AAV Vector-Based Gene Therapy, Progress and Current Challenges

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Abstract Therapeutic efficacy of the adeno-associated virus (AAV) vector gene transfer has been shown by the large number of proof-of-concept studies in animal models. These preclinical studies established a rich pipeline of gene therapy drugs that could be brought to the clinic. Consequently, in recent years, the number of clinical trials in which AAV vectors were used for in vivo gene transfer increased significantly. The excellent safety profile and the high efficiency of transduction of a broad range of tissues promoted AAV vectors as the platform of choice for in vivo gene therapy and they have been successful in the clinic for a variety of indications including hemophilia B, choroideremia and other disorders. Aside from the evidence of clinical success, the recent market approval of the first AAV-based gene therapy drug in Europe represented another important milestone for the field of gene therapy, attracting the interest of investors after a long period of neglect. Nevertheless, clinical translation of novel therapies is a process that involves several bench-to-bedside iterations, during which possible issues of the novel technology may be identified and solved. For the AAV vector gene transfer technology, several hurdles have been highlighted in both preclinical studies and clinical trials; addressing these issues contributed to expand the number of indications in which clinical success was achieved. A lot more need to be carried out, for example to gather crucial information on the interactions between AAV-based therapeutics and

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the host immune system. In this book chapter, we will discuss some of the key approaches to design AAV-based gene therapy strategies and will present the main achievements and emerging issues of the field, using the liver as an example of target tissue.

Keywords Serotypes · Self-complementary · Deficiency · Hemophilia · Immune response · Humoral immunity · Chimeric capsid · Kozak sequence · Codon optimization · Hepatocyte · Genotoxicity

General Features of AAV Vectors

Wild-Type AAV

Adeno-associated virus (AAV) is a small (∼25 nm) virus composed of a non-enveloped icosahedral capsid (protein shell) that contains a linear single-stranded DNA genome of about 4.7 Kb. AAV belongs to the family of *Parvoviridae*, genus *Dependovirus*, as it can replicate in the nucleus of target cells only in the presence of helper viruses such as adenovirus or herpes virus [1]. The AAV genome is flanked by two palindromic inverted terminal repeats (ITR, 145 bp) and includes two open reading frames, *rep* and *cap*. *Rep* encodes proteins involved in: (i) replication of the viral DNA; (ii) transcriptional control of viral genes; (iii) packaging of newly synthetized single-stranded AAV genomes into the capsid; and (iv) site-specific genome integration in the host cell DNA [1]. *Cap* encodes for the VP1, VP2 and VP3 proteins that form the capsid and for the assembly activating protein (AAP) that is required for the starting of capsid assembly in the nucleus [1]. AAV naturally infects humans; usually, exposure to the wild-type virus occurs at around one–three years of age [2–4] and is not associated with any known disease or illness [5]. Importantly, the timing of human exposure to AAVs determines the host immunological response to the recombinant AAV vectors. AAVs infect both dividing and non-dividing cells, integrate into specific chromosomic loci [adeno-associated virus integration sites (AAVS)] and remain latent in the host cell DNA unless a helper virus provides the functions for its replication [1]. Three integration sites for wild-type AAV serotype 2 have been identified: AAVS1-3, respectively, on chromosomes 19, 5 and 3. For AAVS1 the frequency of integration in human and rhesus macaque DNA is estimated to be about 0.5% [1]. More recently, a study on the integration of wild-type AAV in human liver established a link between the insertion of portions of the AAV genome the promoter regions of genes linked to cancer and hepatocellular carcinoma [6]. The relevance of this finding to AAV vectors has been disputed [7], and future studies as well as long-term monitoring of human subjects who received AAV vectors will help assessing the real genotoxicity risk associated with this vector platform.

Recombinant AAV Vector Structure and Production

In the genome of recombinant AAV vectors, the only viral sequences that are retained are the two ITRs (*cis* packaging signals) while the sequences encoding *rep* and *cap* are exchanged with the exogenous DNA of choice (that is flanked by the ITRs and it is referred to as the transgene expression cassette). *Rep* and *cap* are nonetheless required for the production of AAV vectors, and to this end they are provided in *trans* to the packaging cells together with the adenoviral helper functions [8, 9]. AAV vectors can be produced at high yields by transient triple transfection of mammalian cells [10] or infection of packaging eukaryotic [11] and insect cells [12]. The triple transfection method is one of the most commonly used for AAV vector production and it is based on the co-transfection into permissive cells (usually human embryonic kidney 293 cells) of three plasmids:

- one containing the transgene of interest flanked by the viral ITRs;
- a packaging plasmid encoding for the rep and cap proteins;
- a plasmid encoding for adenoviral helper genes [8, 13].

The purification of recombinant AAV vectors for preclinical and clinical applications is performed by either column chromatography or physical methods (CsCl-gradient centrifugation) [8]. Based on the purification method, the removal of both cellular debris contaminants and the AAV empty capsids may vary, and one important focus in the field is to continuously improve the AAV manufacturing processes to increase both vector yield and purity [8, 9, 14, 15].

Cells Transduction with AAV Vectors

Transduction of cells by AAV vectors occurs by a series of sequential events, including: interaction of the viral capsid with receptors on the surface of the target cell, internalization by endocytosis, intracellular trafficking through the endocytic/proteasomal compartment, endosomal escape, nuclear import via the nuclear port complexes [16], virion uncoating and viral DNA double-strand conversion leading to the transcription and expression of the transgene [17].

The conversion of the AAV genome from single-stranded to double-stranded DNA occurs by both de novo synthesis of the complementary DNA strand (second-strand synthesis) and base pairing of complementary single-stranded AAV genomes derived from separate AAVs co-infecting the same cell and carrying plus and minus genomes (strand annealing). The frequency and efficiency of strand annealing have been reported to increase proportionally by increasing the dose of AAV vector per cell [17].

Differently from the wild-type virus, the genome of the recombinant AAV vectors does not undergo site-specific integration in the host DNA but mainly remains episomal in the nucleus of transduced cells, while random integration events are observed with a low frequency (0.1–1% of transduction events) [5, 18, 19].

To date, 12 different AAV serotypes and 108 isolates (serovars) has been identified and classified [1, 20]. The versatility of the AAV production system allows to easily generate hybrid AAV vectors composed by the same transgene flanked by the ITRs from serotype 2 [21] (so far the most commonly used) and any of the available AAV capsid [1]. AAV vectors obtained through this pseudotyping method are often referred as to AAV2/n, where the first number refers to the ITRs and the second to the capsid. Since the capsid interacts with receptors on target cells and impact on the post-entry transduction steps, AAV vectors bearing different capsids have different transduction abilities (i.e., cell tropism and kinetic of transgene expression) and thus one can ideally choose the most appropriate to target the cell of interest [1, 22]. When screening AAV serotypes to identify the best suitable one to target human tissues, the choice of the preclinical model appears to be crucial for success in human trials [23]. This has been exemplified in a recent study in which, for example, mouse model repopulated with human hepatocytes appeared to be more predictive of the outcome of gene transfer in humans [24]; according to these findings, some AAV serotypes like AAV3B do not transduce efficiently mouse hepatocytes but outperform several other serotypes in transducing non-human primate livers [25].

