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Incomplete embryonic lethality and fatal neonatal hemorrhage caused by prothrombin deficiency in mice

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ABSTRACT **Deficiency of blood coagulation factor V or tissue factor causes the death of mouse embryos by 10.5 days of gestation, suggesting that part of the blood coagulation system is necessary for development. This function is proposed to require either generation of the serine protease thrombin and cell signaling through protease-activated receptors or an activity of tissue factor that is distinct from blood clotting. We find that murine deficiency of prothrombin clotting factor 2 (Cf2) was associated with the death of approximately 50% of $Cf2^{-/-}$ embryos by embryonic day 10.5 (E10.5), and surviving embryos had characteristic defects in yolk sac vasculature. Most of the remaining $Cf2^{-/-}$ embryos died by E15.5, but those surviving to E18.5 appeared normal. The rare $Cf2^{-/-}$ neonates died of hemorrhage on the first postnatal day. These studies suggest that a part of the blood coagulation system is adapted to perform a developmental function. Other mouse models show that the absence of platelets or of fibrinogen does not cause fetal wastage. Therefore, the role of thrombin in development may be independent of its effects on blood coagulation and instead may involve signal transduction on cells other than platelets.**

Prothrombin clotting factor 2 (Cf2) is a 72 kDa serine protease zymogen that is made primarily in the liver and secreted into the blood (1). Prothrombin is cleaved and activated during blood coagulation to generate thrombin, a 34 kDa protease with multiple biological activities. Thrombin is required to cleave fibrinogen, and it participates in the feedback regulation of several procoagulant and anticoagulant hemostatic enzyme pathways. Thrombin cleaves and activates G protein-coupled protease-activated receptors (PARs) on platelets, inducing the shape change and secretory reactions that enable platelets to perform their hemostatic function (2). Similar PARs are widely expressed on many cell types, enabling thrombin to induce the secretion of other proteases, adhesive glycoproteins, cytokines, growth factors, prostacyclin, and nitric oxide. In addition, thrombin is chemotactic for macrophages and stimulates mitosis in a variety of mesenchyme-derived cells, including smooth muscle cells and fibroblasts (2).

These diverse cellular effects suggest that thrombin may have important functions besides its role in hemostasis. In particular, thrombin is proposed to participate in inflammation and tissue repair (2), and to regulate the differentiation and survival of both neurons and glia. Such a role for thrombin is consistent with the reported codistribution of mRNA for prothrombin and protease activated receptor-1 (PAR1) in the brain of weanling rats (3). Thrombin at picomolar concentrations induces astrocytes to retract stellate processes and divide (4). Thrombin causes the retraction of neurites in cultured neuronal cells, and this can be reversed by thrombin inhibitors (5, 6). Thrombin inhibition also prevents electrical activity-dependent synapse reduction at neuromuscular junctions (7, 8) and appears to rescue motorneurons from normal developmental or axotomy-induced death *in vivo* (9). These findings suggest that thrombin may be involved in development of the nervous system.

The participation of thrombin-mediated signal transduction in development is supported indirectly by the phenotype caused by the absence of several hemostatic proteins. For example, death *in utero* at embryonic day (E)8.5–E10.5 occurs in mice with targeted inactivation of the genes for FV (10) or tissue factor (TF) (11–13). However, these deficiencies of upstream blood coagulation factors may allow continuing thrombin generation by alternative pathways. Inactivation of the PAR1 gene also causes embryonic death by E10.5 (14), but deletion of this thrombin target does not prevent all thrombin-dependent cell signaling because at least one other thrombin receptor (TR), PAR3, is expressed in mice and humans (15).

A genetic model of prothrombin deficiency would facilitate these studies, but no naturally occurring animal example has been reported. There are humans with autosomal recessive moderate hypoprothrombinemia, but no known cases of total deficiency (16). Therefore, to directly address the biological and pathological functions of prothrombin, mice with prothrombin gene deletions were constructed. The resultant $Cf2^{-/-}$ mice have a severe hemorrhagic disorder that is fatal in the neonatal period and possibly during late gestation. In addition, approximately one-half of the embryos die before E10.5, suggesting an important role for prothrombin in early embryogenesis. Similar results have been obtained by Sun and colleagues (17).

