Fatal haemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V

Jisong Cui*, K. Sue O'Shea†, Anjali Purkayastha‡, Thomas L. Saunders§ & David Ginsburg*‡§

* Howard Hughes Medical Institute and § Departments of Human Genetics, ‡ Internal Medicine and † Anatomy and Cell Biology, University of Michigan Medical School, Ann Arbor, Michigan, USA

COAGULATION factor V is a critical cofactor for the activation of prothrombin to thrombin, the penultimate step in the generation of a fibrin blood clot[1,2]. Genetic deficiency of factor V results in a congenital bleeding disorder (parahaemophilia)[3], whereas inheritance of a mutation rendering factor V resistant to inactivation is an important risk factor for thrombosis[4,5]. We report here that approximately half of homozygous embryos deficient in factor V ($Fv^{-/-}$), which have been generated by gene targeting, die at embryonic day (E) 9–10, possibly as a result of an abnormality in the yolk-sac vasculature. The remaining $Fv^{-/-}$ mice progress normally to term, but die from massive haemorrhage within 2 hours of birth. Considered together with the milder phenotypes generally associated with deficiencies of other clotting factors[6,7], our findings demonstrate the primary role of the common coagulation pathway and the absolute requirement for functional factor V for prothrombinase activity. They also provide direct evidence for the existence of other critical haemostatic functions for thrombin in addition to fibrin clot formation, and identify a previously unrecognized role for the coagulation system in early mammalian development.

The gene-targeting strategy used to generate factor-V-deficient mice is shown in Fig. 1*a*. Southern blot screening of neomycin (*neo*)-resistant embryonic stem cell (ES) colonies indicated targeting efficiency of ~10%. Successful targeting results in replacement of factor V exons 8–11 by a *neo* cassette, introducing a frameshift mutation near the amino terminus of factor V. F_1 mice heterozygous for the targeted allele ($Fv^{+/-}$) were intercrossed to generate homozygous *Fv*-null progeny. Southern blot analysis confirmed the expected structure of the targeted *Fv* locus (Fig. 1*b*, *c*).

Factor-V-deficient animals were immediately evident at birth and generally died within 2 hours of massive intra-abdominal haemorrhage (Fig. 2). Cutaneous bleeding, particularly over the head, was occasionally evident, and scattered microscopic haemorrhages were detected in a variety of tissues. No other gross or microscopic abnormalities were identified. One of 60 homozygous-null pups survived until day 10 and a second pup died immediately following a tail biopsy at day 14. Blood present intra-abdominally was uniformly unclotted and was completely deficient in factor V activity (Fig. 1*d*). $Fv^{+/-}$ mice appeared to be entirely normal and haemostasis was normal after tail biopsy. Factor V activity in the plasma of $Fv^{+/-}$ mice was about half that in wild-type homozygotes (Fig. 1*d*).

The fatal neonatal haemorrhage in $Fv^{-/-}$ mice was unexpected, given the much milder phenotype associated with human factor V deficiency[3]. The severity of the defect in these animals also contrasts with the phenotype of afibrinogenaeimic humans[8] and fibrinogen-knockout mice[7]; both generally survive into adulthood. These results support the idea of further haemostatic functions for thrombin activation in addition to formation of the fibrin blood clot, probably related to the presumed role of thrombin in platelet activation[9,10]. They show that factor V is an essential component of the prothrombinase complex and that factor Xa has little or no prothrombinase activity *in vivo* in the absence of factor V, consistent with previous *in vitro* kinetic studies[11]. They also indicate that there is probably no significant alternative pathway for the generation of thrombin.

Although thrombin activity may be essential for platelet function in haemostasis, mice that completely lack platelets, as a result of targeted disruption of the haematopoietic specific transcription

