The human coagulation system has evolved over the past 450 million years to function through a complex coordinated interaction involving the vessel wall, circulating cells, and a protein cascade of procoagulants that are regulated by a series of anticoagulant mediators. In normal hemostatic responses, these various components combine to generate a protective fibrin clot, limiting blood loss from the circulation. In pathological states, deficiencies, dysfunction, or aberrant regulation of these hemostatic elements result in either bleeding or thrombotic phenotypes. These pathologies are the consequence of either rare genetic traits or, more often, a complex interaction of genetic and acquired influences.

Current Therapeutic Approaches to Coagulation Disorders

Current treatments for bleeding and thrombotic diseases comprise either targeted single factor approaches in which specific elements of the hemostatic process are replaced or inhibited or combination strategies that either restore or interfere with multiple components of the hemostatic response. Although an
initial vasoconstrictive response to vascular damage is crucial for the early phase of hemostasis (eg, reduced blood flow, platelet margination, and unfolding of von Willebrand factor), specific therapies aimed at this early element of hemostasis are not routinely used. In contrast, cellular therapies to enhance the contributions of platelets (adhesion and aggregation) and red cells (contributing to platelet margination and fibrin clot strength) to the generation of an optimal hemostatic response are frequently applied to the treatment of bleeding. Currently, most therapies used for coagulation disorder management are aimed at replacing, enhancing, or inhibiting components of the procoagulant protein cascade and the corresponding anticoagulant and fibrinolytic response.

Procoagulant Treatment Approaches

Interventions aimed at preventing or treating bleeding can involve either single factor strategies or combination replacement therapy. The more common inherited bleeding disorders, hemophilia A (factor VIII [FVIII] deficiency) and B (factor IX [FIX] deficiency), von Willebrand disease, and factor XI deficiency, can be treated with single factor replacement therapies through the infusion of protein concentrates derived from either plasma or recombinant DNA technology. In contrast, most acquired bleeding disorders, such as vitamin K deficiency, liver disease, and consumptive coagulopathies, are managed with the infusion of either defined multifactor concentrates (eg, prothrombin complex concentrates, factors II, VII, IX, and X), cryoprecipitate, von Willebrand factor, factors VIII, XIII, and fibrinogen) or plasma.

The efficacy of these various treatment regimens can be monitored through clinical assessment and by the performance of either global tests of hemostasis, such as the prothrombin, partial thromboplastin, and thrombin times, or by quantification of single factor levels as in the replacement of FVIII and FIX in the hemophilias.

Gene Therapy Concepts and Historical Context

The concept of using genetic approaches to treat and potentially cure disease originated in the early 1980s with the cloning of the first human genes. The first, approved, and successful gene therapy study in a human was conducted in 2 patients with inherited adenosine deaminase deficiency in 1990. Since then, the field of gene therapy has had a chequered history, but in 2012, the European Medicines Agency approved for the first time a gene therapy drug Glybera, a gene therapy treatment for inherited lipoprotein lipase deficiency. Now in 2015, there is realistic optimism that this therapeutic modality can offer significant opportunities for long-term benefits in a range of disorders.

The coagulation genes were among the first to be characterized in the 1980s, with major contributions derived from Earl Davie’s laboratory at the University of Washington (Figure 1A). These discoveries confirmed that the coagulation proteins were derived from genes which encoded modular domains that were shared between related families of coagulation factors (eg, the vitamin K–dependent factors and factors V and VIII). Soon after the cloning of the FVIII and FIX genes, translational application of this knowledge was initiated through the introduction of molecular diagnostics, the development of recombinant clotting factors for replacement therapy, and the initial preclinical trials of gene therapy.

The hemophilias are an ideal model genetic disease for the application of gene therapy. They result from recessive mutations in 2 well-characterized genes with minimal influence from other genetic modifiers. Furthermore, it was well recognized from the natural history of moderately severe and mild hemophilia (clotting factor levels between 1% and 40%) that small increments in plasma clotting factor levels would result in significant benefits in reducing the risk of bleeding. Thus began the 30-year quest to convert what seems to be a simple therapeutic goal, to deliver a normal gene copy to compensate for the existing mutant gene.

