Gene therapy for hemophilia

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Gene Therapy for Inherited Bleeding Disorders

Introduction

- Gene therapy for hemophilia A and B benefited from advancements in the general gene ther field, such as the development of adeno-associated viral vectors, as well as disease-specific breakthroughs, like the identification of B-domain deleted factor VIII and hyperactivefactor IX Padua

- The gene therapy field has also benefited from hemophilia B clinical studies, which revealed for the first time critical safety concerns related to immune responses to the vector capsid not anticipated in preclinical models.

- Preclinical studies have also investigated gene transfer approaches for other rare inherited bleeding disorders, including factor VII deficiency, von Willebrand disease, and Glanzmann thrombasthenia.

Gene Therapy Approaches for Genetic Diseases

- Gene-replacement therapies for genetic disease require long-term expression of the therapeutic transgene (►Fig. 1) without toxicity.

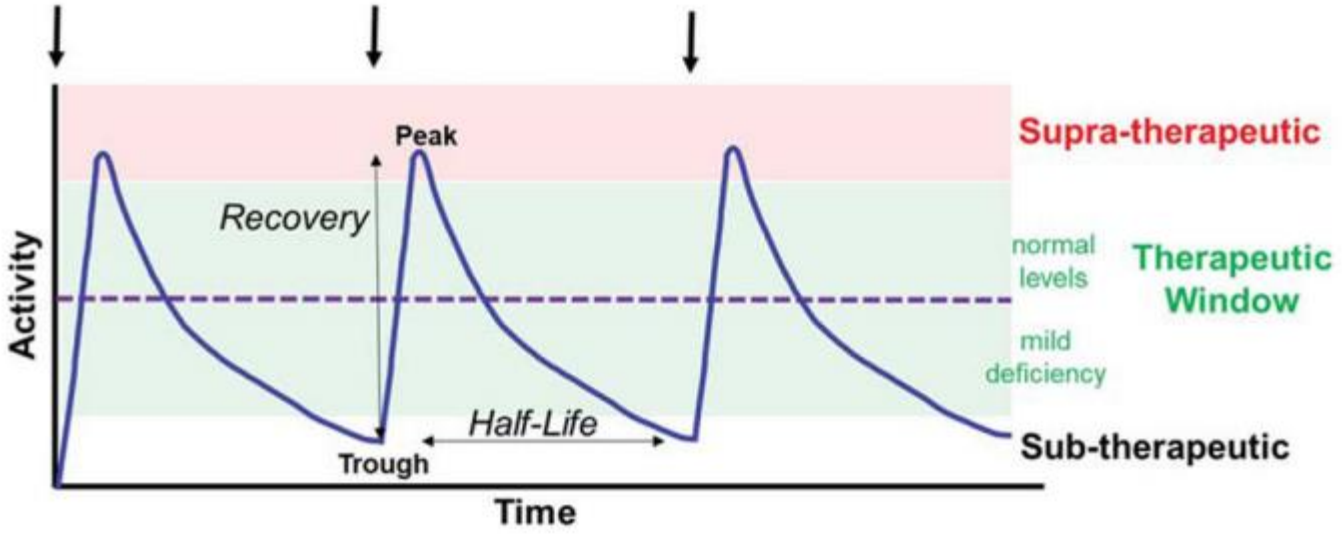

Fig. 1. (Color online) Theoretical pharmacokinetic limitations of replacement therapies compared with gene therapy. Multiple doses (top arrows) of replacement therapies are required to maximize the time within the therapeutic window (green), resulting in a saw-tooth shaped graph of activity versus time (blue solid line). To achieve a clinically feasible administration frequency, replacement therapy may result in peak levels in the supratherapeutic range and trough levels in the subtherapeutic range, which can be associated with thrombosis and bleeding, respectively. The time outside the therapeutic window will depend on the pharmacokinetics of the replacement therapy including the recovery and half-life. Replacement therapies with short half-lives, such as FVII and FVIIa, are especially at risk for this problem. The pharmacokinetics of transgene levels after gene therapy administration (purple dashed line) largely avoid this risk by providing the continuous stable expression levels.

- There are two main strategies:

(1) the use of nonintegrating vectors targeting non- or slowly dividing cells and

(2) the use of integrating vectors in dividing cells

- AAV is a replication-deficient virus that requires another virus for natural infection.
- AAV vectors rely on the transduction of nondividing cells to establish their permanence with the episome acting as an extrachromosomal piece of DNA that is continuously transcribed. This is ideal for hemophilia since both FIX and FVIII are naturally made in the liver, though the cell compartments for each protein is different (►Table 2).

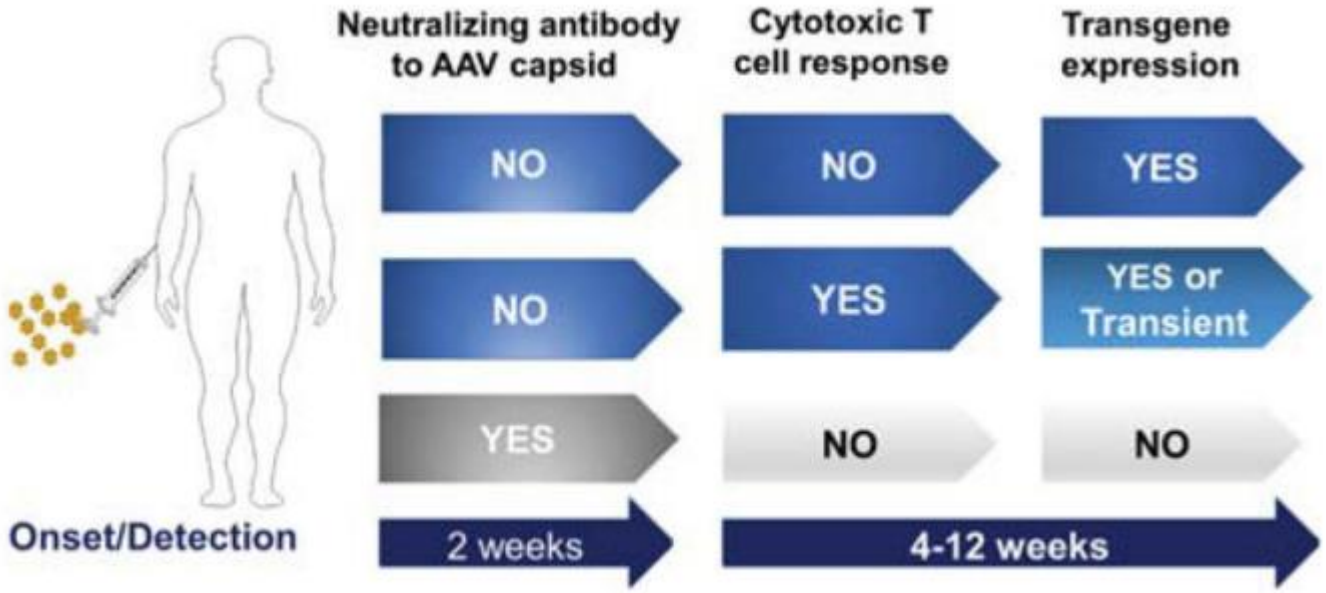

Fig-2.

impact of adaptive immune responses to AAV on gene transfer efficacy. Both neutralizing antibodies and cytotoxic T cells limit AAV-based gene transfer. In the absence of neutralizing antibodies and a cytotoxic T cell response, AAV liver-directed gene therapy results in transgene expression (top). The cytotoxic T cell response can limit transgene expression if not treated with immunosuppression (middle). Neutralizing antibodies to the AAV capsid can prevent efficient transduction for systemically administered AAV and prevent transgene expression (bottom). AAV, adeno-associated virus.

- There are two manufacturing systems used for the production of AAV vectors in ongoing clinical trials for hemophilia. In the mammalian system, cells are adhesive; whereas in the insect cell system, the cells are in suspension.
- AAV episomes can stably express their transgenes for the lifetime of the cell, shown in nonhuman primate (NHP) neurons for more than 15 years22 and HA and HB dog hepatocytes for more than 7 years.

Gene Therapy for Hemophilia

- Activated FIX (FIXa) is a serine protease that requires the cofactor activity of FVIIIa to efficiently activate factor X (FX), which is the rate-limiting step of sustained coagulation. HA and HB affect approximately 1:5,000 or 1:30,000 live-born males worldwide, respectively.