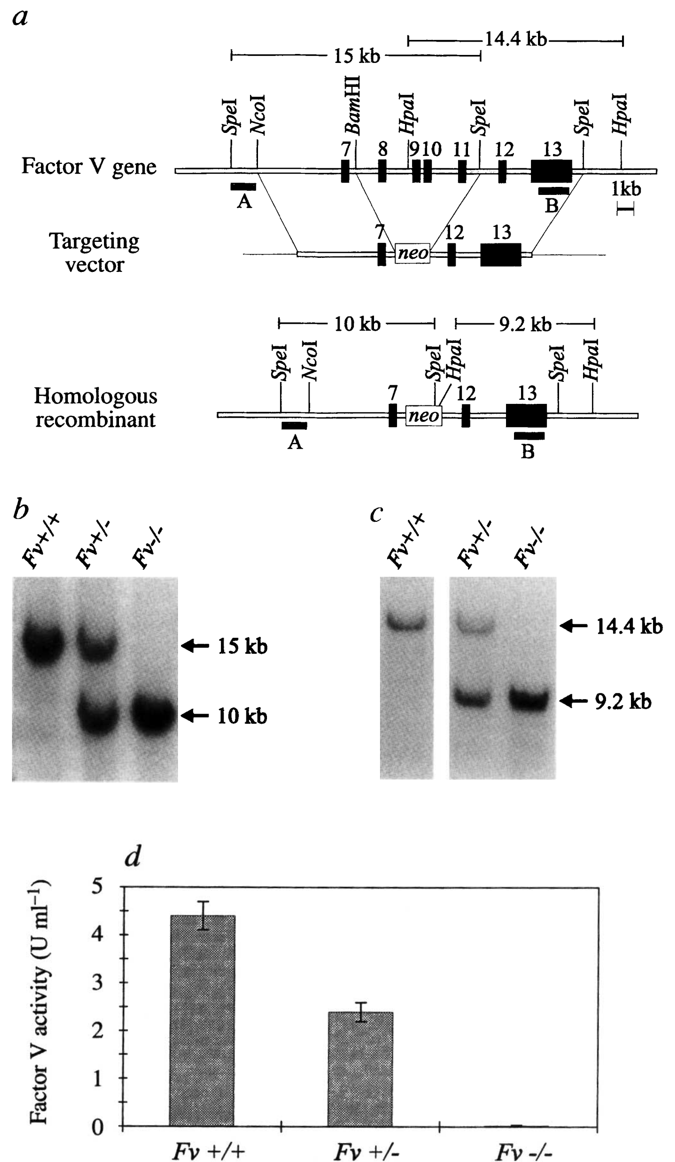

FIG. 1 Targeting of the *Fv* gene by homologous recombination. *a*, Structure of exons 7–13 of the murine *Fv* gene and targeting vector. The targeting vector contains a *neo* cassette, which replaces murine exons 8–11 flanked by ~6.6 kb homologous 5′ and 3′ arms. The *neo* cassette is driven by the *pgk* promoter[24]. The predicted product of successful homologous recombination is shown at the bottom. The location of hybridization probes A and B, used to detect successful targeting, are indicated. The mutant allele carries additional *Spe*I and *Hpa*I sites within the *neo* cassette. Southern blot analysis demonstrating the expected genomic structure at the 5′ end of the locus is shown in *b* and the 3′ end in *c*. Genomic DNA prepared from tail biopsies of $Fv^{+/+}$, $Fv^{+/-}$ and $Fv^{-/-}$ mice was analysed by restriction digestion with *Spe*I and hybridization with probe A (*b*), or by restriction digestion with *Hpa*I and hybridization with probe B (*c*). New genomic fragments of the expected size (10 and 9.2 kb) are seen in $Fv^{+/-}$ and $Fv^{-/-}$ mice and the normal allele (15 and 14.4 kb) is absent from $Fv^{-/-}$ mice. *d*, Plasma factor V functional activity in factor-V-deficient mice. Values are the mean ± s.d. of determinations in 5 ($Fv^{+/+}$ and $^{+/-}$) or 3 ($Fv^{-/-}$) animals. No factor V activity was detectable in the $Fv^{-/-}$ plasma, although $Fv^{+/-}$ plasma contained about half the activity of $Fv^{+/+}$ plasma.

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factor NFE-2 (ref. 12), also bleed less severely than observed here. The profound haemorrhage exhibited in $Fv^{-/-}$ mice probably results from the simultaneous interruption of both platelet activation and fibrin formation.

DNA analysis of ~300 progeny mice at term, derived from an intercross of $Fv^{+/-}$ mice, identified a highly significant decrease in the number of $Fv^{-/-}$ progeny, compared to the expected 25% frequency. Although the expected number of $Fv^{-/-}$ embryos were present at E10.5 and E9.5, a decrease was evident by E11.5, with highly significant differences at E15.5 and E18.5 (Table 1).

