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BRIEF REPORT

Generation of a humanized FXII knock-in mouse—A powerful model system to test novel anti-thrombotic agents

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Abstract

Background: Effective inhibition of thrombosis without generating bleeding risks is a major challenge in medicine. Accumulating evidence suggests that this can be achieved by inhibition of coagulation factor XII (FXII), as either its knock-out or inhibition in animal models efficiently reduced thrombosis without affecting normal hemostasis. Based on these findings, highly specific inhibitors for human FXII(a) are under development. However, currently, *in vivo* studies on their efficacy and safety are impeded by the lack of an optimized animal model expressing the specific target, that is, human FXII.

Objective: The primary objective of this study is to develop and functionally characterize a humanized FXII mouse model.

Methods: A humanized FXII mouse model was generated by replacing the murine with the human *F12* gene (genetic knock-in) and tested it in *in vitro* coagulation assays and in *in vivo* thrombosis models.

Results: These $hF12^{KI}$ mice were indistinguishable from wild-type mice in all tested assays of coagulation and platelet function *in vitro* and *in vivo*, except for reduced expression levels of hFXII compared to human plasma. Targeting FXII by the anti-human FXIIa antibody 3F7 increased activated partial thromboplastin time dose-dependently and protected $hF12^{KI}$ mice in an arterial thrombosis model without affecting bleeding times.

Conclusion: These data establish the newly generated $hF12^{KI}$ mouse as a powerful and unique model system for *in vivo* studies on anti-FXII(a) biologics, supporting the development of efficient and safe human FXII(a) inhibitors.

KEYWORDS
animal models, blood coagulation, factor XII, hemostasis, thrombosis

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1 | INTRODUCTION

Blood coagulation is essential for terminating bleeding upon vascular injury (hemostasis). However, under pathophysiological conditions excessive coagulation can lead to life-threatening ischemic disease states, such as myocardial infarction, pulmonary embolism, and ischemic stroke.

Currently available anticoagulants and antithrombotics have proven beneficial in the prevention of ischemic cardio- and cerebrovascular diseases but are associated with an elevated bleeding risk and therefore often contraindicative. Established anticoagulants target enzymes of the coagulation cascade to prevent or limit fibrin formation. The intrinsic coagulation cascade is initiated through activation of factor XII (FXII) to FXIIa by polyanionic surfaces (such as polyphosphates or nucleic acids *in vivo* or by silica or ellagic acid *in vitro*), finally resulting in fibrin formation. FXII-driven contact system activation has been implicated in various diseases, among those hereditary angioedema (HAE), Alzheimer's disease, and thrombotic and inflammatory disorders.[1] This contact activation process serves as basis of the activated partial thromboplastin time (APTT), a method widely used to determine plasma coagulation in clinics. Furthermore, FXIIa activates the kallikrein--kinin system and the classical complement pathway. In addition, there is growing evidence linking FXII to innate immunity[2] and cytokine-mediated inflammation.[3]

While being dispensable for coagulation *in vivo*, FXII has emerged as a promising antithrombotic target. In patients, hereditary deficiency of FXII is not associated with abnormal bleeding, indicating FXII is not required for hemostasis.[4] Similar to humans, mice lacking FXII do not suffer from spontaneous hemorrhage and do not show prolonged bleeding times, but are protected from pathological thrombosis and thrombo-inflammatory brain infarction.[5,6] These results were reproduced in mice, rats, and rabbits treated with the specific FXIIa inhibitor rHA-Infestin-4 (Infestin-4 derived from the midgut of *Triatoma infestans* recombinantly fused to human albumin).[7–11] Meanwhile, different classes of FXII/FXIIa inhibitors including antibodies, small molecule inhibitors, and oligonucleotides[12–14] were developed with favorable results in a variety of experimental models including extracorporeal circulatory systems, arterial and venous thrombosis, and the transient middle cerebral artery occlusion model of ischemic stroke.[15] Among those, the fully human recombinant monoclonal antibody (mAB) 3F7 directed against the catalytic site of FXIIa was previously described to bind to mouse and human FXIIa in equipotently, low nanomolar range and shown to provide safe anticoagulation in extracorporeal circulation settings.[16] However, for the development of new therapeutics, human FXII(a)-specific agents need a humanized FXII/FXIIa animal *in vivo* model to optimally assess their efficacy and safety. We therefore generated human FXII knock-in mice and show that they are a valuable model system for *in vivo* studies on antithrombotic agents targeting human FXIIa *in vivo*.

