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REVIEW

Role of endothelial progenitor cells in breast cancer angiogenesis: from fundamental research to clinical ramifications

Xuefen Le Bourhis · Rodrigue Romon · Hubert Hondermarck

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Abstract Blood vessel formation (neovascularization) in tumors can occur through two mechanisms: angiogenesis and vasculogenesis. Angiogenesis results from proliferation and sprouting of existing blood vessels close to the tumor, while vasculogenesis is believed to arise from recruitment of circulating cells, largely derived from the bone marrow, and de novo clonal formation of blood vessels from these cells. Increasing evidence in animal models indicate that bone marrow-derived endothelial precursor cells (EPC) can contribute to tumor angiogenesis. This review aims to collate existing literature and provide an overview on the current knowledge of EPC involvement in breast cancer angiogenesis. We also discuss recent attempts to use EPC as biomarker and therapeutic target in clinical trials.

Keywords Endothelial precursor cells · Tumor angiogenesis · Breast cancer · Biomarker

Neovascularization in breast cancer

Breast cancer-induced angiogenesis is first observed at the preinvasive stage of high-grade ductal carcinoma in situ by the formation of microvessels around the ducts that are filled with proliferating epithelial cells [1]. As the tumor progresses, so does the degree of neovascularization. The new vessels not only help to meet the growing metabolic demands of the tumor but also favor tumor dissemination and metastasis. Poor breast cancer prognosis has been shown to correlate with increased microvascular density or production of proangiogenic factors, some of which have been used as therapeutic targets [2]. However, tumor neovasculation can occur not only by angiogenesis (the sprouting of new vessels from existing vessels), but also by vasculogenesis, the embryonic process where blood vessels are formed de novo from bone marrow-derived endothelial precursor cells (EPC). In the later process, EPC is mobilized from the bone marrow, transported through the blood stream to the tumor site where they differentiate into mature endothelial cells to form vascular sprouts and cellular networks, before incorporation into a functional microvasculature (Fig. 1).

Discovery and characterization of EPC

Endothelial precursor cells were initially identified and isolated from the blood of healthy donors in 1997 by Asahara et al. [3]. These cells were found to coexpress both the vascular endothelial growth factor receptor-2 (VEGFR2) and CD34, and to differentiate to mature endothelial cells in culture. Soon after, by using a fluorescent in situ hybridization approach in human recipients of gender-mismatched bone marrow transplants, which allowed to distinguish EPC from the marrow of donor-derived cells and circulating endothelial cells from vessel walls of host-derived cells. Lin et al. [4] found that more than 90% of endothelial cells in the blood were of host origin. When cultured in vitro, donor-derived endothelial cells expanded about 1,000-fold, whereas host-genotype endothelial cells

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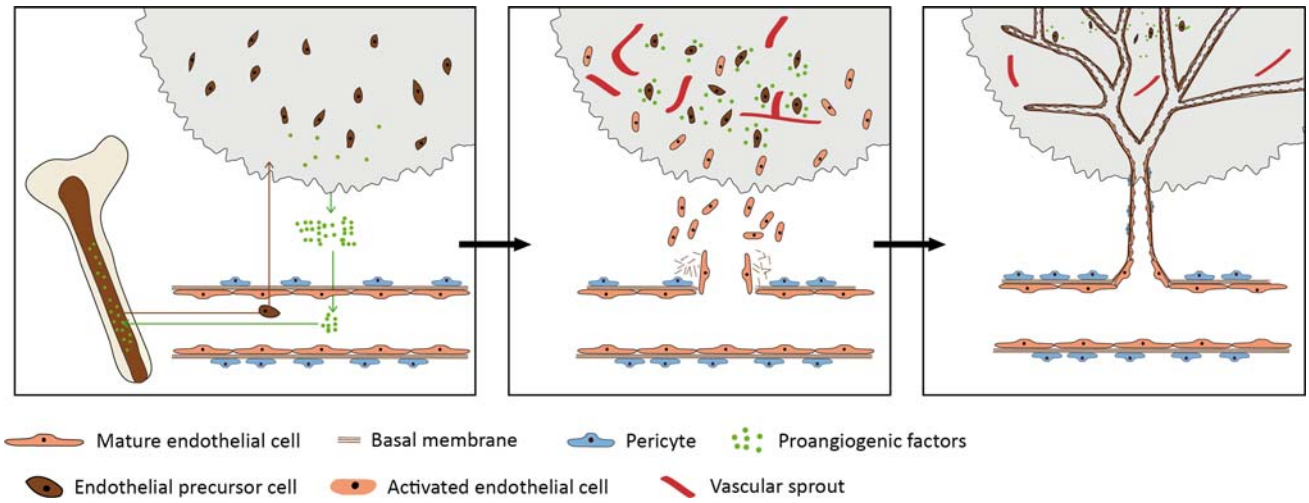

