Disruption of the Endothelial Cell Protein C Receptor Gene in Mice Causes Placental Thrombosis and Early Embryonic Lethality*

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The endothelial cell protein C receptor (EPCR) is a type 1 transmembrane protein found primarily on endothelium that binds both protein C and activated protein C with similar affinity. EPCR augments the activation of protein C by the thrombin-thrombomodulin complex. To determine the physiological importance of EPCR, we generated EPCR-deficient mice by homologous targeting in embryonic stem cells. Genotyping of progeny obtained from EPCR*+/− interbreeding indicated that EPCR*+/− embryos died on or before embryonic day 10.5 (E10.5). Reverse transcriptase-PCR confirmed the absence of EPCR mRNA in EPCR*+/− embryos. EPCR*−/− embryos removed from extra-embryonic membranes and tissues at day E7.5 and cultured in vitro developed beyond E10.5, suggesting a role for EPCR in the normal function of the placenta and/or at the maternal-embryonic interface. Immunohistochemistry revealed the lack of EPCR in trophoblast giant cells of EPCR*−/− embryos. These cells, which normally express EPCR, are in direct contact with the maternal circulation and its clotting factors. In EPCR*−/− embryos, greatly increased fibrin deposition was detected around these cells. To prevent this fibrin deposition, EPCR*+/−-crossed female mice received a daily subcutaneous injection of enoxaparin through pregnancy. Although some EPCR*−/− embryos were rescued from midgestational lethality, this regimen yielded no EPCR*−/− pups. We conclude that EPCR is essential for normal embryonic development. Moreover, EPCR plays a key role in preventing thrombosis at the maternal-embryonic interface.

The protein C anticoagulant pathway is a major regulatory system that controls thrombin generation by inhibiting factors Va and VIIa (1). Thrombin not only activates platelets and clots fibrinogen, but it also binds to thrombomodulin (TM) on the endothelium, at which time the procoagulant activities of thrombin are blocked, whereas the activation of the anticoagulant protein C is potently enhanced (2, 3). The endothelial cell protein C receptor (EPCR) binds protein C and increases the rate of protein C activation on the endothelium (4). In vivo, protein C binding to EPCR enhances the amount of activated protein C (APC) generated in response to thrombin infusion more than 10-fold (5) demonstrating that the receptor plays a key physiological function in the activation complex.

EPCR is a type 1 transmembrane receptor that shares homology with the major histocompatibility class I/CD1 family of proteins involved in the immune response (6, 7). In addition to the sequence homology, the crystal structure of EPCR reveals that, like the CD1 family of molecules, EPCR has a tightly bound lipid located in a groove that is structurally similar to that used by the CD1 family for antigen presentation (8).

In adults, EPCR is primarily localized on endothelial cells of large blood vessels (9), but during development, EPCR is expressed at high levels on the trophoblast giant cells (10). EPCR expression is very low or absent from the microvasculature endothelium of most tissues, with the major exception being the sinusoidal capillaries of the liver (9).

Both cellular and soluble EPCR (sEPCR) appear to have biological functions. sEPCR is present in normal plasma (∼100 ng/ml) (11). It is released from the endothelium by a metalloproteinase (12). A variety of mediators, including interleukin-1, hydrogen peroxide, phorbol esters, and thrombin dramatically increase EPCR shedding from the endothelium. This probably explains the elevated levels of plasma sEPCR found in patients with sepsis or lupus erythematosus (13). The increased shedding of cellular EPCR and the up-regulation of its mRNA in a sepsis model implies that this process may be important for some physiological function of EPCR (14). Consistent with this hypothesis, sEPCR has been shown to retain its binding affinity for protein C/APC (11). sEPCR also binds to activated leukocytes, in a process mediated in part through proteinase 3 (15), the autoantigen of Wegener’s granulomatosis. The proteinase 3-sEPCR complex binds to integrins on the surface of activated neutrophils. Interestingly, in a baboon sepsis model, blocking protein C binding to EPCR exacerbated the coagulation and inflammatory responses that result from low level Escherichia coli infusions (16). Moreover, massive polymorphonuclear cell influx into specific areas of the adrenal gland, liver, and kidneys of these animals suggests that EPCR might also function in neutrophil interactions and leukocyte trafficking.

