DISEASES AND DISORDERS

The endothelial protein C receptor plays an essential role in the maintenance of pregnancy

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Placenta-mediated pregnancy complications are a major challenge in the management of maternal-fetal health. Maternal thrombophilia is a suspected risk factor, but the role of thrombotic processes in these complications has remained unclear. Endothelial protein C receptor (EPCR) is an anticoagulant protein highly expressed in the placenta. EPCR autoantibodies and gene variants are associated with poor pregnancy outcomes. In mice, fetal EPCR deficiency results in placental failure and in utero death. We show that inhibition of molecules involved in thrombin generation or in the activation of maternal platelets allows placental development and embryonic survival. Nonetheless, placentae exhibit venous thrombosis in uteroplacental circulation associated with neonatal death. In contrast, maternal EPCR deficiency results in clinical and histological features of placental abruption and is ameliorated with concomitant Par4 deficiency. Our findings unveil a causal link between maternal thrombophilia, uterine hemorrhage, and placental abruption and identify Par4 as a potential target of therapeutic intervention.

INTRODUCTION

The placenta plays key roles in supporting fetal development and preserving maternal health during pregnancy and parturition. Maternal thrombophilia is a suspected risk factor for placenta-mediated pregnancy complications that include recurrent pregnancy loss, intrauterine growth restriction, stillbirth, placental abruption, and preeclampsia (*1*, *2*). These complications affect a substantial proportion of all pregnancies and are a major cause of maternal and fetal morbidity and mortality (*3*–*5*). The extent of association between inherited thrombophilia and pregnancy complications varies between populations, and causality has not been clearly established. The specifics of placental pathology associated with thrombophilia and whether it can be prevented with antithrombotic therapy remain unclear. Treatment of these disorders with low–molecular weight heparin (LMWH) has shown mixed outcomes; recent meta-analyses suggest absence of significant overall benefit (*6*–*8*). Animal models are necessary to ascertain the importance of anticoagulant gene expression in placental function, identify the nature of thrombophilia-associated adverse outcomes, and determine whether anti-thrombotic approaches prevent these outcomes. The placenta and the yolk sac are characterized by three distinct blood circulations—the uteroplacental, the umbilicoplacental, and the vitelline circulations—each of which could be affected by coagulation abnormalities. Humans and rodents form a hemochorial placenta where maternal blood directly bathes extraembryonic trophoblast cells lining the vascular spaces of uteroplacental circulation. Trophoblast cells are highly invasive and occupy additional endovascular and extravascular locations in the decidua and myometrium. Although epithelial in origin, trophoblast cells regulate local hemostasis by expressing a repertoire of hemostatic and fibrinolytic factors (*9*). Thus, in addition to maternal thrombophilia, fetal gene defects expressed on trophoblast cells are candidate risk factors for coagulation abnormalities at the fetomaternal interface. These abnormalities could activate maternal platelets triggering thrombosis and/or inflammation. Given the low half maximal effective concentration of thrombin-mediated platelet activation, it is unclear whether treatment with LMWH would reduce local thrombin generation to levels below those needed for platelet activation. In this work, we used mice with endothelial protein C receptor (EPCR) deficiency to examine whether maternal platelet activation might play a key role in placental failure due to fetal thrombophilia. We next investigated whether maternal thrombophilia is associated with pregnancy complications and the potential for therapeutic targeting of platelet activation.

EPCR is an anticoagulant protein highly expressed on trophoblast cells (*9*–*10*). EPCR binds to protein C (PC) and facilitates its activation by the thrombin-thrombomodulin complex. This interaction requires localization of EPCR and thrombomodulin on membrane surfaces (*11*). Activated PC (aPC) inhibits excessive thrombin generation by limited proteolytic cleavage and inactivation of coagulation factors Va and VIIIa. EPCR gene polymorphisms are associated with recurrent miscarriages and unexplained pregnancy loss (*12*–*14*). Notably, functional deficiency of EPCR is created by its shedding from the membrane surface by the action of metalloproteases (*15*, *16*). EPCR shedding is favored by certain polymorphisms and increased by thrombin and inflammatory mediators (*17*–*20*). Increased soluble EPCR is observed in the plasma in inflammatory and infectious conditions [examples in (*15*–*21*)] and in association with fetal death (*22*). Inhibitory EPCR autoantibodies have been found in antiphospholipid syndrome and are an independent risk factor for fetal death (*23*, *24*). In mice, genetic inactivation of EPCR in the fetus results in placental thrombosis and midgestational fetal death (*25*). Intrauterine death of EPCR-deficient mice is prevented by concomitant reduction in tissue factor (TF) or by maintaining EPCR expression on trophoblast cells. Yet, treatment of pregnant dams with LMWH prolongs survival of only a small fraction of EPCR-deficient embryos (*26*). Mice carrying a variant of EPCR with impaired ability to bind PC/aPC are viable without reported placental thrombosis and intrauterine death (*27*). Although most well studied for their role in anticoagulation, previously unknown roles of aPC and EPCR in stem cell function (*28*–*31*), inflammation

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(*32*–*34*), and immunity (*35*, *36*) have recently emerged. Many of these roles involve EPCR-mediated intracellular signaling, rather than anticoagulation through enhanced inactivation of factors Va and VIIIa (*37*, *38*). Thus, the mechanism of placental failure and whether it is primarily mediated by the loss of anticoagulation by extraembryonic EPCR remains unclear. EPCR is widely expressed on maternal tissues that include vascular and extravascular cells of the decidua and tissue-resident immune cells. It is unknown whether maternal deficiency of EPCR causes placenta-mediated pregnancy complications. Here, we report severe pregnancy complications in EPCR-deficient female mice and investigate the role of maternal platelets and placental thrombosis in pregnancy complications arising from fetal or maternal EPCR deficiency.

RESULTS

Maternal platelets closely associate with trophoblast cells of early conceptus

Platelet activation is an integral part of thrombosis. Maternal platelets adhere to invading trophoblast cells in human placenta (*39*). Using platelet- and trophoblast-specific antibodies, we identified platelets juxtaposed to extraembryonic trophoblast cells at the implantation site of early murine pregnancy on day 7.5 of gestation (Fig. 1A). By day 9.5 of gestation, maternal blood spaces lined by trophoblast cells were easily visible (Fig. 1, B and C). Platelets were seen adhering to trophoblast cells or in the vicinity of their cytokeratin-stained membranous extensions within these blood spaces. Thus, unfettered activation of maternal platelets could result in granule release, heterotypic interactions with immune cells, and/or thrombotic aggregation contributing to placental failure.

Thrombin activation of maternal platelets kills EPCR-deficient embryos

Analogous to protease-activated receptor 1 (PAR1) and PAR4 on human platelets, murine platelets express dual thrombin receptors Par3 and Par4. Par3 promotes cleavage and activation of Par4 at low thrombin concentration. At higher concentrations, thrombin can activate Par4 independently of Par3. Murine platelets lacking Par4 are unresponsive to thrombin (*40*). We conducted genetic studies to determine whether activation of maternal platelets via thrombin receptor Par3 and Par4 plays a critical role in placental failure of EPCR-deficient mice. Mice lacking EPCR die in utero before 11.5 days post coitum (dpc) in pregnancies from EPCR$^{+/-}$ intercrosses [Table 1, rows 1 and 2; and reported by Gu *et al.* (*25*)]. We analyzed pregnancies

