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INVITED REVIEW

Gene therapy for hemophilia

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Summary. Hemophilia A and B are X-linked monogenic disorders resulting from deficiencies of factor VIII and FIX, respectively. Purified clotting factor concentrates are currently intravenously administered to treat hemophilia, but this treatment is non-curative. Therefore, gene-based therapies for hemophilia have been developed to achieve sustained high levels of clotting factor expression to correct the clinical phenotype. Over the past two decades, different types of viral and non-viral gene delivery systems have been explored for hemophilia gene therapy research with a variety of target cells, particularly hepatocytes, hematopoietic stem cells, skeletal muscle cells, and endothelial cells. Lentiviral and adeno-associated virus (AAV)-based vectors are among the most promising vectors for hemophilia gene therapy. In preclinical hemophilia A and B animal models, the bleeding phenotype was corrected with these vectors. Some of these promising preclinical results prompted clinical translation to patients suffering from a severe hemophilic phenotype. These patients receiving gene therapy with AAV vectors showed long-term expression of therapeutic FIX levels, which is a major step forwards in this field. Nevertheless, the levels were insufficient to prevent trauma or injury-induced bleeding episodes. Another challenge that remains is the possible immune destruction of gene-modified cells by effector T cells, which are directed against the AAV vector antigens. It is therefore important to continuously improve the current gene therapy approaches to ultimately establish a real cure for hemophilia.

Keywords: adeno-associated virus, factor IX, factor VIII, gene therapy, hemophilia, lentiviral.

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Introduction

Hemophilia A and B, both of which are X-linked congenital bleeding disorders, affect ~ 400 000 individuals worldwide, according to the World Federation of Hemophilia [1]. The disorder is characterized by deficiencies of, respectively, factor VIII and FIX, which are essential blood coagulation factors. Therefore, patients suffering from hemophilia have frequent spontaneous bleeding episodes, but they also suffer from chronic damage to joints, muscles, and soft tissues. These symptoms may progress to chronic synovitis, crippling arthropathy, and physical disability. If intracranial hemorrhage occurs, the bleeding can be fatal. Hemophilia A occurs in one in 5000 male births, and affects almost 80–85% of hemophilic patients, whereas hemophilia B affects the remaining 15%, and its occurrence is one in 30 000 male births [2–4]. The levels of clotting factors in the plasma determine the hemophilia category: mild, moderate, or severe. In mild hemophilia, clotting factors are present in the blood at 5–50% of normal levels. It is generally diagnosed only when patients have bleeding problems after serious injury or surgery, but, when a familial history is present, patients will be tested at an early age. In moderate cases, the circulating levels of clotting factor are between 1% and 5% of normal. Patients not only bleed after injuries, but also have some 'spontaneous' bleeding episodes, without an obvious cause. In severe hemophilia, patients have < 1% clotting factor in their blood, which causes them to have recurrent spontaneous bleeding episodes, besides their problems with normal injuries. Current hemophilia treatment consists of parenterally administered plasma-derived or recombinant clotting factor concentrates, also known as protein substitution therapy (PST). Patients benefited significantly from PST, as it greatly improves their quality of life and prolongs life-expectancy. Nevertheless, there are limitations. First, the treatment does not constitute a cure, so patients can still have bleeding episodes or chronic joint damage. Second, the clotting factors have a short half-life, which necessitates repeated infusion of FIX or FVIII prophylactically to maintain therapeutic levels [5]. Third, PST is prohibitively expensive [6], limiting its accessibility mainly to patients in the developed world. Consequently,

there is a substantial unmet need to provide quality treatment to patients suffering from hemophilia in developing countries. Finally, in some patients, neutralizing antibodies (clinically referred to as 'inhibitors') against the injected FVIII or FIX develop, after which treatment is no longer effective. Because of these drawbacks of PST, scientific interest is shifting to a gene-based approach for hemophilia treatment [4].

Hemophilia is a monogenetic hereditary disorder, and is therefore an interesting target for gene therapy, especially as the genetics of the disease are well understood. If a functional copy of the FVIII or FIX gene can be introduced into a target cell via gene therapy, this may provide a cure, eliminating the repeated use of PST. In hemophilia treatment, increasing circulating plasma clotting factor levels to above a threshold of 1% of normal is sufficient to obtain a prophylactic therapeutic effect, which reduces the risk of mortality and morbidity. If gene therapy could slightly increase the clotting factor levels, it could significantly improve the clinical phenotype. Furthermore, the circulating clotting factor levels and bleeding frequency are clear-cut clinical endpoints, which allows for easy determination of the therapeutic efficacy of gene therapy. For preclinical research on gene therapy in hemophilia, several well-validated small and large hemophilic animal models are available.

To improve the clinical phenotype of hemophilia, prolonged high-level expression of clotting factors is needed. Gene therapy aims to achieve this by using two complementary strategies: (i) using integrating (viral or nonviral) vectors to introduce the clotting factor genes into stem cells, which express the genes in their progeny, thereby causing long-lasting expression; and (ii) introducing the genes into long-lived non-dividing cells, such as hepatocytes or skeletal muscle cells. Important to the latter is the persistence of the genes as episomal DNA or their integration into the target cell genome. Although clotting factors are normally expressed in the liver, making it an obvious target for gene therapy of hemophilia, it is important to also consider alternative, ectopic target tissues, particularly for those patients with underlying iatrogenic liver disease and comorbid conditions (e.g. viral hepatitis).