Gene Therapy for Hemophilia B

- The complementary DNA (cDNA) of F9 is approximately 1.6 kb, which is easily accommodated within the approximately 4.7kb packing capacity of AAV.
- Because of the small cDNA size and low risk of inhibitor formation, HB was an early target for AAV-based gene therapy.
- The clinical course of these subjects revealed major immunological obstacles that limit the efficacy of systemic AAV gene transfer (►Fig. 2)

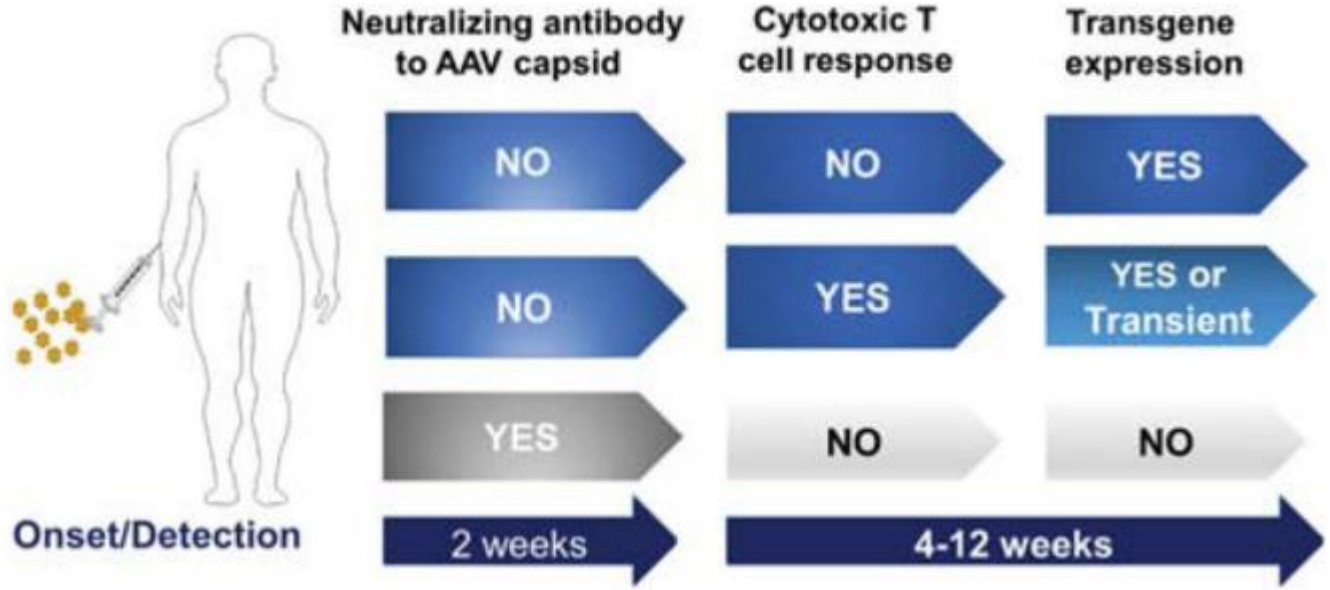

Fig-2.

- The enhanced efficacy of transgene FIX-Padua compared with FIX-WT was demonstrated in 2017 (►Table 1).

Table 1

Durability and dosing differences in published AAV hemophilia gene therapy trials

Transgene	Cell line	AAV serotype	Dose (vg/kg)	Steroids (Y/N)	Average activity (% of normal)	Vector ratio[a]	Follow-up (y)	Reference[b]
Hemophilia B								
FIX-WT	Mammalian	AAV8	2×10^{12}	Y	5	1×	8	44,45,112
FIX-WT	Insect	AAV5	2×10^{12}	Y	7	10×	3.3	56
FIX-Padua	Mammalian	AAV Spark100	2×10^{12}	Y	25	1×	4	54
FIX-Padua	Insect	AAV5	2×10^{12}	N	47	40×	1	55
Hemophilia A								
FVIII-BDD	Insect	AAV-5	2×10^{12}	Y	60		1	58
			2×10^{12}	Y	26		2	59
			2×10^{12}	Y	20		3	59
			2×10^{12}	Y	16		4	
FVIII-BDD	Insect	AAV-5	2×10^{12}	Y	21		1	59
			2×10^{12}	Y	13		2	59

[a] Compared with an empirical standard product defined by 1×.

[b] Published papers and some follow-ups in meeting abstracts and press releases as available.

- In this clinical trial, 10 severe HB subjects were administered an AAV8 vector encoding for FIX-Padua at a single dose of 5 × 1011vg/kg.54 Subjects demonstrated sustained FIX activity levels at approximately 25% of normal without any serious adverse effects.
- Similar benefits of using transgene FIX-Padua55 compared with FIX-WT56 were reported in the clinical development of an AAV5 vector for HB.
- This AAV5 vector was manufactured using an insect cell system, compared with the earlier AAV2 and AAV8 vectors that were manufactured using a mammalian system.
- Three of the 10 HB subjects that received AAV5 FIX-WT (one in the low-dose and two in the high-dose cohort) were treated with steroids for elevated transaminase levels,56 but there is no clear evidence that steroids provided a benefit.
- Currently, utilizing FIX-Padua is the gold standard for current gene therapy trials of HB. FIX-Padua is an example of a disease-specific breakthrough technology that accelerated gene therapy for HB. While initial trials considered even a modest increase of 1 to 5% of FIX to be a success, current trials reaching levels of 30% and higher have redefined what patients can expect in the outcome of such treatments.

Gene Therapy for Hemophilia A

- The F8 cDNA is approximately 7 kb, which exceeds the packaging capacity of AAV vectors.
- The solution to fit F8 within an AAV vector was the result of basic biological studies of FVIII.
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The Future of Gene Therapy for Hemophilia A and B

- The published descriptions and the ongoing pivotal clinical trials that have demonstrated therapeutically relevant factor levels with the resulting amelioration in the bleeding phenotype after AAV-based FVIII and FIX gene therapy without major safety concerns suggest that AAV drugs for HA and HB will likely achieve regulatory approval.
- The risk of integration and genotoxicity after AAV administration is likely low, though how low remains debated.62 However, both the likelihood of a cytotoxic T cell response and genotoxicity increase with increasing vector dose. Although these vector-dose-dependent risks are not well quantified, it does raise the question of how to balance these risks against the diminishing marginal hemostatic benefit of higher factor levels.
- Lastly, access to gene therapy needs to be expanded to all hemophilia patients, including those in developing countries as well as patients currently excluded from clinical trials such as inhibitor patients, children, and females

Gene Therapy for Rare Bleeding Disorders

• *FVII Deficiency*

- FVII deficiency is the most common autosomal recessive bleeding disorder, occurring in approximately two per million individuals.
- Most patients with FVII <2% of normal have a severe bleeding phenotype, encompassing approximately 20% of FVII-deficient patients.
- Menorrhagia and epistaxis are the most common bleeding symptoms, though life-threatening intracranial bleeds occur, especially in infants.
- FVII has the shortest half-life of the coagulation factors (~5 hours), which is only modestly longer than the half-life of FVIIa (~2 hours).

• *Factor VII*

- FVII is a vitamin K-dependent coagulation factor.
- human FVII/FVIIa does not bind mouse TF; thus, the hemostatic efficacy of human FVII/FVIIa therapeutics cannot be evaluated in murine models

Preclinical Studies of Gene Transfer of FVII and FVIIa

- AAV liver-directed FVII gene transfer has produced promising results in large-animal models. Administration of a relatively low dose (4 × 1011 vg/kg) of AAV5 with human FVII to adult NHPs (n = 6) resulted in 7% of normal FVII levels, which are likely therapeutically relevant.77 Interestingly, no difference in expression between female (n = 3) and male (n = 3) NHPs was appreciated.
- administration of an AAV8 vector encoding canine FVII zymogen to FVII-deficient male dogs (n = 4) also resulted in sustained FVII levels without safety concerns.78 There was a vector-dose-dependent increase in plateau FVII levels with clinically relevant vector doses (6 × 1011–2 × 1012 vg/kg) resulting in FVII levels >10% of normal.
- An alternative gene therapy approach to FVII deficiency could be delivery of transgene FVIIa, rather than zymogen FVII.
- The potential advantage of this approach would be that the same gene therapy product could be used to treat HA and HB with and without inhibitors as well as theoretically FVII deficiency

Future Directions of Gene Therapy for FVII Deficiency

- Zymogen FVII levels that are likely therapeutic or near-therapeutic have been expressed with translational doses of AAV vectors in adult large-animal models.
- since FVII deficiency is an autosomal disease, more preclinical studies are needed to address sex as a modifier of AAV liver gene therapy efficacy.

von Willebrand Disease

- Congenital VWD encompasses a spectrum of genetic diseases due to defective or deficient von Willebrand factor (VWF) due to mutations in the VWF gene.
- Patients with a severe bleeding phenotype typically have Type 3 VWD (T3VWD) with a complete absence of VWF due to biallelic VWF mutations.
- Patients with severe disease benefit with decreased bleeding rates from prophylactic therapy with VWF concentrates.

von Willebrand Factor

- Circulating VWF is multimeric glycoprotein responsible for initiating platelet aggregation at the site of injury as well as carrying most circulating FVIII.
- VWF is synthesized primarily in EC as well as megakaryocytes.
- VWF occurs intracellularly throughout the secretory pathway and results in VWF concatemers that are heterogeneous in length ranging from 2 to >100 subunits chained together in an end-to-end fashion.

