

Review Article

Mouse Models in Coagulation

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Introduction

In the past six years, analysis of numerous murine models, enabled by investigator-designed manipulation of the mouse genome, has generated an explosion of new, and sometimes confounding, data. Mice with complete deficiencies in virtually all the known factors involved in hemostasis have been produced by targeted modification of the genes that encode the proteins that generate and regulate coagulation. Mice with altered factors or altered expression of normal factors have also been produced by transgenic techniques. In this review, we summarize the analyses of these mouse models and compare these results to those obtained from people with deficiencies in the same genes (Table 1). Information is available for all the models, but we will focus on those more completely characterized. We present the models in groups (Fig. 1), which places each factor into a single position within a simplified model of hemostasis. The phenotypes of these genetically modified mice reflect a compilation of functions, some of which do not fit within this simplified model of hemostasis. For example, Factor X (FX) not only acts in concert with factor V (FV) to foster clot formation but also acts in concert with tissue factor pathway inhibitor (TFPI) to limit clot formation. Additionally, we know that many factors have functions outside hemostasis; for example, a cleaved fragment of antithrombin III (ATIII) serves as an anti-angiogenic factor (1). Moreover, as shown remarkably by the analysis of these mouse models, many of these proteins also have a critical role in normal embryonic development (Fig. 2). Finally, genetic background significantly influenced the phenotype of some models, and we specify when this variable was examined.

To provide a structure to consider analysis of these mice, we divided the hemostasis proteins into three categories: those that promote the generation of thrombin, those that participate in clot formation, those that curtail the generation of thrombin. To place these proteins in context, we begin with a brief overview of coagulation. Coagulation is initiated when injury to the blood vessel exposes tissue factor (TF) to circulating factor VII (FVII), resulting in the formation of TF/VII complexes. This complex activates factor X (FX), either directly or indirectly though FIX. Activated FX (FXa) converts prothrombin to thrombin. Thrombin catalyzes multiple events in coagulation by activating the platelet integrin αIIbβ3, which mediates fibrinogen- and von Willebrand factor (vWF)-dependent platelet interactions, and by transforming soluble fibrinogen molecules into a fibrin fiber matrix. Thrombin further amplifies its own generation by activating factors (FXI, FX, FIX) and cofactors (FV and FVIII) that promote TF-independent production of the active protease FXa. Anticoagulation is achieved through the inhibition of the various steps of thrombin generation. TFPI forms a complex with TF, FVIIa and FXa to terminate the initiating step. Protein Z forms a complex with FXa and serves as a cofactor in the inhibition of FXa activity. Thrombomodulin (TM), an endothelial cell surface-bound protein, and thrombin complex to activate protein C (PC). With its cofactor protein S (PS), activated protein C (APC) degrades FVa and FVIIIa and thus limits generation of thrombin. The serpin, ATIII, forms complexes with FXa and thrombin to directly inhibit these proteases.

Promoting Thrombin Formation

Tissue factor, Factor VII, Factor X, Factor V, Prothrombin, Factor IX, Factor VIII, Factor XI

Tissue Factor (TF): TF is a cell-surface protein that is not normally found on endothelial cells lining the blood vessel but on cells immediately below the endothelial layer. Following vascular injury, the newly-exposed, subendothelial TF binds to circulating FVII to form the TF/FVII protease complex that activates FX and FIX. People with TF deficiencies have never been described, suggesting this protein is essential for life. In fact, three independent groups had difficulty producing TF -/- mice by gene targeting, indicating embryonic lethality in the absence of TF (3-5). TF -/- embryos arrested development between E8.5 and 10.5 (3) and had pools of red blood cells in the yolk sac cavity (3-5). The embryos lacked the large vitelline vessels that connect the yolk sac and embryonic vasculature, and no blood flow was seen in the yolk sac vessels (4). Thus, TF is essential for the assembly of functional vascular structures and the maintenance of vascular integrity in the yolk sac, although the role of TF in vasculogenesis remains unclear. Inexplicably, the penetrance of the severe TF -/- phenotype varies with genetic background, as a low percentage of embryos survive to birth on a mixed background containing 129/SvJ and C57BL/6. Related experiments demonstrated that low levels of TF activity, approximately 1% of normal, were sufficient to rescue the TF -/- mice from embryonic lethality (6).

Studies with transgenic models have elucidated which of two domains within TF is essential for normal development. The extracellular

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Table 1 List of mutations in murine hemostatic proteins

Category	Gene Mutation	Human deficiency	Embryonic Lethal?	Murine Phenotype	References
Promoting Thrombin Formation	TF -/-	Partial and complete deficiencies unknown	Yes (E8.5-10.5)	Lacked vascular integrity, red blood cell pools in yolk sac cavity	3-6, 9, 113, 115
	FVII -/-	Variable bleeding, thrombosis reported rarely	No	No vascular defects, death caused by hemorrhage in neonates, no survival beyond 24 days	13, 86, 95, 116
	FX -/-	Variable bleeding Deficiencies <1% extremely rare	Partial (E11.5-12.5)	No vascular defects, death caused by hemorrhage in neonates, no survival beyond 20 days	17
	FV -/-	Mild bleeding, complete deficiency unknown	Partial (E9.5-10.5)	Vascular defects, death caused by hemorrhage in neonates, limited survival beyond 2 hours	14, 18, 43
	FII -/-	Severe bleeding, complete deficiency unknown	Partial (E9.5-11.5)	Inconsistent reports of vascular defects; neonatal hemorrhage, no survival beyond several days	19, 20, 117
	FIX -/-	Hemophilia B; severe bleeding, spontaneous joint bleeds	No	Hemorrhagic swelling of feet, death from internal bleeding after injury and normal fighting	22, 27
	FVIII -/-	Hemophilia A; severe bleeding, spontaneous joint bleeds	No	Spontaneous bleeding only after partial tail amputation	23, 24, 26
	FXI -/-	Mild bleeding	No	Tendency for increased bleeding time, prolonged APTT	30, 96, 118
Clot Formation	Fbg -/-	Severe bleeding associated with complete deficiency	No	Neonatal hemorrhage, cannot support pregnancy, strain influences survival	38, 39, 55, 62, 63, 82, 119, 120
	γΔ5 Fbg	Never recorded	No	Impaired platelet aggregation, increased bleeding time	64
	V/F IV Fbg	Venous thrombosis in heterozygotes	No	Heterozygotes appear normal, neonatal hemorrhage in homozygotes	65
	Elevated Fbg	Correlated with various pathologies, thrombosis	No	No gross defects, normal histology in lung, liver, spleen and kidney	66
	NF-E2 -/-	Never recorded	No	Absolute thrombocytopenia, hemorrhage in neonates, less than 10% survive to adulthood	40
	vWF -/-	Type III vWD; severe bleeding, reduced FVIII levels	No	Prolonged APTT, reduced FVIII, neonatal hemorrhage, GI bleeding,	55, 67, 71, 72
Curtailing Clot Formation	TFPI -/-	May be thrombotic (121), complete deficiency unknown	Yes (E9.5-11.5)	Liver fibrin deposition, rarely intravascular thrombosis	84-86, 122
	TM -/-	Thrombosis, complete deficiency unknown	Yes (E9.5)	Overall retardation in growth, maternal environment negatively affects embryonic survival	87-91, 93
	$TM^{Pro/Pro}$	Never recorded	No	Fibrin deposition in lungs, heart, spleen, and liver; phenotype worse upon thrombogenic challenge	88, 91, 92
	PC -/-	Disseminated intravascular coagulopathy (DIC) as neonates	No	DIC by E12.5, no survival beyond 24 hours after birth	94-96
	FV Leiden	Thrombosis	No	APC resistance, fibrin deposition, strain influences survival	99, 101
	AT III -/-	Thrombosis, complete deficiency unknown	Yes (E15.5-E16.5)	Subcutaneous and intracranial hemorrhaging, thrombotic damage to embryonic heart and liver, DIC	100
	PZ -/-	May be thrombotic (103), complete deficiency unknown	No	No overt phenotype	101

domain of TF interacts with FVII to generate FXa and thrombin, and the intracellular domain has recently been implicated in signaling (reviewed in (7, 8)). The transgenic addition of human TF lacking the intracellular domain rescued the TF -/- embryos, suggesting that the signaling activity of the TF intracellular domain is not required for embryogenesis (8, 9). This conclusion was directly demonstrated with a human TF transgene coding for a protein with a mutation in the active site of the extracellular domain; the proteolytic activity of TF was required for rescue of embryonic lethality (9). These results suggest that TF's role in activating proteases, which ultimately results in thrombin generation, is essential for embryogenesis. The function of the intracellular domain remains unclear.

The once simple model of TF function in clot initiation has become quite complex recently. TF expression can be upregulated by inflammatory and angiogenic factors, and TF is implicated in tumor metastasis (reviewed in (7, 10, 11)). Additionally, a ligand for the intracellular domain (actin binding protein 280) has recently been documented (12); further studies with this ligand may identify pathways outside of coagulation in which TF participates. Thus, future studies with the TF mouse models will surely elucidate novel roles for TF beyond the initiation of blood coagulation.

Factor VII (FVII): FVII is the only known ligand for TF's essential extracellular domain. Therefore, the most significant finding from FVII -/- mice is their phenotypic difference from the TF -/- mice. The FVII

-/- mice on the same genetic background as the TF -/- mice were born in a normal Mendelian pattern, demonstrating that FVII deficiency did not affect survival to term (13). The FVII -/- mice had normal vascular development. These data are consistent with the maternal transfer hypothesis that predicts that the loss of a membrane-bound molecule would be more detrimental to the organism than the loss of a soluble one. The investigators speculated that maternal FVII might circulate in the embryo and obscure the FVII -/- phenotype. However, the procoagulant activity of the embryos' murine FVII was undetectable (< 0.05% of adult FVII activity), and human recombinant FVII did not cross the placenta beyond supraphysiological plasma levels (13). Thus, maternal transfer may not explain the phenotypic differences between FVII -/- and TF -/- mice. It remains possible, however, that a critical threshold level, which was below detection in FVII-/- embryos, was sufficient for proper development. Other data do suggest that very low levels of some hemostatic factors are sufficient to support normal development (6, 14). Conversely, it is possible that while TF is important in generating thrombin, it has other roles in development independent of clot initiation. Support for this independent function comes from the observation that TF is abundantly expressed in human embryos prior to accumulation of FVII protein (15).