Both in vivo and ex vivo gene therapy approaches have been considered for hemophilia, each with their own advantages and limitations. In vivo gene therapy allows for the in situ genetic modification of the appropriate target cells, whereas, in ex vivo gene therapy, the target cells are first isolated and genetically modified in vitro. Ex vivo gene therapy obviates concerns associated with vector-associated adaptive and innate immune responses, which is a limiting factor following in vivo gene therapy. However, long-term persistence and efficient in vivo engraftment following transplantation of gene-modified cells can be challenging [7,8], and may require some level of preconditioning [9].

Several comprehensive reviews on gene therapy for hemophilia are available [1–4,10]. The current review focuses on a discussion of the recent developments in this field and the outstanding questions that need to be addressed to achieve a genuine cure for both hemophilia A and B.

Retroviral and lentiviral vectors

Liver-directed gene transfer

FVIII and FIX synthesis primarily takes place in the liver, from where the proteins can easily enter the bloodstream. This makes hepatocytes an eligible target for gene therapy in hemophilia. Additionally, the post-translational modifications needed to process FVIII and FIX resemble the normal pathways in hepatocytes. Mingozzi *et al.* [11] demonstrated that hepatic gene delivery could result in immune tolerance towards the transgene product (FIX). This may be because of the induction of regulatory T cells, which can suppress the formation of antibodies against the product of the introduced transgene [12,13]. This finding has important implications for further research into sustained circulating clotting factor levels after gene therapy.

To treat hemophilia with gene therapy, different vectors can be used. Among the first vectors to be considered were Moloney murine leukemia virus-derived vectors [14]. These are γ-retroviral vectors that are able to transduce several different target cell types, which is only possible when the cells are actively dividing. We previously showed that hepatic gene delivery in neonatal hemophilia A mice with γ-retroviral vector-based gene therapy led to a phenotypic correction of the disorder [15]. In neonatal dogs with either hemophilia A or hemophilia B, efficient γ-retroviral transduction has also been achieved [16,17]. Surprisingly, long-term FVIII expression was obtained in adult rabbits treated by gene therapy with γ-retroviral vectors containing FVIII. The exact FVIII-producing cell was not clearly defined, however. This prompted a phase I clinical trial in patients with severe hemophilia A. Patients tolerated the treatment well; however, FVIII activity was only slightly increased in a few cases, and this expression was transient [18]. As retroviral vectors only transduce dividing cells, this could explain the lack of efficacy in this trial.

Lentiviral vectors resemble γ-retroviral vectors, as both are able to integrate into the target genome efficiently [19], but they do not need cells to be actively dividing. Consequently, they are able to transduce quiescent hepatocytes in vivo, resulting in relatively efficient gene transfer in the liver after systemic or local gene delivery. In addition to hepatocytes, antigen-presenting cells (APCs) are abundantly present in the liver microenvironment. Ectopic expression of FVIII or FIX in these APCs should be avoided, as it increases the risk of an immune

response against FVIII or FIX, thereby inhibiting long-term expression [20]. This often occurs when non-specific promoters are used, causing the production of antibodies against clotting factors. In contrast, the use of a hepatocyte-specific promoter resulted in a lower immunologic risk and long-term FIX expression instead [21,22]. The induction of $CD4^+CD25^+Foxp3^+$ regulatory T cells might be responsible for the immune tolerance visible after hepatic FVIII or FIX delivery [12,13]. It is also possible that cytotoxic T-cell responses against the transduced cells develop, which could lead to their elimination by the immune system. To reduce non-specific expression in APCs, a hematopoietic-specific microRNA (miR) target sequence, miR-142-3p, was incorporated into the vector. This is an additional layer of post-transcriptional control that results in sustained expression of the FIX transgene and the persistence of transduced cells [23,24] (Fig. 1). In contrast to FIX, delivery of FVIII with Vesicular stomatitis virus glycoprotein G (VSV-G)-pseudotyped lentiviral vectors could not prevent inadvertent immune responses to the transgene product, even though a liver-restricted promoter was used and residual transgene expression in APCs could be blocked by post-transcriptional miR-142-3p regulation

