Review

May the remodeling of the Ca^{2+} toolkit in endothelial progenitor cells derived from cancer patients suggest alternative targets for anti-angiogenic treatment?☆

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Francesco Moccia [a,*], Valentina Poletto [b]

[a] Laboratory of General Physiology, Department of Biology and Biotechnology "Lazzaro Spallanzani", via Forlanini 6, 27100 Pavia, Italy
[b] Unit of Clinical Epidemiology and Center for the Study of Myelofibrosis, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Policlinico San Matteo Foundation, Piazzale Golgi 19, 27100 Pavia, Italy

A B S T R A C T

Endothelial progenitor cells (EPCs) may be recruited from bone marrow to sustain the metastatic switch in a number of solid cancers, including breast cancer (BC) and renal cellular carcinoma (RCC). Preventing EPC mobilization causes tumor shrinkage. Novel anti-angiogenic treatments have been introduced in therapy to inhibit VEGFR-2 signaling; unfortunately, these drugs blocked tumor angiogenesis in pre-clinical murine models, but resulted far less effective in human patients. Understanding the molecular mechanisms driving EPC proliferation and tubulogenesis in cancer patients could outline novel targets for alternative anti-angiogenic treatments. Store-operated Ca^{2+} entry (SOCE) regulates the growth of human EPCs, and it is mediated by the interaction between the endoplasmic reticulum Ca^{2+}-sensor, Stim1, and the plasmalemmal Ca^{2+} channels, Orai1 and TRPC1. EPCs do not belong to the neoplastic clone: thus, unlike tumor endothelium and neoplastic cells, they should not remodel their Ca^{2+} toolkit in response to tumor microenvironment. However, our recent work demonstrated that EPCs isolated from naïve RCC patients (RCC-EPCs) undergo a dramatic remodeling of their Ca^{2+} toolkit by displaying a remarkable drop in the endoplasmic reticulum Ca^{2+} content, by down-regulating the expression of inositol-1,4,5-receptors (InsP₃Rs), and by up-regulating Stim1, Orai1 and TRPC1. Moreover, EPCs are dramatically less sensitive to VEGF stimulation both in terms of Ca^{2+} signaling and of gene expression when isolated from tumor patients. Conversely, the pharmacological abolition of SOCE suppresses proliferation in these cells. These results question the suitability of VEGFR-2 as a therapeutically relevant target for anti-angiogenic treatments and hint at Orai1 and TRPC1 as more promising alternatives. This article is part of a Special Issue entitled: 13th European Symposium on Calcium.

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1. Introduction

A finely tuned increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is the ubiquitous mechanism whereby the organism regulates functions as diverse as fertilization, embryonic development, muscle contraction, immune response, learning and memory [1–3]. The indispensable role that calcium signaling has assumed in virtually all life forms across the phylogenetic tree depends of the unmatched versatility of this intracellular messenger as compared to other signaling pathways [1,2]. The spatio-temporal profile as well as the amplitude and duration of intracellular Ca^{2+} elevations may be precisely tailored to target a specific cellular function among the multitude of those endowed with Ca^{2+}-sensitivity, such as proliferation, gene expression, protein folding and processing, motility, nitric oxide (NO) and hydrogen sulphide (H_2S)

synthesis, cytoskeleton remodeling, exocytosis, energy production, programmed cell death or necrosis [1,2,4,5]. It is, therefore, not surprising that any alteration in the delicate balance between the mechanisms feeding Ca^{2+} into the cytoplasm and those terminating the signal to prevent the cytotoxic consequences of a prolonged $[Ca^{2+}]_i$ increase, will perturb cellular homeostasis. It has now been accepted that derangement of the Ca^{2+} machinery is central to the onset of many life-threatening diseases, such as heart failure, neurodegenerative diseases and cancer [6–9]. In particular, the aberrant expression of specific Ca^{2+} channels and transporters, as well as their altered activity, may contribute to establish some of the ten defined cancer hallmarks [8–10], including limitless replicative potential, insensitivity to apoptosis, tissue invasion and metastasis, and angiogenesis, i.e. the sprouting of new capillary branches from adjacent blood vessels. The development of tumor vasculature, however, may impinge on alternative mechanisms, that will be mentioned in the next paragraph and include the recruitment of circulating endothelial progenitor cells (EPCs). While it has long been known that growth factors utilize Ca^{2+} signals to activate

