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PROGRESS IN HEMATOLOGY

Current progress and future direction in the treatment for hemophilia

Advances in gene therapy for hemophilia: basis, current status, and future perspectives

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Abstract

Hemophilia is a congenital hemorrhagic disease caused by genetic abnormalities in coagulation factor VIII or factor IX. Current conventional therapy to prevent bleeding requires frequent intravenous injections of coagulation factor concentrates from early childhood. Accordingly, gene therapy for hemophilia remains an exciting future prospect for patients and their families, due to its potential to cure the disease through a one-time treatment. After a series of successes in basic research, recent clinical trials have demonstrated clear efficacy of gene therapy for hemophilia using adeno-associated virus (AAV) vectors. Although this is likely to alter the paradigm of hemophilia care in the near future, it will be important to overcome immune responses against AAV. Gene therapy for hemophilia cannot be given to patients with anti-AAV capsid-neutralizing antibodies, and cellular immunity with $CD8^+$ T cells should be controlled for sustained expression. Furthermore, long-term therapeutic effects should be closely observed because of the failure of the AAV vector genome to replicate during cell division. This review focuses on the basis of gene therapy, current successes of clinical trials, and the future direction of hemophilia gene therapy.

Keywords Gene therapy · Hemophilia · Adeno-associated virus vector

Introduction

Hemophilia is an X-linked congenital hemorrhagic disease caused by mutations in blood coagulation factor VIII (FVIII) or factor IX (FIX) genes (*F8* and *F9*, respectively). The life expectancy for hemophilia patients was extremely short in the past, but improvements in the quality of blood coagulation factor concentrates have raised the level to one not that different from that of healthy adult males [1]. Therefore, an enhanced quality of life (QOL) is a major goal of current treatment.

The most important factor to determine the QOL is a joint disorder (hemophilic arthropathy) caused by repeated joint bleeding [2]. To prevent this, regular replacement therapy is carried out in which a coagulation factor preparation is administered periodically from early childhood [3]. However, the half-life of the coagulation factor is very limited, so its frequent administration 1–3 times weekly is required for prophylactic replacement therapy [3]. Extended half-life formulations conjugated with an immunoglobulin Fc chain, albumin, and polyethylene glycol (PEG), or emicizumab, a bispecific antibody against FIX and coagulation factor X, have recently been developed [4], but they have not overcome the requirement for lifetime administration of the preparation. In addition, the nature of hemophilia as a hereditary disease causes distress regarding marriage and childbirth in genetic carriers.

To resolve the problems currently faced in hemophilia care, gene therapy is an attractive treatment approach that could provide long-term therapeutic effects with a single treatment. It may have an even greater impact in developing countries, where the use of factor concentrates is often limited. Indeed, only 25% of patients received appropriate treatment globally (World Federation of Hemophilia, https://www.wfh.org/). Here, I will review the basis, history, current state, and future direction of hemophilia gene therapy.

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Hemophilia as a suitable disorder for gene therapy

Traditional gene therapy aims to overcome a genetic problem involving a mutated gene by expressing a normal gene product without affecting the abnormal somatic gene. Hemophilia has long been considered an attractive candidate for gene therapy, because it is caused by a single genetic abnormality. Moreover, even a slight increase in blood coagulation factor levels has a therapeutic effect, and exact gene expression control is unnecessary.

The severity of hemophilia can be divided into severe (< 1%), moderate (1–5%), and mild (> 5%) depending on plasma coagulation factor levels. The risk of bleeding decreases dramatically even if levels rise by only 1–5%, and there is almost no chance of spontaneous joint bleeding if levels are 12% or more [5]. Furthermore, the treatment is not a thrombotic risk unless coagulation factor levels far exceed the normal range [6, 7].

Several animal models have been used to develop gene therapy for hemophilia [8]. As well as the gene targeting of gene-deficient mice, spontaneous hemophilia models in dogs and sheep have been used to confirm therapeutic effects. We previously developed a model of hemophilia A in pigs using gene targeting and cloning technologies [9]. Hemophilia pig is an attractive model to investigate the effects of human hemophilia drugs, because the porcine blood coagulation system is very similar to that of humans, and as hemophilia A pigs develop remarkable hemophilic arthropathy [9]. Preclinical research using larger animal models could resolve the known differences between mice and humans.

Basis of gene transduction

Current gene therapy typically involves the ectopic introduction of a sequence expressing a gene of interest into target cells. The strategies of gene therapy for hemophilia can be divided into two approaches: (1) the direct administration of a vector carrying a therapeutic gene in vivo and (2) the transplantation of transduced cells introducing a gene of interest ex vivo. Current clinical studies are mainly based on direct administration of the vector into the patient's body.

Transgene

Because *F8* cDNA exceeds 7 kb, packaging it into a viral vector is challenging. For this reason, the 4.5 kb B domain-deleted FVIII is used in hemophilia A gene therapy. The B domain is a site cleaved by the activation of FVIII that is not necessary for its function. In the case of FIX, an intronic sequence is often inserted into the cDNA, because the first intron of *F9* contains an expression control sequence [10]. Recent clinical trials have usually applied codon optimization to enhance protein translation. In particular, the codon-optimized *F8* transgene significantly enhanced the expression of FVIII [11]. Several gain-of-function *F9* mutations have been identified. Arginine at position 338 is highly conserved [12], and is important for factor X binding. An FIX substitution at R338A resulted in a threefold increase in factor X-binding activity [13]. A more potent hyperactive mutation involving a substitution at R338L is found in juvenile thrombophilia [14], in which the coagulation factor activity of R338L FIX (Padua mutation) is 5–10 times higher than that of the wild-type sequence.

