Chapter 7

Adeno-Associated Virus (AAV)-Mediated Gene Therapy for Disorders of Inherited and Non-Inherited Origin

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Abstract

Gene therapy is a novel promising approach for treating a spectrum of inherited and non-inherited disorders by delivering therapeutic genes to specific organs or tissues. Of the viral vectors that have been used to date to deliver the genes of interest, the adeno-associated viral (AAV) vector appears to be the most safe and effective vehicle and has the ability to maintain long-term gene and protein expression following a single injection of the vector. Gene therapy studies using AAV vector have shown significant progress not only in animal models but also in human gene therapy with no known pathogenicity. While success has been achieved in gene therapy using AAV vector to deliver the target genes for inherited disorders, however, clinical trials are yet to begin to see whether gene therapy has promise for treatment of non-inherited diseases. This chapter describes AAV biology, viral structure, and cell entry mechanisms, with special emphasis on AAV tissue tropism achieved by manipulating different serotypes and capsid engineering. This chapter also discusses successful application of the AAV vector for non-inherited disorders in animal models with particular reference to liver fibrosis, outlining advantages, disadvantages, and future challenges that this therapy may face.

Keywords: adeno-associated viral vector (AAV), gene therapy, inherited disorders, non-inherited disorders, liver fibrosis

1. Introduction

Gene therapy is a novel promising approach for treating a spectrum of inherited and non-inherited disorders by delivering therapeutic genes to specific organs or tissues. Of the viral vectors that have been used to date to deliver the gene of interest, the adeno-associated viral (AAV) vector appears to be the most safe and effective vehicle and has the ability to maintain long-term gene and protein expression following a single injection of the vector. Gene therapy studies using AAV vector have shown significant progress not only in animal models but also in human gene therapy with no known pathogenicity. Recently, the Food and Drug Administration (FDA) has approved a pioneering gene therapy protocol using an AAV vector for a rare form of childhood blindness, the first such treatment cleared in the United States for an inherited disease. While success has been achieved in this field targeting inherited disorders, however, clinical trials are yet to begin to see whether gene therapy has promise for treatment of non-inherited diseases. This chapter describes AAV biology, viral structure, and cell entry mechanisms, with special emphasis on AAV tissue tropism achieved by manipulating different serotypes and capsid engineering. This chapter also discusses successful application of the AAV vector for non-inherited disorders in animal models with particular reference to liver fibrosis, outlining advantages, disadvantages, and future challenges that this therapy may face.

2. Adeno-associated virus

Adeno-associated virus (AAV) was discovered by Atchison et al. in 1965 from a pooled harvest of rhesus monkey kidney cell (RMK) cultures coinfected with simian adenovirus type 1 (SV15) [1]. This virus that could be observed as small DNA-containing particles was initially discovered as a contaminant of adenovirus preparations, and thus, it was named adeno-associated virus. However, AAV belongs to a genus of the parvoviruses, now known as dependoviruses [2]. AAV is replication defective and depends on a helper virus for effective and productive replication in mammalian cells. Generally, adenovirus or herpes viruses are considered to be the helper viruses for AAV to continue its life cycle. Early research on AAV has shown that this virus does not cause any disease in man even though it appears that it persists in humans along with its helper virus, particularly adenovirus [2]. In 1969, AAV was shown to possess several advantages in experimental systems including its small DNA genome of approximately 5 kb, packaging of plus and minus strands into individual particles, and most importantly, it is present as a defective virus [3]. During the first 20 years after its discovery, its genome structure, growth cycle, and latency were described. In the early 1980s, the genome sequencing of AAV serotype 2 (AAV2) was completed by Srivastava and colleagues [3]. This facilitated the generation of the first recombinant AAV vectors using AAV2 by the mid-1980s. Thereafter, studies using AAV were used for gene transfer in mammalian cell cultures. Subsequently, evidence of clinical safety has encouraged the researchers to use AAV vectors in clinical trials for various inherited disorders [4].

The AAV2 is a non-enveloped virion with a genome consisting of a single-stranded DNA (ssDNA) which is enclosed by a spherical protein shell about 20 nm in diameter [5, 6], with

a density of 1.41 g/cm^3 [6, 7]. The AAV genome is made up of 4675 nucleotides flanked by inverted terminal repeats (ITRs). Each ITR is 145 nucleotides in length and forms a T-shaped hairpin structure by self-base pairing utilizing the first 125 nucleotides [3, 8]. Viral replication (Rep) and capsid (Cap) genes responsible for encoding four non-structural proteins, such as Rep40, Rep52, Rep68, and Rep78, and three structural proteins, such as VP1, VP2, and VP3, respectively, are located between the two ITR regions. The structural proteins, VP1, VP2, and VP3, are arranged in a ratio of 1:1:10 to form the icosahedral symmetrical shape of the virus [6, 9]. It has been reported that the VP1 protein is essential for infection [6, 10], whereas VP2 is the major protein responsible for nuclear transfer of the capsid proteins. Of note, the VP3 subunit is the most abundant protein in the capsid responsible for the binding of the virus to cell surface receptors [6, 11] and viral particle formation in the host cell [12].

3. AAV-host cell interaction

Heparan sulfate proteoglycan (HSPG) is the first identified primary receptor that AAV2 binds when infecting cells [6, 13]. The initial hypothesis was that the HSPG-binding site is located within the capsid protein VP3 [14], and this hypothesis was further supported by a mutational analysis performed by Wu and colleagues in year 2000 [11]. Wu et al. showed that there are two VP3 amino acid clusters of AAV2 that are involved in HSPG binding [11]. HSPG is not the only receptor type involved in AAV2 binding to a host cell, but there are one or more coreceptors which facilitate AAV cellular entry. Interestingly, αvβ5 integrin was identified as a coreceptor for internalization of AAV2 virions by Summerford and colleagues [15]. In cell studies, the chelating agent ethylenediaminetetraacetic acid (EDTA) was used to disrupt integrin function and results showed a notable reduction in AAV2 infection, suggesting that AAV2 uses αvβ5 integrin as a secondary receptor to mediate viral entry. Moreover, Qing and colleagues identified that human fibroblast growth factor receptor 1 (FGFR1) is also essential for viral entry into the host cell [16] and acts as a coreceptor for successful infection by AAV [6].

Although AAV2 is the most extensively studied serotype of AAV, there are several other AAV serotypes which have been evaluated for their binding characteristics to cellular receptors. It was recently shown that AAV serotypes 3 [17] and 13 (VR-942) [18] utilize HSPG as the primary cell surface receptor for cell entry, while AAV serotypes 1, 4, 5, and 6 [18–20] utilize N-linked and O-linked α2–3 and α2–6 sialic acids. AAV9 interacts with N-terminal galactose as the primary receptor [21] and also interacts with secondary coreceptors for facilitating cell entry, such as integrins [15, 22] FGFR1 [16], hepatocyte growth factor receptor (c-Met) [23], and laminin receptor [24]. Despite all these known pathways for AAV infection, no common primary receptor for all the AAV serotypes had been identified. Recently, Pillay and colleagues [25] used a library of mutagenized haploid HAP1 cells to create knockouts of nearly all non-essential genes in the human genome. This knockout library was exposed to recombinant AAV2-RFP (AAV2-red fluorescent protein), and a gene that was most significantly enriched in the screen was identified. This receptor named "AAV receptor (AAVR)" is characterized as a type I transmembrane protein which contains a MANSC domain, five polycystic kidney disease (PKD) domains, and a C6 region near the N terminus. These findings have been validated using an AAVR knockout cell line which demonstrated a resistant to infection by

almost all AAV serotypes, whereas restoring recombinant AAVR gene in the AAVR knockout cells restored the ability of AAVs for successful infection. Furthermore, AAVR gene knockout mice demonstrated robust resistance to AAV9 infection. This important discovery implicates the AAVR as a universal primary receptor for all AAV serotype infection [6].