Spectrum of Gene Therapy Initiatives for Coagulation Disorders

It is clear, for the reasons detailed earlier, that the hemophilias have been leading candidates for the application of gene therapy since the dawn of molecular medicine. However, advances in the development of gene therapy strategies for other pro- or anticoagulant deficiencies has been modest, a fact that relates to the combination of factors, including the infrequent incidence of monogenic inherited bleeding and thrombotic disorders, the existence of safe and effective alternative therapies, and some inadequate biological knowledge to effectively design gene therapy strategies.

Aside from FVIII and FIX gene therapy, the only other procoagulant targets that have been explored to any extent in preclinical studies have been von Willebrand factor and factor VIIa. The former target might find a place for the treatment of the rare type 3 form of von Willebrand disease (incidence 1 per million), but even in this population, with no detectable circulating von Willebrand factor, spontaneous bleeding rates can often be surprisingly low. The limited activated factor VII gene therapy studies have been focused predominantly on the potential for developing a sustainable, long-term strategy for bypassing the presence of FVIII antibodies (FVIII inhibitors) in hemophilia A.

Gene therapy for anticoagulant purposes has been similarly underdeveloped. Again, this relates to the availability of safe and effective alternative treatments and to the fact that monogenic traits directly responsible for initiating thrombosis are rare. Finally, in contrast to the hemophilias, the levels of transgenic protein expression required to produce a phenotypic benefit are likely to be beyond the capability of current gene transfer technologies.

One thrombotic disorder where gene therapy has produced promising preclinical results is the inherited form of...
a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13) deficiency (Upshaw–Schulman Disease). Studies in the mouse model of this condition, using an adenovirus–mediated approach have documented sustained therapeutic levels of transgenic ADAMTS13 expression and a positive effect on the clinical features of the disorder. However, with safe and effective treatment already available for this rare genetic disease through infrequent (every 3–4 weeks) infusion of plasma and the development of a recombinant ADAMTS13 protein, the likelihood of a gene therapy approach reaching the clinic in the foreseeable future is small.

Gene Therapy Strategies for Coagulation Disorders

The therapeutic goal for coagulation factor gene therapy is the delivery of a normal gene copy to produce therapeutically effective levels of the protein that is either deficient or dysfunctional as a result of a germ line mutation (Figure 1B).

Several strategies for genome editing, using technologies such as zinc finger nucleases and transcription activator-like effector nucleases, have the potential for in situ mutation correction. The application of these approaches has proven effective in vitro and in animal studies, but none of the methodologies are close to clinical introduction. Zinc finger nuclease editing has also been applied successfully to target therapeutic transgene insertion to a safe and transcriptionally favorable locus. The latest technology to be applied to genome editing uses guide RNA molecules to target the site for revision, and the Cas9 endonuclease to mediate the double strand cleavage event required for sequence correction with a normal donor molecule (clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeat–associated 9 [CRISPR-Cas9]). There is considerable enthusiasm in the molecular biology community concerning the utility of this technology, and the momentum of CRISPR-Cas9 advances has been dramatic. However, despite the fact that mutant coagulation gene editing has already been reported in vitro, several critical limitations to this technology remain. The achievable efficiency of mutation correction is still low, and perhaps more problematic is the potential for damaging off-target editing events elsewhere in the genome. Similar weaknesses exist for earlier editing approaches involving zinc finger nuclease repair and TALEN–based strategies. For the present, substantial improvements in both efficiency and safety will need to be developed before any genome editing approach could be considered for clinical application as a treatment for coagulation factor deficiency or dysfunction.

Transgene Delivery Approaches

The first critical hurdle to overcome in any gene transfer protocol is the efficient delivery of the therapeutic transgene to somatic cells of the patient (Figure 2A). This goal can be achieved by either in vivo or ex vivo approaches, and there are advantages and weaknesses to both strategies. Ex vivo transgene delivery requires the isolation of a recipient cell population that can be readily transduced and has the potential for prolonged survival after delivery back into the recipient. The advantages of this approach are that efficient rates and
cell-type specificity of transgene delivery can be achieved (Figure 2B) and that the immunologic complications of in vivo vector administration are avoided. However, ex vivo strategies are inherently complex and resource-intensive, and re-establishment of the genetically modified cells may require some form of conditioning to ensure engraftment. In contrast, in vivo gene transfer is straightforward to perform, but ensuring efficient transgene delivery to specific cell types, without inciting immune reactions to the delivery vehicle, is challenging.