Recently, several heterozygous EPCR mutations were identified in an Italian population (17). However, homozygous EPCR deficiency has not been described in humans to date. To better define the physiological and pathophysiological role of EPCR, we disrupted the EPCR gene in embryonic stem (ES) cells and subsequently generated mice that carried the disrupted EPCR gene to their progeny. In this work, we report...
that homozygous EPCR-deficient embryos die in utero on or before embryonic day 10.5 (E10.5). The severe EPCR-null phenotype is associated with fibrin deposition at the materno-embryonic interface of EPCR−/−embryos, particularly around the trophoblast giant cells that normally express high levels of EPCR. This observation provides the first genetic evidence that EPCR plays a critical role in the regulation of blood coagulation.

**EXPERIMENTAL PROCEDURES**

**Disruption of the EPCR Gene—**To construct a gene targeting vector, the vector pL338 (a kind gift from Dr. Tim Ley, Washington University, St. Louis), containing the 1.7-kb neomycin/G418 resistance (neo) gene under control of the mouse FGK-1 promoter and flanked by two directly repeated LoxP sites (5 ‐AACCTCGATAATGATATCCTAGGATTAT-3′) (18) was used. A 3.0-kb NotI/Xho fragment, corresponding to exons 2–4 of the murine EPCR gene, was inserted downstream of the neo sequence. The 3.0-kb HindIII/BamHI fragment (representing 0.8 kb of the EPCR 5′-promoter region, exon 1, and 1.2 kb of intron 1 (19)) was then inserted into the above vector, upstream of the neo sequence. To facilitate the deletion of EPCR exon 1, a third LoxP site was positioned in the EPCR promoter region (−158, Box361 site) (Fig. 1). Restriction enzyme mapping and sequencing of ligation junctions confirmed the structure of the targeting vector. We utilized the Cre/Lox system in order to facilitate generation of mutant mice for future investigations.

The targeting vector was linearized with HindIII and introduced by electroporation into AB2 129 mouse ES cells (Lexicon Genetics). Transfected ES cells were grown on irradiated neonycin-resistant mouse embryonic fibroblasts and LIF (1000 units/ml) (Invitrogen) and selected for in the presence of G418 (300 μg/ml). Initially, PCR was used to screen the G4-18-sensitive clones (primers mP2170, 5′-GGCATAGTG-ACCCCTTACTATAATAC-3′, complementary to a region of the EPCR promoter upstream of the segment used in the targeting vector; Lox5a, 5′-ACATTATAAGATGATAAGCGCTGCCG-3′, complementary to the first LoxP site; PCR conditions involved 3 cycles of 94 °C for 45 s, 65 °C for 45 s, and 72 °C for 3 min, with a final 10-min extension. The PCR-positive ES clones were further confirmed by Southern blot using three different probes: 5′-external probe (probe 1C, 918 bp, Be/I/EcoRV fragment), 3′-external probe (probe “b,” 700 bp, Xho/I/HincII fragment), and a “neo” probe (1 kb).

To delete the region of the EPCR gene flanked by LoxP sites, those ES cells exhibiting correct homologous targeting were transiently transfected with the vector pBS-500/Cre-GFP (a gift of Dr. B. Sauer, Oklahoma Medical Research Foundation, Oklahoma City, OK) and grown in the absence of G-418. Thereafter, PCR was performed to screen for loss of the EPCR exon 1/neo neo gene fragment. A 0.65-kb PCR product was generated by primers for the mutant allele: mP280 and M1 (5′-GCCACTTGGAGAATGGTGGCTG-3′) (in the promoter region of the EPCR construct); mINb, 5′-GAGGCTGAGTACGGCGCT-3′ (intron 1 of the EPCR construct). Southern blotting was employed to further confirm the deletion of this region using probes a and b after digestion of ES cell DNA with either BgIII, HincII, or BclI (Fig. 1).

