

# 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 in 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

Thromb Haemost 2008; 100: 563–575

## 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<sup>-/-</sup> 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-

Correspondence to:

Paul E. Monahan

Dept. of Pediatrics, Hematology/Oncology

CB#7352, 5031 Thurston-Bowles Building

University of North Carolina at Chapel Hill School of Medicine

Chapel Hill, NC 27599-7352, USA

Tel.: +1 919 966 1178, Fax: +1 919 966 0907

E-mail: Paul\_Monahan@med.unc.edu

Financial support:

P. E. M. is supported by NIH P01-HL66973 and NIH R01 HL078944-01.

Received April 26, 2008

Accepted after minor revision July 10, 2008

Prepublished online September 5, 2008

doi:10.1160/TH08-04-0262

**Table 1: Bioengineered mouse models for the study of factor IX and haemophilia B.**

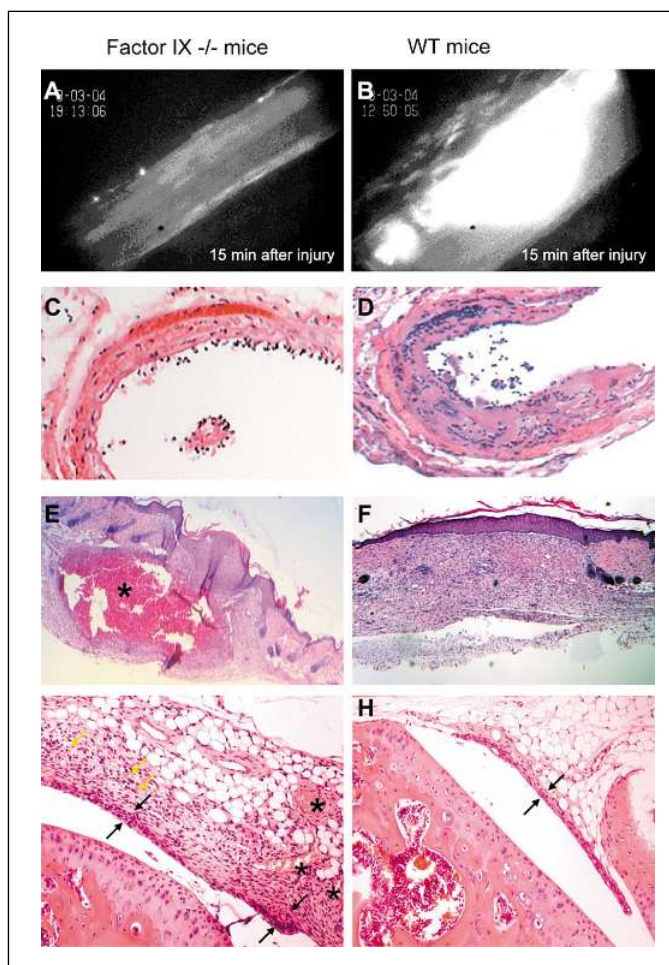
Model	Strain	Genotype	Phenotype/observations of interest	References
Murine factor "Plug and Socket Deletion": Informative crosses: Mouse MHC class II HLA 2 <sup>b</sup> Mouse MHC class II HLA 2 <sup>d</sup> Mouse MHC class II HLA 2 <sup>k</sup> FIX <sup>-/-</sup> cross with CD4 <sup>-/-</sup> mice	C57BL/6 and I29Sv  C57BL/6 BALB/c C3H/HeJ	Promoter through exon 3 of mouse factor IX knocked out ( <i>neo</i> gene inserted)	CRM(-) severe bleeding phenotype Delayed cutaneous wound healing; develops hemophilic arthropathy following joint injury	[4, 12]  [16, 17] [16] [17] [37]
Murine factor IX knockout	C57BL/6 and I29Sv	Exon h of mouse factor IX knocked out ( <i>neo</i> gene inserted)	CRM(-) severe bleeding phenotype	[11]
Murine factor IX knockout	C57BL/6 and I29Sv	Exon g and h of mouse factor IX knocked out ( <i>neo</i> gene inserted)	CRM(-) severe bleeding phenotype	[13]
Human factor IX knock-in Haemophilia B missense mutation	C57BL/6 and I29Sv	human FIX cDNA with Missense glycine for Arg333→gly in catalytic domain; expression from mouse FIX locus, FIX promoter	CRM(+) severe bleeding phenotype Relative immunologic tolerance re: human factor IX	[15] [108]
Human factor IX transgenic haemophilia B: Early stop codon mutation Late stop codon mutation Missense mutation Missense mutation	C57BL/6	(All: random insertion, transthyretin promoter)  human FIX Arg29→stop; 12–68 copies human FIX Arg338→stop; 6–7 copies human FIX Gly381→Glu; 10–13 copies human FIX Arg180→Trp; 1–15 copies	No FIX protein or activity No FIX protein or activity No FIX protein or activity hFIX levels 10–200% of normal; No FIX activity	[27]
Factor IX promoter mutation (factor IX Leyden promoter).	C57BL/6	Normal mouse factor IX gene sequences; normal Human factor IX promoter or FIX promoter with +13 factor IX Leyden mutation drive CAT expression	Leyden mutation abolishes reporter gene expression <i>in vivo</i> in juveniles, followed by age-dependent male-specific gene expression	[33]
Human factor IX transgenic (non-haemophilic) hFIXWTtg	C57BL/6	(Random insertion; transthyretin promoter) human FIX cDNA; 2–14 copies	hFIX protein levels 60–800% of normal; activity >100%	[27]
hFIXWTtg hypersecretor (FIX oversecretors)	C57BL/6 x SJL	mFIXWT AND hFIX cDNA minigenes (various) with or without age-stability element	Express hFIX Mice expressing 200–5,000 ng/ml hFIX have increased incidence early mortality, thromboembolic complications and myocardial fibrosis	[62]
Transgenic (haemostatically normal) mice co-expressing human FIX minigenes with human FIX 5' and 3' untranslated regions		Normal mouse FIX and human FIX with 5' sequences varying in length 416 to 2231 nt	Regulatory elements in the 5' upstream region of the gene and the 3' downstream region control age-related regulation of FIX	[29]
Transgenic mouse expressing increased specific activity hFIX In mammary tissue	KunMing white	goat β-casein promoter-driven human FIX Arg338→Ala cDNA multiple random integrations in WT mouse genome	Express mouse FIX systemically; Normal plasma aPTT; Express hFIXR338A at supra-physiologic levels exclusively in breast milk	[159]
Haemophilia B coexpressing factor V Leiden	C57BL/6	mFIX <sup>-/-</sup> crossed with FVL <sup>-/-</sup> or FVL <sup>+/-</sup>	FVL coexpression partially improves clotting time and haemostatic thrombus formation in microvasculature but not large vessels	[65]
Haemophilia B With Plasminogen deficiency	C57BL/6	mFIX <sup>-/-</sup> crossed with Plg <sup>-/-</sup>	Severity of Plg <sup>-/-</sup> wasting syndrome and mortality improved in the presence of deficient FIX	[58]
C/EBPα knockout		CCAAT/enhancer-binding protein alpha (C/EBPα) knocked out (transcriptional regulatory protein with a binding motif in the FIX promoter)	Deficient factor IX mRNA and factor IX activity; defective energy homeostasis; death on day of life I.	[31]

opment of antibody- or cell-mediated immune responses to the human xenoprotein.