MATERIALS AND METHODS

Cloning of Mouse Prothrombin Genomic DNA. Two fragments of the mouse prothrombin gene were cloned by PCR amplification and used as probes to isolate clones from a mouse genomic library. Oligonucleotide primers were based on the mouse prothrombin cDNA sequence (18): P1 (nt 28–53), GTC CGC GGC CTG GGC CTC CCT GGC TG; P4 (nt 214–237 antisense), CTC AAA GGC CTC CTC ATA GCT GCA; P5 (nt 1159–1182), AAG AGT CCC CAA GAG CTG CTG TGT; and P8 (nt 1453–1476 antisense), TAC TGT CTG CTT GTC TGG CAA ACA. PCR reactions with mouse tail

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Abbreviations: FV, blood clotting factor V; FVII, factor VII; TF, tissue factor; Cf2, prothrombin (clotting factor II); PAR, protease-activated receptor; TR, thrombin receptor (PAR1); E, embryonic day.

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genomic DNA template were performed with primer pairs P1 and P4 (500 bp product) or P5 and P8 (700 bp product). PCR conditions were: initial denaturation for 5 min at 95°C; 30 cycles of denaturation for 1 min at 94°C, annealing for 2 min at 58°C, extension for 2 min at 72°C; final extension for 5 min at 72°C. The PCR products were labeled with [α-^{32}P]dCTP by a random primer method (19) and used to screen a bacteriophage λ mouse genomic DNA library prepared from strain 129/Sv in the λFIX II vector (Stratagene) by standard methods. Clone MT1 contained a 13 kb DNA fragment with prothrombin exons 1–12, as demonstrated by restriction mapping, oligonucleotide hybridization, and partial DNA sequencing.

Construction of a Gene Targeting Vector and Derivation of Mutant Mice. A 12.5 kb *Bam*HI–*Sal*I fragment of the mouse prothrombin gene (Cf2$^{-/-}$) was cloned into plasmid pNTK (20) placing the *Bam*HI site adjacent to the *pgk-tk* cassette of the vector, and the central 3.3 kb *Hin*dIII fragment (containing exons 7–12) was replaced with a *pgk-neo* cassette (Fig. 1*a*). Transfected E14 embryonic stem cells were selected in G418 and gancyclovir, and characterized for correct homologous recombination by PCR analysis and Southern blotting. Cells

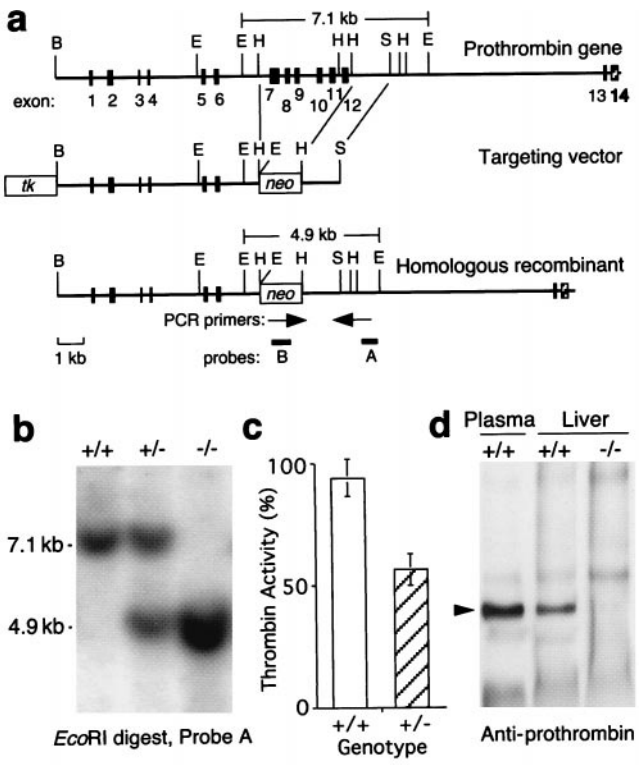

FIG. 1. Targeting of the prothrombin gene by homologous recombination. (*a*) Structure of mouse prothrombin gene and targeting vector. The cloned genomic DNA fragment extended from the first *Bam*HI site to the last shown *Eco*RI site. Solid rectangles represent exons 1–12. Additional exons 13 and 14 (hatched rectangles) are indicated based on the structure of the human prothrombin gene. Cleavage sites are shown for *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), and *Sal*I (S). The targeting vector contains a *pgk-neo* cassette in place of exons 7–12 and a *pgk-tk* cassette. The product of homologous recombination is shown at the bottom. Positions of PCR primers (arrows) and hybridization probes (A and B) used to detect successful gene targeting are indicated. The *Eco*RI digest will generate a 7.1 kb fragment from the untargeted allele and a 4.9 kb fragment from the targeted allele, both of which are recognized by probe A. (*b*) Southern blot of *Eco*RI-digested tail DNA from Cf2$^{+/+}$, Cf2$^{+/-}$, and Cf2$^{-/-}$ littermates hybridized with probe A. (*c*) Prothrombin levels in mouse plasma sampled from 10 mice of each genotype (mean ± SD). (*d*) Western blot of prothrombin in mouse liver extracts. Arrowhead indicates the position of 72 kDa prothrombin.