AAV Vector Engineering

So far, AAV vectors have been generated from many naturally occurring serotypes [1, 22]. More recently, engineered AAV vectors have been generated carrying novel capsids derived from rational design or directed evolution, significantly expanding the AAV vector toolkit [22, 26, 27, 28]. In particular, the increasing knowledge of AAV capsid structure-function [29] allowed to modify specific capsid amino acid residues by rational design while the development of AAV capsid libraries and high-throughput screening methods allowed to generate a huge variety of novel capsids and to select the most efficient at transducing the desired cell type in vivo by directed evolution [26]. Another promising approach to capsid engineering recently proposed [30, 31] consists of reconstructing ancestral AAV capsids to obtain AAV vector variants with the desired tissue tropism.

Additional maneuvers to improve capsid characteristics consist of introducing point mutations that can result in enhanced trafficking of virions to the nucleus and decreased proteasomal degradation [32], leading to higher transduction efficiency and lower immunogenicity [33].

The development of novel "synthetic" AAV vectors responds to the need for improving transduction and efficacy while reducing immunogenicity, toxicity and off-targets. These second-generation vectors are currently being evaluated in several preclinical models and could possibly substitute in the future the vectors derived from the naturally occurring serotypes [22].

AAV engineering has not only involved the capsid but also the genome of the vector. These efforts have been aimed at overcoming some of the key limitations of AAV vectors, such as the slow onset of gene expression (due to the inefficient conversion of single-stranded to double-stranded AAV genome) and the limited DNA cargo capacity (~5 Kb). In particular, McCarty and colleagues showed that the second-strand synthesis step in AAV vector transduction can be circumvented by using self-complementary (sc) AAV vectors [34]. scAAV vectors are produced by mutagenizing one of the two ITRs flanking the transgene so that during the AAV vector production the rep protein cannot solve the replication intermediates [34]. This results in packaging of a "ready to express" complementary dsDNA DNA genome containing both plus and minus vector genome strands. However, since the self-complementary genome cannot exceed the normal AAV packaging capacity (4.7 kb) only transgenes up to ~2400 base pair in length could be used to generate scAAVs, significantly limiting the number of applications of this platform [34]. Notably, scAAV vectors have been demonstrated to drive faster onset and higher levels of transgene expression in a variety of tissues in animal models [34, 35].

The small packaging capacity of AAV vectors (4.7 Kb) precludes the AAV-based delivery of a number of genes that exceed this length and/or the use of large physiological regulatory elements [36]. The AAV genome size limitation can be currently bypassed by using two main strategies: oversized AAV vectors and dual AAV vectors [36–38]. Oversized AAV vectors can be generated by using large (>5 Kb) ITR-flanked transgenes during AAV vector production, and this leads to the packaging of genomes of heterogeneous size, which are mostly truncated at around 5 Kb (the AAV packaging limit) [39–41]. Dual AAV vectors are instead generated by splitting a large transgene expression cassette in two separate halves (5′ and 3′ ends, or head and tail); each half of the cassette is packaged in a single AAV vector of regular size (<5 Kb) [37]. The re-assembly of the full-length transgene expression cassette is achieved upon co-infection of the same cell by both dual AAV vectors followed by: (i) homologous recombination between 5′ and 3′ genomes (dual AAV overlapping vectors); (ii) ITR-mediated tail-to-head concatemerization of 5′ and 3′ genomes (dual AAV trans-splicing vectors); or (iii) a combination of the two mechanisms (dual AAV hybrid vectors) [36]. Notably the use of both oversized and dual AAV vectors in vitro and in vivo results in the expression of full-length proteins and therapeutic efficacy in animal models; however, the efficiency of these systems is still low when compared to canonical single AAV vectors [36], thus requiring high vector doses to achieve therapeutic efficacy.

Additionally, it still needs to be clarified whether the expression of full-length proteins by oversized AAV vectors derives from the delivery of a minority of intact large genomes (>5 kb) or re-assembly of small fragmented genomes in infected cells [39]. The heterogeneous nature of the genomes contained in oversized AAV vectors [39] may constitute an additional challenge to the development of gene therapy products based on this platform. For dual AAV vectors, while promising results have been obtained in several animal models of diseases [37, 42, 43, 44, 45, 46], the need for high vector doses [45–47] and the fact that the efficiency of reconstitution of the full-length genome may vary based on the dual AAV vector system used and cell type (e.g., due to the inherent ability of the cell machinery to drive homologous recombination and/or other DNA repair mechanisms) [43] may represent important constraints to the broad use of this technology. Nevertheless, for some applications, like for gene transfer directed to confined tissues, like the eye, the use of the dual AAV vector platform may represent an efficient and viable gene transfer strategy for transgenes of >5 Kb in size [43, 48]. For other applications, like gene transfer for muscular diseases, promising results are being achieved [45–47]; nevertheless, further improvements of overall transduction efficiency are needed to support clinical development.

Clinical Applications of AAV Vectors

The first gene therapy was introduced in clinics about 20 years ago in order to treat adenosine deaminase deficiency (ADA-SCID) in children [49]. At that time, investigators used a retrovirus for gene delivery, but since then the vector toolkit available to investigators became more diversified. Currently, clinical trials using AAV represent about 6% of all gene therapy trials [50] and are recognized as the most promising in vivo gene delivery tool for treatment of multiple monogenic diseases (e.g., hemophilia, Duchenne muscular dystrophy) as well as by complex mix of genetic and environmental factors (e.g., rheumatoid arthritis, Parkinson's disease).

AAV-based gene therapy has been tested in over 60 clinical trials (http://www.gemcris.od.nih.gov/) showing the most outstanding results when immunoprivileged body sites, like the eye, are targeted [51]; nevertheless, overall safety profile of AAV appears to be impressive. Perhaps the most spectacular results were obtained in several trials in the context of Leber's congenital amaurosis (LCA), where blindness of treated subjects was successfully reversed [52–56]. None of those subjects had adverse effects nor developed antibodies against transgene (*RPE65*) or vector capsid. Another attractive target disease for gene therapy with AAV vectors is hemophilia B, in which stable expression of coagulation factor IX can correct the bleeding diathesis [57, 49]. Hemophilia B has always been considered as a relatively "easy" target, since a very small correction of factor IX

activity (~5% of normal circulating levels of the enzyme) significantly ameliorates symptoms of the disease. Differently from the *RPE65* deficiency trials, in the hemophilia trials conducted thus far, muscle [58, 59] or hepatocytes [57, 60, 61] were targeted with high doses of vector. This highlighted important interactions between AAV vectors and human immune system, which have not been predicted in preclinical studies.