Essentials

- Factor XII (FXII) emerged as a promising antithrombotic target.
- A humanized FXII mouse model was generated and characterized.
- Anti-human FXIIa antibody 3F7 protects $hF12^{KI}$ mice from thrombosis without affecting hemostasis.
- $hF12^{KI}$ mouse is a powerful model system for *in vivo* studies on anti-hFXII(a) biologics.

2 | METHODS

2.1 | Animals

Mice used in this study were matched for age, sex, and genetic background. Animal experiments were approved by the district government of Lower Franconia (Regierung von Unterfranken) and performed in accordance with the current Animal Research: Reporting of *In Vivo* Experiments guidelines (https://www.nc3rs.org.uk/arrive-guidelines).

For the generation of humanized FXII ($hF12^{KI}$) mice, the entire sequence of the mouse *F12* gene was replaced by the human *F12* gene sequence. Mice were genotyped by PCR, with 5' GGA ATC GTG GTG CGG ATA GT3' and 5' ACC ACC ATG CCA GGC TTA AA 3' resulting in 148 bp for wild-type (WT) mice (expressing mouse FXII), no band for hFXII Neo-KI mice (mice bearing the targeted allele with the neomycin resistance cassette), and in 245 bp for $hF12^{KI}$ mice (mice bearing the targeted allele without the neomycin resistance cassette).

2.2 | Human blood samples

For this study, blood samples were obtained from healthy volunteers following written informed consent in accordance with the Declaration of Helsinki and after approval by the Institutional Review Boards of the University of Würzburg and used for comparison with hFXII murine plasma.

2.3 | Chemicals, antibodies, and kits

Midazolam (Roche Pharma AG), Dorbene (Pfizer), and fentanyl (Janssen-Cilag GmbH) were used according to regulations of the local authorities. Iron(III) chloride ($FeCl_3$) was from Roth, Pefabloc®SC from Pentapharm, and polyclonal anti-hFXII antibody (PA5-26672) from Thermo Fisher Scientific. Rat monoclonal antiplatelet antibodies (anti-GPIbα[17]) and (X488[18]), conjugated with DyLight488 or HRP, were obtained from EMFRET Analytics or produced in house.

ELISA kits from Molecular Innovations (#MFXIIKT-TOT) and abcam (#ab192144) were used to determine mFXII and hFXII levels, respectively. Anti-FXIIa antibody 3F7 and isotype control monoclonal antibody huBM4 were provided by CSL Behring GmbH.

2.4 | Coagulation assays

APTT and prothrombin time (PT) measurements were measured on an automated blood coagulation analyzer (BCS® XP; Siemens Healthcare GmbH) as described.[16] For titration assays, plasma samples were preincubated with different concentrations of 3F7 for 15 min.

2.5 | FXII clotting activity

FXIIa enzymatic activity was determined using an APTT-based assay and compared to a reference curve obtained with dilutions of standard human plasma and FXII-deficient plasma, respectively, on a BCS® XP. Fifty µl plasma samples were pre-diluted 1:5 with imidazole buffer and added to 50 µl FXII-deficient plasma. After 30 s incubation at 37°C, 50 µl Pathromtin SL was added and incubated for 120 s at 37°C. Subsequently, 50 µl calcium chloride solution (25 mmol/L) was added to start the reaction. Reagents and protocols were provided by Siemens Healthcare GmbH.

2.6 | Antibody treatment

Mice were intravenously injected with the indicated dose of 3F7, huBM4 (diluted in 0.9% NaCl) or equal volumes of 0.9% NaCl directly before the experiment.

2.7 | Bleeding time

Mice were anesthetized by intraperitoneal (i.p.) injection of triple anesthesia (fentanyl, midazolam, dorbene) and a 2 mm segment of the tail tip was removed using a scalpel. Tail bleeding was monitored by gently absorbing blood on filter paper at 20 s intervals without making contact with the wound site. Bleeding was determined to have ceased when no blood was observed on the paper. Experiments were stopped after 20 min by cauterization.

2.8 | Mechanical injury of the abdominal aorta

Mice were anesthetized by i.p. injection of triple anesthesia. Mechanical injury of the abdominal aorta was performed as described.[5] Blood flow was monitored until complete occlusion occurred or for 30 min.