from a correctly targeted embryonic stem clone were microinjected into blastocysts from C57BL/6J female mice, which were then implanted into pseudo-pregnant ICR mice. A chimeric male transmitted the mutant allele to ≈37% of his progeny, as determined by PCR analysis. Positives identified by PCR were confirmed by Southern blotting of tail DNA (Fig. 1) with probe A (prothrombin intron 12) and probe B (neo). Further backcrosses were always made into the C57BL/6J background.

Purification of Mouse Prothrombin and Antibody Preparation. Mouse prothrombin was purified by the procedure of Mann (21) from 30 ml of citrated mouse plasma (Pel-Freez Biologicals). Active fractions that contained a single band of prothrombin, as shown by SDS/PAGE, were pooled, dialyzed against 25 mM ammonium bicarbonate, and lyophilized. The yield was 1.4 mg prothrombin. Polyclonal antibodies were prepared in rabbits by standard methods (TANA Laboratories, Houston).

Immunoprecipitation and Western Blotting. Rabbit polyclonal anti-mouse prothrombin antibody was bound to a recombinant protein A-Sepharose 4B conjugate (Zymed) by mixing 100 μl of a 50% bead suspension with 4 μl rabbit serum and 400 μl PBS containing 0.05% Tween-20 (PBS–Tween). After 2 hr incubation, the beads were washed and resuspended to 500 μl in PBS–Tween. For each genotype, two livers (postnatal day 1) were homogenized in 200 μl RIPA buffer (20 mM Tris-HCl, pH 7.4/1% Triton/0.1% SDS). After centrifugation, the supernatant was mixed with 100 μl of the protein A-Sepharose 4B-anti-prothrombin antibody suspension and incubated overnight at 4°C. The beads were washed three times with PBS–Tween and immune complexes were eluted with sample buffer for 10% SDS/PAGE. After electroblotting onto nitrocellulose membrane, prothrombin was detected with anti-mouse prothrombin antibody (diluted 1:1,000) and enhanced chemiluminescence reagents (Amersham).

Histological Analyses. Embryos and yolk sacs were fixed in 10% formalin/phosphate buffer overnight at 4°C, dehydrated, embedded in paraffin, and sectioned. Sections (5 μm) were stained with hematoxylin/eosin for microscopy. Portions of yolk sacs or embryos were removed for genotyping by PCR or Southern blotting.

Measurement of Plasma Prothrombin. Mouse blood samples were collected into EDTA microcapillaries from the retroorbital plexus. Plasma was separated by centrifugation for 10 min at 2,500 × *g* and stored at −70°C. Prothrombin concentration was determined by a modified prothrombin time assay. Solutions were preincubated at 37°C. Fifty microliters of rabbit brain cephalin (Sigma), 50 μl of barium-absorbed human plasma (provided by George Broze, Washington University), and 50 μl of diluted standard mouse plasma or test sample were mixed rapidly with 100 μl of 20 mM Hepes-Na (pH 7.4), 0.1 M NaCl, 10 ng/ml human factor Xa, and 12.5 mM $CaCl_2$. Clotting times were measured by using a fibrometer (Becton Dickinson). Clotting times were expressed in terms of the percent of activity in normal mouse plasma (Pel-Freeze Biologicals). Approximately 1.2 μl of normal mouse plasma gave a clotting time of 37 sec.

RESULTS

Construction of Prothrombin Gene Targeting Vector and Derivation of Mutant Mice. A partial mouse prothrombin cDNA probe was used to screen a 129/Sv strain mouse genomic DNA library, and a hybridizing clone was identified containing a 13 kb insert. Exons 1–12 of the mouse prothrombin gene are contained within this fragment, and the gene structure was found to resemble that of human prothrombin (22). The prothrombin (Cf2) gene was inactivated by replacing exons 7–12 with the *neo* gene (Fig. 1 *a* and *b*), thereby deleting kringle 2, the prothrombin activation cleavage site and the

FIG. 2. Newborn mice. Both wild-type (*Lower*) and homozygous prothrombin deficient mice (*Upper*) were able to breath and suckle. Compared with wild type, the $Cf2^{-/-}$ mouse is pale, with extensive bruising over the swollen abdomen and head. Dissection of the $Cf2^{-/-}$ mouse showed massive intraperitoneal hemorrhage with no clotted blood.

active site His and Asp residues. When hybridized with probe B derived from the inserted *neo* gene, mice bearing the mutant allele exhibited only the 4.9 kb band, consistent with a single site of vector integration (data not shown).

