Factor IX: Insights from knock-out and genetically engineered mice

Paul E. Monahan
Department of Pediatrics, Gene Therapy Center, and the Harold R. Roberts Comprehensive Hemophilia Diagnostic and Treatment Center, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

Summary
The study of coagulation factors has been rapidly advanced by studies performed using genetically engineered mouse strains. Investigation of factor IX (FIX) has benefited from excellent gene-deleted mouse models that recapitulate many of the features of human haemophilia B. Moreover, advanced positional cloning techniques and availability of technology to allow not only knock-out mice, but also knock-in and knock-down mice, provide new opportunities to observe genotype-phenotype and structure-function correlations regarding FIX, as well as the interaction of FIX with inflammatory, immune, and tissue repair systems. In this paper, available FIX knock-out mice and additional haemophilia B mouse models are reviewed specifically in regards to observations these models have facilitated concerning factor IX gene expression and factor IX protein pharmacokinetics; the role of FIX in haemostasis, thrombosis and wound healing; insights into coagulation FIX arising out of gene therapy applications in haemophilia mouse models; immunology of tolerance or loss of tolerance of FIX and inhibitor antibody formation.

Keywords
Factor IX, mouse model, transgenic mouse, haemophilia B, gene therapy

Introduction
Biomedical research has been revolutionized by the ability to genetically manipulate the mouse genome, an accomplishment recognized by the award of the 2007 Nobel Prize in Medicine or Physiology to Oliver Smithies, Martin Evans, and Mario Capecchi (1). The study of the procoagulant serine protease factor IX (FIX) and of haemophilia B, the disease that results from deficient activity of FIX, has been considerably advanced by study in vivo in genetically engineered mice. Experimentally induced models of haemophilia B (2) (e.g. infusion of anti-FIX antibodies) and the use of hereditary large animal models of haemophilia (2, 3) have been used for decades to advance evaluation in vivo. Those approaches, however, do not take advantage of the extensive knowledge of the mouse genome and the excellent reproductive capacity of mice, which permit experimental designs to evaluate physiologic and pathologic endpoints that can be statistically evaluated and that have close parallels to the human condition.

The mouse F9 gene open reading frame has an 80% sequence similarity with human F9 ORF (4) (23% in the activation peptide) (5), and mouse factor VIII (FVIII) (FIX’s co-factor in the tenase complex) shares 74% homology overall with the human FVIII gene (6). The coagulation factor domain structures as well as the basic processes leading to coagulation are very similar in humans and mice, and the same global assays of coagulation and specific factor measurements can be adapted to and interpreted in mice. The parallels and the divergences between humans and mice in regards to haemostatic and thrombotic processes have been reviewed (7–9), as have mouse strain-specific differences in normal ranges for basic coagulation screening assays, and are important to consider in experimental design (see the Mouse Phenome Database available at: http://phenome.jax.org/pub-cgi/phenome/mpdcgi?rtn=meas/catlister&req=Cblood%20hematologyqqqcoagulation).

FIX-knockout mice (FIX–/– mice)
By 1997, expression of FIX had been demonstrated in wild-type mouse models (and a handful of large animals) using a variety of gene transfer approaches, including retroviral, adenoviral, and adeno-associated virus (AAV) vectors, as well as naked DNA gene transfer (10). In each of these therapeutic models, the ability to examine phenotypic correction was confounded by background expression of mouse FIX. In addition, examination of long-term efficacy was in most cases complicated by the devel-
### Table 1: Bioengineered mouse models for the study of factor IX and haemophilia B.

<table>
<thead>
<tr>
<th>Model</th>
<th>Strain</th>
<th>Genotype</th>
<th>Phenotype/observations of interest</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine factor IX knockout</td>
<td>C57BL/6 and 129Sv</td>
<td>Exon h of mouse factor IX knocked out (neo gene inserted)</td>
<td>CRM(-) severe bleeding phenotype</td>
<td>[11]</td>
</tr>
<tr>
<td>Murine factor IX knockout</td>
<td>C57BL/6 and 129Sv</td>
<td>Exon g and h of mouse factor IX knocked out (neo gene inserted)</td>
<td>CRM(-) severe bleeding phenotype</td>
<td>[13]</td>
</tr>
<tr>
<td>Human factor IX knock-in Haemophilia B missense mutation</td>
<td>C57BL/6 and 129Sv</td>
<td>human FIX cDNA with Missense glycine for Arg333→gly in catalytic domain; expression from mouse FIX locus, FIX promoter</td>
<td>CRM(+) severe bleeding phenotype Relative immunologic tolerance: human factor IX</td>
<td>[15] [108]</td>
</tr>
<tr>
<td>Human factor IX transgenic haemophilia B: Early stop codon mutation</td>
<td>C57BL/6</td>
<td>(All: random insertion, transthyretin promoter)</td>
<td>No FIX protein or activity</td>
<td>[27]</td>
</tr>
<tr>
<td>Human factor IX transgenic (non-haemophilic) hFIXWTtg</td>
<td>C57BL/6 x SJL</td>
<td>mFIX WT and hFIX cDNA minigenes (various) with or without age-stability element</td>
<td>hFIX protein levels 60–800% of normal; activity &gt;100%</td>
<td>[27]</td>
</tr>
<tr>
<td>Transgenic (haemostatically normal) mice co-expressing human FIX</td>
<td>Normal mouse factor IX gene sequences; normal Human factor IX promoter FIX promoter with +13 factor IX Leyden mutation drive CAT expression</td>
<td>Leyden mutation abolishes reporter gene expression in vivo in juveniles, followed by age-dependent male-specific gene expression</td>
<td>[33]</td>
<td></td>
</tr>
<tr>
<td>Haemophilia B coexpressing factor V Leiden</td>
<td>C57BL/6</td>
<td>mFIX−/− crossed with FVL−/− or FVL +/+</td>
<td>Express mouse FIX systemically; Normal plasma aPTT; Express hFIX338A at supra-physiologic levels exclusively in breast milk</td>
<td>[159]</td>
</tr>
<tr>
<td>Haemophilia B With Plasminogen deficiency</td>
<td>C57BL/6</td>
<td>mFIX−/− crossed with Plg−/−</td>
<td>Severity of Plg− wasting syndrome and mortality improved in the presence of deficient FIX</td>
<td>[58]</td>
</tr>
<tr>
<td>C/EBPtx knockout</td>
<td>C57BL/6</td>
<td>CCAAT/enhancer-binding protein alpha (C/EBPtx) knocked out (transcriptional regulatory protein with a binding motif in the FIX promoter)</td>
<td>Deficient factor IX mRNA and factor IX activity; defective energy homeostasis; death on day of life 1.</td>
<td>[31]</td>
</tr>
</tbody>
</table>
opment of antibody- or cell-mediated immune responses to the human xenoprotein.