**Generation and Breeding of Chimeric Mice—**Heterozygous ES cells (LoxK + Cre, clone 7E10-1A) carrying targeted homologous recombination at the EPCR locus and also exhibiting normal karyotype were microinjected into C57BL/6J blastocysts and surgically implanted into uteri of pseudopregnant foster mothers. 10 chimeric males (50–100% coat color) were generated, and at 6 weeks of age were mated with female Black Swiss mice. From these matings, four chimeras demonstrated germ-line transmission. Tail biopsies were obtained from agouti (F1) mice for genotyping. Starting at F2, the heterozygous EPCR (EPCR+−) mice were interbred to generate homozygous EPCR−/− mice. PCR was performed to genotype embryos and tail biopsies. PCR conditions involved 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min.

**Southern blotting** was employed to confirm the structure of the targeting vector. We utilized the Cre/Lox system in order to facilitate generation of mutant mice for future investigations.

**In Vitro Embryo Culture—**Embryos (n = 12) were dissected from pregnant mice at E7.5. The decidual and embryonic membranes (yolk sac, etc.) were removed and embryos rinsed with phosphate-buffered saline (PBS). Embryos were placed in 6-cm dishes containing Dulbecco's modified Eagle's medium, supplemented with 50% rat serum and 1% non-essential amino acids (Invitrogen) (21, 22). Embryos were cultured in 95% air, 5% CO2 with gentle agitation.

**Oligotex mRNA mini kit (Qiagen).** First strand cDNA was synthesized using Super First Strand Synthesis kit (Invitrogen). PCRs were performed using murine EPCR-specific primers (20), TM-specific primers (sense primers, 5′-GCAGCTTGGAAGATGTGGGCTG-3′; antisense primer, 5′-GAACCTTGGACGGTGATGCG-3′), and murine a-actin primers (Clontech), which amplified 300-bp, 1.0-bp, and 530-bp PCR products, respectively.

**Characterization of Murine EPCR Knockout Embryos**

**In Vivo Embryo Culture—**Embryos (n = 12) were dissected from pregnant mice at E7.5. The decidual and embryonic membranes (yolk sac, etc.) were removed and embryos rinsed with phosphate-buffered saline (PBS). Embryos were placed in 6-cm dishes containing Dulbecco's modified Eagle's medium, supplemented with 50% rat serum and 1% non-essential amino acids (Invitrogen) (21, 22). Embryos were cultured in 95% air, 5% CO2 with gentle agitation.

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**Histology, Immunohistochemistry, and EPCR Antigen Determination—**Timed matings between EPCR−/−mice were set up. The developmental progress of the developing embryos was estimated by the gestational age, with day 0.5 post-coitum defined as the morning that a vaginal plug was detected. Some mice were injected daily to full term, and others were sacrificed, and the embryonic/maternal decidual tissue was removed for histology. Some mice were injected daily to full term, and others were sacrificed, and the embryonic/maternal decidual tissue was removed for histology. Some mice were injected daily to full term, and others were sacrificed, and the embryonic/maternal decidual tissue was removed for histology.
each well. To two wells, 5 units of bovine thrombin was also added. Cells were incubated at 37 °C for 1 h and then fixed and immunostained, using streptavidin-Texas Red for detection. Cells incubated with both mouse fibrinogen and thrombin stained strongly for fibrin, whereas staining was undetectable on those cells incubated with mouse fibrinogen alone (not shown).

For histological analysis, hematoxylin and eosin staining was performed upon sections of mouse maternal/embryonic specimens. Immunostaining using the antibodies described above was performed upon serially cut sections (8 μm) of mouse tissue specimens as reported previously (10).

Plasma sEPCR and tissue EPCR were measured using goat anti-mouse EPCR polyclonal antibodies and an enzyme-linked immunosorbent assay as described previously (14), except plasma samples were used rather than serum.

RESULTS

Disruption of the EPCR Gene in Mouse ES Cells—The murine EPCR gene spans 8.8 kb and is composed of 4 exons and 3 introns (6). To facilitate disruption of the EPCR gene, we generated a targeting vector in which exon 1 (encoding the 5'-untranslated region, ATG start codon, signal peptide, and part of the extracellular domain) and part of intron 1 of the murine EPCR gene (1.56 kb) was flanked by two loxP sites. This vector contained an additional LoxP-flanked neoR cassette for selection purposes. Following introduction of the targeting vector into AB2 ES cells, we identified 17 positive clones (out of a total of 880 G418-resistant colonies) that by PCR screening were found to contain the targeted EPCR gene. Southern blot analysis revealed 6 clones with the correct homologous recombination at the EPCR locus (Fig. 1). Subsequently, 3 positive ES clones were transiently transfected with a Cre recombinase expression vector, and those clones in which the EPCR exon 1/intron 1/neoR had been excised were identified by Southern blotting.