The majority of FVII -/- neonates died from intra-abdominal bleeding within 24 h of birth, and the remaining mice died within 24 days from intra-cranial hemorrhage. The neonatal bleeding observed in the FVII -/- mice seems a good model for the human deficiency. FVII-deficient patients, none of whom totally lack FVII, have variable degrees of severity of bleeding and, rarely, thrombotic tendencies (16). The massive hemorrhage in FVII -/- mice indicates that the human and mouse proteins have similar functions.

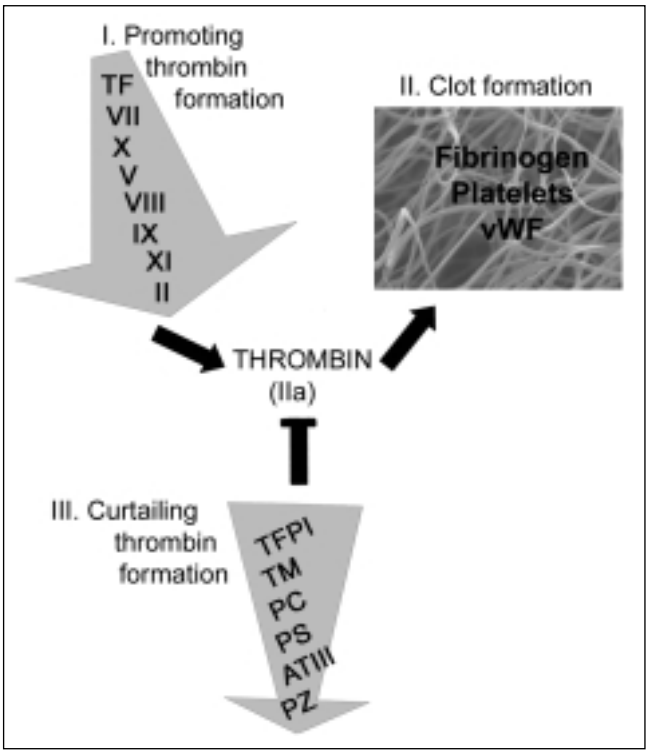

Fig. 1 Proteins involved in hemostasis. For simplicity, each protein is placed under one of three categories: factors that promote thrombin formation, factors that participate in clot formation, and factors the curtail thrombin formation

Factor X (FX): Circulating FX is activated either by TF/VIIa or FIXa/FVIII. Active FX binds to FV to form the protease complex FXa/FV that activates prothrombin to thrombin, and this is the only known activator of thrombin. Since the loss of thrombin in mice is partially lethal (discussed below), it was expected that the absence of FX would also be lethal. Indeed, approximately one-third of the FX -/- mice died in utero by E12.5 (17). The majority of the null embryos were resorbed. Those remaining showed signs of bleeding, including

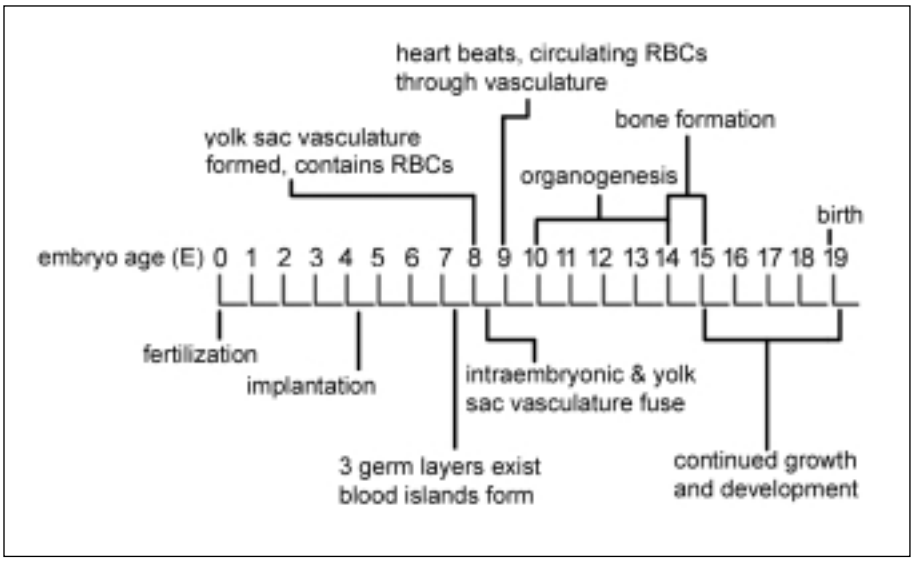

Fig. 2 Mouse development. This succinct description of murine embryogenesis emphasizes vascular development because several murine models are defective in this specific process (for a review of mouse development, see (2)). The gestation period is between 19–20 days. Implantation of the blastocyst occurs at embryonic day (E) 4.5 and shortly thereafter, gastrulation begins. By E7.5 the three germ layers of the embryo have been formed, and the tissues required for embryo/maternal interactions are developing. These tissues go on to form the placenta and the extraembryonic yolk sac. Blood islands are first seen in the yolk sac; these islands give rise to both blood cells and endothelial cells. The yolk sac vasculature is developed at E8 and primitive red blood cells are contained with the yolk sac vessels. Meanwhile, intraembryonic vessels form as the yolk sac vasculature develops, and at E8.5 the two vascular beds fuse. By E9, the heart is formed and beats regularly, circulating red blood cells through the yolk sac and embryonic vasculature. Vascular development progress, and major and minor vessels form. Organogenesis is prominent between days E10-E14, bone formation begins between E14-E15, and the fetus continues to grow and develop until birth at E19

intracranial hemorrhage, but no vascular defects were seen in the yolk sac. The cause of the developmental block between E11.5 and E12.5 remains unresolved. The FX -/- embryos that survived to term died within 20 days, due to severe bleeding.

Factor V (FV): Homozygous disruption of the FV gene also resulted in partial embryonic lethality (18). At E9.5, a subset of FV -/- embryos showed multiple anomalies such as overall growth and developmental delay, defects in cardiac muscle, focal hemorrhage, and collapsed vascular channels in the yolk sac with fewer blood islands than normal. The authors concluded that partial lethality at E9.5-10.5 was likely a result of impaired vasculogenesis in the yolk sac. The FV -/- mice that survived to birth (approximately half) died within 2 h due to massive intra-abdominal hemorrhage. When <0.1% FV was expressed with a FV transgene in FV-/- mice, some survived to adulthood without hemorrhage, demonstrating that quite low levels of FV may be sufficient for hemostasis (14). As discussed in the introduction, activated protein C inactivates FVa to limit thrombin production. A prevalent mutation in FV affecting the efficiency of this inactivation is associated with clinically significant thrombosis in the population and is known as FV Leiden. Targeted replacement of the FV gene with the mouse homolog of FV Leiden is described under the section on anticoagulation.

Prothrombin (FII): Similar to disrupting the activators of prothrombin (FX and FV), the homozygous deletion of the prothrombin gene resulted in partial embryonic lethality between days E9.5-11.5 (19, 20). In the FII -/- embryos, the vessels were not filled with fetal blood cells and free blood was found in the yolk sac cavity (19). These observations suggest a loss of vascular development, but further ultra-structural examination of the cell layers comprising the yolk sac showed no abnormalities in E9.5 FII -/- embryos. In contrast, Xue et al. observed no bleeding in the yolk sac, yet found signs of abnormal vascular development (20). Although these differences remain unresolved, genetic variation may explain the differing phenotypes as mice in the first study were of 129/Black Swiss background and mice in the latter study were C57BL/6J. Nonetheless, both studies showed that the loss of FII resulted in a partial embryonic lethal phenotype with at least 50% of FII -/- embryos dying between E9.5 and E11.5. Furthermore, FII -/- mice that survived to term (approximately 20-25%) died within a few days of birth, due to massive hemorrhagic events.

The partial lethality associated with the FII -/- mice and several other coagulation factors involved in the activation of prothrombin (TF -/-, FX -/-, and FV -/-) suggests that active thrombin is essential for development. These factors may represent the most direct pathway to produce low levels of thrombin in the embryo. The similarities in some of the phenotypes associated with their loss (vascular defects) suggest that active thrombin is essential in vasculogenesis. However, it is unclear, then, why FVII -/- mice do not have an analogous phenotype and why FX -/- mice do not also have vascular defects. Phenotypic differences might be explained if certain players in hemostasis have more (or less) important roles in the embryo than those currently defined for the adult. Embryonic differences are evident in studies of the temporal expression of hemostasis-related proteins in murine embryos (21) and studies using a FVII inhibitor in embryos (13). Further analyses of these mouse models should provide insights into the function of the coagulation factors in developmental processes.

Factor IX (FIX) and Factor VIII (FVIII): Circulating FIX is activated by TF/FVIIa and binds to circulating factor VIII (FVIII) to form the FIXa/FVIII protease complex that activates FX. As in humans, the FVIII and FIX genes are both located on the X chromosome in mice. The FIX -/- mice had hemorrhagic swelling of the feet or footpads due to spontaneous bleeding (22). Normal fighting between littermates often led to massive hemorrhage and death. Partial amputation of the tail was fatal if the wound was not cauterized. Splenomegaly, a sign of anemia, was often observed upon autopsy. In contrast to FIX -/- mice, the FVIII -/- mice had a milder bleeding disorder (23, 24). These mice did not have spontaneous bleeding; a bleeding phenotype was only observed after partial tail amputation, which was fatal in roughly 2/3 of the affected males examined.