In 1997–1998 three different groups created haemophilia B mouse models expressly for the purpose of studying gene therapy (11–13); one of these groups also envisioned the potential value of these mice “for studying structure-function relationships of recombinant factor IX proteins in vivo” (12) (Table 1). The strategy of the latter group was to delete the promoter through the third exons of the mouse FIX gene using a “plug-and-socket” strategy as originally described by Smithies (14). The promoter and the first three exons of the FIX gene are deleted by the insertion of a neo gene plus a partially deleted hypoxanthine phosphoribosyl transferase minigene. The plug-and-socket design potentially allows the subsequent insertion of other sequences into the same locus of correctly targeted embryonic stem cells, consistent with the goal of creating a model for FIX structure-function studies. Recently, a mouse that expresses a mouse FIX gene with a point mutation of Gla domain lysine 5 to alanine has been generated using this strategy (personal communication, Darrel W. Stafford). The other FIX–/– mouse models were generated by the insertion of the neo coding region into either exon g (11) or into exons g and h (13) of the mouse gene.

Each of these FIX–/– models fails to produce any hepatic FIX mRNA, has no circulating FIX protein, bleeds excessively with haemostatic challenges such as tail clipping, and displays pathologic changes of bleeding-induced poor wound healing (as discussed below; see Fig. 1). These mice were initially reported to have FIX procoagulant activity of up to 0.03–0.08 U/ml in a onestage (aPTT-based) clotting assay. This apparent activity in the absence of protein has subsequently been shown to be an artifact that can be reduced to <0.01 U/ml if standards for the clotting assay are constructed with uniform dilutions of the knockout plasma (15). All three FIX–/– models were created on the C57BL/6 (blastocyst) and 129Sv (ES cell) genetic background. Much is known about the murine immune system as studied in inbred strains. To further mechanistic studies of immune responses to FIX, FIX–/– mice have also been bred into pure MHC class II HLA2 C3H/HeJ (17) strain backgrounds.

Figure 1: Thrombus formation, haemostasis, and wound healing in wild-type (WT) and FIX–/– mice. A and B) Platelet adhesion and thrombus formation are complete at 15 minutes after ferric chloride-induced mesenteric arteriolar injury in haemostatically normal mice. Platelet-rich thrombus formation is not supported in the absence of factor IX (FIX). Image courtesy of Drs. Heyu Ni and Adeli Reheman. C and D) Carotid arteries collected at one hour following an injury consisting of mechanically denuding the endothelium using an intraluminal needle. Neutrophils and scant fibrin present in FIX–/– contrast with circumferential thrombus in WT vessel. Images courtesy of Drs. Tong Gui and Darrel Stafford. E and F) Day 7 following skin punch wound biopsy, the WT wound is re-epithelialized and the area of prior wound is identified by lack of hair follicles. Despite epidermal closure of the wound, haemophilia B mice develop persistent haematoma (*) and mononuclear cell infiltrates adjacent to the wound. Images courtesy of Drs. Maureen Hoffman and Dougald Monroe. G and H) Two week following knee joint capsular puncture to induce bleeding, WT mouse joint shows no sign of injury or synovitis, with the joint space well maintained and a thin (~3 cell layer, black arrows), relatively avascular synovial lining. Cartilage lining articular bone is in bottom left of each figure and adjacent synovium in upper and right part of figure. The joint space of the FIX–/– mouse is narrowed by blood-induced proliferative and infiltrative synovial pathology, including frank blood (yellow arrows), mononuclear cell infiltrate, neovascularity (†), and synovial lining cell thickening (black arrows). Images courtesy of Dr. Junjiang Sun.

Spontaneous haemorrhages (occurring in the absence of observed trauma) are relatively rare in these mice, although musculoskeletal bleeding and footpad swelling are observed, especially after fighting with cagemates (Fig. 2). It is worth noting that mice with a complete deletion of FIX expression deliver normal size litters; most pups survive normally to wean and adulthood. As reviewed elsewhere (18) this normal survival of mice with complete deletions of FIX or other intrinsic coagulation pathway factors (factor XII, factor XI, FVIII) contrasts with mice having absent tissue factor (TF) or factor VII and resultant absence of thrombin generation via extrinsic pathway proteins. Deletion of TF results uniformly in embryonic lethality (19). Absent factor VII results in normal embryonic development, followed by fatal intraabdominal haemorrhage during birthing and/or intracranial hemorrhage prior to weaning (20). Deletion of common pathway procoagulants (prothrombin, factor V, or factor X) or of the vitamin K-dependent γ-glutamyl carboxylase results in varying degrees of intrauterine lethality, with all live-births succumbing to
fatal hemorrhage in the peripartum period or prior to weaning (21–25). Consistent with the clinical scenario in human hemophilia B, FIX−/− mice (defective intrinsic pathway) are particularly at risk for musculoskeletal bleeding, i.e. bleeding in sites where TF expression is poor, and are not observed to bleed in tissues with rich TF expression and apparent TF-dependent hemostasis (e.g. myocardium, testis) (18). Additionally, while a potential role in embryonic development, independent from hemostasis, has been postulated for some procoagulants (e.g. prothrombin) (21, 22), experience to date does not suggest any direct function for FIX outside of haemostasis and thrombosis.