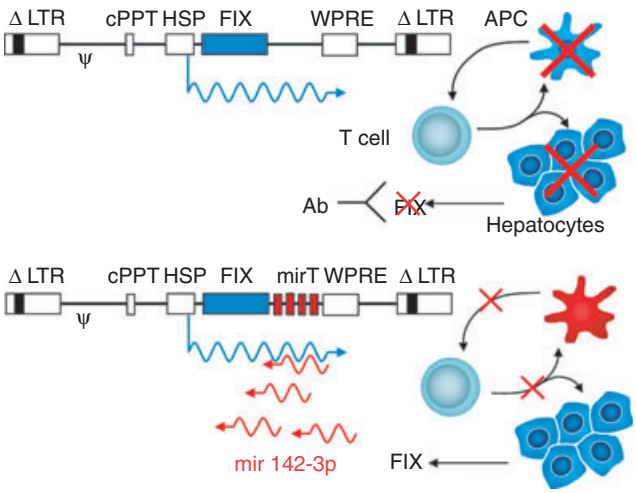

Fig. 1. The use of microRNA (miR)-regulated lentiviral vectors for hemophilia gene therapy. The use of a hepatocyte-specific promoter does not rule out ectopic transgene expression in antigen-presenting cells (APC)s. This results in an antigen-specific T-cell response that eliminates both APCs and hepatocytes that express the FIX transgene product. Ectopic transgene expression can be avoided by the addition of miR target sequences to the vector that are complementary to a hematopoietic-specific miR (e.g. miR-142-3p). In hematopoietic cells, such as APCs, the transcription of the miR and its specific interaction with the target sequence on the mRNA results in the degradation of the mRNA product via the cellular miR-processing pathway and the subsequent inhibition of FIX expression. This FIX mRNA degradation does not take place in hepatocytes, as they do not express miR-142-3p. Because of this treatment, APCs do not induce an antigen-specific T-cell response, resulting in the survival of APCs and hepatocytes, and prolonged expression of FIX in hepatocytes. Ab, antibody; cPPT, central polypurine tract; HSP, hepatocyte-specific promoter; LTR, long terminal repeat; WPRE, Woodchuck post-transcriptional regulatory element.

[25]. This may be a result of residual FVIII expression in the marginal zone of the spleen [23]. Although this off-target transgene expression did not cause an anti-FIX immune response, preventing FVIII transgene expression in the spleen is critical to facilitating long-term expression without the complication of the development of untoward immune responses. Consequently, redirecting the tropism of the lentiviral delivery system with the use of the GP64 envelope glycoprotein and presumably preventing the transduction of cells within the marginal zone of the spleen prevented the anti-FVIII immune response. Further studies are needed to identify the cell type within the spleen that is transduced by the VSV-G-pseudotyped lentiviral vector and the mechanisms by which this cell initiates the anti-FVIII immune response.

Genomic integration by γ-retroviral and lentiviral vectors is essential for expression of the transgene in the host genome. However, this generates some safety concerns, as insertional mutagenesis and activation of oncogenes (or inactivation of tumor-suppressor genes) can take place [19]. In particular, in a recent lentiviral trial for β-thalassemia, most of the therapeutic benefit resulted from a dominant, myeloid-biased cell clone [26]. This dominant clone contained an integrated lentiviral vector that caused transcriptional activation of HMGA2 in erythroid cells, with further increased expression of a truncated HMGA2 mRNA that was insensitive to degradation by let-7 miRs. The clonal dominance that accompanies therapeutic efficacy may result from a hitherto benign cell expansion caused by dysregulation of the *HMGA2* gene in stem/progenitor cells. However, it could also be coincidental and stochastic.

The genotoxic risk of integrating viral vectors can be influenced by the vector design or a transcriptionally active long terminal repeat (LTR) (reviewed in [19]). A combination of an internal moderately active promoter and a self-inactivating vector (SIN design), in which ~ 400 bp are removed in the LTR region to abolish its transcriptional activity, could alleviate the risk of insertional oncogenesis. In general, lentiviral or γ-retroviral vectors show semirandom insertion profiles, with varying degrees of preference for actively transcribed genes [27,28]. Both lentiviral and γ-retroviral vectors show a bias for integration into transcriptional units, indicating that integration is not random. Nevertheless, γ-retroviral vectors integrate preferentially in the immediate proximity of transcription start sites and a small window around DNase I-hypersensitive sites, whereas lentiviral vectors are more likely to integrate further away from the transcription start sites, into active transcription units [29]. Preclinical studies in tumor-prone models suggest that SIN lentiviral vectors have a reduced oncogenic risk as compared with SIN γ-retroviral vectors [30–32]. This is probably because of the bias for preferential sites of integration into cancer genes that is intrinsic to γ-retroviral as opposed to lentiviral vectors. Nevertheless, to date,

there is no evidence from clinical studies that lentiviral vectors have a reduced risk of insertional oncogenesis as compared with γ-retroviral vectors.

