In addition to decreased transduction efficiency in comparison with the mouse brain, a shift in AAV9 tropism from neuronal to glial cells was observed in the monkey brain following IV administration.[64] These findings underscore the importance of cross-species variation in transduction efficiency and tissue tropism of different AAV serotypes. Furthermore, systemically injected AAV9 in cynomolgus macaques was efficient at crossing the blood–brain barrier, with transgene expression being detected in glial cells throughout the brain and dorsal root ganglia neurons and motor neurons within the spinal cord.[65] Systemic injection of AAV9 vectors in macaques also results in robust transduction of skeletal muscle and other peripheral organs. As an alternative strategy, restricted gene expression in the primate CNS has be achieved by AAV9 delivery to cerebrospinal fluid, which efficiently targets motor neurons. These strategies provide the rationale for translation of AAV9-mediated gene transfer to patients with CNS-related disorders. Lastly, a recent study comparing the ability of different AAV strains to traverse the blood–brain barrier in mice demonstrates that AAVrh.10 is at least as efficient as AAV9 vectors in CNS gene transfer following systemic administration.[66]

Gene transfer to the eye: AAV4 and AAV8

Over the past 5 years, AAV vectors have clearly emerged as lead candidates for therapeutic gene transfer in a wide range of eye diseases. The safety and efficacy of AAV2 vectors delivering the *RPE65* transgene in clinical gene therapy of Leber's congenital amaurosis has been unequivocally established.[3–9] An earlier comparison of AAV serotypes 1, 2, and 5 yielded similar transgene expression levels within retinal pigmented epithelium (RPE) as well as photoreceptors upon subretinal administration in a canine model of Leber's congenital amaurosis.[67] Another study has demonstrated successful restoration of vision in RPE65-deficient Briard dogs using AAV4 vectors, which display selective tropism for the RPE.[68] Transduction efficiency of AAV4 was shown to be similar to AAV2 vectors in this canine model.

Subretinal administration of AAV8 vectors in canine models has been shown to mediate transgene expression in the RPE, photoreceptors as well as cells of the inner nuclear layer and ganglion cells.[69] These results suggest that AAV8 vectors might undergo transport along neurons of the visual pathway. Moreover, while no species differences have been noted between rats, dogs and primates with AAV1, AAV2, or AAV5 vectors, AAV8 vectors appear to display greater spread in the canine model. A recent dosage threshold study comparing AAV2 and AAV8 vectors was carried out in a nonhuman primate model.[70] Although both serotypes demonstrated comparable transduction levels, AAV8 vectors transduced photoreceptors with greater efficiency when compared to AAV2.

In general, the pre-existing NAb response is thought to minimally affect AAV transduction efficiency in immune-privileged sites such as the brain or the eye. Nevertheless, it is noteworthy to mention that a transient increase in anti-AAV2 NAbs was seen in two of three subjects in the Leber's congenital amaurosis clinical trial.[9] Whether this transient increase in NAbs will impact vector readministration in the same or contralateral eye remains to be seen. The availability of other AAV serotypes with similar transduction efficiency or broader tropism within the eye might serve as an advantage in such a scenario.

Pulmonary gene transfer

Gene transfer to the lung for treatment of diseases such as cystic fibrosis using AAV vectors has faced several hurdles such as the lack of availability of appropriate animal models, poor transduction efficiency due to various physiological barriers and cross-species variation. Repeated administration of AAV2 vectors encoding the cystic fibrosis transmembrane regulator, although well tolerated, failed to demonstrate any significant improvement in lung function in cystic fibrosis patients.[71] Recent studies have demonstrated that AAV1 vectors mediate more efficient transgene expression than AAV5 following intratracheal delivery in a chimpanzee model.[72] These results corroborated studies carried out in cultured human airway epithelia *in vitro*. Intratracheal delivery of AAV6 vectors in immunosuppressed dogs was recently shown to mediate efficient transduction in airway epithelia.[73] Recent reports describing the development of ferret and porcine models of cystic fibrosis with lung anatomy and cell biology similar to humans are noteworthy,[74–76] as these critical advances in the field will likely enable clinically relevant studies of AAV-mediated lung gene transfer in large animal models.