Additional haemophilia B mouse models

A limitation in the use of FIX−/− mice for the study of new hemophilia therapeutics results from the fact that FIX epitopes are never expressed during the mouse’s development (including thymic development). In modeling gene therapy approaches, and in some cases following FIX protein replacement, delivering human or canine FIX leads to the development of antibodies that inhibit FIX activity (inhibitors). Although it is not surprising that the FIX−/− mice make antibodies against the xenoprotein FIX including human or canine sequences, the FIX−/− mice are also reported to raise inhibitors against recombinant murine FIX (but not following murine plasma infusions) (26). The FIX−/− mice do not model human disease in this respect, as only 2–3% of individuals with haemophilia B develop inhibitors following treatment with plasma-derived or recombinant human FIX.

Jin et al. generated a haemophilia B mouse to reproduce the type of FIX mutation most common in haemophilic patients, which is an underlying missense mutation leading to the production of a circulating defective FIX protein (so-called antigenically cross-reactive material positive (CRM+)) that developed massive left forelimb musculoskeletal bleeding after fighting with a cage mate. Although ambulating, the mouse haemorrhaged to a haemoglobin about 20% of normal. Factor IX−/− mice have been used to model a wide range of strategies for gene correction of haemophilia and other monogenic disorders.

New vitamin K-dependent proteins

For personal or educational use only. No other uses without permission. All rights reserved.
Interestingly, mice carrying the early stop mutation did not respond to the same therapy, and the effect of sequence context of the FIX mutation upon this therapy’s potential is unresolved.

**Engineered mice in the study of FIX expression and pharmacokinetics**

Several groups have used engineered mice to study transcriptional regulation of the FIX protein. In most cases, targets for study have been prompted by clinical observation of patients with haemophilia B and isolated promoter mutations, the effect of which could then be modeled in mice. Although gene transfection studies in relevant cell lines may be used to suggest sequences for study in transgenic mice (29), it has been observed that transcription factor activity observed in cell lines is not always observed in the adult liver, so that study in animals is a more stringent test system (30).

Davies et al. took advantage of a previously generated mouse lacking the CCAAT/enhancer-binding protein alpha (C/EBPα), and showed that homozygous deletion of this transcriptional regulatory element, which binds adjacent to the F9 start site for transcription to transactivate the FIX promoter, leads to significantly deficient FIX transcription (31). Several groups used mice to study age-specific regulation of FIX gene expression. FIX expression increases with age in both sexes in humans and mice (32). Boland et al. generated transgenic mice containing the −189 to +21 FIX promoter segment linked to a chloramphenicol acetyltransferase (CAT) reporter to specifically score the effect of the nucleotide +13 Leyden mutation (numbering here relative to the start site for transcription) (33). The Leyden mutation promoter, but not this segment of wild-type promoter, directed age-dependent and sex-specific post-pubertal increases in FIX expression. Brady et al. examined a different haemophilia B Leyden mutation at nucleotide −26 using normal and testicular feminized mice to demonstrate that the pubertal age-related FIX levels were dependent on the combined binding of androgen receptor and C/EBPα in the region of this mutation (34). Kurachi et al. subsequently expressed FIX throughout the lifetime of normal and testicular feminized FIX−/− mice have also been used to examine pharmacokinetics and potential therapeutic value of variant FIX proteins (mupiens). Three groups have examined in vivo the effect of mutations within the Gla domain that are known to affect the avidity of FIX’s specific binding to extracellular matrix collagen IV. Gui et al. described the increased circulating levels of infused FIX mutants K5A or V10K, apparently resulting from their greatly decreased binding to endothelial collagen IV, avoiding sequestration in the liver and other sites (35). A FIX K5R Gla mutant with increased specific binding to collagen IV disappeared from the circulation rapidly after infusion with greatly decreased area under the curve kinetics relative to wild type FIX. The decreased binding mutant FIXs, delivered as recombinant K5A hFIX protein to FIX−/− mice (36) or as combined K5A/V10K hFIX gene therapy vectors to FIX−/−/T cell CD4 knockout mice (37), demonstrated two-fold greater survival. In addition, Begbie et al. studied the clearance of FIX modified to delete multiple sites of post-translational glycation in the activation peptide (Δ155–177); clearance of this modified FIX was also markedly decreased (36). While haemophilic dogs have been a faithful model for preclinical pharmacokinetic studies of FIX, the scarcity and cost of these animals suggests that the demonstrated utility of pharmacokinetic screening in haemophilic mice will be valuable. This is especially true if improved circulating factor levels in mice can be correlated with haemostatic efficacy in clinically relevant bleeding models (see below).

**FIX in haemostasis: Bleeding and wound healing modeled in haemophilia B animals**

Potential protein and gene therapies are frequently evaluated in haemophilia B models, and it is desirable to correlate correction of circulating FIX with some indication of improved haemostatic protection. Tail transection bleeding time assays (“tail-clip assays”) that measure initial haemostasis have been used to evaluate mouse models of coagulopathy. Nevertheless, the tail-clip assay alone may fail to reliably distinguish factor deficiencies (38–41). Contributors to the discrepant results of tail bleeding assays include the central role of platelets in initial haemostasis (as distinct from soluble clotting factors), as well as variations in central tail artery constriction and dilation that may obscure discrete endpoints. Delayed haemorrhage and persistent oozing of blood resulting in decreased survival are, however, hallmarks of haemophilic bleeding. For this reason, the tail transection bleeding time may be modified to observe persistent as well as initial haemostasis (42). Secondary bleeding time assays have been described to measure this characteristic phenotype in humans with haemophilia (43), as well as in a monkey model of haemophilia (44).