Fig. 1 Schematic representation of neovascularization in tumors. Under low oxygen tension or hypoxia condition, tumor cells secrete a number of proangiogenic factors which activate endothelial cells of existing vessels in neighboring areas to proliferate, migrate, leading to the formation of new vessels from existing vessels. Proangiogenic factors can also initiate the mobilization of bone marrow-derived endothelial precursor cells to the tumor site, where they differentiate into mature endothelial cells to form vascular sprouts and cellular networks before incorporation into a functional microvasculature

expanded only about 20-fold, thus illustrating the high proliferative potential of EPC. Since then, many studies have been performed to determine the phenotypical and functional characteristics of EPC [5–8]. However, as EPC, endothelial cells and haematopoetic stem cells share many cell surface markers including CD45, CD34, CD133 (CD117 for mouse), CD146, CD31, CD105, CD144, vascular endothelial growth factor receptor 2 (VEGFR2) and von Willebrand factor (vWF) [6, 9–15] (Table 1), the term EPC may therefore encompass ranging from relative primitive haemangioblasts to more differentiated endothelial cells. As CD133 is expressed in stem cells and/or progenitors of different tissues [16], it is currently accepted that early EPC (localized in the bone marrow or immediately after migration into the circulation) are CD133+/CD34+/VEGFR2+/CD45− cells, whereas circulating EPC are CD133low/CD34+/VEGFR2+/CD45− and begin to express cell surface markers typical to mature endothelial cells, including CD31 and vWF. The majority of circulating EPC resides in the bone marrow in close association with hematopoietic stem cells and the bone marrow stroma that provides an optimal microenvironment. Clearly, putative precursors and the exact differentiation lineage of EPC remain to be determined. Further and more detailed molecular characterization of EPC could emerge from transcriptomic and proteomic analysis, as illustrated by recent publications [17, 18]. It is also to be noted that proteomics of breast tumors might equally reveal

Table 1 Marker profile of endothelial cell lineage, platelet, and lymphocyte

Markers	Hematopoietic stem cell	Endothelial precursor cell	Circulating endothelial cell	Mature endothelial cell	Endothelial cell microparticule	Platelet	Lymphocyte
DNA	+	+	+	+	−	−	+
CD45	+	−	−	−	−	−	+
CD34	+	+	+/−	+/−	+/−	+/−	−
CD133	+	+	−	−	−	−	−
CD117	+	+	−	−	−	−	−
CD146	−	+/−	+	+	+	−	+/−
CD31	+/−	+/−	+	+	+	+/−	−
CD105	+/−	+/−	+	+	−	−	−
CD144	+/−	+/−	+	+	+	−	−
VEGFR2	+/−	+	+	+	+	−	−
vWF	−	+/−	+	+	+	−	−

Adapted from Bertolini et al. [6]

important molecular trends related to angiogenesis and EPC intervention [19].

Contribution of EPC to mammary tumor vascularization in animal models

The contribution of EPC to tumor vascularization has been mainly evaluated by using tumors grown in chimeric mice which were previously lethally irradiated and reconstituted with LacZ+ or GFP+ tagged bone marrow-derived cells. The first proof-of-principle of bone marrow-derived EPC contribution to cancer-associated blood vessels was reported in 2001 by Lyden et al. [20], by using an angiogenesis-defective Id-mutant mice model. The Id proteins interact with other helix–loop–helix transcription factors, thereby modulating cellular differentiation in early fetal development [21]. Adult mice with reduced Id gene dosages cannot support neo-angiogenesis when challenged with tumor [22]. Bone marrow transplantation from wild-type mice, not from Id-mutant mice, restored the tumor growth and neovascularization in Id-mutant mice [20]. These findings were later confirmed by a clinical study of cancer patients, who developed cancers after bone marrow transplantation with donor cells derived from individuals of the opposite sex [23]. By using fluorescence in situ hybridization with sex chromosome-specific probes, the authors found that the percentage of bone marrow-derived endothelial cells in the tumor vasculatures ranged from 1% to 12% according to tumor types.