4. Cell entry mechanism of AAV

The major cell entry mechanism for AAV is via endocytosis utilizing clathrin-coated pits, although other minor mechanisms are possibly involved in this process. However, these alternative minor mechanisms are yet to be confirmed [6]. Upon AAV binding to its cell surface receptors, it stimulates intracellular signaling pathways, which in turn stimulates internalization of AAV. This phenomenon can be clearly explained using the mechanisms reported for AAV2 host cell interaction. It was shown that attachment of AAV2 to HSPG and $\alpha V\beta 5$ integrin resulted in the activation of Rac1, an intracellular small guanosine triphosphate (GTP)-binding protein, and phosphoinositide 3-kinase (PI3K) in HeLa cells within 5 minutes of AAV2 infection [26]. Furthermore, inhibition of Notch1 by siRNA, a transmembrane receptor known to be involved in the activation of Rac1 and PI3K, was reported to decrease cell transduction by AAV2 [27], suggesting that the Rac1-PI3K pathway is necessary to initiate endocytosis of AAV2. Direct injection of AAV into the cytoplasm and nucleus of cells results in a significant lower infection rate than cells that are simply exposed to virus [28], suggesting that the processing of AAV virion through endosomal compartments is a critical initiating step for transduction following endocytosis.

In addition, transduction efficiency of AAV is largely dependent on the endosomal pH. Changing the pH to acidic (pH 4–6) inside the endosomal compartment facilitates transduction of AAV, whereas blocking acidification during endosomal processing decreases the rate of transduction [29–31]. Also, the application of different classes of proteasome inhibitors such as tripeptidyl aldehydes and N-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL) and the anthracycline compounds such as doxorubicin increases the rate of viral translocation to the nucleus [32]. Furthermore, LLnL appears to increase AAV2 capsid ubiquitination that results in augmented gene transfer in different cell types [33], suggesting a mechanism by which these inhibitors increase transduction may be related to ubiquitination. AAV must exit from the endosome first before translocating to the nucleus. Prior to escape from the endosome, AAV undergoes a conformational change leading to the exposure of the unique N-terminal ends of VP1 and VP2, which contains a domain of phospholipase A2 (PLA2) [34], an enzyme that breaches the endosomal membrane and thereby facilitates efficient endosomal escape of viral particles. Upon endosomal escape, AAV enters the nucleus as an intact particle [28] and uncoating then occurs inside the nucleus. However, nuclear transport of AAV is a slow process, approximately only 1–2% of internalized AAV enters and expresses in the nucleus, and the whole entry process takes about 2–13 h [35]. Thus, most viral particles which fail to translocate are located outside or away from the nucleus.

The viral particles that fail to translocate into the nucleus are eventually degraded by host proteasomes in the cytoplasm and presented as antigen to cytotoxic T cells via the major

histocompatibility complex (MHC) class I pathway [36]. Although there are several studies that have investigated the nuclear entry of AAV, the mechanism by which AAV translocates into the nucleus is still unclear. Because AAV is a small virus with a diameter of around 20 nm, it has been suggested that the virion enters the nucleus using the nuclear pore complex (NPC) [37].

Furthermore, the nuclear entry of AAV is dependent on importin-β, a nuclear import protein that has been shown to play a key role in facilitating the binding of viral particles to host nuclei in other viral infectious pathways [38, 39]. Another study using single-point edge excitation sub-diffraction (SPEED) microscopy, a form of super-resolution imaging, to track single AAV particles revealed that approximately 17% of AAV particles were imported through the NPC successfully to the nucleus [40], reinforcing the importance of the NPC in AAV nuclear transfer. Interestingly, there is further evidence that nucleolin, a protein that shuttles between cytoplasm and nucleus, specifically binds to AAV capsid, which suggests that nucleolin may act as a nuclear receptor for AAV particles as well [41]. Upon entry into the nucleus, the ssDNA of AAV genome is converted to double-stranded DNA (dsDNA) using nuclear machinery of the target cells for transcription of the transgene [42]. The synthesis of second DNA strand has been considered as a rate-limiting factor for the onset and efficiency of transgene expression in ssAAV vectors [43]. As a result, second-generation AAV vectors with a dsDNA, also known as self-complementary AAV (scAAV) vectors, have been developed to improve the transduction and transcription efficiency. In the past decade, several studies have shown that new scAAV vectors provide safe, reliable, and organ-specific transduction both *in vitro* [44–46] and *in vivo* [46–49]. This suggests that the limitations associated with cell transduction using ssAAV genome can be overcome by the use of scAAV vectors in gene therapy.

5. AAV serotypes and tissue tropism

To date, a total of 12 naturally occurring AAV serotypes have been discovered from both human and non-human primates (**Table 1**). These serotypes are able to infect cells of diverse tissue types. Interestingly, the tissue specificity is determined by the capsid serotype. The existence of a variety of serotypes with different infectivity rates and tissue specificity makes AAV one of the most promising candidates in gene therapy research. By development of different AAV pseudotypes, researchers have been able to obtain unique cellular tropism and high transduction efficiency. All AAV serotypes share at least 50% sequence homology. However, serotype AAV5 has the most divergent amino acid capsid sequence, and AAV4 also shows a considerable degree of divergence [50]. Surprisingly, this sequence diversity between serotypes is not scattered but primarily located in the looped out domains of the capsid protein [51]. However, comparative studies of AAV serotypes found that this sequence variability may not be responsible for the differences in infectivity rates and tissue specificity. AAV serotype 2 is most widely used in gene therapy research. Several studies have investigated gene expression and tropism *in vivo* mediated by different AAV serotypes and identified that they differ broadly in transduction efficacies and tissue tropism. A comparative study of AAV serotypes 1–9 mediated transgene expression after systemic

AAV serotype	Characteristics	Tissue tropism
AAV1	Shares 99% homology with AAV6 serotype [50]	Liver, heart, skeletal muscle [52]
AAV2	The most commonly used serotype Close homology to all serotypes except AAV 4, 5, 11, and 12 [50] Low transduction efficiency [52]	Liver, heart, muscle [52]
AAV3	Low transduction efficiency Slow in targeting the tissues [52]	Heart, liver [52]
AAV4	Close homology to AAV11 (82%) and AAV12 (79%) serotypes [50] Low transduction efficiency Slow in targeting the tissues [52]	Lung, heart, liver, central nervous system [52, 134]
AAV5	The most divergent serotype (shares only 53–59% homology to other serotypes) Low transduction efficiency [52]	Liver [52]
AAV6	Shares 99% homology with AAV1 serotype [50]	Liver, heart, skeletal muscle [52]
AAV7	Fast in targeting the tissues [52]	Liver, skeletal muscle [52]
AAV8	93% homology to AAV10 serotype [50]	Heart, liver, brain, muscle (second most efficient serotype reaching the brain) [52]
AAV9	Fast in targeting the tissues [52]	Liver, heart, brain, lung, skeletal muscle (serotype with the broadest tissue tropism and most efficient in reaching the brain) [52]
AAV10	93% homology to AAV8 serotype [50]	Liver, heart, muscle, lung, kidney, uterus (with pseudotype AAV2/10) [135]
AAV11	Close homology to AAV4 serotype [50]	Muscle, kidney, spleen, lung, heart, stomach (with pseudotype AAV2/11) [135]
AAV12	Close homology to AAV4 serotype [50]	Muscle, salivary glands [136]

Table 1. Characteristics and tissue tropism of AAV serotypes in the mouse.

tail vein injection in mice showed that each AAV serotype profoundly differs in its ability to transduce organs, with AAV9 having the highest and fastest onset of transgene expression, highest viral genome copies, and the broadest tissue tropism, as determined by luciferase images [52]. Conversely, AAV3 and AAV4 are the slowest in targeting tissues, and among all the serotypes, AAV2, 3, 4, and 5 have the lowest transduction efficiency. The liver is the most common organ transduced by nearly all AAV serotypes with AAV7 and AAV9 showing the strongest tropism. Moreover, AAV9 is the most efficient serotype in reaching the heart and brain, followed by AAV4 and AAV8, respectively [52]. Of note, AAV serotype 8 (AAV8) shows a significantly greater liver transduction efficiency than the other AAV serotypes, and therefore, this serotype has been developed to use as a gene therapy vector for hemophilia A and familial hypercholesterolemia [53].