Of note, in 2012 the European Commission has granted marketing authorization for the first gene therapy drug, Glybera® (http://www.unicure.com) [62]. This drug is an AAV1 vector encoding for lipoprotein lipase (LPL), injected intramuscularly, that has been shown to ameliorate the condition of patients with LPL deficiency (LPLD) [63–67]. Prior to development of this medicine, there was no cure available for LPLD and patients were obliged to follow a very restrictive low-fat diet and were subjected to frequent, life-threatening pancreatitis. Data from several clinical trials with the gene therapy drug suggest that injection of Glybera can ameliorate the disease phenotype and reduce the pancreatitis episodes.

In Table 1, we summarized the AAV-based clinical trials directed to diseases affecting various organ systems and using different routes of administration, including the lung via the airway, direct injection to muscle, brain and liver via hepatic artery. These examples illustrate well the wide range of possible applications of AAV vectors.

The Liver, a Versatile Platform for Multiple Gene Therapy Approaches

The liver is a particularly attractive organ for the development of gene-based therapeutic approaches for a number of reasons including: (1) It is one of the body's major biosynthetic organs; (2) studies in small and large animal models and in humans have demonstrated that it is possible to target hepatocytes with high efficiency using AAV vectors administered intravenously [57, 35, 61]; (3) despite the predominantly non-integrative nature of AAV vectors [19], multi-year transgene expression after gene transfer to the liver has been documented in large animals and humans [99, 100]; (4) expression of a transgene in hepatocytes induces antigen-specific tolerance mediated by regulatory T cells [101–104]; and (5) several preclinical studies demonstrate that it is possible to treat not only plasma protein deficiencies but also metabolic disorders with liver gene transfer, resulting in long-term cure for many of these disorders in small and large animal models (Table 2).

To date, liver gene transfer with AAV vectors has been tested in the clinic only for few indications, although the landmark results obtained in the context of liver gene transfer for hemophilia B [57, 61] paved the way to a number of clinical

Table 1 Current and completed clinical trials with AAV vectors

Disease		Transgene	Route of administration	AAV serotype	Reference
Familial lipoprotein lipase deficiency	Disorder that impairs breakdown of fatty acids	Lipoprotein lipase—LPL	Intramuscular	AAV1	[64, 65]
Cystic fibrosis	Lethal autosomal disease that affects mostly the lungs but also the pancreas, liver, kidneys and intestine	Cystic fibrosis transmembrane conductance regulator—CFTR	Lung, via aerosol	AAV2	[68–74]
Hemophilia B	Blood clotting disorder leads to an increased propensity for hemorrhage	Factor IX—FIX	Intravenous, intramuscular, hepatic	AAV8	[57, 60, 61]
Batten's disease	Fatal autosomal recessive neurodegenerative disorder that begins in childhood	Neuronal ceroid lipofuscinosis—CLN2	Intracranial	AAV2, AAV rh10	[75]
Canavan's disease	Most common degenerative cerebral diseases of infancy	Aspartoacylase—ASPA	Intracranial	AAV2	[76]
Parkinson's disease	Degenerative disorder of the central nervous system mainly affecting the motor system	Glial cell line-derived neurotrophic factor—GDNF or neurturin—NTN or aromatic L-amino acid decarboxylase —AADC	Intracranial	AAV2	[77–80]
Giant axonal neuropathy	Chronic neurodegenerative disease fatal by the third decade of life	Gigaxonin—GAN	Lumbar intrathecal injection	AAV9	–
Alzheimer's disease	Chronic neurodegenerative disease leading to short-term memory loss and ultimately to death	Beta nerve growth factor—β-NGF	Intracranial (stereotactic injection)	AAV2	[81]
Mucopolysaccharidosis	Lysosomal storage disease associated with developmental delay, severe hyperactivity, spasticity, motor dysfunction, death by the second decade	*N*-acetylglucosaminidase—NAGLU or heparan sulfamidase—SGSH		AAV9	–

(continued)

Table 1 (continued)

Disease		Transgene	Route of administration	AAV serotype	Reference
Galactosialidosis	Autosomal recessive lysosomal storage disorder with a broad spectrum of clinical manifestations	Protective protein cathepsin A—PPCA	Intravenous infusion	AAV8	–
Homozygous familial hypercholesterolemia	Severe elevation of serum LDL leading to premature and lethal coronary artery disease	Low-density lipoprotein (LDL) receptor LDLR	Hepatic artery	AAV8	–
Prader–Willi syndrome	Genetic disorder characterized by low muscle tone, short stature, cognitive disabilities and a chronic feeling of hunger that can lead to life-threatening obesity	Brain-derived neurotrophic factor—BDNF		AAV2	–
Heart failure	Wide range of abnormalities that impair normal cardiac function	Sarcoplasmic endoplasmic reticulum calcium ATPase—SERCA2a	Intracoronary	AAV1	[82, 83]
Becker muscular dystrophy	X-linked recessive inherited disorder characterized by slowly progressive muscle weakness of the legs and pelvis	Follistatin 344—FS344	Intramuscular	AAV1	[84]
Spinal muscular atrophy type 1	Autosomal recessive disease of early childhood leading to progressive muscle wasting and mobility impairment	Survival motor neuron protein—SMN	Intravenous peripheral limb vein	AAV9	[85]
Limb girdle muscular dystrophy type 2D	Progressive muscle wasting which affects predominantly hip and shoulder muscles	Alpha sarcoglycan—SGCA	Intramuscular or femoral artery limb perfusion	AAV1, AAV8	[86]
Duchenne muscular dystrophy	Recessive X-linked disease, which results in muscle degeneration and premature death	Mini-dystrophin or GalNAc transferase—GALGT2	Intramuscular	AAV2 rh74, AAV5	[87, 88]
Dysferlinopathy	Autosomal recessive neuromuscular disorder characterized by progressive muscle wasting	Dysferlin—DYSF	Intramuscular	AAV6	[89]

(continued)

Table 1 (continued)

Disease		Transgene	Route of administration	AAV serotype	Reference
Flexor tendon injury	Inability to bend fingers	Vascular endothelial growth factor—VEGF	Intramuscular	AAV2	[90]
Pompe disease	Glycogen storage disease, which damages muscle and nerve cells throughout the body	Acid alpha glucosidase—GAA	Intramuscular, diaphragm	AAV1	[91]
Alpha-1 antitrypsin deficiency	Genetic disorder provoking respiratory problems and impaired liver function	Alpha-1 antitrypsin—AAT	Isolated Limb Infusion; intramuscular	AAV1	[92, 93]
Leber congenital amaurosis	Autosomal recessive disorder associated with multiple genes and leading to severe vision loss or blindness	Retinal pigment epithelium-specific 65-kDa protein—RPE65	Subretinal	AAV2	[52–56]
Leber hereditary optic neuropathy	Mitochondrially inherited degeneration of retinal ganglion cells and their axons that leads to loss of central vision in young adult males	NADH ubiquinone oxidoreductase subunit 4—ND4	Intravitreal	AAV2	–
X-linked retinoschisis	Early onset retinal degenerative disease leading to juvenile macular degeneration in males	Retinoschisin—RS1	Intravitreal	AAV2tYF	–
Choroideremia	X-linked, recessive, degenerative disease of the retina leading eventually to blindness	Rab escort protein 1 REP1 or retinal pigment epithelium-specific 65-kDa protein—RPE65 or	Intraocular	AAV2	[94, 95, 62]
Neovascular age-related macular degeneration	Disease characterized by a growth of new blood vessels beneath the retina, which leakage causes damage to retinal cells and create blind spots in central vision	VEGF receptor—FLT01 or sFlt-1	Subretinal	AAV2	[96]
Congenital achromatopsia	Retinal disorder characterized absence of daylight vision from birth, photophobia and a slowly progressing loss of cone photoreceptors	Cyclic nucleotide gated channel beta—CNGB3	Subretinal	AAV2tYF	–
Rheumatoid arthritis	Long-lasting disorder that primarily affects joints. It typically results in warm, swollen and painful joints	TNFR-Fc	Intra-articular	AAV2	[97, 98]