By E9.5, $Fv^{+/+}$ and $Fv^{+/-}$ embryos (and ~60% of $Fv^{-/-}$ embryos) had 20-25 somites, with a well developed heartbeat and yolk-sac circulation. However, 17/43 $Fv^{-/-}$ E9.5 embryos showed some degree of developmental delay, many having only 12–16 somites (Fig. 3). Anomalies of yolk-sac organization were also present in a number of null embryos at E9.5. In many null embryos, the yolk-sac circulation was sluggish and the yolk sac had a granular appearance, rather than the smooth surface typical of control embryos. On histological analysis, visceral yolk sacs from these embryos were strikingly abnormal (Fig. 3). By E9.5, the visceral yolk sac consists of an outer endodermal layer and inner mesodermal layer, with large vascular plexes containing haematopoietic stem cells, the blood islands. In 5/11 yolk sacs from null embryos examined histologically, the visceral endoderm appeared flattened, with few blood islands. The additional six null yolk sacs appeared to have slightly fewer haematopoietic precursors, but vascular plexes were present in the mesoderm.

Thus, complete deficiency of factor V results in an incomplete block to murine embryonic development, leading to loss of ~1/2 of $Fv^{-/-}$ embryos at E9.5–10.5, with the remaining animals continuing to develop to term. It is unclear why there is this dichotomy among animals with identical genotypes. The incomplete embryonic-lethal and perinatal haemorrhagic phenotypes were confirmed in animals derived from three independent ES clones (from two different established ES cell lines), excluding an unrelated second mutation or clone-specific effect. The pattern of lethality is not consistent with a genomic imprinting mechanism, and a potential sex-limited modification of the embryonic-lethal phenotype was also excluded by genotyping for the Y-chromosome-specific sequence *SRY*[13]. A contribution from genetic background differences among mouse strains, as reported for targeted deletion of the EGF receptor[14], was excluded by analysis of $Fv^{+/-}$ mice derived from four successive backcrosses to C57BL/6J or backcrosses onto a pure 129Sv background (Table 1).

Two groups have described the targeted deletion of the tissue-factor gene (*TF*) in mice: although both report an embryonic-lethal phenotype between E8.5 and 10.5, Bugge *et al.*[15] conclude that death of $TF^{-/-}$ embryos is secondary to early haemorrhage, whereas Carmeliet *et al.*[16] propose that there is a defect in early blood vessel development. Our results are more consistent with the latter model. Specifically, detailed histological analysis of over 40 $Fv^{-/-}$ E9.5 embryos identified only occasional microscopic haemorrhages, most commonly in the mesenchyme of the face, and were strikingly different from the massive haemorrhage described in $TF^{-/-}$ embryos by Bugge *et al.*[15].

A 50% loss at E9–10 occurs in mice homozygous for disruption of the thrombin receptor (*TR*) gene[17], remarkably similar to our observations of $Fv^{-/-}$ mice, despite the lack of any haeomostatic defect in fetal or adult $TR^{-/-}$ mice. Taken together, these results suggest that factor-V-dependent generation of thrombin and a subsequent signal through the thrombin receptor are both required at a critical step in early development, potentially related to yolk-sac vasculogenesis. Apparently, stochastic compensatory mechanisms permit this block to be overcome in approximately 1/2 of animals, with normal development proceeding past this

TABLE 1 Genotypes of progeny from $Fv^{+/-}$ intercrosses at varying stages

	Total	+/+	+/−	−/−	
Term pups	297	89 (30.0%)	176 (59.2%)	32 (10.8%)	$P < 1 \times 10^{-6}$
E18.5	109	37 (34%)	60 (55.0%)	12 (11.0%)	$P < 0.002$
E15.5	110	32 (29.1%)	65 (59.1%)	13 (11.8%)	$P < 0.01$
E11.5	72	20 (27.8%)	41 (56.9%)	11 (15.3%)	$P \sim 0.16$
E10.5	141	31 (22.0%)	76 (54.0%)	34 (24.0%)	$P > 0.5$
E9.5	137	36 (26.2%)	62 (45.3%)	39 (28.5%)	$P > 0.5$
C57BL6/J background					
Term pups	117	36 (30.8%)	69 (59.0%)	12 (10.3%)	$P < 0.002$
E18.5	50	20 (40.0%)	23 (46.0%)	7 (14.0%)	$P < 0.03$