Promoter

A promoter sequence is required, typically at the 5′ region, to express the coagulation factor from transduced cells. The binding of RNA polymerase and transcriptional factors to the promoter initiates transcription. Ubiquitous promoters including virus promoters have been used in the previous clinical trials, although more tissue-specific promoters are better suited to restrict unwanted and persistent transgene expression [15]. Recent hemophilia gene therapies have attempted to express the transgene in liver hepatocytes, where coagulation factors are physiologically produced. Therefore, promoter sequences derived from secreted proteins synthesized in hepatocytes, including α1 antitrypsin, thyroxine-binding globulin, or transthyretin, have been used to ectopically express coagulation factors in the liver. We and others previously improved the phenotype of hemophiliac mice by expressing coagulation factors in platelets using platelet-specific glycoprotein (GPIbα or GPIIb) promoters [16, 17]. We also found that the plasminogen activator inhibitor-1 promoter was suitable for transducing mesenchymal stromal cells for hemophilic arthropathy [18, 19].

Vectors

Carriers that introduce foreign genes into cells are known as vectors. Gene transfer often involves the use of viral vectors because of their high efficiency at transducing target genes. Adeno-associated virus (AAV) vectors have mainly been used in clinical trials for hemophilia to directly administer the vector, while lentiviral (LV) vectors are often used for the ex vivo gene transduction of cells.

AAV vectors

Current gene therapy clinical trials for hemophilia use AAV vector to transduce coagulation factor genes directly into liver hepatocytes [20]. AAV is a single-stranded DNA virus

of the Parvovirus family that is not associated with any symptoms or disease [21]. AAV vectors have many advantages over other vectors. First, viral-derived DNA is present in the episome after transduction, and is rarely incorporated into chromosomal DNA (Fig. 1). Second, vector safety seems to be high because of a lack of pathogenicity and low immunogenicity, compared with adenovirus vectors. Third, AAVs can introduce the target gene into quiescent cells such as those of the central nervous system, muscle, and adult liver. AAV has a diverse range of serotypes, each with a distinct organ/cell tropism [21]. In particular, serotype 8 (AAV8) allows high gene expression in the liver, even when it is injected intravenously. However, differences in transduction efficacies between mice and humans should be taken into account [22]. Some disadvantages include a limitation of 4.5–5.0 kb on the gene length that can be inserted between the ITRs, that gene transfer cannot be performed if neutralizing antibodies exist, and the fact that the AAV genome is diluted by cell division.

Wild-type AAV has a hairpin structure known as the inverted terminal repeat (ITR) at both ends of the Rep–Cap sequence, which is necessary for virus replication and packaging (Fig. 2). To express the target protein using the AAV

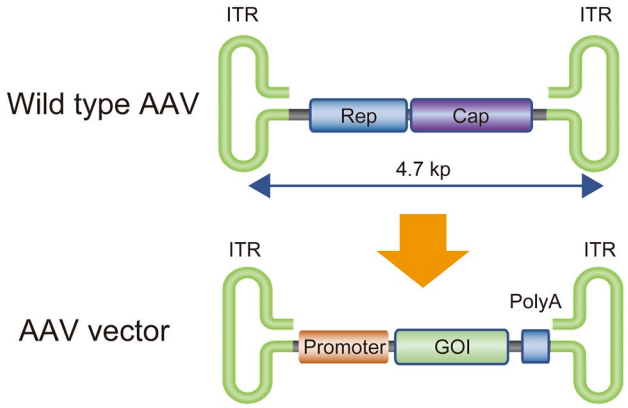

Fig. 2 Structure of an AAV vector. Wild-type AAV is a single-stranded DNA virus-encoding two genes. The rep gene is required for replication and virion assembly, while the cap gene encodes three proteins that assemble to form the viral capsid. AAV vectors carry a therapeutic gene of interest (GOI) under the control of an appropriate promoter, instead of rep and cap genes