The tail-clip model may be useful for evaluating new therapies, but the model itself does not advance understanding of FIX or its role in haemostasis. Two recently described wound models studied in haemophilic mice have stimulated insight into FIX-dependent haemostasis. The first of these is a cutaneous wound model. Normal wound healing entails four overlapping phases: haemostasis, inflammation, proliferation, and remodeling or resolution. In the method of Hoffman et al., the healing of standardized skin punch biopsy wounds of haemophilic mice, as compared to haemostatically normal mice, is delayed and histologically abnormal (45). Specifically, closure of wounds is delayed, neovascularity is prominent, macrophage infiltration is delayed but subsequently prolonged, as is iron contamination; haemorrhage in the tissue near the wound site is seen even after the surface wound closes (Fig. 1). Significantly, restoring haemostasis at the time of injury, without prolonged coagulation protein replacement throughout healing, does not normalize healing in haemophilia B mice (46). Extending the observations using this model, perivascular TF is shown to be down-regulated following cutaneous wounding, with TF expression depressed for a longer period in haemophilic mice than in wild-type mice (47). It appears likely that appropriate wound healing requires...
the coordination of effective haemostasis and modulation of inflammation, and that insights may be gained by modeling the ways this coordination is dysregulated in haemophilia B.

Understanding the role of FIX and FIX replacement therapies in haemostasis and wound healing will also likely be advanced by the study of arthropathy in haemophilic mice. Valentino et al. have evaluated a serial blunt trauma injury to the knee joint of FVIII-deficient haemophilic mice and have shown that the resulting acute and chronic histopathology and radiographic findings closely model those seen in human haemophilic arthropathy, and correlate with functional losses in terms of exercise tolerance (48–51). In the FIX−/− mice, a modification of this technique has been developed, consisting of a needle puncture of the joint cavity resulting in a reproducible major haemarthrosis (Fig. 1) (52). Using this model, the potential for extravascular (intra-articular) FIX to provide protection from sequelae of joint bleeding has been examined. When compared with mice receiving the same or greater doses of human FIX intravenously, FIX−/− mice receiving intraarticular FIX concentrate were protected from synovitis, although no FIX activity could be detected in plasma after intraarticular injection. The apparent amelioration of haemophilic joint destruction by FIX in the joint space, despite absent circulating FIX, supports further investigation in vivo of tissue-specific haemostasis as well as the intersection of coagulation proteins and inflammatory pathways.

Engineered mice and the role of FIX in thrombosis

In-vitro models of coagulation suggest that the processes that initiate therapeutic haemostasis in the injured blood vessel progress along a continuum to produce occlusive thrombus formation. However, observation in vivo of animals having deficiencies in specific intrinsic pathway proteins suggests different roles for these proteins in supporting thrombotic versus haemostatic events. Arterial thrombus formation in F IX−/− mice has been examined in three models (53, 54). Occlusive thrombus resulting from ferric chloride (FeCl₃) injury, using a range of concentrations of FeCl₃ applied to the carotid artery, was measured as loss of doppler blood flow signal distal to the injury. At low and intermediate FeCl₃ concentrations, FIX−/− mice as well as FXI−/− mice were relatively protected from arterial occlusion when compared to wild-type mice (53). In separate experiments using comparable conditions, a similar defect in formation of arterial thrombus formation and stability has been observed in mice deficient in intrinsic pathway factor XII (55). The morphology of the defective carotid artery thrombotic response following a mechanical endothelial injury has also been observed in FIX−/− mice (Fig. 1) (56). Finally, intravital microscopy following mesenteric arteriolar ferric chloride injury has been studied in FIX−/− mice, allowing real-time observation of thrombus formation. A striking inability of platelets interacting with the injured endothelium to form visible platelet aggregates was seen (Fig. 1). Labeled platelets were observed to encounter the vessel wall in normal numbers in the FIX−/− mice, and the number of adherent single platelets was normal in the first 3–5 minutes after arteriolar injury. Although some thrombin generation via TF/VIIa activation of factor X was presumably intact and evidenced by platelet poor fibrin deposits that disrupted linear blood flow thrombin formation sufficient to support even early platelet aggregation was absent in FIX−/− mice, as was subsequent propagation of thrombus (56). Despite the apparent requirement of an intact classical intrinsic coagulation pathway for stable occlusive thrombus formation (as recently reviewed) (57), defective haemostasis is observed only in FIX−/− mice and not in FXI−/− or FXII−/− mice (12, 53, 55).

An additional distinction between the functions of FIX and factor XI is implicit in the results of double knockouts created by crossing these mice with plasminogen-knockout mice. Plasminogen-deficient mice have a phenotype characterized by normal embryonic development but decreased longevity due to disseminated fibrin deposition. Intriguingly, the cross of factor XI deficiency on the Plg−/− background resulted in decreased survival and a pulmonary inflammation with fibrosis, which was not observed in any of the single deficiencies, suggesting a function of factor XI that is independent of FIX activation (58). The cross of FIX deficiency onto the Plg−/− background resulted in improved longevity and weight gain, consistent with disrupting the primary role of FIX in thrombin generation and fibrin formation; there was no pulmonary inflammation, fibrosis or other sequelae observed to suggest a role of FIX outside of coagulation.