6. Molecular engineering of AAV capsid

There are several challenges for AAV serotypes to exert their therapeutic potential in target organs including the need for high vector doses for efficient delivery, pre-existing antiviral immunity in the host, and the lack of cell type-specific tropism leading to off-target transduction [6]. One way to overcome these limitations is to randomly generate capsid mutants from a library to extend the capability of the traditional AAV vector by increasing its cell transduction efficiency for specific cell types and its ability to escape from antibody neutralization.

One approach used to create a mutant library is DNA shuffling, a strategy in which the open reading frame of capsid genes of different AAV serotypes is fragmented by nucleases. This is followed by random ligation, resulting in new and random combinations of capsid sequences. These new molecular-enhanced AAV vectors exhibit a broad range of cell tropism with numerous functional differences between chimeras and their parent serotypes. Consequently, there is potential to produce unlimited numbers of new AAV variants with novel gene delivery properties. This method of AAV capsid engineering was first described in 2008 by Grimm and colleagues [54] and has become a commonly used technique over the years. More recently, Lisowski and colleagues utilized a humanized mouse model to perform serial selection using a human-specific replication competent viral library composed of DNA-shuffled AAV capsids. After four rounds of selection, they identified a novel chimeric capsid variant composed of five different parental AAV capsids [55]. Of these, LK-03, which efficiently transduced human primary hepatocytes both *in vitro* and *in vivo*, was found to be a human liver cell-specific AAV serotype [55]. This study has opened up a new avenue to validate therapeutic potential of an AAV capsid variant in preclinical studies using human primary cell xenotransplanted models prior to commencing clinical studies.

In addition, a study using *in silico* ancestral sequence reconstruction (ASR) of AAV capsid protein generated nine functional putative ancestral AAVs. In this study, Zinn and colleagues also identified Anc80, the predicted ancestral sequence of the widely used AAV serotypes 1, 2, 8, and 9 and showed that Anc80 is a highly potent *in vivo* gene therapy vector compared to AAV2 and AAV8 for targeting liver, muscle, and retina in mice [56]. Nevertheless, Anc80 demonstrated a high stability and no toxicity in several safety studies carried out in mice. This synthetic viral vector has been evaluated in non-human primates (rhesus macaques), which demonstrated a superior expression of Anc80 in monkey liver following Anc80 administration compared to control monkeys injected with AAV8. Hence, future studies may also rely on the use of Anc80, in particular for liver-directed gene therapy studies.

7. AAV as a safe vector in gene therapy

It has been shown that AAV viral proteins cause a minimal immunogenic response, and at the same time, it can yield prolonged expression of therapeutically relevant genes/proteins. Also, when comparing to the other potential viral vectors such as lentiviral vectors, AAV possesses a reduced proinflammatory risk and has been considered as one of the most promising gene

transfer vectors for *in vivo* gene therapy [57]. However, in some experimental settings, it was reported that immune responses generated by AAV administration appear to compromise the outcomes of AAV-mediated gene therapy. Thus, several factors may determine the occurrence of immune responses against the AAV proteins, including the route of administration, dose, serotype, host species, transgene and expression cassettes, and pre-existing immunity to AAV [6, 58].

It has been suggested that AAV activates mouse and human plasmacytoid DCs to produce type 1 interferon via a TLR9-MyD88 pathway, resulting in induction of adaptive immune CD8+ T cell responses to AAV capsid and the transgene [58]. In addition, different administration routes for AAV2-mediated ocular gene therapy induced varying immune responses. For instance, intravitreal administration of an AAV2 vector, which led to transduction of the inner retina, triggered a humoral immune response to AAV2 capsid; however, no effect was observed following subretinal administration and subsequent repeated injections [59]. Animal studies have suggested that the presence of neutralizing antibodies could compromise AAV transduction *in vivo* following systemic administration [60, 61]. These findings are potentially important for translation of AAV gene therapy from animal studies to clinical trials due to the large prevalence of AAV neutralizing antibodies in humans.

Due to natural exposure to wild-type AAV early in life, a significant proportion of human population have humoral immunity to the AAV capsid, primarily AAV1, 2, 3, and 5 [62, 63]. Of note, among the most commonly used AAV vectors, the most prevalent anti-AAV antibodies in humans are AAV2 followed by anti-AAV antibodies to AAV1 [64], while the least prevalent are for AAV7 and AAV8. It has been shown that rAAV vectors, including serotypes 1, 2, and 5 can transduce dendritic cells (DCs) and generate immune responses to transgene products [65, 66]. Interestingly, another study, which evaluated the differential immune responses to the transgene products from rAAV1 and rAAV8 vectors using a hypersensitive autoimmune mouse model, revealed that unlike AAV1 vectors, AAV8 vectors were unable to transduce dendritic cells (DCs) and elicit transgene-specific immune responses efficiently, resulting in induction of immune tolerance to transgene products [67]. Different properties of these vectors imply tremendous potential in different applications, where an immune response to transgene is to be either elicited or avoided.

8. AAV vector transduction efficiency—male versus female

Recombinant AAV vector transduction efficiency clearly depends on the gender. This fact has been specifically shown in the liver and the brain in murine models. A study carried out by Maguire and colleagues has shown that the vector transduction efficiency using AAV serotype 9 was found to be different in the brain and the liver between male and female mice [68]. This study revealed a higher transgene expression in the brain of females compared with male mice, whereas a higher transgene expression was observed in the liver of male mice compared with female mice. In line with this study, Davidoff and colleagues revealed that when compared with female mice, transgene expression after liver-targeted delivery of AAV2 and AAV5 particles was 5- to 13-fold higher in male mice [69]. In addition, they found that transduction efficiency was dramatically reduced by castration in male mice, whereas oophorectomy in female mice did not significantly influence rAAV transduction [69]. Moreover,

administration of 5α dihydrotestosterone in female mice prior to rAAV injection enhanced stable hepatocyte gene transfer to levels observed in male mice, suggesting rAAV vector transduces hepatocytes via an androgen-dependent pathway [69].

In addition, there is evidence to demonstrate the distinctly different patterns of persistence of rAAV-eGFP (enhanced green fluorescent protein) expression across the hepatic lobule in male and female mice. Female mice retained a predominantly perivenous pattern of expression, whereas male mice had shown an inversion of this pattern with preferential loss of perivenous expression and relative retention of periportal expression [70]. Therefore, these sexually dimorphic patterns of genome persistence could have significant implications for the long-term therapeutic efficacy of rAAV-mediated gene transfer in man, particularly in the context of correction of liver functions showing metabolic zonation [70].

9. Production and modification of AAV

The AAV serotype 2 was the first AAV vector used for gene transfer applications. This particular vector was chosen primarily because of its broad tropism, efficient transduction with stable and long-term transgene expression with minimal inflammation, and immune responses in a number of organs, such as the brain [71], retina [72], and skeletal muscles [73]. Liver is the other major organ which is targeted for rAAV2 gene delivery strategy because hepatocytes are easily accessible to vectors injected into the circulation through large pores in liver capillaries. Although results in the liver have been less consistent, a number of studies demonstrate a successful transduction of rAAV2 vector with persistent transgene expression in the liver using a single dose [74], and approximately 5% of hepatocytes were transduced following rAAV2 vector injection [75]. Of note, a study which was undertaken by Snyder and colleagues provided the most impressive results by achieving sustained and therapeutic levels of factor IX in hemophilia B, with no associated toxicity in both canine and murine models [75, 76].