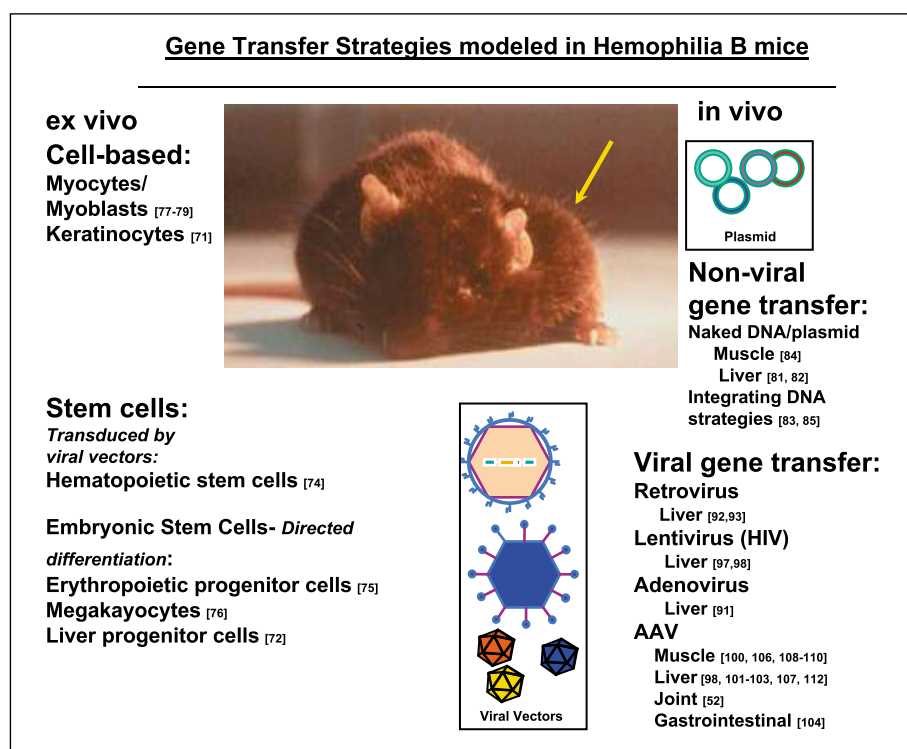
In 1997–1998 three different groups created haemophilia B mouse models expressly for the purpose of studying gene therapy (11–13); one of these groups also envisioned the potential value of these mice “for studying structure-function relationships of recombinant factor IX proteins *in vivo*” (12) (Table 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<sup>-/-</sup> 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<sup>-/-</sup> 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 one-stage (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<sup>-/-</sup> 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<sup>-/-</sup> mice have also been bred into pure MHC class II HLA2<sup>b</sup> C57BL/6 and HLA2<sup>d</sup> BALB/c (16) and HLA2<sup>k</sup> C3H/HeJ (17) strain backgrounds.

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  $\gamma$ -glutamyl carboxylase results in varying degrees of intrauterine lethality, with all live-births succumbing to



**Figure 1: Thrombus formation, haemostasis, and wound healing in wild-type (WT) and FIX<sup>-/-</sup> 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<sup>-/-</sup> 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 epithelial 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 capsule 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<sup>-/-</sup> 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.



**Figure 2: Gene transfer strategies modeled in haemophilia B mice.** Pictured in the center is a C57BL/6 FIX<sup>-/-</sup> mouse (Chapel Hill model) that developed massive left forelimb muscular bleeding after fighting with a cage mate. Although ambulating, the mouse haemorrhaged to a haemoglobin about 20% of normal. Factor IX<sup>-/-</sup> mice have been used to model a wide range of strategies for gene correction of haemophilia and other monogenic disorders.

fatal hemorrhage in the peripartum period or prior to weaning (21–25). Consistent with the clinical scenario in human haemophilia B, FIX<sup>-/-</sup> 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 haemostasis (e.g. myocardium, testis) (18). Additionally, while a potential role in embryonic development, independent from haemostasis, 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<sup>-/-</sup> mice for the study of new haemophilia 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<sup>-/-</sup> mice make antibodies against the xenoprotein FIX including human or canine sequences, the FIX<sup>-/-</sup> mice are also reported to raise inhibitors against recombinant murine FIX (but not following murine plasma infusions) (26). The FIX<sup>-/-</sup> 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 antigeni-

cally cross-reactive material positive (CRM(+) haemophilia B) (15). Created by homologous recombination into the mouse X chromosome, the mouse expresses a single copy human FIX cDNA with a missense mutation R333→Q endogenously expressed under the control of the mouse FIX promoter; the expression is therefore physiologic, a potential advantage for the study of immune responses to the protein. The defective R333Q-hFIX circulates at approximately 15–30% of normal human levels but has <1% activity. This model has been used to evaluate efficacy and risk of gene therapy approaches, in particular to demonstrate the very low risk of inhibitor antibody formation in the setting of CRM+ missense FIX mutation, owing to a relative, but not absolute, state of immunologic non-responsiveness to replacement FIX.

Sabatino et al. have also generated a series of transgenic haemophilia B mouse models to provide a spectrum of mutations to evaluate the degree of tolerance to human FIX conferred by the underlying mutation; in addition, they created haemostatically normal mice expressing human FIX instead of mouse FIX (27) (see Table 1). Mice carrying from 7–68 copies of human FIX genes expressed from a transthyretin promoter and challenged with AAV2 intramuscular gene therapy demonstrated IgG formation against human FIX only in the mice with early (R29X) or late (R338X) stop codon mutations or complete knockout of the gene; mice with CRM(-) or CRM(+) human FIX missense mutations rarely (CRM(-)) or never (CRM(+)) developed humoral immune response to human FIX. This series of mice subsequently proved valuable to evaluate the therapeutic potential of translational bypass therapy. Mice carrying the late nonsense mutation treated with aminoglycoside demonstrated significant translational readthrough of the nonsense sequence with multi-day increases of circulating human FIX protein and activity (28).