An excellent demonstration of EPC as a major determinant of nascent mammary tumor neovascularization was recently reported by Nolan et al. [24]. The authors used MMTV-PyMT transgenic mice, in which the PyMT oncogene was expressed under the transcriptional control of the mouse mammary tumor virus promoter/enhancer specifically in the mammary epithelium [25]. The PyMT transgene activates pathways similar to that induced by ErbB2 [26], and importantly, this murine tumor model recapitulates human breast cancer progression from early nonmalignant hyperplasia (6 weeks of age) and adenoma (8–9 weeks of age), to early and late malignant adenocarcinoma (8–12 weeks of age) [27]. They examined the contribution of bone marrow-derived EPC and luminally incorporated endothelial cells at various stages of these mammary tumors developing in animals previously transplanted with GFP+ bone marrow. They found that early adenomas contained foci of bone marrow-derived GFP+ cells including EPC and exhibited increased vessel density, with 5–10% of host vessels having incorporated bone marrow-derived GFP+ endothelial cells. These findings indicate that bone marrow-derived EPC contribute to neovascularization in early stages of breast tumor progression. In the same study, the authors obtained similar results using other transplanted tumors including lung carcinoma, lymphoma, and melanoma, thus highlighting the general relevance of these cells in tumor neovascularization. The contribution of EPC in mammary tumor neovascularization has also been studied using other animal models [28–31] (Table 2). In general, the percentage of EPC incorporation in neovessels is relatively low (0.5–3.5%). However, Duda et al. [29] reported that as high as 58% of tumor vessels were bone marrow-derived endothelial cells-positive when TG-1 mammary carcinoma cells were injected superficially under the pial surface of mouse brain, while only 1.5% of tumor vessels were from bone marrow-derived endothelial cells when MCa8 breast cancer cells were injected subcutaneously or at mammary fat pad, suggesting that the extent of vasculogenesis may also depend on the specific tumor-stroma/microenvironment interaction.

In addition to the physical contribution of EPC to newly formed capillaries, the angiogenic cytokine release of EPC may improve neovascularization in a paracrine manner [32]. This idea is supported by a recent report by Gao et al. [33] who found that although only 12% of the new blood vessels showed incorporation of EPC, blocking EPC mobilization caused severe angiogenesis inhibition and significantly impaired lung tumor progression. Moreover, in the same study, gene expression analysis of EPC revealed up-regulation of a variety of key proangiogenic genes including vascular endothelial growth factor, platelet-derived growth factor, fibroblast growth factor receptor 1, chemokine ligands and receptors. Similarly, Suriano et al. [31] reported that 17β-estradiol mobilizes bone marrow-derived EPC to orthotropically implanted mammary tumors. The homing of EPC in tumor tissues was associated with enhanced expression of several proangiogenic factors which might contribute to stimulate vessel formation and support tumor growth.

Endothelial precursor cells are not only involved in primary tumor development, but also in metastase formation. This has been recently evidenced by Gao et al. [33]. The authors transplanted syngeneic GFP+ bone marrow into MMTV-PyMT transgenic mice, which develop spontaneous mammary carcinoma and lung metastase. They found that lung metastases initially formed micrometastases which were poorly vascularized, but as the metastatic tumors grew over time (16 weeks), vessels became increasingly abundant and up to 12% of the tumor vascular endothelium contained BM-GFP^{+}CD31^{+} EPC. Similar results were obtained after implantation of Lewis lung carcinoma cells into syngeneic mice reconstituted with bone marrow-derived cells expressing green fluorescent protein (BM-GFP^{+}). Importantly, the blockade of EPC mobilization by short hairpin RNA, directed against the Id1

Table 2 Contribution of EPC in neovessels of mammary primary tumors and metastases

Tumor model	Mice	Time point analysis after BMT	% of EPC incorporation in vessels	EC markers	References
MMTV-PyMT Spontaneous mammary carcinoma	MMTV-PyMT mice	10–12 weeks	1.3	LacZ, CD31	[28]
MCa8 breast cancer cells	C57BL6 mice	3 months	<1	GFP, CD31	[29]
MMTV-PyMT Spontaneous mammary carcinoma	MMTV-PyMT mice	10 weeks	5–10	CD31, GFP, CD144	[24]
MT1A2 and TG1-1 mouse mammary carcinoma cells	FVB mice	>4 weeks	2.5–3.5	LacZ, CD31	[30]
TG1-1 mouse mammary carcinoma cells	Tg(TIE2GFP) 287SATO/J mice	–	0.5–3	GFP, CD133	[31]
MMTV-PyMT Spontaneous mammary carcinoma	MMTV-PyMT mice	12 weeks (lung metastases)	12	GFP, CD31	[33]

BMT Bone marrow transplantation, *EC* endothelial cells

gene, caused severe angiogenesis inhibition at the micrometastatic stage and significantly impaired the formation of lethal macrometastases.

