Targeted deletion of murine coagulation factor XII gene-a model for contact phase activation in vivo

Hans-Ulrich Pauer1, Thomas Renné2, Bernhard Hemmerlein3, Tobias Legler4, Saskia Fritzlar5, Ibrahim Adham5, Werner Müller-Esterl6, Guenter Emons1, Ulrich Sancken5, Wolfgang Engel5, Peter Burfeind5

1Department of Gynecology and Obstetrics, Medical School, University of Goettingen, Germany
2Junior Group at the Institute of Clinical Biochemistry and Pathobiochemistry, University of Wuerzburg, Germany
3Department of Pathology, Medical School, University of Goettingen, Germany
4Department of Transfusion Medicine, Medical School, University of Goettingen, Germany
5Institute of Human Genetics, University of Goettingen, Germany
6Institute of Biochemistry II, Medical School, University of Frankfurt, Germany

Summary
To analyze the biological role of factor XII (FXII, Hageman Factor) in vivo, we generated mice deficient for FXII using a gene targeting approach on two distinct genetic backgrounds, i.e. mixed C57Bl/6j X 129X1/Svj and inbred 129X1/Svj. Homozygous FXII knockout (FXII-/-) mice showed no FXII plasma activity and had a markedly prolonged activated partial thromboplastin time (aPTT). In contrast, coagulation factors XI, VIII, IX, X, VII, V, II and fibrinogen did not differ between FXII-/- mice and their wild-type littermates. Heterozygous matings segregated according to the Mendelian inheritance indicating that FXII deficiency does not increase fetal loss. Furthermore, matings of FXII+ males and FXII+ females resulted in normal litter sizes demonstrating that total FXII deficiency in FXII-/- females does not affect pregnancy outcome. Also, gross and histological anatomy of FXII-/- mice was indistinguishable from that of their wild-type littermates on both genetic backgrounds. Thus it appears that deficiency of murine FXII does not cause thrombophilia or impaired fibrinolysis in vivo. These results indicate that FXII deficiency does not affect hemostasis in vivo and we anticipate that the FXII-/- mice will be helpful to elucidate the biological role(s) of FXII in health and disease.

Keywords
Coagulation, factor XII, gene targeting

Introduction
In the classical view, activation of factor XII (FXII, Hageman factor) is thought to initiate the intrinsic coagulation pathway by triggering the contact phase system. In this scenario, contact of mammalian plasma with negatively charged surfaces (“contact phase”) induces (auto)activation of FXII, possibly due to an allosteric activation and limited proteolysis of the zymogen in the presence of Zn²⁺. The activated serine protease, FXIIa, then cleaves plasma prekallikrein and generates active plasma kallikrein which in turn cleaves and activates FXII to FXIIa, leading to a local burst of protease activity at the surface. At this point, the signaling pathway branches: plasma kallikrein cleaves H-kininogen and liberates bradykinin triggering the NO/cGMP pathway, whereas FXIIa is thought to initiate and enhance fibrin clot formation via factors XI and IX (1, 2). Conversely, activation of FXII may also induce fibrinolysis either directly through the activation of plasminogen or...
indirectly via plasma kallikrein through the activation of pro-urokinase (pro-uPA) (3, 4).

Given the critical roles of FXII in hemostasis that have been postulated on the basis of in vitro studies, one may expect that total FXII deficiency will produce symptoms due to its procoagulant and/or profibrinolytic activities. Human FXII deficiency causes a markedly prolonged activated partial thromboplastin time (aPTT), however, bleeding incidents are not associated with this defect. On the contrary, some studies have identified FXII deficiency as a risk factor for thrombembolism, whereas others failed to find such a relationship (5-10). Also some investigations have reported an association of human FXII deficiency with recurrent abortion suggesting that low FXII levels may negatively affect pregnancy outcome whereas others have failed to reveal such an association (11-14). Here we have used a gene targeting approach to generate FXII-deficient mice to study the effects of FXII-deficiency on murine hemostasis in vivo.