Generation of EPCR-deficient Mice—Mice were raised from the targeted ES cells and shown to transmit the mutant allele to their offspring by two independent PCR strategies. Macroscopic examination of EPCR−/− embryos suggested that the heterozygotes develop normally. By the comparison of the Thieiler stages of EPCR−/− and EPCR+/+ littersmates, we found no obvious developmental abnormalities associated with EPCR heterozygosity (not shown). Adult EPCR−/− mice were of normal size and weight and appeared to have normal viability and fertility. Out of 176 EPCR−/− mice monitored during the initial observation period of 10 months, none died or showed any evidence of thrombotic complications, as determined by gross examination. Microscopic examination of the major organs of adult heterozygous EPCR−/+ mice did not indicate any abnormalities when compared with tissues obtained from EPCR−/− littersmates (not shown). The amount of sEPCR in plasma and tissue EPCR in whole lung extracts from EPCR−/− mice (58 ± 17 ng/ml (n = 16) and 302 ± 145 ng/g (n = 22), respectively) was approximately half that detected in WT mice (106 ± 30 ng/ml (n = 16) and 582 ± 257 ng/g (n = 22), respectively).

EPCR Deficiency Is Embryonic Lethal—Crosses between EPCR−/− mice did not result in any viable EPCR−/+ progeny. Out of 410 mice born to heterozygous breeding pairs of agouti mice (50% Black Swiss/50% 129SvEv), 117 (29%) were identi-
fied as EPCR^{+/+}, 293 (71%) were EPCR^{+/−}, and 0 were homozygous EPCR^{−/−}. Of 109 mice born from EPCR^{+/−} pairs back-crossed with the 129SvEv genetic background, 43 (39%) were EPCR^{+/−}, 66 (61%) were EPCR^{−/−}, and again no EPCR^{−/+} mice were identified. Because of the origin of the ES cells utilized, when the chimeras were bred to 129SvEv mice, the resultant offspring were in a pure 129SvEv background. Genotyping of the resultant offspring were in a pure 129SvEv background. Genotyping of the resultant offspring when the chimeras were bred to 129SvEv mice, the resultant offspring were in a pure 129SvEv background.

### Table I

| Embryo stage | No. litters | No. offspring | No. embryos genotyped | EPCR genotype | No. observed (% offspring) | No. resorptions |
|--------------|-------------|---------------|-----------------------|---------------|----------------------------|----------------|
| E7.5         | 4           | 42            | 42                    | +/+           | 9 (21)                     | 25             |
|              |             |               |                       | +/−           | 23 (55)                    | 10             |
|              |             |               |                       | −/−           | 10 (24)                    | 0              |
| E8.5         | 3           | 32            | 32                    | +/+           | 7 (22)                     | 14             |
|              |             |               |                       | +/−           | 14 (44)                    | 11             |
|              |             |               |                       | −/−           | 11 (34)                    | 0              |
| E9.0         | 4           | 44            | 42                    | +/+           | 11 (25)                    | 23             |
|              |             |               |                       | +/−           | 23 (55)                    | 8^c            |
|              |             |               |                       | −/−           | 8 (21)                     | 2              |
| E9.5         | 5           | 51            | 46                    | +/+           | 12 (24)                    | 25             |
|              |             |               |                       | +/−           | 25 (49)                    | 9^c            |
|              |             |               |                       | −/−           | 9 (17)                     | 5^c            |
| E10.5        | 4           | 29            | 25                    | +/+           | 7 (24)                     | 16             |
|              |             |               |                       | +/−           | 16 (55)                    | 2^b            |
|              |             |               |                       | −/−           | 2 (7)                      | 4^b            |
| E11.5        | 3           | 27            | 18                    | +/+           | 5 (19)                     | 13             |
|              |             |               |                       | +/−           | 13 (48)                    | 0^a            |
|              |             |               |                       | −/−           | 0 (0)                      | 9^c            |
| E12.5        | 4           | 30            | 22                    | +/+           | 8 (27)                     | 14             |
|              |             |               |                       | +/−           | 14 (47)                    | 0^a            |
|              |             |               |                       | −/−           | 0 (0)                      | 8^c            |
| E14.5        | 4           | 32            | 25                    | +/+           | 7 (22)                     | 18             |
|              |             |               |                       | +/−           | 18 (56)                    | 0^a            |
|              |             |               |                       | −/−           | 0 (0)                      | 7^a            |

^a Embryos were visually smaller and developmentally retarded. ^b p < 0.01 (difference between observed and expected frequencies derived by the confidence interval for binomial distributions).