In humans, FVIII deficiency (hemophilia A) and FIX deficiency (hemophilia B) have nearly identical clinical presentations (25). The symptoms vary from person to person, but severe bleeding and spontaneous joint bleeds are common. Bi et al. suggest that the difference between the relatively mild phenotype in FVIII -/- mice and hemophilia A may reflect an inherent difference between the human and mouse proteins, since only 2 of 66 anti-human factor VIII monoclonal antibodies cross-react with mouse FVIII (24). Nevertheless, these mouse models should be useful to explore the feasibility of gene-therapy for humans, as successful studies have been reported with both murine hemophilia models (26-28). The non-essential roles of FVIII and FIX in human and murine development correlate with the observation that there is little or no expression of FVIII and FIX early in murine development, when other factors that generate thrombin are highly expressed (21). Additionally, expression of the factors in human embryos suggests that both factors play less important roles in the embryo than the adult (29). Since these factors serve to amplify thrombin when large amounts of the protease are needed, they are likely not involved in the direct pathway used to generate thrombin during embryogenesis for vasculogenesis.

Factor XI (FXI): Factor XI is activated by thrombin and, as an active protease, it further enhances thrombin production by activating FIX. The amplification role of FXI is somewhat analogous to the functions of FVIII and FIX, and so it might be predicted that the loss of FXI would not result in developmental arrest. Indeed, the FXI -/- mice were viable, and homozygous females were able to deliver pups. The mice had prolonged APTTs and normal prothrombin times (PTs) (30), as expected. A bleeding time assay from tail transections indicated that the median bleeding time was slightly longer in the FXI -/- mice than heterozygous or wild type mice. Because murine and human FXI proteins were found to be quite similar (31), it is not unanticipated that humans with a deficiency in FXI have comparable mild phenotype, with variable symptoms mainly related to bruising and post-operative bleeding (32).

Studies have suggested that FXI has a role outside of clot formation, and may serve to protect clot dissolution (33, 34) and play a role in thrombosis (35). These studies suggest that the loss of FXI could have a protective effect against thrombosis. Indeed, when mice with a combined FXI and protein C (PC) deletion were generated, the loss of FXI improved the survival of thrombotic PC neonates (discussed in more detail below). Future crosses of the FXI -/- mice with other models of spontaneous thrombosis will likely define FXI's role in fibrinolysis and thrombotic disorders.

Discussion/Conclusion

The embryonic lethality in TF -/-, FV -/-, FI I-/-, and FX -/- mice strongly suggests that thrombin generation is critical for normal development. Complete deficiency of TF, FV or FII has never been reported in humans, and cases with less than 1% FX activity are extremely rare (6, 18, 36, 37). However, individuals with low levels of FV, FX or FII have been reported and in these cases there are variable degrees of bleeding. The symptoms are all mild relative to the fatal bleeds ob-

served in the null mice that survived to birth. As mentioned above, together the results indicate that low levels of these factors are necessary and sufficient for life.

Is it their role in coagulation that makes these proteins essential for life? Two clues indicate that it is not. First, mice lacking even the most essential components of a clot, fibrin (38, 39) or platelets (40) have no developmental defects (discussed in more detail below). Second, vascular anomalies were observed in several models, indicating problems independent of coagulation. These findings support the hypothesis that the generation of thrombin is important for vascular development during embryogenesis. Further, it has been speculated that the generation of thrombin may serve as a "leak detector" for developing blood vessels (41). For example, if a vessel is not patent, then TF from underlying, non-endothelial cells may contact FVII in plasma to initiate thrombin generation. This thrombin might serve to signal through a receptor on a nearby endothelial cell that the vessel is not fully developed and result in local morphological responses by the endothelium.

Other *in vivo* evidence also suggests that thrombin signaling through endothelium may be essential to vascular development. The loss of the PAR-1 thrombin receptor, expressed on endothelium, causes a partial embryonic lethal phenotype with abnormal bleeding and yolk sac vasculature (42, 43). Of the four known PARs, both PAR-1 and PAR-2 are expressed on endothelial cells. As PAR-1 is highly expressed in endothelium by E 9.5, it is likely the major mediator of thrombin signaling in vascular cells (44, 45). In fact, when PAR-1 was re-expressed only in the endothelium of PAR-1 -/- embryos, there was a decrease in embryonic lethality, suggesting that the vascular defects in PAR-1 -/- embryos were due to its loss in endothelial cells and not from the perivascular mesenchyme (43). The partial embryonic lethality of the PAR-1 -/- embryos suggests that other PARs, possibly PAR-2, may be able to compensate for the loss of PAR-1. For instance, in the presence of TF and FXa, picomolar concentrations of FVIIa can activate PAR-2 and to a lesser extent, PAR-1 (46). It is possible that the combined loss of both PAR-1 and PAR-2 would have severe consequences for vasculogenesis. The joint loss of PAR-1 and FV results in a more lethal phenotype than the loss of either gene alone; Griffen et al. propose that these data suggest that the PAR-1 and FV pathways interact, yet other targets and agonists in these pathways remain ambiguous (43). Therefore, it is likely that while thrombin signaling through PAR-1 is important in vascular development, there may be other proteases in the cascade that signal through the PARs or yet unidentified receptors.

In vitro studies also provide evidence for a bridge between coagulation and vascular development. The regulation of vascular endothelial growth factor (VEGF), the primary growth factor involved in both vasculogenesis and angiogenesis, and PAR signaling have been linked; this regulation was shown to involve TF, FVII, FXa, and thrombin (47). Studies demonstrating that VEGF can upregulate TF and thrombin expression or conversely, can be regulated by these proteins, indicate the complexity of the interactions between the hemostatic proteins and vascular-specific proteins (48-54). Combined, the insights from the *in vivo* and *in vitro* data reinforce the opinion that the hemostatic proteins, particularly those that regulate thrombin levels, constitute far more than an elaborate system to control the generation of fibrin.

Not all factors that serve to promote thrombin generation play roles in embryogenesis. FVIII, FIX, and FXI are not essential to generate small amounts of thrombin; rather, these factors serve to enhance or magnify its generation. Therefore, it is not unanticipated that the loss of these factors did not cause developmental problems. Nonetheless, variable bleeding phenotypes were associated with the loss of these proteins, as predicted from human phenotypes. Defects in hemostasis were also observed in those mice that survived development. The disparity between early developmental defects and later death secondary to hemorrhage is most clearly evident in mice lacking factors X, V, and II; some embryos escape the developmental bottleneck, but these "survivors" die from massive bleeding in the peri-/postnatal period. These results suggest that the arrest of bleeding in embryos *in utero* is either not essential until the time of birth or is controlled differently in embryos than neonates. Little is still known about hemostatic factors in embryos but evidence that birth is a traumatic situation requiring an intact hemostatic system is demonstrated by the improved long-term survival of embryos lacking platelets if delivered by cesarean section (40).

Clot Formation

Fibrinogen, Platelets, von Willebrand Factor

Fibrinogen: Fibrinogen is composed of two copies of three polypeptides, Aα-, Bβ-, and γ, encoded by three genes. Homozygous disruption of one gene, either Aα or γ, resulted in total loss of fibrinogen expression (38, 39). Embryonic development was normal, and, surprisingly, most homozygous mice survived to adulthood. About one third of Aα -/- neonates had overt bleeding that was often abdominal. Survival varied with the genetic background, and abdominal hemorrhage was the usual cause of death later in life. Aα -/- females could not support pregnancy due to fatal vaginal bleeding. Aα +/- and γ+/- mice were normal. *In vitro* studies with plasma from Aα -/- mice demonstrated complete loss of platelet aggregation. Nevertheless, following ferric-chloride injury to arterioles, *in vivo* platelet deposition and thrombus initiation were normal (55). Unlike normal controls, where vessel occlusion occurred at the site of ferric-chloride injury, vessel occlusion in Aa -/- mice was embolic and occurred downstream from the site of injury, implying that fibrinogen is essential in platelet-vessel wall interactions.

While mouse models of fibrinolytic deficiencies are beyond the scope of this review, it is of interest to mention the plasminogen deficient mice in the context of fibrinogen deficiency. Since plasmin (activated form of plasminogen) is the major protease responsible for lysing insoluble fibrin, the Plg -/- mice represent a situation in which excess fibrin causes a severe phenotype. Plg -/- mice developed normally to birth, but multiple symptoms appeared with maturity (56-58). They had rectal prolapse, fibrin deposition in the liver, runting, and they had severely delayed lysis of induced pulmonary emboli. The Plg -/- mice also had impaired wound healing (59, 60) and conjunctival lesions, analogous to the ligneous conjunctivitis seen in plasminogen deficient humans (61). When Plg -/- mice were bred onto a fibrinogen null background, the mice were phenotypically similar to fibrinogen null mice (62), and the pathologies associated with the Plg -/- mice were corrected. These studies suggested that the major physiological role of plasminogen was to degrade fibrin. Nonetheless, a subsequent study of toxic liver injury in these doubly deficient mice demonstrated that persistent, centrilobular lesions characteristic to the Plg-/- mice were not corrected by the loss of fibrinogen (63), indicating a function for plasmin independent of fibrinolysis.

Mice with specific fibrinogen alterations have been generated by targeted replacements in the γ-chain gene. A five-residue truncation of the C-terminal end of this chain (γΔ5) removed a site identified as critical for fibrinogen mediated platelet aggregation (64). In homozygous mice, bleeding time from a nail bed injury was indefinite, while only minimal bleeding was found from a skin injury. These results indicate that platelet aggregation at injury sites in large vessels requires normal fibrino-

gen, and that this C-terminal γ-chain segment is essential for fibrinogen-platelet interactions. Homozygous females were able to support pregnancy, suggesting that platelet aggregation is not essential for implantation. Mice heterozygous for this deletion were normal. Mice with a two amino acid deletion in the γ-chain (γΔ319,320) were generated to model the human dysfibrinogen Vlissingen/Frankfurt IV (65). As expected from studies of recombinant human fibrinogen with this deletion, homozygous mice had a severe bleeding diathesis and most animals died as neonates with abdominal hemorrhage (KAH and STL, unpublished observation). Mice heterozygous for this deletion appeared normal. Transgenic mice with elevated levels of fibrinogen have also been generated. Although elevated fibrinogen is a strong risk factor for athero-thrombotic disease in people, under normal laboratory conditions these mice appeared no different from their littermates (66).

p45 NF-E2: The deletion of the p45 subunit of the transcription factor NF-E2 demonstrated its necessity in platelet production, resulting in mice with absolute thrombocytopenia (40). As intriguing as the ability of embryos to develop to term without fibrinogen, NF-E2 -/- mice also survived to birth lacking this primary component of hemostasis. Nonetheless, hemorrhage was common in newborns, and less than 10% of liveborn homozygotes survived to adulthood. As stated above, cesarean section delivery significantly decreased bleeding in newborns. Adult homozygous animals were fertile and females successfully supported pregnancy (S. Orkin, personal communication). In contrast to mice, a lack of platelets due to a genetic mutation is likely not compatible with human life. There are no reports of humans born with absolute thrombocytopenia, although a severe reduction in platelet count can be a consequence of a pathological condition (e.g. HIV infection) or a side effect of certain pharmaceuticals.