Preclinical Gene Transfer Studies of VWF

- The inherent complexity of the VWF protein and its large cDNA size pose challenges for gene transfer.
- At 8.4 kb, the cDNA of the VWF limits the use of AAV vectors, though not LV, which is more suitable for dividing cells such as ECs.
- Given the problems of inefficient multimerization of ectopically expressed VWF, alternative targets for VWF gene transfer are attractive.
- An early proof-of-concept study demonstrated successful ex vivo gene transfer of functional VWF using a LV vector into blood outgrowth ECs (BOECs) from T3VWD dogs.

- The cDNA of VWF greatly exceeds the packaging capacity of AAV vectors, though this obstacle in not unique for VWF and several strategies to allow for AAV-based gene transfer of large genes have explored.101 One application of a dual AAV vector system for VWF transfer worked in vitro, but failed in vivo.

Future Directions of VWD Gene Therapy

- The large cDNA of VWF limits the vectors available for VWD gene therapy.
- In contrast to FVIII-BDD,46 there are no truncated variants of functional VWF. LV and dual-vector AAV systems both have potential for VWF transfer.

Glanzmann Thrombasthenia

- GT is an inherited qualitative platelet disorder due to dysfunctional or absent αIIbβ3 integrin that is an instructive example of gene therapy for a primary platelet disorder.
- GT is recessively inherited with pathological variants occurring in either the neighboring αIIb (ITGA2B) or β3 (ITGB3) genes on chromosome 17.
- Integrin αIIbβ3 is essential for platelet plug formation by mediating activation and cross-linking of adjacent platelets through ligand binding.
- The development of antiplatelet alloantibodies is a major risk of platelet transfusions occurring in approximately 30% of all patients, but much higher (up to 80%) in patients with a complete αIIbβ3 deficiency

αIIbβ3 Integrin

- The αIIbβ3 integrin is the most abundant protein on the surface of platelets with ligands including fibrinogen, VWF, and fibronectin.
- Signals originating inside the cell during platelet activation induce a conformational change in α IIbβ3 that enhances ligand binding (inside-out signaling); conversely, ligand binding results in intracellular signal transduction (outside-in signaling) mediating multiple cellular processes for hemostasis.

Preclinical Gene Transfer Studies of αIIbβ3 Integrin

- A limited number of preclinical studies have demonstrated the feasibility of gene therapy for GT by administration of ex vivo LV-transduced HSC after bone marrow conditioning.
- In conditioned β3 knockout mice, administration of transduced HSCs resulted in circulating platelets with approximately 10% of normal αIIbβ3 levels.

Future Directions of GT Gene Therapy

- Although the limited preclinical studies of ex vivo HSC-directed LV gene therapy have demonstrated a phenotypic improvement in GT models, they have also exposed substantial obstacles including major bleeding complications during bone marrow conditioning and the risk of an immune response against the transgene.

Conclusions

- The increasing number of approved gene therapy products and the likely approval of AAV-based gene transfer drugs for HA and HB have engendered optimism that similar treatments could be developed for other monogenic bleeding disorders.
- Though the rationale for gene therapy of other rare inherited bleeding disorders is well established by the benefits of replacement therapies, and especially prophylactic treatment, development of translational gene therapy for these diseases will likely require additional breakthroughs.
- The cellular target needs to be carefully considered.
- Early skeletal-muscle-directed studies of FIX gene transfer established the safety of AAV vectors in human subjects.
- The field has subsequently moved to hepatocyte-directed AAV approaches, in a large part because of the ease of IV administration.

- AAV-based gene transfer for FVIII has also focused on hepatocyte-directed approaches, mostly because of the existing technologies that allowed for successful FIX hepatocyte-directed gene transfer, even though FVIII is endogenously expressed in EC.
- The hypothesis is that the endogenous source of a protein may have unique cellular machinery that facilitates sustainable expression.
- These observations raise the question of whether EC or megakaryocytes could better multimerize transgene-VWF.
- The risk of an immune response to the transgene in another issue.
- Gene therapy for HA and HB have both greatly benefited fromvariant transgenes, FVIII-BDD, and FIX-Padua, respectively, that were identified by studying the basic biology of FVIII and FIX, respectively.
- Recently, FIX-Padua was successfully substituted for FIX-WT in uniQure NV trials for HB gene therapy product with predictable increases in FIX activity levels and without restarting clinical development.
- Likewise, the experiences of systemically administered AAV in infants with SMA15 and Crigler–Najjar will inform on treatment of infants in other diseases, including FVII deficiency. A better understanding of differences between transgene expression in male and female subjects after AAV liver-directed gene therapy will also be critical for autosomal disorders.

A Molecular Revolution in the Treatment of Hemophilia

INTRODUCTION

- Therapy for hemophilia is being completely transformed by diverse,
- disruptive molecular therapies.

- Congenital hemophilia A and B are X-linked bleeding disorders caused by mutations of the F8 gene or F9 gene, which lead to deficiencies of coagulation factor VIII (FVIII) or IX (FIX), respectively.
- Hemophilia is characterized by painful and often spontaneous hemorrhages into joints and soft tissues that are life-threatening if intracranial, gastrointestinal, or in the neck/throat.
- FVIII or FIX level (normal range is 50–150 IU/dL) typically correlates with bleeding severity: <1 IU/dL of normal is classified as severe hemophilia, 1–5 IU/dL as moderate, and 5–50 IU/dL as mild.
- Development of FVIII- or FIX-neutralizing alloinhibitory antibodies (inhibitors) is currently the most serious complication of treatment

- Many novel molecular therapies are currently being developed that promise to transform hemophilia care and patients' quality of life. Gene therapy aims to provide sustained factor levels with a single treatment (Figure 1),

