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Recent Advances and Future Perspectives in Lentiviral Gene Therapy for Hemophilia A and B

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Abstract

Hemophilia A and B are rare incurable hereditary diseases due to deficiencies in clotting factor VIII (FVIII) and factor IX (FIX), respectively. These genetic defects result in potentially life-threatening, uncontrolled bleeding episodes. Current treatment by protein substitution therapy does not constitute a cure making gene therapy an attractive alternative. Lentiviral vectors (LVs) have many distinctive features that make them especially well suited for FVIII or FIX gene delivery. This includes the lack of vector-specific pre-existing immunity, their ability to permanently transduce both dividing and non-dividing cells and their capacity to readily accommodate FIX and FVIII expression cassettes, consistent with their packaging capacity of 10 kb. LVs have been used to achieve sustained therapeutic clotting factor expression levels and hemostatic correction in preclinical hemophilic mouse models. The liver has been the target organ of choice for direct *in vivo* LV transduction of FVIII or FIX genes, resulting in sustained therapeutic effects. Nevertheless, the potential development of neutralizing antibodies to the clotting factors following *ex vivo* or *in vivo* gene therapy with LVs can preclude long-term phenotypic correction. These risks can be minimized by preventing ectopic expression in antigen-presenting cells. LVs are well suited to deliver the clotting factor genes into hematopoietic stem/progenitor cells, allowing for stable FVIII or FIX expression upon hematopoietic reconstitution. In addition, therapeutic FVIII and FIX expression levels have been achieved *in vivo* after transplantation of lentivirally transduced endothelial and mesenchymal stem/progenitor cells. Current challenges relate primarily to the translation of these findings to larger preclinical animal models and ultimately to patients suffering from hemophilia.

Keywords: Hemophilia; Lentiviral vector; HIV; Factor VIII; Factor IX; Liver; Hematopoietic stem cell.

Introduction

Hemophilia A and B are congenital bleeding disorders caused by a deficiency of functional clotting factors, FVIII or FIX, respectively, owing to mutations in the cognate genes. The bleeding diathesis is X-linked and affects an estimated 400,000 people worldwide (according to the World Federation of Hemophilia). Hemophilia A is the more common version, afflicting 80–85% of all hemophiliac patients [1]. Since FVIII and FIX play a key role in the coagulation cascade, patients are suffering from uncontrolled bleeding episodes and chronic damage mostly in soft tissues, joints and muscles. This ultimately results in chronic synovitis, crippling arthropathy and physical disability. Moreover, the bleeding itself can be life-threatening, as in the case of intracranial hemorrhage. Current treatment for hemophilia is based on protein substitution therapy (PST) using plasma-derived or recombinant clotting factors [1-3]. Although, PST has significantly increased the quality of life and prolonged the life expectancy of patients, there remain some drawbacks and limitations. PST is non-curative and it is constrained by the relatively short half-life of the clotting factors [3]. Consequently, repeated infusions of relatively large doses are required either prophylactically or on demand. Moreover, patients remain at risk for life-threatening bleeding episodes and chronic joint damage. Another important drawback of the current therapy is that some patients can develop neutralizing antibodies (i.e. inhibitors) specific for the administered FVIII or FIX proteins. Patients with severe hemophilia (< 1% FVIII or FIX) either do not produce any FVIII or FIX or express dysfunctional proteins instead rendering these patient's immune system intolerant to the therapeutic protein, appearing as neo-antigens. The presence of these inhibitors can render further therapy ineffective and makes bleeding episodes extremely difficult to manage. An additional hurdle for hemophilia patients is that they are dependent on continuous medical care and social support due to the exorbitant costs of the recombinant factors. Not surprisingly therefore, an estimated 75% of patients worldwide receive inadequate therapy or even no treatment at all [3,4]. Given these limitations, gene therapy could provide an alternative for hemophilia treatment. By introducing a functional FVIII or FIX gene copy into the target cells, gene therapy ultimately could provide a cure and obviate the need for repeated clotting factor infusions. Interestingly, hemophilia has been recognized as an ideal target disease for gene therapy since it is caused by a well-known single gene defect and has a broad therapeutic window [1,5]. Indeed, tight regulation is not strictly required. Levels at or slightly above 1% of the physiological clotting factor plasma levels prevents the bleeding episodes and minimize mortality and morbidity. Moreover, sustained supra-physiologic FVIII or FIX expression between 10 to 100-fold normal levels does not significantly increase mortality rates in hemophilic mice undergoing gene therapy [6,7]. Nevertheless, population-based patient-control studies revealed that FVIII levels above 150% are associated with increased thrombotic risk [8].