Table 2 Examples of proof-of-concepts of therapeutic efficacy of AAV-based gene transfer in animal models

Disease	Transgene product	Route of administration	AAV serotype	Reference
Arginase deficiency	Arginase 1	Facial vein	rh10	[105]
Atherosclerosis	Apo-A1	IV (tail vein)	8	[106]
Citrullinemia type 1	ASS	IP, IP (in utero)	8, rh10	[107, 108]
Crigler–Najjar	UGT1A1	IP	8, 9	[109, 110]
Diabetic peripheral neuropathy	mIGF-1	IV (tail vein)	8	[111]
Ethylmalonic encephalopathy	Sulfur dioxygenase	Intracardiac	8	[112]
Fabry disease	Human α-galactosidase A	IV (intraportal and tail vein)	1, 2, 8	[113–115]
Familial amyloidotic polyneuropathy	Transthyretin T119M	IV (tail vein)	8	[116]
Familial hypercholesterolemia	Human LDL receptor	IV (intraportal)	2, 7, 8	[117]
Galactosialidosis	Cathepsin A	IV (tail vein)	8	[118]
Gaucher syndrome	Human glucocerebrosidase	IV (tail vein)	8	[119]
GH deficiency	Growth hormone	IP	8 (sc)	[120]
GSDIa	G6PC	Temporal vein, retro-orbital sinus	1, 2, 7, 8, 9 (sc)	[121–126]
GSDII	hGAA	Retro-orbital sinus	8	[127, 128]
Hemophilia A	m, c, hFVIII, cFVIIIdeleted, codop-hFVIII-V3	IV (intraportal, tail vein and cervical vein)	2, 5, 6, 8	[129–136]
Hemophilia B	c, hFIX, hFIX variants, hFIX-Padua	IV (intraportal and tail vein)	2, 5, 8 (sc)	[35, 137, 138, 139, 140]
Hereditary tyrosinemia	FAH	IV (facial vein, tail vein)	2,8	[141]
Homocystinuria	CBS	IV (intraportal) or IP	2	[142]
Inherited Apo-A1 deficiency	Apo-A1	IV (intraportal and tail vein)	8 (sc)	[143]
Liver fibrosis	HGF	IV (intraportal)	5	[144]

(continued)

Table 2 (continued)

Disease	Transgene product	Route of administration	AAV serotype	Reference
Mitochondrial neurogastrointestinal encephalomyopathy	Thymidine phosphorylase	IV (tail vein)	8	[145]
MPS VII	b-Glucuronidase	IV (tail vein)	2	[146]
MPSII	Iduronate 2-sulfatase	IV (tail vein)	8	[147]
MPSIIIA	Sulfamidase	IV (tail vein)	8, 9	[148, 149]
von Willebrand disease	von Willebrand factor	IV (tail vein)	8	[150]
OTC deficiency	OTC	IP, IV (intraportal and tail vein)	2, 7, 8, 9 (sc)	[151–153]
Phenylketonuria	PAH	IP, IV (intraportal)	5, 8 (sc)	[154–156]
Primary hyperoxaluria type I	AGT	IV (tail vein)	5, 8	[157]
Propionic acidemia	PCC	Intrahepatic injection, IV (tail vein)	8, rh10	[158, 159]
Renal fibrosis	Hepatocyte growth factor	IV (tail vein)	9	[160]
Sialidosis	Cathepsin A	IV (tail vein)	8 (sc)	[161]
Smith–Lemli–Opitz syndrome	DHCR7	IV (temporal and tail vein)	2, 8	[162, 163]
VLCAD deficiency	VLCAD	IV (tail vein)	8	[164]

studies of AAV liver gene transfer that are ongoing (e.g., Clinicaltrials.gov ID# NCT02396342; NCT02484092; NCT02618915; NCT00979238; NCT01687608) or about to start. Hemophilia B is a bleeding diathesis caused by mutations in the gene for blood coagulation factor IX (FIX). Initial results in the dog model of hemophilia B provided a strong rationale for targeting the liver to express the therapeutic FIX transgene [165]. In the first AAV-FIX liver trial, a single-stranded AAV2 vector carrying the human FIX transgene expressed under the control of a liver-specific promoter was administered through the hepatic artery [60]. This trial has been particularly important for the field of in vivo gene transfer, as it demonstrated for the first time that it was possible to transduce the human liver with AAV vectors, leading to therapeutic levels of transgene expression. Additionally, it allowed identifying important limitations of the approach related to vector immunogenicity [166] and preexisting immunity to AAV in humans. Following the results obtained in the AAV2-FIX trial, a second trial was initiated in which a self-complementary AAV8 vector encoding for a codon-optimized version of the FIX transgene was administered intravenously to target the liver of hemophilia B subjects [57, 61]. In this study, a short course of immunosuppression was used to block potentially detrimental immune responses triggered by the viral vector. This approach successfully demonstrated that it was possible to target the liver via the administration of an AAV8 vector delivered through a peripheral vein. Additionally, it showed that transient immunosuppression could be safely applied with gene transfer to avoid detrimental immune responses and leading to long-term expression of the transgene product.

Despite the small number of subjects enrolled in clinical trials conducted thus far, the experience with liver gene transfer with AAV in humans has resulted in important knowledge on the safety and efficacy of this approach and allowed testing of strategies to achieve the goal of safe and long-term correction for a number of genetic and metabolic diseases with liver gene transfer. In the context of hemophilia B, several gene transfer trials have been initiated or are about to start, with some preliminary results being released [167, 168] confirming the findings in the first trials.

Gene Therapy for Children—Question of AAV Persistence in the Developing Liver

Differently from vector administration in neonate animals, the transduction of post-mitotic or slowly replicating adult tissues is more efficient and stable. Long-term evaluation of the persistence of the AAV genome in adult dogs and primates indicates that vector expression persists for more than 10 years [99, 100]. In humans, long-term transgene expression for >5 years after the injection has been

demonstrated [61]; however, at present no human data exist on the persistence of AAV genomes in liver after treatment of pediatric subjects.

