Received: 1 September 2022 | Revised: 26 September 2022 | Accepted: 11 October 2022

DOI: 10.1002/rth2.12841

rpth research & practice in thrombosis & haemostasis

RESEARCH LETTER

A demonstration of factor XI contributing to hemostasis in the absence of factor XII

Factor XIa (FXIa), the activated form of the plasma zymogen FXI, is a trypsin-like protease that contributes to thrombin generation and hemostasis primarily by activating factor IX (FIX).[1] In humans, severe FXI deficiency may cause excessive bleeding with trauma or surgery, particularly when injury involves the mouth, nasopharynx, or urinary tract.[2,3] Bleeding severity varies, and many individuals who are FXI deficient do not experience abnormal hemostasis. A variable trauma-induced bleeding disorder has also been reported in FXI-deficient dogs,[4] cats,[5] and cattle.[6] Bleeding associated with FXI deficiency is considerably milder than with FIX deficiency (hemophilia B), supporting the premise that FIX is primarily activated by the factor VIIa/tissue factor complex in vivo.[7,8] In the cascade/waterfall hypotheses of thrombin generation, FXI is activated by the protease factor XIIa (FXIIa).[1] However, the absence of an obvious hemostatic abnormality in humans and other animals lacking FXII (the zymogen of FXIIa) implies that other proteases activate FXI during hemostasis.[3] For example, thrombin activates FXI in purified protein and plasma-based systems.[9–12] However, to our knowledge, there has not been a demonstration of FXI contributing to hemostasis in the absence of FXII.

Mice lacking FXI (*F11*$^{-/-}$ mice), in contrast to humans who are FXI deficient, do not appear to bleed excessively with trauma.[13,14] Furthermore, murine FXI is primarily bound to blood vessel endothelium rather than circulating in plasma, as in humans.[15] These features would seem to make mice unsuitable for studying the impact of FXI on hemostasis. However, we showed that intravenous infusion of human FXI, or inducing expression of human FXI by hydrodynamic tail vein injection (HTI), decreased the severity of bleeding in male mice lacking FIX (*F9*$^{-}$) in a saphenous vein bleeding (SVB) model.[14] Human FXIa, in addition to activating FIX, activates factors V, VIII, and X, which may explain the beneficial effect in the absence of FIX.[16,17] Now, using mice deficient in both FIX and FXII, this model provides us with a system to specifically address the hypothesis that FXI can contribute to hemostasis in vivo in the absence of FXII.

C57Bl/6 *F9*$^{-/-}$ female mice[18] were crossed with C57Bl/6 male mice lacking FXII (*F12*$^{-/-}$).[19] Female offspring were heterozygous for FIX and FXII null alleles (*F9*$^{+/-}$/F12$^{+/-}$), while males were heterozygous null for FXII and null for FIX. *F9*$^{+/-}$/F12$^{+/-}$ females were crossed with *F9*$^{-}$/F12$^{+/-}$ males, and offspring homozygous null for both genes (*F9*$^{-/-}$/*F12*$^{-/-}$ females and *F9*$^{-}$/*F12*$^{-/-}$ males) were identified (Figure 1A). Western blots of plasma confirmed that the mice with hemophilia also lack FXII (Figure 1B). The animals were crossed to generate sufficient *F9*$^{-}$/*F12*$^{-/-}$ males for testing. In the SVB model, the saphenous vein is exposed and punctured with a needle.[14,20,21] After bleeding stops, clot is gently removed to restart bleeding. The number of clots forming over 30 min and the duration of each bleeding episode are recorded (Figure 1C). C57Bl/6 wild-type (WT) and *F12*$^{-/-}$ mice formed 20.2 ± 3.1 and 18.7 ± 3.6 ($p = 0.26$) clots over 30 min, with bleeding times of 75 ± 14 and 79 ± 19 s ($p = 0.65$), respectively. In contrast, *F9*$^{-}$ mice formed 1.8 ± 1.0 clots, with bleeding times of 1333 ± 589 s ($p < 0.001$ for both parameters compared with WT mice), demonstrating the severe hemostatic defect caused by FIX deficiency. Results with *F9*$^{-}$/*F12*$^{-/-}$ mice were comparable to *F9*$^{-}$ mice (2.0 ± 1.5 clots, bleeding times 1165 ± 622 s).