Clinical observation supports the notion that elevated baseline levels of FIX activity contribute to venous thrombotic risk (59); association of FIX activity levels with arterial thrombosis has been harder to demonstrate as an independent variable (60, 61). Kurachi et al. have generated transgenic mice having normal endogenous mFIX expression but also expressing human FIX at significantly higher levels. FIX-deficient mice receiving FIX−/− had an increased susceptibility to venous thrombosis, independent of FIX levels (62). Surviving mice were found to have a prolonged FIX prothrombin time, which was reversed by recombinant FIX infusion (63, 64). Using genetically engineered mice, Schlachterman et al. modeled the possibility that co-inheritance of factor V Leiden might explain the variation in phenotypic severity that has been observed in individuals with severe FIX or FVIII deficiency (65). The haemophilia B or A phenotype was not improved following large vessel damage (FeCl₃, carotid artery model), and no sustained thrombus formation was observed in any of the activated protein C resistance scenarios modeled. Nevertheless, heterozygosity or homozygosity for factor V Leiden mutation, or the infusion of exogenous activated factor V, all resulted in shortening of the haemophilic clotting times and rescued the ability of
FIX−/− and FVIII−/− mice to generate thrombi in microvascular injury models.

**Gene therapy in haemophilia B mice: What have we learned about FIX?**

The most common experimental use of haemophilia B mice has been to explore the potential for gene therapy, taking advantage of the well-characterized disease phenotype. The advantages of haemophilia B as a model system for exploring gene therapy strategies have been reviewed frequently (66), and include i) disease correction requires expression of a single gene, and only partial correction is needed to improve the phenotype; ii) the FIX protein and gene are well characterized at the molecular and biochemical levels for multiple species and the requirements for post-translational modifications are understood; iii) success of gene therapy and phenotypic correction can be followed longitudinally with standardized coagulation assays; iv) both large and small animal models are available that closely reproduce the human haemophilia phenotype. For this reason, multiple gene therapy strategies have been modeled in haemophilic mouse models. Strategies that have been tested specifically in haemophilia B mice are presented with references in Figure 2 and a brief summary follows; additional approaches for FIX gene correction have been modeled in haemostatically normal mice and will not all be reviewed here. The reader is referred to many excellent reviews of haemophilia gene therapy that are available (66–70); a comprehensive synopsis of approaches to gene delivery is beyond the scope or the purpose of this review.

The strategies studied in haemophilia B mice include approaches *ex vivo*, 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 keratinocytes (71), embryonic stem cells directed toward hepatic endodermal differentiation (72), allogeneic hepatocytes (73), haematopoietic stem cells (74), haematopoietic stem cells directed toward erythroid differentiation (75), allogeneic megakaryocytes (76), and encapsulated primary myoblasts (77–79) have all been modeled 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 (74, 75) and FIX tolerance induction (72, 74, 75).

Alternatively, *in vivo* approaches studied in haemophilia B mice deliver therapeutic nucleic acid sequences directly as either naked DNA (80–86), via a chemically formulated vector (e.g. lipids), or using a virus vector for gene delivery. When naked DNA delivery is used, achieving FIX expression at levels adequate to correct the bleeding phenotype has depended on enhancing delivery of the FIX gene with the concurrent use of hydrodynamic pressure (80, 87), electrical current (84), or ultrasound (81). Each of these mechanical approaches may cause transient cell damage and several groups are investigating whether these enhancements to gene delivery can be scaled for safe use in large animals and humans.

The earliest uses of viral vectors in haemophilic animal models (haemophilia B dogs) employed onco-retroviral (88) and adenoviral vectors (86, 89–91). Although retroviral approaches in mature animals directed relatively low levels of expression, delivery of retroviral vectors in the immediate neonatal period has produced physiologic and persistent levels of expression in mice (92, 93), and the neonatal delivery strategy has converted haemophilia B dogs from severely to mildly deficient. Adenoviral vectors can lead to supraphysiologic levels of FIX expression in mice, although host immune responses to conventional adenoviral vectors limited persistence of expression. Helper-dependent adenovirus vectors (all viral genes deleted) may direct FIX expression in mice lasting for months (94). Nevertheless, the adenovirus capsid (in the absence of adenoviral genes) still elicits a spectrum of interferon-responsive genes; whether these will significantly impact efficacy and safety, or necessitate some concurrent treatment to induce tolerance (95), requires further study (96). More recently, phenotypic correction of FIX-deficient mice has been achieved using lentiviral vectors (97, 98). When specific strategies to avoid expression in antigen-presenting cells are incorporated FIX levels of >10% have been maintained for months in mice (99). Traditional adeno-associated virus (AAV) serotype 2 vectors (100–105), alternative AAV serotypes (98, 105–111), and AAV vectors with self-complementing transgene cassettes (103, 112, 113) can all produce dose-dependent supraphysiologic FIX expression lasting months to years in mice. Although host immune responses to AAV-FIX delivery in mice have been modest in comparison to other vectors (96, 114), the potential for immune response in human hosts with previous exposure to wild-type AAV is an area of active investigation (67, 69, 115).

For the correction of FIX deficiency, only retrovirus and adeno-associated virus vectors (no *ex vivo* approaches) have advanced to clinical application in humans. Two brothers were treated with a retrovirus FIX vector in China in the early 1990s (116). A total of 15 individuals with haemophilia B have been treated in two clinical trials using adeno-associated virus (AAV) serotype 2 vectors delivered either via intramuscular injection or injection to the liver via the hepatic artery (117, 118). Although each of these trials yielded important safety data, long-term correction of FIX activity has not been shown in any trial.

While the haemophilia model has done much to inform the field of gene therapy, the focus of this review is on gene therapy applications that have extended our understanding of FIX and FIX-dependent hemostasis and thrombosis. One large knowledge deficit in the clinical care of FIX deficiency surrounds immunologic tolerance of FIX; because gene therapy potentially offers several insights regarding immune tolerance of FIX, that area will be discussed at length in the next section.