Materials and methods

Targeted deletion of the murine FXII gene

The murine FXII cDNA was generated by RT-PCR on total RNA obtained from mouse liver by using FXII-specific primers (FXII-for: 5’-ATG ACG GCT CTG TTG TCC CTG-3’, FXII-rev: 5’-CAA GTC GCA AAA ATA TCC AGT GTA G-3’; 5’region, FXII-for: 5’-GAG AAG CTG ACA GTG GTA CTT GGT-3’, FXII-rev: 5’-AAG CCT GGT TAG TTA AGC AAT A-3’). For Western blotting we used a polyclonal antibody against human FXII (Nordic Immunological Laboratories, Tilburg, The Netherlands) or rabbit antiserum to human FXII (kindly donated by Dr. A. Kaplan, Charleston, SC, USA), followed by the chemiluminescence assay (Nycomed Amersham Buchler, Braunschweig, Germany).

Activated partial thromboplastin time (aPTT) and determination of coagulation factors

Whole blood was collected from mice by cardiac puncture under general anesthesia (Avertin). Blood (720 μl) was withdrawn using a 25-gauge needle containing 80 μl of 3.8% sodium citrate. All samples were centrifuged twice at 1000 × g for 10 min to separate plasma. Coagulation factors were determined immediately using the Dade Behring coagulation system (BCS) according to the manufacturer’s protocols and guidelines given from the Jackson Laboratory (http://pga.jax.org). Analysis included the activated partial thromboplastin time (aPTT) and single coagulation factors XII, XI, VIII, IX, X, VII, V, II or fibrinogen. Fibrinogen levels are given in g/l and other coagulation parameters are expressed as percentage relative to human plasma (100%).

Histological studies

For histopathological analysis mouse organs were fixed in 4% buffered formalin and embedded in paraffin. Two micrometer thick sections were stained with hematoxyline and eosin and with a modified trichrome stain according to Goldner.

Statistical analysis

Statistical analysis regarding the littersize, sex contribution and genotypes according to the expected Mendelian inheritance (ratio 1:2:1) were performed using the chi2-square for observed and expected distributions. The statistical analysis of the results of coagulation assays of wild-type, heterozygous and homozygous mice were performed using the Mann-Whitney-U-test. All statistical procedures were performed by Statistica, StatSoft Inc., Tulsa, OK, USA.

Animals

All experimental procedures complied with National Regulations for the care and use of laboratory animals.
Results

Targeted disruption of the FXII gene in mice
Using a gene targeting approach we have generated homozygous FXII-/- mice to elucidate the functional role of coagulation factor XII in blood coagulation. The mouse FXII gene was disrupted in ES cells by homologous recombination using a replacement-targeting vector containing pTKNeo and thymidine kinase expression cassettes. The neomycin (Neo) cassette replaces exons 1 and 2 containing the start codon ATG (Fig. 1A). We obtained 4 correctly targeted ES cell clones, which were identified using Southern-blot analysis with an external probe located upstream of the 5’ flanking region of the targeting construct (Fig. 1B). The external probe detected the wild-type allele as a 10 kb fragment and the recombinant allele as a 4.6 kb fragment in genomic DNA digested with the SstI restriction enzyme. The ES cells from one clone were injected into C57Bl/6J blastocysts to generate chimeric mice. Male chimeric mice transmitting the targeted mutation into the germ line were bred with female mice from C57Bl/6J and 129X1/SvJ strains to establish the FXII knockout model in two different genetic backgrounds. Heterozygous animals were identified by PCR analysis of DNA obtained from mouse tails (Fig. 1C) and were intercrossed to generate homozygous mice in the respective genetic backgrounds.

Analysis of FXII expression in knockout mice
The complete absence of FXII transcripts in the liver and of FXII protein in the plasma of FXII-/- mice was demonstrated by Northern blots, RT-PCR and Western blots (Figs. 1D-F).