The discovery of novel strategies for pseudotyping, recombination of AAV constructs into capsids of alternative serotypes, and the development of scAAV vectors which effectively alter tissue tropisms with enhanced transduction efficiency [77] has opened up new avenues to produce more attractive vectors for use in clinical applications including hemophilia B, Parkinson's disease, and rheumatoid arthritis [78]. Among all novel recombinant AAV serotypes, AAV2 genome construct pseudotyped with capsid 8 (AAV2/8) is one of the most efficient vectors for hepatic gene transfer. In addition, it has greater liver transduction efficiency, with fourfold more genomes per transduced cell, when compared with other pseudotyped vectors [6, 79]. Moreover, it has an excellent transduction rate (95%) in hepatocytes of the mouse liver via intraportal vein injection [80]. In line with this, the development of scAAV vectors further enhances the transduction efficiency to the liver [81], suggesting that the conversion of single-stranded AAV genome into double-stranded form for gene therapy studies appears to be beneficial since this procedure can avoid the need to assemble second DNA strand for transgene expression *in vivo* [6, 46, 82].

The most widely used method to produce and purify recombinant AAV particles for preclinical applications is the triple transfection method using HEK293 cells, which requires the use of an

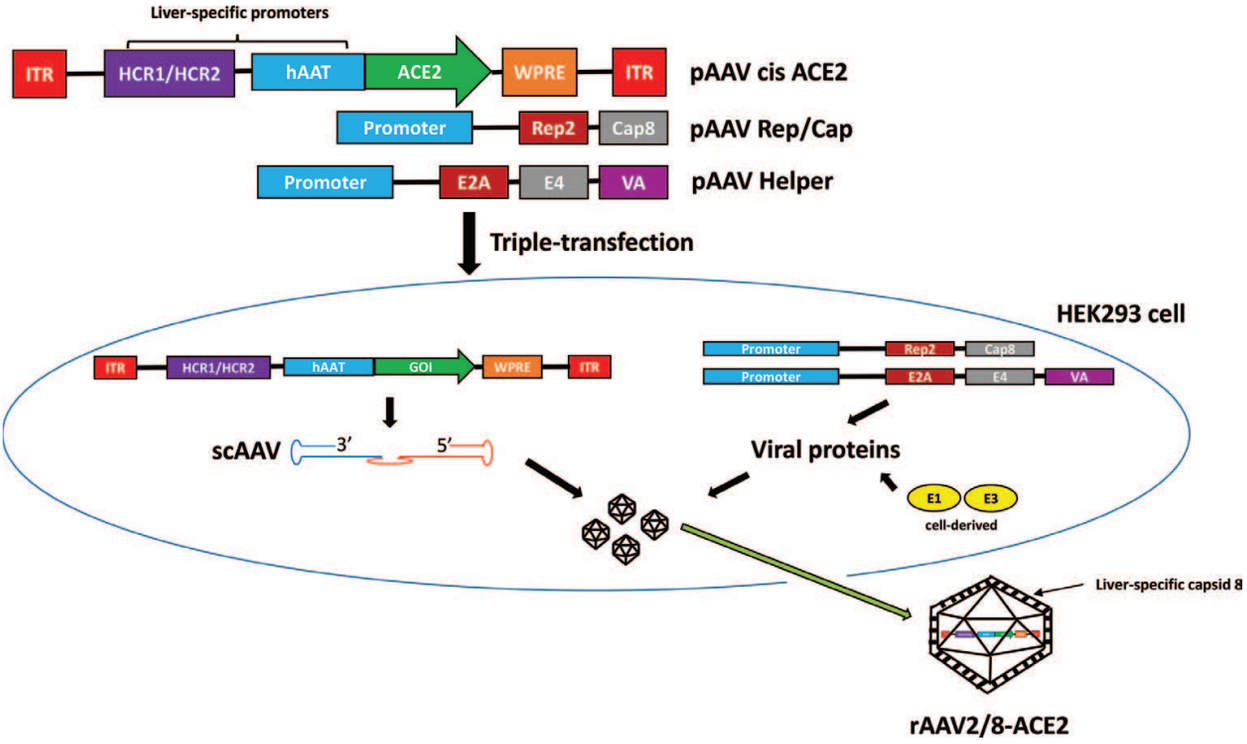

Figure 1. Schematic representation of the assembly of AAV2 genome pseudotyped with liver-specific AAV serotype 8 and liver-specific promoters in HEK293 cells. Liver-specific rAAV2/8-ACE2 viral particles are produced by transfecting HEK293 cells with rep2/cap8 plasmid, Ad helper plasmid, and a plasmid carrying AAV2 inverted terminal repeat-ACE2 cassette with liver-specific promoters. Recombinant AAV2/8-ACE2 viral particles are purified from cell homogenate 48–72 h post transfection, followed by assessment of AAV quality, genome titer, infectious and transducing properties, and integrity of the packaged AAV genome [87].

AAV replication and capsid plasmid that provides Rep78, Rep68, Rep52, and Rep40 proteins necessary for vector genome replication and VP1, VP2, and VP3 capsid proteins, the vector DNA plasmid with the inverted terminal repeat-transgene cassette, as well as the adenovirus (Ad) helper plasmid [83, 84]. In addition, HEK293 cells have been engineered to provide adenovirus helper genes *in trans* such as E1a and E1b55k for AAV assembly. The key advantage of this method is that AAV particles can be efficiently made with genes supplied by Ad helper and HEK293 cells without the need to use replication competent adenovirus [84] (**Figure 1**). In addition, to improve tissue tropism, the AAV genomes can be pseudotyped with a desired capsid protein. Following 48–72 h transfection, the cell homogenate is purified, followed by the assessment of AAV quality control including genome titer [85], infectious and transducing properties, and integrity of the packaged AAV genome [86]. This is schematically illustrated in **Figure 1** where AAV2 genome is pseudotyped with capsid 8 (AAV2/8) to increase liver specificity [87].

10. Pros and cons of AAV gene therapy

A successful gene therapy approach should deliver an appropriate amount of a therapeutic gene into the target tissue without substantial toxicity while achieving long-term gene expression. Of all currently available viral vectors including retroviral, lentiviral, adenoviral, and AAV vectors, the AAV is a unique non-pathogenic viral vector with broad tissue tropism and has the potential

to be the leading vector for future gene therapy studies [88]. Unlike recombinant adenoviral vectors which yield high initial gene expression that diminishes rapidly due to immune clearance, the AAV vector-based gene expression is persistent. In addition, as AAV vectors were derived from a parental virus with no known pathogenesis which is replication defective, they do not carry a risk of infecting patients with a pathogenic wild type virus. In addition, AAV vectors mediate a minimal cell-mediated immune response, which is favorable for the persistence gene transduction to the host cells. At the same time, AAV-based vectors are able to transduce a wide range of host cells including both dividing and non-dividing cell types [88, 89]. A prominent disadvantage associated with AAV compared to the other viral vectors is its small packaging size, which limits the size of the transgene to be delivered using the vector. However, novel molecular engineering methods have the potential to overcome these limitations, and thus, genetically engineered AAV is poised to become the leading vector for future gene therapy in humans.

11. Gene therapy using AAV vectors for inherited disorders

Many studies have explored the therapeutic potential of these engineered AAV vectors for a number of inherited disorders. After several decades of experimental studies, the first successful human gene therapy protocol using AAV serotype 1 vector was approved in 2012 by the European Commission (EU) for the treatment of patients with lipoprotein lipase deficiency (LPLD), an extremely rare genetic disorder [90]. This was a milestone achievement for researchers who have been working to develop successful gene therapy protocols for inherited human disorders. The therapy was introduced under the trade name Glybera® (alipogene tiparvovec) by UniQure. However, after 5 years of the launch of the world's first approved gene therapy, UniQure has not renewed its EU license in 2017 and ceased to produce Glybera for use because of the expensive nature of the treatment protocol [91]. However, it was unfortunate that UniQure has discontinued its production despite the first LPLD patient treated with alipogene tiparvovec showing improvement of quality of life without abdominal pain and pancreatitis attacks for 18 months [92]. UniQure, however, has endeavored to develop gene therapy for hemophilia B.