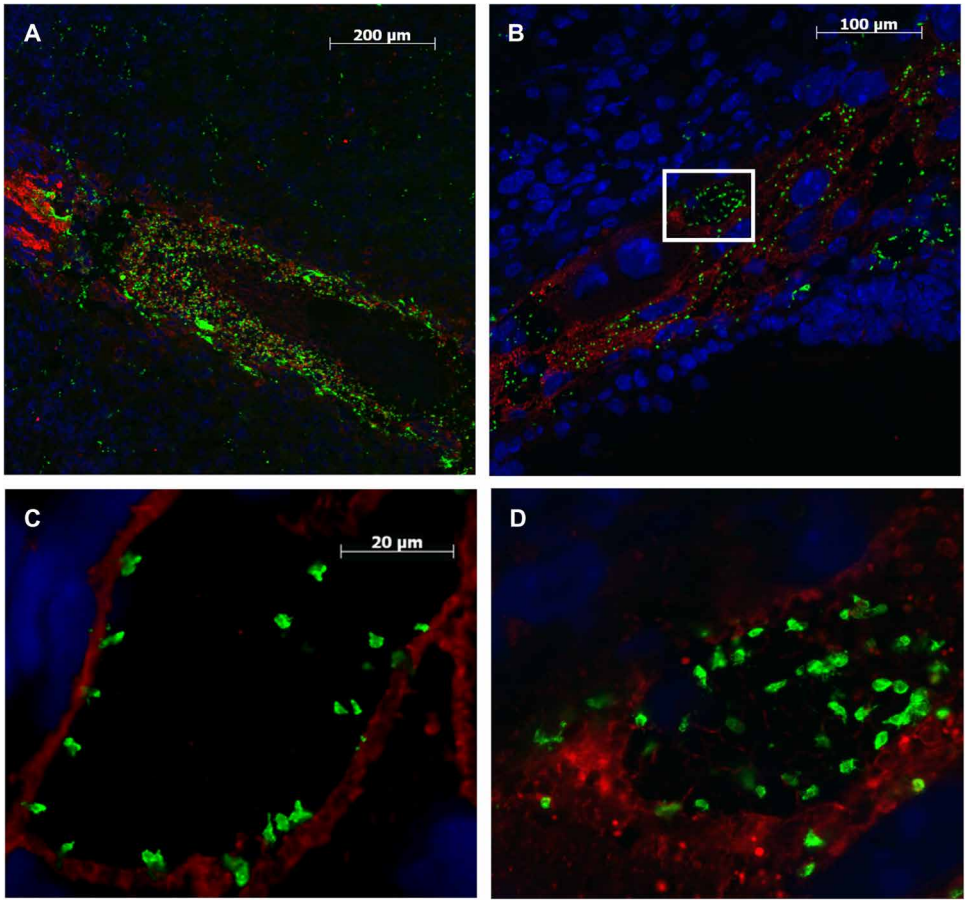

Fig. 1. Platelets closely associate with trophoblast cells in early conceptus. Platelets (stained green with GP1bβ antibodies) surround the implantation site of murine placenta at 7.5 days post coitum (dpc). (**A**) and are seen in close proximity to trophoblast cells (stained red with cytokeratin antibodies) throughout early placental development [(**B** and **D**) 9.5 dpc and (**C**) 10.5 dpc]. Nuclei are stained blue with 4′,6-diamidino-2-phenylindole. (D) is a magnified image of the boxed area in (B). Scale bars, 200 μm (A), 100 μm (B), and 20 μm (C and D).

Table 1. Pregnancy outcomes from EPCR$^{+/-}$ intercrosses. Outcomes of intercrosses with and without inactivation of Par4, Par3, αIIb, or FVIII in the mother, father, or both parents are shown. GOF, goodness of fit. * denotes significant *P* values <0.05.

Row no.	Father	Mother	Stage of analysis	Offspring			% EPCR$^{-/-}$ (95% confidence interval)	*P* value, (χ^2 GOF)	No. aborted	Total no. offspring (pregnancies) analyzed
				EPCR$^{+/+}$	EPCR$^{+/-}$	EPCR$^{-/-}$				
1	EPCR$^{+/-}$	EPCR$^{+/-}$	11.5 dpc	14	27	0	0 (0–8.6)	0.001*	21	41 (7)
2	EPCR$^{+/-}$	EPCR$^{+/-}$	4-week pups	11	19	0	0 (0–11.6)	0.006*	–	30 (5)
3	EPCR$^{+/-}$	Par4$^{-/-}$ EPCR$^{+/-}$	11.5 dpc	8	8	8	33.3 (15.6–55.3)	0.264	0	24 (3)
4	Par4$^{-/-}$ EPCR$^{+/-}$	EPCR$^{+/-}$	11.5 dpc	10	21	0	0 (0–11.2)	0.006*	19	31 (7)
5	EPCR$^{+/-}$	Par3$^{-/-}$ EPCR$^{+/-}$	11.5 dpc	8	14	8	26.7 (12.3–45.9)	0.936	4	30 (4)
6	EPCR$^{+/-}$	αIIb$^{-/-}$ EPCR$^{+/-}$	11.5 dpc	11	26	13	26 (14.6–40.4)	0.887	6	50 (7)
7	EPCR$^{+/-}$	Par4$^{-/-}$ EPCR$^{+/-}$	4-week pups	22	36	8	12.9 (5.7–23.9)	0.039*	–	66 (11)
8	EPCR$^{+/-}$	Par3$^{-/-}$ EPCR$^{+/-}$	4-week pups	14	30	1	2.2 (0.1–11.8)	0.002*	–	45 (9)
9	EPCR$^{+/-}$	αIIb$^{-/-}$ EPCR$^{+/-}$	4-week pups	14	28	4	8.7 (2.4–20.8)	0.038*	–	46 (10)
10	EPCR$^{+/-}$	Par4$^{-/-}$ EPCR$^{+/-}$	18.5 dpc	11	17	6	17.7 (6.8–34.5)	0.479	3	34 (6)
11	EPCR$^{+/-}$	Par4$^{-/-}$ EPCR$^{+/-}$	Neonates	24	41	17	20.7 (12.6–31.1)	0.550	–	82 (14)
12	EPCR$^{+/-}$	Par3$^{-/-}$ EPCR$^{+/-}$	18.5 dpc	11	16	9	25 (12.1–42.2)	0.717	8	36 (6)
13	EPCR$^{+/-}$	Par3$^{-/-}$ EPCR$^{+/-}$	Neonates	22	44	10	13.2 (6.5–22.9)	0.058	–	76 (13)
14	EPCR$^{+/-}$	αIIb$^{-/-}$ EPCR$^{+/-}$	18.5 dpc	15	29	8	15.4 (6.9–28.1)	0.276	5	52 (8)
15	Par4$^{-/-}$ EPCR$^{+/-}$	Par4$^{-/-}$ EPCR$^{+/-}$	4-week pups	27	71	13	11.7 (6.4–19.2)	0.002*	–	111 (30)
16	Par3$^{-/-}$ EPCR$^{+/-}$	Par3$^{-/-}$ EPCR$^{+/-}$	4-week pups	30	47	3	3.8 (0.8–10.6)	3×10^{-5}*	–	80 (16)
17	αIIb$^{-/-}$ EPCR$^{+/-}$	αIIb$^{-/-}$ EPCR$^{+/-}$	4-week pups	24	61	6	6.6 (2.5–13.8)	1×10^{-4}*	–	91 (25)
18	FVIII$^{-/y}$ EPCR$^{+/-}$	FVIII$^{-/-}$ EPCR$^{+/-}$	18.5 dpc	10	26	12	25 (13.6–39.6)	0.779	3	48 (7)
19	FVIII$^{-/y}$ EPCR$^{+/-}$	FVIII$^{-/-}$ EPCR$^{+/-}$	4-week pups	25	41	10	13.2 (6.5–22.9)	0.041	–	76 (15)

of Par4$^{-/-}$ EPCR$^{+/-}$ dams sired by EPCR$^{+/-}$ males at 11.5 dpc (Table 1, row 3). Absence of Par4 in the mother allowed embryonic development of EPCR$^{-/-}$ embryos (Table 1, compare rows 1 and 3; $P << 0.001$, χ^2 test of independence). These were normal in appearance and showed placental development comparable to littermate EPCR$^{+/+}$ controls (Fig. 2, A to F). In this genetic experiment, the mother lacked Par4 and the fetuses (and their extraembryonic tissues) were heterozygous for Par4. Par4 is expressed on a variety of cell types that include trophoblast cells (*9*). To confirm that these results reflect maternal Par4 deficiency rather than fetal haploinsufficiency, we performed reverse genetic crosses where Par4$^{-/-}$ EPCR$^{+/-}$ males sired pregnancies of EPCR$^{+/-}$ dams. The absence of Par4 in the father alone did not protect EPCR$^{-/-}$ embryos from midgestational death; no live Par4$^{+/-}$ EPCR$^{-/-}$ embryos were found at 11.5 dpc in these pregnancies (Table 1, row 4). Thus, maternal, but not paternal, deficiency of Par4 protects EPCR-deficient embryos from placental failure and midgestational demise. We next analyzed pregnancies of Par3$^{-/-}$ EPCR$^{+/-}$ dams sired by EPCR$^{+/-}$ males. The absence of Par3 in the mother restored Mendelian frequency of EPCR-deficient embryos at 11.5 dpc (Table 1, row 5) and conferred significant protection (Table 1, compare rows 1 and 5; $P << 0.001$, χ^2 test of independence). EPCR$^{-/-}$ placentae appeared normal and unremarkable