Human FXI was infused into *F9*$^{-}$/*F12*$^{-/-}$ mice through a tail vein to achieve plasma concentrations twice the normal level in human plasma (approximately 60 nM).[14] As in our prior work with *F9*$^{-}$ mice, human FXI was superimposed on endogenous murine FXI in all mouse lines. The infusions resulted in an increase in the number of clots formed over 30 min (5.8 ± 3.3) and a decrease in bleeding time (393 ± 233 s) compared to *F9*$^{-}$/*F12*$^{-/-}$ control mice (Figure 1C, $p < 0.01$ for both parameters), consistent with our earlier experience with *F9*$^{-}$ mice.[14] Because infused FXI could contain FXIa that may influence hemostasis, we expressed human FXI in *F9*$^{-}$/*F12*$^{-/-}$ mice by HTI.[14] HTI resulted in sustained expression of human FXI for at least 6 weeks, with an average plasma concentration approximately 65% of that achieved with FXI infusion (a concentration slightly above the upper end of the normal plasma FXI concentration range in humans). The number of clots in mice expressing human FXI (7.2 ± 4.5) was significantly greater, and the bleeding times (380 ± 307 s) significantly shorter, than for untreated *F9*$^{-}$/*F12*$^{-/-}$ mice ($p < 0.01$ for both parameters).

Some comments on the model used in these experiments are required. Human FXI was used rather than increasing endogenous expression of murine FXI for two reasons. First, most mouse FXI is bound to blood vessel endothelium, making it difficult to determine the effect of infusing or expressing additional protein.[15] Second, given the absence of a bleeding disorder in FXI-deficient

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2022 The Authors. *Research and Practice in Thrombosis and Haemostasis* published by Wiley Periodicals LLC on behalf of International Society on Thrombosis and Haemostasis (ISTH).

