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Role of the Protein C Receptor in Cancer Progression

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Abstract

The hemostatic system plays pleiotropic roles in cancer progression by shaping the tumor microenvironment and metastatic niches through thrombin-dependent fibrin deposition and platelet activation. Expanding experimental evidence implicates coagulation protease receptors expressed by tumor cells as additional players that directly influence tumor biology. Pro-angiogenic G protein-coupled signaling of TF through protease activated receptor 2 and regulation of tumor cell and vascular integrins through ligation by alternative spliced TF are established pathways driving tumor progression. Our recent work shows that the endothelial protein C receptor (EPCR), a stem cell marker in hematopoietic, neuronal and epithelial cells, is also crucial for breast cancer growth in the orthotopic microenvironment of the mammary gland. In aggressive triple-negative breast cancer cells, EPCR expression is a characteristic of cancer stem cell-like populations that have tumor initiating properties *in vivo*. Blocking antibodies to EPCR attenuate *in vivo* tumor growth and proliferation specifically of $EPCR^+$ cells on defined integrin matrices *in vitro*. We also showed that tumor-associated macrophages are a source for upstream coagulation proteases that can activate TF- and EPCR-dependent cellular responses, suggesting that tumor cells utilize the tumor microenvironment for tumor promoting coagulation protease signaling.

Keywords

cancer stem cell; coagulation; tumor microenvironment; macrophage; protease

Links of coagulation initiation and tumor progression

The tissue factor (TF) initiated extrinsic coagulation pathway is essential for responses to vascular and tissue injury, coupling hemostatic clot formation (1;2) with innate immune host defense (3–5) and initiating repair mechanisms and their pathological consequences (6–13). TF association with cytoskeletal structures (14–17), the interaction of the TF extracellular

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domain with integrins (18;19), and TF-dependent activation of the pro-migratory G protein-coupled receptor, protease activated receptor (PAR) 2 provide molecular connections by which TF supports not only tissue repair, but also cancer progression. TF-FVIIa cleaves PAR2 and stimulates cell migration though chemokine induction (20–23). PAR2 activation also recruits β-arrestin adaptor proteins (24) that act as scaffolds for MAP kinase ERK1/2 and cofilin activation in cell migration (25–28). TF-FVIIa-PAR2 signaling induces phosphorylation of the TF cytoplasmic domain and thereby reverses inhibitory effects of TF on integrin α3β1-dependent migration (18;29). In clinical breast cancer, upregulated TF and PAR2 expression is associated with TF cytoplasmic domain phosphorylation and relapse (30), indicating that pathological TF-FVIIa signaling is turned on in aggressive cancers.

TF-FVIIa signaling elicits a transcriptional program that broadly influences angiogenesis and innate immune cell recruitment (22;31;32). TF-FVIIa-PAR2 signaling can be selectively blocked by an antibody that prevents the association of TF-FVIIa with integrins, but has no major effect on coagulation (19;33). Inhibition of tumor growth and angiogenesis by this antibody provided the first compelling evidence that TF-FVIIa proangiogenic signaling promotes tumor progression independent of coagulation activation *in vivo*, a conclusion confirmed by a delayed “angiogenic switch” in tumor prone mice lacking PAR2, but not PAR1 (34). Clinical (30) and transfection (35;36) studies implicated the TF cytoplasmic domain in tumor progression. Consistently, cytoplasmic domain deleted ($TF^{\Delta CT}$) mice showed the same delay in angiogenesis-dependent spontaneous cancer development as PAR2−/− mice (37). Reconstitution of PAR2−/− breast cancer cells with PAR2 or with a PAR2 mutant defective in β-arrestin recruitment restored proangiogenic signaling and tumor growth (37), supporting the current model of tumor cell TF-FVIIa-PAR2 signaling promoting angiogenesis and tumor progression.

The requirement for TF in embryonic vascular development (38–42) led to diverse studies on coagulation-dependent (43;44) and –independent (45–51;51–55) functions of the TF pathway in angiogenesis. While these studies were largely focused on roles of the TF-FVIIa complex, recent data show that alternatively spliced TF (asTF) regulates angiogenesis independent of initiating coagulation or PAR signaling (56). The TF mRNA is spliced in two gene products, transmembrane-anchored full-length TF (flTF) or asTF that lacks exon 5 encoding an extracellular region involved in substrate FX binding. Instead asTF has a unique carboxyl-terminus translated from an alternative reading frame of exon 6 and is a soluble protein (57). While TF procoagulant function is crucial for tumor cell metastasis, asTF does not contribute to this process (58). Instead, purified asTF was shown to induce endothelial cell sprouting by ligating endothelial cell-expressed integrins α6β1 and αvβ3 (59) and upregulates leukocyte adhesion molecules to enhance monocyte transmigration (60;61). In addition, inhibitory antibodies specific for asTF have shown a role for asTF in regulating tumor cell proliferation *in vivo* (62). Broader roles of coagulation receptors in tumor progression were uncovered by our recent studies in mice deficient of the endothelial protein C receptor (EPCR) (63) which will be reviewed here.

