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Low levels of Tissue Factor rescue Protein C-associated pregnancy failure in mice

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We have previously demonstrated that maternal Protein C (PC) is critical for ensuring a successful pregnancy outcome [1]. Our findings suggested that enhanced coagulation, accompanied by elevated inflammation, due to a severe PC deficiency, were contributing causes of the failed pregnancies. Whether severe thrombosis at the fetal-maternal interface was the direct consequence of pregnancy failure was unclear. Furthermore, it was not apparent whether the enhanced recruitment of inflammatory cells was due to a PC deficiency and/or whether it was secondary to a local accumulation of fibrin or an excess of thrombin. To investigate whether severe coagulation was a significant factor in the pregnancy failure, we generated, by crossmating strategies, mice expressing very low levels of PC, combined with mice with a severe depletion in Tissue Factor (TF), i.e., $PC^{-/-}$PC(tg4)/$TF^{-/-}$hTF(tg). These lines of mice express PC and TF from nontargeted single allelic transgenic (tg) insertions of murine PC and human TF cDNAs, respectively, with the endogenous genes encoding these proteins totally inactivated through breeding strategies. Singly-deficient $PC^{-/-}$PC(tg4) [1] and $TF^{-/-}$hTF(tg) [2] mice each express PC and TF at approximately 1% of WT adult values.

A significant (N = 6, $P < 0.05$) increase in the clottable fibrinogen level was observed in $PC^{-/-}$(tg4) mice compared to WT mice (Figure 1a). However, mice with a combined severe deficiency of PC [$PC^{-/-}$PC(tg4)], along with a ~99% reduction in TF [$TF^{-/-}$hTF(tg)], restored fibrinogen to WT levels (N = 6, $P < 0.05$). Similarly, thrombin-antithrombin (TAT) levels (Figure 1b) responded to these genotypic manipulations in the same fashion as the fibrinogen levels (same samples as in Figure 1a; $P < 0.05$ for each genotype, compared to WT). These data suggest that the coagulopathy observed in $PC^{-/-}$PC(tg4) mice was reversed by the additional severe deficiency of TF.

Whereas $PC^{-/-}$PC(tg4) females were not able to sustain pregnancies beyond midgestation (ED7.5–8.5), independent of the embryonic genotypes, $PC^{-/-}$PC(tg4) mice, crossbred with mice with a severe reduction in TF levels (50% or 99% of WT), led to successful pregnancies. In particular, 15/15 pregnancies from $PC^{-/-}$PC(tg4)/$TF^{-/-}$hTF(tg) females x $PC^{-/-}$PC(tg4)/$TF^{+/-}$hTF(tg) males and 12/12 pregnancies from $PC^{-/-}$PC(tg4)/$TF^{-/-}$hTF(tg) females x $PC^{-/-}$PC(tg4)/$TF^{-/-}$hTF(tg) males, were successfully maintained to term. In these cases, live pups were born and survived at least to weaning age. Interestingly, these pregnancies produced an average of 3 pups/litter, suggesting that some embryos still failed to fully develop despite a reduction in the coagulopathy due to a severe PC deficiency. In addition, when 44 embryos from 10 matings of $PC^{+/-}$PC(tg4)/$TF^{+/-}$hTF(tg) females x $PC^{-/-}$PC(tg4)/$TF^{-/-}$hTF(tg) males were analyzed, 12 live offspring of the genotype

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Disclosure of Conflicts of Interest
None.

$PC^{+/-}PC(tg4)/TF^{-/-}hTF(tg)$ and 9 live offspring of the genotype $PC^{-/-}PC(tg4)/TF^{+/-}hTF(tg)$ were found, numbers close to the theroetical value of 11 for each of these two genotypes. This indicates that elevation of the anticoagulant PC activity in a low-TF embryo, or an increase in procoagulant TF in a low-PC embryo, did not affect embryonic viabilities.

To monitor the $PC^{-/-}PC(tg4)/TF^{-/-}hTF(tg)$ x $PC^{-/-}PC(tg4)/TF^{-/-}hTF(tg)$ pregnancies more closely, we followed the gestational progress from 7.5–13.5 dpc by exploratory surgeries to visualize yolk sacs from the uteri of pregnant mice. As predicted, a significant number of non-viable reabsorbing $PC^{-/-}PC(tg4)/TF^{-/-}hTF(tg)$ fetuses (~6/litter) were detected at this time. The pregnancies spontaneously terminated at 7.5–8.5 dpc, confirmed histologically, which was a similar time of death of non-viable embryos from low-PC mothers [1]. Since these embryos were all of the same genotype, it appears as though the embryonic block in $PC^{-/-}PC(tg4)$ mothers was only partly restored by the additional decrease in TF levels, a nonetheless very significant result. In addition, it appeared that in the absence of overt thrombosis at the ectoplacental cone (EC) region (Figure 1c,d) in the $PC^{-/-}PC(tg4)/TF^{+/-}hTF(tg)$ cohort, inflammation was also reduced, as seen by the significantly smaller numbers of leukocytes in this same area in this mouse line (Figure 1e,f). These findings suggested that the recruitment of inflammatory cells in low-PC mothers was a response to fibrin deposition, or to proteases generated from the TF-dependent coagulation cascade. The data also imply that hypercoagulation alone was not entirely responsible for embryonic death in low PC mothers, since some death persisted in very low TF/PC embryos, despite the absence of severe thrombosis. The evidence that only a fraction of embryos survived beyond the implantation stage in mothers with a compound severe deficiency of PC/TF further implies that improving normal hemostasis was not in itself sufficient to fully rescue embryonic death. We suggest that in addition to balancing hemostasis, activated PC (aPC)-dependent inflammatory signaling is required for sustaining embryonic viability. Findings from studies in mice deficient for thrombomodulin [3] and Endothelial Protein C Receptor (EPCR) [4] also support a role for aPC signaling in favorable pregnancy outcomes.

