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Animal Models of Hemophilia

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Abstract

The X-linked bleeding disorder hemophilia is caused by mutations in coagulation factor VIII (hemophilia A) or factor IX (hemophilia B). Unless prophylactic treatment is provided, patients with severe disease (less than 1% clotting activity) typically experience frequent spontaneous bleeds. Current treatment is largely based on intravenous infusion of recombinant or plasma-derived coagulation factor concentrate. More effective factor products are being developed. Moreover, gene therapies for sustained correction of hemophilia are showing much promise in pre-clinical studies and in clinical trials. These advances in molecular medicine heavily depend on availability of well-characterized small and large animal models of hemophilia, primarily hemophilia mice and dogs. Experiments in these animals represent important early and intermediate steps of translational research aimed at development of better and safer treatments for hemophilia, such as protein and gene therapies or immune tolerance protocols. While murine models are excellent for studies of large groups of animals using genetically defined strains, canine models are important for testing scale-up and for longer-term follow-up as well as for studies that require larger blood volumes.

Keywords

hemophilia; coagulation; factor VIII; factor IX; gene transfer; gene therapy; murine model; canine model; adeno-associated virus; lentivirus; cell therapy; factor VIIa

I. The Hemophilia A mice

A. Hemophilia A

Hemophilia A is an X-linked bleeding disorder due to a deficiency in factor VIII (FVIII). It is the most prevalent form of hemophilia representing 80% of hemophilia cases. Genetic defects in the FVIII gene that cause hemophilia A include point mutations, deletions, insertions and gene inversions.¹ Current treatment for hemophilia is protein replacement given either prophylactic or in response to bleeding episodes. The major complication of this treatment is the development of an immune response to the protein that occurs in 25–30% of patients.

FVIII is a critical component of the intrinsic blood coagulation pathway. The FVIII protein is composed of A1-A2-B-A3-C1-C2 domains. The secreted form of the protein is a heterodimer composed of a 90–200 kDa heavy chain (A1-A2-B) and an 80 kDa light chain (A3-C1-C2). The B-domain is not essential for FVIII activity. Upon cleavage by thrombin, FVIII forms a heterotrimer consisting of an A1 subunit, A2 subunit and A3-C1-C2 subunit that is the active form of the protein. Activated FVIII (FVIIIa) is a cofactor for FIX in the tenase complex. In the presence of FVIIIa, phospholipid and Ca^{2+} , FIXa cleaves FX to generate the activated form (FXa). FVIII is stabilized by vWF in the circulation and the concentration of FVIII is 100–200 ng/ml in the plasma.

The murine FVIII (mFVIII) is 74% identical to human FVIII (hFVIII).² The A and C domains are 84 and 93% identical, respectively, while the B-domain is the most divergent (55% identical). The domain structures as well as the processing and activation of FVIII are similar in mouse and humans. The ability to assess hemostasis in a mouse model is an important tool for preclinical studies for novel therapeutics for hemophilia A.

B. The hemophilia A mouse model

While a colony of the naturally occurring hemophilia A dog model had been established in 1947, hemophilia mouse models were not available until the 1990's (All murine and canine as well as alternative animal models of hemophilia are summarized in Table 1).³ As novel therapeutics including gene therapy approaches were being developed, initial studies were performed in wild type mice or the hemophilia A dog model. The wild type mice were not able to provide information about hemostasis while the studies in the dog model were limited by the expense and the long generation time of the dog. Almost 10 years after the human FVIII (hFVIII) cDNA sequence was described⁴, the mFVIII cDNA sequence was cloned.² Shortly thereafter, Dr. Haig Kazazian and colleagues generated mice with FVIII deficiency using standard gene targeting techniques to establish the first murine model of hemophilia.⁵

After isolation of a genomic clone containing exons 15 through 22 of the mFVIII gene, two separate neomycin cassettes were used to generate the hemophilia A mice, one targeting exon 16 and a second targeting exon 17 (both in the A3 domain of the light chain). The exon 16 knockout mice were generated by insertion of the neomycin cassette into the 3' end of that exon causing abnormal splicing of the RNA.⁵ The exon 17 knockout mice have the neomycin cassette inserted into the 5' end of exon 17 that leads to exon skipping. Mutations in both of these exons cause hemophilia A with a severe phenotype (<1% FVIII residual activity). Analysis of liver RNA demonstrated that the exon 16 knockout mice have a truncated protein and exon 17 mice have a truncated or partially deleted protein.⁶ While there is no detectable light chain in either knockout strain, these mice do have low levels of heavy chain protein in the circulation.^{6, 7}

Phenotypically, the exon 16 and exon 17 knockout mice are indistinguishable. The homozygous knockout female and hemizygous knockout male mice have <1% activity while the carrier females have been reported to express ~75% of normal FVIII activity. In contrast to humans with severe hemophilia, the mice do not appear to have frequent spontaneous bleeding episodes, however, bleeding in response to trauma is often lethal. However, in a subcolony of the exon 17 mice it was reported that spontaneous bleeding that was lethal occurred in 22% of hemizygous males and 10% of homozygous females.⁸ Due to their fragile phenotype, gentle handling of these animals minimizes fatal bleeding events. The use of forceps while handling mice, ear tags and surgical procedures all increase the likelihood of trauma induced internal or external bleeding. After tail transection, affected hemophilia A mice have excessive bleeding and die within several hours, while carrier females and wild type mice survive the procedure. Homozygous females survive pregnancy and delivery of

the litters with rare complications. Thus, breedings between hemizygous affected males and homozygous affected females are feasible with litter sizes of 5–8 pups.

While the original hemophilia A mice were established on a C57BL/6/129sv genetic background, other lines have been established on different strains. The hemophilia A exon 16 mice have been backcrossed onto the C57BL/6, Balb/C, and non-obese diabetic/severe combined immunodeficiency (NOD-SCID) backgrounds. The NOD-SCID hemophilia A mice have impaired B and T cell lymphocyte development and provide the opportunity to evaluate novel therapeutics in the absence of an immune response to FVIII.⁹

C. Assessment of hemostasis in hemophilia A mouse models

Assessment of coagulation function in the hemophilia mice after protein infusion or expression of a FVIII transgene has been hampered by variation in the assays that are performed. The hemophilia A mice have <1% FVIII activity which provides a control background for detection of even low levels of FVIII activity. The human, canine, porcine as well as the murine FVIII protein are efficacious in the hemophilia A mouse, however, differences in the specific activity and/or stability of these proteins from different species has been observed.^{10; 11; 12} Species-specific reagents may provide the most appropriate assessment of FVIII activity.

In vivo assessment of FVIII function is performed using a variety of different assays including the tail vein transection assay as well as more recently developed models of thrombosis and hemostasis.¹³ The tail transection assay measures both venous and arterial bleeding.¹⁴ Variation in the bleeding with this assay is often due to differences in the amount of the tail that is clipped, how the blood is collected and how long the blood is collected.¹⁵ Despite this variation, there is consensus that prolonged bleeding without intervention is lethal. The cremaster injury model measures thrombus formation following laser injury to a small arteriole in the cremaster muscle by monitoring platelet accumulation at the site of injury.¹⁶ The ferric chloride carotid artery occlusion model uses ferric chloride to induce an injury to the vessel wall and then measures blood flow and the time to occlusion using a Doppler probe.¹⁷ The primary difference between these two in vivo injury models is that the cremaster model assesses the microcirculation while the ferric chloride model measures thrombus formation in the macrocirculation. The use of both in vitro and in vivo assays to determine FVIII function in the hemophilia A mouse model provides important tools for development of therapeutics.

D. Arthropathy in the murine model

Hemophilia patients experience arthropathy and impaired wound healing secondary to joint hemorrhage. While these are non-lethal complications of the disease, these sequela have a debilitating impact on the quality of life in these patients. Without regular protein treatments, hemophilia A patients have 30–35 joint hemorrhages per year that causes significant morbidity.¹⁸ By age 25, 90% of hemophilia A patients have chronic degenerative changes in 1–6 joints.¹⁹ The hemophilia A mouse has been used to develop a model of the bleeding into the joints that causes inflammation of the synovial membrane leading to structural damage within the joint termed hemophilic synovitis. In controlled studies a blunt trauma is delivered to one knee using a spring loaded device which causes joint swelling due to bleeding and inflammation. Acute morbidity is observed that is assessed by inactivity, immobility and weight loss. Different parameters that are measured experimentally include: knee diameter, joint capsule bleeding score assessed by visual bleeding score, changes in body mass and histology using a grading scheme.^{20; 21} Two different injury models were developed: one that induced a mechanical trauma to the joint and then required that the animals walk on a rotating rod apparatus and a second model induced hemarthrosis by

puncture with a 30 gauge needle which was thought may mimic blood-induced arthropathy.²² Using these models, recombinant hFVIII and human factor VIIa (hFVIIa) were evaluated for their ability to prevent acute joint bleeding in the hemophilia A mouse.²³ Joint swelling and acute hemarthrosis was reduced along with synovitis, however, the treatments did not completely prevent bleeding into the joint space. Clinically, this correlates with findings that hemophilia A pediatric patients that receive regular rFVIII treatments are 83% less likely to develop bone and cartilage changes compared to episodic treatments for bleeding.²⁴ Using these mouse models to measure the impact of bleeding on the joint is challenging due to the subjective nature of the scoring of the pathology. Furthermore, the mouse model may not mimic the weight bearing impact on the joints in humans. Nonetheless, these models may provide an opportunity to study the pathogenesis of joint disease and evaluate novel therapies designed to prevent joint bleeding.

E. Immune responses and tolerance induction to FVIII in the hemophilia A mouse

Since the major complication in hemophilia A patients is the development of neutralizing antibodies (inhibitors) to FVIII, the mouse model became a valuable tool for investigating the immune response to FVIII. The hemophilia A mouse has several advantages as a model for studying immune responses. First, the immune responses can be studied in a genetically homogeneous strain. Second, there is less influence of environmental factors in mouse studies. Third, murine specific reagents are readily available and studies can be designed to manipulate the immune system to investigate the mechanisms involved in the immune response.

Characterization of the immune response to FVIII in hemophilia A mice—Early studies in the hemophilia A mouse model demonstrated that the immune response to hFVIII in mice was similar to what was observed in hemophilia A patients. It was known that the inhibitors in patients are IgG antibodies that require CD4+ T helper cells.^{25; 26} Initial studies in the hemophilia A mouse (C57BL/6 Exon 16 and Exon 17 knockout mice) demonstrated a dose-dependent IgG antibody response that could be detected after one or two intravenous infusions of hFVIII protein and increased after subsequent doses.²⁷ There was a linear correlation between anti-hFVIII IgG antibody titers representing both neutralizing and non-neutralizing antibodies and the neutralizing antibodies (inhibitors) measured in a functional FVIII assay (Bethesda units). Wild type mice (C57BL/6) may develop an immune response to hFVIII with antibody titers that are significantly lower (or undetectable) than in C57BL/6 hemophilia A mice.²⁷ Since CD4+ T cells specific to FVIII would be eliminated by clonal deletion during ontogenesis of the immune system, these antibody responses may reflect species specific differences in the proteins since the murine and human FVIII protein are 84 and 93% identical in the A and C domains, respectively.^{2; 4} Thus, wild type mice may have a similar immune response to some cross-reacting material positive (crm+) patients that have mutations in FVIII that result in synthesis of a non-functional protein.