Figure 1: Generation of FXII knockout line and FXII expression analyses. (A) Targeting strategy for the FXII construct, representing the wild-type (wt) FXII locus (top) and the recombinant (mut) FXII allele (bottom). The neomycin (Neo) cassette replaces exons 1 and 2. The external probe (ex1) is shown as a block at the 5′ end of the FXII locus. Exons are represented by numbers. Primers used for genotyping of progeny by PCR are indicated by arrowheads (FXII wt-for = 1, FXII wt-rev = 2, pTKNeo-rev = 3). The restriction sites are H, HindIII; S, SalI; Ss, SstI; Sp, Spel; B, BamH I; and C, Clal. (B) Southern blot analyses to identify recombinant ES cell clones. Genomic DNA from ES cell clones was digested with SstI. Hybridization with a [32P]-labeled external probe (ex1) shows a 10 kb band for the wild-type (wt) FXII allele and a 4.6 kb band for recombinant (mut) FXII allele in four different ES cell clones (lanes 1-4). (C) Genotyping of progeny by PCR using genomic DNA isolated from mouse tails and primers FXII wt-for, FXII wt-rev 2 and pTKNeo-rev. The wild-type (wt) FXII allele yields a PCR product of 842 bp and the mutated (mut) FXII allele generates a 492 bp product. +/+: wild-type, +/-: heterozygous null, -/-: homozygous null. (D) Northern blot analysis on total RNA isolated from liver of FXII+/+, FXII+/- and FXII-/- mice using FXII cDNA as the probe. FXII transcripts were observed in FXII+/- and FXII-/- mice using FXII cDNA as the probe (upper panel). RNA integrity and quantity was monitored by rehybridization using a β-actin amplimer set generating a 540 bp product (lower panel). (E) The complete absence of FXII transcripts in the liver and of FXII protein in the plasma of FXII-/- mice was demonstrated by Northern blotting, RT-PCR and Western blotting (Figs. 1D-F). RT-PCR products of 632 (600) bp were generated in FXII+/+ and FXII+/- but not in FXII-/- mice (upper panel). (F) Western blot analysis of mouse plasma probed with polyclonal antibodies to human FXII. The antibody detects a 85 kDa band in FXII+/+ but not in FXII-/- mice. For control an identical volume of human plasma (Hu) was applied and probed under identical conditions.
Northern blot analysis was performed on total liver RNA isolated from FXII+/+, FXII+/-, and FXII-/- mice, respectively. For Northern blot analysis a 632 bp 5'-end FXII cDNA fragment (nucleotide position 21-653) was used as a probe. A strong expression of FXII with a transcript size of 1.9 kb was observed in FXII+/+ and FXII+/- mice, whereas FXII expression was not detectable in liver RNA of FXII-/- mice (Fig. 1D). Rehybridization with a β-actin probe proofed the integrity and the amount of RNA used in this experiment (Fig. 1D). To exclude a possible fusion transcript, RT-PCR analyses was performed using primers located either at the 5'-end (nucleotide position 21-653) or at the 3'-end (nucleotide position 1226-1826) of the FXII gene. By using these RT-PCR approaches PCR products of 632 bp and 600 bp were generated on RNA from FXII+/+ and FXII+/- mice, respectively, but not on RNA obtained from FXII-/- mice. To monitor for RNA integrity, a β-actin amplimer set generating a 540 bp product was used in both RT-PCR experiments (Fig. 1E).

To prove that our targeting strategy had successfully exterminated FXII protein expression we probed for FXII in plasma by Western blot analysis (Fig. 1F). FXII migrating at 88 kDa was detected in human plasma samples (positive controls, Fig. 1F, lane 5, 6) using polyclonal anti-FXII antibodies. These antibodies raised against human protein detect its murine homolog at 85 kDa in plasma from FXII+/+ mice (Fig. 1F, lane 1, 2), but failed to detect any FXII protein in knockout mice (Fig. 1F, lane 3, 4). Heterozygous FXII+/- mice showed FXII plasma levels about half the concentration as compared to wild-type mice (not shown). Similarly, FXII antigen was detectable in homogenized liver from FXII+/+ mice, but was completely absent in FXII-/- liver tissue.