Alternatively, integration-defective lentiviral vectors (IDLVs), which contain an inactivating mutation in the integrase, can also be used to reduce the risk of insertional mutagenesis. However, the use of IDLVs to target FIX expression in hepatocytes was not as efficient as using integrase-competent vectors, as transgene expression levels were reduced. Nevertheless, sustained immune tolerance to FIX was obtained [24]. In hemophilia B mice, induction of neutralizing antibodies, even after an antigen rechallenge, was prevented after IDLV-mediated hepatocyte-targeted FIX expression. Additionally, FIX expression levels were prolonged at ~2% of normal levels. These hepatocyte-targeted IDLVs were able to induce transgene-specific regulatory T cells, thereby contributing to immune tolerance. It was proved by deep sequencing of transduced livers that IDLV did indeed show significantly fewer genomic integrations. To overcome the reduced hepatic transgene expression by IDLVs, the FIX transgene was optimized, so the levels of active FIX protein would be increased [33]. Besides using a codon-optimized FIX, a hyperactivating mutation was incorporated in the transgene. This mutation is an R338L amino acid substitution (FIX Padua) that has been associated with clotting hyperactivity and thrombophilia. After applying this vector in hemophilia B mice, we showed that sustained therapeutic levels of FIX activity could be obtained, which corresponded to a 15-fold increase in gene transfer efficacy, without any detectable adverse thrombogenic effects. Hence, the use of optimized IDLVs is a promising strategy with which to create tolerogenic expression of FIX proteins in the liver, with a reduced risk of insertional mutagenesis. The superior performance of vectors containing the codon-optimized FIX Padua was also extended to conventional integration-competent lentiviral vectors, resulting in unprecedented FIX activity after lentiviral gene therapy and robust phenotypic correction [33]. The Padua mutation in itself did not seem to have increased the FIX immunogenicity, consistent with other studies with adeno-associated virus (AAV) vectors [34]. These encouraging results justify moving forwards with preclinical studies in hemophilia B dogs.

Stem cell-based gene transfer

Besides being used for in vivo gene transfer, γ-retroviral and lentiviral vectors can be used to transfer genes ex vivo into stem cells, such as hematopoietic stem cells (HSCs) and other stem/progenitor cell populations [4,19]. As HSCs can self-renew and differentiate into all blood lineages, they are interesting target cells for gene therapy in hemophilia. They can easily be genetically modified and transplanted. Additionally, it is possible to generate immune hyporesponsiveness against the gene therapy

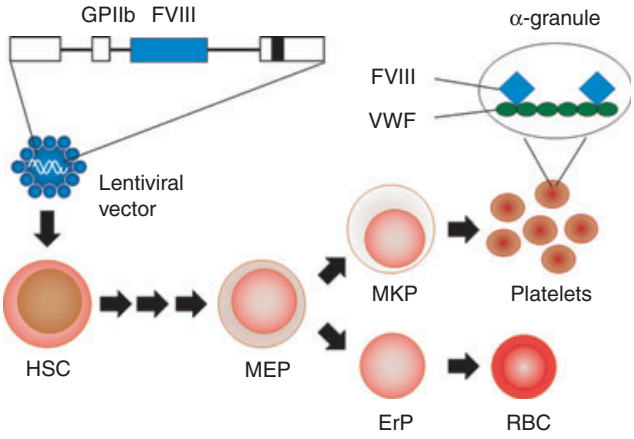

Fig. 2. Platelet-directed gene therapy for hemophilia A. Lentiviral vectors expressing FVIII from a megakaryocyte-specific promoter, glycoprotein (GP)IIb, were used to transduce hematopoietic stem cells (HSC)s. This results in the expression of FVIII in platelets, which also express von Willebrand factor (VWF), the natural carrier of FVIII. VWF and FVIII are then colocalized and stored in α-granules until the complexes are released when hemostatic activity is needed. This avoids the recognition of anti-FVIII inhibitory antibodies, and therefore this treatment is also effective when pre-existing antibodies are present. ErP, erythroid precursor; MEP, megakaryocytic/erythroid progenitor; MKP, megakaryocyte precursor; RBC, red blood cell.

product. With targeting of HSCs, their progeny, such as erythrocytes, megakaryocytes, and platelets, are able to secrete the clotting factors in the circulation.

Transplantation of ex vivo transduced hematopoietic cells will generate immediate access of clotting factors to the circulation. Initial experiments did not show clinically relevant FVIII plasma levels when bone marrow cells were transplanted after ex vivo transduction with γ-retroviral vectors containing FVIII [35]. Nevertheless, Evans and Morgan [36] showed that, although FVIII levels were low, they were sufficient to induce immune tolerance to the transgene product. After advances in methodology, transplantation of bone marrow cells transduced by FVIII containing γ-retroviral vectors into hemophilia A mice resulted in sustained phenotypic correction of the disorder [37,38]. Furthermore, the development of FVIII antibodies, even after an immunologic challenge, was avoided after transduction and transplantation of bone marrow cells [39]. In hemophilia A mice that were transplanted with transduced HSCs encoding a B-domain-deleted (BDD) porcine FVIII transgene, sustained curative FVIII activity could be seen as well, whereas there was no immunologic response to FVIII [40,41].

Lentiviral transduction of HSCs with a hybrid human–porcine FVIII transgene in hemophilia A mice led to long-term expression of FVIII at therapeutic levels [42], which was more efficient than with a regular human FVIII transgene. Hemophilia can be phenotypically corrected by lentivirally transducing HSCs with FVIII or FIX specifically directed to platelets [9,43] (Fig. 2), based

on previous transgenic models of platelet-restricted FVIII expression [44,45]. Remarkably, even in the presence of high-titer antibodies, hemophilia A mice showed phenotypic correction after FVIII expression was targeted to platelets by lentiviral transduction [46,47], which could be beneficial for patients carrying inhibitors against the clotting factors. However, other studies suggest that platelet-delivered FVIII provides limited resistance to anti-FVIII inhibitors [48], which probably reflects differences in sensitivity between the different assays used to assess phenotypic correction. It will be important to translate these finding into large animal models [49], in anticipation of exploiting them in the clinic. In a separate study, releasable FIX could be expressed and stored in the α-granules of the platelets, normalizing hemostasis in hemophilia B mice. However, in this case, hemostatic protection could not be achieved when high-titer anti-FIX antibodies were present [50]. This suggests that, in contrast to FVIII, which binds von Willebrand factor (VWF), FIX does not bind any plasma protein, and is therefore not protected from neutralization when it is released from activated platelets.