Insights regarding FIX have arisen from attempts to achieve FIX expression from organs or tissues outside of the natural site of FIX production, which is the liver. The capability of cells other than hepatocytes in *situ* to perform the complex post-translational modifications required for a fully active FIX protein is clearly essential for successful FIX protein production via recombinant or transgenic animal or plant approaches. Limitations in the ability of rodent producer cells (Chinese Hamster Ovary, CHO cells) used in commercial recombinant FIX production to properly sulfate and phosphorylate the FIX protein, and perhaps also differences in glycation, have been implicated in the lower
initial plasma recovery of FIX activity seen clinically with the use of recombinant FIX, as compared to human plasma-derived FIX. Arruda et al. examined the capacity for cultured human myotubes to perform FIX posttranslational modifications and found that myotube-expressed FIX also differs from human plasma-derived FIX, having limited tyrosine sulfation and serine phosphorylation and a low relative recovery, followed by a relatively normal terminal half-life (119). Although skeletal muscle has been shown to have only about 5–10% of the amount of gamma-glutamyl carboxylase present in the liver, carboxylation of the Gla-domain moieties appears to be sufficient at the sub-physiologic levels of expression that are the usual goal of gene therapy (103, 119), there is evidence to suggest that when gene expression is pushed to supraphysiologic levels from the ectopic site of expression in skeletal muscle, the specific activity of expressed FIX may fall, perhaps exhausting the PTM machinery (120).

The phenomenon of specific binding of FIX to extracellular matrix collagen IV has been described previously (121, 122). A functional role in haemostasis resulting from this FIX binding of collagen IV has not been described, although sequestration of FIX in tissues as a result of this binding does affect the circulating levels of infused FIX protein (35) (see above, “Engineered mice suggest that studying this rare complication in mice is possible, and may guide development of therapies (Table 2)."

Anti-FIX antibodies, inhibitors, and immunomodulation of anti-FIX responses: Characterization in mice

Preclinical data from haemophilic mice has been used to support the conduct of three human gene therapy trials for the correction of FIX deficiency (116–118). The latest of these trials, in particular, has sent investigators back to the FIX models to attempt to understand an apparent immune response against FIX-transduced cells that was not anticipated based on preclinical approaches. Transient partial correction of haemophilia B was observed in a human clinical trial using an AAV2 vector to deliver the FIX gene to the liver. Evidence of asymptomatic hepatocellular inflammation was observed in one subject coincident with loss of FIX expression; subsequent studies have suggested the possibility that cytotoxic T lymphocytes eliminated treated hepatocytes presenting intact AAV vector structural proteins for immune recognition (118). Other possible mechanisms have been suggested, including the possibility that transduced hepatocytes might at some low frequency transcribe an alternative reading frame present in the FIX sequence and present immunogenic peptides (derived from out of frame sequences) for immune recognition (C. Li and R. J. Samulski, personal communication) (123). There was no evidence of immune response directed against wild-type human FIX protein. Nevertheless, neutralizing antibody responses against FIX (inhibitor antibodies) do arise as a rare, potentially devastating, and poorly understood complication of haemophilia B following protein replacement therapy.

Given an incidence of haemophilia B of 1 in 30,000 males, and an incidence of inhibitors of 1.5–3% in haemophilia B, the ability to study pathophysiology and potential therapies in the clinical population is limited. Parallels between the immune responses directed against FIX observed in humans and in bioengineered mice suggest that studying this rare complication in mice is possible, and may guide development of therapies (Table 2).

The haemophilia patient’s FIX genotype is the only strong determinant of FIX inhibitor risk determined to date. Deletions within the FIX gene result in the highest risk for both neutralizing antibody development and allergic and anaphylactoid reactions upon FIX exposure; smaller losses of coding sequence present lesser risk of inhibitor formation (see the Haemophilia B mutation database: www.kcl.ac.uk/ip/petergreen/haemBdata base.html) (124, 125). In humans, the inhibitors that do arise are polyclonal and primarily of the (non-complement-binding) IgG4 subclass, although IgG1 antibodies, which have the potential to bind complement, may be seen and appear to have a higher association with allergic reactions to FIX-containing products (126, 127). FIX-specific IgE has also been demonstrated by RAST reaction in some individuals exhibiting immediate hypersensitivity (128). Epitopes of the FIX protein targeted by inhibitors fall mostly in the serine protease domain and in the γ-carboxyglutamic acid-rich (Gla-) domain, and have not been described in the EGF-like domains or the activation peptide (129). Immune tolerance induction (ITI) to eliminate FIX inhibitors has a markedly lower success rate than ITI for FVIII inhibitors, and carries a risk of the development of nephrotic syndrome (124, 130).

Significant parallels between the human inhibitor responses and those documented in mice are summarized in Table 2. Several groups have reported that anti-FIX IgG that develops in mice after intravenous human FIX (108, 131), intravenous mouse FIX (26), subcutaneous human FIX given with adjuvant (16), or intramuscular AAV-FIX gene therapy (26, 106, 108–110) is mostly IgG1 isotype (which is the mouse homologue of human IgG4). Low titers of IgG2a and IgG2b sometimes have been observed; IgE anti-FIX has not been studied 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 production (108, 132, 133).

As discussed above (see “Additional haemophilia B mouse models”), the underlying FIX mutation (degree of lost coding material and production of CRM) is a principal determinant of antibody response to FIX in mice (15, 26, 27). A weak association of FVIII inhibitor development with major histocompatibility locus (MHC) class II phenotype has been reported from human haemophilia cohort studies, but the number of haemophilia B inhibitor patients studied is too small to establish associations between inhibitors and MHC II or other potential genetic factors (134, 135). For similar reasons, the established links between FVIII inhibitor risk and polymorphisms in cytokine immune response genes interleukin 10 (IL-10) (136), tumor necrosis factor-α (137), and in the CTLA-4 gene (138) have not been observed in relation to FIX inhibitors. Lozier et al. have performed linkage analysis studies in inbred mouse strains and have shown that antibody response following expression of human...
Monahan: Mouse models for study of factor IX

Table 2: Antibody immune response directed against factor IX.