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/EBP $\alpha$ ), 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/EBP $\alpha$  in the region of this mutation (34). Kurachi et al. subsequently expressed FIX throughout the lifetime of transgenic animals expressing varying long segments of 5' and 3' untranslated regions of the FIX gene to identify two essential age-regulatory elements, AE5' and AE3', which act in concert to direct natural gender-independent age-associated increased FIX gene expression (29).

FIX<sup>-/-</sup> mice have also been used to examine pharmacokinetics and potential therapeutic value of variant FIX proteins (mutants). 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<sup>-/-</sup> mice (36) or as combined

K5A/V10K hFIX gene therapy vectors to FIX<sup>-/-</sup> 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 ( $\Delta$ 155–177); 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<sup>-/-</sup> 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<sup>-/-</sup> 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 FIX<sup>-/-</sup> mice has been examined in three models (53, 54). Occlusive thrombus resulting from ferric chloride (FeCl<sub>3</sub>) injury, using a range of concentrations of FeCl<sub>3</sub> applied to the carotid artery, was measured as loss of doppler blood flow signal distal to the injury. At low and intermediate FeCl<sub>3</sub> concentrations, FIX<sup>-/-</sup> mice as well as FXI<sup>-/-</sup> 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<sup>-/-</sup> mice (Fig. 1) (56). Finally, intravital microscopy following mesenteric arteriolar ferric chloride injury has been studied in FIX<sup>-/-</sup> 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<sup>-/-</sup> 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 acti-

vation 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<sup>-/-</sup> 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<sup>-/-</sup> mice and not in FXI<sup>-/-</sup> or FXII<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> 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 a range of levels (0–5,000 ng/ml hFIX) (62). Survival was inversely correlated with the circulating levels of human FIX. Fibrin deposition was demonstrated in the vasculature and fibrosed myocardium of prematurely dying animals. Thromboemboli were also observed in lung, brain, and other organs, supporting human epidemiologic data that persistently high circulating levels of FIX are a risk for thrombosis, and perhaps also for myocardial infarction. The expected age-associated increases in FIX were demonstrated in the mice and postulated to contribute to age-dependent myocardial fibrosis (29). It must be stated that the human epidemiologic data is strongest in regards to risk of venous thromboembolism, which was apparently not a prominent feature in the hFIX over-expressing mice.

Clinical studies have yielded some debate as to whether the converse applies, i.e. can the inheritance of prothrombotic risk factors ameliorate the bleeding phenotype in FIX deficiency? (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 (FeCl<sub>3</sub> 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<sup>-/-</sup> and FVIII<sup>-/-</sup> 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 ade-

noviral 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 in the study of FIX expression and pharmacokinetics.”) In the context of gene therapy, FIX expressed from skeletal muscle has been demonstrated to co-localize with collagen IV in the extracellular matrix of FIX-transduced muscle cells, and this binding creates a sink which must be overcome before efficacious circulating blood levels of the FIX can be achieved (37, 100).

### 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<sup>-/-</sup> 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 input 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.html](http://www.kcl.ac.uk/ip/petergreen/haemBdata.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  $\gamma$ -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- $\alpha$  (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

**Table 2: Antibody immune response directed against factor IX.**

<b>Human clinical data:</b>		
<b>Influence of underlying factor IX mutation:</b>		
High risk: Large deletion, nonsense mutations		
Low risk: Missense mutation (majority of hemophilia B patients); Small deletion/insertion or splice site mutations		
<b>Immunoglobulin subclass of factor IX inhibitor antibody:</b>		
Major: IgG4		
Minor: IgG1 (suggested association with allergy/anaphylaxis)		
Occasional: IgE (suggested association with allergy/anaphylaxis)		
<b>Association with MHC:</b>		
None reported		
<b>Functional mapping of factor IX inhibitor antibody epitopes:</b>		
Catalytic domain		
Gla domain		
<b>Parallels in mouse models:</b>		
<b>Influence of underlying factor IX mutation:</b>		
High risk: Gene deletion, early stop, late stop mutations		
Low risk: Missense mutation		
<b>Immunoglobulin subclass of factor IX inhibitor antibody:</b>		
Major: IgG1 (homologue of human IgG4)		
Minor: IgG2a		
Minor: IgG2b		
Unknown: IgE		
<b>Association with MHC and other genetic loci:</b>		
Significant linkage: <i>D17Mit 62</i> marker, mouse chromosome 17 Near MHC class II (H-2) and/or IaK genes locus		
Suggestive Linkage: Polymorphic markers, mouse chromosomes 1 and 10 Near immunoregulatory genes, incl. IL-10 and IFN- $\gamma$		
Suggestive Linkage: <i>D1Mit218</i> marker, mouse chromosome 1		
<b>Functional mapping of factor IX inhibitor antibody epitopes:</b>		
Not investigated in mice		
<b>Immunodominant epitopes: CD4+ T cells</b>		
<b>Strain:</b>	<b>A.A. Position (Domain):</b>	<b>A.A. Sequence :</b>
C57BL/6 (MHC H-2 <sup>b</sup> ):	272–290 (Catalytic)	LLELDEPLVLNSYVTPIC
C57BL/6 (MHC H-2 <sup>b</sup> ):	264–283 (Catalytic)	NNKYNHDIALLELDEPLVNS
BALB/c (MHC H-2 <sup>d</sup> ):	222–236 (Catalytic)	CVETGVKITYVAGEH
C3H/HeJ (MHC H-2 <sup>k</sup> ):	244–263 (Catalytic)	TEQKRNVIRIIPHHYNAAI
C3H/HeJ (MHC H-2E <sup>k</sup> ):	62–76 (EGF-I)	YTKVSRVYNWIEKEKT
C3H/HeJ (MHC H-2A <sup>k</sup> ):	398–412 (Catalytic)	CKDDINSYECWCPFG
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- $\gamma$  (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 chromo-

some 1 site observed by Lozier (139). These experiments suggest that multiple genetic loci independently influence the isotype-specific immunoglobulin 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<sup>+</sup> T cell-dependent B-cell antibody response to human and mouse FIX in both normal and haemophilic mice of C57BL/6 (MHC H-2<sup>b</sup>) and BALB/c (MHC H-2<sup>d</sup>) 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-2<sup>K</sup> 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 FIX that direct CD8<sup>+</sup> 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<sup>+</sup> T cells that suppress anti-FIX formation (140). In-vivo activation via hepatic FIX gene therapy of regulatory CD4<sup>+</sup>CD25<sup>+</sup> FoxP3-expressing T-regulatory cells suppresses antibody formation to FIX; in-vivo depletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>regs</sub> 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<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> T cells (17). Humans are a particularly “outbred strain,” which confounds prediction of patient-specific FIX immunodominant epitopes as required for translating such a strategy 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 toleragenic 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 enzymology 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 fibrinolysis 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 baboon (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. Adili 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