One challenge of HSC transplantation is that patients will have to be conditioned with conditioning regimens, such as busulfan. This is important to precondition the bone marrow 'niche' and facilitate HSC engraftment. However, preconditioning can cause severe side effects. Therefore, this should be considered carefully in the context of hemophilia gene therapy. Besides HSCs, endothelial progenitors can also be used as target cells for gene therapy, as they express VWF, a plasma protein that stabilizes FVIII and that can therefore improve therapeutic efficacy [51]. Nevertheless, it remains a challenge to generate endothelial progenitors that are able to engraft efficiently and persistently. The development of endotheliotropic lentiviral vectors may potentially overcome this limitation [52], provided that high-titer vectors can be manufactured.

Adeno-associated viral vectors

AAVs are non-pathogenic viruses with a single-stranded DNA genome that occur naturally and are incapable of replicating. Their safety profile is favorable, and the vectors can achieve persistent expression of the transgene. The AAV genomes are episomally retained, and long-term expression can therefore be achieved. The stably transduced vector genomes mostly remain extrachomosomally, organized as high molecular weight concatamers. This reduces the risk of insertional oncogenesis, as does the fact that transduced cells will not expand after hemophilia gene therapy. However, this does not rule out the existence of oncogenic events after gene transfer regulated by AAV vectors [53]. Some caution is warranted in not overinterpreting these results, because, in other model systems, this effect has not been reproduced in large cohorts of hemophilic mice [54], or in dogs or non-human primates. The precise reason for this discrepancy is not fully understood, and is the subject of ongoing debate. The major disadvantage of AAV vectors is that vector particles can only package DNA of a certain size (~ 4.7 kb). This restricts the size of the transgene cassette that can be accommodated in functional vector particles, which poses a significant hurdle for FVIII. AAV has several immunologically distinct serotypes that have been isolated [55], but, in hemophilia gene therapy, most vectors were initially derived from AAV serotype 2, which is the most prevalent. AAV-based gene therapy is a very promising technology, as clinical success has already been achieved in patients with congenital blindness [56].

Muscle-directed gene transfer

AAV-mediated hemophilia B gene therapy was first targeted at skeletal muscle [3], which does not normally express FVIII or FIX. An advantage of using muscles is that they are easily accessible for gene therapy and have a robust secretory capacity. Additionally, muscle cells are able to post-translationally modify the protein to produce a functional transgene product. However, these modifications, such as proteolytic cleavage and glycosylation, are not as efficient as in hepatocytes. Herzog *et al*. [57] demonstrated stable FIX expression after muscle-directed gene transfer in hemophilia B dogs. These dogs had a missense mutation in the FIX gene, but gene transfer with the same vector in dogs carrying an FIX null mutation resulted in the formation of high-titer inhibitors [58]. This shows that the mutation present in the FIX gene influences the risk of inhibitor formation. Also, the AAV vector dose is able to influence the development of neutralizing antibodies in a dose-dependent way, as the dose per site correlated with an increased risk of inhibitor formation [59]. The induced antibody response is largely caused by a local immune response [60]. A critical parameter for this is the local FIX antigen dose in the transduced muscle. The development of anti-FIX antibodies can be avoided by limiting the vector dose per site [59] or applying transient immunosuppression [58,61].

Stable expression of FIX and phenotypic correction of the bleeding diathesis have been shown in translational studies in animal models of hemophilia [3,57,62]. These results justified a phase I clinical trial in severe hemophilia B patients. AAV vectors expressing FIX from a ubiquitous cytomegalovirus promoter were injected repeatedly into the skeletal muscle. Circulating FIX levels did not reach the therapeutic range, although FIX expression was detectable at the injection sites. Interestingly, in one patient, expression was still visible 10 years after the treatment [63], underscoring the long-term expression potential of AAV. None of the patients in this clinical trial developed inhibitory antibodies against the transgenic FIX protein. However, none of them had a history

of neutralizing antibodies prior to gene therapy. Patients were selected for this trial on the basis of the low risk of inhibitor development, consistent with their genotype based on missense mutations in the FIX gene. It is encouraging that muscle-directed gene therapy did not seem to have increased the risk of inhibitor development, at least in this small patient cohort.