Crossing of $Cf2^{+/-}$ and $Cf2^{+/+}$ mice yielded 57 heterozygous and 64 wild-type offspring, indicating that heterozygous prothrombin deficiency is compatible with normal development. Heterozygous mice appeared to have normal viability and fertility, and no excessive bleeding occurred after tail biopsy. The plasma level of functional prothrombin in heterozygous mice (56.4 ± 6.4% SD) was approximately one-half that of wild-type mice (93.8 ± 7.6% SD) (Fig. 1*c*).

Spontaneous Fatal Hemorrhage in Prothrombin-Deficient Mice. Crossing of $Cf2^{+/-}$ mice yielded 620 progeny of which 221 (36%) were $Cf2^{+/+}$, 378 (61%) were $Cf2^{+/-}$, and only 21 (3%) were observed to be $Cf2^{-/-}$ ($P < 0.001$, χ^2 test). The absence of prothrombin in $Cf2^{-/-}$ mice was confirmed by immunoprecipitation and Western blotting of liver extracts from newborn mice (Fig. 1*d*). A 72 kDa band corresponding to prothrombin was detected only in wild-type and heterozygous mice. The rare homozygous $Cf2^{-/-}$ neonatal mice were extremely pale and died during the first day after delivery (Fig. 2). Dissection showed massive intraperitoneal hemorrhage with no clottable blood, consistent with the absence of prothrombin. Hemorrhage into skin, especially over the head, was common. Intracranial bleeding was observed occasionally. Despite their severe bleeding, $Cf2^{-/-}$ mice often had undigested milk in their stomach, indicating they could breathe and suckle. No developmental abnormalities were identified grossly or by routine histologic examination of neonatal $Cf2^{-/-}$ mice.

Table 1. Progeny from the mating of $Cf2^{+/-}$ mice

Day of gestation	Total embryos	Prothrombin genotype		
		+/+	+/−	−/−
8.5	60	14	38	8 (13%)
9.5	129	24	88	17 (13%)
10.5	51	12	38	1 (2%)
11.5	51	10	34	7 (14%)
12.5	100	31	52	17 (17%)
13.5	44	8	31	5 (11%)
14.5	33	9	20	4 (12%)
15.5	74	24	49	1 (1.4%)
16.5	68	16	49	3 (4.4%)
18.5	40	12	26	2 (5%)
Total	650	160	425	65 (10%)