EPCR as a stem cell marker

The procoagulant effects of the TF pathway are counterbalanced by the protein C (PC) anticoagulant pathway to avoid intravascular thrombosis (64). The CD1d–like immune receptor EPCR binds the γ-carboxyl glutamic acid-rich (Gla) domain of PC to markedly improve PC activation by thrombin bound to endothelial cell-expressed thrombomodulin. The protein C pathway has an important role in balancing the prometastatic effects of thrombin generation initiated by tumor cell-expressed TF. For example, vascular overexpression of EPCR or treatment with activated protein C (aPC) reduces metastasis, while protein C blockade, protein C resistance due to the factor V_{Leiden} mutation or thrombomodulin dysfunction increase metastasis (65–68). In contrast, EPCR deficiency has minimal effects on metastasis (58), suggesting that direct neutralization of thrombin by thrombomodulin is a dominant mechanism by which metastatic spread is prevented once tumor cells have entered the bloodstream.

In addition to these roles of the anticoagulant pathway on vascular cells, tumor cell-expressed EPCR has been implicated in tumor progression. EPCR-dependent PAR1 activation by aPC stimulates cell migration of breast cancer cells or prevents apoptosis of lung cancer cells leading to enhanced metastasis (69;70). In contrast to these tumor promoting functions of EPCR in epithelial tumors, EPCR counteracts TF and PAR1 dependent metastasis of mesothelioma in the pleural cavity (71). It is unclear whether EPCR exerts the profound anti-proliferative and pro-apoptotic effects on TF-expressing mesothelioma through anticoagulant or signaling pathways. We became interested in the role of EPCR in breast cancer progression, because EPCR also binds FVIIa and FXa and contributes to TF-dependent and independent signaling by these proteases (72–74). In addition, EPCR is a potential marker for breast cancer stem cells and was used to isolate these subpopulations implicated in cancer recurrence (75;76).

EPCR is expressed by hematopoietic, neuronal and epithelial progenitor populations (77–80), but functional roles of EPCR in stem cell and cancer stem cell biology are poorly understood. EPCR is expressed by highly aggressive basal-like breast cancer subtypes (81). Cancer tissue from patients contain stem cell-like subpopulations that can be enriched by several markers, including a $CD44^{high}/CD24^{-}$ surface phenotype (82), expression of aldehyde dehydrogenase (ALDH1) (83), and EPCR (76). We found that in a triple negative, aggressive breast cancer line (84), EPCR defined a subpopulation of $CD44^{high}/CD24^{-}$ cells (Fig. 1) and went on to characterize the unique properties of these cells.

EPCR expression influences tumor cell biological properties

Comparison of this population with another relatively stable subpopulation with high TF expression revealed distinct differences. The subpopulation with high TF expression showed upregulated PAR2 expression and of a concerted repertoire of genes previously implicated in proangiogenic TF-dependent signaling. In the EPCR expressing subpopulation, we documented expression of markers associated with an aggressive or stem cell-like phenotype, including ALDH1B1 and ALDH1A3 (83), the hematopoietic stem cell marker integrin α4 (85), and the pan stem cell maker integrin α6 (86). However, these cells did not match previously established gene signatures, for example found in integrin $\alpha6^{high}$ skin

cancer stem cells or other stem cells (87;88), suggesting that plasticity of tumors include partial escape from developmentally defined regulation of stem cell gene expression patterns. Importantly, selection for EPCR enriched a subpopulation with improved growth properties in suspension culture and the ability to produce tumor growth *in vivo* at very low doses of injected tumor cells.

Most importantly, we found that inhibitory antibodies to EPCR attenuated the tumor initiation capacity of these cells injected at low doses and also reduced tumor growth of a mixed aggressive breast cancer population. In a mouse model of EPCR deficiency, we further documented diminished oncogene-induced spontaneous tumor progression. In addition, deletion of EPCR from murine mammary tumors reduced tumor growth, providing multiple lines of evidence for a functional role of the stem cell marker EPCR in breast cancer growth. Genetic overexpression of EPCR in endothelial cells reduces lung metastasis (65), indicating a role for EPCR in tumor cell survival in vascular niches. However, we found no differences in spontaneous lung metastasis in the spontaneous breast cancer models when EPCR expression was severely reduced.