A more detailed characterization of the immune response to hFVIII in the hemophilia A mice that were infused intermittently and followed over a long period of time similar to hemophilia A patients revealed similarities between human and murine responses. Hemophilia A mice (exon 17) that were administered hFVIII protein intravenously over the course of months developed anti-hFVIII inhibitory antibodies.²⁸ In contrast to the 25–30% of hemophilia A patients that develop inhibitors, all of the hemophilic mice developed antibodies to the hFVIII which can be attributed to their identical genetic background with the same mutation. However, even in this controlled study there was considerable variation among the antibody titers in the mice. IgG subclasses were homologous to inhibitors in hemophilia A patients with primarily IgG1 (equivalent to human IgG4) and also IgG2a or IgG2c (equivalent to the human IgG1). Cytokine profiles agreed with the IgG subclass

analysis of the anti-hFVIII antibody response with IL-10 as the predominant Th2 cytokine.²⁸ CD4⁺ T cell epitopes were observed throughout all of the domains of the hFVIII protein. In hemophilia A mice the blockade of B7/CD28 co-stimulatory pathways (of T cells) prevented the synthesis of anti-FVIII antibodies, supporting the role of CD4⁺ T cells in the development of the immune response to FVIII.²⁷

The immune response to FVIII is strain dependent. C57BL/6 exon 16 hemophilia A mice have a more robust immune response to FVIII protein than Balb/C mice with higher anti-FVIII IgG antibody titers.²⁹ These strain differences pose a limitation of the mouse model since observations in one strain do not always hold in a different strain. This heterogeneity in immune response is similar to observations in humans.³⁰

Transgenic models in the hemophilia A mouse for immunology studies—While the hemophilia A mouse provides a model to study immune responses to FVIII, it has several limitations. First, humans have different mutations in the factor VIII gene that impact the immune response to the protein. The hemophilic mice make a truncated protein (exon 16 or exon 17) or have a partial deletion (exon 17) while some patients synthesize a full-length protein that is non-functional and others do not make any protein. Second, these studies characterize the immune response to human FVIII peptides that are presented by mouse major histocompatibility complex (MHC) II. In an effort to develop improved mouse models for the study of immune responses to human FVIII, humanized transgenic mice were bred onto the hemophilia A mouse.

One strategy to study the immunology of FVIII has been to express mutations that are found in the hemophilia A population. One missense mutation that causes a mild form of hemophilia is an arginine to cystine mutation at position 593 (R593C). These patients are particularly interesting because a subset of these patients make inhibitors to FVIII. Hemophilia A mice that express a hFVIII-R593C transgene driven by the murine albumin enhancer/promoter were generated and bred onto the murine FVIII knockout mouse.³¹ While these mice have detectable hFVIII mRNA in the liver, no hFVIII protein was detected in the plasma. These mice do not develop antibodies or a T cell response to hFVIII. Thus, these mice do not mimic the inhibitor formation observed in some patients with this mutation. The differences in the mouse and human responses may be due to underlying genetic factors that contribute to the formation of inhibitors.

A mouse model was also created that humanized the HLA MHC Class II molecule in the hemophilia A mice. These mice express the human HLA-DRB1*1501 in the exon 17 knockout mice.³² While this HLA only represents a subset of the human population, this haplotype is associated with an increased risk of inhibitor formation in hemophilia A patients.³³ These mice provide an *in vivo* model for identifying immunodominant epitopes by isolation of T and B cell clones and studies of CD4⁺ T cell responses.

Mice that are knock-out for mFVIII (exon 17 knockout) but express the full-length hFVIII cDNA have also been described.³⁴ The expression of hFVIII is liver specific since it is directed by the murine albumin enhancer/promoter. While a transgenic line had detectable hFVIII mRNA but no detectable hFVIII protein in the circulation, the mice were immunologically tolerant to hFVIII upon challenge with recombinant hFVIII. However, the mice do develop an immune response to a modified form of hFVIII that was shown to be more immunogenic in the exon 17 mFVIII knockout mice. These mice may be a valuable model because they do not make antibodies to the native protein and can be used to evaluate novel FVIII molecules that may represent neoantigens in the setting of protein replacement.

B cell epitope mapping—The study of B cell responses to FVIII in humans is difficult since the lymphoid tissues are not accessible and humans are genetically heterogeneous which influences the immune response. Furthermore, functional and structural studies of B cell epitopes is complicated by the polyclonality of the inhibitory antibodies. The hemophilia A mouse model provides the opportunity to identify epitopes within FVIII and determine their functional significance. After infusion of hFVIII into hemophilia A mice, splenocytes were harvested for generation of anti-FVIII B cell hybridomas. Epitope mapping was performed to determine the domain specificity in the context of a hybrid human-porcine FVIII molecule that retains the structure and function of the molecule. As observed with humans, the predominant epitopes for anti-FVIII inhibitory antibodies reside in the A2 and C2 domains.³⁵ Notably, 40% of the B cell clones that were analyzed were not inhibitory antibodies thus a significant portion of anti-FVIII antibodies may be non-neutralizing but may contribute to the pathogenic effect. Further functional studies demonstrated how these antibodies inhibit hemostasis by blocking the binding of FVIII and FVIIIa to phospholipids and vWF or prevent activation of FVIII by thrombin and factor Xa or blocking release of vWF from FVIII after thrombin activation.³⁶ These findings correlate with the human studies that observe a predominant portion of anti-FVIII antibodies are specific to the A2 and C2 domains, however, it is not known whether the mouse accurately predicts the specific human epitopes and their prevalence. Mutagenesis of specific epitopes in the A2 (or C2) domains may reduce the immunogenicity while maintaining function.³⁷ However, the safety of introducing FVIII variants into the hemophilia population has not been determined.

F. Safety and efficacy of novel protein therapeutics

The development of novel protein therapeutics for hemophilia raises the concern for the immunogenicity of a new treatment product. Plasma-derived purified clotting factors contain different formulations of not only clotting factor but additional proteins; in the case of FVIII, human vWF is present that can influence the immune response to the FVIII. In contrast, recombinant FVIII products are not formulated with vWF. For hemophilia A patients, the only protein that may be foreign to the immune system is FVIII while these patients have normal vWF protein. A comparison of plasma-derived with recombinant full-length FVIII in the hemophilia A mouse (Balb/C) suggests that the prevalence of inhibitors to recombinant FVIII appeared to be higher than the immune response to plasma-derived FVIII.^{29; 38; 39} Notably, the hemophilia A mice develop an immune response to human vWF. The presence of human vWF may provide antigen competition thus impacting the immune response to the FVIII products, however, human vWF administered with rFVIII does not reduce the inhibitory antibody titer. These studies provide insight into the possible mechanisms involved in the differential immune response between recombinant FVIII (rFVIII) and plasma-derived FVIII that may involve antigen competition and the influence of the splenic cytokine environment. In fact, the presence of human FIX spiked into rFVIII diminishes the immune response to the rFVIII protein.²⁹ While the murine model provides an opportunity to characterize immune responses that are not possible in humans, these studies illustrate the challenges of using a murine model to interpret potentially clinically relevant findings. These differential immune responses observed in the mouse model remains to be defined in humans.

Historically, purified plasma-derived porcine FVIII (pFVIII) was developed as an alternative product to hFVIII due to the availability of porcine plasma. Later, it was favored as a bypass agent for the treatment of patients with inhibitors due to the low cross-reactivity of hFVIII inhibitors to pFVIII that provided a treatment for 90% of bleeding episodes in patients with inhibitors.^{40; 41} The side effects to plasma-derived pFVIII included allergic reactions likely due to other porcine proteins in the plasma material and contamination with porcine

parvovirus have led to the development of recombinant pFVIII. No differences were observed in the immunogenicity of plasma-derived (Hyate:C) and recombinant (OBI-1) pFVIII compared in hemophilia A mice with prior exposure to human FVIII.⁴² Further assessment of pFVIII using a recombinant human/porcine FVIII by domain swapping showed that while the overall immunogenicity of pFVIII was similar to hFVIII, there were differences in the neutralizing abilities of the antibodies generated to the two proteins.⁴³ Recombinant hybrid porcine/human FVIII may provide a platform for development of a protein that is less immunogenic for use in protein therapy or exploited in gene therapy approaches.

Recent approaches to improve FVIII protein therapeutics have focused on prolonging the half-life of the FVIII protein to reduce the injection frequency required for prophylaxis in hemophilia A patients.⁴⁴ The half-life of FVIII is 10–12 hours which requires three infusions per week to maintain FVIII above >1% to prevent spontaneous bleeding. While direct PEGylation of random amine residues in the FVIII protein has not produced a functional FVIII product, site-specific PEGylation of cysteine residues that have been engineered into the surface of the FVIII molecule by site-directed mutagenesis has led to identification of a FVIII variant protein that maintains coagulation activity. In the hemophilia A mouse the FVIII with a modest two-fold extension of the half-life that was significantly longer in vWF deficient mice suggesting that the PEGylation does not extend the half-life of FVIII stabilized by vWF.⁴⁵ Another FVIII formulation termed PEGlip non-covalently binds FVIII to the outer surface of PEGylated liposomes. After treatment with PEGlip-FVIII the survival of hemophilia A mice following tail transection was significantly higher than mice treated with standard FVIII formulations.^{46; 47; 48} Interestingly, the enhanced efficacy could not entirely be explained by the increased circulating half-life. The mechanism responsible for these improvements in efficacy appears to rely on the presence of platelets.⁴⁸ These studies supported several Phase I/II clinical trials for PEGylated FVIII or PEGlip-FVIII that have demonstrated that these products are not more immunogenic than current FVIII treatments and appear to provide prolonged bleed-free periods.⁴⁹ Another approach uses monomeric Fc fusion technology to stabilize the FVIII protein (rFVIII-Fc). In vivo studies suggest that a two-fold increase in the half-life of rFVIII-Fc that is currently being evaluated in a Phase II/III clinical trial.⁵⁰ The challenge of developing these approaches is that modification of the FVIII protein can interfere with the interaction of FVIII with vWF, FIX and FX that can result in loss of coagulant activity. Ultimately, a long-acting FVIII molecule that has reduced immunogenicity would provide a new generation of protein products for hemophilia.

G. Tolerance induction strategies in the hemophilia A mouse

Since inhibitor formation is the major complication of hemophilia A, the ability to establish tolerance to FVIII would be vital to inhibitor patients. The current strategy for inducing tolerance in these patients is immune tolerance induction (ITI) that requires frequent high dose infusions of protein over the course of months or years. The success rate of this approach is 50–90%.⁵¹ Studies in hemophilia A mice to elucidate the mechanism of tolerance induction with ITI suggest that an irreversible dose-dependent inhibition rather than stimulation of memory B cells occurs at high FVIII doses.⁵² Alternative approaches that are more cost effective and have a better success rate will be important for providing treatment options for patients.

Since all hemophilia A mice make immune response to hFVIII, it is a stringent model for evaluating tolerance induction approaches. Various strategies to induce tolerance in the setting of autoimmune disease have been investigated in mouse disease models. The immune response to FVIII in hemophilia is different than the setting of autoimmune disease in which an immune response develops to self-antigens. In hemophilia tolerance to the FVIII

protein was never established in some patients since the immune system had no previous exposures to the protein. Several recent studies demonstrate the possibility of using tolerance approaches for hemophilia A.

Mucosal tolerance has evolved as a mechanism to avoid immune responses to food antigens. The administration of antigens via oral or nasal routes has been used to re-tolerize animals in the setting of autoimmune disease.⁵³ The dose of the antigen influences the mechanism of tolerance induction with low doses inducing active suppression while high antigen doses resulting in T cell anergy or deletion.^{54; 55} The oral or nasal administration of the C2 domain of the FVIII protein that contains immunodominant epitopes induces tolerance to C2 but not to the entire FVIII protein.⁵⁶ Thus, the epitopes in other domains contribute significantly to the inhibitor development. Cytokine profiling suggested that an active suppression mechanism involving regulatory CD4⁺ T cells was responsible for the tolerance. This was supported by the observation that the tolerance was not maintained if the treatment was suspended. Thus, using this strategy with multiple domains or the entire full length FVIII may prevent immune responses to FVIII, however, long-term treatment may be necessary to maintain tolerance.

A B cell tolerance approach takes advantage of using B cells as tolerogenic antigen presenting cells. In this approach, activated syngenic B cells are transduced ex vivo with a retrovirus expressing an IgG fusion protein that contains the N-terminus of the IgG heavy chain and a portion of the FVIII protein. The A2 and C2 domains were selected for this approach since inhibitory antibodies react to epitopes in these domains. While the use of only one of these domains had only a modest effect, the effect of using a combination of B cells expressing both domains was tolerogenic even when challenged to the entire FVIII protein.⁵⁷ In addition, mice that were pre-immunized with FVIII to develop FVIII inhibitors also showed a remarkable reduction in immune response to FVIII after B cell transplant with a >90% reduction in inhibitor titer. The mechanism of this modulation relies on MHC Class II presentation on B cells as well as CTLA-4 signaling pathways and regulatory T cells.⁵⁸

Cell-based approaches that utilize dendritic cells for suppression of immune responses to FVIII have been explored. Immature dendritic cells promote an environment that promotes tolerance by decreased production of proinflammatory cytokines. Dendritic cells pulsed with FVIII infused into C57BL/6 hemophilia A mice prior to exposure to hFVIII protein reduced the anti-FVIII antibody response by induction of Tregs.⁵⁹ Expression of hFVIII in autologous apoptotic fibroblasts that are phagocytosed by immature dendritic cells to create a tolerogenic environment that was able to suppress the development of inhibitors to FVIII in both naïve and pre-immunized hemophilia A mice.⁶⁰ Both of these strategies rely on the immature dendritic cell ability to induce a population of Tregs (CD4⁺CD25⁺ T cells).