mature endothelial cells [11,12], vascular endothelial growth factor (VEGF) stimulates their immature progenitors through an oscillatory increase in $[Ca^{2+}]_i$, as demonstrated by a recent series of studies from our group [13–15]. In the present article, we will discuss how the Ca^{2+} toolkit is deranged in EPCs derived from cancer patients, thereby leading to the suppression of the pro-angiogenic Ca^{2+} response to VEGF. This feature might explain the well known failure of anti-VEGF drugs to cause a persistent inhibition of tumor growth and metastatization in cancer patients. The concomitant up-regulation of Stim1, Orai1 and Canonical Transient Receptor Potential channel 1 (TRPC1), that control proliferation and in vitro tubulogenesis both in normal EPCs and in their malignant counterparts, hints at store-operated Ca^{2+} entry (SOCE) as a promising target to devise alternative therapies to treat highly angiogenic tumors, such as renal cellular carcinoma (RCC) and breast cancer.

2. Mechanisms of tumor vascularization and resistance to anti-angiogenic therapy

The development of new blood vessels is an essential step for tumor progression, invasion and dissemination (metastatization) to distant organs, where the primary neoplasm gives rise to secondary lesions. The pioneering work conducted by Judah Folkman provided the first evidence that tumors cannot enlarge beyond the physical constraints imposed by nutrient diffusion rates, i.e. 1–2 mm^3, without having access to peripheral circulation. The formation of a patent vasculature is indispensable for cancer cells to obtain the necessary oxygen and nutrient supplies and to clear out their metabolic waste. Moreover, the "angiogenic switch", the mechanism whereby a dormant hyperplasia is turned into a highly vascularized tumor, paves the way for cancer cell to escape from the primary lesion site and disseminate towards neighboring or even distant sites [16,17]. Disseminated tumor cells may in turn develop the secondary macrometastases that ultimately lead to patient death [18]. The capability of cancer cells to intravasate into peripheral circulation and disseminate to remote organs is exacerbated by the well known chaotic disorganization of tumor vessels. These are featured by a number of morphological, cellular, and molecular abnormalities that all together concur to shape a dilated, tortuous, and hyperpermeable microvascular network inside the neoplasm [19,20]. The remarkable heterogeneity in tumor endothelium is dictated by three main mechanisms, which are not mutually exclusive, but may interact to produce aberrant endothelial cells: 1) the cross-talk with the surrounding microenvironment — featured by hypoxia, low pH, disorganized basement membrane, elevated interstitial fluid pressure, enrichment in growth factors and cytokines — which may impart both genetic and epigenetic modifications to tumor endothelial cells (TECs); 2) the vascular bed of origin, which conveys a site-specific epigenetic footprint to endothelial cells sprouting towards the neoplastic lesion; and 3) the contribution of additional mechanisms to tumor vascularization, including vasculogenic mimicry, intussusceptive angiogenesis, vessel co-option, recruitment and engraftment of hematopoietic and endothelial progenitor cells [19,21]. Relevant to the aims of the present article, a growing number of studies have now established that the concerted interaction between local endothelial cells and circulating EPCs drive the angiogenic switch during tumor growth and metastatic progression [20,22,23]. This discovery opened a new avenue in the investigation of deranged endothelial Ca^{2+} signals during neoplastic transformation and proposed this intracellular pathway as a molecular target for novel anti-angiogenic treatments [15,20,24–26].

