Endothelial Progenitor Cells in Sprouting Angiogenesis: Proteases Pave the Way

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Abstract: Sprouting angiogenesis consists of the expansion and remodelling of existing vessels, where the vascular sprouts connect each other to form new vascular loops. Endothelial Progenitor Cells (EPCs) are a subtype of stem cells, with high proliferative potential, able to differentiate into mature Endothelial Cells (ECs) during the neovascularization process. In addition to this direct structural role EPCs improve neovascularization, also secreting numerous pro-angiogenic factors able to enhance the proliferation, survival and function of mature ECs, and other surrounding progenitor cells. While sprouting angiogenesis by mature ECs involves resident ECs, the vasculogenic contribution of EPCs is a high hurdle race. Bone marrow-mobilized EPCs have to detach from the stem cell niche, intravasate into bone marrow vessels, reach the hypoxic area or tumour site, extravasate and incorporate into the new vessel lumen, thus complementing the resident mature ECs in sprouting angiogenesis. The goal of this review is to highlight the role of the main protease systems able to control each of these steps. The pivotal protease systems here described, involved in vascular patterning in sprouting angiogenesis, are the matrix-metalloproteinases (MMPs), the serine-proteinases urokinase-type plasminogen activator (uPA) associated with its receptor (uPAR) and receptor-associated plasminogen/plasmin, the neutrophil elastase and the cathepsins. Since angiogenesis plays a critical role not only in physiological but also in pathological processes, such as in tumours, controlling the contribution of EPCs to the angiogenic process, through the regulation of the protease systems involved, could yield new opportunities for the therapeutic prospect of efficient control of pathological angiogenesis.

Keywords: Angiogenesis, cathepsins, endothelial progenitor cells (EPCs), matrix-metalloproteinases (MMPs), proteases, uPA/uPAR.

INTRODUCTION

While the term 'vasculogenesis' identifies the process of *de novo* formation of blood vessels, the term 'angiogenesis' connotes expansion and remodelling of existing vessels, referred to as 'sprouting angiogenesis', which has long been thought to be the only mechanism accounting for the formation of new vascular networks in embryonic development, as well as in inflammation, cancer and re-vascularization of ischaemic tissues [1]. Alternative mechanisms of angiogenesis have been identified in tumours. Tumour cells and the tumour microenvironment do indeed produce several angiogenic factors that induce a robust endothelial sprouting angiogenesis, but they show at least five additional methods of vessel formation: vascular mimicry [2], trans-differentiation from tumour stem cells to tumour endothelial cells (ECs) [3], vascular co-option [4, 5], recruitment of circulating bone response to tumour-specific factors [6], and recruitment of endothelial progenitor cells (EPCs) [7-9]. EPC marrow-derived ECs to the tumour vascular bed in recruitment occurs as a response to hypoxia, either in tumours or in ischaemic tissues. EPCs must therefore leave the production site (intravasation step) and enter the vasculogenic site (extravasation step). Each step is characterized by the activity of master protease systems that will be discussed in this review. A few previous reviews have partially covered this topic [10-12], with particular emphasis on the MMP system. This review extends to this topic recent advancements on the uPA/uPAR/plasmin system in EPCs and integrates the whole knowledge on EPC proteases with the aim to widen the possibilities to control single steps of EPC pathophysiology by specific anti-protease tharapies.

Protease Systems Involved in 'Classical' Sprouting Angiogenesis

During the sprouting angiogenesis process, mature ECs initiate a programme of tissue invasion across anatomical barriers and within an extracellular matrix (ECM) comprised mainly of type-IV and type-I collagen. According to this programme, the vascular endothelial growth factor (VEGF)-selected 'tip cell' activates a proteolytic pathway, while other ECs stay behind ('stalk cells') and maintain the integrity of the vessel. Such a new structure invades adjacent tissues and reorganizes into empty patent tubules connected to the

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vessel where they came from [13]. The pivotal protease systems involved in vascular patterning in sprouting angiogenesis are the matrix-metalloproteinases (MMPs), the urokinase-type plasminogen activator (uPA) associated to its receptor (uPAR) and receptor-associated plasminogen/plasmin.

The Matrix-Metalloproteinases

Although the triple-helical molecule of collagens is resistant to almost all forms of proteolysis, mature ECs involved in angiogenesis orchestrate interconnected proteolytic cascades that converge on the MMPs' progelatinase A (MMP2, 72 kDa type IV collagenase), and progelatinase B (MMP9, 92 kDa type IV collagenase) [14]. MMP-2 and MMP-9 are synthesized as latent enzymes [15], which are activated when cells activate tissue-invasive or morphogenetic processes. In the sprouting angiogenesis process, the serine proteinase plasmin has been proposed to act as an activator of both MMP-2 and MMP-9, whereas many members of the membrane-anchored family of MMPs (membrane-type (MT)1-, 2-, 3-, 4-, 5-, and 6-MMP) can process the MMP-2 zymogen to its active form [16] (Fig. **1A**). After synthesis, MT1–MMP is activated by furin-like proteases and reaches the plasma membrane [17], where it localizes in filopodia of migrating tip cells upon interaction with CD44 [18]. Tissue inhibitor of metalloproteinase-2 (TIMP2) is known as an inhibitor of MMPs (together with TIMP1 and TIMP3), but also as a