The most common clinical trials based on AAV therapy in recent years have been in hemophilia B, a blood clotting disorder caused by a defect in the gene encoding coagulation Factor IX (FIX), leading to a deficiency of FIX. The only treatment available for this disease is lifelong intravenous infusion of FIX concentrates. Although this treatment is effective as a preventive medicine, it is not curative. In addition, the treatment is invasive, inconvenient, and very expensive, thus not affordable for most patients with hemophilia B, resulting in a reduction in life expectancy for those patients with a severe bleeding phenotype [93]. Similar to the FIX concentrates, there are clotting formulations with longer half-life which represents a major advance but still require lifelong intravenous administration. Robust preclinical results using AAV-based therapy in two murine [74, 94] and three canine models of hemophilia B [95–97] demonstrated long-term expression of FIX, with no significant liver toxicity and with no FIX-specific antibodies detected following muscle- or liver-directed injections. A follow-up study demonstrated an induction of immune tolerance in mice after hepatic gene transfer by rAAV expressing human FIX (rAAV-hFIX), which is mediated by regulatory CD4+ T cells, resulting in suppression of human FIX antibody formation [6, 98]. Based on the results from

animal studies, the world first clinical trial using rAAV2-hFIX vector in humans via intramuscular route has been conducted [99]. The results indicated that the transduction of muscle tissue was successful; however, circulating plasma FIX levels in all patients were less than the required level for a therapeutic effect (<2% of normal). In a subsequent clinical study, the delivery target was switched to the liver, the normal site of FIX synthesis. Although rAAV2-mediated hFIX gene transfer to the liver-mediated therapeutically relevant expression levels [100], the expression persisted for less than 8 weeks.

Recent study by Nathwani and colleagues demonstrated the AAV8 serotype as a more effective vector for liver-directed hemophilia B gene therapy [101]. In this study, six severe hemophilia B patients received a single injection of pseudotyped AAV2/8-hFIX vector at three escalating doses (high, intermediate and low), with two patients per dose and no immunosuppressive was given. Patients were subsequently followed for up to 16 months. All patients have achieved AAV2/8-mediated expression of FIX at above the therapeutic threshold, ranging between 2 and 11% of normal levels, and the increase in FIX serum level was dose-dependent. Four out of six patients discontinued their prophylactic treatment with hFIX concentrates without having spontaneous hemorrhage, whereas the other two patients continued to receive hFIX concentrates but extended the interval between hFIX treatments. This was the first liver-directed AAV gene therapy trial to show sustained therapeutic FIX levels and improved clinical outcomes in patients with hemophilia B. However, in patients who received the highest dose of vector, T cell-mediated clearance of AAV-transduced hepatocytes was observed, with associated elevation of liver enzyme levels. This response has been overcome by a short course of glucocorticoids, without the loss of hFIX expression.

Nathwani and colleagues later conducted a follow-up study to evaluate the long-term safety and efficacy of AAV2/8-hFIX therapy in the same cohort of hemophilia B patients [93]. Of note, this monitoring study also included addition of four new patients, each of whom received the high dose of vector. Consistent with their previous findings, a single intravenous injection of vector resulted in an increase in plasma FIX activity from less than 1% to sustained level of up to 6% of the normal value in all 10 patients, and this remained stable for up to a period of 4 years. Additionally, substantial clinical improvements were achieved in all patients, including significant reductions in number of spontaneous hemorrhage and annual number of prophylactic treatment with FIX concentrates. Not surprisingly, there was a dose-dependent, asymptomatic increase in both the serum alanine transaminase (ALT) level and increase in anti-AAV capsid neutralizing antibody level, which led to a gradual decline in FIX levels, suggesting transduced hepatocyte destruction. There was a transient increase of ALT levels in all patients which resolved with administration of a single course of prednisolone, after which no recurrent elevation of serum ALT in patients was observed.

A recent clinical trial completed using ssAAV vector consisted of a bioengineered capsid, liver-specific promoter, and FIX Padua (FIX-R338L) in 10 men with hemophilia B who had FIX coagulant activity of 2% or less of the normal also showed a success with no serious adverse events during or after vector infusion [102]. These patients were followed up to 492 days (16 months). The results showed that 8 of 10 patients did not require the regular treatment with FIX concentrates, and bleeding episodes were not reported in 9 patients after the vector treatment. Overall, there was a significant reduction in annual bleeding rate in patients treated with AAV-FIX-R338L. Although there were two patients who developed asymptomatic increase in liver

enzyme levels, they were recovered after a short-term prednisone treatment. Of all participants, only one patient had been treated with FIX concentrates who was diagnosed with an advanced arthropathy at baseline. However, the use of FIX concentrate was reduced to 91% comparing to the status before vector infusion. Additional clinical trials are underway with AAV2/8-hFIX (NCT00979238) and FIX-Padua (NCT01687608), which will provide more information on safety and efficacy of the therapy [103]. Overall, the results from these studies suggest that gene therapy has the potential to significantly improve disease phenotype in hemophilia B patients.

It is of significance that for the first time, the US Food and Drug Administration (FDA) has approved a pioneering gene therapy protocol using an AAV vector for a rare form of childhood blindness in 2017 as the first such treatment cleared in the United States for an inherited disease. The disease known as Leber congenital amaurosis (LCA) develops due to mutations in the RPE65 (retinal pigment epithelium-specific 65-kDa) gene, causing a severe form of inherited retinal blindness in infants and children. Several independent studies [104–106] using rAAV2/2 expressing RPE65 complementary DNA (cDNA) have provided preliminary evidence of short-term safety and efficacy in this disorder. Further studies by Cideciyan and colleagues showed a significant efficacy of human retinal gene transfer with rAAV2-RPE65 vector with transgene expression for up to 1 year post treatment [107]. Also, they have proven the treatment as a safe therapy by evaluating the safety parameters obtained through regular standard eye examinations, physical examinations, routine hematology, serum chemistries, coagulation parameters, and urinalysis. This particular FDA-approved gene therapy (LUXTURNA) (voretigene neparvovec-rzyl) is to be used in patients with confirmed biallelic RPE65 mutation-associated retinal dystrophy. This approval is considered as a milestone of AAV vector-associated gene therapy research and further encourages researchers to develop successful vectors to deliver therapeutic genes for number of diseases where there is no effective medical treatment.

12. Gene therapy for non-inherited disorders

There have been many advances in identification of the mechanisms involved in chronic organ damage which opened up avenues for gene therapy studies [108]. While a plethora of preclinical and clinical studies over past several decades has focused on developing gene therapy for inherited disorders, despite several preclinical studies in animal models, there have been only a few clinical trials that have been undertaken to investigate therapeutic efficacy of gene therapy for non-inherited diseases. A recent study shows that telomerase expression using AAV9 vectors exerts therapeutic effects in a mouse model of pulmonary fibrosis [109]. This therapy targeted idiopathic pulmonary fibrosis. It is known that telomeres act as protective structures at the ends of chromosomes and the presence of short telomeres has been shown to be one of the causes for disease development. In this condition, telomeres become too short, resulting in the cessation of cell division which in turn leads to cell apoptosis. Telomerase is an enzyme that can restructure the telomeres length, and Povedano and colleagues developed a treatment using AAV serotype 9 to deliver telomerase to correct the short telomeres. As AAV9 preferentially targets regenerative alveolar type II cells (ATII), AAV9-Tert-treated mice show improved lung function with reduced inflammation and fibrosis at 1–3 weeks after vector treatment. It is of interest to note that pulmonary fibrosis either improved or disappeared at 8 weeks of gene therapy. AAV9-Tert

treatment lead to longer telomeres and increased proliferation of ATII cells, as well as lower DNA damage, apoptosis, and senescence.