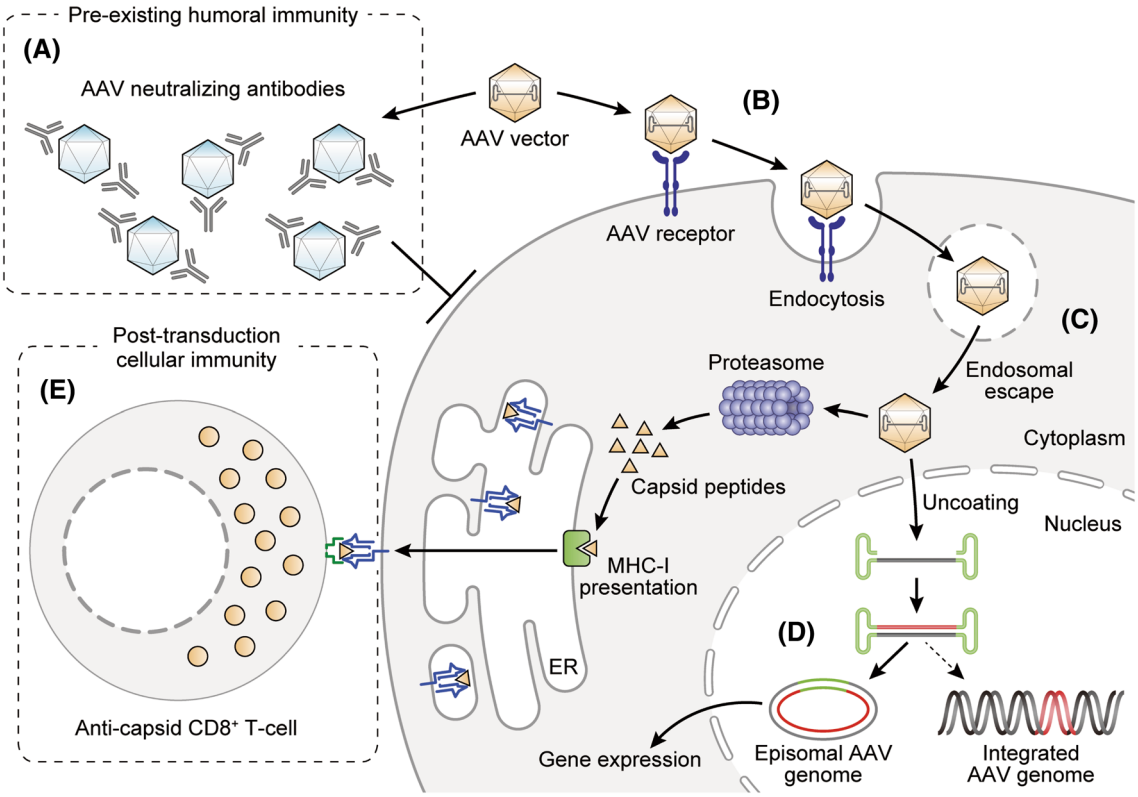

Fig. 1 Transduction, cell entry, and trafficking of the AAV vector: **a** anti-AAV capsid-neutralizing antibodies prevent cell entry, **b** AAV vector enters the cell by endocytosis with receptor binding, **c** vector escapes from the endosome, and enters the nucleus, **d** AAV vector predominantly exists as a non-replicating episome, with a low number of random integrations, and **e** degraded capsid-derived peptides are expressed by MHC class I molecules on transduced hepatocytes, and the cells may be eliminated by $CD8^+$ T-cell immunity

vector system, a promoter sequence and target gene of interest are inserted in place of Rep and Cap sequences (Fig. 2).

In the clinical trial reported by Nathwani et al., a Good Manufacturing Practice level vector was obtained by plasmid transfection into HEK 293 cells using the helper-free method (Fig. 3). To obtain 2×10^{15} vg of AAV vector, a total of 432 independent 10-stack flasks were required [23]. AAV vectors can also be produced from the baculovirus system using insect Sf9 cells (Fig. 3) [24]. This baculovirus production system is capable of obtaining high quantities of vector, and was used in production of the first approved gene therapy product, Glybera [25].

LV vectors

LV vectors are a subclass of retroviral vector that more efficiently transduce quiescent cells, including hematopoietic stem cells. Because proviruses can integrate into the chromosomal DNA of transduced cells, permanent gene expression can be obtained. Therefore, these vectors are suitable for cell therapy. Indeed, chimeric antigen receptor T cells generated by the transduction of LVs for the treatment of hematopoietic malignancies (KYMRIAH®), and hematopoietic stem cell gene therapy targeting adenosine deaminase deficiency (Strimvelis®) have been approved by the US Food and Drug Administration and the European Medicines Agency [26]. In an early gene therapy clinical trial, leukemic transformation by provirus integration of a retroviral vector in the vicinity of *LMO-2* was problematic in the treatment of severe combined immune deficiency [27]. However, recent vector design reduces the risk of transformation by deleting the promoter activity of a long terminal repeat, leading to self-inactivation. LVs also have a high safety level, because they integrate less frequently into transcription start sites compared with retroviral vectors [28]. Bioverativ, a Sanofi company, plans to develop LV treatment for hemophilia, in collaboration with San Raffaele-TIGET (https://www.bioverativ.com/research-pipeline/our-pipeline.aspx).

Non-viral vectors

Naked nucleic acid can be directly introduced into the mouse liver by the hydrodynamic delivery method. This is capable of introducing large genes such as full-length *F8*. For example, an improvement of the bleeding tendency of mice with hemophilia A was reported using the PiggyBac transposon and this procedure [29]. However, there is a need to solve the problem of invasiveness for its use in clinical practice.