Von Willebrand Factor (vWF): In the circulation, vWF is associated with FVIII and protects FVIII from rapid degradation; vWF is also found in the α-granules of platelets and the Weibel-Palade bodies of endothelial cells, and these sources likely contribute to vWF-mediated platelet adhesion and aggregation. vWF bridges platelets with the subendothelium through platelet membrane glycoproteins GP1bα (part of the Ib-IX-V complex) and αIIbβ3. Homozygous disruption of the vWF gene led to complete loss of vWF antigen and a 4-5 fold reduction in FVIII antigen (67). Embryonic development was normal and the mice were viable, although bleeding was not controlled following tail transection. The vWF -/- mice were fertile, and pregnancy was normal with no prenatal or post partum bleeding complications. About 10% of the mice had spontaneous and sometimes fatal neonatal bleeding. Gastrointestinal bleeding was seen in some older animals. Heterozygous mice did not have these bleeding phenotypes.

Evidence for a suggested link between vWF and P-selectin, a protein involved in leukocyte rolling and extravasation, has been supported utilizing the vWF -/- mice. It had been previously shown that P-selectin and vWF are stored in the same places (α granules and Weibel-Palade bodies), and that deletion of P-selectin disrupted hemostasis, as demonstrated by a prolonged bleeding time (68). Furthermore, P-selectin is involved in platelet adhesion and rolling mediated through GpIba (69, 70). A recent study examined leukocytes in vWF -/- mice and found a 70% decrease in leukocyte rolling in stimulated mesenteric venules viewed by intravital microscopy and a significant decrease in leukocyte recruitment utilizing inflammatory models of meningitis and wound-healing (71). Interestingly, the loss of vWF affects the Weibel-Palade body structure, thereby leading to altered storage of P-selectin in vWF -/- endothelial cells (71). To examine the loss of vWF in chronic inflammation, the vWF -/- mice were bred onto an atherosclerotic-prone background (LDR receptor deficiency). When placed on an atherogenic diet, the lesions in the doubly-deficient animals were smaller than LDLR -/-, vWF +/+ at earlier timepoints (8 and 22 weeks) but not at a later timepoint (37 weeks) (72). While P-selectin expression of LDLR -/-, vWF -/- mice was slightly different than the LDLR -/-, vWF +/+ mice, the authors suggest that it is unlikely that this alone leads to the protective phenotype observed. Rather, due to the observations that fewer monocytes were recruited and that lesions were smaller in areas of disturbed flow (branch points) the authors discuss the possibility of a complex role for vWF involved in 1) flow dependent direct and indirect recruitment of monocytes and platelets and 2) shear-stress regulation of endothelial cell gene expression.

Discussion/Conclusion

In mice, some deficiencies in factors that participate in clot formation were associated with phenotypes that could be anticipated from human studies. Cases of afibrinogenemia have been reported (73, 74) and these patients have severe bleeding disorders, as was seen in the mouse models. Women with low or undetectable levels of fibrinogen have difficulty maintaining pregnancy without clinical intervention (75), similar to fibrinogen null mice. Mice lacking vWF essentially recapitulated the analogous human disease; complete deficiency of vWF, or Type III von Willebrand disease (vWD), is associated with life-threatening hemorrhage and hemarthroses, and reduced FVIII levels (76). In some instances, the studies in mice gave insight into a possible basis for previously confounding pathologic consequences. For example, pulmonary emboli have been observed in afibrinogenemic patients (77, 78), in seeming contradiction to severe bleeding, but in agreement with the ferric-chloride injury studies in Aα -/- mice, which showed embolic thrombus formation (55). As humans with an absolute deficiency in platelets have never been identified, the ability of mice lacking platelets to develop to term and survive is remarkable.

These mouse models suggest that some aspects of clot formation are essential in pregnancy. For example, uterine bleeding is evident halfway through gestation in female mice deficient in fibrinogen (38) or with variant fibrinogen incapable of forming fibrin (γΔ319, 320) (KAH and STL, unpublished data). This bleeding coincides with the maternal blood source becoming linked with the fetus through the chorioallantoic placenta. The loss of fibrinogen lacking the domain essential for platelet aggregation or absolute thrombocytopenia is compatible with pregnancy, suggesting that fibrin (not fibrinogen) may be an essential coagulation component for placental function. Nevertheless, the loss of integrin β3, a component of the platelet receptor αIIbβ3, also has consequences on pregnancy; 77% of β3 -/- females experience intrauterine hemorrhage, occurring within the labyrinth of the placenta and under Reichert's membrane (79). The difference in ability to support pregnancy between mice lacking platelets and those lacking a platelet receptor component may be explained by the expression of β3 in other cell types. Therefore, while the loss of some hemostatic factors such as TF, FV, FX, and FII, directly affects embryonic development, loss of others such as fibrinogen affect the placenta that supports this development.

Technology to generate mutant mice is always improving, as are the methods for studying them. One significant breakthrough has enabled the monitoring of thrombus formation *in vivo* in real time. Arteriole thrombus formation induced by ferric-chloride can now be followed by intravital microscopy. Early experiments by Ni et al. showed that the vessel injury is thrombin dependent (80). Multiple studies with this method have helped to identify molecules that operate in vivo to promote platelet plug formation and stability. For example, less stable thrombi, that embolized 100% of the time, were generated in fibrinogen

null mice, indicating the necessity of fibrinogen/fibrin interactions between platelets and the vessel wall (55). In vWF -/- mice, platelet deposition was decreased and thrombus initiation was delayed (55). In contrast to fibrinogen deficiency, these thrombi were stable on the vessel wall, but the adhesive properties of the platelets in these mice were insufficient to terminate blood flow in high shear stress channels of nearly occluded vessels, as with thrombi in wildtype mice. Surprisingly, vWF-independent thrombus formation persisted in the absence of fibrinogen (Aα -/-, vWF -/- mice), although the thrombi were more fragile than in either single deficiency (55). This finding suggests a novel pathway for platelet adhesion/aggregation that does not require vWF or fibrinogen (55). It is likely that at least one other ligand of β3 may be responsible for the residual thrombus formation in the Aα -/-, vWF -/- mice because thrombus formation is ablated in β3 -/- mice (80). Interestingly, this real time model has also been useful in identifying a state of accelerated platelet adhesion and thrombus formation. Mice lacking the platelet membrane glycoprotein V (GPV -/-) have 2.5 fold more platelets interacting with the vessel wall in the first minute, and the time required for appearance of the first clot is significantly decreased. Thrombi from the GPV -/- mice embolized more frequently and were noted to be much larger in size than the emboli from fibrinogen null mice. GPV is a member of the complex that binds vWF, yet interactions of GPV -/- platelets with vWF were normal. The authors conclude that an increased responsiveness to thrombin caused the observed phenotype. In sum, this particular model of thrombus formation and numerous others, provide new and informative information while analyzing the mutant mice *in vivo.*

Clot formation likely plays a major role in atherothrombotic diseases. Following atherosclerotic plaque rupture, thrombus formation on the damaged vessel can occlude blood flow through the vessel. Studies with mouse models have provided some insight into the role of clot formation, and in particular the role of fibrinogen, in promoting this pathological condition (81). The development of atherosclerotic lesions in mice mediated by the loss of apolipoprotein E (ApoE) has been examined in fibrinogen -/- mice (82). Lesions developed to the same extent in the ApoE -/- mice and the ApoE -/-, fibrinogen -/- mice, though fibrinogen was absent from the lesions in the doubly-deficient mice. This result, which suggests that fibrinogen has no role in atherosclerotic disease, must be interpreted with caution (81). As elevated fibrinogen is the known risk factor, a more compelling experiment would examine atherosclerosis in the context of elevated fibrinogen. In recent experiments with transgenically induced elevated fibrinogen, we found that diet-induced lesion development was not altered in transgenic mice relative to their normal littermates, indicating that fibrinogen has no influence on the development of small lesions (Gulledge et al., in preparation). In a different model of atherosclerosis, wherein mice express human apolipoprotein (a), a role for fibrinogen was demonstrated (83). Diet-induced atherosclerosis was markedly reduced in apo(a)+, fibrinogen -/- mice relative to apo(a)+ littermates, suggesting that fibrinogen has a role in lesion development in the presence of apo(a). Future studies will likely further examine the influence of platelets and vWF on the development of atherothrombotic disease in mouse models.

Curtailing Thrombin Formation

Tissue Factor Pathway Inhibitor, Thrombomodulin, Protein C, Factor V Leiden, Antithrombin III, and Protein Z

Tissue Factor Pathway Inhibitor (TFPI): The phenotype of mice with a functional disruption of the TFPI gene was similar to that of TF -/- mice: no embryos survived to birth due to intra-yolk sac hemorrhage and depletion of blood cells in the yolk sac and placental vessels. This vascular insufficiency led to wasting and death between E9.5 and E11.5 (84, 85). Some TFPI -/- embryos survived beyond E12.5, and these embryos had deposition of fibrin in the liver and, rarely, intravascular thrombosis (84). When TFPI +/- mice were bred to FVII +/- mice, the reduced expression of FVII rescued the embryonic lethality associated with the loss of TFPI (86). Although massive hemorrhage was still a problem shortly after birth of these mice, survival to birth was dramatically increased with the loss of either one or both copies of the FVII gene. The authors reasoned that the loss of TFPI alone leaves the activity of TF/FVII unrestrained, so removing the activity (by reducing FVII levels) restored development.