<table>
<thead>
<tr>
<th>Human clinical data:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influence of underlying factor IX mutation:</td>
</tr>
<tr>
<td>High risk: Large deletion, nonsense mutations</td>
</tr>
<tr>
<td>Low risk: Missense mutation (majority of hemophilia B patients); Small deletion/insertion or splice site mutations</td>
</tr>
<tr>
<td>Immunoglobulin subclass of factor IX inhibitor antibody:</td>
</tr>
<tr>
<td>Major: IgG4</td>
</tr>
<tr>
<td>Minor: IgG1 (suggested association with allergy/anaphylaxis)</td>
</tr>
<tr>
<td>Occasional: IgE (suggested association with allergy/anaphylaxis)</td>
</tr>
<tr>
<td>Association with MHC:</td>
</tr>
<tr>
<td>None reported</td>
</tr>
<tr>
<td>Functional mapping of factor IX inhibitor antibody epitopes:</td>
</tr>
<tr>
<td>Catalytic domain</td>
</tr>
<tr>
<td>Gla domain</td>
</tr>
<tr>
<td>Parallels in mouse models:</td>
</tr>
<tr>
<td>Influence of underlying factor IX mutation:</td>
</tr>
<tr>
<td>High risk: Gene deletion, early stop, late stop mutations</td>
</tr>
<tr>
<td>Low risk: Missense mutation</td>
</tr>
<tr>
<td>Immunoglobulin subclass of factor IX inhibitor antibody:</td>
</tr>
<tr>
<td>Major: IgG1 (homologue of human IgG4)</td>
</tr>
<tr>
<td>Minor: IgG2a</td>
</tr>
<tr>
<td>Minor: IgG2b</td>
</tr>
<tr>
<td>Unknown: IgE</td>
</tr>
<tr>
<td>Association with MHC and other genetic loci:</td>
</tr>
<tr>
<td>Significant linkage: D17Mit 62 marker, mouse chromosome 17</td>
</tr>
<tr>
<td>Near MHC class II (H-2) and/or laK genes locus</td>
</tr>
<tr>
<td>Suggestive Linkage: Polymorphic markers, mouse chromosomes 1 and 10</td>
</tr>
<tr>
<td>Near immunoregulatory genes, incl. IL-10 and IFN-γ</td>
</tr>
<tr>
<td>Suggestive Linkage: D1Mit218 marker, mouse chromosome 1</td>
</tr>
<tr>
<td>Functional mapping of factor IX inhibitor antibody epitopes:</td>
</tr>
<tr>
<td>Not investigated in mice</td>
</tr>
<tr>
<td>Immunodominant epitopes: CD4+ T cells</td>
</tr>
<tr>
<td>Strain:</td>
</tr>
<tr>
<td>C57BL/6 (MHC H-2b):</td>
</tr>
<tr>
<td>C57BL/6 (MHC H-2^c):</td>
</tr>
<tr>
<td>BALB/c (MHC H-2^d):</td>
</tr>
<tr>
<td>C3H/HeJ (MHC H-2^e):</td>
</tr>
<tr>
<td>C3H/HeJ (MHC H-2^f):</td>
</tr>
<tr>
<td>C3H/HeJ (MHC H-2^a):</td>
</tr>
</tbody>
</table>

A.A. = amino acid. Numbering is from the first amino acid of the circulating protein (Factor IX Gla domain position 1).

FIX from adenovirus vector is linked to the MHC class II (H-2) locus (on mouse chromosome 17) (131). Somewhat weaker but suggestive linkage was also observed between FIX antibodies and chromosomal regions including genes for immune response modifiers IL-22, interferon-γ (mouse chromosome 10) and IL-10 (mouse chromosome 1). Zhang et al., examining IgG1 anti-FIX response following AAV-canine FIX delivery, also observed a quantitative trait locus that coincided with the chromsome 1 site observed by Lozier (139). These experiments suggest that multiple genetic loci independently influence the isotype-specific immunoglobin response to FIX in mice, similar to the conclusion reached by clinical observation of humans with FVIII deficiency. Study of the phenomenon in mice may facilitate study of this rare clinical complication.

The MHC class II phenotype of a patient should determine which FIX-derived peptides are presented to the T-helper cells.
Greenwood et al. examined the CD4+ T cell-dependent B-cell antibody response to human and mouse FIX in both normal and haemophilic mice of C57BL/6 (MHC H-2b) and BALB/c (MHC H-2k) strains (16). They have identified immunodominant epitopes for T-cell stimulation in the catalytic domain of the protein, with peptide sequences that are specific to each strain (see also Chen et al. (133) and Cao et al. (17) for similar examination in MHC H-2k strains). Importantly, even normal mice, which have the opportunity for clonal deletion of murine FIX-recognizing T cells, contain T cells that recognize specific immunodominant epitopes in the murine FIX sequence (133). This finding (supported by additional information discussed below) suggests that autoreactive T cells, capable of recognizing FIX protein, are neither deleted nor anergic, but are maintained in an unresponsive condition. Understanding the mechanisms that regulate this FIX tolerance is likely to be critical with respect to understanding inhibitor development and therapy. (Additional studies examine in mice the strain-specific class MHC I peptide epitopes for T cells that direct CD8+ T-cell responses. The potential for cytotoxic T-lymphocyte responses following FIX gene expression does not impact current therapy, and will not be discussed here, but is an important consideration for haemophilia B gene therapy (95, 118, 133).