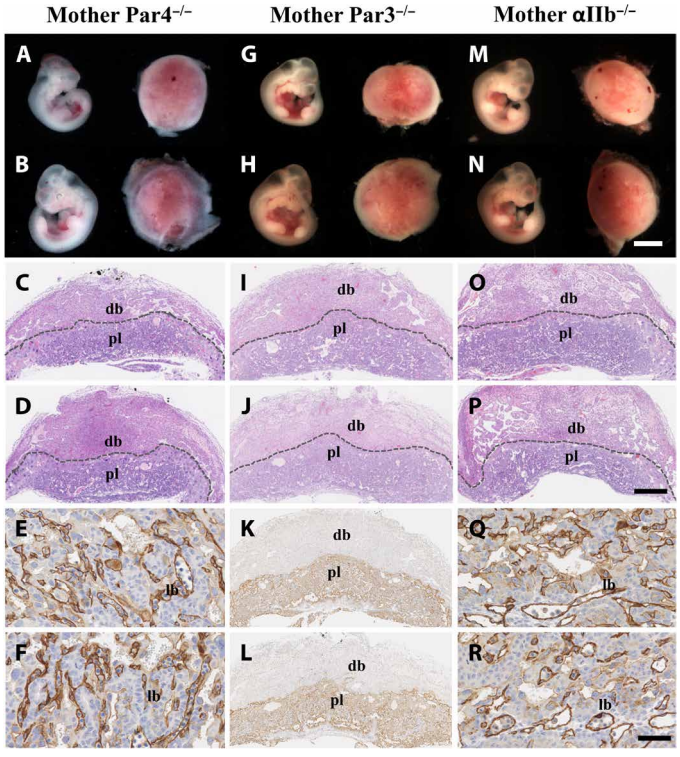

Fig. 2. EPCR-deficient embryos and placentae develop past midgestation. Genetic inactivation of Par4, Par3, or αIIb in the mother allows placental and embryonic development of EPCR-deficient mice past 10.5 dpc. Representative images of progeny analyzed at 11.5 dpc in pregnancies of Par4$^{-/-}$ EPCR$^{+/-}$ (**A** to **F**), Par3$^{-/-}$ EPCR$^{+/-}$ (**G** to **L**), or αIIb$^{-/-}$ EPCR$^{+/-}$ (**M** to **R**) dams sired by EPCR$^{+/-}$ males are shown. Top two rows show whole mounts of EPCR$^{-/-}$ (A, G, and M) and littermate EPCR$^{+/+}$ (B, H, and N) embryos and placentae. Middle two rows show hematoxylin and eosin–stained histological sections of placentae corresponding to EPCR$^{-/-}$ (C, I, and O) and EPCR$^{+/+}$ (D, J, and P) embryos. Dashed lines delineate placenta (pl) from decidua basalis (db). Bottom two rows show amplified images of labyrinth (lb) regions of EPCR$^{-/-}$ (E, K, and Q) and EPCR$^{+/+}$ (F, L, and R) placentae immunostained with CD31 (E, F, Q, and R) or cytokeratin antibodies (K and L). Scale bars, 2 mm (whole mounts), 50 μm [placental sections (E, F, O, and R)], and 500 μm (all other placental sections).

upon histological examination (Fig. 2, G to L). Reverse genetic crosses (pregnancies of EPCR$^{+/-}$ females sired by Par3$^{-/-}$ EPCR$^{+/-}$ males) did not produce any live EPCR$^{-/-}$ embryos at 11.5 dpc and exhibited a high abortion rate [7 EPCR$^{+/+}$, 7 EPCR$^{+/-}$, 0 EPCR$^{-/-}$, 10 aborted, 3 pregnancies analyzed, $P = 0.030$, χ^2 goodness of fit (GOF)]. These results suggested that thrombin-mediated activation of maternal platelets plays a key role in placental failure of EPCR-deficient embryos.

Aspirin has been tested for its ability to improve outcomes in human pregnancy. It inhibits Cox-1 and Cox-2 enzymes and reduces platelet aggregation. In EPCR$^{+/-}$ intercrosses, aspirin treatment of pregnant dams prolonged the survival of EPCR-deficient embryos but did not restore normal placental development (note S1 and fig. S1, A to E). In contrast, depletion of maternal platelets restored placental development and allowed survival of EPCR-deficient embryos (note S2 and fig. S1, F to I). Upon activation by thrombin, platelets convert integrin αIIbβ3 to its high-affinity state. Integrin αIIbβ3 mediates binding to fibrinogen and von Willebrand factor and plays a key role in platelet aggregation. Inhibitors of αIIbβ3 that block this common pathway of platelet adhesion and aggregation have been used in the management of acute coronary syndromes (*41*). We tested the role of integrin αIIbβ3 in placental failure of EPCR-deficient mice. Genetic inactivation of αIIb in the mother fully protected EPCR$^{-/-}$ embryos from midgestational death (Table 1, row 6). Upon histological examination, αIIb$^{+/-}$ EPCR$^{-/-}$ placentae from these crosses were unremarkable at 11.5 dpc (Fig. 2, M to R). In contrast, EPCR-deficient embryos were not found in pregnancies of EPCR$^{+/-}$ dams sired by αIIb$^{-/-}$ EPCR$^{+/-}$ males. Rather, we observed a high rate of abortions (6 EPCR$^{+/+}$, 14 EPCR$^{+/-}$, 0 EPCR$^{-/-}$, 7 aborted, 4 pregnancies analyzed at 18.5 dpc). Thus, inactivation of Par4, Par3, or the integrin αIIbβ3 receptor on maternal platelets prevented placental failure and midgestational death of EPCR-deficient embryos, but aspirin treatment provided marginal benefit. Together, these data provide strong evidence that thrombin-mediated activation of maternal platelets causes placental failure and midgestational death of EPCR-deficient embryos.

Extraembryonic deficiency of EPCR results in placental venous thrombosis and high neonatal lethality

Although the above genetic approaches allowed placental and embryonic development of EPCR-deficient mice past midgestation, we observed that they were present at significantly reduced frequencies among 4-week-old pups genotyped at wean (Table 1, rows 7 to 9). This was an unexpected finding as specifically deleting EPCR expression from epiblast derivatives while maintaining expression on trophoblast cells results in the generation of mice with undetectable EPCR expression. EPCR-deficient mice generated by this approach are born at expected Mendelian frequency and exhibit normal life spans (*26*). We independently repeated this experiment in our laboratory. In pregnancies of EPCR$^{lox/lox}$ females sired by Meox2Cre$^{tg/-}$ EPCR$^{\delta/\delta}$ males, we observed 27 Meox2Cre$^{-/-}$ EPCR$^{lox/\delta}$ and 23 Meox2Cre$^{tg/-}$ EPCR$^{\delta/\delta}$ live pups at 4 weeks of age [$P = 0.85$, $\chi 2$ GOF test; 50% EPCR$^{-/-}$ expected, 46.0% observed, 95% confidence interval (CI) = 31.8 to 60.7%]. These results confirm that epiblast-specific deletion of EPCR (Meox2Cre$^{tg/-}$ EPCR$^{\delta/\delta}$; abbreviated henceforth as EPCR$^{\delta/\delta}$) allows embryonic development and survival comparable to EPCR-expressing littermate controls. Thus, though the absence of Par4, Par3, or αIIb in the mother protected EPCR-deficient (EPCR$^{-/-}$) embryos from midgestational death, longer-term survival of these mice that lack EPCR expression on extraembryonic trophoblast cells was markedly reduced, compared to the survival of EPCR-deficient mice generated by maintaining expression on trophoblast cells (EPCR$^{\delta/\delta}$).

To investigate the time and cause of death of EPCR$^{-/-}$ embryos/pups, we examined pregnancy outcomes at late gestation and within the first 24 hours of birth. Frequencies of late-gestation EPCR$^{-/-}$ embryos (analyzed at 18.5 dpc) and neonates (collected within 24 hours of delivery) from pregnancies of Par4$^{-/-}$ EPCR$^{+/-}$ dams sired by EPCR$^{+/-}$ males did not deviate significantly from expected (Table 1, rows 10 and 11). EPCR$^{-/-}$ offspring were also present in expected Mendelian frequency at 18.5 dpc in pregnancies of Par3$^{-/-}$ EPCR$^{+/-}$ dams sired by EPCR$^{+/-}$ males (Table 1, row 12) but were markedly reduced among 1-day-old neonates (Table 1, row 13, $P = 0.058$, χ^2 GOF). EPCR-deficient mice were fewer at late gestation in pregnancies of αIIb $^{-/-}$ EPCR$^{+/-}$ mothers sired by EPCR$^{+/-}$ males, but the reduction in their frequency was not statistically significant (Table 1, row 14; 15.5% observed, 25% expected, $P = 0.276$, χ^2 GOF). In each of these crosses, 100% of EPCR$^{-/-}$ mice that survived past the first week of birth exhibited normal life spans (>1 year of age). Thus,