As a limited vector dose per site could be administered in this trial, systemic FIX expression levels were low. To avoid multiple localized intramuscular injections, AAV FIX vectors directed to skeletal muscle were designed to be delivered via an intravascular route [64]. In this hemophilia B dog model, intravascular delivery resulted in widespread transduction in the muscles, and the expression of the canine FIX transgene was sustained at therapeutic levels, in a dose-dependent manner. These levels were up to 10-fold higher than those achieved by localized intramuscular delivery. After normal treatment, a transient inhibitor could be detected, but the inhibitory antibody did not develop when transient immunosuppression was applied. The treatment was effective, as the increased levels of FIX and a reduced bleeding time corresponded with significantly fewer spontaneous bleeding episodes. The feasibility of treating hemophilia B with an intravascular approach is demonstrated by this preclinical trial. However, transient immunosuppression is still required to avoid the development of FIX-specific inhibitors. Recently, the previously mentioned FIX Padua (R338L) amino acid substitution, which results in hyperactivation of FIX, has also been incorporated in AAV vectors. Muscle-directed gene delivery of this vector in hemophilia B dogs resulted in eight-fold to nine-fold increased FIX activity and phenotypic correction. These effects lasted for at least 5 years, without the formation of inhibitory antibodies or T-cell responses, confirming that it is a promising strategy with which to improve gene therapy in hemophilia B patients [34].

Liver-directed gene transfer

The liver is another attractive alternative for AAV-mediated gene therapy in hemophilia. In several preclinical studies with murine and canine hemophilia models or non-human primates, it has been shown that AAV vectors are able to induce persistent therapeutic expression, resulting in partial or complete phenotypic correction [64–70]. Moreover, transducing hepatocytes leads to the induction of regulatory T cells, inducing immune tolerance to the FIX antigen [11,71]. When null mutation hemophilia B dogs were treated with AAV2-mediated FIX gene transfer directed to the liver, the bleeding phenotype was almost completely corrected without the development of inhibitory antibodies [65]. More potent FIX-containing expression cassettes were developed to further reduce the dose of the vector. This was achieved by using codon-optimized FIX, stronger promoter/enhancer elements, or self-complementary, double-stranded AAV vectors (scAAVs) [72–75]. This scAAV configuration overcomes one of the limiting steps in AAV transduction, namely the conversion of single-stranded to double-stranded AAV. The vector dose could also be reduced by the use of alternative AAV serotypes, e.g. AAV8, which are able to increase hepatocyte transduction, improve intranuclear vector import, and cause less T-cell activation [76,77]. In mice, the use of AAV8 or AAV9 for liver-directed gene therapy is more efficient than the use of lentiviral vectors, and also reduces inflammation [22]. In a recent study, it has been shown that the vector particles can evade phosphorylation and ubiquitination when surface-exposed tyrosine residues are mutated [78]. This results in the prevention of proteasome-mediated degradation, leading to a 10-fold higher FIX expression level. Because the FVIII transgene is larger than the FIX gene, and the AAV vector has packaging constraints, it was initially not possible to generate an AAV vector to treat hemophilia A. To overcome this limitation, a BDD form of FVIII was used in combination with small promoters [79]. Although higher vector doses were needed, sustained FVIII expression was achieved after AAV vector-mediated gene transfer into mice and dogs, establishing a proof-of-concept that hemophilia A gene therapy with AAV is possible.

These encouraging preclinical studies paved the way towards a liver-directed AAV gene therapy trial in hemophilia B patients. Delivery of AAV FIX vectors by hepatic artery catheterization resulted in maximum transient therapeutic FIX levels of 12% of normal [80]. Unfortunately, AAV capsid-derived antigens were presented to T cells by the transduced hepatocytes [81,82] (Fig. 3).

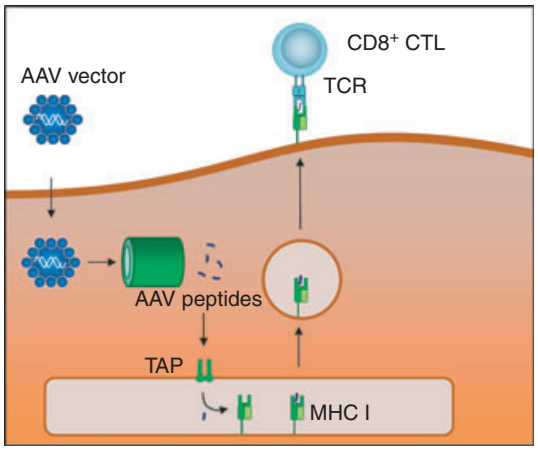

Fig. 3. Hypothetical model of adeno-associated virus (AAV)-specific T-cell immune responses. Following AAV transduction, AAV vector proteins (blue) are degraded by proteasomes, after which they are presented to cytotoxic T cells by major histocompatibility complex (MHC) class I molecules. At high vector doses, this results in the killing of the transfected cell by the cytotoxic T cells. CTL, cytotoxic T lymphocyte; TAP, transporter associated with antigen processing; TCR, T-cell receptor.