Pharmacological approaches utilize immune suppression regimens to induce tolerance to FVIII. An anti-CD3 antibody approach prevents anti-FVIII inhibitor development by upregulation of CD4⁺CD25⁺ regulatory T cells.⁶¹ Immune suppression with rapamycin at the time of FVIII protein infusion suppresses the inhibitor formation and induces a population of FVIII specific Tregs.⁶²

While tolerance approaches and mechanisms have been exploited in hemophilia A mice, the ability to translate these to large animals and patients have been limited. The key feature of a new tolerance strategy would be that it is effective not only as a preventive therapy in patients prior to the onset of anti-FVIII antibodies to prevent this serious complication but also in patients that have already developed inhibitors. Further development of these approaches will be facilitated by new large animal hemophilia A models that are inhibitor prone (see Hemophilia A Dogs).

H. Gene- and cell-based therapy for hemophilia A

Hemophilia A is an excellent candidate for gene-based therapies for several reasons. While the primary site of FVIII synthesis is the liver (hepatocytes and LSEC)⁶³, a variety of cell types can produce functional FVIII including hepatocytes, endothelial cells, fibroblasts and several hematopoietic lineages. The therapeutic window for FVIII expression is wide with even a small amount of clotting factor (>1%) in the circulation being sufficient to improve the disease phenotype. In addition, FVIII can be assayed in the plasma to frequently monitor the effects on coagulation.

The development of gene therapy for hemophilia A has been challenging and lagged behind gene therapy approaches for hemophilia B already in clinical trials. From a technical standpoint, the FVIII cDNA is larger than FIX. Most gene transfer approaches use the B-domain deleted FVIII (BDD-FVIII) cDNA (4.3Kb) rather than the full-length cDNA (7Kb) as a transgene. Furthermore, the risk of inhibitor formation to protein replacement therapy in hemophilia A patients is higher (25–30%) than in hemophilia B (5%) which poses a challenge to develop a therapy that not only achieves efficacy but also induces tolerance to FVIII. Early clinical trials in the late 1990's for gene-based approaches to treat hemophilia A did not achieve long-term expression of FVIII.⁶⁴ Recent preclinical studies using several approaches show promise for the next generation of gene therapy for hemophilia A.

Liver targeted expression of FVIII—Gene-based approaches for hemophilia A initially were based on the non-integrating adenoviral vector that easily accommodated the large FVIII cDNA and efficiently targeted dividing and non-dividing cells. These initial studies were performed by administration of Ad-hFVIII in wild type mice that limited the ability to demonstrate efficacy since these mice were hemostatically normal.^{65; 66} With the generation of the hemophilia A mouse model, the efficacy of gene delivery approaches could be assessed by not only detection of FVIII antigen in the circulation but also FVIII activity. Early generation adenoviral vectors resulted in therapeutic expression of FVIII that was followed by the onset of antibodies to FVIII transgene and the viral vector.^{7; 67; 68} Helper dependent (“gutted”) adenoviral vectors were developed that do not encode any viral sequences. These vectors have reduced immunogenicity, however, intravenous delivery of these vectors still elicits an innate immune response that results in acute toxicity and development of anti-FVIII immune response that precludes long-term expression from these vectors.^{69; 70; 71; 72} Recent studies using hFVIII variants in an effort to reduce the vector dose to provide therapeutic levels of FVIII resulted in development of inhibitors in all of the mice thus sustained FVIII expression was not observed.⁷⁰ As the helper-dependent adenoviral vectors are evaluated in large animal models, vector-associated toxicities and immune responses to the transgene remain a safety concern.⁷³

Adeno-associated viral (AAV) vectors are derived from a non-pathogenic parvovirus. The advantages of AAV vectors are that they can transduce both non-dividing and dividing cells and the DNA primarily remains as an episomal form avoiding risks of insertional mutagenesis. The major disadvantage of this vector is the limited packaging capacity of approximately 5.0 kb. Initial studies focused on using AAV serotype 2 (AAV2) because it was isolated from humans and transduced human cells efficiently. In the last decade major developments in AAV biology has led to: (1) the discovery of alternate serotypes with distinct tissue tropisms and efficacy;⁷⁴ (2) modifications in the vector genomes to overcome the conversion of single-stranded to double-stranded DNA by using self-complementary vectors;⁷⁵ (3) use of directed evolution to generate novel AAV serotypes to overcome neutralizing antibodies to AAV which are prevalent in the human population;⁷⁶ and (4) development of large scale recombinant systems to produce AAV vector.⁷⁷

Early studies of the B-domain deleted hFVIII delivered in an AAV2 vector demonstrated that a >4.7 kb transgene construct could be packaged into rAAV, however, the vector dose was higher than had been demonstrated effective with AAV2-hFIX despite the requirement of 50-fold less antigen to achieve therapeutic levels FVIII.^{78; 79} Since the large FVIII cDNA (4.3 kb) is difficult to utilize in AAV vectors with limited packaging capacity, initial studies with AAV took advantage of the normal post-transcriptional processing that FVIII undergoes to form a heterodimer composed of the heavy chain and light chain, the secreted form of the protein. In vitro studies had demonstrated the feasibility of expressing the heavy chain and light chain from two separate expression constructs within the same cell to secrete functional FVIII.⁸⁰ In this “two-chain” delivery approach the FVIII heavy chain is delivered in one AAV vector and the FVIII light chain is delivered in a second AAV vector. This approach relies on co-transduction of the same cells with both vectors to produce functional FVIII. The use of genetic approaches to dimerize the transgene itself resulted in low levels of FVIII expression.⁸¹ An alternate approach relied on the self-association of the individual protein chains into the heterodimer form of the protein. The initial studies with this approach demonstrated that >100% of normal levels of FVIII in mouse models.⁸² Further refinement of this approach using liver-specific promoters^{83; 84} and alternate AAV serotypes (AAV8 and AAV9)^{85; 86} provided therapeutic levels of FVIII with a lower vector dose that was necessary to move this approach into the hemophilia A dogs.^{85; 86} Despite the inefficiency of this approach with 5–10 fold higher antigen levels than activity, therapeutic levels of >100% activity could be detected in the mouse circulation.^{84; 85} These observations fueled the further development of a single chain delivery approach in which the full length B-domain deleted FVIII is packaged into one AAV vector thus theoretically resulting in the expression of a functional FVIII molecule for each vector particle. This approach was challenging because it required the use of minimal regulatory elements to facilitate packaging of the construct in the AAV vector. Analysis of AAV-FVIII packaging by analysis of fractions of AAV-FVIII using a large 5.7 kb transgene construct demonstrated that a heterogeneous population of transgene sizes was present suggesting that a heterogeneous population of FVIII transgenes was being packaged into the AAV particles.⁸⁷ Nonetheless, the BDD-FVIII can be packaged into AAV albeit not efficiently but sufficiently to express functional FVIII molecules. The development of alternative AAV serotypes that more efficiently transduced liver (AAV8 and AAV9) and identification of additional small liver specific promoters improved the levels of FVIII expression in the circulation. These studies led to large animal studies.^{85; 88} Proteasome inhibitors that facilitate the intracellular trafficking of the AAV particle to the nucleus rather than the proteasome for degradation, resulted in an increase in FVIII expression.⁸⁹

Overall, these studies demonstrated that the mouse was not predictive of the therapeutic dose of AAV in the hemophilia A dogs and that the dose required to achieve therapeutic levels of FVIII in both mice and dogs was comparable for two-chain and single chain delivery which would not be predicted since each AAV vector delivering a FVIII transgene should express a functional FVIII protein. While mouse models provide the opportunity to screen AAV serotypes for tropism and efficacy, the large differences in the efficacy of AAV serotypes observed in mouse models are more moderate in large animals. Despite efforts to minimize the FVIII transgene construct for the single chain delivery, these data eludes to the inefficient packaging as a remaining obstacle for translation of this approach to humans.

The hemophilia A mouse model has provided a valuable tool for comparing AAV serotypes and transgene constructs, however, the immunocompetent hemophilia A mouse develops an immune response to FVIII of murine, human or canine origin. This is in contrast to hepatic delivery of AAV-FIX in the hemophilia B mouse model that appears to induce tolerance on most mouse strain backgrounds.⁹⁰ While AAV is non-pathogenic and is not associated with the pathology of adenoviral infections, the human population has been exposed to wild type

AAV infections that establish immunity to these viruses that impacts the transduction and long-term expression using these vectors.⁹¹ These hurdles must be overcome for translation of AAV-mediated gene transfer to patients.

Retroviral vectors have also been used to target hepatocytes by in vivo delivery in neonatal hemophilia A models. During the neonatal period the rapidly dividing cells of the neonate would provide the cell division required for retroviral integration into the host cell genome and this early period in development may favor immune tolerance. Dose-dependent immune responses to FVIII were observed that suggests that there may be a threshold of FVIII expression required to induce tolerance.^{92; 93} While long-term expression is observed when high FVIII levels (>20ng/ml) are sustained in the absence of an immune response, it is unlikely that a retroviral approach will move forward clinically for hemophilia due to concerns of insertional mutagenesis.

Non-viral gene delivery approaches for hemophilia have focused on plasmid DNA delivery using hydrodynamic delivery or, more recently, cell targeted nanoparticles to the liver. This approach has several advantages in that (1) it circumvents issues related to the viral vector such as immune responses (2) remains episomal thus avoiding insertional mutagenesis (3) ease and low cost of production and (4) ability to deliver large genes such as the FVIII gene. As with viral vectors, optimization of the expression cassettes has improved the success of this approach in mouse models. Hydrodynamic delivery efficiently delivers plasmid DNA to the liver in mouse models, however, delivery of plasmid DNA to large animals remains challenging. Initial high levels of FVIII gene expression in immunocompetent HA mice were detected after hydrodynamic delivery of a FVIII expression cassette, however, a humoral immune response to FVIII was observed that shortens the duration of FVIII expression.⁹⁴ This anti-FVIII immune response was similar to the response observed to FVIII protein with primarily IgG1 along with some IgG2a, IgG2b. Transient immunomodulation with cyclophosphamide resulted in high levels of FVIII expression that declined after removal of the immune suppression. This response was species independent since similar findings whether a mouse, human or canine transgene was delivered. A comparison of additional transient immune suppression regimens demonstrated that CTLA4Ig plus anti-CD40 ligand, inducible costimulator (ICOS) or anti-CD3 were effective at preventing inhibitor formation and ensuring persistent FVIII expression.^{95; 96; 97} Regulatory T cells (CD4+CD25+FoxP3+) are the key to preventing these immune responses to FVIII⁹⁸ and expansion of Tregs by IL-2 and anti-IL-2 antibodies facilitates the modulation of these responses.⁹⁹

Plasmid DNA has been used to deliver the Sleeping Beauty transposon along with the FVIII transgene. In this approach the transposon provides a mechanism to integrate the transgene into the target cell genome. Initial studies using hydrodynamic delivery in hemophilia A mice resulted FVIII expression that was eliminated after development of an immune response to the FVIII protein.¹⁰⁰ Recent studies encapsulating the Sleeping Beauty/FVIII plasmid DNA in nanocapsules that specifically target receptors on hepatocytes by using the asialoorosomucoid (ASOR) ligand or LSECs using hyaluronan corrected the bleeding phenotype in the absence of an immune response to FVIII.¹⁰¹ New approaches that target the liver using encapsulated DNA may improve the feasibility of these approaches in large animals.