EPCR$^{-/-}$ pups are vulnerable in the perinatal period, and many died within the first few days of birth. We examined EPCR$^{-/-}$ neonates from each of these genetic crosses but did not discover any obvious potential cause of death. However, we observed that EPCR$^{-/-}$ embryos in pregnancies of Par3$^{-/-}$ EPCR$^{+/-}$ dams were significantly smaller than their littermates at 18.5 dpc. The reduction in size was also observed for their corresponding placentae. A similar reduction was noted in placental sizes for EPCR$^{-/-}$ concepti in pregnancies of Par4$^{-/-}$ EPCR$^{+/-}$ and αIIb$^{-/-}$ EPCR$^{+/-}$ dams and in sizes of EPCR$^{-/-}$ embryos in pregnancies of αIIb$^{-/-}$ EPCR$^{+/-}$ dams (Fig. 3, A to J). Histological examination of late-gestation placentae in each of these genetic crosses notably revealed thrombi in the junctional zone of EPCR$^{-/-}$ placentae. EPCR is highly expressed in the spongiotrophoblast cells of the junctional zone in normal mouse placentae (Fig. 3, K and M). The junctional zone is traversed by trophoblast-lined venous sinuses that drain maternal blood from the labyrinth to decidua basalis (*42*). Frequent thrombi were observed in these channels in EPCR$^{-/-}$ but not in littermate EPCR$^{+/+}$ placentae (Fig. 3, K to T). In contrast, calcified and infarcted regions were frequently observed in the placental labyrinth, but these did not correlate with any specific genotype. EPCR$^{-/-}$ mice continued to exhibit neonatal lethality even when both maternal and fetal genes for Par4, Par3, or αIIb were inactivated (Table 1, rows 15 to 17). We examined 18.5 dpc EPCR$^{-/-}$ placentae among progeny of αIIb$^{-/-}$ EPCR$^{+/-}$ intercrosses. Thrombi persisted in the junctional zone of these placentae (Fig. 3, U and V). In contrast, thrombi were not present in the junctional zone of placentae corresponding to EPCR$^{\delta/\delta}$ embryos where extraembryonic EPCR expression was maintained (Fig. 3, W to Z). These data show a remarkable association between placental venous thrombosis (due to EPCR deficiency on trophoblast cells) and the high neonatal lethality of EPCR$^{-/-}$ mice. Neonatal lethality is not observed when EPCR expression is maintained on trophoblast cells but removed from the embryo. Inactivation of Par4, Par3, or αIIb significantly improved survival but were each insufficient to completely overcome placental thrombosis and associated neonatal death.

Inactivation of coagulation factor VIII protects EPCR-deficient embryos

Daily injections of LMWH (20 μg/g) has been reported to prolong survival of a small fraction of EPCR-deficient mice; 2 of 48 pups born from heterozygous intercrosses were found to be EPCR$^{-/-}$ (4.2% observed, 25% expected) (*26*). In our laboratory, continuous delivery of LMWH through subcutaneously implanted osmotic pumps also resulted in marked anticoagulation but did not restore normal placental development (note S3 and fig. S2). To rigorously test the role of excess thrombin generation in fetal demise, we conducted breeding experiments using mice in which FVIII was genetically inactivated. FVIIIa supports the propagation of thrombin generation via intrinsic tenase (FVIIIa-FIXa) formation. It is one of the targets of inactivation by aPC. We analyzed pregnancies of FVIII$^{-/-}$ EPCR$^{+/-}$ dams sired by FVIII$^{-/y}$ EPCR$^{+/-}$ males. (Note that FVIII is an X-linked gene with a single copy in males.) EPCR$^{-/-}$ embryos were present at expected Mendelian frequency at 18.5 dpc (Table 1, row 18), but only half survived the neonatal period (Table 1, row 19). EPCR$^{-/-}$ embryos and placentae were normal in size and gross appearance at 18.5 dpc (Fig. 3, D, H, I, and J). Histological examination of placentae once again revealed thrombi in venous channels of the junctional zone (Fig. 3, S and T). Thus, reducing thrombin generation by inactivating FVIII was also effective in preventing early death of EPCR-deficient embryos but insufficient in preventing late placental thrombosis and high neonatal lethality. Nonetheless, FVIII inactivation significantly improved survival of EPCR$^{-/-}$ embryos (Table 1, compare rows 1 and 18; $P < 0.0006$, χ^2 test of independence) and pups (Table 1, compare rows 2 and 19; $P < 0.037$, χ^2 test of independence). The results were comparable to Par4 inactivation that also significantly improved survival of EPCR$^{-/-}$ pups (Table 1, compare rows 15 and 19). About half of the EPCR-deficient neonates continued to die regardless of FVIII or Par4 inactivation. Thus, inhibiting maternal platelet activation or reducing thrombin generation each provided significant but partial protection from neonatal death.

Platelets cause placental failure independent of their ability to enhance thrombin generation

Par4-activated platelets enhance thrombin generation. Given the similar protective effect of FVIII and Par4 inhibition, we asked whether protection from early placental failure seen in the absence of platelet receptors Par4, Par3, or αIIb might reflect their reduced ability to participate in thrombin generation. To address this question, we performed thrombin generation assays with platelet-rich plasma (PRP) from Par4$^{-/-}$, Par3$^{-/-}$, or αIIb$^{-/-}$ mice using recombinant TF as a trigger (Fig. 4). As expected, Par4$^{-/-}$ PRP showed reduced thrombin peak height (45.5 ± 20 nmol for Par4$^{-/-}$ versus 194.6 ± 29.2 nmol for wild-type controls; $P << 0.0001$) and reduced total thrombin generation (1554.3 ± 543.2 nmol for Par4$^{-/-}$ versus 2746.9 ± 594.2 nmol for wild-type controls; $P << 0.0001$), compared to wild-type PRP. We found that Par3$^{-/-}$ PRP also has markedly reduced thrombin peak height (53.2 ± 20.5 nmol; $P << 0.0001$) and reduced total thrombin generation (1300.6 ± 370.0 nmol; $P << 0.0001$) compared to wild-type control. In contrast, the absence of αIIb did not reduce TF-induced thrombin generation by PRP in vitro. Rather, thrombin peak height and total thrombin generation were increased in αIIb$^{-/-}$ PRP compared to wild-type controls (peak height, 289.5 ± 42.6 nmol for αIIb$^{-/-}$; $P << 0.0001$ and total thrombin generation 3304.9 ± 412.5 nmol for αIIb$^{-/-}$; $P = 0.0003$). Thus, the benefit observed with inactivation of platelet Par4 or Par3 is unlikely to be due to reduced platelet participation in thrombin generation but rather involves other pathogenic events downstream of platelet activation.

Maternal EPCR deficiency causes retroplacental hemorrhaging and severe pregnancy complications

During our studies, we discovered severe pregnancy complications in pregnancies of FVIII$^{-/-}$ EPCR$^{-/-}$ females sired by wild-type males. Two of the four pregnancies that we analyzed exhibited midgestational vaginal bleeding. One female died toward term gestation (19.5 dpc) and three were moribund. Surgical evaluation revealed retroplacental bleeding, blanched organs, and undelivered live pups. It was unclear whether this was a phenomenon of concomitant EPCR and FVIII deficiencies. To address this further, we evaluated pregnancy outcome of EPCR-deficient females that were generated by maintaining EPCR expression in the placenta (EPCR$^{\delta/\delta}$). Adult EPCR$^{\delta/\delta}$ mice exhibit plasma markers of thrombophilia without overt thrombosis (*26*). They can serve as a useful model to investigate whether maternal thrombophilia predisposes pregnancies to placenta-mediated complications. We analyzed 18 pregnancies of 12 dams sired by wild-type males. Of these, 13 showed one or more obvious adverse events. These included eight cases of maternal death or moribund mother (44%), eight cases of midgestational vaginal bleeding (44%), two cases of stillbirths, and one pregnancy