A single human clinical trial for ex vivo delivery of a FVIII transgene has been performed for hemophilia A. This study, involving 12 patients, used autologous fibroblasts that were electroporated ex vivo with a FVIII transgene, selected for optimal FVIII expression, expanded, and subsequently injected into the omental membrane. There were no adverse events associated with these studies, but also no evidence of sustained FVIII transgene expression, although several patients showed evidence of transient (2–3 days) minimal increments of plasma FVIII levels (2%–6%). Other preclinical studies of ex vivo transgene delivery for hemophilia have involved hematopoietic stem cells, mesenchymal stem cells, and endothelial progenitors. Results from the hematopoietic stem cell studies have been especially promising and may advance to a phase I/II clinical trial in the near future, but the endothelial progenitor investigations have been complicated by the generation of immune responses to the transgene product.

One particular ex vivo strategy for ectopic hemophilia gene therapy deserves further commentary. The generation of FVIII transgenes that are delivered to and expressed exclusively in megakaryocytes and platelets has shown promise in promoting an effective hemostatic response in standard animal models of hemophilia A and in the presence of FVIII inhibitory antibodies. Whether this strategy can be sufficiently optimized in terms of transgene delivery and protein production remains to be established. One potential approach to circumvent the requirement for bone marrow conditioning is the use of intraosseous vector infusion to mediate in vivo hematopoietic cell transduction. Using a lentiviral-based strategy with a platelet-specific regulatory sequence, long-term persistence of intra-platelet FVIII has been demonstrated at sufficient levels to mediate hemostatic protection in the presence of anti-FVIII antibodies.

### Modes of Transgene Delivery

The goal of transgene delivery is to introduce the therapeutic gene construct as efficiently and safely to the recipient, usually to selected types of somatic cells. Importantly, avoidance of delivery to germ cells is a requirement for all clinical studies because the safety of germ line modification remains unresolved.

There are essentially 2 modes of transgene delivery: the application of viral vectors or nonviral vector–mediated strategies. Some of the latter approaches have been used for in vitro molecular biology studies since the 1970s, but in general, physicochemical protocols are relatively inefficient delivery approaches for achieving long-term persistence of transgenes. Electroporation has also been applied in some ex vivo protocols but again lacks sufficient efficiency for routine clinical translation. Finally, localized application of hydrodynamic gene transfer protocols has been proposed as a further approach to deliver expression plasmids to specific organs or regions of organs. However, the inevitable, albeit transient, vascular and local tissue damage caused by this mode of delivery initiates a strong innate immune response, thus increasing the likelihood of a secondary adaptive response to the neoantigenic transgene protein.

In marked contrast to the limitations of the various nonviral modes of transgene delivery, viral vectors use

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**Figure 2. Methods for transfer of coagulation factor genes.** **A.** Currently used strategies (clinical or preclinical) for transfer of coagulation factor genes include in vivo and ex vivo approaches. In vivo methodologies involve inserting the coagulation factor cDNA into a viral vector genome, with subsequent introduction of the vector into the patient by intravenous or intramuscular injection. Ex vivo gene transfer involves isolation of autologous stem cells. Isolated cells are transduced with the normal coagulation factor gene, selected for protein expression, expanded, and then reintroduced into the patient. **B.** Cellular targets for in vivo transfer of coagulation factor genes include hepatocytes and skeletal muscle. Cellular targets for ex vivo gene transfer approaches involve isolation and transduction of autologous stem cells including hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and endothelial progenitor cells (EPCs). AAV indicates adeno-associated virus.
properties that have evolved over millions of years to facilitate the efficient entry of viruses into cells. The generation of viral vectors involves the modification of the viral genome to eliminate the possibility of viral replication and to provide sufficient space for insertion of the therapeutic transgene in a viral particle that can still be efficiently packaged. Subsequent to the generation of the modified vector genome, the vector particles are produced in cell culture, providing packaging and structural proteins from cotransfected helper plasmids.