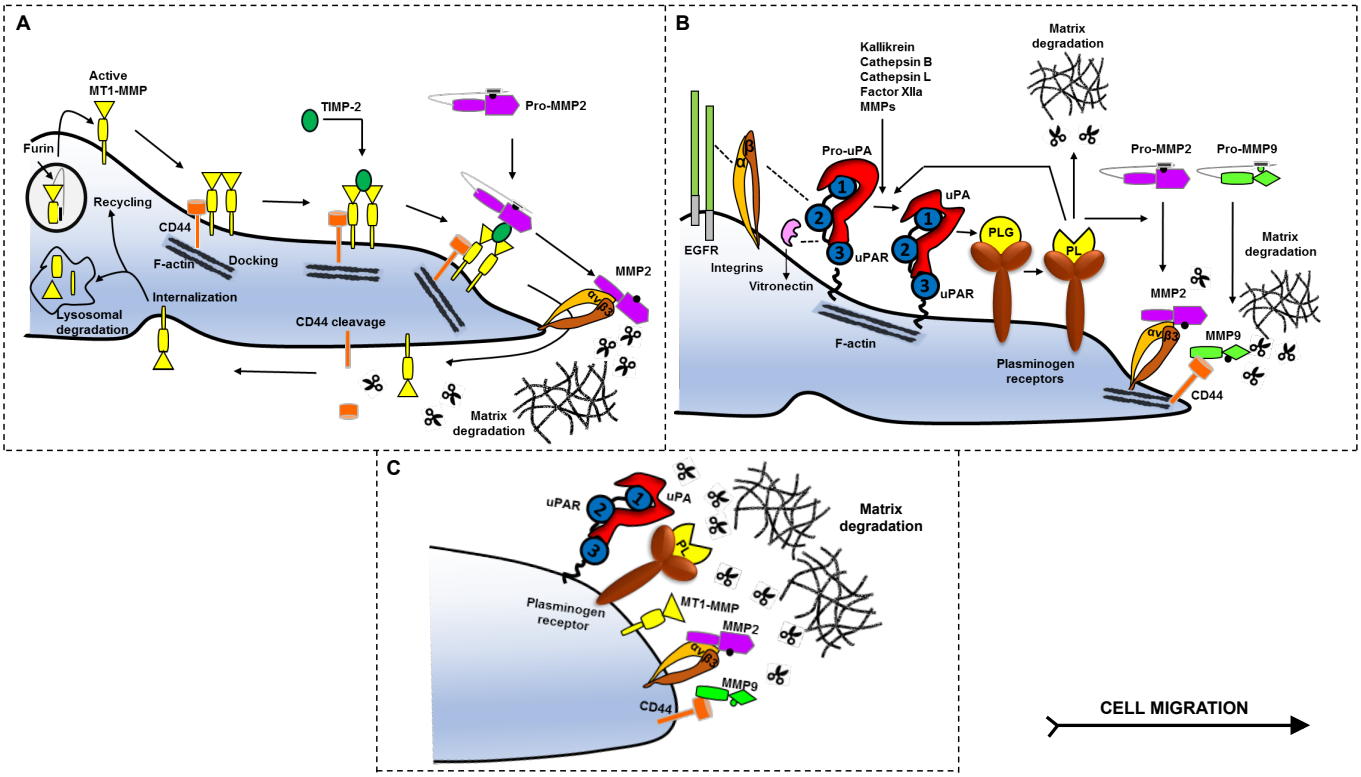

Fig. (1). Protease systems involved in sprouting angiogenesis: pericellular activities of plasmin, MMPs and membrane type-matrix metalloproteinases (MT–MMPs). (*Panel* ***A***) MT1–MMP, after its synthesis, is activated by furin-like proteases and reaches the plasma membrane, where it localizes in the lamellipodia of migrating ECs in association with actin fibres upon interaction with CD44. MT1–MMP is responsible for MMP-2 activation after binding in a complex reaction between these two metalloproteinases and TIMP-2 on the cell surface. This interaction facilitates the activation of MMP-2 by a second adjacent MT1–MMP molecule after di-/oligo-merization. MMP2, anchored to the cell membrane by αvβ3, and MT1–MMP are now able to degrade ECM through their proteolytic activity. MT1–MMP is then internalized *via* caveolae or clathrin-coated pits and degraded within lysosomes or recycled to the plasma membrane. (*Panel* ***B***) uPAR activates uPA on the cell surface, leading to plasminogen activation that generates plasmin and activation of the plasmin-related MMPs cascade. In particular, the inactive precursor pro-uPA interacts with its receptor, uPAR, through its growth-factor domain, resulting into the conversion of uPAR-bound pro-uPA to active uPA. Once activated, uPA cleaves the proenzyme plasminogen (PLG), linked to its receptor on the cell surface, to active plasmin (PL). Plasmin activates pro-MMP2 and pro-MMP9 to their active forms, as well as pro-uPA to active uPA. Pro-uPA activation is also mediated by kallikrein, factor XIIa, cathepsin B, cathepsin L and MMPs [25-27]. Moreover, uPAR interacts through its three dinstinct domains with vitronectin, integrins and receptors, such as the epidermal-growth factor receptor [EGFR]. (*Panel* ***C***) Description of the organization of the activated protease systems in the filopodia of tip cells: uPAR-bound uPA that generates plasmin from plasminogen, MT1–MMP, MMP2 anchored to the cell membrane by αvβ3, and MMP9 anchored to the cell membrane by CD44. Here, we showed the classical way of MT1–MMP, MMP2 and MMP9 activation in ECs, but MMPs can also be activated by other mechanisms as reported by Ra *et al*. [49].

co-factor for MT1–MMP-mediated MMP2 activation. How TIMP2 regulates MMP2 activation in parallel with cleavage of substrates by MT1–MMP is still unclear. In 293 T cells transfected with the MT1–MMP and TIMP-2 genes, it has been shown that co-expression of TIMP2 at an appropriate level increased the amount of cell-surface MT1–MMP, both the TIMP2-bound and free forms, and generated processed MMP2 with gelatin-degrading activity. In contrast, MT1–MMP-specific substrates testican-1 and syndecan-1 were cleaved by the cells expressing MT1–MMP, which was inhibited by TIMP2, even at levels that stimulate MMP2 activation. These results suggest that TIMP2 present in the microenvironment determines MT1–MMP substrate choice between direct cleavage of its own substrates and MMP2 activation [19]. However, surface localization of activated MMPs is critical for allowing cells to migrate within dense connective tissues. Binding interactions between MMP2 and its membrane-docking $\alpha_v\beta_3$ integrin-binding site, and between MMP9 and the cell surface proteoglycan CD44, have been associated with invasive/angiogenic phenotypes [20-22].

The Urokinase-Pslasminogen Activator (uPA)/uPA Receptor (uPAR) System and the Plasminogen/ Plasmin Receptors

This system, also referred to as the plasminogen activation (PA) system, or cell surface-associated fibrinolytic system, is strongly involved in angiogenesis. The proteases belonging to this system are serine proteinases, and their inhibitors are grouped in the so-called 'serpin' (serine proteinase inhibitors) superfamily. The main plasminogen activators, namely the uPA and the tissue-type PAs (tPA) activate plasminogen to plasmin, but only uPA is associated to a *bona fide* receptor (uPAR) on ECs [23]. VEGFA interaction with its type II receptor (VEGFR2) present in mature ECs causes vessel hyperpermeability, and a consequent fluid and protein leakage. Therefore, the presence of fibrin and vitronectin in the provisional ECM of angiogenesis accounts for the need of the uPAR-dependent PA system to perform an efficient angiogenesis programme. In fact, although several molecular interactions between the MMP and the PA system may affect cellular fibrinolysis [24] (Fig. **1B**), the main cell-associated fibrinolytic system hinges on uPAR. uPAR, also known as CD87, is a glycoprotein organized in three domains and tethered to the cell membrane with a glycosylphosphatidylinositol (GPI) anchor [28, 29]. The traditional role of uPAR is the cell surface activation of uPA, leading to plasminogen activation that generates plasmin and activation of the plasmin-related MMPs cascade. Both MMPs and the PA proteases show optimal performance upon association with the cell surface. Such an association is achieved upon interaction with cell membrane receptors and/or binding sites, showing that the process of cell invasion is solid surface-dependent. Besides uPAR, MT1–MMP, CD44 and $\alpha_v\beta_3$ integrin, also receptors for plasminogen have been described. While uPAR is a receptor with low capacity and high affinity for its own ligand, plasminogen/plasmin receptors show high capacity and low affinity for their ligand, and the ratio between the number of uPAR and of plasminogen receptors is about 100 in human ECs [30]. Plasminogen and plasmin tether to the cell surface through a ubiquitously expressed and structurally dissimilar family of proteins, collectively referred to as 'plasminogen receptors'. Some 12 plasminogen receptors have been described so far, but ECs mainly express annexin A2, the P11 protein, actin, the $\alpha_v\beta_3$ integrin, α-enolase, and histone 2B, a quite heterogeneous group of proteins [31]. All these molecules have been shown to facilitate plasminogen activation to plasmin and to protect bound plasmin from inactivation by inhibitors. However, the ability of plasmin to cleave transmembrane molecules and thereby to generate functionally important cleaved products, which induce outside-in signal transduction, has begun to receive attention [32].

uPAR has been shown to contribute to many proteolysis-independent processes, as previously reviewed [33-36]. It can bind directly to vitronectin, which is abundant in angiogenessis provisional ECM, with a domain distinct from its uPA binding site [37] and, although lacking a transmembrane and intracellular domain, uPAR serves as a 'signalosome' organizer, which is triggered upon uPA or vitronectin ligation, and by simultaneous interactions with signalling-competent surface integrins and receptors, such as the epidermal-growth factor receptor (EGFR) [38]. From a general point of view, the inactive precursor pro-uPA interacts with uPAR by its growth-factor domain, allowing the conversion of uPAR-bound pro-uPA to active uPA [39]. Once activated, uPA cleaves the proenzyme plasminogen to yield active plasmin [40] that activates pro-MMPs to active MMPs, as well as pro-uPA to active uPA [33-36, 40, 41]. In the particular case of ECs, VEGF interaction with VEGFR-2 rapidly induces pro-urokinase activation that is dependent on a PI3 kinase-mediated change in integrin affinity, MT1–MMP-mediated activation of MMP2 and subsequent MMP2 uPAR-bound pro-uPA activation and generation of plasmin from receptor-bound plasminogen. This event on ECs is responsible for VEGF-dependent local fibrinolytic activity and might be one of the initial steps in the angiogenic process [42]. As a consequence, stimulation of ECs by VEGFA/VEGFR2 engagement leads to a redistribution of MT1–MMP and uPAR to the leading edge of tip cells, thus focussing the proteolytic activity of the growing vessel to the invasive front of migrating ECs [43] (Fig. **1**).