Thrombomodulin (TM): The complete loss of TM function in mice results in early post-implantation growth arrest of TM -/- embryos, followed by the rapid and complete resorption of the conceptus (87, 88). Failure of TM null embryos occurred at a very early stage in development, and in fact preceded the establishment of cardiovascular function within the embryo. The analysis of mutant embryos at the time of failure suggested that the death of TM null embryos was not associated with overt thrombosis of embryonic blood vessels, or maternal blood vessels supplying the placenta. When only the cytoplasmic domain of TM was disrupted, expression of the remaining gene product was sufficient for early development of the mice (89).

Placenta-specific restoration of TM expression via the tetraploid embryo aggregation technique rescued TM -/- embryos from early abortion and developmental arrest (90). This finding, together with the analysis of TM expression in the developing embryo, showed that the critical site of TM function was not the vascular endothelium, but non-endothelial placental tissue (trophoblast and/or parietal endoderm). Yet, TM -/- mice died during the second half of pregnancy (day 12.5-16.5 p.c.) from a lethal hemorrhagic diathesis associated with focal thrombosis in the liver and brain and subcutaneous, intracranial bleeding. It was concluded that the early death of TM -/- embryos is caused by a placental defect that probably does not involve intravascular thrombosis or vascular dysfunction. In contrast, the secondary phenotype observed in TM -/- embryos rescued by tetraploid aggregation, which closely resembles the phenotype of PC -/- mice described below, was likely caused by a consumptive coagulopathy. While the midgestation coagulopathy of TM -/- mice is consistent with the anticoagulant function of TM in endothelium, the precise role of TM function in the placenta is as yet unknown.

In contrast to the embryonic lethal phenotype seen with a complete loss of TM function, a selective disruption of TM cofactor function was compatible with embryonic development. These mice ($TM^{Pro/Pro}$ mice) were generated by targeted replacement of the TM codon for Glu404 with a codon for Pro (88, 91). In mice homozygous for this substitution, the expression of TM was diminished and thrombin-dependent formation of APC was reduced more than 100-fold. $TM^{Pro/Pro}$ mice exhibited a hypercoagulable state with fibrin deposition in the arterial and venous microcirculation of the lung, heart, liver, and spleen. Although $TM^{Pro/Pro}$ mice did not develop spontaneous thrombosis, experimental challenge with hypoxia, vascular stasis, or endotoxin exacerbated intravascular fibrin deposition in specific organs of $TM^{Pro/Pro}$ mice. Microvascular thrombosis in $TM^{Pro/Pro}$ mice was much more severe on a mixed 129-C57Bl/6 background relative to an inbred C57Bl/6J background, a characteristic seen in other mouse models of thrombosis (92). Much more severe thrombosis is seen in mice generated by the endothelial-specific ablation of the TM gene: since TM expression is preserved in the placenta, the majority of these animals develop normally, but suc-

cumb to massive thrombosis at a young age (93). The penetrance of disease reaches 100%, and the mice experience large vessel arterial and venous thrombosis in their extremities, as well as in internal organs, and eventually die from a consumptive coagulopathy. The disease can be prevented by treating animals with anticoagulants, demonstrating that unfettered coagulation activation is the predominant pathophysiological mechanism underlying the disease.

Protein C (PC): PC -/- mice developed normally to term, yet perished at birth (94). Clottable plasma fibrinogen was undetectable in neonatal PC -/- mice, suggesting a severe consumptive coagulopathy as the cause of death. Symptoms of thrombosis, including fibrin deposition in the brain and liver, bleeding in the brain, and focal liver necrosis, were visible in E12.5 embryos. This phenotype is similar to that observed following tetraploid rescue in TM -/- mice, a coincidence indicating that TM and APC have related functions in embryogenesis. The thrombotic diathesis became progressively more severe until birth; at E17.5, 22% of all pups were PC -/- and by birth only 8% were PC -/-. As delivery by cesarean section did not prolong survival, it is unlikely that the trauma of birth precipitated death of PC -/- mice. PC +/- mice did not develop thrombosis, but did have significantly reduced levels of plasma fibrinogen consistent with increased coagulopathy. An attempt to rescue PC -/- mice with a superimposed deficiency of FVII yielded a surprising result: the coagulopathy of PC -/- was augmented by the loss of FVII (95). That is, the unrestrained generation of thrombin expected with loss of APC was not compensated by the reduced generation of thrombin expected with the loss of FVII.

In contrast, the PC -/- coagulopathy was compensated by the loss of FXI. PC -/-/FXI -/- mice survived the early lethality observed in the PC -/- neonates, the oldest mice living to 3 months of age (96). Growth of these mice was retarded and, on sacrifice or natural death, all had massive systemic fibrin deposition with concomitant hemorrhage and fibrosis. Thus, although the PC -/-/FXI -/- mice survived the lethal perinatal coagulopathy seen in the PC -/- neonates, they nonetheless succumbed to overwhelming thrombotic disease in later life. Thus, limiting thrombin generation through FXI was not sufficient to completely compensate for the loss of the APC-mediated arrest of thrombin generation. It would be of interest to see if the PC -/- coagulopathy could be compensated by loss of either APC substrate, FV or FVIII.

FV Leiden: FV Leiden is the most common inherited risk factor for thrombophilia in humans (97, 98). FV Leiden, which has an R506Q missense mutation, is resistant to inactivation by APC. Targeted mutagenesis of the mouse factor V gene to introduce the R504Q mutation (99) produced mice with resistance to APC. The plasma APC resistance ratio was decreased from 2.1 ± 0.3 in normal mice to 1.5 ± 0.1 in heterozygous (FvQ/+), and to 1.3 ± 0.1 in homozygous (FvQ/Q) mice. Adult FvQ/Q mice appear healthy, and reproduce normally. Biosynthesis, processing and clearance of FV were not altered by this mutation in mice. Nevertheless, fibrin deposition was seen in the lung, heart, spleen, kidney and brain, suggesting chronic, low-grade thrombin generation in the vascular beds of these organs. Despite this latent prethrombotic state, the histopathology of FvQ/Q mice is unremarkable except for occasional focal hepatic fibrosis. Similar to the $TM^{Pro/Pro}$ mice, the genetic background had a profound effect on the phenotypic penetrance of the FvQ/Q mutation: on a C57Bl/6J background, FvQ/Q mice develop normally and survive, while on a mixed 129Sv-C57Bl/6J background, about 50% of FvQ/Q mice suffer from fatal disseminated intravascular thrombosis in the perinatal period. The genetic modifiers that enhance thrombosis in the mixed background remain to be identified.

Antithrombin III (ATIII): The antithrombin III gene was disrupted by targeted deletion of exon II (100). ATIII +/- mice exhibit ~50% reduced plasma antigen and functional activity, yet remain healthy in the absence of thrombotic challenges. In contrast, complete ATIII deficiency causes intra-uterine lethality. ATIII -/- embryos develop normally until day 14.5 of gestation. Thereafter, mice die from severe subcutaneous and intracranial hemorrhage that occurs in 70% of ATIII -/- embryos by E15.5 and 100% of embryos by E16.5. Histologic analysis of embryo tissues revealed tissue necrosis in the myocardium and liver. Extensive fibrin deposition was observed in liver sinusoids and within myocardial lesions, but was not associated with hemorrhagic lesions seen in other organs, including lung, brain, and kidney. The authors conclude that ATIII -/- embryos died from thrombotic organ damage to the heart and liver, and secondary fibrinogen depletion due to consumptive coagulopathy and/or liver damage, causing widespread hemorrhage.

Protein Z (PZ): The role of protein Z in hemostasis remains obscure. PZ -/- mice were phenotypically normal, with no evidence of vascular thrombosis or hepatic fibrin deposition (101). However, when these mice (PZ +/- or -/-) were bred to FV Leiden mice (+/FvQ or FvQ/FvQ), there was a reduction in survival of the FV Leiden mice due to a consumptive coagulopathy (101). These studies demonstrated the multigenic nature of thrombophilia, and predicted that protein Z deficiency in humans could be associated with thrombosis. Indeed, a significant association between ischaemic stroke and PZ deficiency in humans has recently been reported (102, 103).

Discussion/Conclusion

Similar to the factors that promote thrombin generation, the phenotypes associated with factors that curtail thrombin generation can be divided into two categories: those associated with developmental deficiencies and those associated with hemostasis problems, here with thrombosis. A clear distinction between these two groups is difficult in some cases, as the loss of either PC or ATIII led to embryonic thrombotic complications and severely affected the ability of embryos to develop. The TFPI -/-, TM -/- and ATIII -/- mice did not survive to birth. Complete deficiencies of these factors have not been reported in humans, indicating that they have a critical role in human embryogenesis. Analysis of the mouse embryos showed evidence for unchecked coagulation, as would be anticipated for these factors that curtail thrombin generation. Mice deficient in TFPI, the regulator of TF activity, display vascular abnormalities closely resembling the phenotype observed in FV -/-, FII -/-, and TF -/- mice. While the role of the TFPI in vasculogenesis is still ambiguous, these results nevertheless suggest that TF controls vascular development by converting dormant signaling molecules into agonist proteases capable of interaction with specific cell-surface receptors. Further, the rescue of the TFPI -/- mice by reduced levels of FVII is consistent with the conclusion that defective development is associated with unchecked thrombin generation. The more complex developmental phenotypes seen with TM -/- mice suggest that TM has a role in development in addition to the regulation of thrombin generation.

PC -/-, FV Leiden and $TM^{Pro/Pro}$ mice had phenotypes of thrombosis. Both the PC -/- and the factor V Leiden models recapitulate the major hallmarks of human disease associated with these gene defects. The perinatal pathology observed in protein C knockout mice closely resembles the neonatal disseminated intravascular coagulation and purpura fulminans in newborns with minimal protein C levels (104, 105). Similar to the situation in humans, the phenotype associated with the factor V Leiden mutation varied. This mutation was compatible with long-term survival in mice on a C57Bl/6J genetic background, with

only sporadic spontaneous thrombosis. In contrast, with significant contribution from the 129Sv mouse strain, factor V Leiden mice had severe thrombosis. With the first human gene mutation in TM only discovered in 1995 (106), little is known about inherited TM deficiency; data suggest a correlation with reduced TM expression and thrombotic disease in humans (107-110). The phenotype of the $TM^{Pro/Pro}$ mice suggests that disruption of TM cofactor function would be associated with thrombosis in people. Like FV Leiden, expressivity of thrombosis would be expected to vary.