2.9 | $FeCl_3$-induced arterial thrombosis

Mice were anesthetized by i.p. injection of triple anesthesia. Intravital microscopy of mesenteric arterioles was performed as described previously.[7]

2.10 | Data analysis

Results are reported as mean ± standard deviation. Differences between more than two groups were analyzed using the Kruskal-Wallis test for nonparametric tests or one-way analysis of variance with Tukey's multiple comparisons test. The Fisher's exact test was applied to assess variance between non-occluded and occluded vessels. *P*-values <.05 were considered statistically significant.

3 | RESULTS AND DISCUSSION

To enable *in vivo* studies on antithrombotic agents targeting human FXII(a), mice expressing the human instead of the murine version of the FXII ($hF12^{KI}$) were generated. For this, the entire sequence of the mouse *F12* gene was replaced by the human *F12* gene sequence. The replacement of mFXII by hFXII in the newly generated mutant mice was confirmed by western blot analysis (Figure 1A), ELISA (Figure 1B,C), and targeted mass spectrometry online-coupled to liquid chromatography (LC-MS; Figure 1D,E). Of note, hFXII plasma levels (2.4 µg/ml) in $hF12^{KI}$ mice were lower than mFXII levels in WT controls (~15 µg/ml) or hFXII levels in plasma samples of healthy volunteers (~27 µg/ml; Figure 1C). Targeted LC-MS confirmed hFXII levels 80% lower in $hF12^{KI}$ mice compared to healthy human control plasma (Figure 1D). Reduced FXII plasma levels in $hF12^{KI}$ mice could result from less efficient recognition of human regulatory elements in the *F12* gene in mice. However, despite reduced hFXII levels, the APTT was indistinguishable between $hF12^{KI}$ mice and WT controls (Figure 1F), which is in line with previous reports that 10% FXII are sufficient to maintain normal APTT [19]. In line with reduced FXII plasma levels, FXIIa clotting activity was found to be significantly reduced in $hF12^{KI}$ mice compared to WT controls (Figure 1G). Because FXII activation also triggers the kallikrein--kinin pathway, we assessed the impact of hFXII on the murine contact system. Total kallikrein-like and FXIIa activity following aprotinin blockade were indistinguishable between $hF12^{KI}$ and WT plasma, demonstrating comparable hFXIIa--mPKa and mfXIIa--mPKa interactions (data not shown). As expected, the PT (measure of tissue-factor [TF]--initiated coagulation) was indistinguishable between $hF12^{KI}$ mice and WT controls (Figure 1H).

Next, the effect of the anti-FXIIa mAB 3F7 on $hF12^{KI}$ and WT plasma clotting was assessed. Titration experiments revealed a dose-dependent prolongation of the APTT (Figure 1I) with 100 µg/ml 3F7 being sufficient to reduce FXIIa protease activity to less than 5% (baseline values) compared to untreated control groups in both WT