Hematopoietic stem cell approaches for hemophilia A—The ability to express functional FVIII in various cell types has been exploited for hematopoietic stem cell approaches for hemophilia A. Early studies for hemophilia A used murine oncoretroviruses to transduce mouse bone marrow or fibroblasts ex vivo and transplant the cells into irradiated recipient mice, however, FVIII expression was typically short term or not

detected.^{102; 103; 104; 105} The development of lentiviral vectors renewed enthusiasm for using retroviral based ex vivo bone marrow approaches for hemophilia A. These vectors promised the ability to transduce both dividing and non-dividing cells and deliver long-term transgene expression.¹⁰⁶ These vectors combined with bioengineered FVIII transgenes that had increased secretion in immunodeficient hemophilia A mice and then immunocompetent mice with nonmyeloablative conditioning showed no evidence for an anti-FVIII immune response but FVIII levels remained low.^{107; 108}

Ex vivo retroviral delivery approaches of porcine FVIII (pFVIII) to hematopoietic stem cells sought to overcome the low expression observed in early retroviral approaches.¹⁰⁹ Porcine FVIII was found to express 10–14 fold higher than hFVIII when compared alongside each other in exogenous mammalian expression systems.¹¹ This high level pFVIII expression is not at the mRNA level but is due to more efficient post-translational mechanisms that affect movement of the FVIII through the secretory pathway. Long-term FVIII expression and immune tolerance was observed under sublethal conditioning regimens after pFVIII delivery using ecotropic and dualtropic MSCV.¹⁰⁹ These studies also demonstrated sustained FVIII expression in hemophilia A mice with inhibitors to hFVIII, an important issue for evaluating the translation potential. While myeloablative conditioning regimens successfully established long-term FVIII expression without development of an immune response to FVIII, nonmyeloablative regimens did not result in sustained expression of FVIII due to the presence of anti-FVIII antibodies.¹¹⁰ Conditioning regimens that utilize a combination of nonmyeloablative conditioning with transient immune suppression provide long-term expression of FVIII. With expression being driven by the retroviral LTR rather than lineage specific promoters, FVIII expression was detected in the granulocytes and it is not clear if FVIII expression in this lineage has any pitfalls. More recently, the use of a porcine/human chimeric protein that contains key regions of pFVIII that lead to high levels of expression may minimize immune responses to the non-human transgene. However, it is not yet known what portion of the hemophilia A population that has not developed immune responses to recombinant hFVIII protein treatment might be susceptible to development of an immune response to the neo-antigen. While retrovirus based vectors are a promising gene delivery vehicle, the risks of insertional mutagenesis that has been observed in clinical trials and the use of nonmyeloablative conditioning raises safety concerns.

Hematopoietic lineage specific approaches have focused on expressing FVIII in megakaryocytes. While FVIII is not normally synthesized in platelets, FVIII/VWF complexes bind to activated platelets. There would be several advantages to expressing FVIII in platelets. First, the platelets are activated at the site of an injury where the α -granules are released which would provide FVIII at the site to facilitate the ongoing clot formation. Second, the FVIII within the platelets would be protected from circulating antibodies to FVIII that would neutralize its activity. Initial investigations in transgenic mouse models demonstrated that the hFVIII transgene driven by the glycoprotein 1ba promoter¹¹¹ or the glycoprotein IIb promoter¹¹² corrected the bleeding phenotype in hemophilia A/transgenic mice. These studies supported lentivirus delivery of hFVIII into the bone marrow of hemophilia A mice which corrected the bleeding phenotype without any evidence of an immune response to the transgene.¹¹³ However, the hFVIII used in these studies does not produce a stable clot thus FVIII variants with higher specific activity or stability may improve the efficacy of this approach.^{113; 114} These studies led to a large animal study in the hemophilia A dogs.¹¹⁵

FVIII expression in endothelial cells—Ex vivo approaches using lentiviruses to target FVIII expression to endothelial cells have also been explored. Blood outgrowth endothelial cells (BOEC) can be isolated from peripheral blood for ex vivo transduction by lentiviral vectors followed by re-introduction of the modified cells. Since vWF is synthesized in

endothelial cells, expression of FVIII may result in co-storage of FVIII/VWF complexes within Weibel-Palade bodies that would be released at the time of vascular injury.^{116; 117; 118} Transplantation of these cells intravenously¹¹⁶ or subcutaneously¹¹⁷ has been challenging due to an apparent loss of implanted cells. Liver sinusoidal endothelial cells (LSEC) isolated from wild type mice were successfully transplanted into NOD/SCID hemophilia A mice.^{119; 120} However, an endothelial cell toxin was required to engraft significant numbers of transplanted cells for phenotypic correction. The biggest challenge for these approaches may be long-term engraftment of transplanted cells.

Therapeutic approaches in the setting of inhibitors—While one goal of cell- and gene-based approaches is to improve the hemophilic disease phenotype, it will also be critical to establish therapeutic approaches that also achieve hemostasis and facilitate immune tolerance induction in patients that have inhibitors to FVIII. The FVIII delivery approaches that have demonstrated efficacy in the presence of inhibitors are the platelet approaches¹¹⁴, AAV-mediated delivery approaches¹²¹ and retroviral delivery of pFVIII into hematopoietic stem cells¹⁰⁹. Alternative approaches based on bypass treatment strategies rely on achieving hemostasis by sidestepping the intrinsic pathway. One example is FVIIa that has been used clinically as a bypass agent for inhibitor patients for >10 years. FXa mutants that function similarly to the inactive form of the protein may provide another approach to achieve hemostasis by bypassing the intrinsic pathway.¹²²

FVIIa is a protease in the extrinsic pathway that initiates coagulation when complexed with tissue factor. This approach requires that the active form be used to facilitate hemostasis and continuous expression of the active form carries a risk of thrombosis. Transgenic mice were generated that express mouse FVIIa in hepatocytes using a liver specific promoter (mouse transthyretin promoter) that was then crossed onto the hemophilia A mouse (or the hemophilia B mouse) to assess the safety of continuous expression of FVIIa that improves hemostasis but avoids thrombosis.¹²³ Since reports described a differential affinity of human FVIIa and mFVIIa for murine tissue factor, murine FVIIa was used in these transgenic models—pointing to the importance of considering species-specific transgenes. These studies demonstrated the efficacy of continuous expression of FVIIa but also that expression of <1500ng/ml was safe. However, levels of FVIIa >2000ng/ml were associated with premature mortality thus establishing the therapeutic window. The advantages of developing the FVIIa approach is that patients will be tolerant to the expressed protein since the patients' do not have defects in the FVII gene and that it can be used to treat both hemophilia A and hemophilia B with or without inhibitors. Recombinant FVIIa has a very short half-life (2.7 hours) that often requires multiple expensive protein treatments to achieve hemostasis thus continuous expression would overcome this issue. However, the continuous expression of FVIIa has a therapeutic window that raises concerns for the safety of this approach. The initial findings with AAV delivery of FVIIa in hemophilia A (and hemophilia B) mice demonstrated phenotypic correction in the presence or absence of inhibitors which led to large animal studies.^{124; 125} The concerns of this approach lie in the high vector dose to achieve efficacy and the safety concerns of thrombosis. The development of regulatable promoters that would allow the gene expression to be turned off (or turned on) in response to a drug may make this a more appealing approach. In conclusion, the hemophilia A mouse model provides a valuable model for evaluating novel therapeutic approaches prior to more challenging large animal studies.

I. Hemophilia A rat model

A hemophilia A rat model that arose from a spontaneous mutation in a colony of WAG/RijY rats at Yale University was recently described.^{126; 127; 128} These animals have a missense mutation that results in an amino acid substitution, Leu176Pro, which lies in the A1 domain.

The rats have a prolonged clotting time while other hemostatic parameters are normal. Due to the limited availability of rat specific reagents, it is not yet known if these rats have circulating FVIII. It will also be important to determine if this model generates inhibitors to FVIII. Similar to the hemophilia A sheep model, spontaneous joint bleeds have been observed in the rats which has been difficult to clinically diagnose in the mouse and dog models. The rat model will be experimentally advantageous since it will allow for collection of a larger plasma volume for analysis than can be collected in mice and yet will allow for breeding capacities similar to mice. This new model will likely find a valuable niche among the hemophilia A animal models used for translational research.

II. The hemophilia B mouse model

A. Hemophilia B

All hemophilia was thought to have the same pathogenesis, a bleeding tendency resulting from deficiency of “anti-hemophilic factor,” until Pavlovsky reported in 1947 that it was possible in some cases to correct the clotting deficiency in the plasma of one hemophilia patient by mixing the plasma with that of another hemophilia patient.¹²⁹ By 1952 it was clear that the deficiency of a second clotting factor (initially called “Christmas factor” or “plasma thromboplastin component” and subsequently renamed factor IX) causes hemophilia in about 20% of cases (hemophilia B), while FVIII deficiency accounts for the majority of hemophilia (hemophilia A).^{130; 131}

Factor IX (FIX) is produced in the liver. The proper activity and circulating kinetics of the secreted FIX are dependent upon several post-translational modifications (PTMs), the most critical of which is the carboxylation of the 12 glutamic acid residues in the N-terminal portion of the mature protein (the FIX Gla domain) in a vitamin K-dependent process; the majority of the remaining PTMs are to the FIX activation peptide.^{132; 133} FIX circulates as an inactive zymogen protein. The activation of FIX by the cleavage of the FIX activation peptide from the protein (either by the complex of factor VIIa/Tissue Factor or by factor Xia) results in a protein with a light chain and a proteolytic heavy chain linked by disulfide bonds. On the phospholipid surface of activated platelets the active serine protease FIXa can activate factor X in a calcium-dependent process. The catalysis of the activation of factor X by FIXa is increased several logs by the interaction of FIXa with its cofactor, activated factor VIII (FVIIIa), in the so-called “Tenase complex” (FIXa/FVIIIa/PL/Ca⁺⁺). Factor X activated by the tenase complex can subsequently drive the generation of abundant thrombin that is essential to the amplification of adequate hemostatic clot formation. Accordingly, given the requirement for the cooperative function of FVIIIa and IXa in the tenase complex, it is not surprising that the bleeding phenotype that results from a given level of deficiency of either FVIII or FIX is very similar (e.g. <1% activity of either FVIII or IX results in severe hemophilia with a similar bleeding risk).¹³⁴

The mouse and human *F9* gene open reading frames share ~80% sequence homology.¹³⁵ As in humans, the mouse *F9* gene is present on the terminus of the q arm of the X chromosome (as are the mouse and human *F8* genes) and the expected X-linked transmission of hemophilia B seen in humans is observed in mice. The domain structure of FIX and the basic interactions of the protein in hemostasis are the same in mouse and human and the same global assays of coagulation and specific factor measurements that are universally used in clinical coagulation laboratories throughout the world can be adapted for use in mice. For these reasons, the study of human FIX and potential human FIX therapeutic approaches, using engineered mouse models that are either mFIX and hFIX deficient or engineered to express modified FIX variants, has been widely pursued and recently reviewed.¹³⁶ It needs to be stated that in addition to multiple informative parallels, multiple divergences exist between humans and mice in regards to hemostatic and thrombotic

processes, as do differences between strains of mice, and these must be considered in experimental design. Comprehensive considerations of comparative mouse and human hemostasis are available^{137; 138; 139} and strain specific differences in normal ranges for coagulation assays are available in the Mouse Phenome Database (available at: <http://phenome.jax.org/pub/cgi/phenome/mpdcgi?rtn=meas/catlister&req=Cblood%20hematologyqqcoagulation>).