Molecular mechanism of EPC mobilization

Although the molecular pathways involved in EPC mobilization remain to be determined, several stimuli including VEGF, stroma cell derived factor-1α (SDF-1α), placental growth factor (PlGF), granulocyte colony stimulating factor (G-CSF) and estrogens were described to be involved in the mobilization of EPC from the bone marrow to tumor sites [31, 34–39]. VEGF can activate matrix metalloproteinase-9 which that cleaves the membrane-bound stem cell cytokine mKit Ligand in bone marrow stromal cells, to liberate soluble sKit Ligand, which then stimulates cKit-positive EPC to migrate from a quiescent bone marrow niche to a permissive microenvironment, activating EPC from a quiescent to a proliferative state [40]. Furthermore, VEGF has been found to upregulate SDF-1 (also known as CXCL12) and CXCR4 (the SDF-1 receptor) [41, 42]. SDF-1α plays a key role in both the release and the homing process of EPC. SDF-1α is present at high concentrations in the bone marrow, where it holds the stem cells within their niche. In response to different proangiogenic factors, such as VEGF, the level of SDF-1α in bone morrow is decreased, leading to the egress of stem cells in the circulation. In contrast, the concentration of SDF-1α within the tumor is increased in response to VEGF, and the progenitor cells are subsequently trapped in the tumor [36]. Other chemokines, including CCL2 and CCL5, were also reported to be produced by tumor cells to attract the progenitor cells from the circulation [43]. Suriano et al. [31] have reported that bone marrow-derived EPC initiate the neovascularization of TG1-1 mammary cells implanted in the inguinal mammary gland of Tie-2 GFP transgenic mice. In this study, 17-β estradiol supplementation of ovariectomized mice significantly enhanced EPC-induced neovascularization, which was accompanied by enhanced expression of proangiogenic paracrine factors, such as VEGF, bFGF, angiopoetin-1, angiopoetin-2, thrombospondin-1, and matrix metalloproteases-2 and -9. Similarly, adiponectin, an adipocyte-specific secretory protein, was reported to stimulate migration and differentiation of EPC [44, 45]. Adiponectin exerts also a biphasic effect on mammary tumor angiogenesis in MMTV-PyMT mouse model, with an increase of EPC mobilization in mice developing more aggressive tumors [46]. Thus, other growth factors and cytokines produced by tumor cells and/or surrounding normal cells could be also involved in EPC mobilization. For example, brain-derived neurotrophic factor (BNDF) was described to promote revascularization of ischemic and nonischemic mouse tissues by both local recruitment of endothelial cells and systemic mobilization of hematopoietic progenitors [47]. These findings, together with the recent demonstration of the involvement of nerve growth factor (NGF) and its tyrosine kinase receptor TrkA in breast tumor growth and angiogenesis [48, 49], suggest that neurotrophins-promoted angiogenesis could also involve the mobilization of hematopoietic progenitors such as EPC. Finally, systemic hormones and/or factors which are known to regulate angiogenesis in both physiol-pathological situations [50] could also contribute to EPC mobilization.

Clinical applications: EPC as biomarker and therapeutic target?

In light of the aforementioned results, showing the role of EPC in mammary tumor angiogenesis in animal models,

attempts have been made to determine their potential use in clinical oncology. Particularly, several investigations have been undertaken to establish whether variations exist in EPC according to breast cancer type, stage or response to therapy. Higher levels of circulating EPC were found in both preclinical breast cancer xenograft model in mice [51, 52] and in breast cancer patients [53, 54]. As shown in Table 3, 3 of 6 studies failed to show significant difference in EPC among varying stages of breast cancer [53, 55, 56]. In contrast, Richter-Ehrenstein et al. [57] found increased number of EPC in tumors of large size (over 2 cm). Naik et al. [58] showed increased number of EPC in stage 3 and 4 breast cancer patients, compared to stage 1 and 2 breast cancer patients. These conflicting results may be explained by the very limited number of patients analyzed, as well as different markers, used for enumerating EPC (Table 3). Accordingly, a recent report by Mancuso et al. [54], who used a more standardized method, with limited intrareader and interreader variability, showed more EPC in metastatic breast cancers than locally advanced ones. Interestingly, in patients with pediatric solid malignancies, including medulloblastoma, neuroblastoma, sarcoma, and lymphomas [59], the levels of circulating bone marrow-EPC were found to be significantly higher, compared to no metastatic diseases. Furthermore, higher circulating levels of EPC were also seen in patients with advanced unresectable hepatocellular carcinoma when compared to patients with resectable hepatocellular carcinoma [60]. Thus, the significantly higher levels of EPC paralleling clinical severity suggest the possible relevance of these cells in metastatic progression of tumors, and point out their potential use as biomarker and/or target in cancer therapy.