This resulted in liver inflammation, owing to the destruction of transduced hepatocytes by the T cells. Recently, patients with severe hemophilia B were treated with intravenously injected scAAV8 vectors containing codon-optimized FIX and a liver-specific promoter [10,83]. The AAV8 serotype is used because there is less cross-reactivity to anti-AAV2 antibodies, which are highly abundant in humans. Additionally, the uptake of AAV8 by dendritic cells may be lower than that of AAV2 vectors, thereby potentially reducing T-cell activation [77]. However, this may not suffice to prevent T-cell responses against AAV8-transduced hepatocytes, as shown recently in an AAV8-based clinical trial for hemophilia [83]. The use of AAV8 vectors in mice, as compared with AAV2, could lead to a substantial increase in hepatic transduction. However, in higher species, such as dogs, non-human primates, and humans, this advantage was apparently not preserved. In the trial by Nathwani *et al.* [83], patients received the scAAV8 FIX vector in escalating doses, with two participants per dose. All participants showed sustained FIX expression levels above the therapeutic threshold for several months after treatment. This was the first liver-directed AAV trial that resulted in sustained therapeutic FIX levels after gene therapy. However, in some patients, there was, again, T-cell-mediated clearance of hepatocytes transduced with AAV, which correlated with elevated liver enzyme levels. This response is vector-specific, and could be reduced by treating the patients with glucocorticoids to cause transient immune suppression.

Although this study showed promising results, some questions remain. Patients receiving the intermediate vector dose had an increased T-cell response, which was directed at the AAV8 capsid, although their liver enzyme levels were not elevated. This suggests that a T-cell response against AAV8 does not necessarily result in the elimination of transduced hepatocytes and the subsequent liver toxicity. An explanation for this could be that differences in the liver microenvironment and cytokine state impact on this T-cell response. Another possible explanation is that the intermediate vector dose was not high enough to induce T-cell-mediated destruction of the hepatocytes, in contrast to the highest dose, which was cytotoxic. Finally, it should be considered that the increase in liver enzyme levels was not a result of the AAV-specific T-cell response, but had other causes. To address these questions, future clinical trials should be designed.

One possible strategy to overcome the AAV-specific T-cell immune response is to engineer the AAV vectors themselves. We have shown recently that the incorporation of motifs that prevent proteasome-mediated processing of AAV prevent T-cell-mediated rejection of transduced hepatic cell lines in vitro [84]. This concept was recently confirmed independently by Martino *et al.* [85], who engineered an AAV2 capsid in which the surface-exposed tyrosine residues are mutated to phenylalanines. This mutation of possible phosphorylation sites should reduce the chance of proteasomal degradation. Indeed, these AAV2 Tyr→Phe mutants show less effective capsid presentation to $CD8^+$ T cells, resulting in reduced killing of transduced hepatocytes.

Other vector systems

Immune complications caused by the vector itself are less likely to occur when non-viral vectors are used, although the risk of an innate immune response to such a non-viral vector is not entirely eliminated. Nevertheless, just as in the case of viral vectors, the therapeutic transgene product itself may evoke immune reactions. Gene transfer mediated by viral vectors is generally more efficient than non-viral gene transfer. Additionally, non-viral transfection typically results in transient expression of the transgene. This can be overcome by enriching the stably transfected clones following in vitro selection [86]. Plasmid-based gene transfer has been tested in a phase I clinical trial with hemophilia A patients [87]. Autologous fibroblasts of these patients were stably transfected with FVIII-expressing plasmids by electroporation, after which they were selected, expanded, and implanted into the patient. Unfortunately, FVIII activity was only slightly and transiently improved. Recently, a vector system of human artificial chromosomes (HACs) has been developed. These vectors are capable of packaging large gene inserts, and are maintained as an episomal minichromosome. This minimizes the risk of insertional mutagenesis. HACs containing FVIII under control of a megakaryocyte-specific promoter were introduced into induced pluripotent stem (iPS) cells. In vitro differentiation of these iPS cells into megakaryocytes and platelets resulted in FVIII expression in the differentiated progeny [88]. However, translating iPS cell-based cellular therapies to the clinic raises some safety concerns, as undifferentiated iPS cells carry an intrinsically high risk of teratomagenesis. Moreover, the differentiation of iPS cells into transplantable cells that are functionally equivalent to their normal counterparts requires a better understanding of these processes, and is still very challenging.

Although non-viral vectors can be relatively easily delivered ex vivo (e.g. by electroporation), in vivo gene delivery is more problematic. The main limitation of non-viral vectors is how to achieve safe and robust gene delivery to the liver. Typically, hydrodynamic plasmid injection is performed to achieve transgene delivery into the liver. This method relies on rapid injection, which enhances hepatic gene delivery [89]. This approach is currently being adapted for potential clinical applications in large animal models by the use of computer-assisted devices or by reducing the injection volume and maintaining the local hydrodynamic pressure needed for gene transfer [90,91]. A different approach that is being explored is based on the use of targetable nanoparticles

to deliver FVIII or FIX into hepatocytes or endothelial cells [92].

To enhance sustained transgene expression following in vivo DNA transfection, bacterial backbone sequences can be removed. These so-called DNA minicircles are safer, more efficient on a molar basis, and less likely to cause innate immune responses. Alternatively, transposons could be used to stabilize transgene integration in the genome. We and others have demonstrated that hyperactive transposons are able to increase the stability of FVIII or FIX expression after either ex vivo or in vivo gene therapy [92–96].