- Manis JP. Knock out, knock in, knock down--genetically manipulated mice and the Nobel Prize. *N Engl J Med* 2007; 357: 2426–2429.
- Ovlisen K, Kristensen AT, Tranholm M. *In vivo* models of haemophilia – status on current knowledge of clinical phenotypes and therapeutic interventions. *Haemophilia* 2008; 14: 248–259.
- Rawle FE, Lillicrap D. Preclinical animal models for hemophilia gene therapy: predictive value and limitations. *Semin Thromb Hemost* 2004; 30: 205–213.
- Wu SM, Stafford DW, Ware J. Deduced amino acid sequence of mouse blood-coagulation factor IX. *Gene* 1990; 86: 275–278.
- Sarkar G, Koeberl DD, Sommer SS. Direct sequencing of the activation peptide and the catalytic domain of the factor IX gene in six species. *Genomics* 1990; 6: 133–143.
- Elder B, Lakich D, Gitschier J. Sequence of the murine factor VIII cDNA. *Genomics* 1993; 16: 374–379.
- Emeis JJ, Jirouskova M, Muchitsch EM, et al. A guide to murine coagulation factor structure, function, assays, and genetic alterations. *J Thromb Haemost* 2007; 5: 670–679.
- Matsuo O, Lijnen HR, Ueshima S, et al. A guide to murine fibrinolytic factor structure, function, assays, and genetic alterations. *J Thromb Haemost* 2007; 5: 680–689.
- Jirouskova M, Shet AS, Johnson GJ. A guide to murine platelet structure, function, assays, and genetic alterations. *J Thromb Haemost* 2007; 5: 661–669.
- Walter J, High KA. Gene therapy for the hemophilias. *Adv Vet Med* 1997; 40: 119–134.
- Wang L, Zoppe M, Hackeng TM, et al. A factor IX-deficient mouse model for hemophilia B gene therapy. *Proc Natl Acad Sci USA* 1997; 94: 11563–11566.
- Lin HF, Maeda N, Smithies O, et al. A coagulation factor IX-deficient mouse model for human hemophilia B. *Blood* 1997; 90: 3962–3966.
- Kundu RK, Sangiorgi F, Wu LY, et al. Targeted inactivation of the coagulation factor IX gene causes hemophilia B in mice. *Blood* 1998; 92: 168–174.
- Detloff PJ, Lewis J, John SW, et al. Deletion and replacement of the mouse adult beta-globin genes by a „plug and socket“ repeated targeting strategy. *Mol Cell Biol* 1994; 14: 6936–6943.
- Jin DY, Zhang TP, Gui T, et al. Creation of a mouse expressing defective human factor IX. *Blood* 2004; 104: 1733–1739.
- Greenwood R, Wang B, Midkiff K, et al. Identification of T-cell epitopes in clotting factor IX and lack of tolerance in inbred mice. *J Thromb Haemost* 2003; 1: 95–102.
- Cao O, Armstrong E, Schlachterman A, et al. Immune deviation by mucosal antigen administration suppresses gene-transfer-induced inhibitor formation to factor IX. *Blood* 2006; 108: 480–486.
- Mackman N. Tissue-specific hemostasis in mice. *Arterioscler Thromb Vasc Biol* 2005; 25: 2273–2281.
- Bugge TH, Xiao Q, Kombrinck KW, et al. Fatal embryonic bleeding events in mice lacking tissue factor, the cell-associated initiator of blood coagulation. *Proc Natl Acad Sci USA* 1996; 93: 6258–6263.
- Rosen ED, Chan JC, Idusogie E, et al. Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. *Nature* 1997; 390: 290–294.
- Sun WY, Witte DP, Degen JL, et al. Prothrombin deficiency results in embryonic and neonatal lethality in mice. *Proc Natl Acad Sci USA* 1998; 95: 7597–7602.
- Xue J, Wu Q, Westfield LA, et al. Incomplete embryonic lethality and fatal neonatal hemorrhage caused by prothrombin deficiency in mice. *Proc Natl Acad Sci USA* 1998; 95: 7603–7607.
- Cui J, O'Shea KS, Purkayastha A, et al. Fatal hemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V. *Nature* 1996; 384: 66–68.
- Dewerchin M, Liang Z, Moons L, et al. Blood coagulation factor X deficiency causes partial embryonic lethality and fatal neonatal bleeding in mice. *Thromb Haemost* 2000; 83: 185–190.
- Zhu A, Sun H, Raymond RM, Jr., et al. Fatal hemorrhage in mice lacking gamma-glutamyl carboxylase. *Blood* 2007; 109: 5270–5275.
- Fields PA, Arruda VR, Armstrong E, et al. Risk and prevention of anti-factor IX formation in AAV-mediated gene transfer in the context of a large deletion of F9. *Mol Ther* 2001; 4: 201–210.
- Sabatino DE, Armstrong E, Edmonson S, et al. Novel hemophilia B mouse models exhibiting a range of mutations in the Factor IX gene. *Blood* 2004; 104: 2767–2774.
- Yang C, Feng J, Song W, et al. A mouse model for nonsense mutation bypass therapy shows a dramatic multiday response to geneticin. *Proc Natl Acad Sci USA* 2007; 104: 15394–15399.
- Kurachi S, Deyashiki Y, Takeshita J, et al. Genetic mechanisms of age regulation of human blood coagulation factor IX. *Science* 1999; 285: 739–743.
- Inoue Y, Peters LL, Yim SH, et al. Role of hepatocyte nuclear factor 4alpha in control of blood coagulation factor gene expression. *J Mol Med* 2006; 84: 334–344.
- Davies N, Austen DE, Wilde MD, et al. Clotting factor IX levels in C/EBP alpha knockout mice. *Br J Haematol* 1997; 99: 578–579.
- Sweeney JD, Hoernig LA. Age-dependent effect on the level of factor IX. *Am J Clin Pathol* 1993; 99: 687–688.
- Boland EJ, Liu YC, Walter CA, et al. Age-specific regulation of clotting factor IX gene expression in normal and transgenic mice. *Blood* 1995; 86: 2198–2205.
- Brady JN, Notley C, Cameron C, et al. Androgen effects on factor IX expression: *in-vitro* and *in-vivo* studies in mice. *Br J Haematol* 1998; 101: 273–279.
- Gui T, Lin HF, Jin DY, et al. Circulating and binding characteristics of wild-type factor IX and certain Gla domain mutants *in vivo*. *Blood* 2002; 100: 153–158.
- Begbie ME, Mamdani A, Gataiance S, et al. An important role for the activation peptide domain in controlling factor IX levels in the blood of hemophilia B mice. *Thromb Haemost* 2005; 94: 1138–1147.
- Schuettrumpf J, Herzog RW, Schlachterman A, et al. Factor IX variants improve gene therapy efficacy for hemophilia B. *Blood* 2005; 105: 2316–2323.
- Dejana E, Callioni A, Quintana A, et al. Bleeding time in laboratory animals. II – A comparison of different assay conditions in rats. *Thromb Res* 1979; 15: 191–197.
- Broze GJ, Jr. Protein Z-dependent regulation of coagulation. *Thromb Haemost* 2001; 86: 8–13.
- Gailani D, Lasky NM, Broze GJ, Jr. A murine model of factor XI deficiency. *Blood Coagul Fibrinolysis* 1997; 8: 134–144.
- Tsakis DA, Scudder L, Hodivala-Dilke K, et al. Hemostasis in the mouse (*Mus musculus*): a review. *Thromb Haemost* 1999; 81: 177–188.
- Broze GJ, Jr., Yin ZF, Lasky N. A tail vein bleeding time model and delayed bleeding in hemophilic mice. *Thromb Haemost* 2001; 85: 747–748.
- Borchgrevink CF, Waaler BA. The secondary bleeding time. A new method for the differentiation of hemorrhagic diseases. *Acta Med Scandinav* 1958; 162: 361–374.
- Tomokiyo K, Nakatomi Y, Araki T, et al. A novel therapeutic approach combining human plasma-derived Factors VIIa and X for hemophiliacs with inhibitors: evidence of a higher thrombin generation rate *in vitro* and more sustained haemostatic activity *in vivo* than obtained with Factor VIIa alone. *Vox Sang* 2003; 85: 290–299.
- Hoffman M, Harger A, Lenkowski A, Hedner U, Roberts HR, Monroe DM. Cutaneous wound healing is impaired in hemophilia B. *Blood* 2006; 108: 3053–3060.
- McDonald A, Hoffman M, Hedner U, et al. Restoring hemostatic thrombin generation at the time of cutaneous wounding does not normalize healing in hemophilia B. *J Thromb Haemost* 2007; 5: 1577–1583.
- McDonald AG, Yang K, Roberts HR, et al. Perivascular tissue factor is down-regulated following cutaneous wounding: implications for bleeding in hemophilia. *Blood* 2008; 111: 2046–2048.
- Mejia-Carvajal C, Hakobyan N, Enockson C, et al. The impact of joint bleeding and synovitis on physical ability and joint function in a murine model of hemophilic synovitis. *Haemophilia* 2008; 14: 119–126.