After birth the liver starts increasing its size depending on the metabolic requirements of the developing organism, reaching its limit before adulthood. In rodents, the better characterized model, most of the cells in the liver develop during the first 28 days of life, during which the number of hepatocytes increases proportionally with the size of the liver [169]. In adult mice, hepatocytes divide every 100–200 days, whereas in rats the division appears to be faster [169, 170], although the documented difference may be due to the different methods used for the evaluation of the hepatocyte turnover in the two species. In humans, data reported that the liver size increases during childhood and became stable approximately at 10–15 years of age [171]. Additional factors that may influence vector genome stability following AAV gene transfer to the liver are related to the intrinsic characteristics of the pathology, in particular whether gene transfer is performed in a fibrotic/cirrhotic liver [172] or in a liver with increase turnover [173].

The early demonstration that AAV vectors do not integrate in significant proportion in the host genome comes from experiments in which partial hepatectomy was performed after gene transfer, resulting in loss of transgene expression [174]. Similarly, in neonate mice the transduction of actively replicating cells with AAV leads to partial vector dilution over time, with reduction in transgene expression levels. This was recently described by Bortolussi and colleagues, who injected a neonate mouse model of Crigler–Najjar syndrome [109] with a therapeutic AAV vector at day 2 after birth; in these animals, partial loss of transgene expression was observed over time, resulting in lower levels of phenotype correction 17 months after gene transfer (measured by the levels of circulating total bilirubin in serum), which still remained within the therapeutic range [110]. Nevertheless, it is a known fact that proliferation of the neonate liver over time leads to dilution of the effect of gene transfer [175]. For some diseases like Crigler–Najjar syndrome or hemophilia, where the amount of transgene expression needed to rescue the diseased phenotype is low (about 5% of normal levels of enzyme activity is sufficient to convert both diseases from severe to mild [176, 177]), a single administration of an AAV vector at an appropriate dose may be sufficient to achieve lifelong correction of the disease phenotype also when gene transfer is performed in young pediatric subjects. For other diseases, requiring more robust transgene expression, vector re-administration is likely required [178]. Finally, it should be noted that important loss of transgene expression in mice is observed only when AAV vectors are given very early after birth (day 1 or 2). Vectors given at a later time point (>day 4) result in more persistent effect; similarly, higher vector doses result in better persistence of the therapeutic effect [175]. Thus, the issue of vector re-administration may be more relevant to those diseases with early lethality and no therapeutic options available [179].

To overcome the vector genome dilution following AAV treatment, several strategies have been developed in preclinical studies, all based on the enhancement of a stable integration of the viral genome in the host genome. For instance, Wang and colleagues [180] obtained 30-fold increase of the vector integration and

increased persistency in the mouse liver by inserting in the AAV genome a sequence derived from 28S ribosomal RNA present in multiple copies in the host.

Another method to stably integrate AAV genome is the utilization of nucleases, like meganucleases, zinc-finger nucleases or the most recent CRISPR/Cas9 system, that are able to cut a specific sequence in the host genome, thus inducing an homologous recombination with the vector genome. Examples of in vivo gene editing with AAV vectors exist [181, 182], in which robust expression of the transgene was achieved in neonate and adult animals throughout the liver development and even after partial hepatectomy. Another similar approach, relying on homologous recombination driven by homology arms flanking the transgene to be integrated in the genome, has been recently proposed, in which an AAV vector has been used to transfer a promoterless transgene downstream of the albumin gene [183]. However, all the approaches described at the moment suffer from the limitation of low efficiency, as logs higher doses of AAV vectors were needed (compared to "classic" gene addition strategies) to achieve detectable transgene expression levels, thus making translation of these results to the clinic challenging.

Nevertheless, the approaches described above demonstrate that the stable integration of a transgene into the liver DNA increases the persistence of expression in mice. There are still some limitations for the application of these methods to the clinics, and they are related in particular to (i) the high levels of transduction that are required to obtain the transgene integration and (ii) the possibility that off-target integration events could cause insertional mutagenesis. Notably, the issue of insertional mutagenesis is of particular relevance in a context of a rapidly dividing liver, like that of neonates.

Liver Mediates Induction of Tolerance to the Transgene Product

It has been shown that AAV vector-driven expression of human FIX (hFIX) in hepatocytes leads to tolerance to the transgene product [101], a phenomenon that is not entirely surprising, given the unique immunological environment of the liver, placed at the crossroads between the gut and the bloodstream [184]. The lack of responsiveness observed in liver-directed gene transfer with AAV vectors appears to be mediated by antigen-specific $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) [103], which play a central role in liver-mediated tolerance induction [102, 185]. Importantly, liver-mediated tolerance induction can be achieved for various transgenes [103, 186, 187] and can be used to eradicate ongoing antibody responses to antigens [188, 189] and ongoing autoimmune disease [190]. Thus far, preclinical results on the induction of tolerance mediated by gene transfer seem to be confirmed in clinical trials, in which, for example, in the case of the hemophilia B trials, no response to the FIX transfer transgene was noted, despite the fact some of them were carrier of null mutations in the FIX gene [57, 60, 61]. However, it should be

noted that thus far all subjects at risk of having a response to the transgene product have been systematically excluded from enrollment in gene therapy trials, thus underestimating transgene immunogenicity in gene therapy.

While animal models offer a valuable tool to estimate the risk of anti-transgene immune responses, some factors may complicate the evaluation of transgene immunogenicity in humans: (1) species specificity, as human transgenes may be highly immunogenic in lower mammalians [191, 192] but not in humans; (2) genetic background, which in case of models obtained by disrupting a gene, it may be associated with complete lack of tolerance to the expressed transgene [193, 194], thus not fully reflective of the heterogeneity of mutations found in humans; (3) some immunomodulatory drugs used in immunosuppression protocols for highly immunogenic transgenes [195] only work in non-human primates and humans (e.g., several monoclonal antibody-based immunosuppressive drugs).

The Interactions Between AAV Vectors and the Immune System

Cellular Immune Responses to AAV Vectors

AAV vectors are complex multi-component biological entities, composed by both a protein capsid and a nucleic acid. Each of these components may contribute to shaping the host immune response to gene transfer [196]. One key concept to keep in mind when studying immune responses to AAV vectors is that the viral capsid in AAV vectors is identical or nearly identical to the capsid of the wild-type virus, to which humans are exposed [3, 197, 198]. Thus, it is expected that the host immune responses triggered by vector administration will be similar to those associated with a natural infection with AAV, although high quantity of viral particles administered and their route of administration may contribute to the unique features on immune responses observed in gene transfer with AAV vectors [67, 196].

It is known that the DNA and capsid structure of AAV can be readily detected by the immune system, via Toll-like receptor (TLR)9 and TLR2, respectively, triggering adaptive immune responses to capsid and/or transgene product [193, 199, 200, 201, 202]. Recent findings also suggest that CpG sequences, a ligand for TLR-9, contribute to transgene immunogenicity [203]; although these observations are limited to preclinical animal models, factors influencing transgene immunogenicity should be carefully evaluated when approaching the clinic.