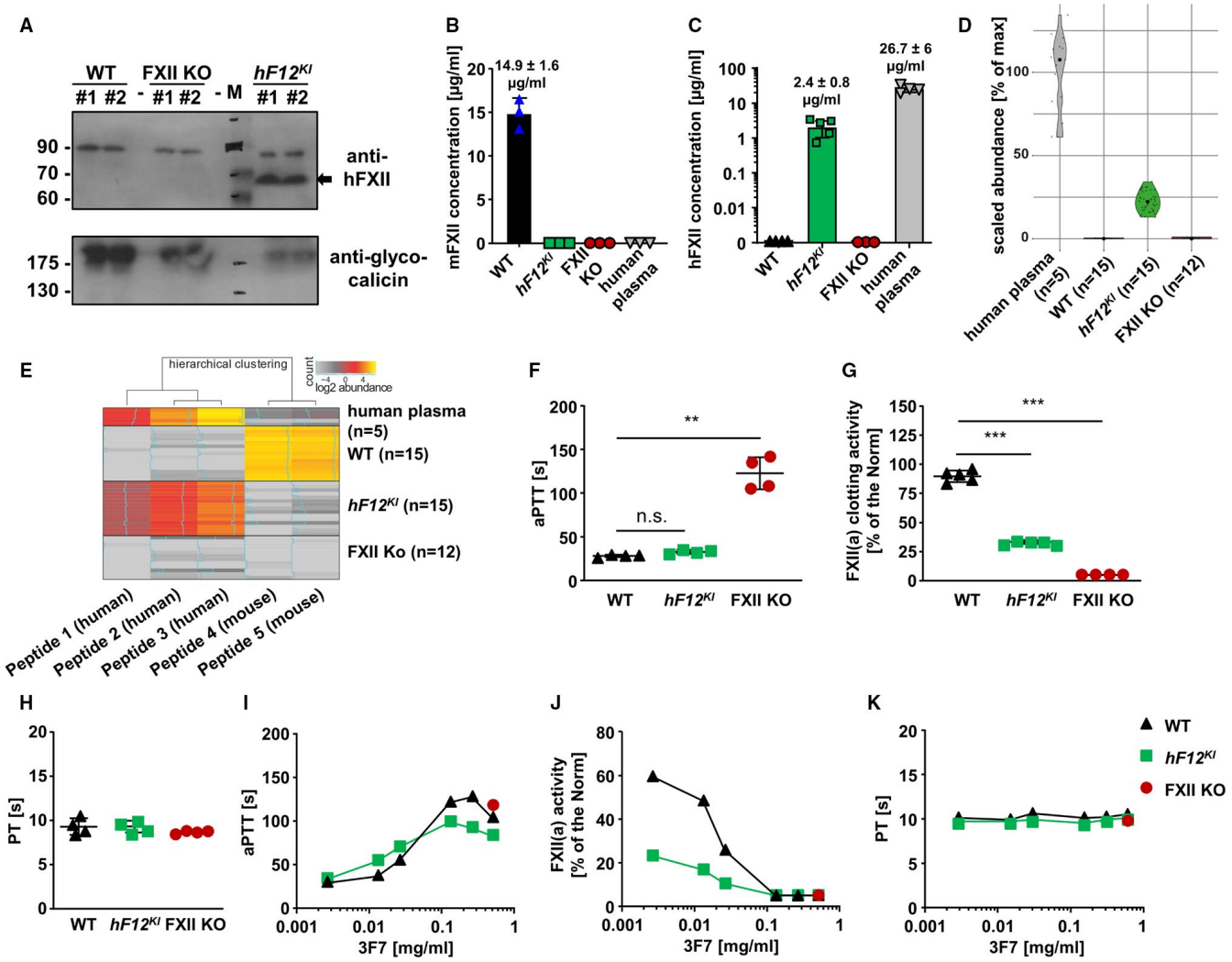

FIGURE 1 3F7 inhibits factor XII (FXII)a activity in hF12KI mouse plasma. A, hFXII expression in plasma was assessed by western blot analysis ($n = 2$). hFXII was detected at ~68 kDa. Glycocalicin served as a loading control. B, mFXII-specific ELISA confirmed absence of mFXII in hF12KI mice ($n = 3$). C, hFXII expression in hF12KI plasma was confirmed by an hFXII-specific ELISA ($n = 3$). D, E, Targeted mass spectrometry online-coupled to liquid chromatography (LC-MS) allowed clear differentiation between hFXII and mFXII using three and two stable isotope-labelled reference peptides, respectively. Peptide 1 (human FXII): TTLSGAPCQPWASEATYR, peptide 2 (human FXII): VVGGLVALR, peptide 3 (human FXII): LHEAFSPVSYQHDLALLR, peptide 4 (mouse FXII): GLSSFMR, peptide 5 (mouse FXII): WTVEATYR. Quantification revealed ~80% lower hFXII levels in hF12KI mice compared to healthy humans analyzed by targeted LC-MS ($n = 5$–15). F, APTT (activated partial thromboplastin time) and (G) FXIIa activity in citrated plasma ($n \geq 4$). H, Unaltered prothrombin time (PT) in hF12KI mice. I, J, 3F7 inhibits FXII-driven clotting activity without affecting PT in wild-type and hF12KI mice ($n \geq 4$). Plasma was incubated with a serial antibody dilution starting at a maximum of 0.5 mg/ml. I) APTT, (J) FXIIa activity, and (K) PT were measured on an automated blood coagulation analyzer

and *hF12*^{KI} mice (Figure 1J). 3F7 (in concentrations of up to 0.5 mg/ml) did not affect PT in both mouse lines (Figure 1K), which is in line with previously published data[16] supporting the specificity of 3F7 for interference with FXIIa-mediated clotting. Of note, low hFXII expression in *hF12*^{KI} mice might make them more sensitive to the antithrombotic effect of an FXII inhibitor than would a mouse with a physiologic (normal) level of FXII.