Gene therapy clinical trials for hemophilia have used 3 types of viral vector (Figure 3A). One patient received an adenoviral-mediated treatment for hemophilia A; there has been one trial of retroviral gene transfer of FVIII, and the remaining studies have used AAV vectors to deliver FIX transgenes (Table).

In contrast to AAV vectors that are discussed later, adenoviral vectors are not appropriate for the delivery of coagulation transgenes, in large part because of the significant proinflammatory innate immune response that their capsid proteins incite. This response can be limited to the development of fever, thrombocytopenia, and the elevation of liver transaminases, but can, under certain circumstances, advance to a fatal systemic inflammatory state such as that which killed Jesse Gelsinger during a trial of ornithine transcarbamylase gene therapy. Although γ-retroviral vectors are no longer being pursued for hemophilia therapy, the development of lentiviral vectors, capable of transducing both dividing and nondividing cells, has continued. Lentiviral vectors can be used as the means for transgene delivery either in ex vivo strategies for modifying stem cell populations or via systemic in vivo delivery. Both strategies have been used successfully in animal models of hemophilia, and some form of lentiviral-based treatment may well enter the clinic in the future. Nevertheless, in 2015, the clearly favored vector system for clinical coagulation gene therapy was AAV.

### AAV-Mediated Gene Therapy for Hemophilia

The development of AAV-based protocols for gene transfer of FIX has been one of the highlights of clinical gene therapy advances in the past 5 years. AAVs are small infectious agents (~20 nm) that belong to the family of paroviruses. In humans, infection with AAV is asymptomatic. AAV has a small single-stranded DNA genome of ≈4.7 kb, with 2 open reading frames that encode capsid and other structural proteins and proteins facilitating integration of the wild-type virus into a specific region on chromosome 19. In the construction of AAV vectors, both these coding regions are deleted, allowing the insertion of transgenes of ≤5 kb without adversely influencing the packaging efficiency of vector particles. This packaging limitation has not affected the generation of FIX transgenes, in which the coding sequence spans ≈1.3 kb. In contrast, the FVIII cDNA occupies ≈8.5 kb, and even after deletion of the nonessential B domain–encoding sequence, the cDNA is still ≤5 kb. Thus, promoter/enhancer elements for AAV FIX VIII constructs have to be compact, and even then, a reduction in vector packaging efficiency is a significant concern (Figure 3B).

To date, human clinical trials of skeletal muscle and liver-directed AAV FIX gene therapy have been conducted. In each instance, strong tissue-specific promoter/enhancer

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**Figure 3. Viral vectors for in vivo gene transfer.** A. Gene therapy clinical trials for hemophilia have used adenovirus (1 patient), γ-retrovirus (12 patients), and adeno-associated virus (AAV: 31 patients). B. Adenovirus-associated inflammation and inadequate transduction and transgene expression by γ-retrovirus have discontinued their use in coagulation factor gene therapy. One challenge of AAV-mediated gene therapy is the limited packaging size of the recombinant vector genome. Strategies for overcoming this limitation include cDNA contraction and the use of compact promoter/enhancer elements. ITR indicates inverted terminal repeat.
elements have been used, and the vectors have been delivered by either intramuscular injection or portal or peripheral vein injection for the skeletal muscle and liver studies, respectively. Tissue tropism of the AAV vectors is influenced by the differential efficacy for transduction mediated by serotype-specific capsids.\textsuperscript{56–58} Thus, AAV8 possesses strong hepatotropism and can be delivered efficiently to the liver via peripheral vein infusion without the risk of significant transduction of other tissue types.