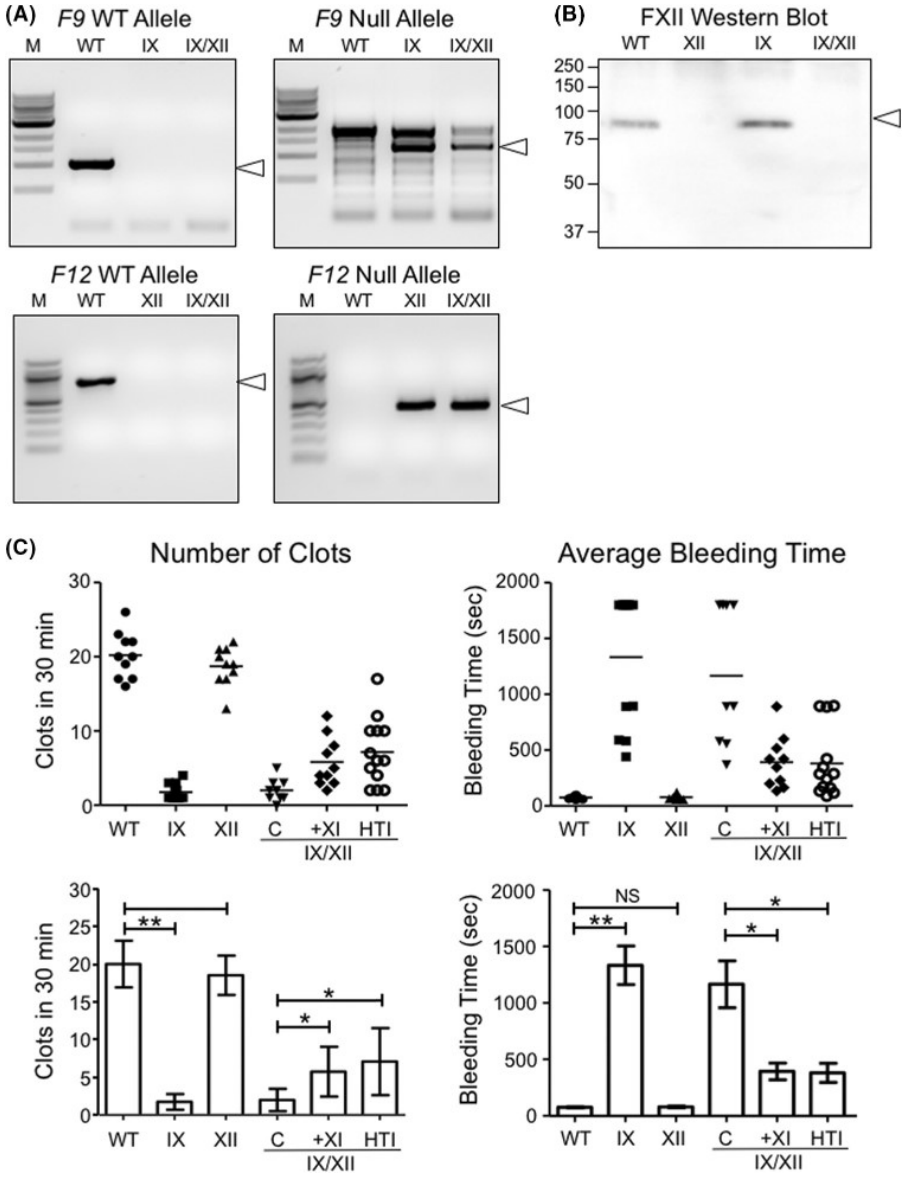

FIGURE 1 The effects of human FXI on hemostasis in mice lacking FIX and FXII. (A) PCR analysis of the *F9* (upper row) and *F12* (lower row) alleles in C57Bl/6 mice. Genomic DNA was prepared from ear punches for wild type (WT) mice, mice lacking fIX (IX) or fXII (XII), or mice lacking both fIX and fXII (IX/XII). Descriptions of the oligonucleotides used to copy the WT (left column) or disrupted null alleles (right column) for the *F9* and *F12* genes are described in references 18 and 19, respectively. The positions of bands specific for the relevant alleles are indicated by the white arrow heads. M indicates lanes containing molecular mass standards. (B) Western blot for FXII in plasma. One-microliter plasma samples from mice were size fractionated by SDS-PAGE under nonreducing conditions. Proteins were transferred to nitrocellulose membranes and incubated with an antihuman FXII IgG conjugated to horseradish peroxidase. Detection was with chemiluminescence. The position of bands representing mouse FXII is indicated by the white arrowhead. (C) Saphenous vein bleeding model. Shown are results for numbers of clots formed (left column) and average bleeding times (right column) for WT ($N = 10$), FIX-deficient (IX, $N = 9$), FXII-deficient (XII, $N = 10$), or FIX and FXII-deficient (IX/XII) C57Bl/6 mice. Mice with combined FIX and FXII deficiency were tested without supplementation (C, $N = 9$), after infusion of human FXI (+XI, $N = 10$) or after induction of human FXI expression by hydrodynamic tail vein injection (HTI, $N = 13$). In the graphs in the top row, symbols each represent the results for one mouse, and horizontal bars are averages for the group. The bar graphs in the bottom row show averages for each group ± 1 standard deviation. Pairs of groups were compared with Welch's *t*-test (two-tailed, unequal variances). *P*-values are indicated by asterisks ($^{*}p < 0.001$, $^{**}p < 0.01$). NS indicates not significant ($p \geq 0.05$). The data set was evaluated for skewedness by Shapiro test and visual inspection. There was sufficient evidence to assume normality for the data set as a whole. Groups were also compared with parametric one-way ANOVA testing, and Dunn's test with Bonferroni adjustment for post hoc multiple comparison testing. The results supported those obtained with the Welch's *t*-test.

mice, murine FXI may not be a hemostatic protease.[10,14] Indeed, FXI may not be involved in hemostasis in all mammal species. We also used a relatively high plasma concentration of human FXI. This was necessary to get a hemostatic signal in the absence of FIX. While these features of the model raise legitimate concerns regarding the physiologic relevance of FXIa activating targets other than FIX, our

sole purpose in this study was to demonstrate that FXI can have an impact on hemostasis in the absence of FXII. The results shown here imply that FXI is being converted to FXIa in this model independently of FXII.