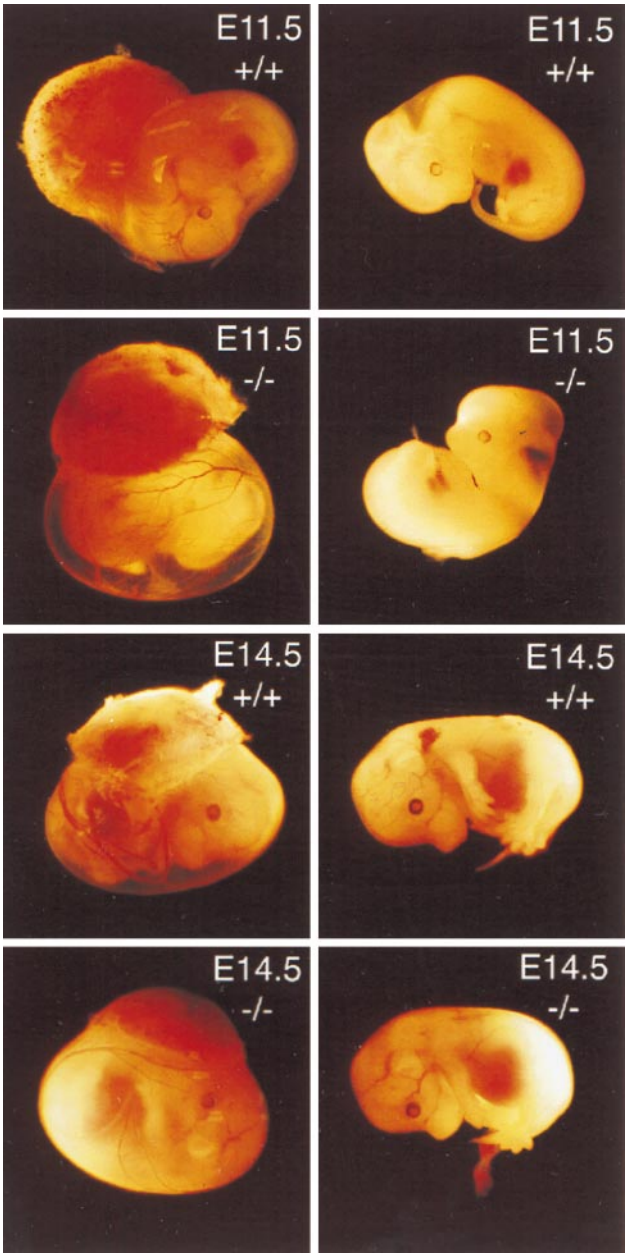

FIG. 3. Prothrombin-deficient embryos. Embryos from a single pregnancy at either E11.5 or E14.5 are shown with their placentas and extraembryonic membranes (*Left*) and without (dissected) (*Right*). For all genotypes, the yolk sac vasculature and embryos appear grossly normal.

Prothrombin Deficiency Causes an Incomplete Block in Embryonic Development. The low yield of $Cf2^{-/-}$ neonatal mice suggested that prothrombin deficiency is lethal during gestation. To determine when $Cf2^{-/-}$ embryos were lost, $Cf2^{+/-} \times Cf2^{+/-}$ matings were analyzed on specific days after conception (Table 1). Two distinct times of fetal death were identified. Approximately one-half of the null embryos were missing by E9.5–E10.5 ($P < 0.01$ at E9.5, $P < 0.001$ at E10.5, χ^2 test). The remainder survived until E14.5, but almost all died by E18.5 ($P < 0.001$ for E9.5–E14.5 versus E15.5–E18.5, χ^2 test). Determination of fetal sex by PCR of Y chromosome-specific *Zfy-1* sequences (23) indicated that there was no

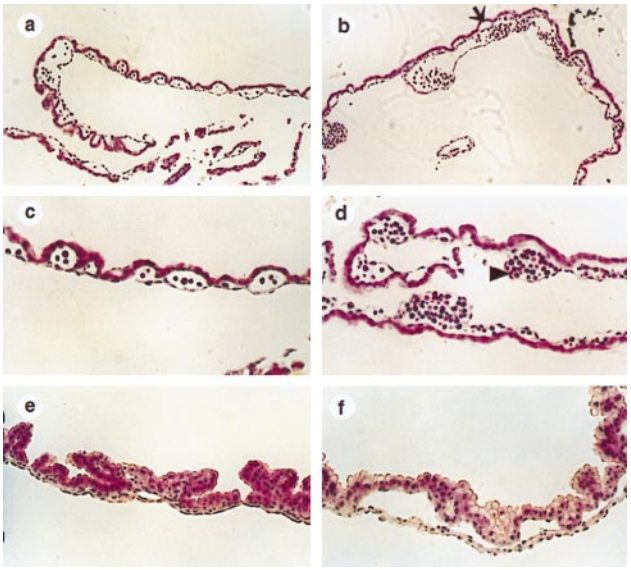

FIG. 4. Histological analysis of the yolk sac. Sections are shown for normal $Cf2^{+/+}$ embryos (*a*, *c*, and *e*) and null $Cf2^{-/-}$ embryos (*b*, *d*, and *f*). At E9.5 (*a–d*), 100× magnification of a wild-type yolk sac (*a*) shows a normal outer layer of visceral endoderm and inner layer of small capillaries surrounded by endothelium, whereas the prothrombin-deficient yolk sac (*b*) shows greatly dilated, enlarged vascular spaces. In some places the visceral endoderm and mesothelial layers appear to be separated (arrow). Under higher (200×) magnification, the normal small yolk sac capillaries (*c*) contain erythrocytes and hematopoietic cells; the yolk sac of the $Cf2^{-/-}$ embryo has normal appearing blood islands (triangle) adjacent to dilated vascular spaces. The difference in appearance of yolk sac capillaries is evident also at E11.5 (200× magnification (*e* and *f*). Capillary channels in the normal yolk sac (*e*) are small, whereas those in $Cf2^{-/-}$ yolk sac are greatly enlarged (*f*).

preferential survival of $Cf2^{-/-}$ males or females at any time of gestation. The fraction of $Cf2^{-/-}$ embryos at E15.5–E18.5 (6/182, 3.3%) was similar to the observed fraction of $Cf2^{-/-}$ neonates (21/620, 3%).