AAV vector-derived cardiac gene therapy is emerging as an entirely new platform to treat cardiac disorders [110]. AAV gene therapy for heart failure have been validated in preclinical studies using animal models, and the vast majority of these approaches have been undertaken to improve calcium handling by cardiomyocytes. The therapeutic protein used in the majority of these studies was sarcoplasmatic calcium ATPase (SERCA2a). Based on the positive preclinical findings, the first clinical trial (CUPID trial: calcium upregulation by percutaneous administration of gene vector in cardiac disease, NCT02346422) was carried out to deliver SERCA2a using AAV serotype 1 vector to treat patients with advanced heart failure [111, 112]. The outcome of this phase 1 trial was successful with no adverse events and was progressed to phase 2a study, providing promising outcomes with significantly low rate of adverse events. However, the results of phase 2b clinical trial (CUPID2b trial, NCT01643330) using the same vector were disappointing with no significant change between the treatment group and the placebo group [113]. This has led to the cessation of patient recruitment for two additional trials using AAV1. SERCA2a [110]. Interestingly, there are two new upcoming trials aimed to deliver S100A1 with an AAV9 vector and a constitutively active form of the protein phosphatase 1 inhibitors, I1c, with a chimeric capsid with AAV2 and AAV8 serotypes [114, 115]. In addition, AAV1, AAV6, and AAV9 have emerged as the most promising AAV serotypes for cardiac gene transfer, which provides hopes for successful gene therapy approaches to treat heart failure in the future.

AAV-mediated gene therapy approaches to treat neuropathic pain in rodents have also been reported [116]. Fischer and colleagues have shown that administration of rAAV expressing Ca^{2+} channel-binding domain 3 (CBD3) gene significantly reduced pain behavior such as hyperalgesia after touch with a pin or sensitivity to acetone stimulation in animal models of inflammatory and neuropathic pain [117]. Another study using AAV9 vector encoding short hairpin RNA (shRNA) against vanilloid receptor 1 (TRPV1), which is an important target gene for acute pain, demonstrated that the therapy attenuated nerve injury-induced thermal allodynia (increased response of neurons) 10–28 days after treatment in a mouse model of spared nerve injury (SNI) [118]. These results provide positive evidence to encourage gene therapy researchers to develop AAV vector-based treatments for patients with chronic/diabetic neuropathic pain.

Considerable progress has been made in gene therapy approach to treat chronic liver fibrosis. Although angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) are widely used as treatments in patients with hypertension, they have been trialed in patients with chronic liver disease; however, the outcomes were not convincing mainly because they produce adverse systemic side effects [119]. Because of the lack of medical treatments, liver transplantation has inevitably become the only option for patients with end stage liver disease, resulting from chronic hepatic fibrosis and/or cirrhosis. Moreover, increasing incidence of chronic liver disease, lack of donor organs, post-transplantation complications, and the high cost in liver transplantation mean that there is a major need to discover and formulate specific, effective, safe, and inexpensive novel therapies for liver fibrosis/cirrhosis.

One possible approach to circumvent this is to develop organ-targeted antifibrotic strategies. Studies from our laboratory suggested that one possible target is the "alternate axis" of the renin-angiotensin system (RAS), comprising its key enzyme angiotensin-converting enzyme

2 (ACE2), which breaks down the potent profibrotic octapeptide, angiotensin II (Ang II) to an antifibrotic heptapeptide, angiotensin-(1–7) (Ang-(1–7)) [120, 121]. Evidence from experimental animal studies showed that recombinant human ACE2 (rhACE2) is beneficial for prevention of hypertension in cardiovascular disease [122] and to improve kidney function in diabetic nephropathy [123]. Interestingly, rhACE2 was well tolerated by a group of healthy human volunteers in a phase 1 clinical trial, without exerting any unwanted cardiovascular side effects [124]. There is one study that reported therapeutic effects of recombinant ACE2 in experimental liver fibrosis, in which liver injury was surgically induced by cholestasis or by hepatotoxic carbon tetrachloride injection [125]. They demonstrated that recombinant ACE2 significantly reduced hepatic fibrosis in both animal models of liver disease [125]. However, a major drawback of this systemic approach is that the treatment inevitably produces off-target effects, which in many cases are undesirable. Thus, there are several disadvantages with systemic administration of recombinant ACE2. This includes daily injections of ACE2, a procedure that is invasive in a clinical setting and expensive approach with unwanted effect on blood pressure regulation [125, 126]. To circumvent this problem, an ideal approach would be to increase tissue-specific ACE2 levels in the target organ. Thus, organ-specific increased ACE2 activity using a liver-specific recombinant AAV vector is expected to produce therapeutic effects confined to the targeted organ while minimizing unwanted off-target effects.

In addition to the use of liver-specific capsid serotype, specificity can be further enhanced by engineering the vector with ACE2 gene under the transcriptional control of a strong liver-specific promoter, apolipoprotein E/human α1-antitrypsin. Studies published by our laboratory used a pseudotyped liver-specific AAV vector (rAAV2/8) for preclinical evaluation and found that hepatic overexpression of murine ACE2 gene delivered into the mice lasted for up to 6 months following a single intraperitoneal injection [87]. We then treated mice with a range of liver disease models, which included biliary fibrosis induced by bile duct ligation (BDL), toxic injury induced by carbon tetrachloride (CCl4) injections, and fatty liver-associated liver fibrosis induced by feeding methionine- and choline-deficient (MCD) diet using a single intraperitoneal injection of rAAV2/8-ACE2 [87]. The treatment produced a major increase in ACE2 expression and protein activity, which was confined to the liver without affecting other major organs. Unlike inherited disorders, for example, hemophilia B where a relatively low level of transgene expression in the liver may be sufficient for subsequent small increases in FIX levels in the blood [48, 81], the magnitude of the expression of transgene required for therapeutic intervention in non-inherited disease may be substantially higher. This, in turn, may pose a challenge for gene therapy researchers. Interestingly, however in our liver-targeted therapeutic approach with rAAV2/8-ACE2, we found that increased hepatic ACE2 expression reduced hepatic level of profibrotic Ang II by more than 50% compared to those treated with a control vector that carried human serum albumin (rAAV2/8-HSA) [87]. A reduction of Ang II, which was accompanied by increases in hepatic levels of antifibrotic Ang-(1–7) peptide, resulted in a marked reduction in inflammatory cytokine expression, leading to a profound reduction in hepatic fibrosis in all three models (**Figure 2**) [87]. These studies with short-term animal models have been further validated to provide evidence that in long-term animal models of biliary fibrosis and fatty liver disease, which produce hepatic lesions more comparable to those seen in patients with such diseases, a single intraperitoneal injection of rAAV2/8-ACE2 caused a profound reduction in hepatic fibrosis (**Figure 3**). In marked contrast to other studies using