The development of gene therapy for hemophilia could serve as a trailblazer for other, more complex or multifactorial diseases. Several

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vectors have been developed for gene therapy of hemophilia including adenoviral (Ad), adeno-associated viral (AAV), lentiviral (LV) and non-viral vectors (reviewed by: [1-3,9-11]). The aim of this review is to highlight the most recent advances, benefits and risks of LV vector-based gene therapy for hemophilia A and B. This review complements several other reviews on LVs and on hemophilia gene therapy [1,2,12-22].

Lentiviral vectors

LVs have become one of the most widely used vectors for gene therapy. LVs resemble γ-retroviral vectors (γ-RVs) in their ability to stably integrate into the target cell genome, resulting in persistent expression of the gene of interest. However, in contrast to γ-RVs, LVs can also transduce non-dividing cells [23]. This distinctive feature paves the way toward many applications for which γ-RVs are not suitable. Moreover, LV can accommodate larger transgenes [up to ~10 kilobases (kb)] compared to when γ-RVs are used though vector titers tend to decrease with larger inserts [14,24,25]. It is critically important to ensure that the LVs are replication-defective because HIV-1 is a human pathogen. The latest generation LV technology has several built-in safety features that minimize the risk of generating replication-competent wild-type human HIV-1 recombinants. Typically, LVs are generated by trans-complementation whereby packaging cells are co-transfected with a plasmid containing the vector genome and the packaging constructs that encode only the proteins essential for LV assembly and function, *gag-pol*, *rev*, and *env*, respectively. Typically, the LV design is based on the so-called self-inactivating (SIN) LV configuration. This SIN configuration reduces the likelihood that cellular coding sequences located adjacent to the vector integration site will be aberrantly expressed by abolishing the intrinsic promoter/enhancer activity of the HIV-1 LTR or will be mobilized by wild type HIV-1 [14,26]. The vesicular stomatitis virus G glycoprotein (VSV-G) is the most commonly used viral envelope protein used for LV pseudotyping, but other envelopes have also been used [4,14,27,28]. Pseudotyping HIV-1 vectors obviate safety concerns associated with the use of HIV-1 gp120, which has known pathogenic consequences. Moreover, pseudotyping has a dramatic impact on the biodistribution and vector tropism. In particular, HIV-1 gp120 restricts transduction of HIV-1 vectors to CD4+ cells that limits its usefulness for gene therapy applications to CD4+ cells, like T cells or macrophages. In contrast, heterologous envelopes, like VSV-G, typically broaden the tropism and allow gene transfer into target cells that are relevant for hemophilia gene therapy, such as CD34+ hematopoietic stem/progenitor cells and hepatocytes. On the other hand, the choice of envelope can also restrict transdcution to the desired target cell or tissue. For instance, baculovirus GP64 and hepatitis C E1 and E2 pseudotyping enhances specific hepatic transduction [4,27,28].

Proof of concept has been established in a variety of preclinical animal models that LVs can yield long-term therapeutic effects following *ex vivo* or *in vivo* gene therapy [1,2,12,14,18-21,27,29-37]. Moreover, LVs have now moved beyond the preclinical stage into the clinic with multiple gene therapy trials ongoing or approved [16,17,19,38-40], particularly for hereditary diseases including β-thalassemia [41], Parkinson's disease [20], Wiskott–Aldrich syndrome [18], and most recently adrenoleukodystrophy [37,42,43]. These results and achievements further underscore the potential of using LVs for gene therapy of hemophilia.