### Figure 2

A. Macroscopic examination of EPCR^{+/+}, EPCR^{+/−}, and EPCR^{−/−} embryos at E7.5, E8.5, E9.5, and E10.5 (A) and results of embryo genotyping by Southern blot (B) and RT-PCR (C). A. Gross appearance of the EPCR^{−/−}, EPCR^{+/−}, and EPCR^{+/+} embryos at E7.5, E8.5, E9.5, and E10.5 embryo stage. Embryos from a single pregnancy at the same stage are shown. Note the obvious developmental arrest and pale appearance observed in EPCR^{−/−} embryos at E9.5 (this was the largest EPCR-null embryo observed at this time point) and the onset of degeneration by E10.5. Morphologically, WT and EPCR^{−/−} embryos were indistinguishable throughout development. B, genotype determination in E9.5 embryos by Southern blot analysis. Genomic DNA extracted from embryos was digested with HindIII and hybridized with probe b. Bands corresponding to the WT allele (4.2 kb) and those corresponding to the targeted allele (5.8 kb) are identified. C, RT-PCR analysis of EPCR mRNA in total RNA extracts prepared from E9.5 embryos. Note that EPCR mRNA levels in EPCR^{−/−} are ~50% that of WT embryos and undetectable in EPCR^{−/−} embryos. TM and actin mRNA levels are comparable in all three genotypes.
termates was more apparent. The extra-embryonic membranes, including the yolk sac, amnion, and Reichert's membrane/parietal endoderm were present and appeared normal in all genotypes. In addition, islands of nucleated red blood cells could be clearly seen within the yolk sac, indicating that EPCR is unlikely to have a major function in the development of the

**Fig. 3.** The first signs of any abnormality in the EPCR−/− embryos were observed from about E8.0. At this time, EPCR-null embryos were slightly smaller than EPCR+/− littermates (compare A and B, cross-sections of EPCR+/− and EPCR−/− termates; images depict embryos in utero, in approximately the same orientation and depth through the tissue). Although EPCR−/− embryos had no apparent developmental defect, they were consistently growth-retarded. Immunoperoxidase staining for EPCR in WT and EPCR−/− at E9.5 (C–F). Staining for EPCR in EPCR+/− embryo at E9.5 (C and E), showed that EPCR was detected in the trophoblast giant cells of the trophectoderm, surrounding the embryonic space. These cells, identified by their large nuclei (gc arrows), are in direct contact with the maternal circulation and decidual cells, which in EPCR−/− mothers also stained strongly for EPCR. The parietal endoderm, yolk sac, and embryo proper exhibited no staining for EPCR at this time. E shows an enlargement of the boxed area in C. Note the preferential staining for EPCR on the surface of the trophoblast giant cells, consistent with its membrane location. Staining for EPCR of an E9.5 EPCR+/− littermate on the same slide (D) highlighted the absence of EPCR from the trophoblast giant cells (enlarged black boxed area, F). At E9.5, the heart of the EPCR−/− embryo was visible (red box). However, the onset of resorption is also evident. Note the highly diminished embryonic space surrounding the embryo, normally filled with amniotic fluid. Often beyond E10.5, EPCR−/− embryos could not be genotyped by PCR. Immunostaining for EPCR (G) revealed that the trophoblast giant cells (not staining for EPCR) were the last remnants of resorbed embryos. Bars, 400 μm (unless stated). Key: gc, trophoblast giant cell; dc, decidual cells; ys, yolk sac; E, embryo; pe, parietal endoderm; A, antimesometrial pole; M, mesometrial pole.
yolk sac vasculature. This hypothesis corroborated our previous finding that EPCR is undetectable in the yolk sac of WT embryos (10). At E9.5, EPCR-null embryos were markedly smaller than their EPCR+/− and EPCR−/− littermates (Fig. 2A). Furthermore, they appeared developmentally retarded. While by E9.5, EPCR+/− and EPCR−/− embryos had generally reached Theiler stage 14–15 (optic vesicle and limb bud formation), homozygotes did not seem to progress past Theiler stage 13. Indeed, no EPCR−/− embryos examined were found to have completed axial rotation (normally completed ~E9.5; Theiler stage 15). Despite their reduced size at E9.5, the heart of EPCR−/− embryos was identifiable (Fig. 3D, red box). This sagittal section depicts an EPCR−/− embryo that exhibits signs of the onset of resorption, highlighted by the disappearance of the embryonic space, which is normally filled with amniotic fluid. Furthermore, the extra-embryonic membranes are discontinuous, and the embryo appears compacted. The trophoblast giant cells at the feto-maternal border (identifiable by their large nuclei) were the only cell type of embryonic derivation found to stain positive for EPCR in either EPCR+/− (Fig. 3, C and E) or EPCR−/− embryos at E9.5. No staining for EPCR was seen in the trophoblast giant cells of EPCR−/−/− embryos (Fig. 3, D and F). It therefore seems likely the absence of EPCR expression from the giant cells contributes to the embryonic lethality at this developmental stage.