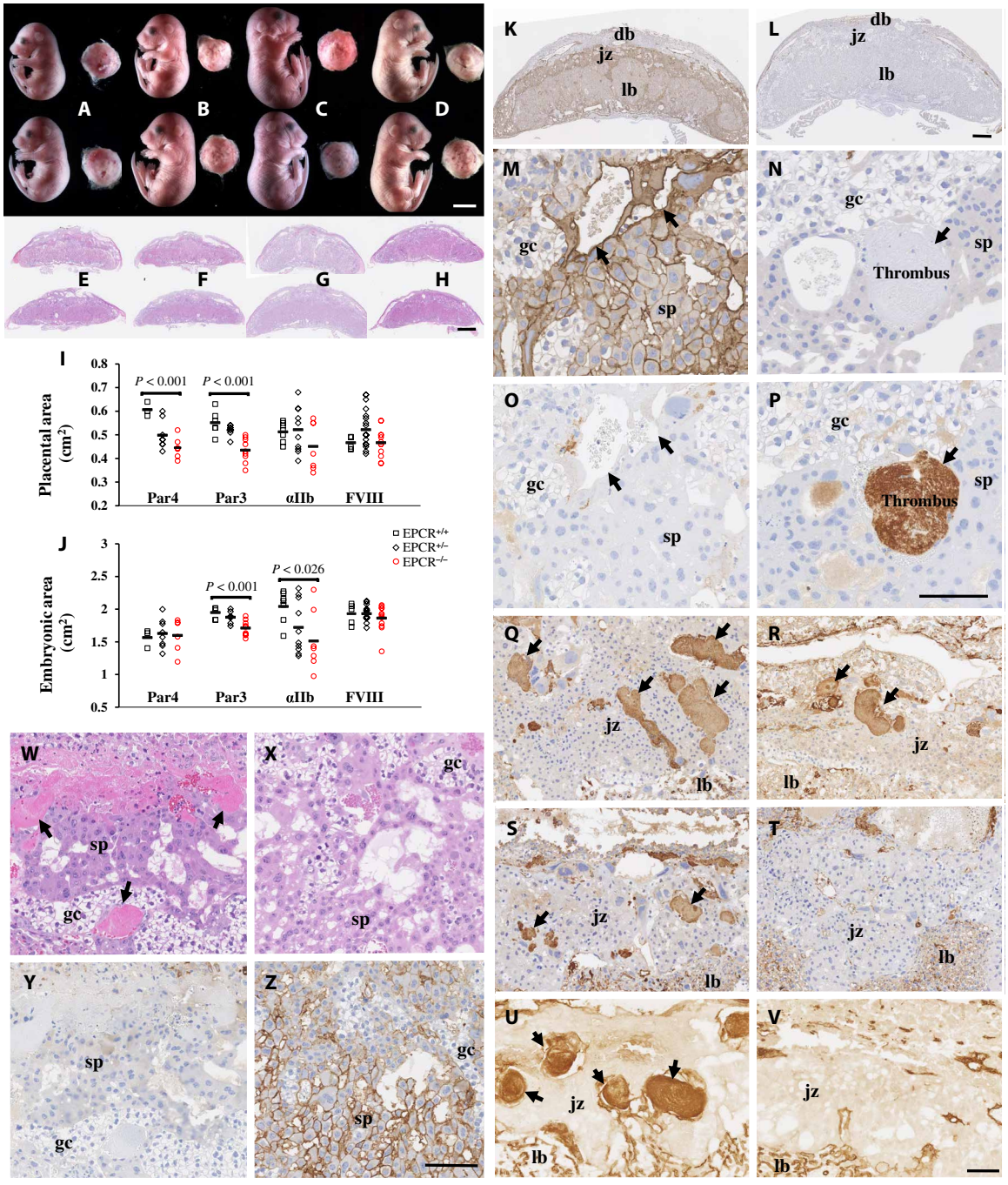

Fig. 3. EPCR-deficient embryos survive to late gestation, but placentae show thrombi in venous sinuses of the junctional zone. Representative images of embryos and placentae analyzed at late gestation from pregnancies of Par4$^{-/-}$ EPCR$^{+/-}$ (**A**, **E**, **Q**, and **W** to **Z**), Par3$^{-/-}$ EPCR$^{+/-}$ (**B**, **F**, **R**, and **T**), αIIb$^{-/-}$ EPCR$^{+/-}$ (**C**, **G**, and **K** to **P**), or FVIII$^{-/-}$ EPCR$^{+/-}$ (**D**, **H**, and **S**) dams sired by EPCR$^{+/-}$ males or from pregnancies of αIIb$^{-/-}$ EPCR$^{+/-}$ intercrosses (**U** and **V**) are shown. Analysis was done with embryos and placentae harvested at 18.5 dpc (A to V) or 15.5 dpc (W to Z). (A) to (D) are whole mounts of EPCR$^{-/-}$ embryos and placentae (top row) and littermate EPCR$^{+/+}$ controls (bottom row). (E) to (H) are hematoxylin and eosin–stained histological sections of placentae corresponding to (A) to (D) (top row, EPCR$^{-/-}$; bottom row, EPCR$^{+/+}$). Cross-sectional area of the placental disc (**I**) and lengthwise cross-sectional area of the embryos (**J**) measured from images of whole mounts are shown, grouped by maternal genes that were inactivated. Histological sections shown in (K) to (Z) are EPCR$^{-/-}$ (L, N, P, Q to S, U, W, and Y) or littermate EPCR$^{+/+}$ controls (K, M, O, T, V, X, and Z). (M) and (N) are enlarged images of the junctional zones (jz) from (K) and (L), respectively. Sections are hematoxylin and eosin–stained (W and X) or immunostained with antibodies for EPCR (K to N, Y, and Z), fibrin(ogen) (O to T), or CD31 (U and V). Black arrows point to venous channels that traverse the junctional zone. EPCR is highly expressed on spongiotrophoblast (sp) cells that line these channels (M). Thrombi are seen in these venous channels in EPCR$^{-/-}$ placentae from all pregnancies analyzed, but not in littermate EPCR$^{+/+}$ controls. Scale bars, 5 mm (A to D), 1 mm (E to H), 500 μm (K and L), and 100 μm (M to P, Q to V, and W to Z). gc, glycogen cells.

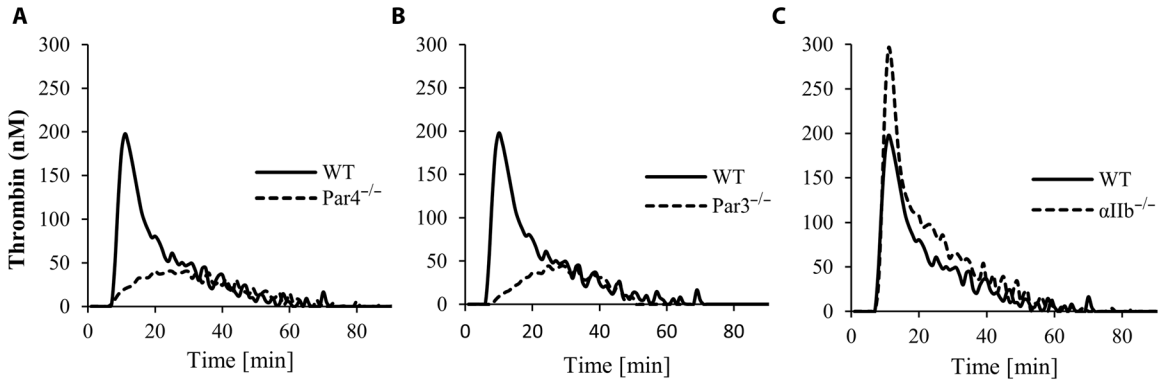

Fig. 4. Differential effect of platelet receptor inactivation on thrombin generation. Thrombin generation triggered with recombinant TF was significantly reduced in Par4$^{-/-}$ (**A**) and Par3$^{-/-}$ (**B**) but was enhanced in αIIb$^{-/-}$ PRP (**C**) PRP. The assays were performed as described in Materials and Methods. WT, wild type.

loss. One maternal death occurred on 13.5 dpc and two on 15.5 dpc. The remaining five cases of maternal death or moribund mother occurred past the expected delivery date; autopsies of four of these showed dead or live pups that had not been delivered. These data showed that vaginal bleeding and maternal morbidity observed in pregnancies of FVIII$^{-/-}$ EPCR$^{-/-}$ dams was primarily due to EPCR deficiency.

Surgical evaluation of pregnant EPCR$^{\delta/\delta}$ dams at term revealed extensive retroplacental hemorrhaging paired with blanched appearance of maternal organs. Uterine hemorrhaging was already present at 12.5 dpc. Upon histological evaluation, the placentae appeared normally developed, but large thrombi were observed in the decidua basalis, both at mid- and late gestation (Fig. 5, A to D, I, and J). In contrast, the placental layers (giant trophoblast cells, junctional zone, and labyrinth) were remarkably devoid of thrombotic pathology and comparable to wild-type controls (Fig. 5, E to H, K, and L). Immunohistochemical staining revealed massive infiltration of neutrophils into the decidua, release of myeloperoxidase, and positive staining for citrullinated histone 3, indicative of neutrophil extracellular trap formation (Fig. 5, M to R). Thus, genetic inactivation of EPCR in female mice results in decidual thrombosis and retroplacental hemorrhaging that leads to vaginal bleeding, complications of parturition, and frequent maternal and/or fetal death. Overall, these findings are consistent with placental abruption and consumptive coagulopathy, suggesting that maternal death may be caused by hemorrhagic shock. We evaluated pregnancy outcome of EPCR-deficient mice with a concomitant deficiency of Par4. In notable contrast, five pregnancies of Par4$^{-/-}$ EPCR$^{-/-}$ dams sired by wild-type males resulted in uneventful pregnancies with normal maternal outcomes. These data demonstrate an essential role of maternal EPCR in uterine hemostasis. Inactivation of Par4, but not clotting factor VIII, prevented pregnancy-induced hemorrhage and maternal morbidity.