VEGF-challenged sprouting angiogenesis originates from mature vessels, where ECs are in a confluent state. The response of ECs to VEGF stimulation is reduced by cell density due to increased activity of density-enhanced tyrosine phosphatase DEP1. High levels of DEP1 impair extracellular signal-regulated kinase 1/2 (ERK1/2) activation, a downstream signalling event of the VEGF/VEGF-receptor system, leading to uPAR synthesis down-regulation, which blocks angiogenesis [44]. Sparsely growing cells over-

express uPAR as a consequence of DEP1 down-regulation, and it is therefore likely that over-expression of uPAR in invading tip ECs may be related to the absence of cognate ECs at the leading edge of the sprouting vessel, with inhibition of DEP1 in the tip cell [45]. Other growth factors, such as fibroblast growth factor-2 (FGF2), epidermal growth factor (EGF) and hepatocyte growth factor (HGF), have been shown to induce over-expression of proteolytic activity and angiogenesis in ECs by PI3 kinase pathway-dependent activation of uPAR-bound pro-uPA [46]. Through its domains II and III, uPAR also interacts with α5β1 integrin in ECs, leading to integrin activation and redistribution required to disclose integrin affinity to ECM substrates and providing a further support to the 'grip' of invading ECs [47]. uPAR has been implicated also in zymogen coagulation Factor XII-dependent angiogenesis [48]. Due to VEGF-dependent vascular hyper-permeability, coagulation Factor XII (FXII) is present within the provisional ECM of the sprouting vessel. FXII binds to domain II of uPAR on EC membranes. FXII engagement induces uPAR's communication with the cell through a β1 integrin. Cell stimulation through uPAR and the integrin also includes EGFR. These pathways lead to ERK1/2 and Akt phosphorylation, which stimulates EC growth, proliferation and angiogenesis [48].

Organization of the Protease Systems in Filopodia of the Tip Cells

The accepted model for the mechanistic involvement of MMPs and of the PA system in sprouting angiogenesis is as follows. MT1–MMP is localized in the filopodia of migrating tip cells upon interaction with CD44. In this location, MT1–MMP activates MMP2 (Fig. **1C**). The filopodium of the tip cell is now provided with MMP2 (activated by plasmin and by MT1–MMP and anchored to the cell membrane by $\alpha_v\beta_3$ integrin), MMP9 (activated by plasmin and anchored to the cell membrane by CD44), and by MT1–MMP itself. This proteolytic machinery is further implemented by uPAR-bound uPA, which generates plasmin from plasminogen, both bound to plasminogen receptors. MT1–MMP is then internalized *via* caveolae or clathrin-coated pits, and degraded within lysosomes or recycled to the plasma membrane. A possible reason for the concentration of tip cell proteolytic activity in selected spots of the cell membrane could be the need for spatial and temporal control of proteolytic activities required for an efficient angiogenesis. In fact, excessive proteolysis can cause unwanted damage to the provisional ECM, by degrading and dissolving the 3D structure required for anchoring the migrating cell, as we originally proposed in the 'grip-and-go' model of cell migration [28] on the basis of previous experiments performed in plasminogen activator inhibitor type-1 (PAI-1)-deficient mice showing severely impaired angiogenesis [50, 51]. Despite overwhelming evidence supporting a role for MMP2, MMP9, plasminogen/plasmin, or MT1–MMP activity in angiogenic events *in vivo* [14, 52], the means by which these proteinases exert their effects still remain undefined. MT1–MMP has many proteolytic functions, ranging from MMP2 activation, ECM degradation, release of ECM-bound cytokines and generation of bioactive neo-peptides. To identify the proteolytic systems required for neovessel formation within a physiologically relevant interstitial matrix, mainly composed of three-dimensional gels of cross-linked type I collagen, Tae-Hwa Chun *et al*. have shown that neovessel formation proceeds in an unperturbed fashion in the absence of either plasminogen, MMP2, MMP9, β_3 integrin, or CD44. Instead, the membrane-anchored collagenase MT1–MMP plays a role in conferring ECs with the ability to both proteolytically remodel type I collagen and express a collagen-invasive phenotype critical to the tubulogenic process [53, 54]. Furthermore, the proteolytic activity of MT1–MMP seems to be indispensable for the vacuolization/cavitation process giving origin to patent lumens in the tubulogenesis process [54].

WHAT IS AN EPC?

Although the classification of EPCs is not the aim of the present review, it is important to underline their main features. Haematopoietic progenitors and EPCs share many cell-surface markers in the developing yolk sac and embryo. Genetic knock-out of several genes affects both haematopoietic cells and EC development, suggesting they originate from a common precursor, the haemangioblast [55, 56]. Differently from haematopoietic stem and progenitor cells, hierarchies of stem and progenitor cells (based on differences in proliferative potential) for the endothelial lineage have not yet been firmly established. As reviewed elsewhere [11], EPCs are a subtype of stem cells with high proliferative potential, capable of participating to neovascularization upon differentiation into mature ECs. Although EPCs are detectable in bone marrow and liver, the study of peripheral and cord blood has allowed the characterization of four categories of circulating EPCs: (1) early and (2) late-EPCs, obtained from peripheral blood as an adherent population of EPCs on a gelatin monolayer after 4–7 days and more than 2 weeks, respectively, both capable of being incorporated in capillaries [57]; (3) CFU-ECs (endothelial cell colony-forming units), obtained from non-adherent cells of peripheral blood and producing EC colonies at day 5 after plating [58]; (4) endothelial colony-forming cells (ECFCs) obtained from cord blood adherent cells after 2–3 weeks of culture [59] (Table **1**). From a general stand point, during trafficking and differentiation to ECs, EPCs progressively loose stem cell markers (CD133 and CD34) and gain EC markers, such as CD31 (platelet endothelial cell adhesion molecule-1), VEGF-receptor type 2, CD146 (melanoma cell adhesion molecule), CD144 (VE-cadherin), eNOS (nitric oxide synthase), CD-105 (endoglin) and vWF (von Willebrand factor). In particular, ECFCs isolated from cord blood have been identified as a unique population of high proliferative potential-endothelial colony-forming cells that can achieve at least 100 population doublings (PDs), replate into at least

Table 1. Characteristics of Four Categories of Circulating EPCs.

	EPC		CFU-EC†	ECFC‡
	Early EPC*	Late EPC*		
Endothelial antigens	Yes [60]	Yes [60]	Yes [61]	Yes [61]
Haematopoietic antigens	Yes [60]	No [60]	Yes [61]	No [61]
Proliferative potential	Low [62]	High [62]	Low [61]	High [61]
Paracrine augmentation of angiogenesis	High [60]	Low [60]	High [63]	Low [63]
In vitro tube formation	No [60]	Yes [60]	Low [64]	High [64]
In vivo de novo vessel formation	Low [60]	High [60]	No [61]	Yes [61]

*Early and late-EPCs, obtained from peripheral blood as an adherent population of EPCs on a gelatin monolayer after 4–7 days and more than 2 weeks, respectively.
†CFU-EC, obtained from non-adherent cells of peripheral blood and producing EC colonies at day 5 after plating.
‡ECFCs obtained from cord blood adherent cells after 2–3 weeks of culture. During trafficking and differentiation to ECs, EPCs progressively loose haematopoietic antigens (CD133, CD34 and CD45), but gain typical endothelial antigens, such as CD31 (platelet endothelial cell adhesion molecule-1), VEGF-receptor type 2, CD146 (melanoma cell adhesion molecule), CD144 (VE-cadherin), eNOS (nitric oxide synthase), CD-105 (endoglin) and von Willebrand factor (vWF).

secondary and tertiary colonies, and retain high levels of telomerase activity [61].