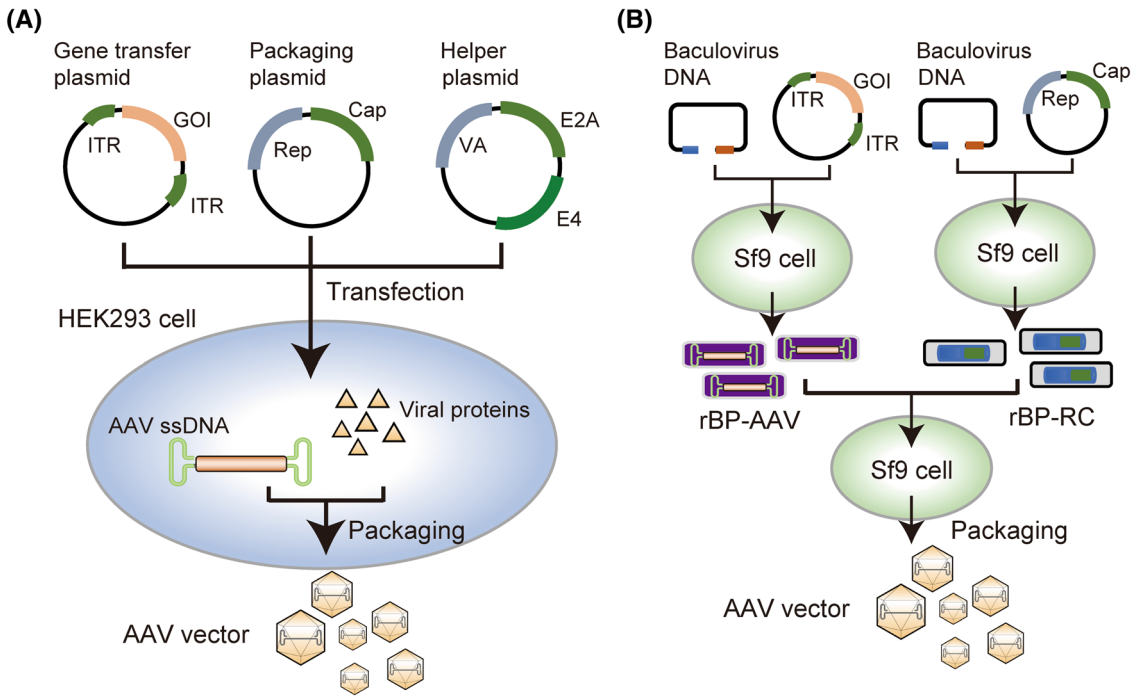

Fig. 3 Two AAV vector production systems. **a** Helper-free system to produce AAV vectors without the existence of a helper virus, including adenoviruses or herpesviruses. Specific adenovirus gene products require virus replication. The gene transfer plasmid containing the therapeutic gene of interest (GOI) and the rep and capsid protein (rep/cap) expression plasmid are simultaneously introduced into HEK293 cells by transfection. The AAV vector can then be produced from the cells. **b** Baculovirus expression system to produce the AAV vector. Baculovirus containing a GOI or rep/cap of AAV is produced from transfection into Sf9 cells. These cells are further infected with two baculoviruses to produce the AAV vectors

Recently, the systemic delivery of *F9* mRNA by lipid nanoparticles was reported in the treatment of a mouse model of hemophilia B [30]. This method requires repeat administration, but it could be used as an alternative to replacement therapy.

Lessons learned from early clinical trials using AAV vectors

Hemophilia gene therapy utilizing AAV vectors has achieved promising results in clinical trials in the almost 20 years that have passed since the first human trial. The challenges faced in overcoming the failures of the initial clinical trials have led to the current success of gene therapy.

Initial clinical trials for the treatment of hemophiliac patients used the first-generation serotype AAV2. Kay et al. reported the intramuscular injection of an AAV2 vector to three hemophilia B patients as a Phase I study [31]. However, FIX expression did not reach necessary treatment levels, despite the fact that local FIX expression persisted at the injection site for at least 3.7 years after treatment [32]. In another study, liver-directed gene therapy was performed to express coagulation factors by administering AAV2 into the hepatic artery [33]. Elevated FIX activity was obtained in the high-dose vector group (2×10^{12} vg/kg), but its expression was decreased according to increases in liver transaminases [33]. This could be explained by the fact that degraded capsid-derived peptides are expressed by major histocompatibility complex (MHC) class I molecules on transduced hepatocytes and that these cells are eliminated by $CD8^+$ T-cell immunity [33]. Therefore, for long-term expression of the coagulation factor, $CD8^+$ T-cell-mediated cellular immunity should be overcome.

The discovery of various new viral serotypes, especially those with hepatic specificity, provided additional approaches for efficient hemophilia gene therapy. A newer serotype (AAV8) discovered from monkeys can efficiently transduce genes of interest to the liver [34], and the efficacy of liver transduction was unchanged following portal vein administration or intravenous administration [35]. Nathwani et al. reported the success of hemophilia B Phase 1/2 clinical studies using the AAV 8 vector in 2011 [36]. Intravenous injection of AAV8 vectors allows the production of coagulation factor from the liver. During a follow-up period of up to 3.5 years, no important adverse effects were reported, and the therapy was shown to have long-term efficacy [37]. Liver dysfunction, reflecting the reaction of $CD8^+$ cytotoxic T cells, occurred in the high-dose vector group (2×10^{12} vg/kg), but the transient administration of high-dose corticosteroids was successful in controlling this [36].

In summary, current gene therapies for hemophilia are focusing on the intravenous administration of AAV vectors directed to the liver, with cellular immunity against the capsid peptide being controlled by the administration of a high dose of corticosteroid.