As stated above, immunogenicity of FIX as a secreted transgene (e.g., following gene therapy) involves different mechanisms from immune response to exogenous protein therapy. Nevertheless, transgenic FIX expression modeled in mice may provide lessons regarding shared mechanisms for tolerance induction. Multiple groups have shown that induction of tolerance is more likely to occur if high levels of FIX are achieved; when very low levels are achieved (in the context of a large deletion FIX mutation) immunity against the antigen is promoted (108, 109). In general, immune responses against FIX expressed from the liver are less frequent and weaker than when FIX is expressed from other sites (e.g., muscle) and expression from the liver is more likely to induce regulatory CD4+ T cells that suppress anti-FIX formation (140). In vivo activation via hepatic FIX gene therapy of regulatory CD4+CD25+FoxP3-expressing T-regulatory cells suppresses antibody formation to FIX; in vivo depletion of CD4+CD25+Treg has led to loss of tolerance evidenced by antibody formation (141). It has been suggested from studies of T-lymphocyte populations of individuals with or without FVIII inhibitors (or following successful immune tolerance) that failure to activate regulatory CD4+ T cells specific for certain immunodominant FVIII sequences results in a pathogenic inhibitor response to FVIII (142, 143). The studies in haemophilia B and haemostatically normal mice suggest the same mechanisms may apply to FIX tolerance.

In one intriguing application, repeated intranasal exposure of haemophilic C3H/HeJ mice to a specific FIX peptide, known to be an immunodominant FIX epitope in this strain, reduced the incidence of subsequent inhibitor development via immune deviation to a T-helper cell response, with activation of regulatory CD4+CD25+ T cells (17). Humans are a particularly “outbred strain,” which confounds prediction of patient-specific FIX immunodominant epitopes as required for translating such an approach to the clinic; the study of mechanisms of tolerance in mice may lead to design of more rational therapeutics than are currently available for inhibitors in haemophilia B. Finally, several groups have demonstrated that life-long tolerance of FIX can be induced in mice by in utero or neonatal gene delivery of FIX (92, 93, 144–148). Although the process of thymic selection of T-cell epitopes is supposed to be complete in larger animals prior to the newborn period, the strategy of neonatal FIX gene therapy has produced tolerance in dogs as well (92, 149). The authors speculated that gene therapy or frequent protein injections in the healthy neonate might induce tolerance to subsequent injections of FIX protein. The suggestion is particularly timely in light of two recent epidemiologic studies that document an association of lower relative risk of FVIII inhibitors in severe haemophilia A patients who begin FVIII continuous prophylactic infusions instituted early in life (150, 151). It remains to be seen whether eventually a tolerogenic strategy may emerge involving neonatal or early institution of regular physiologic FVIII or FIX treatments, avoiding major peaks of treatment dose or intensity. As relates to haemophilia B but not haemophilia A, such an approach needs to be balanced by recognition of the possibility of anaphylactic reactions to FIX and caution for the relative fragility of the infant.

Future: Will findings in mice translate to large animals and humans?

Rapid advances in the understanding of FIX have been made taking advantage of genetically engineered mice to manipulate both normal and abnormal mammalian haemostasis. Extrapolating data from mice to humans requires caution, especially in light of strain-specific phenotypic variations in mice. Lessons learned in mice regarding the mechanisms that control expression of FIX have translated relatively well to larger mammals, as has the ability to examine relative specific activity and pharmacokinetics of FIX and FIX mutants. Notwithstanding the shorter half-life of FIX in mice when compared with dogs and primates, screening of enzyonomics and kinetics of variant FIX proteins in mice appears to be a valid approach to determine which candidate therapeutics to confirm in hemophilic dogs and man (37, 152, 153). While in many respects the parallels between the coagulation and fibrinolyis systems of humans and mice are striking, limitations must be acknowledged and considered in experimental design. Murine platelets, in particular, differ greatly from human platelets, e.g., in number, in response to some agonists, and in some specific receptors. Nevertheless, in regards to the effects (described above) of FIX and of FXI deficiency upon murine platelet-rich thrombus formation, results seen in mice appear to be consistent with those observed using neutralizing antibodies against human FIX or factor XI antibodies in rabbit (154) and ba-boon (155) models, respectively. The histopathologic changes of FIX-deficient haemophilic synovitis in mice appear strikingly similar to human blood-induced synovitis, but the extent to which mechanisms of wound healing in coagulation-deficient mice recapitulate that in humans likewise requires careful correlation; for example, the development of blood-induced arthropathy in non-haemophilic canines has been reported to depend on weight-loading the affected joint, a stress that might not be modeled well in the 25–35 gram mice.
As more is learned about the interactions of the haemostatic systems with the systems of immunity, inflammation, and tissue repair, animal modeling is essential for interpreting physiologic and clinical relevance. It can be hoped that emerging strategies to “humanize” components of the mouse immune system (156, 157) may lead to the development of models to study FIX gene therapy and immunology with greater confidence in direct translation of the results to human applications. The ability to genetically manipulate individual pieces of the jigsaw puzzle image that emerges from these interactions allows sometimes surprising new perspectives. While the complexity of observation in vivo of the interactions of FIX in coagulation and other systems potentially confounds interpretation, the ability to observe these elements acting in concert is the greatest strength of in-vivo modeling (158), and genetically engineered mice have proved to be valuable tools for elucidating potential clinically relevant mechanisms and therapeutic approaches.

Acknowledgements

The author thanks Dr. Tong Gui for assistance with graphics and manuscript preparation and Dr. Darrel Stafford for insightful discussions. Dr. Tong Gui, Dr. Aditi Reheman, Dr. Heyu Ni, Dr. Maureen Hoffman, and Dr. Junjiang Sun contributed data for images. The author’s work is supported by the National Institutes of Health.

References

4. Wu SM, Stafford DW, Ware J. Deduced amino acid sequence of mouse blood-coagulation factor IX. Gene 1990; 86: 275–278.