EPCR was detected in comparatively high amounts in endothelial cells and the cells of the compact decidual layer of the
EPCR-/- maternal uterus. Moreover, as maternal blood (containing sEPCR) irrigates the surfaces of the trophoblasts, and very possibly enters the embryo in trace amounts, most likely it is a function of cellular EPCR that is essential for embryonic survival.

By E10.5, EPCR-null embryos were largely resorbed (Fig. 3G). Although the remains of resorbed embryos could not be genotyped effectively by PCR, the last remnants corresponded to the trophoblast giant cells. The absence of detectable EPCR by immunostaining served to confirm the original genotype of these cells (Fig. 3G).

In Vitro Embryo Culture—As a strategy to determine the basis of embryonic lethality, we isolated 12 embryos at E7.5 and cultured them in vitro for 4 days. Extra-embryonic membranes were removed from the embryos before culture. Embryos developed without a placenta and obtained nutrients by direct absorption from the culture medium. All cultured EPCR-/- embryos appeared to develop beyond E10.5 in a similar fashion to EPCR+/+ littermates cultured in parallel. Similarly, all embryos developed a heart and were of comparable size, irrespective of their genotype. There seemed to be no discernable developmental block associated with the EPCR-null embryos. As the embryos were cultured in the absence of maternal influence and without a developing placenta, these data might suggest that the embryonic lethality induced by complete EPCR deficiency may be attributable to dysfunctional materno-embryonic interactions or impaired placental function.

EPCR Deficiency Results in Deposition of Fibrin and Infiltration of Leukocytes—To examine the possible cause of lethality of EPCR-/- embryos, 40 uterine/embryonic specimens (E8.5 and E9.5) from EPCR+/+ crosses were harvested and simultaneously analyzed by double immunofluorescence for EPCR and fibrin (Fig. 4, A–D). In specimens in which EPCR (green) was detected in the giant cells, corresponding to EPCR+/+(Fig. 4, A and C) or EPCR-/- embryos, very little staining for fibrin (red) was observed. Occasionally, small, isolated patches of fibrin were seen, but as a general observation, this was minimal. Of the 40 embryos, 8 were identified as EPCR+/+(visualized by the lack of EPCR in the giant cells). In all of these specimens staining for fibrin was observed in the extracellular milieu surrounding these giant cells (Fig. 4, B and D). Frequently, the developing placenta also exhibited particularly intense staining for fibrin (Fig. 4B). This strongly suggested that thrombosis at the materno-embryonic interface might contribute to EPCR-deficient embryonic death. The deposition of fibrin around the null embryos was also associated with the infiltration of leukocytes, identified by staining for CD45 (Fig. 4E). However, whether this was a direct consequence of EPCR deficiency or indirectly as a downstream effect of fibrin deposition or impending resorption could not be ascertained.