In conclusion, this study shows that in the presence of a limited level of the anticoagulant, PC, placental homeostasis can be restored by reducing TF expression, thus facilitating proper embryonic implantation, similar to the TF-mediated rescue observed in $EPCR^{-/-}$ embryo [5]. The evidence of limited viable embryos in the low-PC mother supports our previous work showing that embryonic survival is highly dependent on the availability of maternal PC. This correlates to, and extends, the finding that EPCR expression only on placental giant trophoblasts allows embryos to be maintained to term [5]. We show here that this cannot be accomplished without maternal aPC. Thus, interfering with maternal aPC/giant trophoblast EPCR-mediated signaling is detrimental to the development of the embryo.

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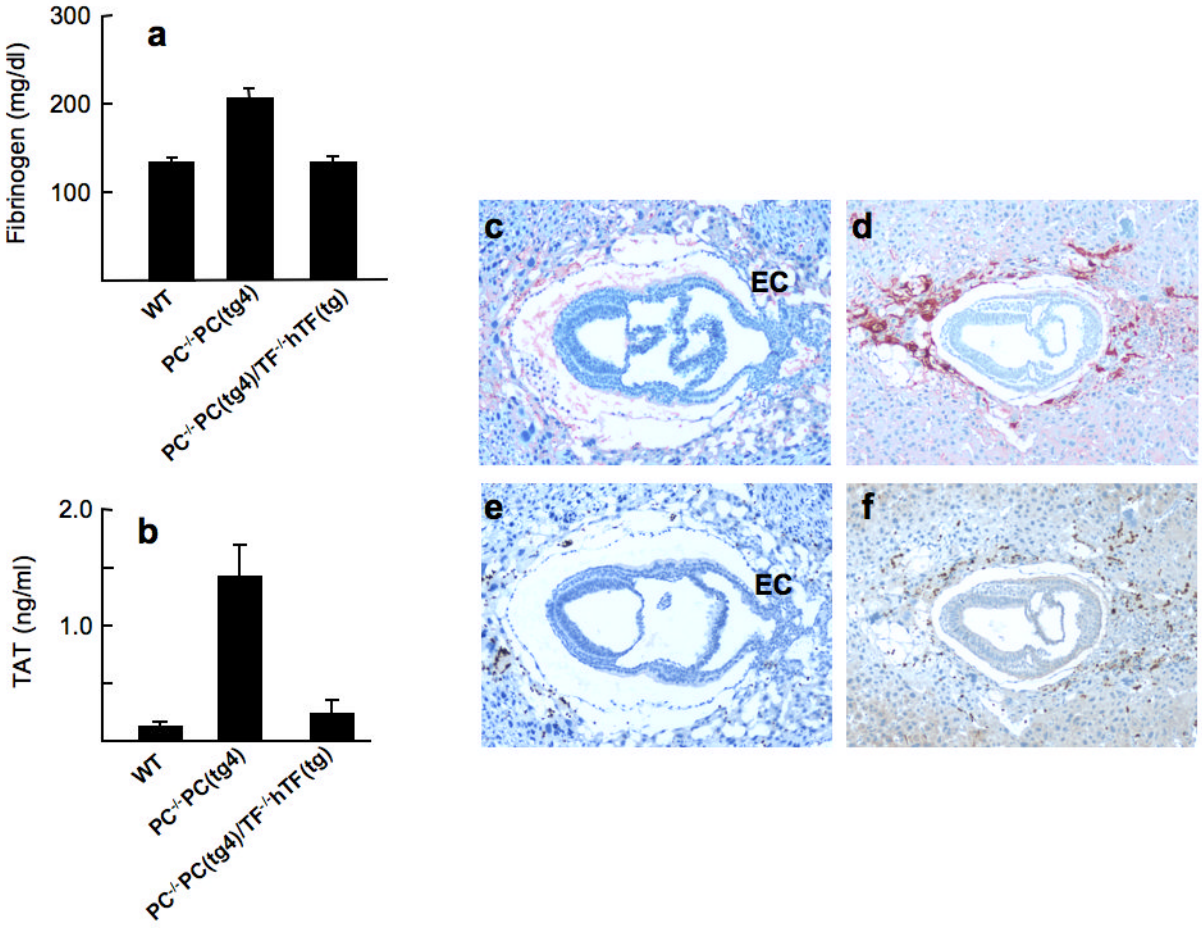

Figure 1. Coagulopathy and hyperinflammatory responses in the ectoplacental cone regions of low-PC pregnant females are corrected by a simultaneous deficiency of TF

Circulating fibrinogen (a) and TAT (b) levels were determined in citrated plasma using the clotting time assay with Fibritest. (c–f). Immunohistochemical detection of fibrin/fibrinogen and leukocytes in 8.5 dpc placentas. A female with compound severe deficiencies in PC and TF [$PC^{-/-}$PC(tg4)/$TF^{-/-}$hTF(tg)] (panel c) showed no discernable fibrin deposition (red staining) around the ectoplacental cone (EC) the compared to those carrying a single deficiency of PC (panel d). Similarly, a significant reduction in leukocytes (brown staining) were observed in the EC region in $PC^{-/-}$(tg4)/$TF^{-/-}$hTF(tg) mothers (panel e) compared to mice with a single deficiency of PC (panel f).