To test functional consequences of replacing mouse FXII by human FXII *in vivo*, WT and *hF12*^{KI} mice were subjected to an experimental model of $FeCl_3$-induced thrombosis in the mesenteric arterioles. Here, time to vessel occlusion was comparable between both genotypes (WT = 16.00 ± 5.24 min, *hF12*^{KI} = 17.77 ± 4.59 min), while FXII-deficient mice did not form occlusive thrombi within the observation period (Figure 2A,B). In a second *in vivo* thrombosis model, similar results were obtained after mechanical injury of the abdominal aorta (Figure 2C). These results indicate that human FXII can compensate for the loss of murine FXII in arterial thrombosis, which is in line with previous studies.[5] Of note, *hF12*^{KI} mice showed unaltered tail bleeding times compared to WT animals indicating normal hemostatic function (Figure 2D).

To analyze the *in vivo* effects of the hFXIIa inhibitory antibody 3F7, *hF12*^{KI} mice received up to 10 mg/kg antibody intravenously

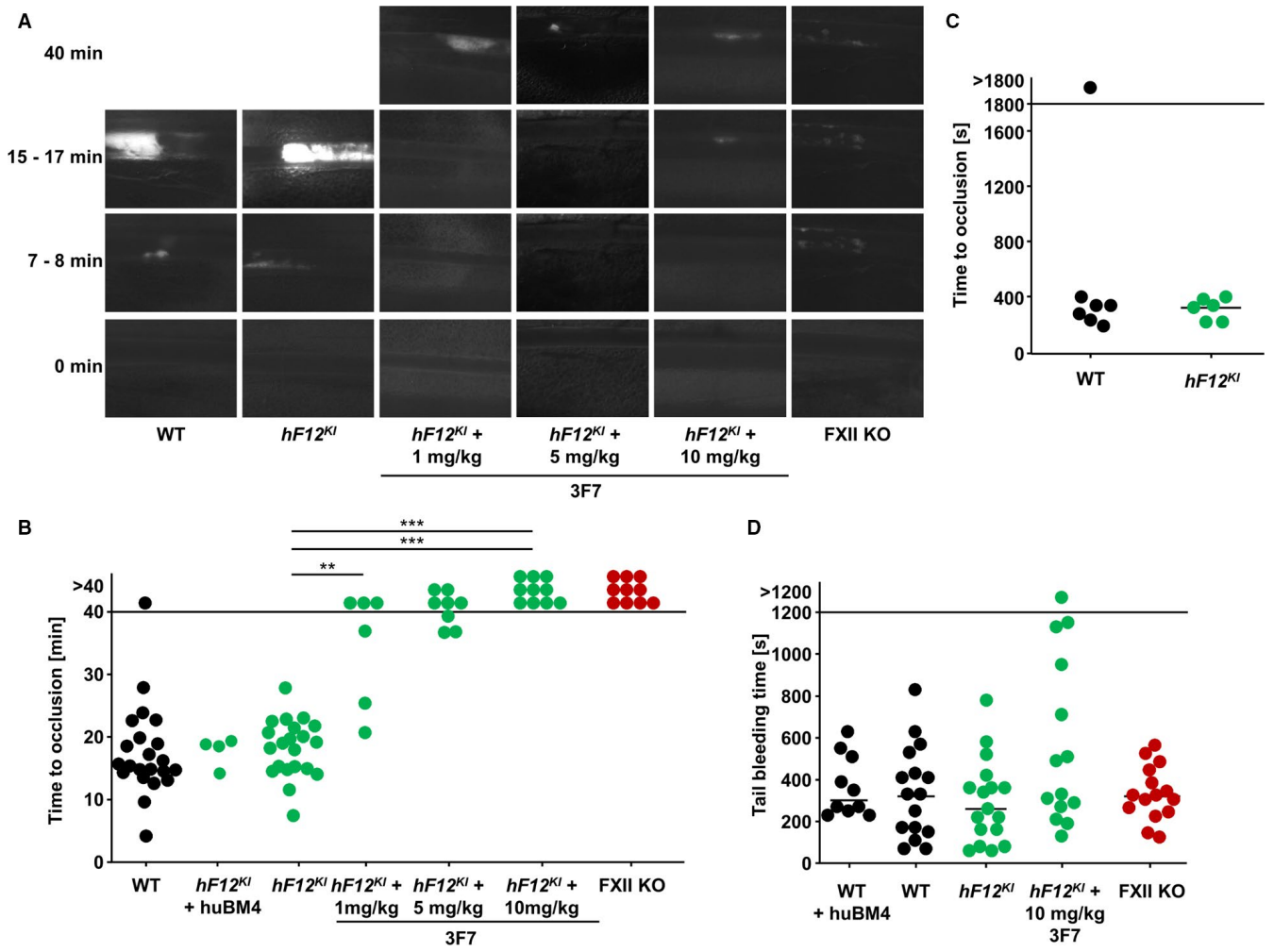

FIGURE 2 3F7 inhibits factor XII (FXII)-driven thrombosis in a hF12KI mouse. A, B, 3F7 protects from arterial occlusive thrombosis upon FeCl3-induced injury on mesenteric arterioles. A, Representative microscopic images of mesenteric arterioles of the indicated genotypes and treatments. B, Each dot represents one vessel. C, The abdominal aorta was mechanically injured by a single firm compression with a forceps and blood flow was monitored with a Doppler flowmeter. Time to final occlusion is shown. Each symbol represents one individual mouse. D, 3F7 does not alter the hemostatic function in wild-type and hF12KI mice using a tail bleeding time assay on filter paper. Each symbol represents one animal. huBM4: control mAB (10 mg/kg)

directly before $FeCl_3$-induced injury of the mesenteric arterioles. In this assay, 3F7 dose-dependently decreased thrombus formation and prolonged occlusion times (Figure 2A,B). This dose-dependent prolongation was comparable to that of 3F7-treated WT mice (data not shown). At the highest tested dose of 10 mg/kg 3F7, mice were completely protected from thrombosis, resembling the phenotype of FXII-deficient mice,[5] which were tested in direct comparison. Of note, isotype control or vehicle treatment were inactive in all assays. These data are in line with a previous report of 3F7-treated WT mice.[16] Notably, even at a high concentration of 10 mg/kg, tail bleeding times were statistically not significantly altered by 3F7-mediated inhibition of FXIIa in *hF12*^{KI} mice (Figure 2D), indicating that the antithrombotic effect of 3F7 is not associated with impaired hemostasis.