In vivo LV gene therapy: hepatocyte targeting

Since FVIII and FIX are mainly synthesized in the liver, hepatocytes are well suited as primary targets for hemophilia gene therapy. This guarantees that the necessary post-translational modifications of the transgenic clotting factors faithfully mimic natural FVIII and FIX processing. Initially, we had established proof-of-concept that hemophilia A mice could be cured by gene therapy using γ-RVs. We demonstrated that stable therapeutic levels of FVIII could be achieved following intravenous γ-RV injection in neonatal hemophilia A mice [44]. Similarly, efficient γ-RV transduction could be obtained in neonatal hemophilia dogs [45]. Though this approach may ultimately be useful for treating pediatric hemophilia it was not possible to achieve efficient gene delivery in adults using these γ-RVs since hepatocytes are largely non-dividing in adults. To overcome this restriction of γ-RVs, LVs could be used instead since they can efficiently transduce non-dividing hepatocytes in adult liver [46,47]. Therapeutic FVIII or FIX levels could be achieved in adult hemophilic mice following intravenous injection with LVs derived from either HIV-1 or feline immune deficiency virus (FIV) [27,28,48,49]. However, these vectors are also efficient at transducing antigen-presenting cells (APCs) [47]. This interaction with APCs can trigger a rapid but self-limiting pro-inflammatory response that involves a transient cytokine surge (*e.g.*, interleukin-6) and IFNαβ response due to engagement of toll-like receptor (TLR) 7 and/or TLR9 [6,47,50]. This innate immune response is less robust compared to when adenoviral vectors are employed [1], but may still influence the adaptive immune response to the vector, the transgene product, and/or the LV-transduced cells [4]. It is also possible, in principle, that pre-existing antibodies to the heterologous envelope protein (Env) used to pseudotype the LVs may interfere with viral transduction. However, by carefully selecting the type of envelope used for pseudotyping, this risk can be significantly reduced. The use of LVs may therefore potentially overcome some limitations associated with the use of AAV vectors. Because most human subjects have not been pre-exposed to LV components, this is likely to be less of a concern with LVs than with AAV [14].

The induction of an antigen-specific antibody response against secretable transgene products that are encoded by the LVs, particularly FVIII or FIX, typically requires antigen processing via the exogenous pathway. Consequently, APCs take up the FVIII or FIX proteins and present the cognate antigenic peptides in association with major histocompatibility complex class II [51,52]. This, in turn, triggers a T-helper response that ultimately activates B cells to produce antibodies specific to the clotting factors encoded by the LV. In addition, gene transfer may result in the presentation of endogenously synthesized peptides derived from the transgene product in the context of major histocompatibility complex class I molecules potentially resulting in cytotoxic T-cell responses (CTL) that could consequently eliminate the LV-transduced target cells [31]. The magnitude of these antigen-specific humoral and cellular adaptive immune reactions following LV administration depends on several parameters, including target cell type, the transgene product, vector design, vector dose, route of vector administration and genotype of the recipient [1,2,4,31,47,53-56].

Ectopic expression of the transgene product (i.e. FVIII or FIX) in APCs may cause immune response that curtails long-term gene expression [50,51]. To avoid the clearance of the transgene product and the cells that express it, it is warranted to use cell type–specific promoter/enhancers to restrict transgene expression to the liver while preventing inadvertent ectopic transgene expression in APCs [47]. Nevertheless, even with highly tissue-specific promoters, immune response cannot always be prevented due to "leaky" transgene expression in APCs. Despite the use of LVs expressing FIX from a strong liver-specific synthetic transthyrethin (ET) promoter/enhancer, FIX expression