About 1/2 of the $Fv^{-/-}$ embryos are lost after E10.5, with the remaining half surviving to term. 'C57BL6/J background' indicates the results of an intercross between $Fv^{+/-}$ mice derived from four successive backcrosses to C57BL6/J. In addition, the original chimaeric founders were backcrossed to 129Sv to produce $Fv^{+/-}$ mice on a pure 129Sv background. Analysis of an intercross between these 129Sv $Fv^{+/-}$ mice identified only 3/26 $Fv^{-/-}$ progeny at E18.5, consistent with the patterns observed on the C57BL6/J background, as well as the original intercross. To detect a potential sex-limited modification of the embryonic-lethal phenotype, 15 $Fv^{-/-}$ term pups were typed by PCR for the Y-chromosome-specific sequence *Sry*[13]. Eight pups were identified as male and seven as female, excluding a modifying effect of the sex chromosomes as the explanation for these unusual survival data. Statistical significance was calculated using the χ^2 test to compare the observed and expected frequencies.

FIG. 2 Newborn progeny of $Fv^{+/-}$ intercross. The litter depicted here contained a single homozygote $Fv^{-/-}$ (middle animal in the lower row). Genotypes of the remaining six mice were two $Fv^{+/+}$ and four $Fv^{+/-}$. the single homozygote-null animal has suffered massive intra-abdominal haemorrhage and is a pale colour over the upper body because of blood loss.

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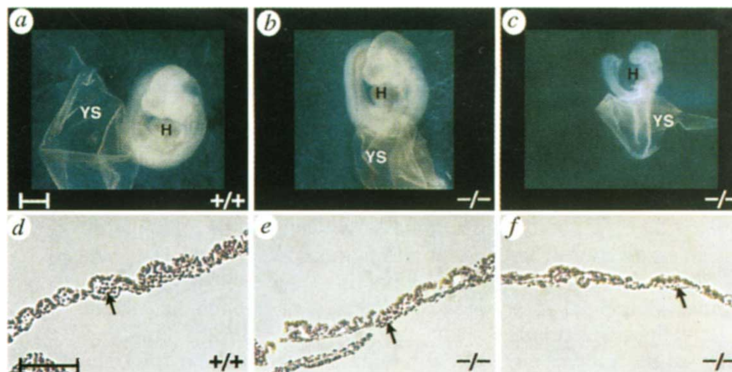

FIG. 3 Embryos isolated at E9.5. The wild-type (+/+) embryo shown in *a* and the null (−/−) embryo in *b* have developed to the 20–24-somite stage, whereas the null embryo in *c* is developmentally delayed. The most common anomalies in these embryos were focal haemorrhages (14/43 null embryos), anomalous positioning of the first branchial arch, as well as cardiac and pericardial anomalies. Both developmentally delayed and 20–25-somite-stage null embryos were characterized by anomalies of the 'turning' process, in which the early murine embryo reverses its inverted U shape to adopt the dorsal curvature (C shape) typical of the day-10 embryo. Defects of axial rotations were observed in 23/43 null embryos and positional defects were also seen in 12/108 embryos identified as heterozygous (+/−) or wild-type (+/+). When examined histologically, $Fv^{-/-}$ embryos had defective development of cardiac muscle: specifically trabeculation of the myocardium was delayed or absent. Small haemorrhages had occurred in the mesenchyme, particularly in the cephalic region. In these embryos, the mesenchyme was often abnormally condensed, and it was common to observe a lack of development of the posterior region. Histological section of a wild-type visceral yolk sac (*d*) illustrates the normal appearance of outer endodermal layer and blood islands containing haematopoietic stem cells (arrow) within the extraembryonic mesoderm. A similar pattern is seen in yolk sacs isolated from the subset of null embryos that had developed to the 20–24-somite stage (*e*). In contrast, vascular channels (arrow) in yolk sacs from a population (5/11) of null embryos appeared to have collapsed (*f*). Scale bars: 500 μm in *a–c*, and 100 μm in *d–f*. H, heart; YS, yolk sac.

bottleneck. The previous demonstration of widespread thrombin receptor messenger RNA expression in mesenchymal cell populations at E9.5 (ref. 18) is consistent with this model. The well known teratogenic effect of warfarin, an inhibitor of several coagulation factors, including factor Xa and thrombin[19], may perhaps occur through a related mechanism[20,21]. Although the thrombin receptor is abundantly expressed in the early embryo, prothrombin gene expression has not been detected in the mouse embryo before E12.5 (ref. 18), suggesting that the target for factor V in early development may be maternally derived prothrombin, very low levels of fetal prothrombin, or another as-yet unknown protease.