OTHER NATURAL AAV ISOLATES

The discovery of several hundred human and nonhuman primate isolates of AAV has been reviewed earlier.[10] Other studies have reported the isolation of new AAV isolates in ATCC (Manassas, VA) simian adenovirus stocks.[77–79] The AAV12 serotype isolated in aforementioned studies, which displays 78% identity to AAV4, was found to be particularly resistant to neutralization by anticapsid antibodies in human serum. In addition, AAVhu.37 and AAVrh.8 have been noted for their enhanced liver transduction efficiency in mouse models.[26] Furthermore, successful gene transfer following intracranial as well as intrapleural administration of AAVrh.10 in mouse models has also been demonstrated.[62,80,81] The City of Hope Medical Center recently reported the isolation of new AAV9 variants from $CD34^+$ human hematopoietic stem cells.[82] Notably, AAV-HSC15 and AAV-HSC17 displayed improved liver transduction efficiency in mouse models when compared to AAV9. In

another study, the UMass Gene Therapy Center reported isolation of novel AAV5 variants from chimpanzee tissues such as liver, lung, and heart. One variant, CHt-P6, was found to target alveoli and airway epithelia and transduce the murine lung at modestly higher levels than AAV5 vectors following intranasal instillation.[83] Evaluation of these AAV isolates in large animal models is forthcoming.

REENGINEERED AAV STRAINS: NEXT GENERATION VECTOR CANDIDATES FOR THE CLINIC

Naturally occurring AAV serotypes and isolates show promise for gene transfer in a number of clinical indications. However, studies outlined above have posed the question whether synthetic AAV strains engineered with defined properties will demonstrate significant advantages in a clinical setting. New AAV variant or mutant strains have thus been engineered—either by rational design or directed evolution—to address clinically relevant challenges such as dose-related toxicity and gene expression in off-target tissues. Such reengineered AAV strains have displayed the potential to markedly reduce vector dose administered owing to enhanced transduction efficiency in target tissues (summarized in **Table 2**). Having established their efficacy in mouse models, these next generation vectors are poised for translational studies in preclinical large animal models and subsequently add to the growing pipeline of clinical grade vectors.

Tyrosine-mutant AAV vectors

In an effort to reduce dose-related toxicity of AAV vectors, Zhong, Srivastava and others[84] have developed a series of next generation tyrosine-mutant vectors that display improved gene transfer efficiency. Briefly, phosphorylation of surface-exposed tyrosine residues on AAV2 capsids is correlated with decreased transduction efficiency and thought to occur due to increased ubiquitination resulting in proteasomal degradation. Mutagenesis of tyrosines into phenylalanines on the AAV2 capsid was demonstrated to bypass this critical barrier to transduction and improved gene transfer efficiency by as much as 30-fold at a log lower vector dose in mice. Subsequent studies in mice have shown that this strategy can be broadly applied to different tissues such as liver, retina, and skeletal muscle and has been expanded to include AAV serotypes such as AAV3, AAV6, AAV8, and AAV9.[85–89] A recent report has also demonstrated that mutation of capsid surface-exposed serines prone to phosphorylation can enhance transduction in a similar fashion.[90] These exciting new reagents are poised for preclinical dose-variation studies in large animal models. Within this framework, it is noteworthy to mention that enhanced gene transfer by AAV vectors has been achieved following concurrent administration of the proteasome inhibitor, bortezomib, in a canine model.[22] Successful translational studies with tyrosine-mutant vectors would not only corroborate the aforementioned studies, but also enable reduction in vector dose needed for clinical trials.

AAV2.5

The first example of a hybrid AAV vector to proceed to clinical trials is AAV2.5, a rationally engineered AAV strain designed to graft the muscle tropism determinants of AAV1 onto parental AAV2.[91] As shown in preclinical studies and in a phase I clinical trial of Duchenne muscular dystrophy, AAV2.5 is capable of robust gene transfer in skeletal muscle. Consistent with an antigenically distinct profile, this hybrid vector has also been shown to evade NAbs against both AAV1 and AAV2 capsids. While this clinical study highlighted the need to consider T-cell immunity to self and foreign dystrophin epitopes in Duchenne muscular dystrophy patients,[92] the AAV2.5 vector demonstrated an excellent safety profile and remains a promising vector candidate for clinical gene transfer in musculoskeletal diseases.