Recent clinical trials

Successful treatment of the hemophilia B gene by Nathwani et al. suggested that the cure for hemophilia will soon become a reality [36, 37]. Several pharmaceutical companies are currently working on the development of gene therapies, and promising results have been reported. Table 1 summarizes the current state of clinical trials for hemophilia gene therapy.

Hemophilia B

A phase 1/2 study for hemophilia B patients conducted by Spark Therapeutic Inc. reported the most promising results [38]. Ten hemophilia B patients were treated with a single intravenous injection of new AAV serotype Spark-100 [38]. This clinical trial had two important attributes: (1) the use of highly active mutant FIX (Padua mutation) and (2) a newly-developed serotype. As mentioned above, the activity of the Padua mutation can be expected to be eight times higher than that of wild type. FIX activities of around 30% were obtained with a lower vector dose (5×10^{11} vg/kg), compared with the AAV8 vector from a previous clinical trial [36]. During the trial, most patients did not require the administration of coagulation factor concentrates. More importantly, only two patients were required to administer corticosteroid to treat liver injury from cytotoxic T cells [38]. In another study, UniQure Inc. administered AAV 5 vector (AMT-060) expressing wild-type FIX to 10 patients at a vector dose of $0.5–2\times10^{13}$ vg/kg [39]. This increased coagulation factor activity by around 4–7%, while annualized factor concentrate use was reduced by 73–81% [39]. UniQure Inc. published a press release to change wild-type FIX (AMT-060) to the Padua mutation (AMT-061) in a Phase 3 trial (http://www.uniqure.com/gene-therapy/hemophilia.php). Shire plc. conducted a clinical trial on humans using the AAV8 vector (BAX335) carrying the Padua mutation; however, the expression of clotting factor activity was only transient and the test has now been discontinued [40].

Hemophilia A

Although the normal blood molar concentration of FVIII is considerably lower than that of FIX, the expression efficiency of FVIII by AAV in vivo has been poor compared with that of FIX [41]. One possible reason is that the large size of *F8*, even in B domain-deleted FVIII, cannot be effectively incorporated into the AAV vector. Its large

Table 1 Summary of current gene therapy clinical trials for hemophilia

Identifier	Serotype	Transgene	Ages	Sponsor/University	Locations	Status	References
NCT00979238	AAV8	hFIXco	18 years or older	University College London, St. Jude Children's Research Hospital	United States United Kingdom	Active, not recruiting	[36, 37]
NCT01687608	AAV8	hFIX Padua (R338L) co	18–75 years	Shire plc	United States	Active, not recruiting	[40]
NCT02484092	Srpark100	hFIX Padua (R338L) co	18 years or older	Spark Therapeutics (Pfizer Inc.)	United States Australia Canada	Active, not recruiting	[38]
NCT02971969	rAAVrh-10	hFIXco	18 years or older	Dimension	United States	Completed	–
NCT02396342	AAV5	hFIXco	18 years or older	UniQure	Denmark Germany Netherlands	Active, not recruiting	[39]
NCT02695160	AAV6	Zn finger nuclease	18 years or older	Sangamo Therapeutics	United States	Recruiting	–
NCT02576795	AAV5	BDD-FVIII	18 years or older	Bio Marin Pharmaceuticals		Active, not recruiting	[42]
NCT03003533	Spark200	BDD-FVIII	18 years or older	Spark Therapeutics	United States Australia Canada	Recruiting	–
NCT03001830	AAV8	BDD-FVIII-V3	18 years or older	University College London	United States United Kingdom	Recruiting	–
NCT03061201	AAV6	BDD-FVIII	18 years or older	Sangamo Therapeutics	United States	Recruiting	–

co codon-optimized, *hFIXco* human coagulation factor IX

size also means that other DNA sequences, including the promoter and poly A, must be limited to up to 300–500 bp. The significant enhancement of FVIII production in vivo by codon optimization in the treatment model of macaques may facilitate the development of gene therapy for hemophilia A [11]. BioMarin Pharmaceutical Inc. reported a successful Phase 1/2 study for hemophilia patients treated with an AAV5 vector [42]. Nine patients with severe hemophilia A were treated with an AAV5 vector harboring B domain-deleted FVIII at a low dose (6×10^{12} vg/kg), medium dose (2×10^{13} vg/kg), and high dose (6×10^{13} vg/kg) [42]. Following vector transfer, FVIII levels in the blood increased by 12–237%, and annual bleeding and the use of coagulation factor also drastically decreased in the high-dose group. Of note, six of seven patients in the high-dose group maintained their coagulation factor levels of at least 50% [42]. Corticosteroids to prevent liver toxicity by the $CD8^+$ T-cell response were administered to all high-dose patients.

The success of gene therapy for hemophilia A has a greater impact, because the number of target patients is higher than those with hemophilia B, and the half-life prolongation of the FVIII preparation by fusion with Fc or PEG is not as long as expected in a clinical situation. Other companies such as Spark Therapeutic Inc., Sangamo Therapeutics Inc., and Shire plc. are also developing hemophilia A gene therapy.