DISCUSSION

Mice lacking EPCR die in utero from placental failure associated with thrombosis. LMWH treatment prevents thrombosis and prolongs the survival of only a small fraction of EPCR-deficient embryos (*26*). We used this murine pregnancy model of fetal thrombophilia to examine the contribution of maternal platelets in placental failure. We demonstrate that genetic deletion of molecules involved in thrombin generation or in the activation of maternal platelets allows placental development and embryonic survival. Our studies make a strong case that thrombin-mediated activation of maternal platelets is the primary driver of early placental failure and midgestational death of EPCR-deficient embryos. Multiple lines of evidence support this case. Genetic inactivation of maternal Par4, Par3, or αIIb were each equally effective in allowing placental development and intrauterine survival of EPCR-deficient embryos. Of these, integrin αIIb is predominantly expressed on platelets, megakaryocytes, and a subset of hematopoietic progenitors. Platelet depletion also allowed placental development to proceed, consistent with the requirement of these receptors on platelets, rather than other cell types. Genetic inactivation of paternal Pa4, Par3, or αIIb did not protect EPCR-deficient embryos from midgestational death. Thus, protection is conferred by the loss of these receptors in the maternal compartment rather than haploinsufficiency in the fetal compartment. While several proteases can activate Par4, thrombin is the only known agonist for Par3-mediated platelet activation (*44*). Protection conferred by inactivation of maternal Par3 provides a strong argument that pathological activation of maternal platelets is likely to be thrombin driven. Together, these data underscore the tight regulation of thrombin generation at the fetomaternal interface and suggest that inadvertent activation of maternal platelets causes early demise of EPCR-deficient embryos.

We next used the murine pregnancy model with fetal EPCR deficiency to evaluate the role of FVIII in placental failure. The anticoagulant function of EPCR is mediated by augmented activation of PC and inactivation of factors V and VIII. We found that the genetic inactivation of FVIII restored Mendelian frequency of EPCR-deficient embryos at late gestation. These results are consistent with a critical anticoagulant role of EPCR in the placenta. Curiously, inactivation of FVIII was far more effective than treatment with LMWH in preventing placental failure. Our results suggest that inactivation of FVIII by aPC may be necessary for maintaining placental hemostasis by keeping thrombin generation at levels below those that trigger platelet activation.

EPCR-deficient mice generated by inactivating FVIII, Par4, Par3, or integrin αIIb unexpectedly showed high neonatal lethality. Careful examination of late-gestation placentae revealed thrombi in maternal venous sinuses of the placenta as a distinct pathology of EPCR deficiency on trophoblast cells. Inherited thrombophilias, including polymorphisms in the gene encoding EPCR, increase the risk of

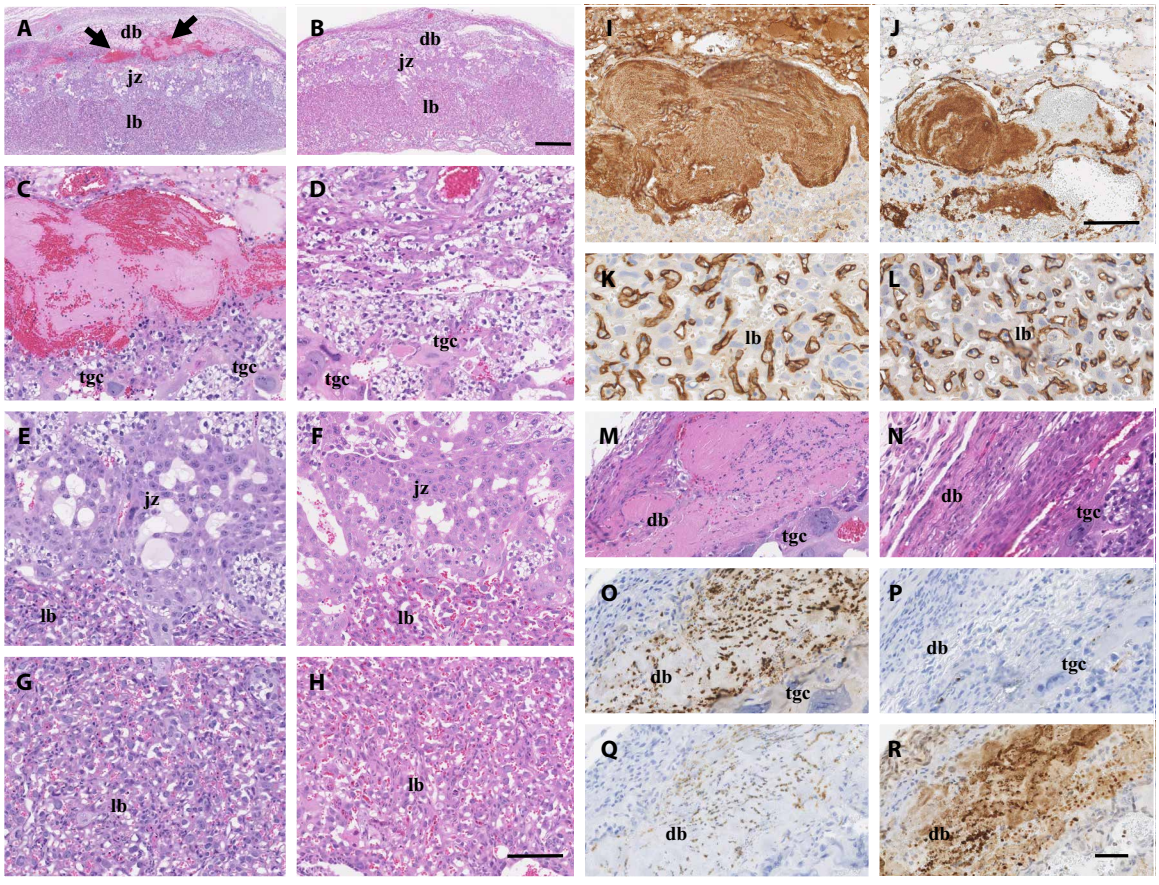

Fig. 5. EPCR-deficient dams exhibit frequent thrombosis in the decidua basalis but not in the placenta. Hematoxylin and eosin–stained histological sections of 15.5 dpc placentae harvested from wild-type intercrosses (**B**) and from pregnancies of EPCR$^{\delta/\delta}$ dams sired by wild-type males (**A**) are shown. Arrows in (A) point to regions of decidual thrombosis. Magnified views of decidua basalis (**C** and **D**), junctional zones (**E** and **F**), and labyrinth regions (**G** and **H**) of placentae in (A) and (B) are shown. Decidual thrombosis was confirmed by immunostaining with fibrin(ogen) (**I**) and CD31 (**J**) antibodies. CD31 immunostaining confirmed normal elaboration of placental labyrinth (**K**) comparable to placenta from control wild-type pregnancies (**L**). Decidual thrombosis was also readily seen in hematoxylin and eosin–stained histological sections of 12.5 dpc placentae from pregnancies of EPCR$^{\delta/\delta}$ dams (**M**) but not in gestational age-matched wild-type pregnancies (**N**). Strong immunostaining was observed with myeloperoxidase antibodies [(**O**); myeloperoxidase-stained placenta from control pregnancy shown in (**P**)], neutrophil marker Ly6G (**Q**), and antibodies against citrullinated histone 3 (**R**). tgc, trophoblast giant cells. Scale bars, 500 μm (A and B),100 μm (C to J), or 50 μm (K to R).

venous thrombosis. They are hypothesized to also increase the risk of thrombosis in low-flow uteroplacental circulation. The murine study tests this hypothesis and demonstrates an increased propensity of uteroplacental venous circulation to thrombophilia-associated thrombosis. Notably, in this model, venous thrombosis was observed in association with fetal gene defect manifest on extraembryonic trophoblast cells. Maternal blood reaches the base of the mouse placenta through one to three arterial channels and drains back into decidual and uterine veins through multiple venous channels (Fig. 6). The venous channels of the junctional zone of mouse placenta are analogous to venous channels in the basal plate of human placenta (Fig. 6). Our observations draw attention to venous channels as potential sites of thrombotic pathology in human pregnancies. It was recently shown that human extravillous trophoblasts not only invade uterine arteries but also line decidual and uterine veins in early pregnancy (*45*, *46*), extending potential sites of pathology to these regions.

The murine study further shows an association between placental venous thrombosis and significantly reduced neonatal survival, supporting the notion that placental pathology is an important contributor to neonatal death. It is noteworthy that inhibition of thrombin-mediated platelet activation or reduction of thrombin generation each prevented placental thrombosis in early gestation but was insufficient to fully overcome late-gestation placental thrombosis and neonatal death. Our observations thus draw attention to gestation age-specific differences in thrombophilia-associated placental pathology and its potential response to treatment. The full scope of platelet-driven and platelet-independent activities that precipitate placental pathology associated with neonatal death of EPCR-deficient mice remains to be identified.