About 0.002% of the circulating mononuclear cells are EPCs. When required, EPCs enter peripheral circulation to increase the circulating pool and follow cytokine gradients to traffic into sites of neovascularization [65].

MORPHOLOGIC AND FUNCTIONAL CONTRIBUTION OF EPCs TO SPROUTING ANGIOGENESIS

In sprouting angiogenesis, the main type of angiogenesis, the vascular sprouts connect each other to form new vascular loops [66]. Vasculogenesis during embryogenesis produces a primary capillary plexus, thanks to the accumulation of EPCs that aggregate into a blood island and differentiate *in situ* to mature ECs [67, 68]. Vasculogenesis in the adult tissue is similar to that occurring during embryogenesis—both are dependent upon EPC aggregation and differentiation *in situ*, but the difference is that in the adult the vasculogenic contribution to sprouting angiogenesis seems to occur only in the anastomotic site of angiogenic sprouts. Zhan *et al*. showed that bone marrow mobilized-EPCs leave blood vessels, migrate and aggregate to form a cell cord near the site of angiogenic sprout tips and differentiate to ECs *in situ* during cell vacuolization and vacuole fusion, forming an anastomosis segment that connects the vascular sprouts [69]. In tumour neovascularization, EPCs regulate the angiogenic switch *via* secretion of pro-angiogenic growth factors and by direct incorporation into sprouting nascent vessels. At an early stage of tumour growth, EPCs have a paracrine role that seems to be critical in EC migration and proliferation. Indeed, it was shown that EC-produced VEGF, even more than the plentiful extracellular VEGF, is necessary for the homeostasis of blood vessels [70]. Since it was shown that approximately only 10–20% [71, 72] of tumour vessels have incorporated BM-derived EPCs, we should also underline that the contribution of EPCs is tumour stage-specific and also dependent on tumour type. It was found that the number of EPCs incorporated into the vasculature of ischaemic tissues [73-75] and tumour bed [8, 71, 76-78] was very low compared to the total progenitor cells mobilized. Nevertheless, there is an improvement in neovascularization that has been related not only to a direct structural function (vessel incorporation and stabilization), but also to the indirect essential role of EPCs in secreting numerous pro-angiogenic factors able to enhance the proliferation, survival and function of mature ECs, and other surrounding progenitor cells.

PROTEASES IN THE MOBILIZATION AND INTRAVASATION OF EPCs FROM BONE MARROW TO PERIPHERAL BLOOD

Under normal conditions, bone marrow harbours the stem cell niche, which consists of EPCs and primitive haematopoietic cells, fibroblasts, osteoblasts and ECs referred to as bone marrow stromal cells (BMSCs) [79]. Several reports suggest that the release of EPCs from the stem cell niche into the peripheral circulation is the result of perturbation of adhesive interactions with BMSCs, which normally restrict EPCs within the bone marrow [11, 80].

Adhesive interactions are perturbed by a variety of growth factors, ligands, cell surface receptors and proteases. Several cytokines produced by tumours, as well as by tissue injury or hypoxia, are able to unhook EPCs from BMSCs. Once disengaged, EPCs enter the bloodstream and undergo incorporation in the new vessel lumen, thus complementing the resident mature ECs in sprouting angiogenesis. VEGF, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), fibroblast growth factor type-2 (FGF2), placental growth factor (PLGF), erythropoietin (Epo), and stromal-derived growth factor-1 (SDF-1, also referred to as CXCL12) are released from tumours and ischaemic tissues, reach the stem cell niche in high concentrations [11, 81, 82] and weaken EPC/BMSC interactions.

Neutrophil Elastase and Cathepsin-G

The interaction between VCAM-1 (CD106), which is expressed by BMSCs, and its counter receptor integrin α4β1 (or very late antigen-4, VLA-4), which is expressed at the EPC surface, is critical to the homing and retention of EPCs, and of other haematopoietic stem and progenitor cells [HPCs] [80, 83-85]. VCAM-1 expression is profoundly decreased in the bone marrow of mice mobilized with GCSF or with the chemotherapeutic drug cyclophosphamide, and the subsequent EPC mobilization and decrease of VCAM-1 expression are synchronous with accumulation within the bone marrow of neutrophil elastase and cathepsin-G [86, 87]. These enzymes cleave VCAM-1 on BMSCs, preventing VLA-4 molecules expressed on the cell surface of EPCs from binding to their VCAM-1 ligand on BMSCs. Moreover, the same proteases also cleave the cytokine SDF-1, which is highly produced by BMSCs and maintains stem cells within the niche by interacting with its cognate receptor CXCR4 expressed by stem cells, as well as the CXCR4 receptor itself [87]. The double cleavage can strongly improve the inactivation of the chemotactic properties of the SDF-1/CXCR4 axis, thus inducing EPCs to leave the stem cell niche and to look for alternative homing sites, such as ischaemic tissues (Fig. **2**).

Neutrophil elastase, proteinase 3 and cathepsin G are three proteases stored in large quantities in neutrophil cytoplasmic azurophilic granules. They act in combination with reactive oxygen species to help degrade engulfed micro-organisms inside phagolysosomes. These proteases are also externalized in an active form during neutrophil activation at inflammatory sites, thus contributing to the regulation of inflammatory and immune responses. In view of their activity as multifunctional proteases, they also play a regulatory role in non-infectious inflammatory diseases and their modulation has been proposed as a therapeutic tool [88].

MMP9

VEGF is one of the most important regulators of EPC mobilization. It is over-expressed in hypoxic tissues and tumours under the effect of hypoxia-inducible factor-1 [89]. Upon binding to its receptor VEGFR-2, VEGF stimulates maturation from the haemangioblast to early/late EPCs [90]. Stem cells