Future challenges

Anti-AAV capsid-neutralizing antibody

Neutralizing antibodies against the AAV capsid, probably caused by a previous latent infection, could prevent transduction via intravenous administration of the AAV vector in vivo [41]. Current hemophilia gene therapy, therefore, targets patients who are negative for anti-AAV neutralizing antibodies. The prevalence of anti-AAV neutralizing antibodies differs among reports, but the overall prevalence is thought to be 30–50% [43]. However, it should be noted that laboratory tests for its detection have not been standardized. We previously examined its prevalence in Japan, and found it to be 32.9–37.6 and 28.8–35.6% in the general population and in hemophilia patients, respectively [44]. Younger individuals have a lower prevalence [44], and the prevalence may also vary among countries. One study found that nearly, all Chinese individuals carried neutralizing antibodies against AAV2 and AAV8, while the prevalence of anti-AAV5 antibodies was as low as 40% [45]. In addition, patients cannot receive repeated intravenous injections of the same AAV serotype, because AAV vector treatment leads to the emergence of high titers of neutralizing antibodies.

To overcome the inhibition of transduction by neutralizing antibodies, several approaches including capsid

modification, chemical modification, pharmacological inhibition, and plasmapheresis have been reported [43]. The simplest approach is the selection of other AAV serotypes, with UniQure Inc. reporting the possibility of re-administering the AAV vector by changing its serotype [46]. In addition, work has focused on the creation of AAV variants with resistance to neutralizing antibodies [43]. However, the cross reactivity of antibodies to other serotypes was reported to exceed 50% [47]. Structural analysis revealed that antibody recognition sites on AAVs might be evolutionarily conserved [48], and the generation of an AAV variant by a structure-guided evolutionary approach effectively evaded polyclonal anti-AAV neutralizing serum [48]. Moreover, exosome-enveloped AAV vectors were shown to evade pre-existing neutralizing antibodies [49]. We developed an administration method that enables AAV gene therapy in macaques, even in the presence of neutralizing antibodies [50]. After the temporal halting of blood flow using a balloon in the portal vein, we flushed the blood with saline and administered the vector to minimize interactions between blood and the vector [50]. An alternative approach used an empty capsid to adsorb neutralizing antibodies as "decay", even at high titers, thus overcoming their inhibitory effect [51].

Long-term expression and safety

Recent human clinical trials using AAV vectors reported a continuation of the therapeutic effect and no major adverse effects other than transient transaminitis. We confirmed the maintenance of coagulation factor levels and safety for up to 10 years in cynomolgus monkeys following a single administration of an AAV vector (unpublished data). However, AAV vectors predominantly exist in the nucleus as non-replicating episomes [52], and transgene expression would be expected to be gradually lost over time because of the failure of the vector genome to replicate with cell division. Long-term therapeutic effects should, therefore, be carefully monitored.

The possible risk of genotoxicity has been indicated in a mouse model of AAV-mediated gene therapy [53]. A low number of random integrations occur after AAV gene transduction [52], although the chances of insertional mutagenesis are greatly reduced compared with LV and retroviral vectors. The previous work has reported relationships between AAV vector transduction and hepatocellular carcinoma (HCC) in mice (reviewed in [54]). The AAV vector genome was shown to integrate into the *Rian* locus and enhance *Rtl1* and microRNA expression, leading to HCC development [55]. Genotoxicity is known to be influenced by the age, dose, serotype, promoter, and transgene [55]. Although the current safety profile of AAV vectors in human clinical applications remains faultless, long-term adverse events should be carefully monitored in the future.

Coagulation factor inhibitors

Because the immune system of hemophiliacs, especially those with severe hemophilia A, recognizes the administered coagulation factor as a foreign substance, neutralizing antibodies (inhibitors) against coagulation factor are produced in 20–30% of patients [56]. The emergence of inhibitors mainly occurs during early administrations of the coagulation factor concentrates [56]. Therefore, current clinical trials have enrolled hemophilia patients with a history of multiple administrations of coagulation factor preparations. Interestingly, inhibitor development may be suppressed by expressing the coagulation factor in the liver through AAV vectors in mice and dogs [57, 58]. Immune tolerance induction therapy is applied as first treatment for patients with inhibitors in clinical practice [56]. If inhibitor disappearance is confirmed in liver-directed gene therapy, this could become an alternative approach to inhibitor control.

Genome-editing approach

The ideal gene therapy would repair the abnormal gene at the DNA level. As genome-editing technologies are significantly improving, future applications of the technology are anticipated. Genome editing could theoretically be performed at the embryonic stage [59], but germline genome editing is currently not acceptable because of ethical and safety concerns. Therefore, genome-editing components should be delivered to somatic cells for the treatment of genetic diseases.

Engineered nucleases for genome editing

Genome editing has been realized through the development of artificial nucleases that cause double-strand breaks (DSBs) at specific DNA sites. These include first-generation zinc-finger nucleases (ZFNs), second-generation TAL effector nucleases (TALENs), and third-generation clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas)9 (Fig. 4). ZFN and TALEN systems have a *Fok*I enzyme at the 3′ end of their DNA binding which forms a dimer to cleave DNA, while the Cas9 protein interacts with and cleaves a specific DNA site recognized by guide RNA (gRNA) close to a protospacer adjacent motif (Fig. 4). CRISPR/Cas9 has been identified as the adaptive immune system against phage infection in prokaryotes [60]. It can induce DSBs at an arbitrary DNA site only by changing the gRNA sequence, and has been used in various fields as a breakthrough technology to modify