These results demonstrate that human FXII is functional in mice, as suggested by previous reports in which *in vivo* injection of human FXII into F12-deficient mice reversed protection from arterial occlusive thrombus formation in KO mice lacking endogenous FXII.[5] This model is clearly advantageous to current protocols, that is, using transfer of recombinant FXII or hydrodynamic tail vein injections of respective plasmid DNA having less off-target effects and being less invasive, therefore ethically advantageous. In addition, recombinant FXII or tail vein injections have comparable short half-times and lead to a higher variability than a genetic knock-in.

Collectively, our study underscores the potential of FXII as an efficient and safe antithrombotic target and demonstrates that our newly generated mouse line is a valuable and unique tool for *in vivo* studies on antithrombotic agents or bradykinin-related angioedema research targeting human FXIIa. Thus, these newly generated *hF12*^{KI} mice will allow for indispensable translation of findings on mouse FXII studies to the human system, paving the way to an efficient and safe FXII(a) inhibitor.

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CONFLICTS OF INTEREST

FM and MWN are employees of CSL Behring GmbH (Marburg, Germany) and CP is an employee of CLS Ltd. (Melbourne, Australia). Furthermore, BN has a consultancy agreement with CSL Behring GmbH. All other authors state that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

S. Beck, A. A. Baig, V. Göb, S. Loroch, C. Schumbrutzki, E. Eilers, and D. Stegner performed experiments and analyzed data. Employees of CSL Behring GmbH and CSL Ltd. provided vital reagents, contributed to *in vitro* work, and analyzed data. A. Sickmann and B. Nieswandt supervised research. S. Beck, D. Stegner, and B. Nieswandt wrote the manuscript with input from all authors. The manuscript has been read and approved for submission to JTH by all authors.

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