resulted in inhibitory antibodies in hemophilia B mice [50,51], partly due to FIX expression in APCs. However, we demonstrated that sustained FIX expression could be achieved using other potent synthetic hepatocyte-specific promoter/enhancers, such as the chimeric a1-antitrypsin/hepatocyte control region (AAT/HCR) [6,31], suggesting that this promoter/enhancer may be more specific than the ET promoter/enhancer. To avoid transgene expression and presentation in APCs using the ET promoter/enhancer, an additional layer of regulation was built into the LVs. This was achieved by incorporating a target sequence for the hematopoietic-specific microRNA, namely miR-142-3p [4,31,56-58]. These studies demonstrate that hepatocyte-restricted expression of foreign antigens, including intracellular or secreted, therapeutically relevant (FVIII, FIX) and model antigens (GFP and OVA), by LVs can result in a state of transgene-specific immunological tolerance due to a strong contraction of the transgene-specific effector compartment and the induction of the transgene-specific regulatory compartment, induced FoxP3+ T regulatory cells (iTreg), in particular. Consequently, FIX-specific immune tolerance was induced resulting in sustained expression of FIX in a hemophilia B mouse model [31,58]. However, several outstanding questions remain [4,52,56,57,59], and have been reviewed elsewhere [60]. For instance, it is difficult to interpret how the generation of iTreg occurs using miR-142 regulated LV since the primary expressing cell (hepatocytes) inefficiently express MHC-II necessary for priming CD4+ T cells, and the transgene is not expressed in the professional APC. It has been proposed that CD4+ T cells are primed by APC that have engulfed the naturally secreted Ag (like FIX) or intracellular antigens expelled from transduced dead or dying hepatocytes (and/or by engulfing the dead cells and cross-presenting the Ag) [60]. After presentation by APC, the Ag can prime naive CD4+ T cells under a tolerogenic cytokine milieu in the liver micro-environment that promotes the induction of transgene-specific iTreg cells.

However, miR-142-3-regulation may not necessarily suffice to induce immune tolerance in all circumstances and may vary depending on the transgene product, the target organ (e.g. liver *versus* muscle), the vector design and the underlying mutation of the defective endogenous gene. It is typically more challenging to induce immune tolerance in the context of a null mutation than when a missense mutation occurred in the affected gene. Moreover, FVIII is more immunogenic than FIX, consistent with the higher incidence of inhibitors in patients suffering from hemophilia A (40%) than hemophilia B (5%). Consequently, it is more challenging to induce FVIII than FIX-specific immune tolerance [4]. Not surprisingly, in contrast to LV gene therapy for hemophilia B, systemic LV mediated delivery of FVIII in mouse models has been largely ineffective due to the emergence of inhibitory anti-FVIII antibodies, even when applying the miR-142-3 regulated vector design in conjunction with a robust hepatocyte-specific promoter (i.e. ET) [4]. However, this impediment was successfully overcome by combining miR-142-3 regulation with pseudotype switching from VSV-G to the more hepatotropic GP64 envelope. In contrast, in the absence of miR-142-3 regulation, GP64 pseudotyping was insufficient to prevent the induction of FVIII-specific antibodies even when the HRC/hAAT promoter was used [4,27,28]. These observations further support the prevailing hypothesis that increasing hepatocyte-specific expression of clotting factors at the expense of undesired ectopic expression in APCs decreases the risk of developing inhibitory antibodies, possibly via induction of Treg-mediated immune tolerance. As a safety concern, it is important to ensure that the miR-142-3p target sequence does not compromise the normal function of its cognate complementary endogenous miR-142-3p sequence. This potential risk may depend on qualitative and quantitative variables, such as the type of miR and the relative expression levels of a given miR *versus* its cognate miR target. Though forced overexpression of miR-142-3p target does not seem to downregulate or titrate the endogenous miR-142-3p, other miR targets may repress their cognate endogenous miR [58,61,62]. Additional studies are required to address these outstanding issues including experiments on large animal models.