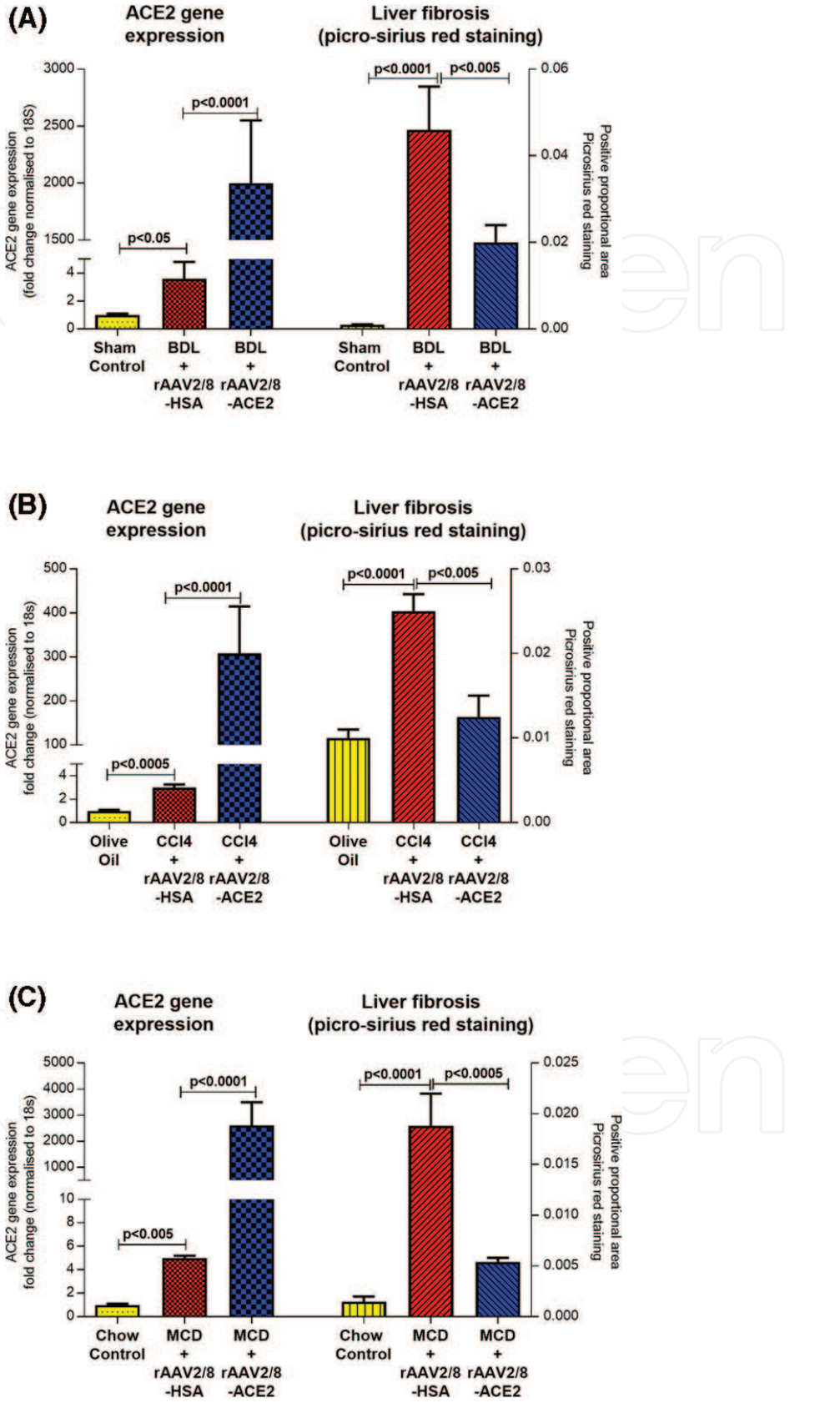

Figure 2. Hepatic ACE2 gene expression and fibrosis in three short-term models of liver fibrosis with rAAV2/8-ACE2 therapy. ACE2 gene expression (A–C) was significantly increased ($p < 0.0001$) in ACE2-treated diseased mice compared to control vector (rAAV2/8-HSA) injected diseased mice of BDL, CCl4, and MCD. As a result, rAAV2/8-ACE2 gene therapy has markedly reduced the liver fibrosis in each mouse model (BDL, CCl4, and MCD).

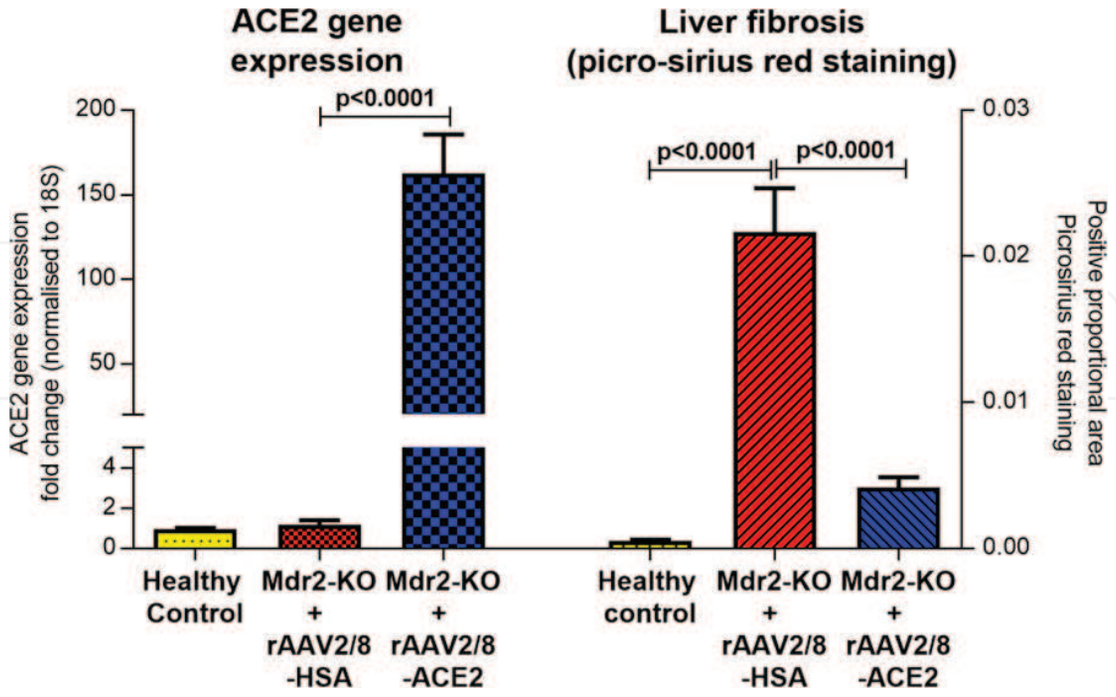

Figure 3. rAAV2/8-ACE2 therapy in Mdr2-KO mice with hepatic fibrosis. rAAV2/8-ACE2 gene therapy has markedly increased the ACE2 gene expression in Mdr2-KO mice, whereas liver fibrosis was significantly reduced by the therapy in ACE2-treated mice compared to the control vector-injected Mdr2-KO mice.

AAV vectors [93], we found that rAAV2/8-ACE2 reduced serum alanine transaminase (ALT) levels in diseased animals compared to those that received the control vector (rAAV2/8-HSA), suggesting that the vector itself is safe in the liver. Moreover, rAAV2/8-HSA (up to 10 days) or rAAV2/8-ACE2 (up to 24 weeks) vector injected into healthy mice produced no change in plasma ALT level, confirming that the vector itself is unlikely to cause liver injury [6, 87]. The schematic representation of molecular mechanism associated with ACE2 gene therapy using rAAV2/8 vector in hepatic fibrosis is shown in **Figure 4**.

Liver-targeted gene delivery using rAAV2/8 vector has shown to be therapeutically promising in adult liver, but their effects have not been extensively investigated in the immature liver. Although rAAV2/8 transduces neonatal mouse liver with high efficiency, the vector is not persistent in the liver and declines rapidly with liver growth [127]. Therefore, the successful use of rAAV2/8-mediated therapy to treat liver disease in early childhood may require readministration [128]. In line with this, another study demonstrated that the treatment of ornithine transcarbamylase (OTC)-deficient neonatal mice with AAV2/8-OTC therapy failed to protect mice from hyperammonemia in adulthood [129]. Thus, producing stable transduction in the developing liver remains one of the biggest challenges for liver-specific rAAV2/8 gene therapy, and readministration of vectors may be necessary to maintain therapeutic efficacy in adulthood after early neonatal treatment.