49. Valentino LA, Hakobyan N. Histological changes in murine haemophilic synovitis: a quantitative grading system to assess blood-induced synovitis. *Haemophilia* 2006; 12: 654–662.
50. Hakobyan N, Kazarian T, Valentino LA. Synovitis in a murine model of human factor VIII deficiency. *Haemophilia* 2005; 11: 227–232.
51. Valentino LA, Hakobyan N, Kazarian T, et al. Experimental haemophilic synovitis: rationale and development of a murine model of human factor VIII deficiency. *Haemophilia* 2004; 10: 280–287.
52. Sun J, Hakobyan N, Valentino LA, et al. Intra-articular factor IX protein or gene replacement protects against development of hemophilic synovitis in the absence of circulating factor IX. *Blood* 2008; epub ahead of print.
53. Wang X, Cheng Q, Xu L, et al. Effects of factor IX or factor XI deficiency on ferric chloride-induced carotid artery occlusion in mice. *J Thromb Haemost* 2005; 3: 695–702.
54. Gui T, Reheman A, Funkhouser WK, et al. In vivo response to vascular injury in the absence of factor IX: examination in factor IX knockout mice. *Thromb Res* 2007; 121: 225–234.
55. Renne T, Pozgajova M, Gruner S, et al. Defective thrombus formation in mice lacking coagulation factor XII. *J Exp Med* 2005; 202: 271–281.
56. Gui T, Reheman A, Funkhouser WK, et al. In vivo response to vascular injury in the absence of factor IX: examination in FIX knockout mice. *Thromb Res* 2007; 121: 225–234.
57. Gailani D, Renne T. Intrinsic pathway of coagulation and arterial thrombosis. *Arterioscler Thromb Vasc Biol* 2007; 27: 2507–2513.
58. Cheng Q, Zhao Y, Lawson WE, et al. The effects of intrinsic pathway protease deficiencies on plasminogen-deficient mice. *Blood* 2005; 106: 3055–3057.
59. van Hylckama Vlieg A, van der Linden IK, Bertina RM, et al. High levels of factor IX increase the risk of venous thrombosis. *Blood* 2000; 95: 3678–3682.
60. Lowe GD. Factor IX and thrombosis. *Br J Haematol* 2001; 115: 507–513.
61. Woodward M, Lowe GD, Rumley A, et al. Epidemiology of coagulation factors, inhibitors and activation markers: The Third Glasgow MONICA Survey. II. Relationships to cardiovascular risk factors and prevalent cardiovascular disease. *Br J Haematol* 1997; 97: 785–797.
62. Ameri A, Kurachi S, Sueishi K, et al. Myocardial fibrosis in mice with overexpression of human blood coagulation factor IX. *Blood* 2003; 101: 1871–1873.
63. Shetty S, Ghosh K, Quadros L. Amelioration of clinical severity of similar mutations severe factor IX deficiency by coinherited thrombophilia. *Eur J Haematol* 2008; 80: 87–89.
64. Arbini AA, Mannucci PM, Bauer KA. Low prevalence of the factor V Leiden mutation among „severe“ hemophiliacs with a „milder“ bleeding diathesis. *Thromb Haemost* 1995; 74: 1255–1258.
65. Schlachterman A, Schuettrumpf J, Liu JH, et al. Factor V Leiden improves in vivo hemostasis in murine hemophilia models. *J Thromb Haemost* 2005; 3: 2730–2737.
66. White G, Roberts H. New Approaches for the therapy of bleeding disorders, including gene therapy. In: *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*. 5th Edition ed: Lippincott, Williams, and Wilkins; 2005.
67. High KA. Update on progress and hurdles in novel genetic therapies for hemophilia. *Hematology Am Soc Hematol Educ Program* 2007; 2007: 466–472.
68. Pierce GF, Lillicrap D, Pipe SW, et al. Gene therapy, bioengineered clotting factors and novel technologies for hemophilia treatment. *J Thromb Haemost* 2007; 5: 901–906.
69. Chuah M, Vandendriessche T. Gene therapy for hemophilia "A" and "B": efficacy, safety and immune consequences. *Verh K Acad Geneesk Belg* 2007; 69: 315–334.
70. Miao CH. A novel gene expression system: non-viral gene transfer for hemophilia as model systems. *Adv Genet* 2005; 54: 143–177.
71. White SJ, Page SM, Margaritis P, et al. Long-term expression of human clotting factor IX from retrovirally transduced primary human keratinocytes in vivo. *Hum Gene Ther* 1998; 9: 1187–1195.
72. Fair JH, Cairns BA, Lapaglia MA, et al. Correction of factor IX deficiency in mice by embryonic stem cells differentiated in vitro. *Proc Natl Acad Sci USA* 2005; 102: 2958–2963.
73. Tatsumi K, Ohashi K, Kataoka M, et al. Successful in vivo propagation of factor IX-producing hepatocytes in mice: potential for cell-based therapy in hemophilia B. *Thromb Haemost* 2008; 99: 883–891.
74. Bigger BW, Siapati EK, Mistry A, et al. Permanent partial phenotypic correction and tolerance in a mouse model of hemophilia B by stem cell gene delivery of human factor IX. *Gene Ther* 2006; 13: 117–126.
75. Chang AH, Stephan MT, Sadelain M. Stem cell-derived erythroid cells mediate long-term systemic protein delivery. *Nat Biotechnol* 2006; 24: 1017–1021.
76. Zhang G, Shi Q, Fahs SA, et al. Ectopic expression of human FIX in mouse platelets can store releasable FIX in platelets and may be a potential strategy for gene therapy of hemophilia B. *Blood* 2007; 110: 65a–66a.
77. Van Raamsdonk JM, Ross CJ, Potter MA, et al. Treatment of hemophilia B in mice with nonautologous somatic gene therapeutics. *J Lab Clin Med* 2002; 139: 35–42.