B. Hemophilia B mouse models - FIX knockout mice

Three different groups generated hemophilia B mouse models in rapid succession in 1997–1998. For decades the use of hemophilia B dogs for the evaluation of novel treatment approaches had provided translationally accurate insights.¹⁴⁰ Nevertheless, the expanding investigation of strategies aimed to correct the genetic defect in hemophilia B demanded a small animal model in which hemostatic correction could be modeled without being confounded by the background expression of mouse FIX. The usefulness of the hemophilia B model to pattern gene therapy approaches for ultimate application in other genetic defects of protein synthesis also accelerated the development of this model. Two groups generated large deletion mutations in the mouse FIX locus by deleting either exon h (which encodes the catalytic domain of the serine protease) or both exons g and h.^{141; 142} The mice generated by these two approaches have no circulating FIX protein (no antigenic “cross-reactive material” in immunologic assays to detect FIX, “CRM(-)”). The remaining group pursued a “plug-and-socket” strategy as originally described by Oliver Smithies’s lab.¹⁴³ The promoter and the first three exons of the FIX gene were deleted by the insertion of a *neo* gene plus a partially deleted hypoxanthine phosphoribosyl transferase minigene. The plug and socket design allows the subsequent insertion of other sequences into the same locus of correctly targeted embryonic stem cells, consistent with the investigators’ goal of creating a reagent for studying structure-function relationships of recombinant FIX proteins *in vivo*.¹⁴⁴ This latter knockout model has in fact been used to generate a number of additional strains each of which expresses a single copy *F9* gene from the “socket” located in the endogenous single copy mouse *F9* gene locus. These strains include:

1. mouse FIX expressing a mutation that alters the FIX Gla domain interaction with endothelium (K5AFIX)¹⁴⁵
2. defective human FIX having a missense mutation at a critical arginine in the catalytic domain (R333QFIX)^{146; 147}
3. defective human FIX having a nonsense mutation in the Gla domain that results in an early stop mutation (R29XFIX)¹⁴⁸
4. human FIX wild type (FIX-WT mouse)¹⁴⁷
5. human FIX carrying three mutations each of which increases the specific activity of the protein (“FIX Triple”).¹⁴⁷

The three knockout models (FIX^{-/-}) produce no hepatic FIX mRNA, have no circulating FIX protein (CRM-), and bleed excessively with hemostatic challenges such as tail transection. The original reports of these mice stated that 0.03–0.08 U/ml of FIX coagulant activity was detected in plasma in one-stage human FIX activity assays. This activity has been demonstrated to be artifactual and can be reduced to <0.01 U/ml if standards for the clotting assay are constructed using uniform dilutions of the knockout mouse plasma.¹⁴⁶ FIX^{-/-} mice breed normally and deliver normal size litters. Colonies can be maintained with breeding females having both X chromosome *F9* genes knocked out, with the dams only occasionally lost to hemorrhage in the peripartum period. Most pups survive normally to wean and into adulthood without bleeding. It is worth noting that this normal survival of mice having deleted procoagulants of the intrinsic pathway FIX contrasts with the

intrauterine or early post-partum lethality that is observed in animals that fail to express the vitamin K γ -glutamyl carboxylase or procoagulant common pathway or extrinsic pathway proteins (prothrombin, factor V, factor X, Tissue Factor, factor VII) (as reviewed by Mackman).¹⁴⁹ Spontaneous bleeding in the FIX^{-/-} mice is rarely observed, although musculoskeletal bleeding is seen, in particular after fighting with cagemates. FIX^{-/-} mice will generally hemorrhage to death after tail clip, however.

C. Additional hemophilia B mouse models

None of the FIX^{-/-} strains discussed above transcribe FIX mRNA and therefore they do not have antigenically detectable FIX protein (i.e. they have a CRM(-) phenotype). As a result, there is no opportunity for potential epitopes derived from FIX to be expressed or presented during mouse immunologic (thymic) development. FIX^{-/-} mice have been reported to make antibodies directed against recombinant mouse FIX following parenteral challenge, although not after exposure to mouse plasma (containing FIX).¹⁵⁰ It is desirable to study potential new human hemophilia B therapies in the FIX^{-/-} mice, however, the human FIX protein (hFIX) stimulates anti-hFIX antibodies in some contexts, given that it appears to be a cross-species protein (xenoprotein). Importantly, inhibitor antibody formation clinically in humans with hemophilia B occurs in only 2–4% of patients exposed to FIX replacement, so that this clinical complication is almost ten-fold less commonly observed than in hemophilia A. To study issues related to immunogenicity of FIX therapies, or to circumvent the potential for antibody formation to confound *in vivo* evaluation of FIX therapies, hemophilia B mouse models have been generated that do not express mouse FIX but instead express human FIX cDNAs having a variety of underlying FIX mutations.

As mentioned above, homologous recombination strategies derived from the “plug-and-socket” FIX^{-/-} strain have been used to generate mice to model immunologic “good risk” and “poor risk” scenarios as regards FIX inhibitor antibody formation. In the first case, a human FIX cDNA having a single nucleotide change results in a missense substitution of glycine for arginine at amino acid 333 (R333QhFIX mouse).¹⁴⁶ The defective R333QhFIX is physiologically expressed from the mouse FIX promoter and a single copy gene in the endogenous mouse *F9* locus. The defective protein circulates at 15–30% of normal human FIX levels (CRM+ phenotype) but has <1% activity and a severe bleeding phenotype. To contrast with this, a mouse was created by homologous recombination into the *F9* locus of a human FIX cDNA having a single nucleotide change that results in nonsense termination of transcription and a very early stop mutation (R29XhFIX mouse).¹⁴⁸ R29X is the most common mutation that leads to inhibitor formation in humans, as recorded in the database of hemophilia B mutations (Available at <http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html>). However, complete gene deletions lead to inhibitors with somewhat greater frequency than the stop mutation). The antibody responses in the R29XhFIX and R333QhFIX strains mimic many expected features of human FIX inhibitors, and the models can be used to evaluate efficacy and risk of protein and gene therapy approaches. As one example, there is very low risk of inhibitor antibody formation in the setting of CRM+ missense R333QhFIX mutation, owing to a relative state of immunologic non-responsiveness of CD4+ T lymphocytes to replacement FIX.¹⁵¹ These good and poor risk mutation hFIX-expressing mice have now been crossed onto the HLA background of a mouse strain that does not express mouse Major histocompatibility class II antigens, but instead expresses a human HLA-DRB1*1501 MHC class II allele and these “double humanized” hemophilia B mice are being used to characterize new therapeutic approaches (Personal communication, Dr. Genlin Hu, University of North Carolina at Chapel Hill).

Similarly, a series of transgenic hemophilia B mice have been created by Sabatino and colleagues and provide a spectrum of mutations to examine the degree of tolerance to human FIX conferred by the underlying mutation.¹⁵² In addition to transgenic mice expressing

early (R29X) and late (R338X) stop mutations and missense mutations (R180W, G381) these investigators generated hemostatically normal mice that do not express mouse FIX but instead express 2 or more copies of a human FIX cDNA. An example of the value of these mice has been to demonstrate the preclinical therapeutic potential of an aminoglycoside translational readthrough strategy to generate circulating FIX from a nonsense mutation F9 gene sequence.¹⁵³ A human Phase I clinical trial of a translational readthrough strategy for hemophilia has been initiated.^{154; 155} (See <http://www.clinicaltrials.gov/ct2/show/NCT00947193?term=hemophilia+B&rank=34>)

D. Engineered mice in the study of FIX expression and circulating kinetics

Clinical observation of patients with unusual phenotypic expression of hemophilia suggests that mechanistic insights may be found by studying the genotype: phenotype expression. Expressing the genotype of interest in cell culture may allow partial characterization of the effect of mutated gene sequences, however, in some cases the expression observed in cell lines does not correlate well with the expression observed from adult liver, so that study in animals is a more accurate or revealing test system.^{156; 157} Transcriptional regulation of FIX gene expression has been modeled extensively in engineered mouse models. Mapping of transcription factor binding and regulation of the FIX promoter has been modeled in mice that recapitulate the hemophilia B Leyden phenotype, demonstrating age-dependent and sex-specific post-pubertal increases in FIX expression.^{158; 159; 160} Elements in the 5' and 3' untranslated regions of the FIX gene that direct natural gender-independent age-associated increases in FIX expression have also been elucidated in mice with engineered FIX gene sequences.¹⁵⁶

Hemophilic dogs have been used as an excellent resource for the preclinical testing of replacement clotting factors over the last several decades, demonstrating circulating kinetics for FVIII, FIX, and factor VIIa that are very similar to the kinetics in humans.^{140; 161} In recent years there has been a tremendous increase in the investigation of clotting proteins that are either genetically or chemically modified to achieve improved therapeutic potential.¹⁶² The most common efforts are to increase the circulating survival of the hemostatic protein and/or to increase the specific activity. FVIII and FIX knockout mice have been increasingly used for preclinical pharmacokinetic/pharmacodynamic screening of the *in vivo* properties of candidate proteins to inform rational choice of protein to examine in the more scarce hemophilic dog resource. In these applications it is clear that the mouse pharmacokinetics are not the same as human. For instance, the circulating half-life of FIX is reproducibly shorter in mice than in humans. Nevertheless, the *relative* kinetics of candidate proteins can be compared to the commercially available replacement protein (e.g. plasma-derived or recombinant FIX) in adequately sized cohorts of mice to generate statistically meaningful data. There has been consistency in the relative kinetics that have been demonstrated in mice and those subsequently confirmed in the dog model (i.e. agreement that the novel therapeutic is biologically equivalent, inferior, or superior to approved FIX in small and large species).¹⁶³

E. Engineered mice in the study of FIX hemostasis, thrombosis, and response to injury

Hemophilia B mice have also been used for preclinical testing of relative pharmacodynamics of candidate FIX or factor VIIa proteins in hemostatic challenges.^{15; 164} Tail transection bleeding time assays measure initial hemostasis after tail vessel wounding. Nevertheless, the tail-clip assay alone may fail to reliably distinguish factor deficiencies.^{14; 165; 166; 167} [[Dejana E et al Contributors to the discrepant results of tail bleeding assays have been recently reviewed by the Animal Models Scientific Subcommittee of the International Society for Thrombosis and Hemostasis.¹⁵ These include the central role of platelets in initial hemostasis (as distinct from soluble clotting factors), as

well as variations in central tail artery constriction and dilation that may obscure discrete endpoints. Delayed hemorrhage and persistent blood loss, however, are hallmarks of hemophilic bleeding and result in decreased survival. For this reason, the tail transection bleeding time may be modified to observe persistent as well as initial hemostasis.^{14; 145; 168} Secondary bleeding time assays have been described to measure this characteristic phenotype in humans with hemophilia,¹⁶⁹ as well as in a monkey model of hemophilia.¹⁷⁰

Another bleeding challenge has been described recently by Whinna and colleagues that involves observation of the average time to hemostasis following a standardized saphenous vein incision.¹⁷¹ The assay appears to have greatly increased sensitivity when compared to the tail clip assay; differences in hemostasis can be demonstrated in the range between 1% to 10% FIX activity level, corresponding to the ability to demonstrate the correction from a severe hemophilia phenotype to a mild hemophilia phenotype. The model has been applied to the *in vivo* study of FIX mutations that result in gain or loss of proteolytic function as well as to the comparative *in vivo* investigation of alternative recombinant activated factor VIIa therapeutics as bypassing agents for hemophilia care.^{171; 172}

Hoffman and Monroe and colleagues have examined hemophilia B mice to demonstrate that wound healing is defective in hemophilia. Normal wound healing entails four overlapping phases: hemostasis, inflammation, proliferation, and remodeling or resolution.¹⁷³ The healing of standardized skin punch biopsy wounds of hemophilia B mice, as compared to hemostatically normal mice, demonstrates delayed wound closure, prominent neovascularization, delayed but subsequently prolonged iron contamination and macrophage/monocyte infiltration of the wound tissue.¹⁷⁴ Late hemorrhage into the tissue near the wound site is seen even after the surface wound closes. Restoring hemostasis at the time of injury, without prolonged coagulation protein replacement throughout healing, does not normalize healing in hemophilia B mice.¹⁷⁵ Extending the observations using this model, perivascular tissue factor (TF) is shown to be down-regulated following cutaneous wounding.¹⁷⁶ TF expression is depressed for a longer period in hemophilic mice than in wild type mice. It appears likely that appropriate wound remodeling requires the coordination of effective hemostasis with modulation of inflammation and appropriate vasculogenesis and that insights may be gained by modeling the ways this coordination is dysregulated in hemophilia B mice.

The cutaneous wound model suggests a number of paradigms for the study of healing in the presence of impaired hemostasis, however clinically the most common site of hemophilic bleeding and degeneration is in the joints, resulting in chronic synovial and osteochondral arthropathy. The distinctive pattern of hemophilic bleeding can be understood by recognizing that hemostatic potential is tissue-specific. Extrinsic pathway tissue factor (TF), the primary initiator of blood coagulation, is expressed at high levels in the brain and heart, as well as in the testis, uterus, placenta and kidney^{149; 177}. Tissue factor is expressed at low levels in healthy skeletal muscle and joints^{178; 179}. At baseline the potential for extrinsic pathway (TF/FVIIa) initiation of coagulation is compromised by low TF in joints and when intrinsic pathway proteins are also deficient (e.g. FVIII or IX in hemophilia A and B) joints are particularly vulnerable to local hemorrhage.