At each stage of gestation the remaining $Cf2^{-/-}$ embryos usually appeared to be grossly normal (Fig. 3) and microscopic examination did not show tissue hemorrhage or bleeding into yolk sac. At E11.5, embryos had 32–39 somites regardless of genotype, suggesting that prothrombin deficiency does not delay development. However, the yolk sacs of normal appearing $Cf2^{-/-}$ embryos showed enlargement of the capillary vessels and flattening of the visceral endoderm (Fig. 4). At E9.5, the visceral yolk sac has a prominent outer endodermal layer and a thinner mesodermal layer. The mesodermal layer includes capillaries and larger vascular spaces that contain hematopoietic cells, referred to as blood islands. In $Cf2^{-/-}$ yolk sacs, the capillaries appeared to fuse into a venous plexus and thin, fibrous connections were seen between the separated mesothelial and endodermal layers in some sections. Blood islands were present and the enlarged capillaries were filled with blood cells. This appearance persisted through E11.5 (Fig. 4), suggesting a sustained defect in the formation or integrity of the yolk sac vasculature.

Prothrombin-deficient embryos at later stages (E14.5) had grossly normal placentas and extraembryonic membranes (Fig. 3), and examination of serial sections throughout the embryos showed no abnormalities in the appearance of any organ system (data not shown). In particular, the skeleton, central nervous system, heart, liver, lungs, and kidneys appeared normal.

In addition to incomplete embryonic lethality by E10.5, prothrombin deficiency appeared to cause the death of almost all remaining $Cf2^{-/-}$ embryos late in gestation, between E14.5 and delivery (Table 1). Because prothrombin deficiency causes neonatal death from hemorrhage, bleeding may explain these late embryonic deaths as well. However, $Cf2^{-/-}$ embryos with hemorrhage have not yet been identified during the interval between E14.5 and birth.

DISCUSSION

As expected from the characterization of many blood coagulation factor deficiencies in humans and other animals, prothrombin deficiency in mice causes severe bleeding that is fatal in the neonatal period. A more remarkable finding is that prothrombin deficiency causes the death of approximately 50% of embryos by E10.5 (Table 1). Sun and colleagues (17) have prepared prothrombin-deficient mice and have obtained similar results. The differences between these studies concern mainly the incidence of bleeding and of vascular abnormalities *in utero*. Sun and colleagues observed bleeding without vascular changes in 9 of 18 embryos at E10.5 (17), whereas we found no bleeding in the yolk sac of 26 embryos from E8.5 to E10.5 and identified changes in yolk sac vasculature. These points have not yet been explained, but may be due either to mouse strain differences or technical considerations. However, the major conclusions of these studies are congruent: approximately 50% of $Cf2^{-/-}$ embryos die by E10.5 and the remaining embryos survive until late in gestation.

Incomplete embryonic lethality also is observed for deficiencies of other proteins that are closely linked to prothrombin activation during blood coagulation or to thrombin-induced cell signaling, particularly FV (10), TF (11–13), and PAR1 (14). FV is a cofactor that interacts with the blood clotting protease factor Xa, thereby accelerating the activation of prothrombin several thousand-fold. Targeted inactivation of FV in mice results in the death of about one-half of $FV^{-/-}$ embryos by E10.5, associated with gross and microscopic abnormalities of yolk sac vessels (10). TF is a membrane cofactor that binds the blood clotting protease factor VIIa, initiating the major physiological pathway of thrombin generation *in vivo*. Deficiency of TF results in the death of almost all mouse embryos during the interval of E8.5–E10.5 (11–13), and the yolk sac vessels of $TF^{-/-}$ embryos exhibit abnormalities like those observed for our $Cf2^{-/-}$ embryos (Fig. 4) with enlarged capillaries that form a disordered vascular plexus (11). These similarities suggest that the fetal loss associated with deficiency of TF, FV, or prothrombin is because of a common mechanism involving the inadequate generation of active thrombin.

Unexpectedly, deficiency of factor VII (FVII), the only known TF ligand, was found not to cause death *in utero*, suggesting that the fetal death in TF deficiency may not be caused by defective thrombin generation, but instead may reflect a function of TF in cell signaling, adhesion, or chemotaxis (24). However, transplacental passage of exogenous FVII in $FVII^{-/-}$ embryos at E11.5 was shown to achieve fetal plasma FVII levels that were 0.06% of the maternal level, and in $FVII^{\pm}$ embryos the level of endogenous FVII at E11.5 was only 0.1% of adult levels (24). Therefore, rescue by maternal FVII still may account for normal embryogenesis in FVII deficiency. If so, the function of TF during development may depend on its ability to promote thrombin generation.