### Fate of AAV Vectors

Transduction by AAV vectors involves an interaction with a range of serotype-specific cell surface receptors.\textsuperscript{56–58} The vector particles are taken into endocytic vesicles, where, in the acidic milieu of the endosome, the capsid is removed and the nucleic acid released for transport to the nucleus.\textsuperscript{59} The capsid proteins are then degraded by the proteasome, and the resultant peptides presented on the transduced cell’s surface, in association with major histocompatibility complex class I molecules (Figure 4B). The vector genome enters the nucleus and persists long term, largely as circular extrachromosomal concatemers. However, AAV-based transgenes will integrate into host cell genomes at sites of natural chromosome fragility, a process that is accompanied by small adjacent insertion/deletion events.\textsuperscript{60} Although integration into the host genome is a rare phenomenon for AAV, the large vector particle doses that are administered in clinical gene therapy (\( \approx 10^{12} \) particles/kg) results in millions of transduced cells with AAV insertions. There has been a single report of increased tumor incidence (hepatocellular carcinoma) in a mouse AAV gene transfer model,\textsuperscript{60} but these observations were made in old normal and mucopolysaccharidosis VII mice after injection of the vectors in neonates. This outcome has not been replicated in any other long-term small or large animal model of AAV therapy.

### Results of AAV-Mediated Gene Therapy for Hemophilia

To date, \( \approx 31 \) hemophilia B patients have been treated in 5 AAV gene therapy trials (Table). A clinical study using AAV to deliver FVIII has just enrolled its first patient. In the hemophilia B trials, initial studies involved intramuscular injection of the vector, showing that long-term expression of the FIX transgene was achievable.\textsuperscript{43,62,63} Latterly, hemophilia gene therapy trials have targeted the liver for transgene delivery using either portal vein infusion or, more recently, peripheral vein injection of hepatotropic AAV8 vectors.

The FIX transgene constructs that have been generated have used small and potent tissue-specific enhancer/promoter elements, and one study has used a codon optimized FIX cDNA.\textsuperscript{45} In the most recent FIX clinical trial, the FIX transgene also contains a gain-of-function missense variation (Arg338Leu–FIX Padua\textsuperscript{64,65}) that increases the specific coagulant activity of the transgenic protein by \( \approx 7 \)-fold.

For native single-stranded AAV transgenes to mediate mRNA expression, a second complementary DNA strand has first to be synthesized. In efforts to accelerate this process, some investigators have constructed self-complementary AAV transgenes that mediate earlier expression after delivery.\textsuperscript{57} Vector doses in the clinical trials have ranged between \( 2 \times 10^{11} \) and \( 5 \times 10^{12} \) vector genomes/kg.

After transgene delivery, FIX plasma levels start to increase after 2 to 3 weeks and have, until the most recent study using the FIX Padua transgene, resulted in FIX levels of 2% to 10% in the short term and persistent levels of 1% to 6%.\textsuperscript{43,45,46} Preliminary results from the FIX Padua study, in patients receiving one of the higher vector doses, have shown transient levels of FIX \( > 50 \) that have subsequently fallen to levels similar to the other studies that have been reported.\textsuperscript{66} In the context of hemophilia, these relatively low levels of FIX have had significant benefits in terms of
Challenges to Gene Therapy for Hemophilia

The first obvious fact is that progress with FIX gene therapy has been significantly advanced compared with FVIII. This is because of a combination of limitations presented by FVIII. First, the FVIII cDNA, even with the B domain—encoding region deleted, is ≈4.5 kb, and thus, generation of FVIII transgene constructs that can be efficiently packaged in AAV particles is difficult. Second, achieving high-level expression of FVIII has been notoriously problematic, for reasons that have been only partially explained. Finally, the immunogenic potential of FVIII is significantly greater than that of FIX, and preclinical studies of FVIII gene transfer have frequently been complicated by the development of an anti-FVIII immune response. In contrast, liver-directed AAV gene therapy in hemophilic dogs with anti-FVIII antibodies has also shown potential for inducing immunologic tolerance to FVIII. Despite these various challenges, a new AAV-mediated FVIII gene therapy study has just been initiated, and the outcome of all of these trials is ongoing.

In contrast to the challenges presented by FVIII gene transfer, the studies of FIX delivery have not been complicated by immune responses to the transgenic FIX protein, and in the latest clinical trials, plasma levels of transgenic FIX have been adequate to produce a significant long-term clinical benefit. Nevertheless, the predictability and consistency of these FIX levels are still not well understood.