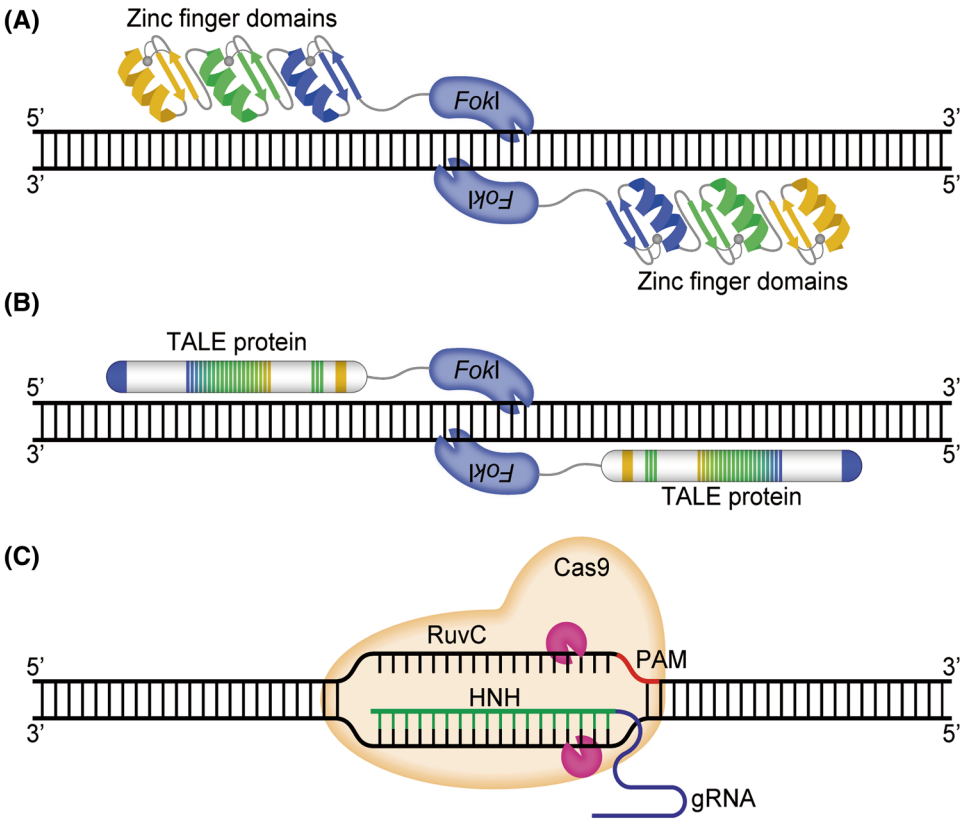

Fig. 4 Schematic of the engineered nucleases for genome editing. **a** Zinc-finger nuclease (ZFN) dimer bound to DNA. Each monomer comprises three zinc-finger domains fused to the cleavage domain of *Fok*I. **b** Transcription activator-like effector nuclease (TALEN) dimer bound to DNA. DNA-binding domains consist of a variable number of amino acids (TALE protein), followed by the cleavage domain of *Fok*I. **c** CRISPR/Cas9 system. Cas9 protein interacts with and cleaves a specific DNA site recognized by guide RNA (gRNA) close to a protospacer adjacent motif (PAM)

genomes. Several Cas9 orthologs have also been identified in various prokaryotes [60].

Mechanisms to modify DSBs

After DSBs, genome editing utilizes the DNA repair pathway. DSB induction by nucleases mainly stimulates two DNA repair pathways: non-homologous end joining (NHEJ) and homologous-directed repair (HDR) (Fig. 5). NHEJ is the main DSB repair mechanism, involving mutations and insertions at the DSB site, resulting in gene product frameshifts, and the disruption of gene expression. HDR occurs in the S/G_2 phase of the cell cycle, and can repair (or modify) DNA using additional DNA as template. Because the efficacy of DNA modification using HDR is extremely low, the knock-in technique for efficiently inserting a target gene into the DSB site has been developed (Fig. 5). Yamamoto et al. developed a knock-in technique based on microhomology end coupling known as precise integration into target chromosome (Fig. 5) [61]. Furthermore, the homology-independent targeted integration technique has also been reported, in which the knock-in direction is guaranteed by inserting the Cas9 recognition sequence in opposite directions at both ends of the sequence (Fig. 5) [62]. These knock-in techniques are important strategies for editing the genome to treat genetic diseases.

Application of AAV vectors for genome editing

The length of DNA that can be inserted into AAV vectors is limited, so relatively short ZFNs are easy to mount (1.2 kb). High et al. succeeded in improving the bleeding tendency of hemophilia B mice by inducing a DSB into intron 1 of *F9* with a ZFN and inserting the cDNA of *F9* exons 2–8 [63]. In another study, a DSB was introduced into the albumin locus, in which target cDNA sequences for the treatment of several diseases including hemophilia A, hemophilia B, and mucopolysaccharidoses, were inserted [64]. This technology has already been launched by Sangamo Therapeutics Inc. as a phase 1/2 trial in humans using AAV6, and a patient with mucopolysaccharidosis has been already treated (https://investor.sangamo.com/press-releases, press released at November 2017). This is the first example of in vivo human genome editing.