2.1. Evidence for EPC contribution to tumor vascularization

The angiogenic switch is initiated by the increased expression and/or activation of hypoxia-inducible factor-1α (HIF-1α), which is consequent to the dramatic decrease in oxygen tension (P_{O2}) occurring in growing tumors. HIF-1α is, in turn, a transcription factor that induces the transcription of genes encoding for several growth factors and cytokines, such as VEGF, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-2 (IGF2), and stromal derived factor-1α (SDF-1α) [12,22,27]. These mediators are released into circulation: 1) to stimulate adjacent capillaries to sprout towards the avascular hyperplasia and 2) to stimulate the bone marrow niche to switch from a quiescent state to a pro-tumorigenic environment [15,20,22,27]. While tumor-associated angiogenesis has long been investigated, the contribution of both hematopoietic progenitor cells (HPCs) and EPCs has only recently come of age [12,22,27]. Angiogenesis is a complex and multistepped process that is initiated when local endothelial cells are stimulated by VEGF to synthesize and secrete matrix proteases (MMPs) for the breakdown of the membrane basement surrounding the capillaries they belong to. As a consequence, endothelial cells are detached from the vascular wall and migrate into the extracellular matrix, where they proliferate and assemble into patent tubes that provide the expanding tumor with blood, nutrients, and oxygen. The stabilization of the immature vessel requires the attraction of accessory cells, such as mural cells and pericytes, which contribute to the formation of a new basement membrane and a firm extracellular matrix [17]. In addition to sprouting angiogenesis, tumor vascularization is facilitated by the engagement of bone marrow-derived HPCs, including Tie2-expressing monocytes, F4/80$^+$ CD11b$^+$ tumor-associated macrophages (TAMs), CXCR4$^+$ VEGFR1$^+$ hemangiocytes, GR1$^+$ CD11b$^+$ "myeloid-derived suppressor cells" (MDSCs), CD45$^+$/CD11b$^+$ myeloid cells, and infiltrating neutrophils and mast cells [22,28]. These cells may either adopt an endothelial phenotype, thereby directly lining tumor vessels, or exert perivascular functions by emitting paracrine signals, such as growth factors and cytokines [22,28]. Nevertheless, the precise biological role of bone marrow-derived HPCs in neovessel formation remains to be elucidated. Conversely, the contribution of circulating EPCs to the angiogenic switch has now been widely accepted [15,20,22,23,29,30]. EPCs are mobilized from both bone marrow and vascular wall stem cell niches by tumor cells-derived VEGF and SDF-1α: once these mediators are released into circulation, they establish a concentration gradient that paves the way for EPC recruitment both to the primary tumor lesion and to the more distant pre-metastatic niches. Herein, EPCs may both emanate angiogenic factors to stimulate local angiogenesis and physically engraft within tumor vasculature, thereby inducing the transition from a dormant undetectable lesion to a deadly metastatic cancer [15,20,22,28]. A number of studies hint at the vital role served by circulating EPCs in supporting tumor growth and metastatization. First, the transplantation of bone marrow-derived cells expressing the β-galactosidase gene (lacZ) under the control of an endothelial-specific promoter recapitulated angiogenesis in a xenograft model of B6RV2 lymphoma. The subsequent histological examination revealed lacZ staining throughout tumor vasculature, thereby suggesting that bone marrow sustains the angiogenic switch [31]. Second, sex chromosome fluorescence in situ hybridization (FISH) analysis of secondary malignancies in patients who were previously transplanted with HPCs from a sex-mismatched donor disclosed the presence of bone marrow-derived cells in the associated vessels [30]. Third, the physical engraftment of bone marrow-derived GFP$^+$ EPCs into tumor-associated bed has been demonstrated by assessing luminal cells positive for the following markers in three transplanted tumors (B6RV2 lymphoma, Lewis lung carcinoma or LLC, and melanoma) and one transgenic breast cancer mouse model (MMTV-PyMT): 1) GFP, which indicates bone marrow origin; 2) isolectin IB4, which confirms luminal integration; 3) CD31, which is a typical endothelial antigen; and 4) CD11b, which rules out any hematopoietic contamination [22,23,32]. Fourth, EPCs transduced with a construct encoding for GFP inserted under the control of the transcription factor inhibitor of DNA binding 1 (Id1), which is selective for EPCs, promoter, have been tracked during their path from the bone marrow towards tumor vasculature in a xenograft model of LLC [33]. Fifth, the conditional suppression of Id1, a helix–loop–helix transcriptional repressor that drives EPC egression from bone marrow, did not affect tumor onset in mice transplanted with

LLC, but prevented the macro-metastatic transformation that causes patient death [32]. Similarly, selective elimination of Dicer, which leads to the suppression of MiR-10b and MiR-196b, has recently been shown to impair EPC-mediated vascularization of LLC [34]. Table 1 summarizes all the evidences in favor of EPC contribution to tumor growth. Consistent with the role they play in cancer neovascularization, the higher frequency of circulating EPCs correlates with enhanced angiogenesis and tumor volume and is associated with decrease rate of patient survival [20,22,28]. It should, however, be pointed out that the extent of EPC incorporation within tumor vasculature is extremely variable, ranging from 0% to more than 90% (Table 1) [22,23,32,34]. This heterogeneity may be easily accounted for a number of intersecting factors that will be discussed in the next paragraph.