AAV6.2

Another interesting subset of hybrid AAV vectors are the singleton vectors developed by Vandenberghe, Wilson and others.[93] For instance, the engineered AAV6.2 strain contains a single F129L mutation in the phospholipase A2 domain originally present in the closely related AAV1. The AAV6.2 vector has also been shown to mediate efficient gene transfer in comparison with other related strains within the same clade following intravenous administration in a mouse model.[93] More importantly, this hybrid vector outperformed several AAV serotypes in the mouse conducting and nasal airways as well as cultured human airway epithelia.[94] Functional correction of cystic fibrosis transmembrane regulator expression cultured cystic fibrosis human airway epithelia has also been demonstrated.[95] Evaluation of AAV6.2 in large animal models is likely to shed more light on the translational potential of this hybrid vector for gene therapy of cystic fibrosis and other lung diseases.

AAV2i8

The UNC Gene Therapy Center has recently developed reengineered AAV vectors demonstrating attenuated liver sequestration following intravenous administration.[96] Liver-detargeting potential of mutant AAV2 vectors was originally observed as a consequence of mutating heparin-binding arginine residues on the AAV capsid by Kern, Kleinschmidt and others.[97] While reengineering the heparin-binding footprint of AAV2 with corresponding domains from other AAV serotypes, we generated the hybrid AAV2i8 vector harboring a linear epitope from AAV8. In addition to being detargeted from the murine liver, this hybrid strain displayed the ability to traverse the blood vessel barrier and transduce cardiac and skeletal muscle tissue with high efficiency. Since then, we have successfully carried out isolated limb infusion studies[98] as well as intravenous administration resulting in robust gene expression in primates (McPhee SW, Asokan A, Tarantal A, Samulski, RJ, unpublished results). Notably, low volume injections of AAV2i8 are significantly more efficient in transducing primate skeletal muscle during isolated limb infusion studies, in contrast to AAV8, which only performs well at high injection volumes. Also noteworthy against the backdrop of AAV2i8 vector development studies are recent clinical studies of transvenous limb perfusion with saline[99] in muscular dystrophy patients at the UNC Wellstone Center. These studies suggest that high-pressure retrograde transvenous limb perfusion with saline up to 20% of limb volume at above infusion parameters is safe and feasible in adult human muscular dystrophy. Taken together with the aforementioned results, AAV2i8 is a promising lead candidate for correction of musculoskeletal diseases through isolated transvenous limb infusions.

AAVrh32.33

Studies with a hybrid AAV isolate, rh32.33, have demonstrated that specific capsid domains on this strain augment the $CD8^+$ T-cell responses to both capsid proteins and transgene product.[100] Thus, AAVrh32.33 appears to be distinct from other AAV serotypes, which have been shown induce a functionally impaired T-cell response to transgene product in mouse models as well as the clinic.[100,101] Based on the aforementioned studies, AAVrh32.33 has been proposed as a new genetic vaccine platform. Evaluation of the immunogenicity of this hybrid strain in large animal models is likely to enable development of a robust AAV-based clinical vaccine platform.

CHIMERIC AND MUTANT STRAINS: EMERGING CANDIDATES FROM COMBINATORIAL AAV LIBRARIES

The earliest AAV libraries were random peptide display libraries generated by Müller *et al.*[102] Targeted vectors derived from the aforementioned libraries continue to be developed and validated in mouse models.[103–106] Additional work in preclinical large animal models will be needed to determine whether these targeting strategies will translate across species. Combinatorial protein engineering strategies such as error-prone PCR and DNA shuffling followed by directed evolution have yielded several AAV vectors with important functional mutations relative to a parent serotype, or chimeras of several serotypes. Several recent studies have assessed the potential applications of such vectors in rodent models. Further evaluation of this chimeric AAV portfolio in large animal models would unequivocally establish their position in the clinical pipeline. The NAb profile of different AAV strains in commonly used animal models is also noteworthy in this regard.[107] Major advances in vector design addressing short-term and long-term clinical needs that are poised for cross-species characterization are listed below (summarized in **Table 2**).

NAb-escape mutants

Amongst the earliest examples of promising vector candidates developed using such combinatorial library approaches are the AAV2-derived mutants, AAV2.15 and AAV2.4.[108] Both these strains harbor mutations at critical antigenic sites, thereby efficiently evading NAbs in human serum. These strains have the potential for immediate translation as candidates for vector readministration in ongoing clinical trials. Another approach involves randomization of previously mapped immunogenic epitopes on the AAV2 capsid to evolve NAb-escape mutants.[109] More recently, a structure-based approach to reengineer surface-exposed antigenic epitopes previously identified using cryo-EM has been proposed.[31] This strategy was used to engineer novel AAV8 NAb-escape variants suitable for administration in the presence of pre-existing humoral immunity to the AAV8 capsid.