Cell-mediated immunity directed against the AAV capsid plays an important role in terms of both safety and efficacy of AAV gene transfer in humans. This was first evidenced in a clinical trial in which an AAV2 vector was introduced into the liver of severe hemophilia B subjects [60]. In this study, upon AAV gene transfer to liver, two subjects developed a transient and asymptomatic elevation of liver enzymes associated with loss of FIX transgene expression around week 4 after

vector delivery. These observations were associated with the expansion of capsid-specific $CD8^+$ T cells, which likely were responsible for the immune rejection of transduced hepatocytes [166]. More recently, similar set of observations was made in the context of a clinical trial of AAV8 gene transfer to the liver of subjects affected by severe hemophilia B [57, 61]. This study showed that AAV8 vector administration in humans resulted in activation of capsid-specific $CD8^+$ T cells and increased liver enzymes in 4 out of 6 subjects from the high-dose cohort, who received 2×10^{12} vg/kg of vector, ~7–9 weeks after vector delivery. In this study, timely intervention with oral corticosteroids was key to ablate the detrimental effect of the ongoing immune response on transgene expression, and even a short delay in the administration of immunosuppression resulted in a fast and significant loss of transduced hepatocytes [57].

Notably, results from both the AAV2 and AAV8 hemophilia B clinical trials highlight important differences in the biology of AAV vectors of different serotypes, as, for example, the timing of detection of the T cell responses differed in the two trials, which was significantly delayed for AAV8 versus AAV2 (weeks 6–9 vs. weeks 2–4, respectively).

As more data emerge from AAV gene transfer trials for hemophilia, the complexity of interactions between AAV vectors and the host seems to gain complexity. It is now evident that what appears to be an immune response to the AAV capsid can be triggered by many serotypes, including AAV2, AAV8, and AAV5 [57, 60, 61, 166, 167, 168]. However, what also is becoming evident is that other factors may concur in the determination of the outcome of gene transfer, as loss of transgene expression and increase in liver enzymes in some cases have not been associated with an immune response to the capsid [168]; furthermore, what complicates the overall picture is that in some instances intervention with steroids did not succeed in preventing/reversing loss of transgene expression [168].

Animal models fail to predict capsid immunogenicity in humans. One major limitation of all animal models (mice, rats, dogs, non-human primates, etc.) used to evaluate the safety and efficacy of gene transfer with AAV vectors is that they failed to predict the issue of T cell reactivity to the capsid in humans. Among multiple studies in mouse models [204–206], though some of them highlighted presentation of AAV antigens in vivo [207, 208], only one managed to recapitulate the human findings [57, 60, 61] showing that AAV8-transduced hepatocytes remain susceptible for $CD8^+$ T cell-mediated lysis longer than those transduced with AAV2 vectors [33].

Despite difficulties, the efforts to develop a murine model continue in a hope to answer remaining questions about safety and efficacy of gene transfer. For instance, such model could help to evaluate the possible detrimental interactions of immunosuppressive regimens applied in the context of AAV gene therapy [102, 209].

Lessons learned from the clinic. The results in the AAV8 hemophilia trial [57, 61] represent an important stepping-stone in the management of unwanted immune responses in AAV gene therapy as they show that it is possible to monitor liver enzymes and administer transient immunosuppression with steroids only if required. However, it should be kept in mind that the ease of endpoint monitoring

characteristic of this trial (i.e., follow-up of liver enzymes and FIX expression levels to guide intervention with steroids) is unlikely to apply for all gene therapy scenarios. For example, for certain disease indications in which liver enzymes are constitutively elevated the use of immunosuppression "on demand" will not be feasible for the lack of endpoints to follow. One solution to the issue could be to administer an immunosuppression regimen upfront to all subjects. However, this is not an ideal solution to the problem, as not all individuals may have an immune response to the vector, the timing of immune responses may vary with the vector dose, serotype, etc. [57, 60, 61, 67], and immunosuppression may change the outcome of gene transfer by decreasing transduction efficiency [209] or triggering unwanted reactions to the donated transgene [102]. Finally, to date it is not entirely clear whether steroid administration will be effective in blocking T cell-mediated immunity to AAV in all gene transfer settings; in fact, it is becoming obvious that this intervention may not be effective in all cases [168].

Immune responses to AAV depend on the vector dose. One important aspect of T cell-mediated immune responses to AAV is that they seem to be detected in a dose-dependent fashion, a result consistent with published in vitro antigen presentation data [207, 208]. Above a certain threshold of capsid antigen load, activation of capsid-specific T cells may result in hepatotoxicity and loss of transgene expression; however, it is not clear at this point, what is the proportion of subjects that will mount a detrimental T cell response. Data from the AAV8 hemophilia B trial suggest that only a subset of subjects will require immunosuppression [57, 61], but the individual differences (HLA type, exposure to the wild-type virus, etc.) accounting for the different outcome of gene transfer between subjects remain unknown.

High purification of vector preparations decreases immune response. The influence of vector manufacturing on the immunogenicity of AAV vectors is currently being discussed. Important open questions include the role of empty capsids, which are found in variable proportions in vector preparations. While empty particles may act as decoys for anti-AAV antibodies [210], they may also contribute to the overall amount of capsid antigen being presented onto MHC class I [208]. The presence of contaminants deriving from the process used for AAV manufacturing (e.g., host cell DNA contaminants, plasmid DNA) is also a possible factor influencing the immunogenicity of AAV vectors.

Humoral Immunity Directed Against the AAV Capsid

The impact of neutralizing antibodies (NAbs) directed against AAV on vector transduction has been first evidenced in the AAV2-FIX liver gene transfer trial [60], in which one subject enrolled in the high vector dose cohort (2×10^{12} vg/kg) had a NAb titer to AAV2 of 1:2 and expressed peak levels of F.IX transgene of $\sim$11% of normal, while another subject in the same dose cohort with a pretreatment NAb titer of 1:17 did not have any detectable circulating FIX following vector administration.

These results were also confirmed by experiments in non-human primates, a natural host for AAV8 [211], which showed that NAb titers as low as ~1:5 can completely block transduction of the liver following AAV8-FIX vector administration at doses of 5×10^{12} vg/kg [212].

After exposure to wild-type AAV, a significant proportion of individuals develop humoral immunity against the capsid, usually starting around 2 years of age [2–4]; however, maternal anti-AAV antibodies can be already found in newborns, disappearing a few months after birth before exposure to the virus later in life [2–4]. Thus, the window of time in which the majority of humans appears to be naïve to anti-AAV antibodies is narrow. Additionally, due to the high prevalence of anti-AAV antibodies in humans, and the cross-reactivity of these antibodies across AAV serotypes [2, 3, 4, 197, 213, 214], anti-AAV neutralizing antibodies can have a profound impact on the efficacy of gene transfer and should be carefully measured prior to enrollment of subjects in clinical trials.

Prevalence of anti-AAV antibodies in the target patient population should be carefully evaluated when designing a gene transfer clinical trial with AAV vectors, particularly when the vector is delivered intravenously. Aside from using highly sensitive assays to measure anti-AAV NAbs [215], preclinical studies should be used to assess the tolerance to anti-AAV antibodies based on the specific characteristics of the vector preparations (e.g., content of empty capsids [210] and doses administered (small vector doses are more prone to neutralization by NAbs). To this end, the use of in vivo models passively immunized with antibodies against AAV vectors [210, 216] can be helpful as it allows to consistently dosing animals with IgG to obtain the desired NAb titers.