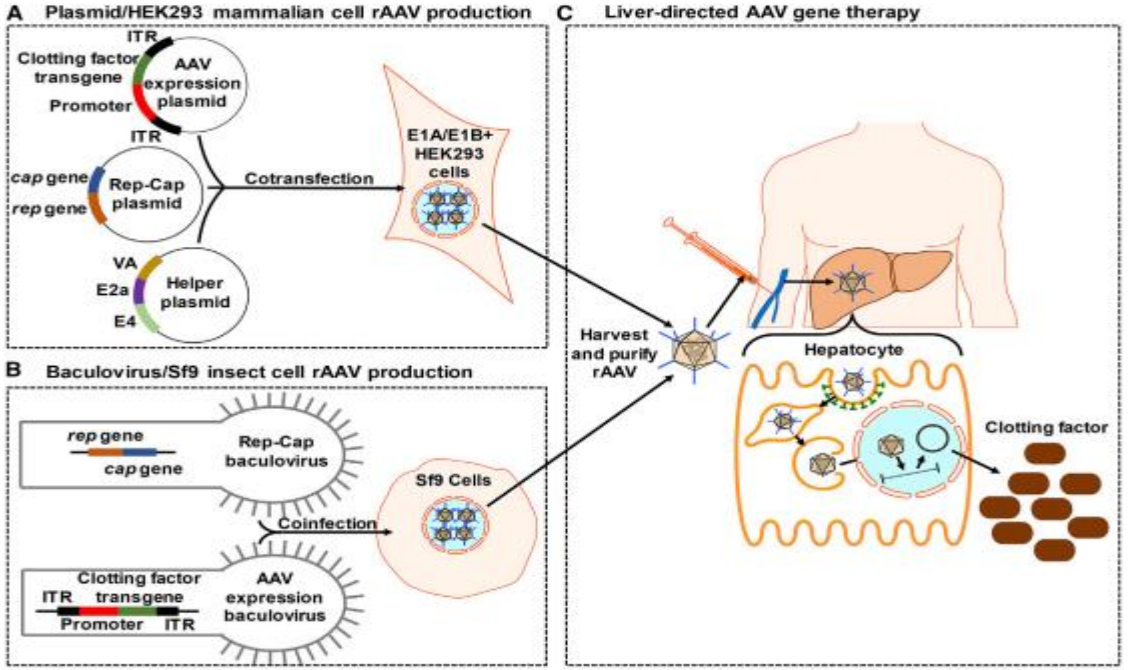

Figure 1. AAV Gene Therapy

(A) Plasmid/HEK293 mammalian cell rAAV production: HEK293 cells are cotransfected with (1) AAV expression plasmid containing the clotting factor transgene and tissue-specific promoter, flanked by ITRs, (2) plasmid containing the *rep* and *cap* genes, and (3) helper plasmid containing adenovirus genes. (B) Baculovirus/Sf9 insect cell rAAV production: Sf9 cells are coinfected with (1) AAV expression baculovirus containing the clotting factor transgene and tissue-specific promoter, flanked by ITRs, and (2) baculovirus containing the *rep* and *cap* genes. (C) Liver-directed AAV gene therapy: rAAV is harvested by freeze-thawing transfected/infected cells, purified, and then injected intravenously. The transgene expression is targeted to hepatocytes using a liver-specific promoter and capsid with strong liver tropism. rAAV binds to a serotype-specific host cell receptor and is internalized by a clathrin-coated dynamin-dependent pathway into the endosomal compartment.[33–38] The rAAV is quickly transported using microtubules to the perinuclear region and undergoes conformational changes that expose regions of its capsid to allow for escape from the endosome and nuclear localization signals for trafficking into the nucleus.[36–38] The clotting factor is expressed after AAV uncoats its capsid and converts to a circular double-stranded DNA episome by annealing of plus and minus strands delivered to the same cell or second-strand synthesis using host polymerase.[39,40] rAAV, recombinant adeno-associated virus; ITR, inverted terminal repeat.

- while non-replacement therapies mimic procoagulant activity of the missing clotting factor or enhance coagulation by inhibiting physiological anticoagulants (Figure 2).

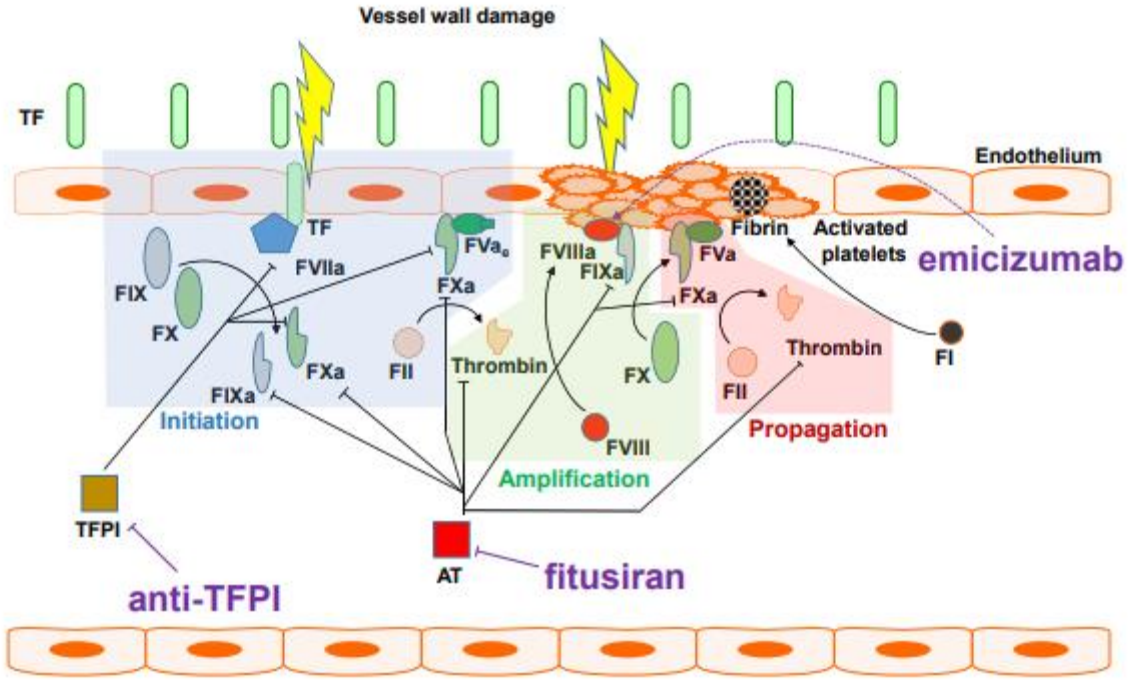

Figure 2. Key Events and Physiologic Inhibitors of Secondary Hemostasis Targeted by Non-replacement Hemophilia Therapies

Upon vessel wall injury, normally subendothelial tissue factor (TF) is exposed to blood and binds to activated FVII (FVIIa), thus enhancing its catalytic activity. The TF-FVIIa complex generates small amounts of FIXa and FXa. FXa and early (partially activated) forms of FV (FVa_e) associate on negatively charged phospholipids (via calcium ions) exposed on damaged endothelial cells and activated platelets, where they form an early prothrombinase complex, which generates minute amounts of thrombin (initiation phase) by cleavage of FII (prothrombin). In the amplification phase, thrombin activates multiple coagulation factors, including cell surface-bound FVIII, FV, and FXI. FVIIIa enhances the catalytic activity of FIXa, which activates FX. FXa and thrombin-activated FV generated during the amplification phase now propel a thrombin burst on the surface of activated platelets (propagation phase), leading to cleavage of FI (fibrinogen) and formation of the fibrin clot.[41,42–44]

Emicizumab: FVIIIa in Disguise of an Antibody

- Emicizumab is a subcutaneous humanized bispecific IgG4 monoclonal antibody that mimics a key function of activated FVIII (FVIIIa):
- bridging FIXa and the FX zymogen to accelerate activation of the latter.
- To that end, the antibody recognizes FIX/IXa with one Fab arm and FX/Xa with the other (Figure 3).