In all mice where the proteins curtailing thrombin formation are completely disrupted, fatal hemorrhage associated with consumptive coagulopathy occurs in midgestation, roughly the same time that fatal bleeding occurs with deficiencies of the proteins that promote thrombin formation. The site and severity of hemorrhage in individual organs is rather distinct in these mice, suggesting that organ specific differences in the control of blood coagulation (91, 111, 112) are already established during intrauterine development. Disruption of the pathways that curtail thrombin formation, including the TM-PC system, ATIII, PZ, and TFPI, has shown that thrombin levels must be effectively controlled. Of these proteins, only TFPI deficiency is associated with vascular defects in the yolk sac or the embryonic vasculature. It therefore appears that uncontrolled thrombin formation is not detrimental to yolk sac blood vessel function/formation and does not impair other morphogenetic or differentiation processes. The placental defects in TM -/- mice might be a special case. Further investigations of these mice are warranted, as the placental abnormalities in mice with minimal TF (113) could potentially lead to novel insights about the pathogenesis of fetal growth retardation and other pregnancy complications in females with familial or acquired thrombophilia.

Additional investigations of thrombophilic mice with heterozygous gene defects also appear warranted, as current work suggests that these investigations will provide insight into the molecular basis of vascular bed-specific differences in the regulation of hemostasis. These animals also provide good experimental means to define the genetic and epigenetic risk factors that modify the phenotypic manifestation of inherited defects in coagulation factor/regulator genes. The demonstration that deficiency of protein Z is a thrombotic risk factor in factor V Leiden mice has preceded the discovery that protein Z may be a risk factor in humans. This represents a nice example to show that results obtained in mice might facilitate the rational design of studies in human populations. One can also envision development of "sensitized" screens in these deficient mice to quantitatively assess suspected risk factors or modifiers of thrombotic disease under precisely controlled environmental conditions. It appears safe to predict that an increasing number of studies of the in vivo role of hemostasis in complex processes like atherosclerosis, cancer, inflammation, aging, and reproduction will utilize many of the mouse models described here.

Conclusion

The observation that some, but not all, hemostasis proteins are essential regulators of morphogenetic processes during early embryogenesis has provided a novel link between developmental biology and blood coagulation. To date, the correlation between hemostasis and embryonic development is seen only with proteins that control thrombin levels, and not with the proteins that participate in clot formation.

Murine models have been revolutionary tools in modern science, and there are some clear advantages to using these models. We can do experiments in mice that would not be feasible in humans, and we can control genetic background and most environmental factors. Nevertheless, we must remember that animal model studies have limitations. As summarized here, some murine factors have different roles than the comparable human factors. These differences may limit interpretation of some experiments, but they should not take away from the numerous discoveries in defining new roles for proteins and confirming the roles of other proteins in specific systems. These differences can provide us with subtle clues that apply globally to various biological functions. All in all, the analysis of mice altered by gene targeting has not produced evidence for dramatic differences in hemostasis between mice and men. In fact, coagulation appears qualitatively very similar in both species (114). Nevertheless, analysis of these pathways in a different species has provided a fresh look at hemostasis, and allowed us to ask new questions. We are looking forward to Zebrafish!

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References

1. O'Reilly MS, Pirie-Shepherd S, Lane WS, Folkman J. Antiangiogenic activity of the cleaved conformation of the serpin antithrombin. Science 1999; 285: 1926-8.
2. Hogan B, Beddington R, Costantini F, Lacy E. Manipulating the Mouse Embryo. A laboratory Manual. Plainview, NY: Cold Spring Harbor Laboratory Press 1994.
3. Bugge TH, Xiao Q, Kombrinck KW, Flick MJ, Holmback K, Danton MJ, Colbert MC, Witte DP, Fujikawa K, Davie EW. Fatal embryonic bleeding events in mice lacking tissue factor, the cell-associated initiator of blood coagulation. Proc Natl Acad Sci USA 1996; 93: 6258-63.
4. Carmeliet P, Mackman N, Moons L, Luther T, Gressens P, Van Vlaenderen I, Demunck H, Kasper M, Breier G, Evrard P, Muller M, Risau W, Edgington T, Collen D. Role of tissue factor in embryonic blood vessel development. Nature 1996; 383: 73-5.
5. Toomey JR, Kratzer KE, Lasky NM, Stanton JJ, Broze GJ, Jr. Targeted disruption of the murine tissue factor gene results in embryonic lethality. Blood 1996; 88: 1583-7.
6. Parry GCN, Erlich JH, Carmeliet P, Luther T, Mackman N. Low levels of tissue factor are compatible with development and hemostasis in mice. J Clin Invest 1998; 101: 560-9.
7. Chen J, Bierhaus A, Schiekofer S, Andrassy M, Chen B, Stern DM, Nawroth PP. Tissue factor – a receptor involved in the control of cellular properties, including angiogenesis. Thromb Haemost 2001; 86: 334-45.
8. Ruf W, Mueller BM. Tissue factor signaling. Thromb Haemost 1999; 82: 175-82.
9. Parry GCN, Mackman N. Mouse embryogenesis requires the tissue factor extracellular domain but not the cytoplasmic domain. J Clin Invest 2000; 105: 1547-54.
10. Ruf W, Fischer EG, Huang HY, Miyagi Y, Ott I, Riewald M, Mueller BM. Diverse functions of protease receptor tissue factor in inflammation and metastasis. Immunol Res 2000; 21: 289-92.
11. Mackman N. Regulation of the tissue factor gene. Thromb Haemost 1997; 78: 747-54.
12. Ott I, Fischer EG, Miyagi Y, Mueller BM, Ruf W. A role for tissue factor in cell adhesion and migration mediated by interaction with actin-binding protein 280. J Cell Biol 1998; 140: 1241-53.
13. Rosen ED, Chan JC, Idusogie E, Clotman F, Vlasuk G, Luther T, Jalbert LR, Albrecht S, Zhong L, Lissens A, Schoonjans L, Moons L, Collen D,