When designing preclinical studies in preclinical animal models, it should be kept in mind that some preclinical animal models like humans are natural host for wild-type AAVs. For instance, anti-AAV NAb can be found in non-human primates [211] and have been documented in dogs and other species [217–219]; thus, prescreening of animals for anti-vector antibodies may be required for some animal species and AAV serotypes.

A comprehensive review of strategies to overcome presence of Nab is presented in [220].

Strategies to Improve Efficacy of AAV-Based Gene Therapy

The objective of gene therapy strategies based on gene transfer is to achieve long-term stable transgene expression at levels that are therapeutic. Based on this, one important lesson learned from the outcome of the two AAV clinical trials for hemophilia B targeting the liver is that therapeutic levels of transgene expression can be achieved in humans in a dose-dependent manner [99, 57, 60]. Unfortunately, vector doses positively correlate with unwanted anti-capsid immune responses that, if not counteracted, may decrease or even abolish transgene expression [99, 57, 60, 67, 166]. This issue is particularly relevant considering that therapeutic efficacy for

hemophilia B may be achieved by restoring only 1% of FIX activity while for other diseases (and transgenes) the threshold may be significantly higher. Despite the fact that therapeutic transgene expression levels vary depending on disease and the nature of the transgene product (e.g., intracellular vs. extracellular, with structural vs. enzymatic function), the goal of gene therapy strategies should be to maximize vector potency in order to decrease the vector dose and reduce the risk of immune response and toxicity. For example, vector potency for liver gene therapy can be increased by: (i) optimizing the design of the vector (capsid and/or genome) and of the transgene expression cassette (sequence and regulatory elements) and (ii) facilitating vector trafficking toward the nucleus.

Additional improvements in the efficacy profile of gene transfer can also be achieved by devising optimized vector delivery methods, as it has been shown for muscle [221]. In the case of liver, some studies have shown that catheterization of liver vasculature allows for more efficient delivery of AAV vectors in the presence of anti-capsid neutralizing antibodies [222]; however, clinically feasible and non-invasive delivery methods (e.g., intravenous infusion via peripheral vein) remain preferable to more invasive and potentially risky procedures.

Enhancement of Capsid Transduction Efficiency

During recent years the AAV serotype 8 has emerged as the most efficient natural AAV serotype for liver transduction upon systemic delivery in preclinical models [99, 20, 211, 223] and human hemophilia B trials [99, 57]. Recently, rational design of AAV capsid leads to the identification of novel capsid variants showing increased transduction efficiency as compared to their natural counterpart in animal models. In particular, the point mutations of specific tyrosine [32], serine, threonine and lysine [224] residues on the AAV2 capsids avoid the targeting of viral particles to the proteasome within the cells, thus increasing the vector load that reaches the nucleus [32, 224]. Interestingly, the intravenous delivery of these AAV2 capsid mutants to wild-type mice resulted in higher and more widespread liver transduction and also faster and higher transgene expression as compared to natural AAV2 [32, 224, 225]. Similar results have been reported by mutagenizing serine, threonine and lysine residues on AAV capsids 8, 5 and 1 [226]. However, a recent study reported minimal effect on the transduction efficiency of mouse liver upon systemic delivery of K137R mutant AAV capsids 7 and 9, in addition to the previously reported K137R AAV8 mutant [227]. Improved liver transduction and faster onset of transgene expression were also reported for the novel AAV2G9 capsid variant that has been generated by inserting the galactose-binding domain of AAV9 on the AAV2 capsid [228].

One of the limitations of testing the liver transduction ability of AAV vectors in mouse (and other small animal models) is that the results achieved may not be always extrapolated to the human liver. To overcome this problem, Lisowsky et al. [24] used a chimeric human-mouse liver model. In this model human hepatocytes

are transplanted in immune-deficient *Fah−/−* mice and repopulate the mouse liver having a selective advantage over the murine hepatocytes (5–40% repopulation efficiency). Using this model, Lisowsky and colleagues screened a library of novel capsid variants and identified one chimeric capsid, AAV-LK03, composed of five different natural AAV capsids, able to transduce human hepatocytes more efficiently than AAV2 and AAV8 [24].

One limitation of this humanized liver model is that the transduction of human hepatocytes is still tested in non-physiological conditions while the extracellular environment (e.g., extracellular matrix, blood composition, immune system) may impact on human liver transduction by AAV vectors in a real-life scenario.

Ultimately, the therapeutic advantage deriving from the use of these capsid mutants will have to be evaluated in the context of clinical trials.

Vector Enhancement at the Genome Level

The AAV genome size constraint is an important limit to the transfer of large transgenes to the liver and other tissues. As reported for tissues like the muscle and the retina, both oversized and dual AAV strategies may be used to express large (5 kb) transgenes in a given target tissue. A promising strategy is to use a truncated and engineered FVIII cDNA encoded by an expression cassette of 5.2 Kb that drives therapeutic expression of FVIII in animal models [229]. Interestingly, AAV8 vectors encoding for canine FVIII (5.8 Kb genome) were reported to correct the bleeding phenotype of HA dogs [36]. Then, subsequent studies in wild-type mice, using a reporter transgene, showed that oversized AAV2/8 vectors are about 25-fold less efficient than regular size AAV vectors [39]. Demonstration of transgene expression in mouse hepatocytes by systemic delivery of dual AAV trans-splicing vectors has also been reported [230], but it would be interesting, at present, to compare the efficiency of all the available dual AAV vector systems and to evaluate their possible side effects (e.g., related to the expression of the truncated proteins deriving from each individual vector injected) in the contest of liver gene transfer. Another possible approach to produce full-length FVIII in the liver is to express two separate FVIII-derived peptide chains (each encoded by regular AAV vector) that re-associate within the cells [231, 232].

Modifications to the vector genome, and in particular, the use of self-complementary (sc) AAV vectors [34] has shown to increase vector potency as compared to single-stranded (ss) AAV in the liver of small and large animal model upon systemic delivery [35, 233, 234, 235], suggesting that hepatocytes are, to some extent, inefficient in *de novo* second-strand synthesis [34]. Notably, scAAV vectors seem to provide faster and stronger transgene expression as compared to ssAAV, allowing reducing vector doses while maintaining efficacy [34]. Other studies have shown that transgene expression in mouse hepatocytes can be increased of more than tenfold by co-injection of scAAV2 vectors encoding for either the T cell protein tyrosine phosphatase (TC-PTP) or the protein

phosphatase-5 (PP5) with an ssAAV encoding for the transgene of interest [203]. If no toxicity would result from the expression of these phosphatases in the liver, this approach could be possibly useful to increase the expression of transgenes that are larger than 2 Kb and thus cannot be easily packaged in scAAV vectors [236].