The phenotype of PAR1 deficiency (14) suggests that the embryonic lethality observed for prothrombin deficiency is not caused by defective blood coagulation. PAR1, also referred to as the thrombin receptor (tr), is a G protein-coupled receptor that is proteolytically activated by thrombin. Approximately 50% of $tr^{-/-}$ embryos die at E9–E10, a percentage similar to that observed for prothrombin deficiency. The remaining embryos show transient growth retardation, but recover to normal size by the end of gestation. In contrast to prothrombin deficiency, they do not die as neonates but survive and

reproduce normally and they have no demonstrable hemostatic defect (14). This result appears to be explained by the expression of a distinct thrombin receptor, PAR3, on mouse platelets (15). Fibroblasts of $tr^{-/-}$ mice, however, express no PAR3 and are unresponsive to thrombin (14). The embryonic phenotype in PAR1 deficiency therefore may be caused by the failure of thrombin-dependent signaling in cells other than platelets. Disruption of the prothrombin gene should prevent thrombin-dependent signaling through all protease-activated receptors. Because the phenotype of prothrombin deficiency is similar to that caused by disruption of PAR1, signaling mediated by other thrombin-activated receptors such as PAR3 may be relatively unimportant during embryogenesis.

Dissociation of embryonic lethality from hemorrhage also is seen in deficiencies of other hemostatic proteins, but with a reversed pattern. The absence of factor VIII or factor IX in mice (or humans with hemophilia A or B, respectively) causes bleeding, but not fetal loss (25, 26). This is consistent with a potential role for thrombin in embryonic life, because thrombin generation can proceed independent of factors VIII and IX through reactions initiated by TF. Fibrinogen deficiency also predisposes to hemorrhage but does not cause fetal wastage in mice (27), even though no blood clotting can occur. The absence of platelets, produced by deficiency of transcription factor NF-E2, causes fatal neonatal hemorrhage but not embryonic death (28). Thus, conditions in which thrombin cannot act on either of its major hemostatic targets, fibrinogen or platelets, do not cause death *in utero*, suggesting that any developmental role of prothrombin is independent of its hemostatic function.

The timing of PAR1 expression is consistent with its apparent role in embryogenesis, because PAR1 mRNA is abundant by E9.5 in many mesenchymal tissues, especially blood vessels (29). Several tissues are potential sources of functional prothrombin during the critical interval around E9.5. In particular, Sun and colleagues have detected prothrombin mRNA in yolk sac by *in situ* hybridization as early as E9.5 (17) and it is expressed in liver by E12.5 (29). Prothrombin expression has been reported in other adult tissues, including the central nervous system of the rat (3). Because prothrombin is a zymogen, action of thrombin at E9.5 also may depend on the extrahepatic expression of proteins that mediate prothrombin activation, such as FV, and possibly factor X and TF. Although maternal blood coagulation factors do not cross the placenta efficiently (30, 31), and prothrombin levels of $Cf2^{-/-}$ embryos are extremely low at E18.5 (17), the possibility has not been excluded that some deficient embryos could be rescued, at least partly, by transplacental passage of soluble proteins. If so, this process could contribute to the apparently stochastic death by E10.5 of only one-half of the embryos deficient in prothrombin or FV.

Deficiency of TF, FV, or prothrombin causes the death of mouse embryos by E10.5, and these deficiencies converge on a common pathway for thrombin generation. Defects in blood clotting and platelet activation do not account for this embryonic function because the absence of thrombin's major hemostatic targets (fibrinogen or platelets) does not cause fetal death. Furthermore, deficiency of one of the two known thrombin-activated receptors, PAR1, causes a similar embryonic phenotype without affecting hemostasis. These studies suggest that a portion of the blood coagulation system is adapted to a developmental purpose related to the growth or stability of yolk sac vasculature, and this activity is dependent on cell signaling through one or more thrombin-activated G protein-coupled receptors.