Although the AAV vectors employed for preclinical studies may be effective in human liver, it is important to select an AAV vector specific for human hepatocytes with enhanced transduction efficiency [6, 55]. Recently, two groups have proposed using humanized mice such as the immunosuppressed FRG ($Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}$) mouse model to identify the best rAAV serotype for liver-directed gene therapy [55, 130]. The studies in humanized

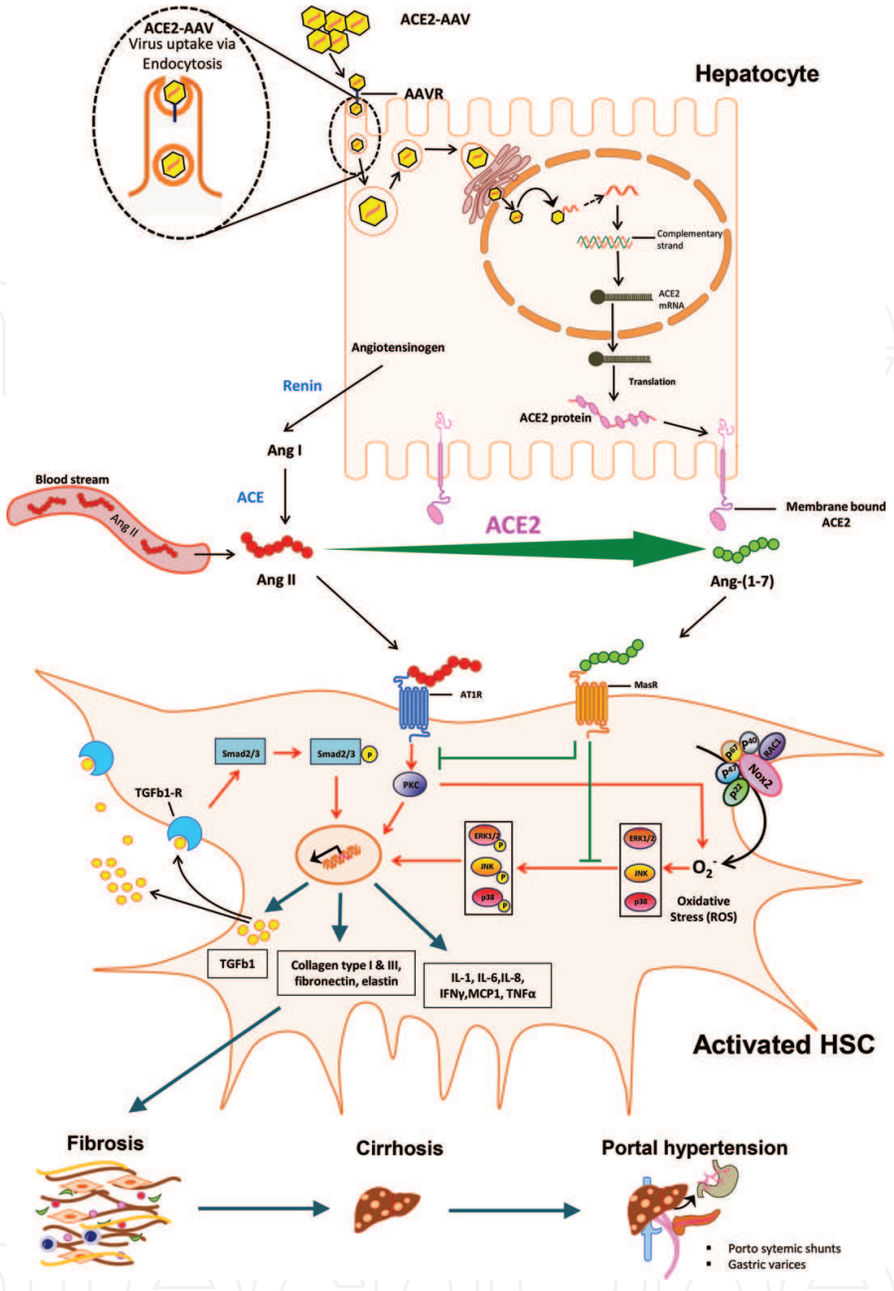

Figure 4. rAAV2/8-ACE2 uptake by hepatocytes and a cascade of events triggered by ACE2 protein in activated hepatic stellate cells (HSCs) during fibrosis. rAAV-ACE2 particles use AAV receptor (AAV-R) on hepatocyte membrane to enter the cytoplasm, followed by translocation into nucleus where uncoating and releasing of single-stranded viral genome occurs. The complementary strand will then be synthesized to transcribe ACE2. Membrane bound ACE2 protein has an exclusive role of cleaving potent profibrotic peptide angiotensin II (Ang II) to antifibrotic peptide angiotensin-1-7 (Ang-(1–7)). While a reduction in local Ang II levels leads to a significant reduction in the activation of its receptor, Ang II type 1 (AT1-R), Ang-(1–7) working through its receptor, Mas (Mas-R), inhibits the AT1-R activated downstream signaling such as PKC- and NADPH-mediated ROS production in activated HSCs. This in turn inhibits the phosphorylation of MAPKs such as ERK1/2, JNK, and p38, leading to a reduction in proinflammatory cytokines such as IL-1, IL-6, IL-8, IFNγ, MCP-1, and TNFα and profibrotic cytokine TGFβ1. A reduction in the activity of TGFβ1 leads to a reduction in phosphorylation of its transcription factors, Smad2/3, resulting in the inhibition of secretion of matrix proteins such as collagens and fibronectins. Thus, rAAV-ACE2 helps improving hepatic fibrosis and thus, intrahepatic vascular tone, leading to an improvement in portal hypertension. PKC, protein kinase C; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; IL, interleukin; IFNγ, interferon γ; MCP-1, monocyte chemotactic protein 1; TNFα, tumor necrosis factor α; TGFβ1, transforming growth factor-β1; ERK1/2, extracellular regulated kinase1/2; JNK, C-Jun N-terminal kinase.

mouse model repopulated with over 25% human hepatocytes allowed the researchers to identify human liver-specific AAV vectors such as LK-03 derived from capsid DNA-shuffled AAV library. This library was generated using 10 AAV capsid genes. LK-03, which is composed of five different parental AAV capsids, was able to transduce human primary hepatocytes at higher efficiency *in vitro* and in a hepatocellular carcinoma xenograft model *in vivo* when compared to AAV serotype 8 [55]. Wang and colleagues also reported a higher liver transduction level in FRG mice using capsid of AAVrh10, a clade E AAV derived from rhesus macaque, and AAV3B and have shown that AAV-LK-03 vectors may be superior to either AAV3B or AAV8 [131]. It is expected that researchers will increasingly use humanized animal models for diseases other than liver disease, which will allow them to identify novel variants of engineered AAV vectors, transduction efficiency, and immune reactions specific to the human tissue under investigation. Moreover, it has been reported that AAV3B-eGFP vector, which was able to cause liver-specific robust GFP expression in the livers of non-human primates, is significantly better than AAV8 with no apparent hepatotoxicity [132].

13. Conclusions

Much of preclinical studies which employed a diverse range of naturally occurring as well as engineered AAV vectors in the last decade provided ample evidence that therapeutic gene transfer certainly holds a great promise for patients with inherited disorders such as those that developed as a result of blood clotting factor deficiency and mutated retinal genes causing blindness. Moreover, it is now becoming clear that the findings of preclinical studies of non-inherited disorders suggest that clinical studies utilizing therapeutic gene transfer is feasible.

Currently active clinical trials in patients with inherited disorders using a diverse range of AAV vector types will be expected to provide valuable insights into the safety and efficacy of AAV vectors [133]. Since the FDA as well as the EU has now endorsed human gene therapy, there is every possibility that the volume of gene therapy research employing next-generation AAV vectors for both inherited and non-inherited disorders in both preclinical and clinical settings would be expected to increase in the coming years. Moreover, a rapidly evolving technology of AAV vector engineering and the use of humanized animal models would be a key for rapid translation of preclinical findings to clinical studies. The findings from our ongoing liver fibrosis/cirrhosis work using human liver-specific AAV-LK-03 vector in humanized FRG mice would be expected to provide valuable information before we commence clinical studies in patients with chronic liver disease.

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