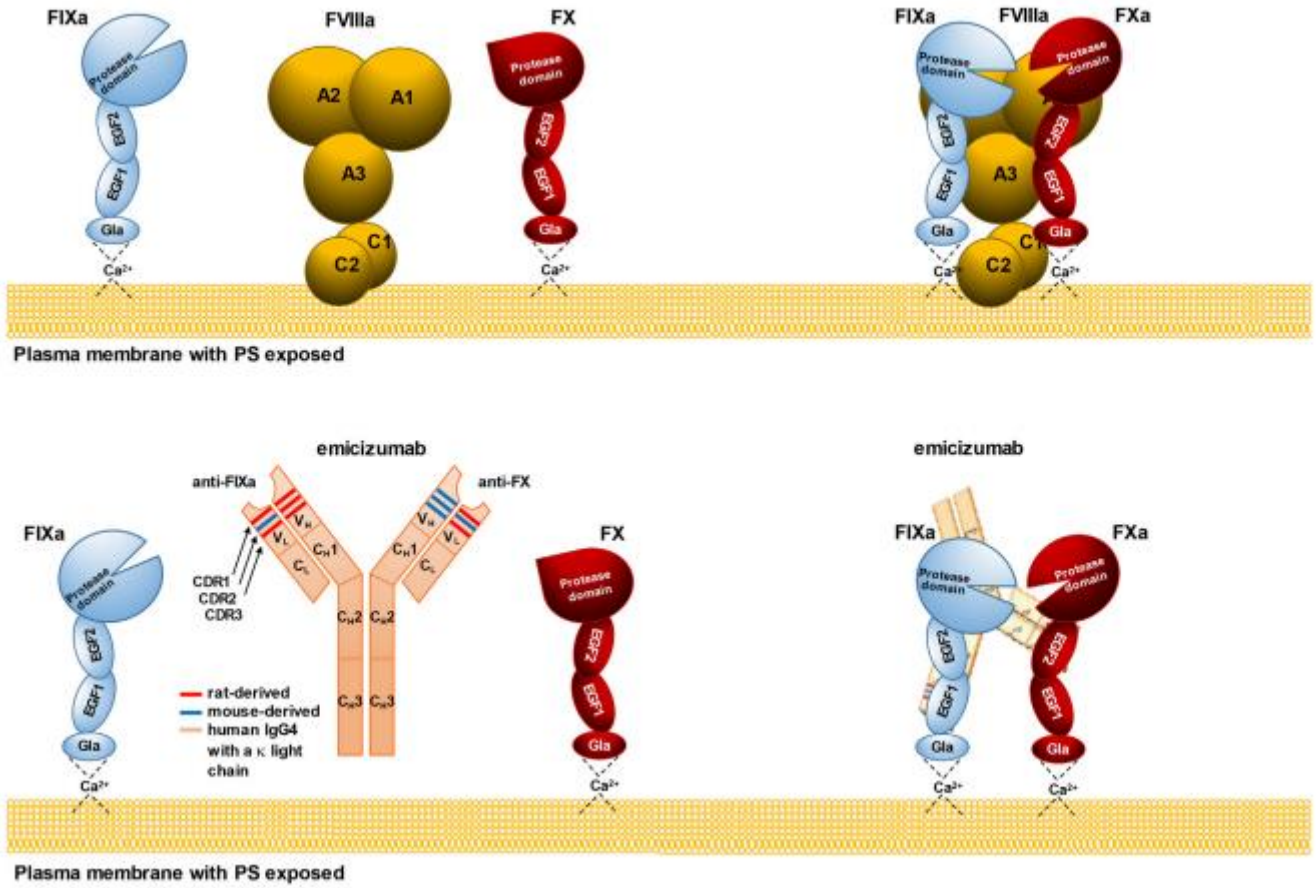

Figure 3. Structure and Activity of FVIIIa and Emicizumab

FVIIIa forms a complex with activated FIX (FIXa, a serine protease) and FX (the zymogen), so that FIXa can activate the latter. In this reaction, FVIIIa functions as a molecular scaffold for FIXa, whose protease activity toward FX is otherwise 10^5- to 10^6-fold lower and insufficient to drive the thrombin burst and, ultimately, optimal blood coagulation. Emicizumab is an asymmetric humanized bispecific anti-FIXa/anti-FX antibody with mouse and rat complementarity determining regions (CDRs) grafted on a human immunoglobulin (IgG4) with a kappa light chain. While FVIIIa binds multiple sites on FIXa and FX, emicizumab recognizes single epitopes within epidermal growth factor-like domain 1 (EGF1) of FIXa and EGF2 of FX. Unlike FVIIIa, emicizumab does not bind negatively charged phospholipids (e.g., PS [phosphatidylserine]), but their presence is necessary for its procoagulant activity, which suggests that bridging FIXa and FX in proper orientation requires that they sit on the cell surface.[47,48]

- Despite being able to mimic FVIIIa, emicizumab differs from it in several important ways that have implications for its efficacy, safety, and laboratory monitoring (Table 1).

Table 1. Differences between FVIII and Emicizumab

Molecule Name	Structure	Sites of Interaction with Substrate (FIXa and FX)	Affinity	Binding Specificity	Role of Phospholipids	Activation and Inactivation	Route of Administration	Immunogenicity	Half-Life (days)	Relationship between Dose and Activity	Relative Procoagulant Activity
Emicizumab	asymmetric bispecific humanized mAb; consists of a rat anti-FIX V_H, a mouse anti-FX V_H, and a common rat-mouse hybrid V_L grafted on the human IgG4 framework with a κ light chain[53,56]	binds single sites with each Fab arm within the EGF-like domain 1 of FIX/IXa and EGF-like domain 2 of FX/Xa[48]	low (K_D = 1.85 µM for FX and 1.52 µM for FIXa)[48]	binds both zymogens (FIX and FX) and activated FIX and FX (FIXa and FXa)[48]	does not bind phospholipids but its procoagulant activity still requires presence of phospholipids[47]	activation not required (always "on"); not susceptible to inactivation by dissociation, cleavage, or by alloinhibitory FVIII-neutralizing antibodies[47]	s.c. (86% bioavailability)[53]	in clinical trials, 14/398 (3.5%) trial participants developed neutralizing anti-drug antibodies, 3 of whom showed decline in response to therapy and 1 of whom discontinued emicizumab and resumed pre-study treatment due to a loss of efficacy[47]	21[47]	bell-shaped (peak at 265 µg/mL, >5-fold the plasma concentration on prophylaxis with emicizumab)[47]	50 µg/mL maintained in patients on prophylaxis is equivalent to ~15 IU/dL FVIII (1 IU FVIII activity per ~333 µg of emicizumab but the relationship is non-linear)[47,48]
Factor VIII	heterodimeric glycoprotein consisting of metal ion-linked heavy (A1-A2-B domains) and light (A3-C1-C2 domains) chain[49]	binds FIXa and FX at multiple sites across several domains	high (K_D = 165 nM for FX and 7.3 nM for FIXa)[57,58]	binds FIXa and FX (zymogen) only	binds negatively charged phospholipids via the light chain (C1 and C2 domain)[49]	activated upon the onset of bleeding; inactivated by activated protein C, spontaneous dissociation of A2 domain, or by alloinhibitory FVIII-neutralizing antibodies	i.v.	30% of patients with hemophilia A develop alloinhibitory FVIII-neutralizing antibodies (inhibitors)	0.5	linear	1 IU of FVIII activity per ~0.2 µg of FVIII protein

V_H, heavy chain variable region; V_L, light chain variable region; Ig, immunoglobulin; mAb, monoclonal antibody; PK, pharmacokinetics; PD, pharmacodynamics; EGF, epidermal growth factor; s.c., subcutaneous(ly); i.v., intravenous(ly).

- In clinical trials, emicizumab was administered prophylactically once a week, every other week, or every 4 weeks, and these three regimens have been used after licensure.

Targeting Physiological Anticoagulants

- Individuals with hemophilia who co-inherit prothrombotic mutations
- may show milder bleeding phenotypes.

Anti-TFPI Antibodies

- Several TFPI-targeting molecules have been investigated, four of which are subcutaneous anti-TFPI antibodies that entered clinical trials including both hemophilia A and B patients with and without inhibitors.
- TFPI operates in a negative feedback loop that regulates the generation of FXa.

- The four antibodies that entered the clinic target K2 or both K1 and K2 domains of TFPI (Table 2).

Table 2. Anti-TFPI Antibodies that Entered Clinical Trials

Anti-TFPI Antibody (Company)	Characteristics	Bioavailability (for s.c. Administration)	Ongoing Clinical Trials	Completed Clinical Trials	Frequency, Route, and Dose in Active Trials	Mean ABR	Immunogenicity
Concizumab, mAb 2021 (Novo Nordisk)	murine mAb, humanized (IgG4); epitope within the K2 domain of TFPI	93%[74,75]	phase 2 (ClinicalTrials.gov: NCT03196297): efficacy and safety of once daily prophylaxis in patients with severe hemophilia A without inhibitors phase 2 (ClinicalTrials.gov: NCT03196284):efficacy of once daily prophylaxis in hemophilia A and B patients with inhibitors	phase 1 (ClinicalTrials.gov: NCT02490787): multiple dose, safety, PK, and PD of concizumab in hemophilia A subjects	once daily s.c., loading dose 0.5 mg/kg followed by 0.15 mg/kg (with potential stepwise dose escalation to 0.25 mg/kg)	7 (ClinicalTrials.gov: NCT03196297, hemophilia A without inhibitors) 4.5 (ClinicalTrials.gov: NCT03196284, hemophilia A and B with inhibitors)	ADAs detected in 6/53 (3 NAbs) participants of ClinicalTrials.gov: NCT03196297 and NCT03196284 studies, none of which caused a loss of efficacy[75]
Marstacimab, PF-06741086 (Pfizer)	fully human (IgG1) mAb, selected from a phage display library of scFvs derived from non-immunized human donors and converted to IgG1; epitope within the K2 domain of TFPI	100% (in cynomolgus)[76]	phase 2 (ClinicalTrials.gov: NCT03363321): safety, tolerability, and efficacy of long-term treatment in subjects with severe hemophilia A and B with or without inhibitors	phase 1 (ClinicalTrials.gov: NCT02531815): safety, tolerability, PK, and PD of single ascending dose in healthy subjects only; phase 2 (ClinicalTrials.gov: NCT02974855): safety, tolerability, PK, and PD multiple doses in subjects with severe hemophilia A and B, with or without inhibitors	once weekly s.c., 3 doses tested: 150 mg (after the first loading dose of 300 mg), 300 mg (no loading dose), and 450 mg (no loading dose)	–	ADAs detected in 15/32 (3 NAbs) participants of ClinicalTrials.gov: NCT02531815 study; all NAbs (3) detected 42 days after administration, when the drug effects had already dissipated, so impact on PK and PD unknown[77]
BAY 1093884 (Bayer)	fully human (IgG2) mAb, selected from a phage display library of scFvs derived from non-immunized human donors and converted to IgG2; epitope encompasses K1 and K2 domains of TFPI[78]	52% or 61% depending on the assay (in cynomolgus)[79]	none (phase 2 ClinicalTrials.gov: NCT03597022 study has just been terminated due to serious adverse events)	phase 1 (ClinicalTrials.gov: NCT02571569): safety, tolerability, and PK of increasing single doses and multiple doses in subjects with severe hemophilia A or B, with or without inhibitors phase 1 (ClinicalTrials.gov: NCT03481946): PK and PD in patients with severe hemophilia A and B, with or without inhibitors	–	–	no data
MG1113 (Green Cross)	murine mAb, humanized (IgG4)[80]	no data	phase 1 (ClinicalTrials.gov: NCT03855696): safety and tolerability of a single ascending dose in hemophilia A and B patients without inhibitors and healthy subjects	N/A	single dose s.c. and i.v., six doses: 0.5 mg/kg (s.c.), 1.7 mg/kg (s.c.), 3.3 mg/kg (s.c. and i.v.), 6.6 mg/kg (i.v.) in healthy subjects; 3.3 mg/kg (s.c.), 6.6 mg/kg, and 13.3 mg/kg (i.v.) in hemophilia subjects	–	N/A