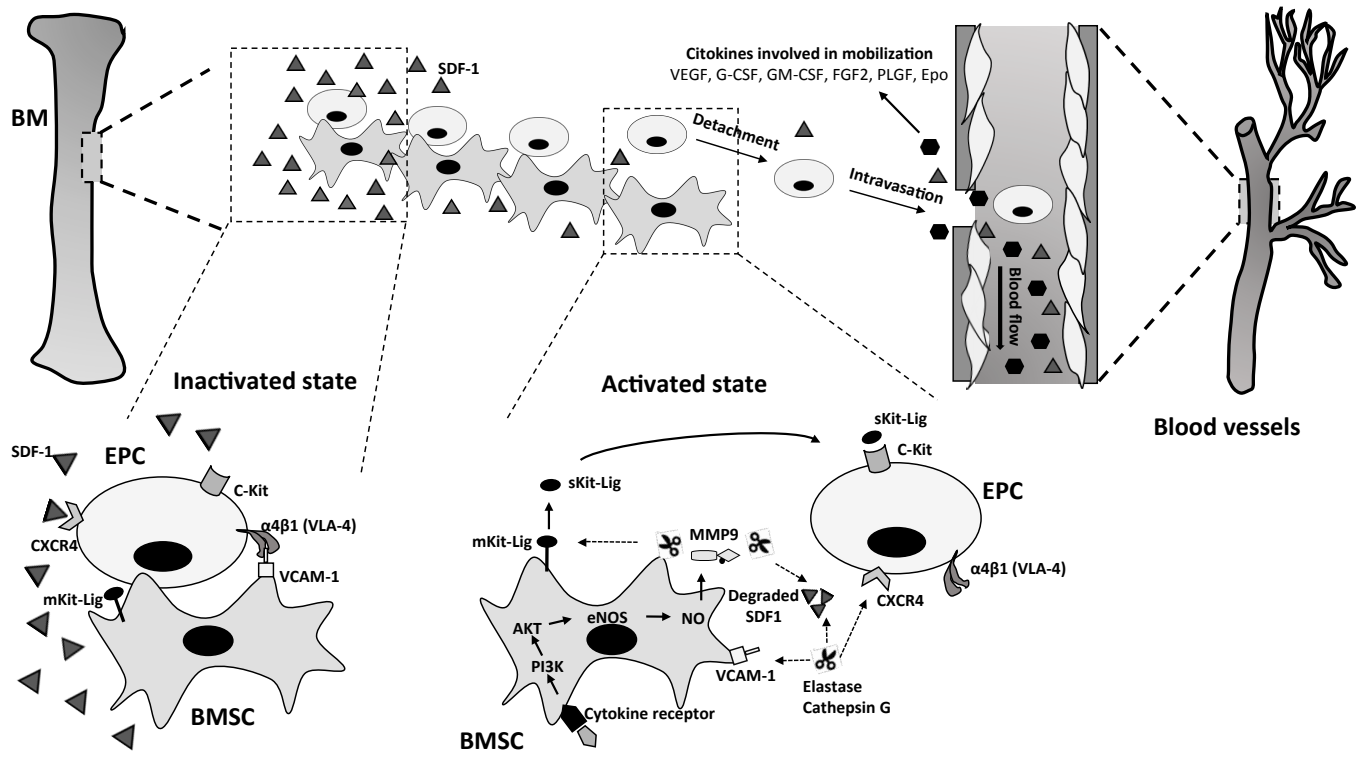

Fig. (2). Mobilization and intravasation of EPCs from bone marrow to peripheral blood. *Inactivated state:* in the stem cell niche SDF-1 interacts with its receptor CXCR-4 on EPCs and VCAM-1 [CD106], expressed by BMSCs, interacts with α4β1 [VLA-4] on EPC surfaces, inducing the retention of EPCs in bone marrow. *Activated state:* neutrophil elastase and cathepsin-G, accumulated within the bone marrow, cleave VCAM-1, preventing VLA-4 molecules expressed on the cell surface of EPCs from bind their VCAM-1 ligand on BMSCs, weakening EPC/BMSC interactions. Mobilizing factors, such as VEGF, G-CSF, SDF-1, produced by hypoxic tissues, reach the stem cell niche and activate BMSCs through the phosphoinositide 3-kinase (PI3K/AKT) signalling pathway and eNOS, leading to an over-production of nitric oxide which stimulates over-production of MMP-9. MMP9, in concert with neutrophil elastase and Cathepsin-G, degrades SDF-1 within the cell niche thus weakening the adhesive chemo-attraction of CXCR4-expressing EPCs in the bone marrow. Furthermore, MMP9, in addition to its collagenolytic activity on the basal membranes of bone marrow vessels, cleaves the membrane–bound Kit ligand (mKit-Lig) to a soluble Kit ligand (sKit-Lig), which binds to the EPC receptor c-kit, allowing EPC intravasation from the bone marrow to peripheral blood.

stimulated by VEGF in the stem cell niche over-produce the 92 kDa type IV collagenase MMP9 [73] which, in turn, mediates the release of EPCs from the stem cell niche. All the EPC mobilizing factors act according to a well-known cascade of events. BMSCs are stimulated by mobilizing factors, such as VEGF, G-CSF and SDF-1, which activate the phosphoinositide 3-kinase (PI3K/AKT) signalling pathway and eNOS [73], leading to an over-production of nitric oxide, which stimulates over-production of MMP9 [91]. MMP9, in concert with neutrophil elastase and cathepsin-G, degrades SDF-1 within the cell niche thus weakening the adhesive chemo-attraction of CXCR4-expressing EPCs in the bone marrow [65]. This event induces a prevalence of SFD-1 produced by other sources, such as tumours or inflammation sites, thereby deflecting the EPCs' chemo-attraction toward alternative sites. At the same time, MMP9 cleaves the membrane–bound Kit ligand (mKit-Lig) to a soluble Kit ligand (sKit-Lig), which binds to the EPC receptor c-kit, allowing translocation of bone marrow EPCs from the quiescent bone marrow niche to the proliferative vascular niche, and their intravasation from the bone marrow to peripheral blood [11, 65, 73, 91]. MMP9, required for EPCs mobilization, is also likely to open a path to EPCs through basement membranes in the process of intravasation by its collagenolytic activity [14] (Fig. **2**).

It is noteworthy that EPCs are also mobilized from the bone marrow in response to myocardial infarction [92] and become incorporated into sites of new vessel growth in the ischaemic tissue [93-95]. The pharmacological CXCR4 antagonist AMD3100 rapidly mobilizes EPCs from bone marrow by reversibly blocking the interaction between SDF-1, produced by BMSCs, and CXCR4 present on the stem cell surface. Administered in combination with G-CSF, AMD3100 enhances angiogenesis in a mouse model of limb ischaemia and in ischaemic areas of myocardial infarction [96, 97]. MMP9 and VEGF expression was elevated in infarcted AMD3100-treated mice for a long period following administration, indicating that MMP9 is involved in AMD3100-dependent EPC mobilization and intravasation [97]. Previous reports indicate that MMP9 expression in a variety of cells is regulated by VEGF [98-100]. Collectively, these data indicate that a VEGF-dependent increase in MMP9 levels in bone marrow, related to EPC mobilization, is a positive factor for post-infarction myocardial revascularization.

Much recent evidence suggests that the function of MMPs in angiogenesis is more intricate than just degrading the extracellular matrix to facilitate the invasion of endothelial cells. For example, MMP2 and MMP9 gelatinases are up-regulated in angiogenic lesions and play a significant role in EPC differentiation [101] and in initiating angiogenesis, respectively [102, 103]. In particular, MMP9 can regulate angiogenesis, promoting the release of extracellular matrix-bound cytokines, such as VEGF. *In vitro* VEGF-induced EPC migration was suppressed after the addition of a selective MMP9 inhibitor, blocking matrix degradation and the release of matrix-bound pro-angiogenic growth factors. *In vivo* MMP9 deficiency impaired ischaemia-induced angiogenesis in mice with unilateral hind limb ischaemia [102]. In such a model, it was demonstrated that the lack of MMP-9 reduced the release of sKit-Lig, which binds to the EPC receptor c-kit, impairing adhesion and migration capacities, probably blocking the EPCs' intravasation from bone marrow to peripheral blood [102]. Similarly, MMP-9 deficiency decreased EPC release from the bone marrow after focal cerebral ischaemia, and the angiogenic ability of late EPCs from ischaemic MMP9-deficient mice was reduced, thus compromising the formation of vascular networks *in vitro*. Exogenous MMP9 did not reverse the impaired angiogenic function in MMP9/KO late EPCs, suggesting that only endogenously secreted MMP9 is essential for accurate EPC function in terms of *de novo* vessel formation [104]. MMP9-mediated EPC mobilization, together with VEGF, also plays a critical role in infantile proliferation haemangiomas [105]. As reported above, the stimulation and support of MMP9's activity is dependent on nitric oxide (NO), whose production is increased in BMSCs by the PI3K/AKT pathway, activated by EPC-mobilizing factors and eNOS [73, 91]. This observation suggests that both eNOS and MMP9 could be two important downstream molecules involved in EPC mobilization. The severe outcome of cardiovascular complications in diabetic patients is linked to augmented endothelial injury and reduced neo-vessel formation [106, 107]. Li Dong *et al*. showed that insulin, besides blood glucose control, induces an increase in EPC mobilization and post-ischaemic vasculogenesis in diabetic mice through a VEGF/eNOS-related pathway increasing NO production [106]. Recent data indicate that, in ischaemic tissue after myocardial infarction and also in tumours, oestradiol is able to increase the mobilization and incorporation of bone marrow-derived EPCs into sites of ischaemia-induced neovascularization by an increment of MMP9 expression in bone marrow mediated by the eNOS pathway [108, 109]. Debanjan Chakroborty *et al*. indicated, for the first time, a new regulatory role in MMP9 synthesis in tumours driven by dopamine. During malignant tumour growth, they observed a decrease of dopamine concentration in bone marrow that was inversely correlated with EPC mobilization to the peripheral blood vessels. The inhibitory role of dopamine in EPC mobilization, exerted after the interaction with its D2 receptors, was associated to the suppression of VEGFA-induced ERK1/ERK2 phosphorylation and, consequently, MMP9 activation. These data suggest a novel use for dopamine and D2 agents in the treatment of cancer and other diseases in which MMP9 mediated-EPC mobilization plays an essential pathogenic role [110].