Airway-tropic AAV vectors

As outlined earlier, airway gene transfer faces major hurdles such as the lack of availability of appropriate animal models, poor transduction efficiency due to various physiological barriers and cross-species variation. Keeping these challenges in mind, novel vector candidates for airway gene transfer have been developed by subjecting combinatorial AAV libraries to directed evolution in cultured human airway epithelia. One study yielded an AAV2/AAV5 chimeric harboring a single point mutation (AAV2.5T)[110] that was 100-fold more efficient than AAV5 vectors in mediating gene transfer to human airway cultures and rescued chloride ion transport following cystic fibrosis transmembrane regulator gene transfer in diseased human airway cultures. These results are currently being evaluated for translation to a porcine model of cystic fibrosis.[75,76] Another contemporary study yielded two chimeric strains, AAV-HAE1 and AAV-HAE2, containing capsid components derived from AAV1, AAV6 and AAV9 that were more efficient than AAV6 and efficiently corrected the cystic fibrosis defect in diseased human airway cultures.[111] Both studies corroborate the notion that AAV5, AAV6, and mutants thereof constitute lead candidates for evaluation in large animal models of lung disease.

Muscle-tropic AAV vectors

Directed evolution of the myocardium-tropic AAVM41, a chimeric capsid derived from AAV1, AAV6, AAV7 and AAV8, was recently achieved by Yang, Xiao and others[112] at the UNC Gene Therapy Center. AAVM41 was shown to be more efficient than AAV6 in transducing the murine heart. In addition, the mutant also demonstrated remarkably attenuated tropism for liver, skeletal muscle amongst other organs. Efficient rescue of cardiac functions were demonstrated following administration of AAVM41 vectors delivering delta-sarcoglycan in a hamster cardiomyopathy model. The UNC Gene Therapy Center also recently reported a subset of liver-detargeted AAV9 mutant vectors, which allowed efficient gene expression in cardiac and skeletal muscle similar to parental AAV9.[113] The aforementioned vectors, when combined with other transcriptional and translational regulation strategies are likely to yield optimal vector candidates for selective cardiac or musculoskeletal gene transfer. Evaluation of these vectors in canine and primate models is forthcoming.

Chimeric AAV vectors for CNS and retinal gene transfer

Directed evolution of chimeric AAV vectors for transducting astrocytes[105] has been demonstrated *in vitro*. Chimeric AAV vectors derived from the latter approach, particularly a variant of AAV6 named ShH10, have been shown to transduce Müller glia following intravitreal administration in a rat model with high efficiency. Investigators at UC Berkeley used these variants to test the hypothesis that intravitreal vector administration can provide strong therapeutic efficacy across the retina while avoiding the risks inherent in more delicate subretinal injections, and they recently found that ShH10 mediated delivery of GDNF significantly ameliorated degeneration and vision in a rat retinitis pigmentosa model.[114] These new vectors are likely candidates for further evaluation in animal models of Alzheimer's disease, amyotrophic lateral sclerosis in the CNS and a broad range of retinal diseases. Similar strategies have been applied toward evolution of new AAV vectors capable of transducing neural stem cells and glioma cell lines.[106,115] While the neural stem cell-tropic AAVr3.45 vector shows promise as a reagent for applications involving gene targeting and stem cell reprogramming, the potential for exploiting oncotropic AAV vectors in preclinical models of glioma remains to be determined.

Another interesting development within the framework of CNS applications is the directed evolution of chimeric AAV vectors that can cross the seizure-compromised blood–brain barrier in a rat model of epilepsy.[116] Mosaic clones 32 and 83 derived from AAV serotypes 1, 3, 8, and 9 demonstrated the ability to efficiently transduce CNS regions damaged during induced seizures, via the compromised blood–brain barrier at those sites. Although similar to AAV8 in transduction efficiency, these clones displayed biodistribution profiles markedly reduced peripheral organ tropism compared to the parental AAV serotypes. Studies evaluating the therapeutic efficacy of these vectors in a rat model of epilepsy are forthcoming.