We next focused our attention on pregnancy outcome in EPCR-deficient mothers. EPCR-deficient mice show decidual thrombosis, retroplacental hemorrhaging, frequent midgestational vaginal bleeding, and pregnancy-induced maternal death. Our findings thus support the epidemiological association between maternal thrombophilia and placental abruption (*47*). Decidual necrosis has been reported to be higher in women with thrombophilia and placental abruption (*48*). Neutrophil infiltrates frequently colocalize with decidual fibrin deposits in cases of placental abruption (*49*). Our study corroborates the idea that recruitment of neutrophils is a component of disease

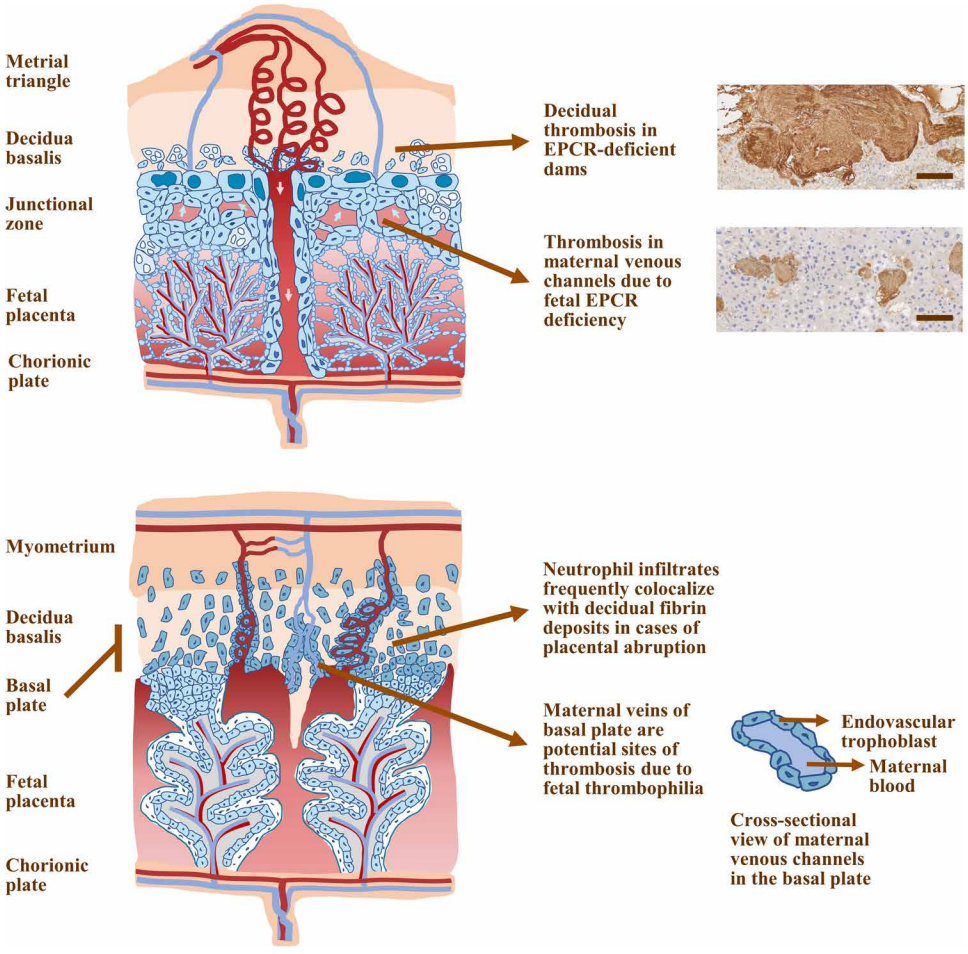

Fig. 6. Schematic showing location of thrombosis due to maternal or fetal EPCR deficiency in murine placenta and equivalent sites in human placenta. In a developing placenta, maternal blood interfaces with fetal trophoblast cells at multiple sites. Thrombosis due to fetal EPCR deficiency is specifically observed in venous channels of the junctional zone of murine placenta (top) equivalent to the basal plate of the human placenta (bottom). Maternal EPCR deficiency results in massive thrombi in decidua basalis. Thrombi immunostained with fibrinogen antibodies are shown. Scale bar, 100 μm.

pathogenesis. The murine model demonstrates chronic etiology of thrombophilia-associated placental abruption and opens the potential for therapeutic intervention before catastrophic hemorrhaging. In a recent meta-analysis of randomized controlled trials, it was noted that LMWH treatment benefitted women with previous abruption but no other subgroup (*6*).

The maternal cell types where EPCR expression is essential for the maintenance of pregnancy remains to be determined. Further studies are also needed to identify relative contributions of excess thrombin generation, reduced PC activation, activation of maternal platelets, recruitments of immune cells, and potential NETosis in disease pathogenesis. Our study has nonetheless identified Par4 as playing a critical role in disease pathogenesis. Vaginal bleeding and maternal death were not observed in pregnancies of EPCR-deficient mothers with a concomitant deficiency of Par4. In contrast, reduced thrombin generation achieved by inactivation of VIII was ineffective in this scenario. The exact role of Par4 and the cell type/s through which it aggravates placental hemorrhage remains to be investigated.

EPCR-deficient mice are a particularly suitable model of maternal thrombophilia as they reduce, but do not eliminate, the generation of aPC. These mice develop normally, appear healthy, and exhibit elevated levels of intravascular thrombin [measurable as increase in thrombin-antithrombin (TAT) complex formation] without spontaneous thrombosis or hemorrhage (*26*). In contrast to EPCR deficiency, mice with thrombomodulin deficiency display signs of inflammation and early onset thrombosis of extremities. Notably, transgenic expression of hyperactivatable PC substitutes for the function of endogenous PC in preventing overt thrombosis and extends survival, but female thrombomodulin-deficient mice exhibit pregnancy-induced death and uterine hemorrhage (*50*). The similarity in maternal outcome of EPCR and thrombomodulin-deficient mice strengthens our observations and warrants an examination of placental and decidual pathology in pregnancies of thrombomodulin-deficient mice.

We have assessed pregnancy complications in EPCR-deficient mice generated by maintaining placental EPCR expression (*26*). A different model of EPCR deficiency was generated in 2002 and is being used by researchers (*51*). These mice carry an intact EPCR gene but express low levels of detectable message due to aberrant insertion of the targeting vector in a region 5′ to the endogenous gene. No pregnancy complications have been reported in these mice. It is unclear whether residual or tissue-restricted EPCR expression in this model precludes these complications. Mice harboring a variant

form of EPCR with impaired ability to bind PC were recently reported (*27*). These mice are predicted to have reduced activation of PC. Our findings prompt an investigation into whether mice with variant EPCR expression exhibit pregnancy complications.

In summary, our study draws on the strengths of murine models to identify key pathological processes that might mediate adverse pregnancy outcomes associated with maternal or fetal thrombophilia. Our experiments use genetic inactivation, rather than pharmacological inhibition, of specific platelet receptors during pregnancy. Further experimentation is necessary to determine whether pharmacological targeting of platelet thrombin receptors will efficiently prevent or reverse pathology in the murine model. We have tested models with complete EPCR deficiency that may not reflect outcomes and/or disease mechanisms associated with specific EPCR polymorphisms or thrombophilia mutations involving other genes. Our studies also suffer from several limitations inherent to the use of animal models. They are best interpreted as proof-of-concept studies that lend credence to the notion that thrombotic processes mediate adverse pregnancy outcomes associated with maternal or fetal thrombophilia. Second, they provide evidence that sites of uteroplacental thrombosis and the response to antithrombotic intervention are likely to differ with gestational age and maternal versus fetal thrombophilia (Fig. 6). These differences can be expected to be further compounded by the heterogeneous primary etiologies of maternal or fetal thrombophilia. Further studies are needed to elaborate disease mechanisms that may allow a more precise patient stratification to interrogate outcomes and effects of interventions (*2*). Third, they identify maternal venous channels in uteroplacental circulation as potential sites of fetal thrombophilia-associated placental thrombosis. Maternal veins in the basal plate of the placenta (part of decidua basalis juxtaposed to the placenta) are lined by endovascular trophoblasts of fetal origin (Fig. 6). Fourth, they draw attention to thrombin-mediated maternal platelet activation as a critical event in precipitating placental failure and a potential target of therapeutic intervention. Conversely, our results indicate that polymorphisms associated with increased Par4 reactivity (*52*, *53*) should be evaluated for a potential association with gestational complications, particularly in populations of African ancestral descent where both conditions are more prevalent. Fifth, our studies establish a causal link between maternal thrombophilia and uterine hemorrhaging that is, in turn, associated with adverse fetal outcomes. Hemorrhage is a leading cause of maternal death worldwide. These studies suggest that EPCR polymorphisms or functional EPCR deficiency due to inflammation-induced receptor shedding or the presence of inhibitory autoantibodies might disturb uterine hemostasis. The murine model provides a unique opportunity to examine thrombo-inflammatory mechanisms in uterine hemorrhage and identify targets of therapeutic intervention.

MATERIALS AND METHODS

Mice

Par4 [Mutant Mouse Regional Resource Center (MMRRC) stock no. 15231], Par3 (MMRRC stock no. 15232), αIIb, FVIII, EPCR loxP, and Meox2Cre (the Jackson laboratory, stock no. 003755) mice have been previously described (*26*, *40*, *54*–*58*). All mouse strains were maintained on a C57BL/6 genetic background. Double mutants were generated by breeding and identified by polymerase chain reaction (PCR)–based genotyping on tissue obtained by tail or ear biopsy. Where noted, the PCR primers recommended by the MMRRC or the Jackson laboratory were used. For other strains, PCR primers described in the original referenced publications were used. All animal experiments were conducted following standards and procedures approved by the Animal Care and Use Committee of the Medical College of Wisconsin.