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1. Mann, K. G. (1994) in *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, eds. Colman, R. W., Hirsch, J., Marder, V. J. & Salzman, E. W. (Lippincott, Philadelphia), pp. 184–199.
2. Coughlin, S. R., Vu, T. K., Hung, D. T. & Wheaton, V. I. (1992) *J. Clin. Invest.* **89,** 351–355.
3. Weinstein, J. R., Gold, S. J., Cunningham, D. D. & Gall, C. M. (1995) *J. Neurosci.* **15,** 2906–2919.
4. Cavanaugh, K. P., Gurwitz, D., Cunningham, D. D. & Bradshaw, R. A. (1990) *J. Neurochem.* **54,** 1735–1743.
5. Gurwitz, D. & Cunningham, D. D. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3440–3444.
6. Farmer, L., Sommer, J. & Monard, D. (1990) *Dev. Neurosci.* **12,** 73–80.
7. Liu, Y., Fields, R. D., Festoff, B. W. & Nelson, P. G. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 10300–10304.
8. Zoubine, M. N., Ma, J. Y., Smirnova, I. V., Citron, B. A. & Festoff, B. W. (1996) *Dev. Biol.* **179,** 447–457.
9. Houenou, L. J., Turner, P. L., Li, L., Oppenheim, R. W. & Festoff, B. W. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 895–859.
10. Cui, J., O'Shea, K. S., Purkayastha, A., Saunders, T. L. & Ginsburg, D. (1996) *Nature (London)* **384,** 66–68.
11. Carmeliet, P., Mackman, N., Moons, L., Luther, T., Gressens, P., Van Vlaenderen, I., Demunck, H., Kasper, M., Breier, G., Evrard, P., *et al*. (1996) *Nature (London)* **383**, 73–75.
12. Bugge, T. H., Xiao, Q., Kombrinck, K. W., Flick, M. J., Holmbäck, K., Danton, M. J., Colbert, M. C., Witte, D. P., Fujikawa, K., Davie, E. W. & Degen, J. L. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 6258–6263.
13. Toomey, J. R., Kratzer, K. E., Lasky, N. M., Stanton, J. J. & Broze, G. J., Jr. (1996) *Blood* **88,** 1583–1587.
14. Connolly, A. J., Ishihara, H., Kahn, M. L., Farese, R. V., Jr., & Coughlin, S. R. (1996) *Nature (London)* **381,** 516–519.
15. Ishihara, H., Connolly, A. J., Zeng, D., Kahn, M. L., Zheng, Y. W., Timmons, C., Tram, T. & Coughlin, S. R. (1997) *Nature (London)* **386,** 502–506.
16. Roberts, H. R. & Lefkowitz, J. B. (1994) in *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, eds. Colman, R. W., Hirsch, J., Marder, V. J. & Salzman, E. W. (Lippincott, Philadelphia), pp. 200–218.
17. Sun, W. Y., Witte, D. P., Degen, J. L., Colbert, M. C., Burkart, M. D., Holmbäck, K., Xiao, Q., Bugge, T. H. & Degen, S. J. F. (1998) *Proc. Natl. Acad. Sci. USA* **95,** in press.
18. Degen, S. J., Schaefer, L. A., Jamison, C. S., Grant, S. G., Fitzgibbon, J. J., Pai, J. A., Chapman, V. M. & Elliott, R. W. (1990) *DNA Cell Biol.* **9,** 487–498.
19. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
20. Mortenson, R. (1993) in *Current Protocols in Molecular Biology*, eds. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (Wiley, New York), Vol. Suppl. 23, Section IV, pp. 9.15.1–9.15.6.
21. Mann, K. G. (1976) *Methods Enzymol.* **45,** 123–156.
22. Degen, S. J. & Davie, E. W. (1987) *Biochemistry* **26,** 6165–6177.
23. Ashworth, A., Swift, S. & Affara, N. (1989) *Nucleic Acids Res.* **17,** 2864.
24. Rosen, E. D., Chan, J. C. Y., Idusogie, E., Clotman, F., Vlasuk, G., Luther, T., Jalbert, L. R., Albrecht, S., Zhong, L., Lissens, A., *et al.* (1997) *Nature (London)* **390,** 290–294.
25. Bi, L., Lawler, A. M., Antonarakis, S. E., High, K. A., Gearhart, J. D. & Kazazian, H. H., Jr. (1995) *Nat. Genet.* **10,** 119–121.
26. Wang, L. L., Zoppe, M., Hackeng, T. M., Griffin, J. H., Lee, K. F. & Verma, I. M. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 11563–11566.
27. Suh, T. T., Holmbäck, K., Jensen, N. J., Daugherty, C. C., Small, K., Simon, D. I., Potter, S. & Degen, J. L. (1995) *Genes Dev.* **9,** 2020–2033.
28. Shivdasani, R. A., Rosenblatt, M. F., Zucker-Franklin, D., Jackson, C. W., Hunt, P., Saris, C. J. & Orkin, S. H. (1995) *Cell* **81,** 695–704.
29. Soifer, S. J., Peters, K. G., O'Keefe, J. & Coughlin, S. R. (1994) *Am. J. Pathol.* **144,** 60–69.
30. Terwiel, J. P., Veltkamp, J. J., Bertina, R. M. & Muller, H. P. (1980) *Br. J. Haematol.* **45,** 641–650.
31. Forestier, F., Daffos, F., Rainaut, M., Solé, Y. & Amiral, J. (1985) *Thromb. Haemostasis* **53,** 401–403.