As discussed above (Arthropathy in the murine model – Hemophilia A) when a reproducible trauma is used to induce bleeding into the joint of hemophilic mice the characteristic histologic and radiologic changes of human hemarthropathy are reproduced.^{20; 180} The clinical, histopathologic, and radiologic changes initially described in hemophilia A mice by Valentino and colleagues are also seen in the hemophilia B mice.^{180; 181} Importantly, these hemophilic changes are not seen in hemostatically normal mice. The effect of potential therapies, including FIX gene therapy, to reduce or prevent the most relevant end-organ

pathology of hemophilia B (i.e. the degenerative effect of bleeding into joints), can be used as the endpoint of interest in preclinical modeling. Even more intriguing is the potential to study the action of FIX to effect wound healing within the joint. For example, coagulant proteins are present in biological fluids including synovial fluid^{182]}, pleural fluid^{183]}, and lymph^{183; 184]}. While it is conceivable that these proteins exist in these locations as merely an ultrafiltrate relative to their plasma concentrations, as merely an ultrafiltrate, the hypothesis that synovial fluid FIX could protect the hemophilic joint via an extravascular hemostatic function has been investigated in hemophilia B mice. When FVIII or IX was injected directly into the joint at the time of induced joint bleeding, the joints developed minimal synovitis. This protection was seen despite the fact that no factor activity was detectable in the circulation throughout 72 hours following the intraarticular (I.A.) dose. In fact, hemophilic mice subjected to the same induced joint hemorrhage and treated intravenously with FIX developed more joint degeneration than those treated directly into the synovial space.¹⁸⁰ Subsequent investigations have established that intraarticular FIX expressed from an AAV gene therapy vector expressing exclusively in the joint can also protect the joint from progression of bleeding-induced synovitis, whether used to treat a joint with pre-existing hemophilic synovitis or a joint that is naïve to previous blood exposure.¹⁸⁵ Correlating findings in the mouse joint injury with the cutaneous injury may yield valuable mechanistic insights of the action of clotting factors in locations outside of the circulation, as well as general mechanisms of wound healing.

Clotting factor knockout mice have been used to study occlusive thrombus formation and pathologic clotting. Not surprisingly, an intact classical intrinsic coagulation pathway is required for stable occlusive thrombus formation, as demonstrated in ferric chloride carotid artery large vessel injury and in laser-induced mesenteric arteriolar small vessel injury. (Recently reviewed)^{186; 187}

An example that demonstrates several of these uses of mouse models of FIX expression to elucidate FIX biology is the study of the effect of substitutions in the FIX Gla domain and their effect upon *in vivo* FIX function. Darrel Stafford and colleagues described FIX single amino acid substitution mutants K5A or V10K, and the effect of these mutations to greatly decrease the binding of FIX apparently resulting from their greatly decreased binding to endothelial collagen IV, an endothelial basement membrane protein.^{188; 189} The substitution of an arginine (K5R) instead of an alanine (K5A) at amino acid 5 resulted in a FIX mutant with increased specific binding to collagen IV. Recombinant FIX protein stocks of each of these variants were generated, purified, and infused into hemophilia B mice for *in vivo* pharmacokinetic comparison.¹⁹⁰ The K5R FIX disappeared from the circulation rapidly after infusion with greatly decreased area under the FIX activity curve kinetics relative to wild type FIX. The decreased binding mutant FIXs, delivered as recombinant K5A hFIX protein to FIX^{-/-} mice¹⁹¹ or as combined K5A/V10K hFIX gene therapy vectors to FIX^{-/-} / T cell CD4 knockout mice¹⁹², demonstrated 2-fold greater survival. These findings suggested, for example, that there might be value to incorporate the K5A, for instance, into recombinant FIX protein, if indeed the level of plasma FIX activity is a surrogate measure for hemostatic protection, as is often assumed in clinical practice. Nevertheless, the possibility of a functional purpose underlying the observed interaction of FIX with collagen IV was explored by Stafford and colleagues by generating a mouse that expressed low-binding K5AFIX instead of wild type FIX.¹⁴⁵ Although the K5AFIX mouse has 20% higher circulating FIX protein *and* activity than wild type, the mouse displays a mildly hemophilic phenotype. This phenotype was demonstrated using several of the methods discussed above including the demonstration of prolonged primary and secondary bleeding in the tail transection challenge, slow time to occlusive thrombus formation in the ferric chloride-treated mesenteric arterioles, cutaneous wound closure time that was intermediate between that of FIX^{-/-} mice and hemostatically normal mice, all suggesting that there is likely a

physiologic role for the binding of FIX to collagen IV.¹⁴⁵ These insights would certainly have been difficult to achieve relying upon *in vitro* studies or upon studies in hemostatically normal animals.

F. Tolerance and immunogenicity of FIX studied in hemophilia B mice

As has been mentioned earlier in this chapter, the development of antibodies that neutralize the activity of replacement clotting factor is the most common serious complication that arises from hemophilia treatment. The development of “inhibitor” antibodies occurs at a much lower incidence in severe hemophilia B (~2–4% of patients) when compared to severe hemophilia A (~20–30% incidence of low and higher titer inhibitors), but can be a devastating complication, in particular because FIX inhibitors are difficult to eliminate with immune tolerance induction regimens and can be associated with life-threatening anaphylactic responses.¹⁹³ The ability to extract clues about inhibitor etiology or treatment through examination of large population epidemiologic data or registries is being vigorously pursued in regards to FVIII inhibitors, but is unrealistic for FIX inhibitors due to their rarity. The ability to mechanistically study FIX neutralizing antibodies in a mouse model that faithfully models the human FIX inhibitor phenotype could be valuable – if only enough were understood about human FIX inhibitors to judge how representative such a mouse model were. Some areas in which the hemophilic mouse inhibitor phenotype appears to mimic FIX inhibitor in humans will be reviewed in brief; more comprehensive discussions are available.^{136; 193; 194}

The only strong determinant of FIX inhibitor risk that is unequivocally determined in hemophilia B is the patient’s FIX genotype (family history is an established risk, however, clearly associated with the underlying shared FIX mutation). As discussed above, the underlying FIX mutation is a principal determinant of antibody response to FIX in hemophilia B mice: greater degrees of loss of FIX coding sequence and lack of circulating CRM(+) protein correlate with greater inhibitor risk.^{146; 148; 151; 152; 195; 196} Anti-FIX IgG has been reported to develop in some strains of mice after exposure to intravenous human FIX¹⁵¹¹⁹⁷, intravenous mouse FIX¹⁵⁰, subcutaneous human FIX given with adjuvant¹⁹⁸, or intramuscular AAV-FIX gene therapy¹⁵⁰¹⁹⁹¹⁵¹²⁰⁰²⁰¹. In each of these contexts the antibodies are mostly IgG1 isotype. Mouse IgG1 is the homologue of human IgG4; human FIX inhibitors are reported to be mostly IgG4 isotype, and so the mouse inhibitor response appears to parallel the human response. Low titers of IgG2a and IgG2b sometimes have been observed in mice. Taken together, the results are consistent that FIX elicits a Th2 lymphocyte-dependent immunoglobulin response which is associated with the high affinity antibody production²⁰²¹⁵¹²⁰³. FIX-specific IgE has also been demonstrated by RAST reaction in some individuals (although not all) who exhibit immediate hypersensitivity to FIX exposure.²⁰⁴ IgE anti-FIX has recently been described in hemophilia B mice crossed onto the particularly immunologically intolerant C3H background (see below).²⁰⁵

Human hemophilia A cohort studies suggest associations of FVIII inhibitor development with polymorphisms in cytokine immune response genes interleukin 10 (IL-10)²⁰⁶, tumor necrosis factor- α ²⁰⁷, and in the CTLA-4 gene²⁰⁸ and weaker associations with major histocompatibility locus (MHC) class II phenotype. The number of studied hemophilia B inhibitor patients studied is too small to establish the same associations between FIX inhibitors and MHC II or other potential genetic factors³³²⁰⁹. Nevertheless, genetic studies in inbred mouse strains that have developed antibodies after exposure to species-mismatched FIX demonstrate linkage to genes for immune response modifiers including IL-10.^{197; 210}

The MHC class II phenotype of a patient should determine which FIX-derived peptides are presented to the T-helper cells. Very striking differences exist between strains of hemophilic mice having different MHC backgrounds in regards to tolerance of human FIX. For

example, C57Bl/6 (MHC H-2^b) FIX^{-/-} mice tolerate repeated recombinant human FIX protein infusion, and following liver-directed human FIX gene therapy and they develop tolerance to the protein that is not broken by subsequent challenge with skeletal muscle-directed FIX gene therapy.²¹¹ At the other end of the spectrum, C3H (MHC H-2^k) FIX^{-/-} mice develop IgG1 inhibitors after liver-directed FIX gene therapy and may also develop IgE-associated anaphylactic reactions and death following repeated intravenous human FIX challenges.²⁰⁵ CD4⁺ T-cell dependent B-cell antibody response to FIX has been demonstrated in several strains of mice and immunodominant epitopes for T-cell stimulation that are specific to each strain have been identified. Immunodominant epitopes for each strain have been mapped in the catalytic domain of the protein.^{194; 198; 203} In this respect, as well, it appears that mice may be useful models for studying human FIX inhibitors, because epitopes targeted in humans are primarily in the catalytic domain.²¹² A final suggestion that consistencies between the mouse and human epitopes support the use of the hemophilia B mice to model inhibitor development is that immunodominant FIX epitopes in very similar regions of the catalytic domain are recognized by a novel hemophilia B mouse model that is engineered to express human HLA-DRB1*1501 MHC class II allele and human FIX gene sequence. (Personal communication, Dr. Genlin Hu, University of North Carolina at Chapel Hill.)

These and other investigations in mouse models are consistent with a mechanism wherein autoreactive T cells, capable of recognizing FIX protein, are neither deleted nor anergic, but are maintained in an unresponsive condition. Regulatory CD4⁺ CD25⁺FoxP3-expressing T-regulatory cells suppress antibody formation to FIX. *In vivo* depletion of CD4⁺CD25⁺T_{regs} has led to loss of tolerance evidenced by antibody formation.⁹⁰ Two recent investigations explored the potential to use mucosal tolerance to achieve immune deviation towards a regulatory T cell immunologic environment in inhibitor-prone C3H FIX^{-/-} mice. The first approach demonstrated that intranasal peptide administration of a human FIX-specific CD4⁺ T-cell epitope reduced inhibitor formation.¹⁹⁴ The second approach fed bioencapsulated cholera toxin β -subunit-fused FIX, resulting in control of inhibitor formation and preventing anaphylactic reactions.²⁰⁵ These experiments, along with others that seek to pharmacologically deviate the immune response following FIX exposure towards IL-10 and TGF- β dominated toleragenic immune responses, present intriguing challenges for translation of mouse results to care of humans.²¹³

Somewhat more disappointing have been attempts to use hemophilia B mice to model an apparent CD8⁺ cytotoxic T-lymphocyte (CTL) response that was seen in a human clinical trial of FIX gene therapy (see below).^{214; 215; 216; 217} Mouse strain-specific class MHC I responses that direct CD8⁺ T cell responses to FIX epitopes on FIX can be mapped, if present.²¹⁸ Nevertheless, there has proved to be limited ability to study a human trial subject's CTL response against a viral gene therapy vector derived from a virus, the normal host of which is human, as will be discussed below.

G. Engineered mice for the study of FIX gene and cell based therapy

The most common use of hemophilia B mice has been to explore potential gene correction strategies. Preclinical studies in the hemophilia B mice have been central to the approaches used in two completed and two ongoing human clinical trials, and the ways in which hemophilia B mouse studies are driving current investigations will be the focus of this section. The reader is also referred to multiple reviews of hemophilia B gene therapy preclinical modeling and clinical translation.^{91; 136; 219; 220; 221}

The strategies studied in hemophilia B mice include *ex vivo* approaches, in which the corrective gene is transferred to cells (autologous or allogeneic) outside the body and the cells expressing the therapeutic gene subsequently delivered to the subject. Autologous

keratinocytes²²², embryonic stem cells directed toward hepatic endodermal differentiation²²³, allogeneic hepatocytes²²⁴, hematopoietic stem cells²²⁵, hematopoietic stem cells directed toward erythroid differentiation²²⁶, allogeneic megakaryocytes²²⁷ and encapsulated primary myoblasts^{228,229,230} have all been investigated for phenotypic correction of FIX-deficient mice. In general, the degree of correction achieved by these strategies has been from <1% to 10% of normal FIX levels; stem cell approaches have in some cases achieved advantageous longevity of expression^{225, 226} and FIX tolerance induction^{223, 225, 226}. In addition, the definition of efficacy of the megakaryocyte approach needs special consideration, given that the plasma FIX activity may not reflect the relevant endpoint. In the megakaryocyte approach, it is estimated that 90% of the total FIX is stored in the platelets, and is releasable upon activation of the platelet, and is therefore immediately available at the required surface for thrombin generation.