The *F11* gene arose early in the evolution of mammals from a duplication of the *Klkb1* gene encoding the contact protease prekallikrein, a substrate of FXIIa.[22] FXI combines features of prekallikrein with a mechanism for efficient FIX activation, allowing it to form a bridge between FXIIa-initiated contact activation and thrombin generation.[1] This bridge is required for normal FXII-initiated clotting in the activated partial thromboplastin time assay used in clinical practice. However, while complete deficiency of FXII does not compromise hemostasis in humans, FXI deficiency does.[2,3] This suggests that there are FXII-independent mechanisms for FXI activation operating in vivo. The data shown here demonstrate that human FXI can contribute to hemostasis in mice in the absence of FXII, supporting this hypothesis. The findings are also in line with the observation that members of the mammalian infraorder Cetacea (whales, porpoises, and dolphins) have FXI in their plasmas, despite lacking FXII.[22]

It is important to note that we have not demonstrated that thrombin is the sole, or even the major, FXI activator in the SVB model. In most placental mammals, the P2′ residing at the FXI activation cleavage site is proline, which is a common feature in thrombin substrates.[23] FXI differs in this regard from prekallikrein, which is not activated by thrombin.[23] However, it is possible that proteases other than thrombin activate FXI in our system. On a more controversial point, our observations should not be construed as a demonstration that FXII does not contribute to hemostasis. Patients who are FXII deficient and FXII-deficient mice do not bleed abnormally during surgery,[3,19] which usually involves clean wounds. However, Juang and colleagues[24] showed that exposing wounds to silicate-containing earth (as may occur often in nature) shortens the duration of bleeding in WT mice but not in FXII-deficient mice. Thus, depending on circumstances, FXI may be activated by different proteases, including FXIIa, at a wound site, consistent with FXI's dual nature as a contact factor and a hemostatic protein.

AUTHOR CONTRIBUTIONS

BMM and QC generated the mice and performed experiments, and contributed to the preparation of the manuscript. DG oversaw the project and writing of the final manuscript.

ACKNOWLEDGMENTS

The authors wish to acknowledge the generous support for this work from the Ernest W. Goodpasture chair in Experimental Pathology for Translational Research, and award R35 HL140025 from the National Heart, Lung, and Blood Institute.

RELATIONSHIP DISCLOSURE

BMM and QC have no conflicts to declare. DG is a consultant for pharmaceutical companies (Anthos Therapeutics; Aronora, Inc.; Bayer Pharma; Bristol-Myers Squibb; Ionis Pharmaceuticals; Janssen Pharmaceuticals) with interests in targeting factor XI, factor XII, and prekallikrein for therapeutic purposes.

Bassem M. Mohammed PhD[1,2]
Qiufang Cheng MD[2]
David Gailani MD[2]

[1]*Department of Biochemistry, St. Louis University, Missouri, St. Louis, USA*
[2]*Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Tennessee, Nashville, USA*

Correspondence
David Gailani, Vanderbilt University Medical Center, Room 4918 The Vanderbilt Clinic, 1301 Medical Center Drive, Nashville, TN, USA.
Email: dave.gailani@vanderbilt.edu

Handling Editor: Prof. Yotis Senis

ORCID

Bassem M. Mohammed https://orcid.org/0000-0001-7227-3663
Qiufang Cheng https://orcid.org/0000-0003-1804-3079
David Gailani https://orcid.org/0000-0001-8142-8014