ABR, annualized bleeding rate; mAb, monoclonal antibody; s.c., subcutaneous(ly); Ig, immunoglobulin; scFv, single-chain variable fragment; TFPI, tissue factor pathway inhibitor; PK, pharmacokinetics; PD, pharmacodynamics; NAb, neutralizing antibody; ADA, anti-drug antibody; N/A, not applicable.

- blocking the K2 domain unleashes both factors, extends the initiation phase of coagulation, and generates more FXa (Figure 4).

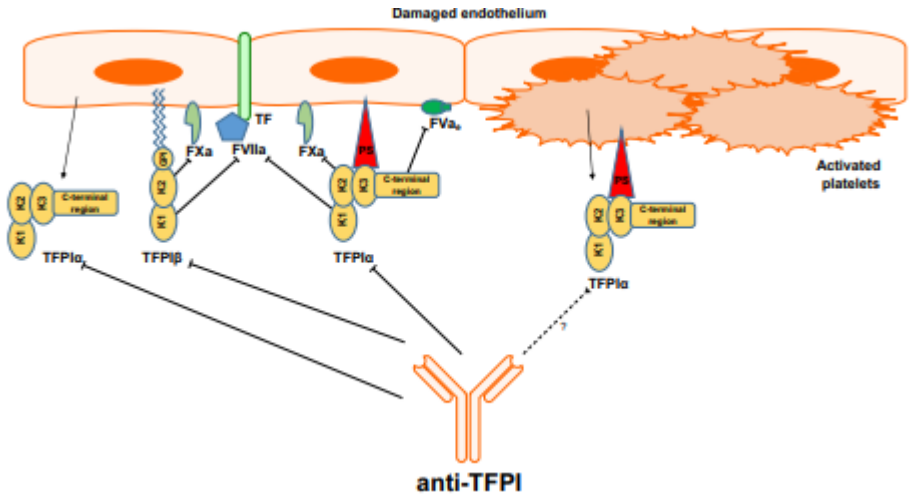

Figure 4. Key Isoforms of Tissue Factor Pathway Inhibitor and Their Activities

Two predominant isoforms, tissue factor pathway inhibitor (TFPI)α and TFPIβ, emerge through alternative splicing and so differ in structure and localization. TFPIα is the full-length isoform consisting of three Kunitz-type domains, one of which (K3) shows no inhibitory function, but it binds protein S (PS), which localizes the circulating TFPIα to cell membrane surfaces upon blood vessel injury. TFPIβ is a truncated form of TFPIα, missing part of the C terminus including the K3 domain, but instead it contains a glycophosphatidylinositol (GPI) anchor, which moors it to the cell membrane. Both isoforms block FXa and the TF-FVIIa complex with K2 and K1 domains, respectively, but TFPIα displays one more mode of FXa inhibition, which is by blocking its association with early (partially activated) forms of FVa (FVa_{ai}), and it operates in different mise-en-scènes. Endothelial cells and megakaryocytes are the main sites of TFPI expression. Megakaryocytes express TFPIα only, while endothelial cells produce both TFPIα and TFPIβ, so the GPI-anchored pool of TFPI resides on the endothelium. The plasma pool of TFPIα also comes from the endothelium, because the TFPIα synthesized by megakaryocytes is stored in platelets, which only release it upon activation. Although TFPIα is altogether the more abundant isoform, TFPIβ represents most of the TFPI that is readily available.[41] Anti-TFPI antibodies currently evaluated in clinical trials block K2 or both K1 and K2 domains of TFPI.

Fitusiran

- Fitusiran (ALN-AT3) is a subcutaneous double-stranded small interfering RNA (siRNA)
- targets the transcript of the SERPINC1 gene
- leading to its degradation by RNA-induced silencing complex (RISC)
- prevente translation (Figure 5).

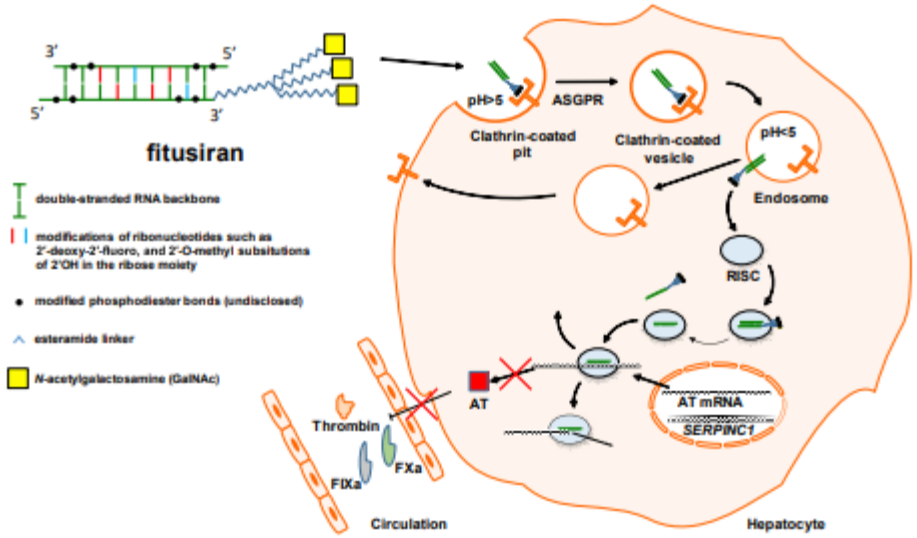

Figure 5. Antithrombin Knockdown by Fitusiran

Fitusiran is a double-stranded small interfering RNA (siRNA) with multiple chemical modifications, which protect it from degradation by nucleases and prevent innate immune sensing. The triantennary GalNAc moiety targets fitusiran to hepatocytes through asialoglycoprotein receptor (ASGPR, also known as Ashwell-Morell receptor) and clathrin-mediated endocytosis. Upon a drop in pH, the siRNA departs from ASGPR and exits the endosome. The cytosolic RNA-induced silencing complex (RISC) captures the molecule and ejects one strand, leaving the antisense strand to bind to antithrombin (AT) mRNA and induce its sequence-specific cleavage and degradation. This thwarts translation of the AT transcript, leading to a drop in blood AT levels.[93,94,95]

Phase 3 trials of fitusiran are ongoing (Table 3).

Table 3. Fitusiran Trials

Trial	Status	ClinicalTrials.gov Identifier	Frequency, Route, and Dose	AT Reduction (%)	Mean (median) ABR
Phase 1, single-ascending and multiple-ascending dose, safety, tolerability, and PK in patients with moderate or severe hemophilia A and B	completed	NCT02035605	once weekly 0.015–0.075 mg/kg s.c. or monthly 0.225–1.8 mg/kg s.c.	70–89	–
Phase 1/2, long-term safety and tolerability in patients with moderate or severe hemophilia A and B with or without inhibitors	ongoing	NCT02554773	once monthly s.c., fixed dose 50 or 80 mg	78–88	1.7 (1)
Phase 3 (ATLAS-INH): efficacy (ABR, HRQOL) of fitusiran relative to prior on-demand treatment with standard BPA in patients with hemophilia A and B with inhibitors	ongoing	NCT03417102	once monthly s.c., fixed dose 80 mg	–	–
Phase 3 (ATLAS-A/B): efficacy (ABR, HRQOL) of fitusiran relative to prior on-demand treatment with CFC in patients with hemophilia A and B without inhibitors	ongoing	NCT03417245	once monthly s.c., fixed dose 80 mg	–	–
Phase 3 (ATLAS-PPX): ABR and HRQOL relative to prior prophylaxis with CFC or standard BPA in patients with severe hemophilia A and B with or without inhibitors	ongoing	NCT03549871	once monthly s.c., fixed dose 80 mg	–	–

ABR, annualized bleeding rates; s.c., subcutaneous(ly); PK, pharmacokinetics; BPA, bypassing agent; ABR, annualized bleeding rate; HRQOL, health-related quality of life; CFC, clotting factor concentrate.