In vivo gene correction approaches studied in hemophilia B mice have delivered the FIX gene either as naked or chemically-formulated DNA^{231,168; 232; 233; 234} or using a virus vector for gene delivery. Naked DNA delivery is inefficient. Achieving FIX expression at levels adequate to achieve hemostatic correction has required driving target cell entry by the therapeutic nucleic acid using hydrodynamic pressure^{168; 235}, electrical current²³³ or ultrasound²³⁶. Several groups are examining whether these enhancements to gene delivery can be scaled for efficient and safe use in large animals and humans.

Early approaches to viral vector-mediated FIX correction used onco-retroviral vectors and adenovirus vectors. Primarily due to safety concerns related to retrovirus and adenovirus vectors, lentivirus and adeno-associated virus (AAV) vectors have progressed for hemophilia B applications. Proficient and pantropic transduction by lentivirus vectors led to initial enthusiasm for these vectors. With time, the efficient cell entry by lentiviral vectors was recognized to have disadvantages, because efficient transduction and expression of FIX by antigen presenting cells led to neutralizing antibody formation against the therapeutic transgene. This problem was overcome by the incorporation in the lentivirus of micro-RNA sequences to silence FIX expression in a cell-specific fashion in the APCs, avoiding inhibitor formation while allowing continued FIX expression from the liver.²³⁷ In recent years, however, concerns regarding the occurrence of insertional mutagenesis (oncogenesis) have prompted the development of integration-defective lentiviral vectors (IDLV). Although this innovation undoubtedly increases the safety of the lentivirus, a very substantial loss of efficiency of expression is incurred, and substantial optimization will be required before the IDLV are likely to move beyond the mouse model.^{238; 239}

Preclinical data derived in hemophilia B mice has been central to the development of two completed human clinical trials using AAV serotype 2 vectors to deliver the FIX gene to skeletal muscle and liver. The wild-type AAV2 upon which most AAV vectors have been based commonly infects most individual in childhood but causes no human disease. On the first of these two trials, no safety concerns were raised and expression of FIX in transduced skeletal muscle tissue was demonstrated for as long as 3.7 years after AAV2 delivery in one subject, although none of 8 subjects had measurable circulating FIX activity.²⁴⁰ Transient partial correction of hemophilia B with FIX activity >10% was seen at the highest dose level in a subsequent trial of AAV2.FIX directed to the liver. Evidence of asymptomatic hepatocellular inflammation was observed, however, by six weeks post-administration and coincided with the loss of FIX expression.²⁴¹ Subsequent study has determined that AAV capsid epitopes presented on the surface of successfully transduced cells were most likely the target of memory T cell CTL-mediated elimination.²⁴²

The results of this trial have prompted the return to study of the hemophilia B mice with results that highlight both the usefulness and the limitation of the mouse model. As the

investigators of the liver-directed clinical trial have modeled in subsequent studies, hemophilia mouse models do not predict the occurrence of the CTL response. Presumably the CTL response was mediated by preexisting memory CD8+ T cells: such cells will be present in humans as the natural host of wild type AAV, but mice do not naturally host this primate virus.²⁴² Although artificial mouse systems have been bioengineered that confirm the proposed mechanism (CTL-mediated elimination of AAV capsid-presenting cells), these systems will not predict parameters to allow the complication to be avoided in future trials. The investigators further concluded that alternative serotypes of AAV would be unlikely to evade capsid-specific immune responses. Unfortunately, this conclusion also cannot be tested in the hemophilia B mouse.²⁴²

Other groups responded to the clinical trial results by returning to the hemophilia B mice to examine and optimize AAV vector design and to address the hypothesis that more highly efficient FIX vectors might direct hepatic FIX expression without requiring as large a load of AAV capsid as precipitated the CTL response in the clinical trial. In 2001, novel AAV vectors were reported that incorporated a “self-complementary” genomic form (scAAV) that bypasses the rate-limiting requirement for DNA second-strand synthesis prior to expression and could be used to deliver therapeutic transgenes.²⁴³ Optimization of elements for incorporation in a FIX expression cassette for liver-targeting scAAV were subsequently determined *in vivo* in mice, and the potential for significant reduction of AAV particle number confirmed.^{244; 245} Optimization of the mammalian codon usage in the FIX cDNA further improved expression and allowed further reduction of AAV particle number. Immunohistochemical staining of the pattern of hepatic expression of endogenous human FIX from the native mouse promoter could be examined in the R333QhFIX mouse. This demonstrated that physiologic FIX expression occurred in a homogeneous FIX expression across the liver parenchyma.²⁴⁴ scAAV vectors also demonstrated this physiologic FIX expression pattern, whereas expression from the conventional single-strand AAV2.FIX vector occurred in an intense pattern in only a small subset of cells, in which expression was intense. In 2002, AAV8 serotype vectors were described and quickly shown to have enhanced tropism for liver, further enhancing the efficiency of FIX gene delivery.⁷⁴ Preclinical studies of a self-complementary AAV serotype 8 codon-optimized FIX vector (scAAV8FIXco) in hemophilia B mice and hemostatically normal primates led to a human clinical trial sponsored by St Jude Children’s Research Hospital and conducted at the University College London.²⁴⁶ In 2010, the first report of sustained partial correction of human hemophilia with a gene therapy vector was reported from this trial,²⁴⁷ with least 5 of 6 patients on this trial converted from severe to moderate hemophilia B.

H. Hemophilia B mice and future directions

An interesting development on the most recent scAAV8FIXco trial is that after safety was demonstrated at lower doses, AAV dose was once again escalated to the range used in the previous single-strand AAV serotype 2 trial. Once again an apparent immune-mediated transient inflammation of vector-transduced liver was observed, although in contrast to the previous trial persistent FIX expression was not eliminated.²⁴⁸ Taken together, these important trials define a threshold load of AAV capsid that has consistently stimulated capsid-specific cytotoxic lymphocyte recognition and potential transaminitis in humans. The limitation of hemophilia B mice to predict risk of CTL response to the AAV vector is demonstrated. On the other hand, the tasks for the next cycle of investigation - from preclinical application in mice to human application in gene therapy, and back to mice again - may now include:

1. Improve further the efficiency of delivery of FIX correction, so that true cure can be achieved using doses that do butt up against known toxicity. This is particularly important for the hemophilia patient population, which has an extremely high

incidence of underlying hepatic disease. A bioengineered FIX mouse has recently been reported by Shu-Wha Lin's group that has a gene knock-in of a *F9* gene incorporating an Arg338Ala (previously characterized)^{249,192; 250} and two additional gain of function mutations.^{147; 251} Mice with endogenous expression of this increased specific activity "FIX Triple" have a hemostatic phenotype that is not complicated by inappropriate activation of coagulation. Incorporating similar gain of function FIX variants into AAV vectors may permit improved correction of hemophilia while further decreasing vector capsid load. Alternatively, tyrosine capsid mutant AAV vectors have already been demonstrated to correct in hemophilia B mice while permitting dose-reduction, potentially decreasing immunogenicity, using a capsid improvement that is globally applicable and not directed to any one target organ.²⁵²

2. Test therapeutic approaches for the population that is not served by current hemophilia gene therapy approaches, e.g. those with neutralizing antibodies directed against either FIX or the AAV capsid. Multiple cell-based strategies would circumvent widely prevalent naturally-occurring antibodies against AAV and adenovirus vectors.
3. Explore the potential hemostatic role that FIX plays at sites of action within tissues. Although our laboratory assays are standardized to measure plasma factor activity as a marker of hemostatic potential, experimental results in hemophilia mice suggest that understanding the potential for FIX function at cell surfaces has therapeutic implications, whether it be the surface of the FIX gene-expressing platelet²²⁷ or the surface of the FIX-expressing fibroblast-like synoviocyte.¹⁸⁰
4. Further develop the recent approach of targeted integration for safe and persistent gene therapy.²⁵³

In summary, engineered FIX mice have become an essential reagent to the study of the intersection of coagulation, inflammation, and immunity in hemophilia and hemostasis.

III. The hemophilia A and B dogs

A. Background to the canine models

The primary benefits, value, and significance of dogs with hemophilia A and B is that they have provided the investigational basis to study the pathophysiology of blood coagulation, hemostasis, and thrombosis, to safely and successfully translate many experimental therapies into clinical practice,^{254; 255; 256; 257; 258; 259} and to continue preclinical evaluation of promising new strategies and therapies.^{201; 260; 261; 262; 263; 264; 265; 266; 267; 268; 269; 270; 271; 272} Several therapeutic agents, developed by performing research in the dogs with hemophilia, have been successfully introduced into human clinical practice and have a multi-year track record of safety and efficacy worldwide.^{254; 255; 256; 257; 258; 259} This means that these bleeder dogs are recognized as being a valid animal model of human-like hemophilia. It also means that when novel investigative treatments are shown to be safe and efficacious in the dogs with hemophilia, the data will reliably have a strong positive pre-clinical predictive value for subsequent safe and successful translation to human medicine. Because of this history of successful translational research, many investigators and advisory boards regard these dogs as an essential for preclinical testing of new treatments for hemophilia A and hemophilia B (MASAC Recommendations #137 and #160, http://www.hemophilia.org/research/masac/masac_all.htm).

B. Hemophilia A dogs

The Chapel Hill hemophilia A colony, established in 1947, appears to be the longest maintained strain of a serious genetic disease in dogs.²⁷³ The prototype animals were Irish Setters, but outbreeding was required to attain hybrid vigor. Both homozygous females and hemizygous males are maintained for breeding.²⁷⁴ The causative molecular defect appears to be due to an aberrant transcript in exon 22 that is reminiscent of the inversion defect that accounts for nearly half of human hemophilia A.²⁷⁵ A separate strain of hemophilia A dogs maintained at Queens University, Kingston ON also has an intron 22 inversion type defect.²⁷⁶ The plasma level of FVIII:Ag in both strains is <0.005 U/ml. Much of the basic understanding of hemophilia as a disease was first made in these animals. As in humans, severe disease is associated with levels of FVIII less than 1% of normal coagulant activity and the clinical severity is inversely proportional to the circulating FVIII level in plasma. Likewise, spontaneous bleeding typically occurs in joints and soft tissues but can occur at any anatomic site.^{277; 278; 279} Their availability played a key role in developing an understanding of the hemophilic clotting defect, which led to the establishment of modern methods for diagnosis and treatment including the partial thromboplastin time test, the one-stage factor assay system for FVIII, and the "home" treatment management system consisting of an early alert at the first sign of hemorrhage with immediate intensive plasma or concentrate treatment.^{280; 281; 282; 283; 284} These advances for detection, characterization, treatment, and prevention of hemophilic disorders over the past 6 decades significantly improved the quality and duration of life for patients with hemophilia from likely death at an age of < 20 years with concurrent painful and crippling arthritis to a nearly normal life span with relative sparing of joints.^{285; 286}

Despite the improved life expectancy and quality of life currently enjoyed by humans and animals with bleeding disorders, significant limitations in treatment persist. There are several interrelated areas of ongoing translational research in hemophilia A dogs that address these limitations. The first exploits strains of hemophilia A dogs that develop inhibitory antibodies to FVIII, the most common and most feared complication of replacement therapy.^{287; 288} These studies focus on mechanisms of inhibitor development and strategies for treatment and prevention. The second is a recent major advance that allows for the production of large amounts of recombinant canine FVIII (rcFVIII).²⁸⁹ This rcFVIII has provided a much needed new treatment for bleeding as well as a consistent antigen stimulus for the inhibitor dogs to test the hypothesis that polymorphisms in FVIII protein induce inhibitors in susceptible patients.²⁹⁰ In a third area of research, liver (and other organ) transplantation has provided a phenotypic cure for canine and human hemophilia.^{291; 292; 293; 294; 295; 296; 297} Fourth, novel approaches that modulate FVIII gene expression are in pre-clinical and clinical testing.^{298; 299}