Anticoagulant Treatment Extends Survival of EPCR-/- Embryos—To examine further the possibility that thrombosis contributed to early embryonic lethality, we administered LMWH (enoxaparin) to pregnant EPCR+/+ mothers as an anticoagulant strategy to rescue or prolong EPCR-/- embryo survival. As shown in Table II, only a subset of EPCR-/- embryos could be rescued from mid- to late embryonic death by LMWH (4 µg/g). On this regimen, EPCR-/- embryos could not be brought to term and still died in utero despite the absence of detectable fibrin (see below). Interestingly, the time of death of EPCR-/- embryos in the same litter did not necessarily occur at the same developmental stage, despite their exposure to the same anticoagulant conditions. At E12.5 and E13.5, although most of the EPCR-/- embryos were undergoing/had undergone resorption, some appeared morphologically similar to EPCR+/+ and EPCR-/- litters. These EPCR-/- embryos had completed axial rotation, had a functional vascular system (heart containing nucleated red blood cells), and prominent limb buds. It therefore appears unlikely that the absence of EPCR gene expression dramatically affects early organogenesis.

EPCR-null embryos found at E14.5 however, seemed to be developmentally retarded. Extensive characterization of EPCR-deficient embryos was not possible due to the low frequency of their survival to this stage.

Analysis of fibrin deposition in/around EPCR-/- embryos (E12.5) receiving LMWH treatment revealed the absence of fibrin around the giant cells (Fig. 4G). Furthermore, leukocytes were not detected among the trophoblast giant cells (not shown). The chorioallantoic placenta, which at this time is highly vascularized and represents the primary site of exchange of oxygen, nutrients, and waste product between the embryonic and maternal circulation, similarly displayed no staining for fibrin (Fig. 4G). These data further suggest that thrombosis is a contributing factor to early embryonic lethality of EPCR-/- embryos.

**DISCUSSION**

In this study we demonstrate that disruption of the EPCR gene in mice results in early embryonic lethality on or before E10.5. High level EPCR expression is detected solely on the embryonic trophoblast giant cells of the trophoectoderm between E7.5 and E10.5 (10). Thrombosis is observed surrounding the trophoblast giant cells derived from the EPCR-/- embryos but not around those derived from the EPCR+/+ or EPCR-/- cells. These results provide the first genetic evidence that EPCR plays a critical role in the control of blood coagulation at the feto-maternal boundary.

We provide several lines of evidence that link thrombin generation in the microenvironment of the trophoblast giant cells with death and retarded development of EPCR-/- embryos. First, EPCR-/- embryos removed from maternal deciduas and cultured in vitro developed beyond E10.5, suggesting materno-embryonic interaction influences the fate of this genotype in utero. Second, at E9.5 and E9.5 we detected a dramatic increase in the amount of fibrin around the trophoblast giant cells and in the primitive placenta of EPCR-/- embryos. Finally, the daily administration of LMWH to EPCR-/- interbred
pregnant female mice was able to prolong the survival of some EPCR-null embryos. Although LMWH prevented detectable fibrin deposition at the materno-embryonic interface, only a subset (~25%) of EPCR−/− embryos survived ~E15.5. Taken together, we speculate that impaired APC generation at the interface between the embryo and maternal circulation contributes to thrombosis and the midgestational developmental arrest of these embryos. Although a defect in trophoblast function may be the underlying cause of uniform lethality of EPCR-deficient embryos on or before E10.5, it seems likely that the absence of essential EPCR function(s) at later developmental stages might prevent the effective rescue of EPCR-null embryos by anticoagulation. Consistent with this possibility, EPCR expression in embryonically derived cells other than the trophoblast giant cells first becomes detectable in the parietal endoderm from ~E10.5. Only at later time points is it detectable in the embryo proper, first in the aortic endothelium from ~E13.5 and in certain other vessels after E16.5 (10). The expression of EPCR at these different sites may indicate that this expression is critical and accounts for the inability of heparin to prevent embryonic death. Alternative approaches will be required to identify the defects responsible for later embryonic death.