Gene Therapy

AAV Gene Therapy for Hemophilia B

- Adeno-associated virus (AAV) is a non-pathogenic single-stranded DNA parvovirus that is naturally replication deficient in the absence of helper virus co-infection.
- Recombinant AAV (rAAV) vectors lack sequences encoding Rep, Cap, and AAP and generally elicit only mild and transient innate immune responses compared to other viral vectors.
- A 2015 phase 1/2 clinical trial infused 15 hemophilia B patients with low-dose AAV-Spark100 vector expressing FIX-Padua (Table 4)

- A 2015 phase 1/2 clinical trial infused 15 hemophilia B patients with low-dose AAV-Spark100 vector expressing FIX-Padua (Table 4)

Table 4. Current and Planned Gene Therapy Clinical Trials for Hemophilia B

Sponsor	Treatment	Capsid Serotype	Promoter	Transgene Product	Dose (vg/kg)	Phase	Estimated Enrollment	Status	ClinicalTrials.gov Identifier
Freeline	FLT180a	synthetic	liver-specific	FIX-Padua	6e11–2e12	1	18	recruiting	NCT03369444
					LTFU	2, 3	50	recruiting	NCT03641703
Pfizer	fidanacogene elaparvovec (SPK-9001/PF-06838435)	Spark100	ApoE/hAAT	FIX-Padua	5e11	2	15	active, not recruiting	NCT02484092
					n.d.	3	55	recruiting	NCT03861273
Sangamo	SB-FIX: integration of corrective FIX transgene into albumin locus by AAV6-delivered ZFN	AAV6	–	–	n.d.	1	12	active, not recruiting	NCT02695160
SGIMI	YUVA-GT-P901: autologous HSC/MSC, modified with lentivirus encoding FIX	–	–	–	–	1	10	not yet recruiting	NCT03961243
Takeda	TAK-748 (SHP648/ AskBio009/BAX 335)	scAAV8	TTR	FIX-Padua	2e11–3e12	1, 2	30	active, not recruiting	NCT01687608
SJCRH	scAAV2/8-LP1-FIX	scAAV2/8	LP1	FIX	2e11–2e12	1	14	active, not recruiting	NCT0979238
UniQure	AMT-060	AAV5	liver-specific	FIX	5e12–2e13	1, 2	10	active, not recruiting	NCT02396342
	AMT-061		liver-specific	FIX-Padua	2e13	2	3	recruiting	NCT03489291
						3	56	recruiting	NCT03569891

FIX, clotting factor IX; LTFU, long-term follow-up; n.d., not disclosed; AAV, adeno-associated virus; SGIMI, Shenzhen Geno-Immune Medial Institute; TTR, transthyretin; ApoE/hAAT, apolipoprotein E enhancer/human alpha 1-antitrypsin promoter; ZFN, zinc finger nuclease; SJCRH, St. Jude Children's Research Hospital.

- ***AAV Gene Therapy for Hemophilia A***
- AAV vectors with genomes exceeding the _5-kb capsid packaging limit are truncated and have substantially reduced efficiencies, which has made the _7-kb length of FVIII coding sequence a significant challenge in the pursuit of gene therapy for hemophilia.

In Vivo Gene Editing Therapies for Hemophilia

A 2018 phase 1 trial infused SB-FIX, a hemophilia B gene therapy consisting of three

liver-tropic AAV2/6 vectors, each delivering one of the three components:

- a right or left zinc finger nuclease (ZFN)
- or normal F9 transgene.

The ZFNs are designed to place the normal copy of the clotting factor gene within the albumin intron 1, under control of the endogenous albumin locus promoter (Figure 6).

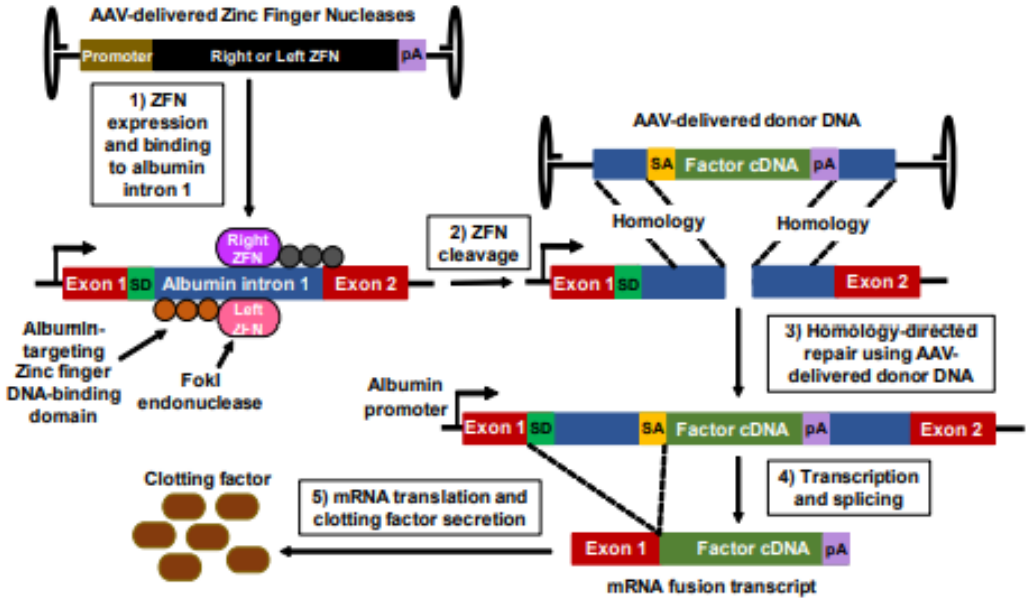

Figure 6. Gene Editing by AAV-Delivered ZFN and Donor DNA Template

The zinc finger nucleases (ZFNs) are designed to place the normal copy of the clotting factor gene within the albumin intron 1, under control of the endogenous albumin locus promoter. Three adeno-associated virus (AAV) vectors are delivered, each providing one of the three components: a right or left ZFN or clotting factor cDNA donor template.[140,141,143] (1) The AAV-expressed ZFN fuses a DNA-cleavage domain (FokI endonuclease) to a zinc finger DNA-binding domain. (2) The ZFN binding domain targets a specific sequence and then the cleavage domain induces a double-strand break. (3) The double-strand break can be repaired by homologous recombination if a DNA donor template is present with flanking arms homologous to the DNA at the ZFN cleavage site. (4) Flanking the clotting factor cDNA is a poly(A) sequence (pA) and a splice acceptor signal (SA), which splices the transcribed clotting factor RNA to the splice donor (SD) of albumin exon 1 RNA to produce an mRNA fusion transcript. (5) The mRNA fusion transcript is translated into secreted clotting factor protein.

- The double-strand break can be repaired by homologous recombination if a DNA donor template is present that has flanking arms homologous to the DNA at the Cas9 cleavage site (Figure 7).