78. Wen J, Vargas AG, Ofosu FA, et al. Sustained and therapeutic levels of human factor IX in hemophilia B mice implanted with microcapsules: key role of encapsulated cells. *J Gene Med* 2006; 8: 362–369.
79. Wen J, Xu N, Li A, et al. Encapsulated human primary myoblasts deliver functional hFIX in hemophilic mice. *J Gene Med* 2007; 9: 1002–1010.
80. Miao CH, Thompson AR, Loeb K, et al. Long-term and therapeutic-level hepatic gene expression of human factor IX after naked plasmid transfer in vivo. *Mol Ther* 2001; 3: 947–957.
81. Miao CH, Brayman AA, Loeb KR, et al. Ultrasound enhances gene delivery of human factor IX plasmid. *Hum Gene Ther* 2005; 16: 893–905.
82. Ye X, Loeb KR, Stafford DW, et al. Complete and sustained phenotypic correction of hemophilia B in mice following hepatic gene transfer of a high-expressing human factor IX plasmid. *J Thromb Haemost* 2003; 1: 103–111.
83. Mikkelsen JG, Yant SR, Meuse L, et al. Helper-Independent Sleeping Beauty transposon-transposase vectors for efficient nonviral gene delivery and persistent gene expression in vivo. *Mol Ther* 2003; 8: 654–665.
84. Liu F, Sag D, Wang J, et al. Sine-wave current for efficient and safe in vivo gene transfer. *Mol Ther* 2007; 15: 1842–1847.
85. Keravala A, Chavez CL, Woodard LE, et al. Hemophilia gene therapy with PhiC31 integrase: A novel approach. *Mol Ther* 2008; 16: Abstract 877.
86. Jacobs F, Snoeys J, Feng Y, et al. Direct comparison of hepatocyte-specific expression cassettes following adenoviral and nonviral hydrodynamic gene transfer. *Gene Ther* 2008; 15: 594–603.
87. Olivares EC, Hollis RP, Chalberg TW, et al. Site-specific genomic integration produces therapeutic factor IX levels in mice. *Nat Biotechnol* 2002; 20: 1124–1128.
88. Kay MA, Rothenberg S, Landen CN, et al. In vivo gene therapy of hemophilia B: sustained partial correction in factor IX-deficient dogs. *Science* 1993; 262: 117–119.
89. Fang B, Wang H, Gordon G, et al. Lack of persistence of E1-recombinant adenoviral vectors containing a temperature-sensitive E2A mutation in immunocompetent mice and hemophilia B dogs. *Gene Ther* 1996; 3: 217–222.
90. Yao SN, Farjo A, Roessler BJ, et al. Adenovirus-mediated transfer of human factor IX gene in immunodeficient and normal mice: evidence for prolonged stability and activity of the transgene in liver. *Viral Immunol* 1996; 9: 141–153.
91. Ehrhardt A, Kay MA. A new adenoviral helper-dependent vector results in long-term therapeutic levels of human coagulation factor IX at low doses in vivo. *Blood* 2002; 99: 3923–3930.
92. Zhang J, Xu L, Haskins ME, et al. Neonatal gene transfer with a retroviral vector results in tolerance to human factor IX in mice and dogs. *Blood* 2004; 103: 143–151.
93. Xu L, Mei M, Haskins ME, et al. Immune response after neonatal transfer of a human factor IX-expressing retroviral vector in dogs, cats, and mice. *Thromb Res* 2007; 120: 269–280.
94. Brunetti-Pierri N, Palmer DJ, Mane V, et al. Increased hepatic transduction with reduced systemic dissemination and proinflammatory cytokines following hydrodynamic injection of helper-dependent adenoviral vectors. *Mol Ther* 2005; 12: 99–106.
95. Dobrzynski E, Fitzgerald JC, Cao O, et al. Prevention of cytotoxic T lymphocyte responses to factor IX-expressing hepatocytes by gene transfer-induced regulatory T cells. *Proc Natl Acad Sci USA* 2006; 103: 4592–4597.
96. McCaffrey AP, Fawcett P, Nakai H, et al. The host response to adenovirus, helper-dependent adenovirus, and adeno-associated virus in mouse liver. *Mol Ther* 2008; 16: 931–941.
97. Brown BD, Cantore A, Annoni A, et al. A microRNA-regulated lentiviral vector mediates stable correction of hemophilia B mice. *Blood* 2007; 110: 4144–4152.
98. Vandendriessche T, Thorrez L, Acosta-Sanchez A, et al. Efficacy and safety of adeno-associated viral vectors based on serotype 8 and 9 vs. lentiviral vectors for hemophilia B gene therapy. *J Thromb Haemost* 2007; 5: 16–24.
99. Brown BD, Gentner B, Cantore A, et al. Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. *Nat Biotechnol* 2007; 25: 1457–1467.
100. Herzog RW, Hagstrom JN, Kung SH, et al. Stable gene transfer and expression of human blood coagulation factor IX after intramuscular injection of recombinant adeno-associated virus. *Proc Natl Acad Sci USA* 1997; 94: 5804–5809.
101. Snyder RO, Miao C, Meuse L, et al. Correction of hemophilia B in canine and murine models using recombinant adeno-associated viral vectors. *Nat Med* 1999; 5: 64–70.
102. Wang L, Takabe K, Bidlingmaier SM, et al. Sustained correction of bleeding disorder in hemophilia B mice by gene therapy. *Proc Natl Acad Sci USA* 1999; 96: 3906–3910.
103. Wu Z, Sun J, Zhang T, et al. Optimization of self-complementary AAV vectors for liver-directed expression results in sustained correction of hemophilia B at low vector dose. *Mol Ther* 2008; 16: 280–289.
104. Peng J, Wang H, Ma Y, et al. Non-invasive viral gene transfer of factor IX to colonic epithelial cells in hemophilia B mice. *J Thromb Haemost* 2008; 6: 1033–1035.