Other MMPs in Bone Marrow EPC Mobilization

In addition to the above, the 72 kDa type IV collagenase MMP2 also regulates EPC mobilization. Bone marrow EPCs (c-Kit+ stem cells) obtained from MMP2(–/–) mice exhibit reduced invasion and proliferation, compared with age-matched MMP2(+/+) mice, and flow cytometry performed on the peripheral blood shows that the numbers of EPC-like CD31(+)/c-

Kit(+) cells are markedly decreased in MMP2-deficient mice [111]. These data suggest that MMP-2 deficiency is associated with a reduction of EPC invasive and/or proliferative activities and bone marrow mobilization.

MT1–MMP (MMP14) has been shown to play a role in G-CSF-induced progenitor cell mobilization. G-CSF-activated MMP14 cleaves the protein CD44 on progenitor cells and thus disrupts their CD44-mediated binding to the basement membrane in the bone marrow, thereby promoting progenitor cell mobilization and egress [112]. CD44 protein is a monomeric high glycosylated transmembrane protein, also expressed by stem cells, whose function is the binding of hyaluronic acid and other extracellular matrix glycoproteins [113].

EPC EXTRAVASATION, ARRIVAL WITHIN HYPOXIC AREAS AND ORGANIZATION IN VASCULAR NETWORKS

Once they enter the blood stream, EPCs home to tumour tissues or to ischaemic areas. Homing appears to depend on chemotactic-dependent attraction of EPCs, operated by several chemokines whose cognate receptors are expressed on the EPC membrane [11]. Such ligand-receptor systems include VEGF/VEGFR-2 [114], SDF-1/CXCR4 [115], IL-8/CXCR2 [116], GRO-α/CXCR1 [117], CCL2/CCR2 and CCL5/CCR5 [118]. Expression of many ligands is controlled by HIF-1, produced in hypoxic tissues [114]. Extravasation, the trans-endothelial passage of EPCs from peripheral blood to sites of active vascular formation, occurs in a way similar to leukocyte recruitment to sites of inflammation. The 'rolling' step is mediated by P-selectin protein ligand-1 (PSGL-1), expressed on EPCs, and P and E-selectin expressed on ECs [119], while the 'adhesion' step occurs by interaction of EPC β1 and β2 integrins with EC cell-adhesion-molecules (CAMs) and fibrinogen [120]. Although the trans-endothelial migration of EPCs is inhibited by blocking of the chemokine receptors CXCR4 or CXCR7 [121], many EPC integrins, including α5β1 (fibronectin receptor), α6β1 (laminin receptor), αvβ3 and αvβ5 are major determinants of EPC homing and invasion, while β2 integrins are the major regulators of EPC trans-endothelial migration *via* their interaction with ICAM-1 [11, 122, 123]. While integrins provide a 'grip' to EPC migration, proteases open the way across anatomical barriers to cells migrating toward the chemotactic source [14, 33]. Of course, EPCs need to migrate through the blood vessel basement membrane and the ECM to home to sites where new vessels are required. Three major protease families account for extravasation and vascular tunnel excavation within the provisional angiogenic matrix, namely MMPs, serine-proteinases of the uPA/ uPAR system and cathepsins [11, 14, 33] (Fig. **3**).

The Role of MMPs

VEGF is required for the initiation of angiogenesis and VEGF-induced MMP production is regulated by matrix stiffness. Reduced elasticity of the ECM and reduced matrix stiffness (from rigid to yielding) affect the progression of tubulogenesis. Several studies have shown that tube formation is implicated in the development of vacuoles [124, 125]. The tube morphogenesis mechanism involves coordinated signalling pathways: (1) VEGF initiates angiogenesis inducing the production of MT1–MMP, MMP1, MMP2 and MMP9 in EPCs; (2) MT1–MMP on the cell membrane degrades the ECM directly and through activation of pro-MMPs at the pericellular area, creating a physical space to facilitate the development of cords of EPCs and lumen formation [53, 126]; (3) EPCs can elongate on the substrate within 2–6 hours; (4) the interactions between the ECM and integrin induce a cascade of downstream signalling; (5) the subsequent activation of Rac 1 and Rho GTPase Cdc42 leads to the formation of intracellular vacuoles [50], extension and fusion to lumens after 12 hours [127] (Fig. **3**).

Yoon *et al*. demonstrated that the transplantation of mixed EPCs (early and late EPCs) results in a synergistic increase in angiogenesis in athymic nude mice with hind-limb ischaemia [59]. Both early and late EPCs express VEGFR and IL-8 receptors, but only early EPCs secrete large amounts of both cytokines able to increase not only migration, proliferation and tube formation [128, 129], but also MMP secretion in ECs [129, 130]. Therefore, early EPC-secreted cytokines act in an autocrine and paracrine manner. While early EPCs contribute to neovascularization secreting pro-angiogenic cytokines and MMP-9, late EPCs provide building blocks and secrete MMP-2 that mediates the MMP-9 activation [131].

MMP9 secretion is known to be stimulated by various cytokines such as VEGF, SDF1, TNFα and TGFβ [91, 132], but peroxisome proliferator activated receptor-δ (PPAR-δ) also plays a crucial role in MMP9 production. PPAR-δ is an important modulator of angiogenesis, mediating a paracrine network between EPCs and ECs or skeletal muscle cells. PPAR-δ directly enhances EPC vasculogenesis through the activation of the Akt pathway. PPAR-δ can also induce MMP9 secretion from EPCs through direct transcriptional regulation leading to degradation of insulin-like growth factor-binding protein-3 (IGFBP-3) in serum. This degradation drives to the liberation of insulin-like growth factor–1 (IGF-1) from the complex IGF-1/IGFBP-3. The free IGF-1 is able to activate IGF-1R in both the surrounding ECs and skeletal muscle, inducing angiogenesis and skeletal muscle regeneration. These effects are MMP9-mediated as indicated by their inhibition after MMP9 knock-out [133]. In addition, insulin-like growth factor-2 (IGF-2)/IGF-2-receptor plays a new role in recruiting and incorporating EPCs into ischaemic sites. IGF-2R is highly expressed on human cord blood EPCs. IGF-2, expressed under hypoxic conditions, induces EPC recruitment and incorporation through an IGF-2R-dependent signalling pathway, and also promotes MMP9 expression [134].