TWITTER

Bassem M. Mohammed @TheCoagMavrick

REFERENCES

1. Mohammed BM, Matafonov A, Ivanov I, et al. An update on factor XI structure and function. *Thromb Res*. 2018;161:94-105.
2. James P, Salomon O, Mikovic D, Peyvandi F. Rare bleeding disorders - bleeding assessment tools, laboratory aspects and phenotype and therapy of FXI deficiency. *Haemophilia*. 2014;20(suppl 4(04)):71-75.
3. Gailani D, Wheeler AP, Neff AT. Rare coagulation factor deficiencies. In: Hoffman R, Benz EJ, Silberstein LE, et al., eds. *Hematology: Basic Principles and Practice*. 7th ed. Elsevier; 2018:2034-2050.
4. Knowler C, Giger U, Dodds WJ, Brooks M. Factor XI deficiency in Kerry blue terriers. *J Am Vet Med Assoc*. 1994;205(11):1557-1561.
5. Kuder H, Dickeson SK, Brooks MB, et al. A common missense variant causing factor XI deficiency and increased bleeding tendency in Maine coon cats. *Genes (Basel)*. 2022 Apr 28;13(5):792.
6. Marron BM, Robinson JL, Gentry PA, Beever JE. Identification of a mutations associated with factor XI deficiency in Holstein cattle. *Anim Genet*. 2004 Dec;35(6):454-456.
7. Smith SA, Travers RJ, Morrissey JH. How it all starts: initiation of the coagulation cascade. *Crit Rev Biochem Mol Biol*. 2015;50(4):326-336.
8. Grover SP, Mackman N. Tissue factor: an essential mediator of hemostasis and trigger of thrombosis. *Arterioscler Thromb Vasc Biol*. 2018;38(4):709-725.
9. Naito K, Fujikawa F. Activation of human blood coagulation factor XI independent of factor XII. Factor XI is activated by thrombin and factor XIa in the presence of negatively charged surfaces. *J Biol Chem*. 1991;266(12):7353-7358.
10. Gailani D, Broze GJ Jr. Factor XI activation in a revised model of blood coagulation. *Science*. 1991;253(5022):909-912.
11. von dem Borne PA, Meijers JC, Bouma BN. Feedback activation of factor XI by thrombin in plasma results in additional formation

of thrombin that protects fibrin clots from fibrinolysis. *Blood*. 1995;86(8):3035-3042.
12. Kravtsov DV, Matafonov A, Tucker EI, et al. Factor XI contributes to thrombin generation in the absence of factor XII. *Blood*. 2009;114(2):452-458.
13. Gailani D, Lasky NM, Broze GJ Jr. A murine model of factor XI deficiency. *Blood Coagul Fibrinolysis*. 1997;8(2):134-144.
14. Mohammed BM, Cheng Q, Matafonov A, Monroe DM, Meijers JCM, Gailani D. Factor XI promotes hemostasis in factor IX-deficient mice. *J Thromb Haemost*. 2018;16(10):2044-2049.
15. Mohammed BM, Cheng Q, Matafonov A, et al. A non-circulating pool of factor XI associated with glycosaminoglycans in mice. *J Thromb Haemost*. 2019;17(9):1449-1460.
16. Whelihan MF, Orfeo T, Gissel MT, Mann KG. Coagulation procofactor activation by factor XIa. *J Thromb Haemost*. 2010;8(7):1532-1539.
17. Matafonov A, Cheng Q, Geng Y, et al. Evidence for factor IX-independent roles for factor XIa in blood coagulation. *J Thromb Haemost*. 2013;11(12):2118-2127.
18. Lin HF, Maeda N, Smithies O, Straight DL, Stafford DW. A coagulation factor IX-deficient mouse model for human hemophilia B. *Blood*. 1997;90(10):3962-3966.
19. Pauer HU, Renné T, Hemmerlein B, et al. Targeted deletion of murine coagulation factor XII gene-a model for contact phase activation in vivo. *Thromb Haemost*. 2004;92(3):503-508.
20. Monroe DM, Hoffman M. A mouse bleeding model to study oral anticoagulants. *Thromb Res*. 2014;133(suppl 1(01)):S6-S8.
21. Mohammed BM, Cheng Q, Ivanov IS, Gailani D. Murine models in the evaluation of heparan sulfate-based anticoagulants. In: Balagurunathan K, Nakato H, Desai U, Saijoh Y, eds. *Glycosaminoglycans: Methods and Protocols*, *Methods in Molecular Biology*. Vol 2303. Springer Nature; 2022:789-805.
22. Ponczek MB, Shamanaev A, LaPlace A, et al. The evolution of factor XI and the kallikrein-kinin system. *Blood Adv*. 2020;4(24):6135-6147.
23. Matafonov A, Sarilla S, Sun MF, et al. Activation of factor XI by products of prothrombin activation. *Blood*. 2011;118(2):437-445.
24. Juang LJ, Mazinani N, Novakowski SK, et al. Coagulation factor XII contributes to hemostasis when activated by soil in wounds. *Blood Adv*. 2020;4(8):1737-1745.