Though concerns remain about the potential long-term adverse effects of LV integration due to insertional mutagenesis, advanced LV design potentially reduces this risk [26,42,63-65]. Moreover, we and others have recently shown that hepatocytes can be successfully transduced *in vivo* with integration-deficient LVs (IDLVs) [56,67]. IDLVs are typically generated by packaging the vector with catalytically inactive human immunodeficiency virus (HIV) integrase [68]. The class I D64V mutation in the integrase catalytic site substantially reduces integration (10^2- to 10^3-fold) without compromising other steps in the transduction pathway [56,69]. Hepatocyte-targeted expression using miR-142-3p-regulated IDLVs resulted in the sustained and robust induction of immune tolerance to both intracellular (e.g. GFP) and secreted proteins, like FIX, despite the reduced transgene expression levels in comparison with their integrase-competent vector counterparts. Most importantly, IDLV-mediated hepatocyte-targeted FIX expression prevented the induction of neutralizing antibodies to FIX even after antigen rechallenge in hemophilia B mice, accounting for the relatively prolonged therapeutic FIX expression levels. Upon the delivery of intracellular model antigens, hepatocyte-targeted IDLVs induced transgene-specific regulatory T cells that contributed to the observed immune tolerance. Deep sequencing of IDLV-transduced livers showed only rare genomic integrations that had no preference for gene coding regions. The non-canonical molecular signatures of these rare integrants confirmed that genomic integrations occurred mostly, if not exclusively by an integrase-independent, cellular recombination mechanisms. Hence, IDLVs provide an attractive platform for the tolerogenic expression of clotting factors in the liver with a substantially reduced risk of insertional mutagenesis. Nevertheless, since FIX expression levels were reduced compared to integrating LV, it will be essential to increase FIX expression levels either by engineering the transgene itself and/or the vector backbone.

FIX and FVIII expression levels achieved by LV mediated gene delivery are still lower compared to AAV, at comparable vector doses [1,6,14,56,70,71]. To increase FVIII or FIX expression levels attempts were made to engineer the FVIII or FIX genes themselves [3]. Remarkably, codon optimization resulted in an unprecedented 44-fold increase of plasma FVIII levels in hemophilic mice following neonatal LV delivery [70]. Alternatively, the efficacy could be increased by altering the specific activity, intracellular transport, secretion or stability of the clotting factors. In particular, this could be achieved by deleting the B-domain from FVIII, by generating human-porcine FVIII hybrids or by incorporating specific mutations in FVIII or FIX [55,72-76].

Ex vivo LV gene therapy: HSC

Although liver is the primary organ for FVIII and FIX production, FVIII and FIX can also be expressed ectopically in other target cells. In particular, hematopoietic stem cells (HSCs) merit consideration as target cells for hemophilia gene therapy since they can self-renew and differentiate into all the distinct lympho-hematopoietic lineages. An additional benefit of targeting HSCs is the possibility to induce immune hypo-responsiveness or, ideally, immunological tolerance to the transgene product, as was demonstrated for FVIII [1,77]. Though

integrating vectors like γ-RVcould trigger insertional oncogenesis or clonal dominance [78-81], this potential risk is significantly reduced in the context of SIN LV mediated gene transfer [26,42,63-65]. Moreover, LV safety could be increased further by directing transgene expression to specific lineages using lineage-specific promoters [5,34,53,54,66,82-86]. Moreover, these studies underscores the benefits of lineage-specific promoters (e.g. glycoprotein IIb, β-globin, GPIbα) to avoid promoter silencing and assure sustained clotting factor levels [5,34,82,84].