Fine-Tuning of Transgene Expression Cassette

In addition to vector optimization, improvements in the design of the transgene expression cassette have also been widely reported to increase transgene expression and the therapeutic efficacy of AAV vectors [203]. To this aim, the design of various elements can be modified and improved such as transcriptional and post-transcriptional regulatory elements, GC content and codon usage. The regulatory elements include: promoter, enhancer, Kozak sequence, intron, UTRs, polyadenylation signal [237, 238]. So far, the promoters used in the liver of hemophilia B patients treated in the two AAV-based clinical trials are the hepatocyte-specific ApoE/hAAT promoter [consisting of human apolipoprotein E/C-I gene locus control region (HCR) combined with the human α1 antitrypsin promoter] [239] and the LP1 promoter (consisting of core liver-specific elements from the HCR and the hAAT promoter) [35]. While in preclinical settings, AAV-mediated transgene expression in the liver is achieved using both constitutive and tissue-specific promoters, it is important to highlight that the success of liver-targeted gene therapy is dependent so far on the selective expression of transgenes in hepatocytes. The restriction of transgene expression to hepatocytes is chosen to avoid the expression of the transgene product in antigen-presenting cells (that may boost anti-transgene immune responses) and to favor induction of immune tolerance to transgene products [101].

Recently, novel hepatocyte-specific transcriptional cis-regulatory modules (CRMs) have been identified containing evolutionary conserved clusters of binding sites for tissue-specific transcription factors. When the CRMs are used upstream of minimal liver promoters either strong (transthyretin, TTR) or weak (paralemmin, Palm), they enhance gene expression in mouse and NHP liver [240]. It is expected that these regulatory elements will potentiate transgene expression for liver-targeted gene therapy while, due to their small size, maintaining the overall transgene expression cassette within the packaging capacity of AAV vectors.

Codon optimization increases both mRNA stability and protein translation [241], and it has been successfully applied to increase expression of therapeutic hFIX and hFVIII proteins by the liver upon AAV-mediated gene transfer [35, 136, 234, 242, 243]. Ideally, the design of an optimal expression cassette for transgene expression should be performed before any experiment in animal models as the optimization of multiple elements in the transgene expression cassette can significantly increase the potency and efficacy of AAV vectors for liver gene transfer. This is exemplified by recent preclinical studies in which the optimization of multiple elements of the expression cassette has been combined with the use of a hyperactive hFIX protein

(obtained by introducing a gain-of-function mutation in the hFIX coding sequence (R338L; FIX Padua) [140, 244, 245]). Similarly, design of codon-optimized, engineered version of FVIII has allowed to achieve therapeutic levels of transgene expression at AAV vector doses that are safe and justify clinical development of a gene therapy for hemophilia A [135, 229, 243].

Based on the recent advances in vector design, it is expected that the development of optimized next-generation AAV vectors with higher potency for liver gene transfer will allow using lower and thus potentially safer vector doses while maintaining efficacy and will promote the clinical translation of the existing proof-of-concepts in animal models (Table 2).

Genotoxicity

One main advantage of AAV vectors, as gene therapy vehicles, consists in the low frequency of vector genome integration in the host DNA and the low risk of related genotoxicity [1]. Despite this, the issue of AAV-related genotoxicity is important in the context of gene therapy as random integration of vector genomes into the host DNA may lead to both loss- and gain-of-function mutations that may alter cell functionality and homeostasis leading to malignant transformation and tumorigenesis. Several studies showed that in the adult and neonatal mouse liver transduced by AAV vectors, the viral genomes remains mainly extra-chromosomal [239, 246] while a minority of them integrate into the host DNA [247] with a preference for sites that are close to active genes, ribosomal DNA and CpG sequences [246, 248, 249, 250, 251, 252]. The potential of AAV-induced genotoxicity in the context of systemic or liver-directed gene therapy has been investigated in the recent years. So far, insertional mutagenesis by AAV vectors has been reported in mouse after neonatal gene delivery [251, 253] but not after AAV administration to juvenile (6–8 weeks) or adult mice [19, 133, 254, 255, 256, 257]. In particular, two independent studies showed that systemic AAV administration to neonatal mice predisposes them to hepatocellular carcinoma (HCC) [251, 253] due to the insertion of viral genomes in the RNA imprinted and accumulated in nucleus (*Rian*) locus, encoding for many regulatory non-coding RNA (snoRNAs, microRNAs and lincRNAs). This integration leads to the misregulation of genes flanking the insertion site (*Rlt1* and various microRNAs), which promotes HCC [251]. Notably, in humans the upregulation of delta-like homolog 1–deiodinase type 3 (DLK1-DIO3), the orthologous genomic imprinted cluster of the *Rian* locus microRNA, has been also associated with poor survival in patients with HCC [251]. Interestingly, Chandler et al. also showed that the preference of viral genome insertion in specific loci (such as *Rian*, *albumin and α-fetoprotein*) is favored by their high transcriptional activity and positively correlates with AAV vector doses [251]. Chandler et al. [251] also reported that the upregulation of genes, which are close to the insertion site, depends on the strength of the promoter included in the transgene expression cassette. In particular, the strong chicken beta actin (CAG) and (thyroxin-binding

globulin) TBG promoters, but not the liver-specific hAAT promoter, induced the dis-regulation of gene expression leading to tumor formation [251]. Based on these findings, it becomes crucial to design and optimize the regulatory elements contained in the transgene expression cassette to find a balance among potency and possible genotoxic side effects. The tumor-initiating potential of scAAV vectors in the liver of adult mice and newborn rats has also been recently assessed [258, 259]. Surprisingly, no integration hot spots of scAAV genomes in the liver DNA and no increased frequency of tumors were found in adult and newborn-treated animals [259]. However, a side-by-side comparison of the profile and efficiency of viral genome integration in the liver upon administration of ssAAV and scAAV vectors is still missing.

It should be noted that while some of the studies conducted thus far in rodents on the insertional mutagenesis of AAV vector revealed their potential genotoxicity, studies in larger animal models such as dogs [100] and non-human primates [99], in which animals were followed for extended periods of time, raised no concerns over the genotoxicity risk of AAV vectors in liver. Similarly, studies in humans also support the safety of the approach, as no tumor formation has been documented $\sim$5 years post-gene transfer [57]. However, numbers are still small, and long-term follow-up in more AAV-treated subjects over an extended period of time is needed. Additionally, follow-up of subjects treated with AAV at a pediatric age will help clarify the genotoxicity risk in this patient population.

Concluding Remarks

As the field of in vivo gene transfer with AAV vectors proceeds toward a more mature state, a growing number of applications of the technology are reaching the clinic. Today investigators are generally more familiar with the use of AAV vectors as therapeutic tools; however, a number of potential issues associated with the technology have yet to be addressed. These include in particular concerns related to the immunogenicity of AAV vectors and the ability to define the optimal combination of capsid and transgene to maximize levels and persistence of therapeutic efficacy. Additionally, unknowns related to the potential genotoxicity risk of AAV vectors and the issue of gene transfer in young individuals will have to be defined.

While human studies will ultimately answer most of the questions above, preclinical studies will remain crucial in guiding the design of clinical trials and testing the safety and efficacy of the future generation of gene therapeutics.

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