Proteases ligands for EPCR and TF are provided by innate immune cells in the tumor microenvironment

The tumor promoting roles of EPCR in murine and human breast cancer growth and the attenuation of tumor initiation and growth by inhibitory antibodies to EPCR suggested that cancer stem cell-like populations sense EPCR ligands in the tumor microenvironment. EPCR binds three coagulation proteases, activated PC, FVIIa, and FXa that could conceivably enter the tumor microenvironment due to the hyper-permeability of the tumor vasculature. However, this pathway was difficult to rationalize when very low numbers of EPCR-expressing cancer stem cells were injected for tumor growth. Although FVII is synthesized by certain cancers (89;90), the TF-and PAR2-dependent breast cancer models studied by us showed no cell autonomous production of FVII or FX. However, we detected mRNA expression of FVII and FX in tumor-associated macrophages recovered from the tumor stroma of the PyMT breast cancer model of spontaneous breast cancer progression (63). It is important to point out that human lung macrophages have been shown previously to synthesize FVIIa macrophages (91), excluding that extrahepatic synthesis of upstream coagulation factors is a peculiarity of rodent models. Thus, these experiments uncovered a potential pathway by which cancer stem cell expressed EPCR may transmit cues from the innate immune system to regulate stem cell survival, retention, and/or differentiation (Fig. 2). These reciprocal links between tumor cells and the host innate immune system in tumor initiation and angiogenesis will require validation by additional genetic mouse models with altered expression of coagulation proteases and their receptors in the tumor microenvironment.

These experiments should also stimulate further research in the pharmacological modulation of coagulation protease activity in preclinical models and potentially in clinical cancer therapy. The mechanisms of novel small molecule oral anticoagulants targeting specific coagulation proteases are finding wider acceptance for thrombosis indications. Identifying EPCR's protease ligand will be instrumental to select an optimal anticoagulant strategy that

may be beneficial in cancer therapy by interrupting the function of EPCR-bound proteases in maintaining cancer stem cell population.

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Abbreviations

EPCR	endothelial Protein C Receptor
TF	tissue factor
TME	tumor microenvironment
TAM	tumor associated macrophages

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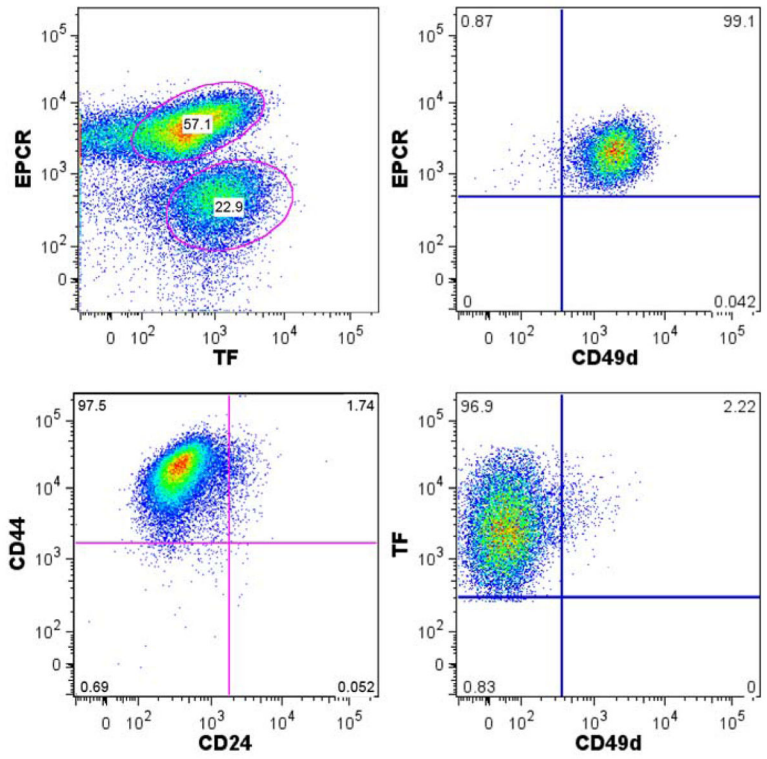

Fig. 1.

TF and EPCR expression levels reveal distinct subpopulations of highly aggressive triple negative MDA-MB-231mfp breast cancer cells.

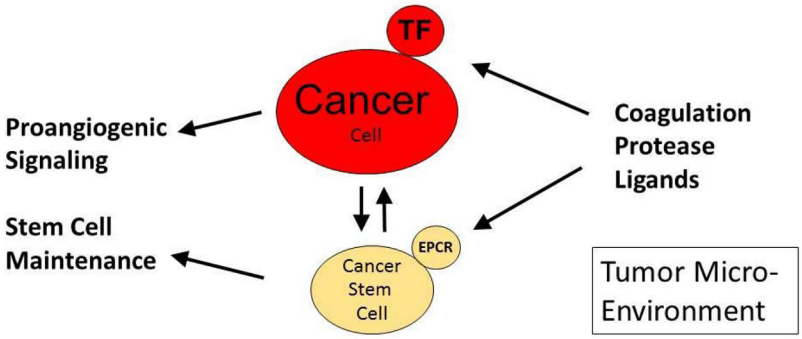

Fig. 2.

Upstream coagulation protease interactions with tumor cell populations in the tumor microenvironment.