In a three-dimensional system, it was shown that VEGF is able to stimulate the secretion and activation

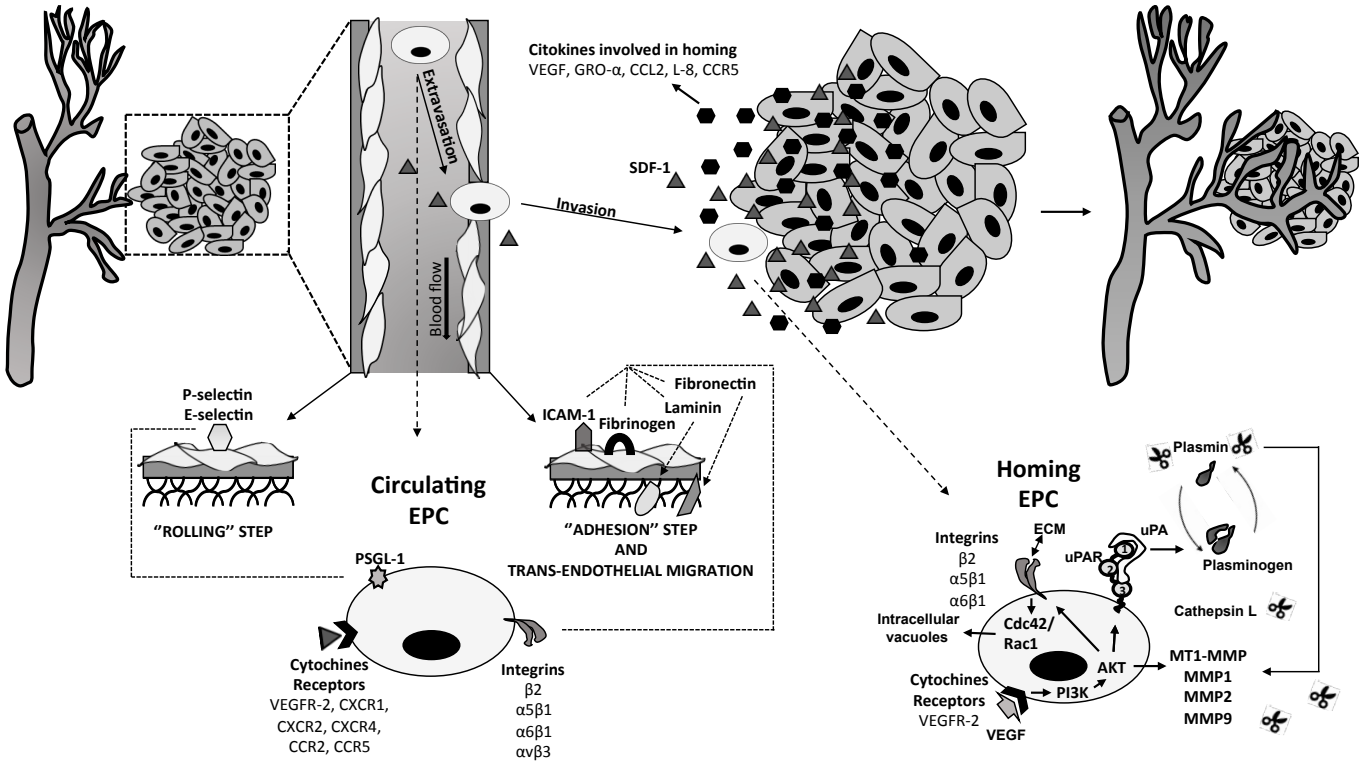

Fig. (3). EPC extravasation, **homing to hypoxic areas and organization in the vascular network.** *Extravasation:* during the trans-endothelial passage of EPCs, the 'rolling' step is mediated by P-selectin protein ligand-1 (PSGL-1), expressed on EPCs, and P and E-selectin expressed on EC, while the 'adhesion' step occurs *via* the interaction of EPC β1 and β2 integrins with EC cell-adhesion-molecules (CAMs) and fibrinogen [102]. α5β1 (fibronectin receptor), α6β1 (laminin receptor) and αvβ3 are responsible for trans-endothelial migration through the basement membrane and extracellular matrix (ECM). *Homing/invasion:* cathepsin L, plasmin activated after uPAR/uPA interaction, and MMPs produced after the VEGF cascade and activated by plasmin, are able to degrade the ECM allowing EPC migration through the blood vessel basement membrane and incorporation in the new vessel lumen. *Organization in the vascular network:* VEGF initiates angiogenesis inducing the production of MT1–MMP, MMP1, MMP2 and MMP9, the up-regulation of integrins α5β1 and α6β1, and the up-regulation of uPAR on EPC cell membranes in a PI3K/AKT pathway-dependent way. MT1–MMP on the cell membrane degrades the ECM directly and through activation of pro-MMPs at the pericellular area, creating a physical space to facilitate the development of a cord of EPCs and lumen formation. The activation of Rac 1 and Rho GTPase Cdc42, after interaction between ECM and integrins, leads to the formation of intracellular vacuole extensions and fusion to lumens after 12 hours.

of MMP-2, but not MMP9. MMP2 and MMP9 have different functions in EPC vasculogenesis, but both are selectively required for a correct process. The vacuole and tube formation mediated by EPCs were blocked after gene ablation of MMP2, but not MMP9. Cleaved high molecular weight kininogen (HKa), an activation product of the plasma kallikrein-kinin system (KKS) was able to block the conversion of pro-MMP2 to MMP2, impeding the association between pro-MMP2 and αvβ3 integrin, thus inhibiting the tube forming capacity of EPCs. Since the KKS can be activated during inflammation and thrombosis, HKa can be responsible for the induction of EPC dysfunction in various pathological conditions [101].

The Role of the uPA/uPAR System

uPA/uPAR expression and its function in tubulogenesis has been studied in EPCs isolated and outgrown from human umbilical cord blood [135]. Such EPC-derived cells (EPDCs), present typical features of late outgrowth ECs and were compared to mature human umbilical vein endothelial cells (HUVECs) in terms of their tubulogenic activity and their uPA/uPAR proteolytic potential. In conditioned culture media, uPA-dependent proteolytic activity was found to be significantly increased in EPDC and this activity was paralleled by a higher secretion of pro-MMP2. Proliferation, migration and capillary-like tube formation was significantly reduced by antibody inhibition of uPA activity and receptor binding. Moreover, TNFα and VEGF further increased the proteolytic potential of EPDCs by up-regulating uPA and uPAR expression, respectively. Overall, these observations indicate that EPDCs are characterized by a high intrinsic uPA/uPAR-dependent proteolytic potential that could contribute to their invasive and angiogenic behaviour. Mature uPAR is organized into three domains (D1-D2-D3, from the N-terminus), with homologous cysteine repeats characteristic of the Ly-6 protein superfamily. The domains are connected by short linker regions. The D1 domain provides about 50% of the sequence that binds uPA and the C-terminal D3 domain is anchored to the cell membrane by a glycosylphospha-

tidylinositol (GPI) tail (Fig. **1**) [136, 137] that favours partitioning of uPAR in lipid rafts in leucocytes [138] and in EPCs [139]. Solved structures of uPAR-ligand complexes show that the three domains form a concave structure that provides, in its centre and at the rim, the remaining 50% of uPA binding ability. Therefore, these data confirm previous observations showing the predominant role of D1 in uPA binding and the requirement of the native D1–D2–D3 form of uPAR for efficient binding [140]. Cell membrane uPAR can be cleaved in the D1–D2 linker region by many proteases, including uPA, plasmin and MMPs, thereby generating a soluble D1 fragment and a cell membrane-GPI-anchored D2-D3 form, which is unable to bind uPA and vitronectin (VN), suggesting that uPAR cleavage may be a regulatory mechanism to unhook cells from the ECM and to inhibit the degradation of anatomical barriers, thus inhibiting the overall uPAR-dependent 'grip-and-go' activity [23, 33]. Recent results show that the native form of uPAR is required, even in cancer cells, to perform a protease-independent amoeboid movement and that the membrane-anchored D2–D3 form is unable to support such amoeboid migration [141].

In light of these data, an interesting role for uPAR in EPCs has been proposed in a study of the pro-angiogenic role of uPAR in ECFCs, a cell population isolated from human umbilical vein blood, which embodies all of the properties of EPCs matched with a high proliferative rate [139]. Upon VEGF challenge, ECFCs over-expressed both caveolin-1 and the full-length form of uPAR, and promoted their co-localization in caveolar lipid rafts (LRs). Inhibition of uPAR expression produced a redistribution of caveolin-1, similarly to the effect of methyl-β-cyclodextrin (β-MCD), a well-known disrupting agent of caveolar-LRs. These data indicated uPAR as an inducer of caveolar-LR organization. Antibody inhibition of uPAR reduced ECFC matrigel invasion and capillary morphogenesis induced by VEGF, thus showing that the pro-angiogenic programme triggered by VEGF involves uPAR. Genetic loss-of-function of uPAR abolished caveolae formation, ECFC matrigel invasion and capillary morphogenesis, similar to silencing of caveolin-1. Such uPAR-dependent ECFC pro-angiogenic activities required the presence of full-length uPAR in caveolar-LRs: over-expression of MMP12, which cleaves full-length uPAR between D1 and D2, inhibited ECFC-dependent vascularization *in vitro* and *in vivo* [139, 142]. VEGF was shown to significantly reduce basal MMP12 expression in ECFCs, indicating a previously unknown role of VEGF in maintaining native uPAR integrity [139]. These data strongly suggest that uPAR becomes functionally important in fostering angiogenesis in EPCs upon recruitment in caveolar-LRs.