Other coagulation-factor deficiencies in mouse and humans, including those of factors VIII and IX (found in haemophilias A and B), and afibrinogenaemia, are not associated with death of the embryo[6–8,22]. In addition, the moderately severe bleeding phenotype of human factor V deficiency (parahaemophilia)[3] sharply contrasts with the dramatic early-lethal phenotype we find in mice lacking factor V. Based on these observations, we suggest that complete factor V deficiency in humans may also be an early-embryonic-lethal condition. Consistent with this, nearly all reported cases of human factor V deficiency are associated with some degree of residual factor V activity and no *FV* gene deletions or null mutations have been identified in patients. These observations also suggest that complete deficiency of other components of the common coagulation pathway, such as factor X or thrombin, may likewise lead to lethal neonatal phenotypes. □

Methods

Factor V gene targeting. λ phage clones spanning ~30 kb of mouse *Fv* genomic sequences were previously isolated from a 129Sv genomic library (Stratagene) and the structure of exons 7–12 characterized (J.C. *et al.*, manuscript in preparation). The targeting vector was constructed by assembling a 6.7-kb *Nco*I–*Bam*HI 5′ *Fv* genomic fragment, a 1.4-kb *Bam*HI–*Xba*I fragment containing a neomycin (*neo*) expression cassette driven by the *pgk* promoter, and a 6.6-kb *Spe*I–*Spe*I 3′ fragment of the *Fv* gene, all cloned into the plasmid vector pSL301 (Invitrogen). The targeting vector was linearized with *Sfi*I, introduced into the 129Sv-derived D3 (from T. Doetschman) and CJ7 (ref. 23) ES cell lines by electroporation, and stable transfectants selected as described[24]. Individual ES clones were screened for homologous targeting by Southern blot analysis using probes A and B (Fig. 1). Ten of 100 *neo*-resistant ES clones carried the expected allele, for a targeting efficiency of 10%. By Southern blotting, each targeted clone contained only a single *neo* gene insertion at the expected site (data not shown). Eight independent ES clones (7 from D3 and 1 from CJ7) were injected into C57BL/6J blastocysts as described[24] and the resulting chimaeric males were bred to C57BL/6J females to generate F_1 $Fv^{+/-}$ offspring.

Determination of factor V clotting activity. Whole blood from anaesthetized $Fv^{+/+}$ and $Fv^{+/-}$ mice was collected into sodium citrate (0.0129 M final) by cardiac puncture at 6 weeks of age. $Fv^{-/-}$ blood was collected directly from the abdominal cavity of newborn $Fv^{-/-}$ mice. Clotting was assayed as described (J. Cui *et al.*, manuscript in preparation) using factor-V-deficient human plasma. Units of factor V activity are defined relative to human factor V.

Genotyping by PCR. Genotypes of mice were established using DNA prepared from tail biopsies analysed by either Southern blotting as already described or by polymerase chain reaction (PCR). In the PCR assay, the wild-type and targeted *Fv* alleles were distinguished using primers specific for exon 10 of the murine *Fv* gene (5′ACGATCAGACCAGTTCAACC3′ and 5′CCTTGTAACGTCCACATCAC3′) and the *neo* gene (5′GGACTGGCTGCTATTGGGCGAAGTG3′ and 5′GAAGAACTCGTCAAGAAGGCGATAGAAGG3′).

Histological analysis. Embryos were photographed at autopsy, fixed in 1% glutaraldehyde in 0.1 M phosphate buffer for 1 h at room temperature, embedded in paraffin and sectioned at 6 μm (ref. 25). Sections were stained with haematoxylin and eosin and examined and photographed using a Leitz Orthoplan photomicroscope. Portions of yolk sacs or of embryos were removed for PCR genotyping.

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CORRESPONDENCE and requests for materials should be addressed to D.G. (e-mail: ginsburg@umich.edu).

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