Castellino FJ, Carmeliet P. Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. Nature 1997; 390: 290-4.
14. Yang TL, Cui J, Taylor JM, Yang A, Gruber SB, Ginsburg D. Rescue of fatal neonatal hemorrhage in factor V deficient mice by low level transgene expression. Thromb Haemost 2001; 83: 70-7.
15. Luther T, Flossel C, Mackman N, Bierhaus A, Kasper M, Albrecht S, Sage EH, Iruela-Arispe L, Grossmann H, Strohlein A, Zhang Y, Nawroth PP, Carmeliet P, Loskutoff DJ, Muller M. Tissue factor expression during human and mouse development. Am J Pathol 1996; 149: 101-13.
16. Cooper DN, Millar DS, Wacey A, Banner DW, Tuddenham EG. Inherited factor VII deficiency: molecular genetics and pathophysiology. Thromb Haemost 1997; 78: 151-60.
17. Dewerchin M, Liang Z, Moons L, Carmeliet P, Castellino FJ, Collen D, Rosen ED. Blood coagulation factor X deficiency causes partial embryonic lethality and fatal neonatal bleeding in mice. Thromb Haemost 2000; 83: 185-90.
18. Cui J, O'Shea KS, Purkayastha A, Saunders TL, Ginsburg D. Fatal haemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V. Nature 1996; 384: 66-8.
19. Sun WY, Witte DP, Degen JL, Colbert MC, Burkart MC, Holmback K, Xiao Q, Bugge TH, Degen SJ. Prothrombin deficiency results in embryonic and neonatal lethality in mice. Proc Natl Acad Sci USA 1998; 95: 7597-602.
20. Xue J, Wu Q, Westfield LA, Tuley EA, Lu D, Zhang Q, Shim K, Zheng X, Sadler JE. Incomplete embryonic lethality and fatal neonatal hemorrhage caused by prothrombin deficiency in mice. Proc Natl Acad Sci USA 1998; 95: 7603-7.
21. Ong K, Horsfall W, Conway EM, Schuh AC. Early embryonic expression of murine coagulation system components. Thromb Haemost 2000; 84: 1023-30.
22. Lin HF, Maeda N, Smithies O, Straight DL, Stafford DW. A coagulation factor IX-deficient mouse model for human hemophilia B. Blood 1997; 90: 3962-6.
23. Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian HH, Jr. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. Nat Genet 1995; 10: 119-21.
24. Bi L, Sarkar R, Naas T, Lawler AM, Pain J, Shumaker SL, Bedian V, Kazazian HH, Jr. Further characterization of factor VIII-deficient mice created by gene targeting: RNA and protein studies. Blood 1996; 88: 3446-50.
25. High KA, Roberts HR. FIX In: Molecular Basis of Thrombosis and Hemostasis. High KA and Roberts HR, eds. New York: Marcel Dekker, Inc. 1995; 215-37.
26. Fakharzadeh SS, Zhang Y, Sarkar R, Kazazian HH, Jr. Correction of the coagulation defect in hemophilia A mice through factor VIII expression in skin. Blood 2000; 95: 2799-805.
27. Kung SH, Hagstrom JN, Cass D, Tai SJ, Lin HF, Stafford DW, High KA. Human factor IX corrects the bleeding diathesis of mice with hemophilia B. Blood 1998; 91: 784-90.
28. Chuah MK, Collen D, VandenDriessche T. Gene therapy for hemophilia. J Gene Med 2001; 3: 3-20.
29. Hassan HJ, Leonardi A, Chelucci C, Mattia G, Macioce G, Guerriero R, Russo G, Mannucci PM, Peschle C. Blood coagulation factors in human embryonic-fetal development: preferential expression of the FVII/tissue factor pathway. Blood 1990; 76: 1158-64.
30. Gailani D, Lasky NM, Broze GJ, Jr. A murine model of factor XI deficiency. Blood Coagul Fibrinol 1997; 8: 134-44.
31. Gailani D, Sun MF, Sun Y. A comparison of murine and human factor XI. Blood 1997; 90: 1055-64.
32. Bloom AL. Inherited disorders of blood coagulation. In: Haemostasis and Thrombosis. Bloom AL and Thomas DP, eds. New York: Churchill Livingstone 1981; 321-70.
33. Bouma BN, Meijers JC. Role of blood coagulation factor XI in downregulation of fibrinolysis. Curr Opin Hematol 2000; 7: 266-72.
34. 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: 3035-42.
35. Meijers JC, Tekelenburg WL, Bouma BN, Bertina RM, Rosendaal FR. High levels of coagulation factor XI as a risk factor for venous thrombosis. N Engl J Med 2000; 342: 696-701.
36. Roberts HR, White GC, Jr. Inherited disorders of prothrombin conversion. In: Hemostasis and Thrombosis Basic Principles and Clinical Practice. Colman RW, Hirsh J, Marder VJ, Clowes AW, George JN, eds. Philadelphia: Lippincott Williams and Wilkins 2001; 839-53.
37. Degen SJF. Prothrombin. In: Molecular Basis of Thrombosis and Hemostasis. High KA and Roberts HR, eds. New York: Marcel Dekker, Inc. 1995; 75-99.
38. Suh TT, Holmback K, Jensen NJ, Daugherty CC, Small K, Simon DI, Potter S, Degen JL. Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. Genes Dev 1995; 9: 2020-33.
39. Ploplis VA, Wilberding J, McLennan L, Liang Z, Cornelissen I, DeFord ME, Rosen ED, Castellino FJ. A total fibrinogen deficiency is compatible with the development of pulmonary fibrosis in mice. Am J Pathol 2000; 157: 703-8.
40. Shivdasani RA, Rosenblatt MF, Zucker-Franklin D, Jackson CW, Hunt P, Saris CJ, Orkin SH. Transcription factor NF-E2 is required for platelet formation independent of the actions of thrombopoietin/MGDF in megakaryocyte development. Cell 1995; 81: 695-704.
41. Coughlin SR. How the protease thrombin talks to cells. Proc Natl Acad Sci USA 1999; 96: 11023-7.
42. Connolly AJ, Ishihara H, Kahn ML, Farese RVJ, Coughlin SR. Role of the thrombin receptor in development and evidence for a second receptor. Nature 1996; 381: 516-9.
43. Griffen CT, Srinivasan Y, Zheng YW, Huang W, Coughlin SR. A role for thrombin receptor signaling in endothelial cells during embryonic development. Science 2001; 293: 1666-70.
44. Coughlin SR. Thrombin signalling and protease-activated receptors. Nature 2000; 407: 258-64.
45. Soifer SJ, Peters KG, O'Keefe J, Coughlin SR. Disparate temporal expression of the prothrombin and thrombin receptor genes during mouse development. Am J Pathol 1994; 144: 60-9.
46. Camerer E, Huang W, Coughlin SR. Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. Proc Natl Acad Sci USA 2000; 97: 5255-60.
47. Ollivier V, Chabbat J, Herbert JM, Hakim J, de Prost D. Vascular endothelial growth factor production by fibroblasts in response to factor VIIa binding to tissue factor involves thrombin and factor Xa. Arterioscler Thromb Vasc Biol 2000; 20: 1374-81.
48. Clauss M, Grell M, Fangmann C, Fiers W, Scheurich P, Risau W. Synergistic induction of endothelial tissue factor by tumor necrosis factor and vascular endothelial growth factor: functional analysis of the tumor necrosis factor receptors. FEBS Letters 1996; 390: 334-8.
49. Clauss M, Gerlach M, Gerlach H, Brett J, Wang F, Familletti PC, Pan YC, Olander JV, Connolly DT, Stern D. Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. J Exp Med 1990; 172: 1535-45.
50. Zucker S, Mirza H, Conner CE, Lorenz AF, Drews MH, Bahou WF, Jesty J. Vascular endothelial growth factor induces tissue factor and matrix metalloproteinase production in endothelial cells: conversion of prothrombin to thrombin results in progelatinase A activation and cell proliferation. Int J Cancer 1998; 75: 780-6.
51. Takano S, Tsuboi K, Tomono Y, Mitsui Y, Nose T. Tissue factor, osteopontin, alphavbeta3 integrin expression in microvasculature of gliomas associated with vascular endothelial growth factor expression. Br J Cancer 1967; 82: 1967-73.
52. Abe K, Shoji M, Chen J, Bierhaus A, Danave I, Micko C, Casper K, Dillehay DL, Nawroth PP, Rickles FR. Regulation of vascular endothelial

growth factor production and angiogenesis by the cytoplasmic tail of tissue factor. Proc Natl Acad Sci USA 1999; 96: 8663-8.
53. Zhang Y, Deng Y, Luther T, Muller M, Ziegler R, Waldherr R, Stern DM, Nawroth PP. Tissue factor controls the balance of angiogenic and antiangiogenic properties of tumor cells in mice. J Clin Invest 1994; 94: 1320-7.
54. Tsopanoglou NE, Maragoudakis ME. On the mechanism of thrombin-induced angiogenesis. Potentiation of vascular endothelial growth factor activity on endothelial cells by up-regulation of its receptors. J Biol Chem 1999; 274: 23969-76.
55. Ni H, Denis CV, Subbarao S, Degen JL, Sato TN, Hynes RO, Wagner DD. Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. J Clin Invest 2000; 106: 385-92.
56. Bugge TH, Flick MJ, Daugherty CC, Degen JL. Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction. Genes Dev 1995; 9: 794-807.
57. Ploplis VA, Carmeliet P, Vazirzadeh S, Van Vlaenderen I, Moons L, Plow EF, Collen D. Effects of disruption of the plasminogen gene on thrombosis, growth, and health in mice. Circulation 1995; 92: 2585-93.
58. Ploplis VA. Gene targeting in hemostasis. Plasminogen. Front Biosci 2001; 6: D555-69.
59. Carmeliet P, Moons L, Ploplis VA, Plow E, Collen D. Impaired arterial neointima formation in mice with disruption of the plasminogen gene. J Clin Invest 1997; 99: 200-8.
60. Romer J, Bugge TH, Pyke C, Lund LR, Flick MJ, Degen JL, Dano K. Impaired wound healing in mice with a disrupted plasminogen gene. Nat Med 1996; 2: 287-92.
61. Drew AF, Kaufman AH, Kombrinck KW, Danton MJS, Daugherty CC, Degen JL, Bugge TH. Ligneous conjunctivitis in plasminogen-deficient mice. Blood 1998; 91: 1616-24.
62. Bugge TH, Kombrinck KW, Flick MJ, Daugherty CC, Danton MJ, Degen JL. Loss of fibrinogen rescues mice from the pleiotropic effects of plasminogen deficiency. Cell 1996; 87: 709-19.
63. Bezerra JA, Bugge TH, Melin-Aldana H, Sabla G, Kombrinck KW, Witte DP, Degen JL. Plasminogen deficiency leads to impaired remodeling after a toxic injury to the liver. Proc Natl Acad Sci USA 1999; 96: 15143-8.
64. Holmback K, Danton MJ, Suh TT, Daugherty CC, Degen JL. Impaired platelet aggregation and sustained bleeding in mice lacking the fibrinogen motif bound by integrin alpha IIb beta 3. EMBO J 1996; 15: 5760-71.
65. Hogan KA, Maeda N, Kluckman KD, Lord ST. Synthesis of a mouse model of the dysfibrinogen Vlissingen/Frankfurt IV. Ann NY Acad Sci 2001; 936: 117-21.
66. Gulledge AA, Rezaee F, Verheijen JH, Lord ST. A novel transgenic mouse model of hyperfibrinogenemia. Thromb Haemost 2001; 86: 511-6.
67. Denis C, Methia N, Frenette PS, Rayburn H, Ullman-Cullere M, Hynes RO, Wagner DD. A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. Proc Natl Acad Sci USA 1998; 95: 9524-9.
68. Subramaniam M, Frenette PS, Saffaripour S, Johnson RC, Hynes RO, Wagner DD. Defects in hemostasis in P-selectin-deficient mice. Blood 1996; 87: 1238-42.
69. Frenette PS, Johnson RC, Hynes RO, Wagner DD. Platelets roll on stimulated endothelium in vivo: an interaction mediated by endothelial P-selectin. Proc Natl Acad Sci USA 1995; 92: 7450-4.
70. Romo GM, Dong JF, Schade AJ, Gardiner EE, Kansas GS, Li CQ, McIntire LV, Berndt MC, Lopez JA. The glycoprotein Ib-IX-V complex is a platelet counterreceptor for P-selectin. J Exp Med 1999; 190: 803-14.
71. Denis CV, Andre P, Saffaripour S, Wagner DD. Defect in regulated secretion of P-selectin affects leukocyte recruitment in von Willebrand factor-deficient mice. Proc Natl Acad Sci USA 2001; 98: 4072-7.
72. Methia N, Andre P, Denis CV, Economopoulos M, Wagner DD. Localized reduction of atherosclerosis in von Willebrand factor-deficient mice. Blood 2001; 98: 1424-8.
73. Duga S, Asselta R, Santagostino E, Zeinali S, Simonic T, Malcovati M, Mannucci PM, Tenchini ML. Missense mutations in the human β fibrinogen gene cause congenital afibrinogenemia by impairing fibrinogen secretion. Blood 2000; 95: 1336-41.
74. Neerman-Arbez M, Honsberger A, Antonarakis SE, Morris MA. Deletion of the fibrinogen alpha-chain gene (FGA) causes congenital afibrinogenemia. J Clin Invest 1999; 103: 215-8.
5. Lak M, Keihani M, Elahi F, Peyvandi F, Mannucci PM. Bleeding and thrombosis in 55 patients with inherited afibrinogenaemia. Br J Haematol 1999; 107: 204-6.
76. Montgomery RR. Structure and function of von Willebrand Factor. In: Hemostasis and Thrombosis Basic Principles and Clinical Practices. RW Colman, Hirsh J, Marder VJ, Clowes AW, George JN, eds. Philadelphia: Lippincott Williams and Wilkins 2001; 250-74.
77. Cronin C, Fitzpatrick D, Temperley I. Multiple pulmonary emboli in a patient with afibrinogenaemia. Acta Haematol 1988; 79: 53-4.
78. Ingram GI, McBrien DJ, Spencer H. Fatal pulmonary embolus in congenital fibropenia. Report of two cases. Acta Haematol 1966; 35: 56-62.
79. Hodivala-Dilke KM, McHugh KP, Tsakiris DA, Rayburn H, Crowley D, Ullman-Cullere M, Ross FP, Coller BS, Teitelbaum S, Hynes RO. Beta3-integrin deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. J Clin Invest 1999; 103: 229-38.
80. Ni H, Ramakrishnan V, Ruggeri ZM, Papalia JM, Phillips DR, Wagner DD. Increased thrombogenesis and embolus formation in mice lacking glycoprotein V. Blood 2001; 98: 368-73.
81. Green FR. Taking the hype out of hyperfibrinogenemia. Thromb Haemost 2001; 86: 509-10.
82. Xiao Q, Danton MS, Witte DP, Kowala MC, Valentine MT, Degen JL. Fibrinogen deficiency is compatible with the development of atherosclerosis in mice. J Clin Invest 1998; 101: 1184-94.
83. Lou XJ, Boonmark NW, Horrigan FT, Degen JL, Lawn RM. Fibrinogen deficiency reduces vascular accumulation of apolipoprotein(a) and developmentof atherosclerosis in apolipoprotein (a) transgenic mice. Proc Natl Acad Sci USA 1998; 95: 12591-5.
84. Huang ZF, Higuchi D, Lasky N, Broze GJ, Jr. Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice. Blood 1997; 90: 944-51.
85. Huang ZF, Broze G, Jr. Consequences of tissue factor pathway inhibitor gene-disruption in mice. Thromb Haemost 1997; 78: 699-704.
86. Chan JC, Carmeliet P, Moons L, Rosen ED, Huang ZF, Broze GJ, Jr., Collen D, Castellino FJ. Factor VII deficiency rescues the intrauterine lethality in mice associated with a tissue factor pathway inhibitor deficit. J Clin Invest 1999; 103: 475-82.
87. Healy AM, Rayburn HB, Rosenberg RD, Weiler H. Absence of the blood-clotting regulator thrombomodulin causes embryonic lethality in mice before development of a functional cardiovascular system. Proc Natl Acad Sci USA 1995; 92: 850-4.
88. Rosenberg RD. Thrombomodulin gene disruption and mutation in mice. Thromb Haemost 1997; 78: 705-9.
89. Conway EM, Pollefeyt S, Cornelissen J, DeBaere I, Steiner-Mosonyi M, Weitz JI, Weiler-Guettler H, Carmeliet P, Collen D. Structure-function analyses of thrombomodulin by gene-targeting in mice: the cytoplasmic domain is not required for normal fetal development. Blood 1999; 93: 3442-50.
90. Isermann B, Hendrickson SB, Hutley K, Wing M, Weiler H. Tissue-restricted expression of thrombomodulin in the placenta rescues thrombomodulin-deficient mice from early lethality and reveals a secondary developmental block. Development 2001; 128: 827-38.
91. Weiler-Guettler H, Christie PD, Beeler DL, Healy AM, Hancock WW, Rayburn H, Edelberg JM, Rosenberg RD. A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. J Clin Invest 1998; 101: 1983-91.
92. Weiler H, Lindner V, Kerlin B, Isermann BH, Hendrickson SB, Cooley BC, Meh DA, Mosesson MW, Shworak NW, Post MJ, Conway EM, Ulf-