Gangliosides are typical components of LRs. In a recent study [143], we checked the interaction of uPAR with membrane models enriched with GM1 or GM3 gangliosides, relying on the adoption of solid-supported mobile bilayer lipid membranes with LR-like composition formed onto solid hydrophilic surfaces, and evaluated by surface plasmon resonance (SPR) the extent of uPAR recruitment. We estimated the apparent dissociation constants of uPAR-GM1/GM3 complexes. These preliminary observations, indicating that uPAR binds preferentially to GM1-enriched biomimetic membranes, were validated by identifying the pro-angiogenic activity of GM1-enriched ECFCs, based on GM1-dependent uPAR recruitment in caveolar-LRs. We have observed that the addition of GM1 to ECFC culture medium promotes matrigel invasion and capillary morphogenesis, as opposed to the anti-angiogenic activity of GM3. Moreover, GM1 also stimulates MAP kinase signalling pathways, typically associated with an angiogenic programme. Caveolar-LR isolation and western blotting of uPAR showed that GM1 promotes caveolar-LR partitioning of uPAR, as opposed to control and GM3-challenged ECFCs. By confocal microscopy, we have shown that, in ECFCs, uPAR is present on the surface in at least three compartments, respectively, associated to GM1, GM3 and caveolar-LRs. Following exogenous addition of GM1, the GM3 compartment is depleted of uPAR, which is recruited within caveolar-LRs, thereby triggering angiogenesis.

An original study on endothelial microparticles (EMPs) produced by TNFα-stimulated EPCs has shown that they serve as a surface for plasmin generation, triggered by uPAR-bound uPA. While low amounts of EMPs promote tube formation in capillary morphogenesis assays, higher amounts result in inhibition of capillary morphogenesis [144]. These data show that the proteolytic activities involved in ECM remodelling require spatial and temporal control. Excessive proteolysis can dissolve the matrix needed for anchoring the migrating cell and destroy the foothold of ECs or EPCs engaged in the angiogenic process.

The Role of Cathepsin L

Urbich *et al*. [145] have reported that cathepsin L is required for EPC-enhanced angiogenesis. After studying the expression profiles of various proteases in EPCs, ECs and monocytes, they found higher expression of a number of cysteine cathepsins in EPCs, including cathepsins D, H, L, K and X/Z. In particular, the antigen and activity of cathepsin L was considerably higher in EPCs than in monocytes and ECs. Mature cathepsin L remains active extracellularly by the chaperone action of a splice variant of the MHC class II-associated invariant chain, which is strongly expressed in EPCs [145]. Cathepsin L activity promotes EPC invasion, remodelling of collagens and gelatin, and neovascularization. Deficiency of cathepsin L in mice impaired blood flow restoration in ischaemic limbs, indicative of impaired neovascularization. However, although several cathepsin L inhibitors have been shown to be effective as inhibitors of angiogenesis and tumour invasion, the molecular mechanism exploited by cathepsin L to stimulate angiogenesis has not yet been identified.

Table 2. Proteases involved in EPC sprouting angiogenesis.

EPC Mobilization and Intravasation	EPC Extravasation and Homing	EPC Organization in Vascular Networks
MT1-MMP (MMP14) [112]	**MT1-MMP (MMP14)** [114]	**MT1-MMP (MMP14)** [53]
MMP2 [101, 111]	**MMP2** [112]	**MMP1** [127]
MMP9 [11, 14, 65, 91, 102–104, 108–110]	**MMP9** [14, 134]	**MMP2** [101]
Cathepsin-G [86, 87]	**Cathepsin-L** [145]	**MMP9** [133]
Neutrophil Elastase [86]	**uPA/uPAR/Plasmin system** [135]	**Cathepsin-L** [145]
		uPA/uPAR/Plasmin system [135, 139, 142, 144]

Schematic table representing the key protease systems implicated in each step of EPC sprouting angiogenesis: EPC mobilization and intravasation from bone marrow to peripheral blood, extravasation and homing within hypoxic areas and organization in vascular networks. The main references for each protease are reported in square brackets.

CONCLUSION AND PERSPECTIVES

Based on the evidence outlined above, it is clear that there is a striking difference between sprouting angiogenesis operated by mature ECs and the vasculogenic contribution to the sprouting angiogenesis of EPCs. In fact, while sprouting angiogenesis by mature ECs develops entirely within hypoxic areas and involves resident ECs, the role of EPCs requires the overcoming of a series of hurdles. EPCs must detach from the stem cell niche, intravasate in the bone marrow vessels, extravasate to hypoxic areas and organize to form joining segments between the vascular loops of sprouting mature ECs. Each one of such steps is characterized by the activity of master proteases that have been discussed in the present review (Table **2**). Here, we have not considered the established role of proteases in the release of various ECM fragments, which regulate important functions of ECs including adhesion, migration, proliferation, cell survival and cell–cell interactions [146], called matrikines or matricryptins. Such fragments are able to act as endogenous angiogenesis inhibitors, in concert with proteases, at any step of the angiogenesis process. The putative targets of matrikine control are the proliferative and invasive properties of tumour or inflammatory cells, and the angiogenic and lymphangiogenic responses [147].

Some observations indicate that, besides the direct role of EPCs in neovascularization, other bone marrow-derived cells act by producing factors able to directly induce tumour vascularization and indirectly activate EPC angiogenic properties [148]. In addition to the recruitment of BM-derived cells into the tumour site, the co-recruitment of other lineages supports capillary sprouting and stabilizes immature cancer capillaries by releasing additional pro-angiogenic factors. For example, mast cells (MCs), in addition to the expression of IL-8, basic FGF, TNFα and VEGF, are also able to produce MC-specific serine proteases that activate pro-MMPs, histamine and heparin which can stimulate EC sprouting [149]. In addition, tumour-associated macrophages (TAMs) infiltrate the tumour mass with the potential to kill tumour cells, but exhibit on the other hand pro-tumour functions, such as the secretion of angiogenic molecules [150, 151]. Jin Hur *et al*. identified for the first time a specific subset of T cells, denominated angiogenic T cells (CD3 + CD31 + CXCR4 +), at the centre of EPC colonies, as a potential therapeutic target for ischaemic disease. Clinical studies showed that the number of angiogenic T cells correlates with EPC colony numbers in the peripheral blood. Angiogenic T cells are able to enhance the differentiation of early EPCs, and to improve EPC and EC function by secreting angiogenic cytokines, such as VEGF, IL-8 and MMPs. CXCR4-expressing angiogenic T cells home to the ischaemic area where a high level of SDF-1 is present. At these sites, angiogenic T cells make contact with ECs through CD31, transmigrate and then, by using MMP9, invade the ischaemic tissue [152]. Since angiogenic T cells secrete high amounts of MMP-9, it is possible that their role in neovascularization could depend also on the improvement of EPC mobilization from the stem cell niche mediated by endogenous MMP-9, through the cleavage of both CXCR4 on the EPC surface and stromal SDF1. Overall, these data indicate that a complex, and not yet clarified, network of accessory cells and cytokines regulate the contribution of EPCs to the angiogenic process, thus widening the therapeutic horizon for the efficient control of pathological angiogenesis.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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