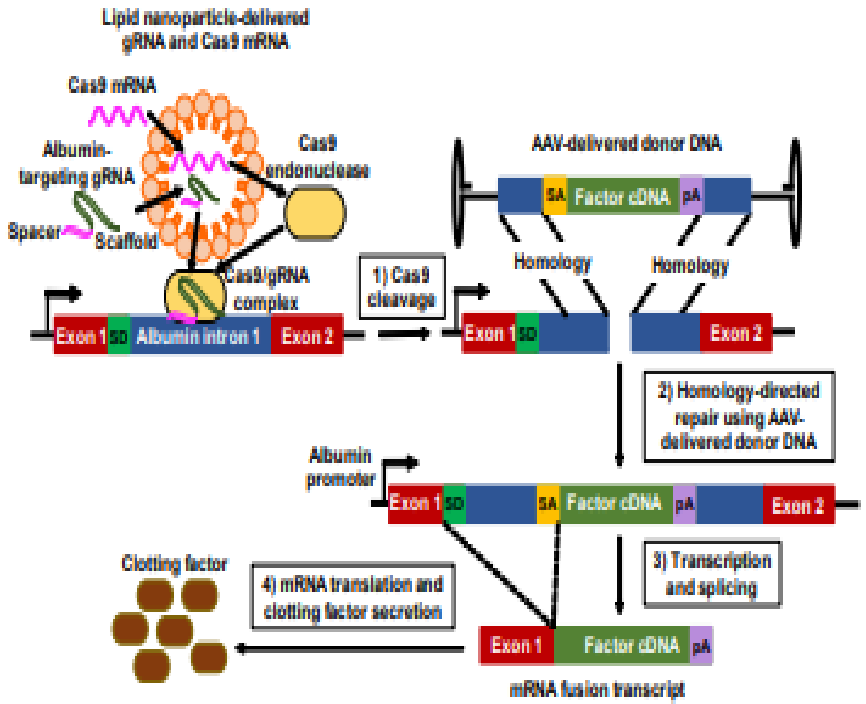

Figure 7. Gene Editing by Lipid Nanoparticle-Delivered CRISPR/Cas9 and AAV-Delivered Donor DNA

The CRISPR/Cas9 system consists of guide RNA (gRNA), which is a combination of two single-stranded RNAs, CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA), and a nuclease (Cas9) that form the Cas9-gRNA complex.[144] The CRISPR/Cas9 components can be delivered by lipid nanoparticle in RNA form.[145] (1) The gRNA scans the genome for a complementary sequence known as the protospacer adjacent motif (PAM) site, and cleavage by Cas9 occurs if the adjacent DNA sequence also matches the remaining gRNA.[144] (2) The double-strand break can be repaired by homologous recombination if a DNA donor template, delivered by adeno-associated virus (AAV), is present with flanking arms homologous to the DNA at the Cas9 cleavage site. (3) Flanking the clotting factor cDNA is a poly(A) sequence (pA) and a splice acceptor signal (SA), which splices the transcribed clotting factor RNA to the splice donor (SD) of albumin exon 1 RNA to produce an mRNA fusion transcript. (4) The mRNA fusion transcript is translated into secreted clotting factor protein.

Immune Tolerance Induction by Liver Gene Transfer

liver-directed gene therapy can induce tolerance against FVIII or FIX, through regulatory T cell induction.

Hepatic gene transfer with AAV2/8-expressing FIX was shown to reverse preexisting anti- FIX NAbs and desensitize anaphylaxis-prone hemophilia B mice.

Ex Vivo Gene Therapies for Hemophilia

employ lentiviral vectors in studies have several advantages over AAV:

- avoidance of gene dilution as the transgene is replicated with the host genome
- lower prevalence of preexisting anti-vector Nabs
- increased packaging limits (_8–10 kb)

Future Directions in Hemophilia Treatment

Inhibitors of natural anticoagulants, including:

- fitusiran and
- anti-TFPI antibodies

are far along in clinical trials, and, if licensed, will greatly facilitate prophylaxis in hemophilia A and B in general, and patients with inhibitors in particular.

Future treatment options may include prophylactic immune tolerance induction to prevent development of FVIII and FIX inhibitors.

Oral tolerance induction using transplastomic lettuce expressing FVIII or FIX fused to a transmucosal carrier is at the forefront of research in this area.

Platelet-Targeted FVIII Gene Therapy Restores Hemostasis and Induces Immune Tolerance for Hemophilia A

INTRODUCTION

- Platelets are the second most common type of cells found in blood, with approximately 10^{11} newly produced daily to replenish the old platelets in the body.
- Aged platelets undergo apoptosis and are phagocytosed by scavenger cells in the spleen and liver
- platelets play fundamental roles not only in hemostasis and thrombosis but also in innate and adaptive immunity.
- Platelets are loaded with abundant bioactive proteins and circulate in the blood, serving as both a storage "depot" and trafficking "vehicle" in circulation so they be a unique target for gene therapy of diseases.
- ectopic expression of factor VIII (FVIII) in platelets directed by either the glycoprotein (GP) Ib or the GPIIb (aIIb) promoter can lead to the storage of FVIII in platelet a-granules and that platelet-derived FVIII can improve hemostasis in hemophilia A mice even in the presence of anti-FVIII inhibitory antibodies.

PLATELETS SHIELD NEOPROTEIN FROM BEING RECOGNIZED BY THE IMMUNE SYSTEM

Platelets could be an ideal target for gene therapy of hemophilia A as they can store neoprotein FVIII together with its carrier protein von Willebrand factor (VWF) in a-granules and act as delivery vehicles in blood circulation.

Plasma:

FVIII is undetectable in 2bF8-transduced recipients even with a platelet-FVIII level as high as 30–35 mU/108 platelets.

Neoprotein:

- FVIII stored in platelets may avoid direct exposure to the immune system during the normal physiological condition.
- effectiveness of platelet-targeted gene therapy has been further confirmed in hemophilia A rats and hemophilia A dogs

targeting FVIII expression to hematopoietic cells under a constitutively active promoter may trigger anti-FVIII immune responses.

Besides the promoter, other factors, e.g., protein properties, may also affect the efficacy of gene therapy.

THE PRESENCE OF PROTECTIVE PROTEIN VWF TO FVIII IN PLATELETS IS CRITICAL FOR OPTIMAL PLATELET GENE THERAPY OF HEMOPHILIA A

- VWF binds with FVIII non-covalently, which affects the expression and stability of FVIII.
- FVIII colocalizes with endogenous protein VWF in platelet a-granules when FVIII is targeted to platelets.

- Platelettargeted

• FIX gene therapy in hemophilia B mice (33, 34). When FIX expression is targeted to platelets under control of the same platelet-specific aIIb promoter used in the FVIII studies, greater than 90% of FIX is stored in platelets and is releasable upon platelet activation.

• The protective role of VWF in platelet-targeted FVIII gene therapy is revealed in its hemostatic function and in immune responses.

• in our platelet-targeted gene therapy protocol, the association of VWF and FVIII is pivotal for clinical efficacy in hemophilia A with inhibitors.

• The VWF/FVIII complex protect FVIII from being inactivated by the inhibitors after a burst of VWF/FVIII complex released at the site of injury.

PROPER PRECONDITIONING BEFORE GENE TRANSFER IS IMPORTANT FOR ACHIEVING SUSTAINED PLATELET-FVIII EXPRESSION AND IMMUNE TOLERANCE INDUCTION IN PLATELET GENE THERAPY

- Proper preconditioning is essential for immune tolerance induction in our platelet-targeted FVIII gene therapy protocol in restoring hemostasis and inducing immune
- tolerance in hemophilia A.
- The optimal preconditioning regimen for platelet-FVIII gene therapy to establish immune tolerance while achieving sustained platelet-FVIII expression is more stringent than that used to achieve sustained platelet-FVIII expression alone in unprimed hemophilia A mice.
- even after rhFVIII immunization, none of the recipients developed inhibitors in the groups preconditioned with an optimized preconditioning regimen, 6.6Gy TBI or busulfan plus ATG.
- a lethal dose of irradiation (11Gy TBI) may severely disrupt the intestinal immune system (42), which may impact Treg cell homeostasis in the body.

PERIPHERAL TOLERANCE IS ESTABLISHED AFTER PLATELET-TARGETED 2bF8 GENE THERAPY

- both primary and secondary anti-FVIII immune responses are CD4 T celldependent
- immune tolerance is transferable when splenocytes from 2bF8- transduced recipients were infused into naive FVIIInull mice.
- Immune tolerance established in 2bF8 lentivirus-transduced recipients is mediated by the CD4 T cell compartment.
- antigen-specific CD4 T cells were deleted in peripheral lymphoid organs (spleen and lymph nodes), but not in the thymus, and antigen-specific Treg cells were expanded after platelet-targeted OVA gene transfer.
- The OVA model study reveals that there are
- dual underlying mechanisms that are responsible for establishing antigen-specific immune tolerance after platelet-targeted gene therapy.

CONCLUSION

Platelets play fundamental roles not only in hemostasis

- and thrombosis but also in innate and adaptive immunity.
- platelet-targeted FVIII gene therapy is effective in treating hemophilia A mice even with inhibitors.
- Platelettargeted gene therapy is a unique approach for gene therapy of hemophilia A even with inhibitors as it can provide not only therapeutic protein but also induce antigen-specific immune tolerance.

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