Adenoviral vectors have been investigated intensively for gene therapy in hemophilia. To improve their efficacy, high-capacity adenoviral vectors have been developed that do not contain any viral genes, and therefore result in more stable transgene expression and a lower risk of adaptive immune responses. Additionally, these vectors are essentially non-integrating, thereby minimizing the risk of oncogene activation. However, adenoviral vectors show a strong interaction with APCs, eliciting, in turn, robust innate immune responses. When these FVIII-containing or FIX-containing high-capacity adenoviral vectors carrying a liver-specific promoter were administered to hemophilic mice, clotting factor expression levels increased to supraphysiologic levels, without any toxicity [97–99]. The same results were obtained with experiments on hemophilic dogs and other large animal models. This was followed by a phase I clinical trial on a severe hemophilia A patient who received systemic administration of high-capacity adenoviral vectors encoding FVIII from a liver-specific promoter. However, this trial was stopped when the patient showed a transient inflammatory response with hematologic and liver abnormalities. Given the intrinsic narrow therapeutic index of adenoviral vectors, it is important to further reduce the vector-induced activation of the innate immune system. A study by Brunetti-Pierri *et al.* [100] in non-human primates demonstrated that localized hepatic delivery of these vectors minimized the immune response and resulted in efficient, sustained transgene expression.

Future considerations

Gene therapy research has progressed significantly in the past few years. The initial phase of scepticism is being replaced by cautious optimism. The use of different vectors and target cells in animal models of hemophilia has demonstrated a sustained therapeutic effect of gene therapy. AAV and lentiviral vectors result in relatively efficient gene transfer and high transgene expression levels. In addition, only limited immune responses are apparent as compared with adenoviral vectors. On the basis of their efficacy and safety profile, they are therefore among the most favorable vectors for gene therapy of hemophilia. Research on target cells for gene therapy and has shown that hepatocytes, muscle cells and HSCs are all capable of maintaining stable clotting factor expression levels. Additionally, clinical trials in hemophilia B patients have demonstrated that gene therapy can result in sustained therapeutic FIX levels.

Nevertheless, some difficulties remain to be solved. The sustained therapeutic FIX levels achieved in an AAV-based trial cannot prevent trauma or injury-induced bleeding episodes. This can be overcome by further improving the vector design so that hemophilia B patients will show full hemostatic correction. Additionally, the level of empty virus particles could be reduced and the viral AAV capsids could be adapted to reduce dose-limiting T-cell responses and liver toxicity. Generating functional AAV FVIII vectors to treat hemophilia A is less straightforward. As AAV vectors have limiting packaging capacity, only small regulatory elements can be used to drive expression of the relatively large BDD FVIII transgene. The incorporation of a codon-optimized FVIII transgene into the AAV vector, which increases the expression nearly 50-fold, overcomes, at least partially, this bottleneck [101].

Owing to the prevalence of AAV in the population, the pre-existing immunity and ensuing T-cell response remains a concern. This risk will be lower when lentiviral vectors are used, because HIV has a lower prevalence than AAV. Additionally, as HIV can incorporate larger transgenes, FVIII delivery should be more straightforward. Shi *et al.* [9] demonstrated, for the first time, that gene therapy could correct hemophilia even in the face of high-titer pre-existing anti-FVIII antibodies. This required platelet-directed FVIII expression after lentiviral transduction of HSCs. If this treatment could be improved further, it might be possible to treat patients with inhibitors that are refractory to immune tolerance induction. Before clinical trials can take place, it is important to further test the lentiviral vector-based approaches in large animal models. The reason for this is the difficultly in translation from mice to humans. Preliminary experiments suggest that lentiviral vectors, equipped with an miR-regulated hepatocyte-specific FIX expression cassette, can give rise to sustained therapeutic effects in the canine hemophilia model without inducing inhibitory antibodies (L. Naldini, personal communication). Nevertheless, this requires the manufacture of relatively large vector doses, which remains challenging and warrants the development of improved and more efficient lentiviral vector production systems.

The risk of inhibitor formation remains a challenge in gene therapy with the different vector approaches. Hemostatic correction will be difficult, especially in patients who carry pre-existing neutralizing FVIII antibodies. A potential solution to this is the expression of FVIII in platelets [41,44] or liver-directed gene therapy, which results in the induction of regulatory T cells and immune tolerance to the clotting factors [11]. It is particularly

encouraging that tolerance to FVIII could be established in the setting of pre-existing inhibitory antibodies following AAV-based gene therapy in an adult large animal model of disease [102]. The sustained expression of canine FVIII from the transgene after inhibitor eradication recapitulates the secondary prophylactic replacement protocols required to maintain immune tolerance after successful inhibitor eradication. This indicates that gene therapy can result in inhibitor eradication and complete normalization of the pharmacokinetics of FVIII protein infusion while improving the disease phenotype. The exact mechanism of the immune tolerance induction in this hemophilia A dog study is currently unknown, but may possibly also involve regulatory T cells, as suggested by the murine studies. The use of other vector systems, such as transposons or engineered nucleases, could overcome some of the immune limitations of AAV and lentiviral vectors, although gene delivery efficiency should be further improved [93,103,104]. In conclusion, it is important to explore different gene therapy modalities in the hope of developing safe and effective treatments for both hemophilia A and hemophilia B.

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