Megakaryocytes and their platelet progeny are attractive targets for hemophilia A gene therapy, because platelets play a crucial role in primary hemostasis and it is beneficial to express FVIII in cells that synthesize and store its natural carrier protein VWF [1,82,83,86-89]. Consequently, ectopic FVIII expression in megakaryocytes resulted in co-localized storage of FVIII with VWF in the α-granules of platelets, constituting a releasable pool of FVIII/VWF complexes [1,83]. Transduction of HSCs with LVs encoding FVIII in platelets resulting in phenotypic correction of the bleeding diathesis in hemophilia A mice. Targeting FVIII or FIX production to platelets that act in the immediate vicinity of the site of vascular injury could potentially reduce the risk of evoking inhibitory antibodies by restricting clotting factor exposure in a spatial and temporal manner. Remarkably, transgenic mouse studies revealed that platelet-directed FVIII expression resulted in correction of the bleeding diathesis even in the face of pre-existing high-titer anti-FVIII inhibitory antibodies. FVIII expression was restricted to platelets in these mice, while plasma FVIII remained undetectable [34,53,86,90]. These findings would need to be further confirmed using LV transduced HSC. Hence, platelets conferred some level of protection of FVIII from immune attack, suggesting that this paradigm may be beneficial to treat hemophilia patients with inhibitors. However, the precise mechanism for the immune protection of platelet-derived FVIII is not understood. Presumably, FVIII produced and released by the activated platelets may be shielded from anti-FVIII antibodies inside the platelet plug. Moreover, the rapid interaction of FVIII with VWF may interfere with anti-FVIII antibody binding. This is consistent with the recent demonstration that VWF is essential to achieve hemostatic correction in the presence of inhibitors following platelet-FVIII gene therapy in hemophilia A mice (Q. Shi personal communication). In another study, releasable FIX could be expressed and stored in platelet a-granules, normalizing hemostasis in hemophilia B mice [53] but in this case no protection was seen in the face of anti-FIX antibodies. This indicates that FIX was inhibited quickly once released by platelets. Unlike FVIII, which binds to and is protected by the VWF carrier protein in plasma no protein protects FIX in plasma, so antibodies can bind freely to FIX once they encounter each other in the plasma when FIX is released from activated platelets. Alternatively, ectopic expression of activated factor VII (FVIIa) in platelets was demonstrated using SIV expressing FVIIa from the platelet-specific GPIbα promoter. This resulted in an efficient bypass therapy, correcting the bleeding phenotype in hemophilia A mice even in the presence of FVIII-neutralizing antibodies [54,86]. In conclusion, transduction of HSCs with LVs encoding FVIII, FIX or FVIIa in platelets resulting in phenotypic correction of the bleeding diathesis in hemophilia A or B mice and is an attractive paradigm for ex vivo gene therapy.

As an alternative strategy, clotting factor expression could be coaxed towards the erythroid lineage using β-globin regulatory elements to drive FIX after LV transduction of HSC. Erythroid-specific FIX delivery resulted in long-term therapeutic FIX expression sufficient to phenotypically correct hemophilia B mice. Following non-myeloablative conditioning and *in vivo* methyl-guanine methyltransferase (MGMT) drug selection, FIX levels rose sharply and eventually reached a level that is considered curative in hemophilia B therapy (>500ng/ ml) for 18 months in hemophilic mice, representing one of the most robust HSC-based approaches for hemophilia [85]. Moreover, erythrocyte-mediated protein delivery may result in immune tolerance and reduced the risk of insertional oncogenesis since the differentiated mature erythrocytes are enucleated [5]. However, ectopic FIX expression in the erythroid lineage may not yield fully active proteins because of limiting post-translational modifications [2,84].

Most recently, HSC were transduced with SIV-based LV that were transplanted into hemophilic mice after busulfan pre-conditioning. The SIV vectors were equipped with insulators to reduce the risk of insertional oncogenesis and were specifically designed to express FVIII in the B cell lineage by virtue of the immunoglobulin heavy chain enhancer-promoter [55]. FVIII was synthesized primarily in splenic B220+ B cells and CD138+ plasma cells resulting in sustained therapeutic FVIII levels and correction of the bleeding diathesis in hemophilic mice. Subsequent challenge with recombinant FVIII elicited at most a minor anti-FVIII antibody response, demonstrating induction of immune hypo-responsiveness. Therapeutic levels of FVIII could be obtained in secondary transplant recipients, confirming gene transfer into long-term repopulating HSCs.

Though these preclinical studies support the use of HSC-based gene therapy of hemophilia, moving this forward towards the clinic remains challenging: (i) pre-conditioning is required to facilitate HSC engraftment. Though mild conditioning regimen would be preferred based on safety considerations, it may compromise the overall efficiency and consequently result in lower clotting factor levels; (ii) the use of MGMT as a selection paradigm requires the administration of cytostatic drugs that are quite toxic and are used for cancer therapy; (iii) though the risk of insertional oncogenesis can be reduced, it cannot formally be excluded; (iv) ectopic clotting factor expression may not faithfully mimic all of the necessary post-translational modifications; (v) a large number of LV-transduced HSC may be required to obtain circulating clotting factor levels.