### Phenotypical analyses of FXII knockout mice

FXII+/+ mice were phenotypically normal and fertile, and heterozygous matings yielded the predicted Mendelian ratio of offspring on both genetic backgrounds (Table 1). Matings of FXII+/- males and FXII+/- females resulted in normal litter sizes and sex distribution of littermates as compared to wild-type FXII+/+ matings. FXII-/- mice on both genetic backgrounds appeared healthy and viable, and they survived for more than

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#### Table 1: Genotype distribution of progeny of FXII+/+ matings at 3 weeks of age.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Observed count</th>
<th>Expected count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>25.32</td>
<td>25.00</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>46.84</td>
<td>50.00</td>
</tr>
<tr>
<td>Homozygous</td>
<td>27.85</td>
<td>25.00</td>
</tr>
</tbody>
</table>

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**Figure 2: FXII activity and aPTT in FXII+/+, FXII+/- and FXII-/- mice.**

(A) The aPTT was markedly prolonged in homozygous knock-out mice as compared to heterozygous and wild-type mice. (B) FXII+/- mice showed virtually no FXII plasma activity, whereas FXII+/- mice display intermediate and FXII+/+ full FXII plasma activity. Data are presented in box whisker-plots showing the median (dark square), 25th to 75th centiles (box), and 5th to 95th centiles (whiskers).
12 months in our laboratory. These results indicate, that severe FXII deficiency neither results in a prenatal selection by increased intrauterine fetal death nor in an increased postnatal mortality. Furthermore, severe FXII-deficiency of the mother did not affect pregnancy outcome.

Activated partial thromboplastin time (aPTT) and coagulation assays
Using the automatic coagulometer (BCS, Dade Behring, Marburg, Germany) homozygous FXII-/- mice showed no FXII plasma activity, whereas heterozygous FXII +/- mice display intermediate FXII plasma activity. The aPTT was also markedly prolonged in homozygous FXII-/- mice (Figs. 2A and B). These results would be expected with the absence of a protein from the intrinsic pathway and are consistent with the results of plasma coagulation assays in humans with severe FXII deficiency. However, all other coagulation factors including factors XI, VIII, IX, X (intrinsic pathway) and VII, V and II (extrinsic pathway) did not differ between homozygous FXII-/- mice and wild-type mice on an identical genetic background (Table 2).

Histological examination
To test for potential changes in the microcirculation of FXII-/- mice, we performed histological analyses of highly vascularized organs such as lung, kidney, liver, small bowel, heart, and spleen with particular emphasis on thrombosis and infarction. Therefore, histopathological analyses were performed on organs from 58 adult mice (mean age 110 days) of different genotypes and different genetic backgrounds. The organs of twelve wild-type mice (n=10, background C57Bl/6Jx129/SvJ; n=2, background 129/SvJ) were compared to organs of 21 homozygous FXII-/- mice (n=16, background C57Bl/6Jx129/SvJ; n=5, background 129/SvJ). Furthermore, organs of 25 heterozygous FXII +/- (n=23, background C57Bl/6Jx129/SvJ; n=2, background 129/SvJ) were investigated for histopathological changes. We did not find vascular thrombosis, infarcts or tissue scars due to ischemia in the tissue sections analyzed. No evidence of microcirculatory disturbances such as erosions were observed in the small bowel. The lung showed a regular branching of the bronchi and regular evolvement of the alveola without evidence of alveolar distress. The kidneys were well developed with regular differentiation of the tubules and collecting ducts. Also megakaryocytes in the spleen of FXII-/- mice were unaffected (data not shown).