NEW DIRECTIONS: MAINTAINING THE CLINICAL PIPELINE AND ONGOING TRIALS

The AAV vector toolkit is currently well-equipped with a broad portfolio of naturally occurring serotypes, reengineered variants, and chimeric and mutant strains. While evaluation of these strains continues on both in preclinical animal models and in the gene therapy clinic, it is critical to maintain a robust pipeline of AAV vector candidates engineered for specific, future applications. New strategies to generate combinatorial AAV libraries that will expand the current portfolio and enable generation of synthetic AAV strains are required. Increased focus on evolving AAV strains in clinically relevant settings has only just begun. For instance, exciting new directed evolution studies were recently carried out in a pig model and hold considerable potential for developing lead vector candidates for cystic fibrosis gene therapy.[117] In addition, evolution of liver-tropic AAV strains derived from AAV3, AAV6, AAV8 and AAV9 in the humanized $Fah^{-/-}/Rag2^{-/-}/IL2rg^{-/-}$ mouse model repopulated with over 25% human hepatocytes was recently reported.[118] Directed evolution of AAV strains in other humanized mouse models as well as canine and primate models will thus not only help expand the AAV portfolio, but also help understand cross-species variation in tissue tropism, transduction efficiency and immune response, ultimately leading to successful translation of safe gene therapy modalities to the clinic.

Recently, the American Society of Cell and Gene Therapy held an National Institutes of Health (NIH) symposium showcasing numerous clinical success stories that have accrued in the last couple of years.[2] With respect to AAV vectors, mounting data in a number of therapeutic areas clearly supports continued exploration of this vector for gene therapy of monogenic disorders. For instance, five AAV clinical trials for Leber's congenital amaurosis (three in United States, one in United Kingdom, one in Israel) treating 35 patients have been carried out. The longest follow-up extending over 3.5 years, reported no vector related safety issues with efficacy in most patients with some patients demonstrating over 63,000-fold improvement in rod photoreceptor-based vision (Hauswirth and others, ASGCT NIH symposium 2011).[7] These outcomes were highlighted by the fact that pseudo-fovea in patients developed exactly at the site of vector administration, strongly supporting the notion that next generation vectors may overcome these limitations by intravitreal delivery. Another clinical highlight at the meeting was the ongoing study with AAV8 vectors packaging self-complementary FIX administered in hemophilia B patients. Unlike preceding trials with AAV2 vectors packaging single-stranded FIX trials,[2] the study carried out by groups at St. Jude's Hospital and University College London demonstrated long-term expression (18 months and counting) with FIX levels ranging from 1 to 8% (Reiss and others, ASGCT NIH Symposium 2011).[25] The latter study corroborates earlier predictions that AAV vector development will continue to resolve rate-limiting steps observed in earlier trials (e.g., lower vector dose and higher potency will overcome the dose-dependent immunotoxicity proposed in the earlier U Penn trial).

The latest clinical results were particularly striking against the backdrop of discussions held during a breakout session, where Dr Kathy High discussed the lack of persistent FIX expression in an immunosuppressed hemophilia B patient administered AAV2 vectors, contrasting results from an earlier preclinical study in rhesus macaques.[20] Although obtained from a small patient cohort, results from the recent St Jude/UCL hemophilia clinical trial,[25] in conjunction with earlier studies, suggest that capsid-specific cytotoxic T lymphocyte response appears to be a minor concern in the clinical setting. Clearly as we accumulate more information in patients, we can hope to sort these disparities with preclinical studies and better determine which animal models are of value in guiding the clinical community forward in utilizing different vectors. Thus, the long-term therapeutic benefits observed in the St Jude/UCL studies clearly highlight the fact that continued AAV vector development is and most likely will be the primary path forward toward solving these early clinical conundrums. These clinical reports were complemented by data describing other first generation AAV serotypes, namely, AAV1 in phase II studies for congestive heart failure, AAV9 in early Pompe trials (Byrne and others, ASGCT NIH symposium 2011) as well as chimeric AAV vectors in Duchenne muscular dystrophy studies.[91] Overall, extensive safety data with AAV vectors in multiple target tissues (brain, ocular, heart, liver, muscle, etc.) continues to accumulate, with early efficacy being acknowledged in a majority of these studies. Resolving the issue of pre-existing NAbs to AAV capsid along with the continued development of tissue-specific AAV vectors, as described in this review, should usher in a new generation of AAV vector candidates. Taken together with the current portfolio, the newly synthesized AAV strains, which are poised to enter the clinical arena, promise a similar-to-improved safety profile.

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