Analysis of pregnancies

Paired mice were checked every morning for the presence of vaginal plug as evidence of coitum. On the day of the plug, 12:00 p.m. was counted as 0.5 dpc. Uteri were surgically removed and dissected in phosphate-buffered saline. Embryos and placentae were examined and photographed with a Nikon SMZ1000 Zoom stereomicroscope (Nikon, Melville, NY) equipped with a high-resolution, five-megapixel digital camera and NIS Elements F4.3 imaging software. Live embryos were identified by the presence of heartbeats, breathing, or limb movements. Embryos and placentae were observed for any phenotypic abnormalities. DNA was prepared from the yolk sacs or tail biopsies of embryo. The presence or absence of genes of interest was determined by PCR using gene-specific primers. Embryos found at advanced stages of resorption were not genotyped. The placentae were marked with ink to identify the center of the disc in histological sections. To estimate relative sizes, the cross-sectional area of placental discs and lengthwise cross-sectional area of the embryos was measured with ImageJ (version 1.52a, National Institutes of Health, Bethesda, MD) from images of whole mounts as shown in Fig. 2 (A to D).

Histology and immunohistochemistry

For most experiments, tissues were fixed in zinc formalin for 72 hours and transferred to 70% alcohol for future processing. Fixed tissues were later embedded in paraffin, sectioned, and stained with hematoxylin and eosin using standard protocols. For immunostaining, antigen retrieval was performed in BOND epitope retrieval solution 1 (pH 6.0) or solution 2 (pH 9.0) (Leica Biosystems, Buffalo Grove, IL). EPCR immunostaining was done with anti-EPCR primary antibodies at a dilution of 1:200 (catalog no. AF2749, R&D systems, Minneapolis, MN) and biotinylated rabbit anti-goat secondary antibodies at a dilution of 1:500 (catalog no. BA-5000, Vector Laboratories, Burlingame, CA). Cytokeratin, CD31, and fibrin(ogen) immunostaining was done with 1:500 dilution of anticytokeratin (catalog no. Z0622, Dako, Santa Clara, CA) or 1:100 dilution of anti-CD31 (catalog no. AF3628, R&D systems, Minneapolis, MN) or 1:2000 antifibrinogen (catalog no. A0080, Dako, Santa Clara, CA) primary antibodies and 1:500 dilution of biotinylated donkey anti-rabbit secondary antibodies (catalog no. 711-066-152, Jackson ImmunoResearch Laboratories, West Grove, PA). Neutrophils were identified with anti-Ly6G primary at 1:75 (catalog no. 14-5931-85, eBioscience, San Diego, CA) and biotinylated goat anti-rat immunoglobulin G (IgG) secondary antibodies at a dilution of 1:300 (catalog no. BA-9401, Vector Laboratories, Burlingame, CA). In all of the above immunostaining protocols, streptavidin/horseradish peroxidase was used at a dilution of 1:300 for 15 min (catalog no. P039701-2, Dako, Santa Clara, CA), and DAB+ kit (catalog no. K346811-2, Dako, Santa Clara, CA) was used for detection. Immunostaining for myeloperoxidase and citrullinated histone 3 was done with anti-MPO (myeloperoxidase) or anti–Cit H3 (citrullinated histone 3) primary antibodies (catalog no. ab208670, Abcam, Cambridge, MA; catalog no. ab5103, Abcam, Cambridge, MA) at 1:500 dilution each and developed with the

BOND polymer refine detection kit (catalog no. DS9800, Leica Biosystems, Buffalo Grove, IL). Stained histological sections were photographed and viewed using a Nanozoomer HT 2.0 slide scanner and NDP.view2 Viewing software (Hamamatsu, Japan). For some experiments, placentae were embedded and flash-frozen in O.C.T. compound, fixed in acetone after sectioning, and immunostained with rabbit anti-cow cytokeratin (catalog no. Z0622, Dako, Santa Clara, CA) and rat anti-mouse glycoprotein Ibβ (GPIbβ) (catalog no. M050-0, EMFRET Analytics GmbH & Co. KG, Germany) primary antibodies at 1:100 dilution each. Primary cytokeratin antibodies were visualized with goat anti-rabbit IgG Alexa Fluor 633 (catalog no. A-21071, Invitrogen, Carlsbad, CA). Primary GPIbβ antibodies were visualized with 4plus Biotinylated Goat Anti-Rat IgG (catalog no. GR607, Biocare Medical) and streptavidin–Alexa Fluor 488 (catalog no. S11223, Invitrogen, Carlsbad, CA). Images were taken on a Zeiss LSM510 confocal system (Zeiss International, Germany).

Drug and antibody treatments

To achieve continuous anticoagulation with LMWH, pregnant dams were implanted with subcutaneous osmotic pumps (Model 1002, Durect Corp, Cupertino, CA) filled with LMWH (100 μg/μl) (enoxaparin, brand name Lovenox; Aventis Pharmaceuticals Inc., Bridgewater, NJ) with release rates of 0.25 μl/hour. Treatment was initiated at 5.5 dpc and continued until the time of analysis at 11.5 dpc. Anticoagulation was measured in terms of Xa inhibitory activity with Coatest heparin (Chromogenix, Lexington, MA) in plasma. Plasma was prepared from citrated whole blood collected from the inferior vena cava at the time of analysis of pregnancies. To deplete platelets, pregnant dams were intravenously injected with anti-GPIbα antibodies (R300, EMFRET Analytics GmbH & Co. KG, Wurzburg, Germany) at 7.5 dpc at a dose of 4 μg/g body weight. Platelet depletion was confirmed 1 hour after treatment by flow cytometry on 5-μl whole blood collected from the tail vein, diluted in citrated Tyrode's buffer, and stained with anti-CD41 eFluor 450–labeled antibodies (catalog no. 48-0411-80, eBioscience, San Diego, CA) in the dark for 15 min. In a separate set of experiments, aspirin (acetylsalicylic acid, catalog no. A5376, Sigma-Aldrich, St. Louis, MO) was administered daily at 40 or 80 mg/kg via oral gavage from 5.5 dpc to the day of analysis of pregnancies. At the time of analysis, citrated whole blood collected from the inferior vena cava was used to prepare washed platelets. Whole blood was centrifuged at 150*g* for 5 min, and the supernatant was collected and centrifuged again at 800*g* for 8 min to sediment platelets. Platelets were washed with Tyrode's buffer containing bovine serum albumin (2.5 mg/ml) and glucose (1 mg/ml) and centrifuged at 800*g* for 8 min and lastly resuspended in the same buffer. All centrifugation steps were done at room temperature and without brakes. To inhibit platelet activation, prostaglandin E_1 (PGE_1) (catalog no. P5515, Sigma-Aldrich, St. Louis, MO) was added before each centrifugation step at a final concentration of 50 ng/ml. To inactivate any residual PGE_1, platelet suspension was rested for 30 min at room temperature before the aggregation experiment. Before measuring aggregation response, calcium was added to the platelet suspension at a final concentration of 1 mM. The aggregation response of platelets was measured following stimulation with arachidonic acid (catalog no. P/N 390, Chrono-log, Havertown, PA) or U46619 (Enzo Life Sciences, Farmingdale, NY) using the Chrono-log 700 instrument (Chrono-log, Havertown, PA).

Thrombin generation assay

To prepare PRP, citrated whole blood collected from the inferior vena cava was centrifuged at 150*g* for 5 min without brakes. The supernatant was collected, and platelet counts were measured on a scil Vet abc Plus hematology analyzer (scil animal care company, Ontario, Canada). Platelet counts were adjusted to 600×10^3 platelets/μl using platelet-poor plasma prepared from the same whole-blood sample using a second spin at 800*g* for 8 min. In vitro thrombin generation assays were conducted using a modified Technothrombin Thrombin Generation Assay (catalog no. 5006010, DiaPharma Group, West Chester Township, OH). Thrombin generation was initiated by the addition of recombinant TF (1.0pM) (RecombiPlasTin 2G, Instrumentation Laboratory, Bedford, MA). Fluorescence was immediately measured with a SpectraMax Gemini EM fluorimeter and SoftMax Pro software (Molecular Devices, San Jose, CA) at 355 nm/460 nm (excitation/emission) for 2 hours at 37°C with measurements collected at 1-min intervals. Raw fluorescence data were analyzed using Technothrombin TGA evaluation software.

Statistical analysis

The $\chi 2$ GOF test was used to determine deviation from expected Mendelian proportions. The $\chi 2$ test of independence was used for comparing two separate treatment groups. Exact binomial 95% CI were computed where appropriate. The Student's *t* test (two-tailed with unequal variance) was used for comparing embryo and placental sizes between groups and for comparing fluorescence values in thrombin generation assays. For all experiments, $P < 0.05$ was considered significant.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/45/eabb6196/DC1

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