Discussion
Our data showing that FXII deficiency does not induce bleeding disorders in mice are consistent with the lack of clinical symptoms in FXII-deficient patients (5, 10). We were unable to find any symptoms of a hypofibrinolytic state as may be expected for the deficiency of a profibrinolytic effector (17, 18). Thus, apart from the reduced plasma activity of FXII and a prolonged partial thromboplastin time, FXII-deficient mice do not show obvious symptoms of their hemostatic system. Furthermore, the determination of all other coagulation factors revealed equal results for FXII-/- mice and their wild-type littermates. Taken together, these results highlight the specificity of the FXII gene targeting approach.

Statistical analysis revealed that the mode of inheritance is in agreement with Mendelian segregation (Table 1), and that litter sizes were normal for matings with FXII-/- male and FXII-/- female mice on both genetic backgrounds as compared to wild-type mice. No significant differences could be found for C57Bl/6J X 129X1/SvJ (p=0.20) and for 129X1/SvJ (p=0.14). These results imply that the fetal genotype plays a minor – if any - role in reproductive outcome and that the genetic background does not affect phenotypic expression, as has been shown e.g. in the factor V knock-in mouse model (19). These conclusions are consonant with previous observations in wild-type mice that FXII transcripts were detectable only until later developmental stages of gestation (20).

On the other hand, a genome-wide linkage analysis has identified two gene loci located on chromosome 5 (in proximi-

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**Table 2: Results of coagulation assays in wild-type and FXII-/- mice.**

<table>
<thead>
<tr>
<th>Coagulation factor</th>
<th>Wild-type (129X1/SvJ), n=10 average activity (%)</th>
<th>FXII-/- (129X1/SvJ), n=10 average activity (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor II</td>
<td>155</td>
<td>176</td>
<td>n.s.</td>
</tr>
<tr>
<td>Factor V</td>
<td>1403</td>
<td>1545</td>
<td>n.s.</td>
</tr>
<tr>
<td>Factor VII</td>
<td>645</td>
<td>714</td>
<td>n.s.</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>48</td>
<td>53</td>
<td>n.s.</td>
</tr>
<tr>
<td>Factor IX</td>
<td>103</td>
<td>104</td>
<td>n.s.</td>
</tr>
<tr>
<td>Factor X</td>
<td>603</td>
<td>686</td>
<td>n.s.</td>
</tr>
<tr>
<td>Factor XI</td>
<td>88</td>
<td>95</td>
<td>n.s.</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>1.32</td>
<td>1.18</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

n.s.: not significant
ty to the human FXII gene) and on chromosome 10 affecting FXII protein levels. Combined analysis of FXII levels and thrombosis risk revealed an enhanced linkage signal on chromosome 5 implying that this quantitative trait locus has pleiotropic effects concerning the risk for thrombosis (21).

Our findings with FXII<sup>-/-</sup> mice are similar to those observed with the murine model of FXI deficiency where null mice were virtually indistinguishable from their wild-type littermates, except for a discretely prolonged bleeding time (22). Combined, these findings suggest that proteins of the intrinsic pathway do not play a significant role in the initiation of mammalian blood coagulation. In an effort to analyze the evolution of the coagulation system, Jiang and Doolittle have compared the vertebrate coagulation. In an effort to analyze the evolution of the coagulation system, Jiang and Doolittle have compared the vertebrate coagulation. In an effort to analyze the evolution of the coagulation system, Jiang and Doolittle have compared the vertebrate coagulation. In an effort to analyze the evolution of the coagulation system, Jiang and Doolittle have compared the vertebrate coagulation. In an effort to analyze the evolution of the coagulation system, Jiang and Doolittle have compared the vertebrate coagulation. In an effort to analyze the evolution of the coagulation system, Jiang and Doolittle have compared

Together, the data presented herein and the results from previous studies indicate that FXII does not play an essential role in coagulation and fibrinolysis in vivo. We anticipate that FXII<sup>-/-</sup> mice as well as theircrossbreds with mice deficient in other contact factors will provide fresh insights into the biological role(s) of FXII and related proteins in health and disease.

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**References**