Unlike the EPCR−/− mice, heterozygous EPCR-deficient mice develop normally, appear healthy, unchallenged, and do not experience any apparent thrombotic complications. Furthermore, to date, the life expectancy of EPCR−/− mice does not seem to differ from that of WT mice. Although heterozygous mutations of EPCR have been identified in humans who develop thrombosis and myocardial infarction (17), it remains to be determined whether this represents an underlying risk factor. It will be of interest to determine whether EPCR−/− mice are at greater thrombotic risk when challenged. If so, these mice might provide a model for studying the mechanisms that lead to the complications experienced by EPCR-deficient humans.

Interestingly, the time of death of EPCR−/− embryos occurs much earlier than in mice deficient in protein C (24), a natural ligand of EPCR. A partial explanation probably lies in the key roles played by the trophoblast giant cells in regulating thrombosis. In this location hemostasis is modulated through the interplay of embryonic cell surface proteins (e.g. tissue factor, TM, and EPCR) and circulating maternal clotting factors. The materno-embryonic boundary in the protein C−/− embryos would have about half-normal levels of protein C contributed by the heterozygous mother. Given normal levels of TM and EPCR in the developing placenta, protein C activation at the materno-embryonic interface would be only marginally compromised in this location. However, with either TM or EPCR deficiency, normal levels of protein C activation would be severely compromised favoring the thrombosis that is observed. Given the normal localization of EPCR on trophoblast giant cells and, as revealed by fibrin deposition, the dysfunction of these cells in the absence of EPCR, it would seem that the cause of early EPCR−/− embryonic lethality is most likely attributable to a defect in trophoblast anticoagulant function, as opposed to impaired protein C activation within the embryo itself.

The fate of EPCR-null embryos is similar to that described previously (22) for TM−/− mice, which die around E8.5. Recently, reconstituted TM expression in the extra-embryonic tissue (corresponding to the trophectoderm) was found to prolong TM-null embryo survival (25). Moreover, the reported rescue of early embryonic lethality of TM−/− embryos also deficient in tissue factor has implicated increased thrombin generation (and fibrin deposition) at the fetomaternal border as a contributing factor in the death of TM−/− embryos at E8.5. However, a second developmental block in rescued TM-null embryos resulted in death at later embryonic time that likely results from a consumptive coagulopathy (25). The latter death seen in the LMWH-treated EPCR−/− embryos may reflect a similar second developmental block that exists in EPCR-null embryos, and the similar time frame of the embryonic loss could suggest that it is a process dependent on expression of both TM and EPCR.

If one assumes that the sole role of EPCR is in the augmentation of protein C activation by the thrombin-TM complex, then it would seem remarkable that EPCR deficiency would lead to death. On cultured cells and in reconstituted systems, EPCR promotes APC generation by less than 20-fold (4, 26). However, EPCR has the unusual property of increasing protein C activation rates catalyzed by the thrombin-TM complex roughly in proportion to the EPCR concentration, even when the EPCR is in vast excess over the TM (26). Like EPCR, TM is expressed at relatively high levels on the trophoblast giant cells, probably indicating that the combination of high level EPCR and TM expression provides a potent anticoagulant phenotype to the surface of these cells. This may be critical as the placenta represents a hypercoagulable environment. Indeed, embryonic placental tissue factor procoagulant activity in the trophoblasts is essential for normal placental development (27). Furthermore, it seems that maternal fibrinogen and its conversion to fibrin is also required (in controlled quantities) for placental stabilization and the anchoring of placental trophoblasts to the maternal decidua (28). These data serve to highlight the requirement for tightly coordinated hemostatic regulation (i.e. anticoagulant roles of TM and EPCR) in the trophoblast cell layers. Interestingly, mice homozygous for a mutant form of TM that reduces protein C activation rates more than 20-fold remain viable (29). Coupled with the observation that fibrin deposition can be blocked without rescuing EPCR−/− mice, these studies suggest that other EPCR-dependent processes are critical. Some candidates for other roles include EPCR nuclear translocation (30) with concomitant alteration of gene expression, EPCR interaction with leukocytes (15), or EPCR-dependent signaling by APC (31, 32). Recently, the crystal structure of EPCR revealed a tightly bound phospholipid and a remarkable three-dimensional homology to the CD1 family of molecules (8), most of which are involved in lipid antigen presentation. This suggests a potential additional function for EPCR in immune regulation that could contribute to the observed embryonic lethality. Appropriate mutations in mice should allow these latter hypotheses to be tested.

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