Ex vivo LV gene therapy: MSC and BOEC

MSCs have several characteristics that make them attractive targets for *ex vivo* gene therapy [91-95]. They are relatively easy to obtain, culture, expand into adequate numbers for clinical application and to transduce *in vitro*. MSCs have mild immunogenic profile, they are able both to self-renew and differentiate into the osteogenic, adipogenic and chondrogenic lineages and to contribute to both the parenchyma and perivascular zones of the engrafted organs. In contrast to the use of HSCs, there is no evidence of clonal dominance or insertional oncogenesis when MSCs are used, though this has not been addressed as rigorously as with HSCs [78,94]. In addition, the expanded cells can be transplanted without the need for preconditioning and without evidence for any adverse effects [94-97]. In the context of MSC-based hemophilia gene therapy, LV transduced MSCs were reported to express FVIII for over a 5-month period *in vitro*. Implantation of the LV transduced MSCs using collagen scaffolds into immune-deficient mice resulted in efficient engraftment of gene-engineered cells and long-term GFP expression *in vivo* [93]. In a recent study, autologous FIX-producing MSCs were loaded on porous scaffolds. When implanted in hemophilic mice, these scaffolds supported long-term engraftment and systemic FIX delivery by MSCs that corrected the hemophilic phenotype of most animals for up to 12 weeks [95]. In another study, paternal MSCs were transduced with a porcine FVIII-encoding LV and transplanted via the intraperitoneal route without preconditioning into hemophilic sheep. Although factor-independence and complete joint

recovery was achieved, a sharp rise in anti-FVIII inhibitors occurred following transplantation, decreasing the effectiveness and duration of this therapeutic regimen [94].

Similarly, blood outgrowth endothelial cells (BOECs) are abundantly available, can easily be isolated form peripheral blood, cultured, expanded, transduced and re-implanted. An additional advantage of BOECs as hemophilia gene therapy targets that they express and store the von Willebrand factor (VWF) in their cytosolic Weibel-Palade bodies. VWF is a natural carrier protein for factor FVIII, therefore co-storage and subsequent release of the VWF/FVIII complex has the benefit of secreting large amounts of FVIII at sites of vascular injury as well as directly increasing FVIII half-life by protecting FVIII from premature clearance and proteolytic degradation [89,98]. Intravenous administration of genetically modified BOECs resulted in sustained therapeutic or even supra-physiologic (1174 ng/mL) levels of FVIII over the 5 months *in vivo* in NOD/SCID mice [99]. This prompted another study, whereby BOECs were transduced with LV encoding FVIII. The transduced BOECS were implanted subcutaneously into NOD/SCID mice or into immunocompetent hemophilic mice using matrigel scaffolds. Therapeutic FVIII expression levels could be achieved for several months before they eventually returned to baseline levels, depending on the promoter used [100].

Conclusions and Future Perspectives

The recent advances in LV gene transfer technology have accelerated the development of safe and efficient gene therapy approaches that recently moved towards clinical applications. Though LV have not yet been used in the clinic for hemophilia gene therapy, long-term expression and phenotypic correction in pre-clinical hemophilic mouse models was achieved either by direct *in vivo* transduction of hepatocytes or by *ex vivo* gene therapy. In particular, hepatic LV gene delivery in hemophilic mice resulted in sustained clotting factor expression and induction of immune tolerance specific for the clotting factors, even in the face of active immunization. This may require fine-tuning of clotting factor expression to increase hepatocyte-specific expression, while preventing ectopic expression in APCs. This could be achieved through the use of hepatocyte-specific promoter/enhancers in conjunction with an additional layer of miR-regulation. Additionally, to further improve the clotting activities of FVIII and FIX, codon-optimization and protein engineering technologies can be applied [66,70,101,102]. LVs are well suited to achieve sustained hemostatic correction of hemophilia after genetic modification of HSC, MSC or BOEC. Although the use of integrating vectors may prompt concerns regarding the potential risks of insertional oncogenesis, clonal dominance and/or insertional oncogenesis has not been observed in adult hemophilic mice treated by LV gene therapy. The use of SIN LVs equipped with tissue-specific promoters and insulators further reduced this risk. Moreover, it is even possible to target LV integration into so-called "safe harbours" using engineered zinc-finger nucleases in conjunction with IDLV [5,103-107], though efficiency remains a bottleneck. To ultimate translate some of these principles towards the clinic, it will be important to first validate the efficacy and safety of these LV approaches in large animal models.

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