C. Gene therapy in hemophilia A dogs

Gene transfer remains an important and active area of research and clinical investigation. All three initial human trials for gene therapy hemophilia A were stopped due to low-level expression or vector toxicity.^{300; 301; 302} Two of these three trials were tested in the Chapel Hill hemophilia A dogs and their outcomes were presaged by the results of these preclinical trials.^{303; 304} Fortunately, no inhibitor to FVIII was detectable after gene transfer in these patients. Several gene therapy strategies have been tried using retroviral, adenoviral^{305; 306; 307}, lentiviral³⁰⁸, and AAV vectors^{309; 310}. Currently, novel serotypes of AAV are showing continued progress with expression up to 20% of normal, levels that would prevent spontaneous bleeding in hemophilia A patients.³¹¹ Importantly, a strategy that co-administered the proteasome inhibitor bortezomib with AAV vectors expressing the relatively oversized FVIII transgene have documented multi-year expression.³¹² This breakthrough has the potential to expand the applicability of AAV vectors for gene therapy

in general but especially for large cDNAs such as FVIII. Most importantly, all animals undergoing gene transfer continue to be monitored for the long-term (i.e., years) safety and efficacy of all of these new methods. An unexpected but a highly desirable outcome in these gene transfer studies has been transgene expression over 10 years albeit at low levels but without detectable toxicity.^{161; 313} These observations are particularly important when considering the occurrence of hepatocellular carcinoma and angiosarcomas in mice with the lysosomal storage disease mucopolysaccharidosis type VII (MPSVII) after treatment with rAAV vectors.^{314; 315} Insertional mutagenesis is probably the operative mechanism but other possibilities include overexpression of a human transgene in rodents and colony contamination by oncogenic viruses. To address this issue we are screening all AAV-treated Chapel Hill hemophilia A (or hemophilia B described below) dogs for evidence of tumor formation or other pathology at the sites of gene transfer. The availability of relevant animal models that survive for 10 years after gene therapy with a species-specific transgene has proven invaluable to assess toxicity over time.

D. Hemophilia B dogs

The Chapel Hill strain of hemophilia B strain of dogs, maintained since 1966, was derived from the Guelph strain. The prototype animals were Keagles, a mixed beagle breed. As in hemophilia A, both the affected male and female genotypes are maintained for breeding. These animals have the severest bleeder state in the colony, with no detectable FIX:Ag in their plasma.³¹⁶ This characteristic makes them an outstanding model for gene therapy studies. The genetic defect is a missense mutation in a highly conserved residue of the catalytic domain of FIX; it results in a complete lack of detectable protein in the circulation of the affected animals. This mutation (G(A at nucleotide 1477) results in the substitution of glutamic acid for glycine-379 (G379E), a mutation that occurs at a residue that has remained invariant in serine proteases from bacteria to humans. The profound effect of the mutation on FIX levels suggest that the glycine at position 379 (position 211 in the chymotrypsinogen numbering system) plays a critical role in the integrity of the trypsin-like serine proteases in general.³¹⁷ A second strain of hemophilia B dogs, originally described in Lhasa Apso dogs at Auburn, has a deletion mutation and is prone to inhibitor formation.^{318; 319} The well-described phenotype and genotype of these hemophilia B dogs make them very desirable for developing widely-used basic assays for FIX³²⁰, studying the pathophysiology of hemophilia B, and testing replacement therapies and gene therapy strategies. Several broad current areas of research in the hemophilia B dogs include muscle- and liver- based strategies for gene transfer and the associated immune response, novel delivery strategies, and new FIX molecules with prolonged half-life.^{321; 322}

E. Gene therapy in hemophilia B dogs and in humans

The first two Phase I clinical trials in humans with severe hemophilia B, utilizing muscle- or liver- directed gene therapy with AAV vectors, were safe and successfully completed after having originally been approved based on the safety and efficacy demonstrated in several animal models including the FOBRL hemophilia B dogs.^{323; 324; 325} The intramuscular (IM) trial tested for safety, which was documented at the expense of dosing for efficacy.³²⁴ The ultimate doses of rAAV used in the human trials were chosen after consideration of data obtained in the hemophilia B dogs.^{319; 326; 327} Also based on studies in hemophilic dogs, the initial muscle trial was limited to subjects whose disease was due to a missense mutation (i.e., the trial excluded those with nonsense mutations or gene deletions). As observed in the dogs, none of the eight patients who received IM gene therapy has shown evidence of an inhibitor to date or any other significant side effects.^{323; 324} Muscle biopsies of injection sites performed 2 to 42 months after vector administration to patients confirmed gene transfer (as evidenced by Southern blot) and transgene expression (as evidenced by immunohistochemical staining).³²⁸ Pre-existing high-titer antibodies to AAV did not

prevent gene transfer or expression. Plasma levels of FIX were mostly less than 1–2% of normal. The vector was administered by separate needle injections and higher dosing seemed impractical; in addition, studies in the dogs suggested that higher doses might induce inhibitor formation.³²⁶ The preclinical information on vector dosing and patient selection provided by the hemophilia dogs were essential data for safely translating gene therapy to the clinic and achieving the goal of safety in these Phase 1 studies.

The liver-based trial of AAV delivery of FIX was initially promising and exciting as it resulted in FIX levels of 10–12%.³²⁵ Unfortunately, there was a gradual decline in FIX accompanied by a transient asymptomatic elevation of liver transaminases that resolved without treatment, both of which were associated with expansion of AAV capsid-specific CD8+ T cells in the circulation. The increased presence of these cells suggested that the transduced hepatocytes were being destroyed by cell-mediated immunity-targeting antigens of the AAV capsid, an event that was not predicted by the preclinical studies in any other species including rodents, dogs, and non-human primates.

While these studies have been safe, the major barrier remains the immune responses to vector capsid. Further studies are in progress in the hemophilic dogs to develop a model of this immune response.³²⁹ The development of reagents that rigorously characterize the canine immune response have enabled the identification of operative mechanisms in the hemophilia B dogs as well as the development of methods for preventing inhibitor formation.^{201; 316; 326; 329} Future liver-based studies in humans may require optimization of the expression cassette³³⁰ or immunomodulation to achieve long-term expression. The US Food and Drug Administration (FDA) has recently approved an immunomodulation protocol. As with hemophilia A, there is a significant need for additional preclinical and clinical studies to achieve successful gene therapy of hemophilia B in humans.

F. Development of alternative treatment modalities in hemophilia B dogs

Related to gene therapy, novel delivery strategies for muscle based gene transfer have been studied in the hemophilia B dogs.^{266; 331; 332} These methods have achieved muscle transduction in entire limbs and reasonable expression levels of at least 10%. This novel method also holds great promise for treating many genetic disorders including muscle disorders such as muscular dystrophy.

Furthermore, novel recombinant FIX preparations are being tested that have significantly prolonged half lives.^{321; 333} If shown to be safe and efficacious in human trials, the immediate practical outcome is that hemophilia B patients may be able to go from twice a week intravenous injections to weekly or monthly injections, possibly even subcutaneously.^{334; 335} For a patient or a parent of a young child with hemophilia B, the improvement in quality of life associated with less frequent needle punctures would be profound.

Finally, as with hemophilia A, organ transplantation produces a phenotypic cure.^{336; 337; 338} While curative, this treatment option is limited by the number of donors and the need for immunosuppression to prevent rejection of the transplanted organ.

G. Normal donor dogs

Normal dogs are maintained as a “walking blood bank” with compatible blood types for preparation of replacement products for treatment of bleeding. The affected dogs of all strains are severe bleeders with an average of approximately 5 spontaneous bleeds per year.^{161; 279; 339} Clinical bleeding requires immediate replacement therapy with pooled normal canine plasma for the hemophilic animals. Without transfusion therapy hemophilia A and B are fatal at an early age. The new capacity to produce rcFVIII has been an

important addition to the treatment options for the hemophilia A dogs.²⁸⁹ At present, recombinant canine FIX is not available in sufficient quantities for treatment. The maintenance and breeding programs require constant close attention by specially trained personnel for the detection of early hemorrhages and their management.

H. Summary of the benefits of research with large animal models of hemophilia

Work at the FOBRL over the past six decades and in other laboratories includes studies in dogs and humans with hemophilia that have contributed to considerable progress in the basic understanding of these diseases and the development of safe and successful treatments. But their development has not been without setbacks, and limitations persist. For hemophilia patients, anti-hemophilia products are expensive (typically \$100,000 per year or more for an adult) and not readily available in less developed countries.³⁴⁰ For most patients worldwide, replacement therapy, if available at all, is usually used to treat acute bleeding on demand rather than to provide the benefits of continuous prophylactic coverage.^{286; 341} Furthermore, the products' half-life in the circulation is relatively short and quite variable among patients (e.g., 8 to 23 hours for FVIII), mandating repeated venipunctures or port implantation for prophylactic administration.^{257; 342} Last, for the plasma-derived material, despite the introduction of viral inactivation techniques that have eliminated the risk of HIV transmission, there are ongoing concerns about other transmissible agents (e.g., prions) that may not be eliminated.^{343; 344; 345; 346; 347; 348} While there has been considerable progress in gene therapy for hemophilia, several barriers remain that are being addressed with the dogs with hemophilia A and hemophilia B.³⁴⁹ The success with correction of defects due to single genetic mutations such as hemophilia dogs may presage great expansion of the role of gene therapy in clinical medicine, both for genetic and acquired diseases. Additional large animals models (such as canine and ovine) are available and are being further characterized to aid in this task.^{350; 351} For example, the recently re-established line of hemophilia A sheep shows a severe bleeding phenotype with no circulating FVIII antigen due to a premature stop codon and frameshift in exon 14.³⁵¹ Sheep models have been particularly useful for *in utero* gene and gene therapy studies, thereby adding to the range of potential studies in hemophilic animals for development of novel approaches to therapy.

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Table 1

Animal models and strains with severe hemophilia A or B.

Species	Strain	Genotype	Reference
Mouse	Hemophilia A	exon 16 knockout	Bi et al. 1995
	• C57BL6/129		Bi et al. 1995
	• BALB/c		Waters et al. 2009
	• CD4-deficient		Sabatino et al. 2010
Mouse	Hemophilia A	exon 17 knockout	Bi et al. 1995
Mouse	Hemophilia A	Transgenic for human F.VIII with missense mutation R593C; exon 17 knockout	Bril et al. 2006
Mouse	Hemophilia A	Transgenic for human MHC II allele HLADRB1* 1501; exon 17 knockout	Reipert et al. 2009
Mouse	Hemophilia B	Targeted deletion	Lin et al. 1997
	• C57BL6		Mingozzi et al. 2003
	• BALB/c		Mingozzi et al. 2003
	• C3H/HeJ		Mingozzi et al. 2003
	Hemophilia B	Knock-in for human F.IX with crm+ missense mutation R333Q	Jin et al. 2004
	Hemophilia B	Knock-in for human F.IX with early stop codon at amino acid residue 29	Hu et al. 2010
Mouse	Hemophilia B (C57BL6 or C3H/HeJ)	Targeted deletion plus transgenic for human F.IX: <ul style="list-style-type: none"> • crm- missense mutation G381E • crm+ missense mutation R180W • Early stop codon at residue 29 • Late stop codon at residue 338 	Sabatino et al. 2004 Cao et al. 2009
Rat	Hemophilia A	Missense mutation L176P	Booth et al. 2010
Dog	Hemophilia A (Chapel Hill)	Exon 22 inversion	Graham et al. 1949 Lozier et al 2002
Dog	Hemophilia A (Queens)	Exon 22 inversion	Hough et al. 2002
Dog	Hemophilia A (Alabama)	unknown	Connelly et al. 1996
Dog	Hemophilia B (Chapel Hill)	CRM- missense mutation G379E	Evans et al. 1989
Dog	Hemophilia B (Alabama)	Partial deletion/early stop codon/low mRNA	Mausser et al. 1996
Sheep	Hemophilia A	Early stop codon/frameshift	Porada et al. 2009