man LH, von Andrian UH, Weitz JI. Characterization of a mouse model for thrombomodulin deficiency. Arterioscler Thromb Vasc Biol 2001; 21: 1531-7.
93. Isermann B, Hendrickson SB, Zogg M, Wing M, Cummiskey M, Kisanuki YY, Yanagisawa M, Weiler H. Endothelium-specific loss of murine thrombomodulin disrupts the protein C anticoagulant pathway and causes juvenile-onset thrombosis. J Clin Invest 2001; 108: 537-46.
94. Jalbert LR, Rosen ED, Moons L, Chan JC, Carmeliet P, Collen D, Castellino FJ. Inactivation of the gene for anticoagulant protein C causes lethal perinatal consumptive coagulopathy in mice. J Clin Invest 1998; 102: 1481-8.
95. Chan JC, Cornelissen I, Collen D, Ploplis VA, Castellino FJ. Combined factor VII/protein C deficiency results in intrauterine coagulopathy in mice. J Clin Invest 2000; 105: 897-903.
96. Chan JC, Ganopolsky JG, Cornelissen I, Suckow MA, Sandoval-Coope MJ, Brown EC, Noria F, Gailani D, Rosen ED, Ploplis VA, Castellino F. The characterization of mice with a targeted combined deficiency of protein C and factor XI. Am J Pathol 2001; 158: 469-79.
97. Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velden PA, Reitsma PH. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 1994; 369: 64-7.
98. van der Meer FJ, Koster T, Vandenbroucke JP, Briet E, Rosendaal FR. The Leiden Thrombophilia Study (LETS). Thromb Haemost 1997; 78: 631-5.
99. Cui J, Eitzman DT, Westrick RJ, Christie PD, Xu ZJ, Yang AY, Purkayastha AA, T.L. Y, Metz AL, Gallagher KP, Tyson JA, Rosenverg RD, Ginsburg D. Spontaneous thrombosis in mice carrying the factor V Leiden mutation. Blood 2000; 96: 4222-6.
100. Ishiguro K, Kojima T, Kadomatsu K, Nakayama Y, Takagi A, Suzuki M, Takeda N, Ito M, Yamamoto K, Matsushita T, Kusugami K, Muramatsu T, Saito H. Complete antithrombin deficiency in mice results in embryonic lethality. J Clin Invest 2000; 106: 873-8.
101. Yin ZF, Huang ZF, Cui J, Fiehler R, Lasky N, Ginsburg D, GJ, Jr. Prothrombotic phenotype of protein Z deficiency. Proc Natl Acad Sci USA 2000; 97: 6734-8.
102. Broze GJ, Jr. Protein-Z and thrombosis. Lancet 2001; 357: 900-1.
103. Vasse M, Guegan-Massardier E, Borg JY, Woimant F, Soria C. Frequency of protein Z deficiency in patients with ischaemic stroke. Lancet 2001; 357: 933-4.
104. Berdeaux DH, Abshire TC, Marlar RA. Dysfunctional protein C deficiency (type II). A report of 11 cases in 3 American families and review of the literature. Am J Clin Pathol 1993; 99: 677-86.
105. Nizzi FA, Jr., Kaplan HS. Protein C and S deficiency. Semin Thromb Hemost 1999; 25: 265-72.
106. Ohlin AK, Marlar RA. The first mutation identified in the thrombomodulin gene in a 45-year-old man presenting with thromboembolic disease. Blood 1995; 85: 330-6.
107. Ohlin AK, Marlar RA. Thrombomodulin gene defects in families with thromboembolic disease – a report on four families. Thromb Haemost 1999; 81: 338-44.
108. Salomaa V, Matei C, Aleksic N, Sansores-Garcia L, Folsom AR, Juneja H, Chambless LE, Wu KK. Soluble thrombomodulin as a predictor of incident coronary heart disease and symptomless carotid artery atherosclerosis in the Atherosclerosis Risk in Communities (ARIC) Study: a case-cohort study. Lancet 1999; 353: 1729-34.
109. Norlund L, Zoller B, Ohlin AK. A novel thrombomodulin gene mutation in a patient suffering from sagittal sinus thrombosis. Thromb Haemost 1997; 78: 1164-6.
110. Kunz G, Ireland HA, Stubbs PJ, Kahan M, Coulton GC, Lane DA. Identification and characterization of a thrombomodulin gene mutation coding for an elongated protein with reduced expression in a kindred with myocardial infarction. Blood 2000; 95: 569-76.
111. Christie PD, Edelberg JM, Picard MH, Foulkes AS, Mamuya W, Weiler-Guettler H, Rubin RH, Gilbert P, Rosenberg RD. A murine model of myocardial microvascular thrombosis. J Clin Invest 1999; 104: 533-9.
112. Rosenberg RD, Aird WC. Mechanisms of disease: vascular-bed-specific hemostasis and hypercoagulable states. N Engl J Med 1999; 340: 1555-64.
113. Erlich J, Parry GC, Fearns C, Muller M, Carmeliet P, Luther T, Mackman N. Tissue factor is required for uterine hemostasis and maintenance of the placental labyrinth during gestation. Proc Natl Acad Sci USA 1999; 96: 8138-43.
114. Tsakiris DA, Scudder L, Hodivala-Dilke K, Hynes RO, Coller BS. Hemostasis in the mouse (mus musculus): a review. Thromb Haemost 1999; 81: 177-88.
115. Mackman N. Gene targeting in hemostasis. Tissue factor. Front Biosci 2001; 6: D208-15.
116. Chan JC. Gene targeting in hemostasis. Factor VII. Front Biosci 2001; 6: D98-104.
117. Sun WY, Degen SJ. Gene targeting in hemostasis. Prothrombin. Front Biosci 2001; 6: D222-38.
118. Gailani D. Gene targeting in hemostasis. FXI. Front Biosci 2001; 6: D201-7.
119. Drew AF, Liu H, Davidson JM, Daugherty CC, Degen JL. Wound-healing defects in mice lacking fibrinogen. Blood 2001; 97: 3691-8.
120. Degen JL. Genetic interactions between the coagulation and fibrinolytic system. Thromb Haemost 2001; 86: 130-7.
121. Ariens RAS, Alberio G, Moia M, Mannucci PM. Low levels of heparin-releaseable tissue factor pathway inhibitor in young patients with thrombosis. Thromb Haemost 1999; 81: 203-7.
122. Chan JC. Gene targeting in hemostasis. Tissue factor pathway inhibitor. Front Biosci 2001; 6: D216-21.
123. Bock SC. Antithrombin III and Heparin Cofactor II. In: Thrombosis and Hemostasis Basic Principles and clinical Practice. Colman RW, Hirsh J, Marder VJ, Clowes AW, George JN, eds. Philadelphia: Lippincott Williams and Wilkins 2001; 321-33.

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