Surprisingly, Shaked et al. [38] found that treatment of tumor-bearing mice with vascular disrupting agents, which target the established but abnormal tumor vasculature, leads to an acute mobilization of EPC. These cells subsequently colonize the viable tumor rim that usually remains after such therapy, and drives "rebound revascularization" and tumor regrowth/recovery. Another study [61] showed that chemotherapy drugs, such as taxanes administered at maximum tolerated doses, can also induce a rapid EPC mobilization. Prevention of the EPC spike by concurrent treatment with targeted antiangiogenic drugs enhanced the antitumor activity of chemotherapeutic drugs. These findings raise the possibility that therapeutic strategies aiming to reduce EPC mobilization might enhance the efficacy of certain cytotoxic anticancer therapies. Accordingly, addition of an antibody against CXCR4 to block EPC mobilization enhanced the antitumor effect of docetaxel in a murine breast cancer model [62]. In line with this, the efficacy of metronomic chemotherapy was thought to be mainly antiangiogenic [63, 64]. Metronomic chemotherapy is defined as regular administration of a chemotherapeutic drug at relatively low (nontoxic) doses, over prolonged periods, with no extended drug-free break periods [63]. Using different preclinical breast cancer models, several studies showed a strong relationship between decreased number of circulating EPC and efficiency of various metronomic chemotherapy regimens [65–68]. These studies demonstrated that EPC can be used successfully as a surrogate marker in mice for determining the optimal biological dose for various metronomic chemotherapies, thus avoiding empiricism (at least in mice), with respect to determining the optimal biological dose in metronomic chemotherapy regimens. However, clinical studies have yielded promising but still contradictory results, likely due to the choice of patients, therapeutic regime and enumeration technique of EPC. It has been reported that circulating EPC are increased in breast cancer patients receiving neoadjuvant chemotherapy [69]. In contrast, Dellapasqua et al. [70] have not observed any correlation between the number of EPC and clinical outcome in patients with advanced breast cancer receiving metronomic chemotherapy with or without Bevacizumab. Several clinical studies are currently under investigation to determine whether EPC can be used as biomarker for patient selection and for defining the optimal biological dose in metronomic chemotherapies.

Table 3 Circulating EPC and breast cancer

Number of patients	Cancer stages	EPC definition	Result	References
46	–	CD45−/CD133+	Low EPC in healthy donors and breast cancer patients	[55]
19	1/2 vs. 3/4	CD34+/VEGFR2+/CD144+	No difference	[53]
47	1 to 4	CD34+/Flk1+	Increased EPC in tumors ≥2 cm	[57]
25	1/2 vs. 3/4	CD133+/VEGFR2+	Increased EPC in stage 3/4 vs. stage 1/2	[58]
160	1 to 3/4	CD45−/CD133+/CD34+	No correlation with disease stage	[56]
56	Locally advanced and metastatic breast cancer	DNA+/syto16+/CD31+/CD133+	Increased EPC in metastatic breast cancer	[54]

Conclusion and perspectives

Most recent findings point out the relevance of EPC in tumor progression. Therefore, these cells might serve both as surrogate marker for cancer progression or response to therapy and as target for therapy. Further studies and consensus are required concerning the phenotype and enumeration of these cells, to better define their exact role in clinical oncology. Furthermore, many questions related to EPC biology and functional impact remain to be answered. For instance, what is the exact lineage of EPC in the context of the hematopoietic system in the adult? Can reliable culture and expansion methods be developed to address the origin and functional definition of EPC? To what extent do EPC contribute to neovessel formation and/or vessel maintenance? These issues will be the important subjects of future investigation to dissect out the critical players promoting tumor angiogenesis.

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