Adeno-associated virus vectors can also deliver CRISPR/Cas9 tools. Because the length of the most common Cas9, deriving from *Streptococcus pyrogens* (SpCas9), exceeds 4 kb, it cannot be inserted into one AAV vector together with a gRNA expression cassette. However, *Staphylococcus aureus* Cas 9 (SaCas9) is 1 kb shorter than SpCas9, so can easily be incorporated into an AAV vector [65]. For in vivo genome editing, it was reported that cholesterol levels were reduced by disrupting mouse *Pcsk9* by SaCas9 [65].

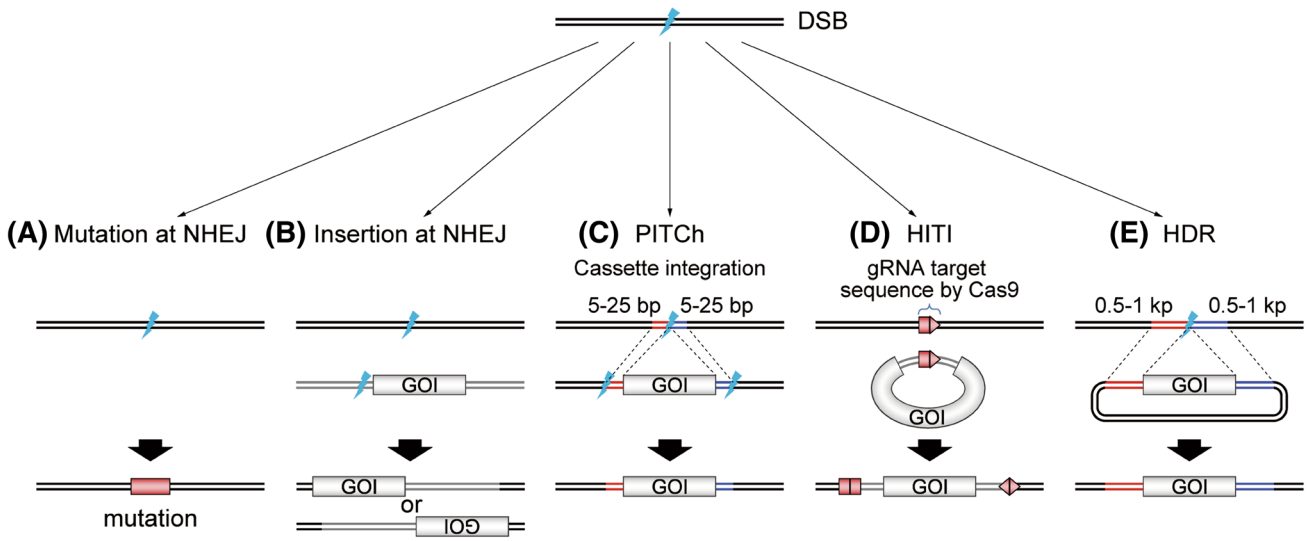

Fig. 5 Several pathways modify genome sequences following double-strand breaks (DSBs). **a** Non-homologous end joining (NHEJ) causes gene disruption in the absence of exogenous DNA. **b** Linear DNA can be inserted at the NHEJ site, though the direction of gene of interest (GOI) cannot be ensured. **c** Precise integration into target chromosomes (PITCh) system is a knock-in strategy using microhomology-mediated end joining (MMEJ). The addition of 5–25 bp microhomology regions enables precise sequence insertion at DSBs. **d** Homology-independent targeted integration (HITI) is an NHEJ-mediated knock-in strategy. The recognition site of gRNA is inserted into target DNA in the reverse direction to ensure the direction of GOI. **e** Homology-directed repair (HDR) corrects the original DNA sequence using donor DNA as a template

Moreover, a mouse model of Duchenne muscular dystrophy has been treated by exon skipping [66–68], while ornithine transcarbamylase deficiency, an abnormality of the urea cycle, was treated by administering two AAV vectors [69]. We also found that Cas9 could be expressed in almost all liver parenchymal cells and that the bleeding tendency in hemophilia B mice was improved by inserting cDNA using the knock-in technique [70]. Of note, the genome-editing approach could treat neonate mice with hemophilia B, a situation that cannot be achieved using conventional gene therapy with an AAV vector. A major advantage of genome editing is that its therapeutic effects will persist even if the AAV genomes are diluted through cell division [70]. Other improvements to reduce the off-target effects of Cas9 by transient expression include liposomal transfer of *Cas9* mRNA [71]. Additional investigations are required to further reduce off-target effects and to increase the efficacy of cDNA insertion or HDR for the treatment of genetic diseases.

Conclusion

Recent successes of gene therapy for hemophilia indicate the possibility of curing the disease, and will lead to a paradigm shift in its treatment. The liver-directed strategy for hemophilia gene therapy could be applied to other congenital metabolic diseases including ornithine transcarbamylase deficiency and phenylketonuria. Suitable gene therapy for hemophilia products is expected to be commercially available within the next decade, and could provide an alternative to coagulation factor preparations. However, a number of issues remain to be resolved, such as variable treatment effects, anti-AAV capsid-neutralizing antibodies, and its indication for children. Moreover, a system for the long-term observation of treated patients is required to confirm extended safety and therapeutic effects.

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Compliance with ethical standards

Conflict of interest T.O. received research support from Bayer, Daiichi Sankyo, Novo Nordisk, and CSL Behring outside of this study.

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