105. Grimm D, Zhou S, Nakai H, et al. Preclinical in vivo evaluation of pseudotyped adeno-associated virus vectors for liver gene therapy. *Blood* 2003; 102: 2412–2419.
106. Chao H, Monahan PE, Liu Y, et al. Sustained and complete phenotype correction of hemophilia B mice following intramuscular injection of AAV1 serotype vectors. *Mol Ther* 2001; 4: 217–222.
107. Mingozzi F, Schuttrumpf J, Arruda VR, et al. Improved hepatic gene transfer by using an adeno-associated virus serotype 5 vector. *J Virol* 2002; 76: 10497–10502.
108. Zhang TP, Jin DY, Wardrop RM, 3rd, et al. Transgene expression levels and kinetics determine risk of humoral immune response modeled in factor IX knock-out and missense mutant mice. *Gene Ther* 2007; 14: 429–440.
109. Cohn EF, Zhuo J, Kelly ME, et al. Efficient induction of immune tolerance to coagulation factor IX following direct intramuscular gene transfer. *J Thromb Haemost* 2007; 5: 1227–1236.
110. Arruda VR, Schuettrumpf J, Herzog RW, et al. Safety and efficacy of factor IX gene transfer to skeletal muscle in murine and canine hemophilia B models by adeno-associated viral vector serotype 1. *Blood* 2004; 103: 85–92.
111. Monahan PE, Sun J, Valentino LA, et al. Adeno-associated Virus (AAV) mediated intraarticular expression of clotting factor IX protects from hemophilic arthropathy. *Haemophilia* 2008; 14: 407.
112. Nathwani AC, Gray JT, Ng CY, et al. Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. *Blood* 2006; 107: 2653–2661.
113. Wu Z, Duan H, Samulski RJ. Self-complementary AAV2 vectors transduce liver with the same efficiency as AAV8: the critical role of second-strand synthesis in AAV biology. *Mol Ther* 2005; 11 (S1): 5.
114. Stilwell JL, Samulski RJ. Role of viral vectors and virion shells in cellular gene expression. *Mol Ther* 2004; 9: 337–346.
115. Li C, Hirsch M, Asokan A, et al. Adeno-associated virus type 2 (AAV2) capsid-specific cytotoxic T lymphocytes eliminate only vector-transduced cells coexpressing the AAV2 capsid in vivo. *J Virol* 2007; 81: 7540–7547.
116. Qiu X, Lu D, Zhou J, et al. Implantation of autologous skin fibroblast genetically modified to secrete clotting factor IX partially corrects the hemorrhagic tendencies in two hemophilia B patients. *Chin Med J (Engl)* 1996; 109: 832–839.
117. Manno CS, Chew AJ, Hutchison S, et al. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood* 2003; 101: 2963–2972.
118. Manno CS, Pierce GF, Arruda VR, et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* 2006; 12: 342–347.
119. Arruda VR, Hagstrom JN, Deitch J, et al. Posttranslational modifications of recombinant myotube-synthesized human factor IX. *Blood* 2001; 97: 130–138.
120. Zhang TP, Jin DY, Wardrop RM, 3rd, et al. Transgene expression levels and kinetics determine risk of humoral immune response modeled in factor IX knock-out and missense mutant mice. *Gene Ther* 2007; 14: 429–440.
121. Cheung WF, van den Born J, Kuhn K, et al. Identification of the endothelial cell binding site for factor IX. *Proc Natl Acad Sci USA* 1996; 93: 11068–11073.
122. Wolberg AS, Stafford DW, Erie DA. Human factor IX binds to specific sites on the collagenous domain of collagen IV. *J Biol Chem* 1997; 272: 16717–16720.
123. Recombinant DNA and Gene Transfer Meeting. *Advances in Clinical Gene Therapy*. June 19, 2007. Available at <http://www4.od.nih.gov/oba/RAC/meeting.html>. NHGRI 2007; Last accessed August 12, 2008.
124. DiMichele D. Inhibitor development in hemophilia B: an orphan disease in need of attention. *Br J Haematol* 2007; 138: 305–315.
125. Thorland EC, Drost JB, Lusher JM, et al. Anaphylactic response to factor IX replacement therapy in hemophilia B patients: complete gene deletions confer the highest risk. *Haemophilia* 1999; 5: 101–105.
126. Pike IM, Yount WJ, Puritz EM, et al. Immunohistochemical characterization of a monoclonal G4, lambda human antibody to factor IX. *Blood* 1972; 40: 1–10.
127. Sawamoto Y, Shima M, Yamamoto M, et al. Measurement of anti-factor IX IgG subclasses in hemophilia B patients who developed inhibitors with episodes of allergic reactions to factor IX concentrates. *Thromb Res* 1996; 83: 279–286.
128. Dioun AF, Ewenstein BM, Geha RS, et al. IgE-mediated allergy and desensitization to factor IX in hemophilia B. *J Allergy Clin Immunol* 1998; 102: 113–117.
129. Christophe OD, Lenting PJ, Cherel G, et al. Functional mapping of anti-factor IX inhibitors developed in patients with severe hemophilia B. *Blood* 2001; 98: 1416–1423.
130. Tengborn L, Hansson S, Fasth A, et al. Anaphylactoid reactions and nephrotic syndrome—a considerable risk during factor IX treatment in patients with hemophilia B and inhibitors: a report on the outcome in two brothers. *Haemophilia* 1998; 4: 854–859.
131. Lozier JN, Tayebi N, Zhang P. Mapping of genes that control the antibody response to human factor IX in mice. *Blood* 2005; 105: 1029–1035.
132. Fields PA, Kowalczyk DW, Arruda VR, et al. Role of vector in activation of T cell subsets in immune responses against the secreted transgene product factor IX. *Mol Ther* 2000; 1: 225–235.