The most significant problem that has arisen in the recent AAV clinical trials has been the development, in ≈25% of patients, of an anti-AAV capsid immune response that has resulted in transduced hepatocyte cytotoxicity. This complication is the result of CD8+ cytotoxic T cell responses to AAV capsid peptides being presented on the surface of transduced hepatocytes (Figure 4A). The timing of the subsequent transaminitis that develops has varied from as early as 3 weeks to as late as 10 weeks post vector delivery, probably reflecting either a secondary recall response or a later primary immune response to the AAV capsid. With the aim of minimizing this immunologic problem, patients with evidence of preexisting anti-AAV immunity, as demonstrated by the presence of anti-AAV antibodies, are currently excluded from participation in these clinical trials. This affects different numbers of patients for the different AAV serotypes but ranges from ≈30% of the population with antibodies to AAV8 to ≈60% of the population with preexisting anti-AAV2 immunity. Unfortunately, even with the generation of novel AAV capsids, the issue of cross reactivity of the immune response may still be an obstacle to avoiding the subsequent cellular immune attack.

With the development of a cytotoxic T cell response to AAV capsid peptides, transduced liver cells are killed and the transgene is lost, thus prevention or rapid intervention of this immune attack is essential to achieve long-term transgene expression. To date, mitigation and eventual extinction of the immune response has been possible with transient corticosteroid administration started after early recognition of liver transaminase increases. Although the use of prophylactic immunosuppressive therapy for this complication has been discussed repeatedly, this strategy has not yet been used. Finally, in addition to the cell-mediated immune response to the AAV capsid, a potent humoral response is consistently documented after AAV vector administration that prevents effective repeat AAV delivery. This obstacle to vector readministration might be circumvented by strategies such as infusing capsid decoys to divert the antibody blockade.
Future Considerations for the Application of Gene Therapy for Coagulation Disorders

After a 30-year period of in vitro experimentation and pre-clinical assessment, the field of gene therapy is beginning to show robust evidence of clinical benefit in a range of genetic disorders. The recent success of gene transfer for hemophilia B highlights the potential of this therapeutic modality for management of coagulation pathologies. As further evidence of the promise of gene therapy initiatives, the partnership in hemophilia gene therapy trials by the biopharmaceutical industry has increased dramatically in the past 2 years. Nevertheless, before gene therapy can be extended to widespread clinical utility, several critical hurdles need to be overcome. First, is the development of gene therapy vectors that can be produced in large scale with high and reproducible quality. Evidence that the AAV vectors used in a recent FIX clinical trial contained only 10% of transgene containing particles illustrates the need for improved and more efficient vector production protocols. With current clinical trials being limited to study populations of 5 to 10 patients, there is a long way to go before the widespread application of gene transfer can be envisaged.

Next is the issue of immune obstacles to successful gene transfer. With levels of preexisting immunity to current AAV-based vectors ranging from 30% to 60%, many otherwise eligible patients are excluded from this form of treatment. Whether AAV capsid modifications or the use of novel AAV serotypes will circumvent this obstacle remains to be seen. Similarly, the use of other vector types, such as lentiviral constructs, would substantially reduce this problem. Aside from the problems posed by immune responses to the vector, immune reactions to the novel transgenic protein may also complicate some applications of gene transfer, particularly when the transgenic protein presents peptide sequences that are novel to the recipient. Although immediate adverse effects of gene transfer using AAV and lentiviral vectors have been negligible, the long-term outcome of gene therapy will require formal monitoring, particularly for genotoxicity outcomes. Studies performed in small and, more critically, large animal models with greater longevity have not shown any evidence of an enhanced incidence of chronic pathologies (most importantly, no evidence of increased cancer development). Nevertheless, these observations will need to be strengthened by formal long-term, multi-year surveillance in human gene therapy recipients. Finally, the efficacy of gene therapy in contrast to currently available or next-generation infused factor replacement therapies will need to be evaluated in randomized clinical trials.

The promise of genetic therapies for improved management of coagulation disorders is now beginning to be realized. Although gene replacement strategies are the most promising of these approaches, the application of inhibitory oligonucleotides and small inhibitory RNA molecules to alter the hemostatic balance has demonstrated how other nucleic acid–based strategies have shown considerable potential in recent clinical trials. With enhanced access to genome editing technologies, this momentum toward translational benefits is likely to continue.

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