133. Chen J, Wu Q, Yang P, et al. Determination of specific CD4 and CD8 T cell epitopes after AAV2- and AAV8-hFIX gene therapy. *Mol Ther* 2006; 13: 260–269.
134. Hay CR, Ollier W, Pepper L, et al. HLA class II profile: a weak determinant of factor VIII inhibitor development in severe hemophilia A. UKHCDO Inhibitor Working Party. *Thromb Haemost* 1997; 77: 234–237.
135. Oldenburg J, Picard JK, Schwaab R, et al. HLA genotype of patients with severe hemophilia A due to intron 22 inversion with and without inhibitors of factor VIII. *Thromb Haemost* 1997; 77: 238–242.
136. Astermark J, Oldenburg J, Pavlova A, et al. Polymorphisms in the IL10 but not in the IL1beta and IL4 genes are associated with inhibitor development in patients with hemophilia A. *Blood* 2006; 107: 3167–3172.
137. Astermark J, Oldenburg J, Carlson J, et al. Polymorphisms in the TNFA gene and the risk of inhibitor development in patients with hemophilia A. *Blood* 2006; 108: 3739–3745.
138. Astermark J, Wang X, Oldenburg J, et al. Polymorphisms in the CTLA-4 gene and inhibitor development in patients with severe hemophilia A. *J Thromb Haemost* 2007; 5: 263–265.
139. Zhang HG, High KA, Wu Q, et al. Genetic analysis of the antibody response to AAV2 and factor IX. *Mol Ther* 2005; 11: 866–874.
140. Mingozzi F, Liu YL, Dobrzynski E, et al. Induction of immune tolerance to coagulation factor IX antigen in vivo hepatic gene transfer. *J Clin Invest* 2003; 111: 1347–1356.
141. Cao O, Dobrzynski E, Wang L, et al. Induction and role of regulatory CD4+CD25+ T cells in tolerance to the transgene product following hepatic in vivo gene transfer. *Blood* 2007; 110: 1132–1140.
142. Reding MT. Immunological aspects of inhibitor development. *Haemophilia* 2006; 12 (Suppl 6): 30–36.
143. Hu G, Guo D, Key NS, et al. Cytokine production by CD4+ T cells specific for coagulation factor VIII in healthy subjects and hemophilia A patients. *Thromb Haemost* 2007; 97: 788–794.
144. Monahan PE. Neonatal immune tolerance for hemophilia: can we „tolerate“ new paradigms for gene therapy trials? *J Thromb Haemost* 2007; 5: 1801–1804.
145. Xu L, Gao C, Sands MS, et al. Neonatal or hepatocyte growth factor-potentiated adult gene therapy with a retroviral vector results in therapeutic levels of canine factor IX for hemophilia B. *Blood* 2003; 101: 3924–3932.
146. Schneider H, Muhle C, Douar AM, et al. Sustained delivery of therapeutic concentrations of human clotting factor IX—a comparison of adenoviral and AAV vectors administered in utero. *J Gene Med* 2002; 4: 46–53.
147. Waddington SN, Nivsarkar MS, Mistry AR, et al. Permanent phenotypic correction of hemophilia B in immunocompetent mice by prenatal gene therapy. *Blood* 2004; 104: 2714–2721.
148. Sabatino DE, Mackenzie TC, Peranteau W, et al. Persistent expression of hFIX After tolerance induction by in utero or neonatal administration of AAV-1-FIX in hemophilia B mice. *Mol Ther* 2007; 15: 1677–1685.
149. Xu L, Nichols TC, Sarkar R, et al. Absence of a desmopressin response after therapeutic expression of factor VIII in hemophilia A dogs with liver-directed neonatal gene therapy. *Proc Natl Acad Sci USA* 2005; 102: 6080–6085.
150. Santagostino E, Mancuso ME, Rocino A, et al. Environmental risk factors for inhibitor development in children with hemophilia A: a case-control study. *Br J Haematol* 2005; 130: 422–427.
151. Gouw SC, van den Berg HM, le Cessie S, et al. Treatment characteristics and the risk of inhibitor development: a multicenter cohort study among previously untreated patients with severe hemophilia A. *J Thromb Haemost* 2007; 5: 1383–1390.
152. Chang J, Jin J, Lollar P, et al. Changing residue 338 in human factor IX from arginine to alanine causes an increase in catalytic activity. *J Biol Chem* 1998; 273: 12089–12094.
153. Brunetti-Pierri N, Grove N, Zuo Y, et al. Bioengineered factor IX molecules with increased catalytic activity improve the safety and efficacy of helper-dependent adenoviral vectors (HDAd) for hemophilia B gene therapy. *Mol Ther* 2008; 16 (S 1): S152.
154. Refino CJ, Jeet S, DeGuzman L, et al. A human antibody that inhibits factor IX/IXa function potently inhibits arterial thrombosis without increasing bleeding. *Arterioscler Thromb Vasc Biol* 2002; 22: 517–522.
155. Gruber A, Hanson SR. Factor XI-dependence of surface- and tissue factor-initiated thrombus propagation in primates. *Blood* 2003; 102: 953–955.
156. Reipert BM, Hausl C, Sasgary M, et al. Humanized E17 hemophilic mice are a major breakthrough in the design of new preclinical models for developing factor VIII products with reduced immunogenicity. *Blood* 2007; 110: Abstract #782.
157. Jiang Q, Zhang L, Wang R, et al. FoxP3+CD4+ Treg cells play an important role in acute HIV-1 infection in humanized rag2-/-gC-/- mice *in vivo*. *Blood* 2008 June; epub ahead of print.
158. Monahan PE. Experimental animal use in the study of hemophilic bleeding. *Haemophilia* 2008; 14: 112–116.
159. Yan JB, Wang S, Huang WY, et al. Transgenic mice can express mutant human coagulation factor IX with higher level of clotting activity. *Biochem Genet* 2006; 44: 349–360.