2.2. The controversy about EPC contribution to tumor vasculature: hematopoietic vs. non-hematopoietic EPCs

The controversy about EPC involvement in malignant transformation has been engendered by the wide variety in the methods of tissue collection, in the number of cells injected and in the animal models investigated to examine the composition of cancer vessels [22]. In addition, EPC-mediated neovascularization depends on type, stage and location of the tumor. This feature has been unveiled by utilizing mice heterozygous for the tumor suppressor Pten (Pten$^{+/-}$), that may exhibit different malignant neoplasms, including uterine carcinomas and lymph hyperplasia. EPC-induced angiogenesis was found in the former, while it was undetectable in the latter [35]. A series of studies from the same group revealed that EPCs home to implanted LLC, B6RV2 lymphoma and melanoma and to spontaneous breast cancer occurring in MMTV-PyMT mice and in developing pulmonary metastases before vessel formation [23,32]. As a consequence, they are diluted both by local endothelial cells and bone marrow-derived hematopoietic cells, a feature that might underestimate their contribution at later stage of tumor progression [22,28]. Finally, and perhaps the most important source of variability in the evaluation of their involvement in the angiogenic switch, EPC phenotype is far from being clearly identified (Fig. 1 and Table 2) [36,37]. The term EPC cannot be referred to a unique cell population featured by specific surface markers that make it easy to detect and quantify in vivo, but encompasses at least two separate subsets that may be classified as hematopoietic and non-hematopoietic [37]. The confusion in EPC definition has been engendered by: 1) the ability of hematopoietic progenitor and stem cells to sustain vessel growth

Table 1
Summary of the evidences in favor or against EPC contribution to tumor vascularization.

Tumor type	Tumor stage	% of contribution	BM tracking	Endothelial cell marker	BM-EC detection	
Colon cancer (MCA38) s.c.	1–3 weeks	Positive	Flk-lacZ or Tie2-LacZ		IHC	Asahara et al. [181]
Lymphoma cell (B6RV2) s.c.	2 weeks	90%	Rosa26-LacZ	vWF	IHC	Lyden et al. [31]
Neuroblastoma cell (NXS2) s.c.	ND	5%	BM cells expressing MSCV-tsFlk-1-IGFP	CD31and CD34	IF	Davidoff et al. [182]
Lewis lung carcinoma (LLC) s.c.	2 weeks	35%	Human β2-microglobulin	CD31 and vWF	IF	Reyes et al. [183]
LLC, s.c., Melanoma (B16) s.c.	Late stage	Negative	Tie2-GFP	CD31	IF, 3D microscopy	De Palma et al. [184]
Fibrosarcomas (MCA/129) s.c.	2 weeks	50%	Rosa26-LacZ	vWF	IHC	Garcia-Barros et al. [185]
Pten$^{+/-}$ mouse (uterine carcinoma and lymph hyperplasia)	Spontaneous	16% — uterine carcinoma Negative — lymph hyperplasia	Rosa26-LacZ	CD31	IHC	Ruzinova et al. [35]
MMTV-PyMT mouse	10–12 weeks	1.3%	Rosa26-LacZ	CD31	IHC	Dwenger et al. [186]
B16F1 s.c.	2–3 weeks	Negative	Actb-GFP	CD31 and vWF	IHC	Rajantie et al. [187]
LLC s.c., B6RV2 s.c.	2 weeks	Negative	Endothelial-SCL-Cre-ER × R26R or R26REYFP	CD31	IF	Goethert et al. [188]
RIP1-Tag5 mice AlbTag	10–16 weeks	3–38%	Tie2Cre × RAGE-EGFP	Lectin staining, CD31	IHC, FACS	Spring et al. [189]
Various human tumors	ND	1–12%	X or Y chromosomes	vWF	FISH	Peters et al. [30]
LLC s.c., B16 s.c., Breast cancer cell (MCa8) orth.	ND	<1%	Actb-GFP, Tie2-GFP	CD31	IF, FACS	Duda et al. [190]
LLC s.c., B16F0 (ortho); MMTV-PYMT mouse	2 weeks	2–20%	Actb-GFP	CD31, VE-cadherin, Lectin staining	FACS, 3d microscopy	Nolan et al. [23]
LLC s.c., MMTV-PYMT mouse	Lung metastasis	12%	Actb-GFP	CD31	IF, FACS	Gao et al. [32]
B16 s.c.	2–3 weeks	Negative	Actb-GFP, VEGFR2-LacZ	CD31, vWF	IF	Purhonen et al. [191]
Dorsal skinfold chamber model xenograft of rat C6 cells (glioblastoma) c.a.	1–2 days	Positive	DiI-labeled ECFCs	DiI staining	Intra-vital multifluorescence microscopy	Bieback et al. [40]
LLC s.c.	5 days	Positive	DiI-labeled CFU-ECs	CD31, DiI staining	IHC	Jung et al. [192]
	2 weeks	Positive	GFP$^+$ bone marrow-derived cells	CD31	IHC	
Human U87 glioma, flanks	28 weeks	Positive	CFU-ECs	CD31	IHC	Zhang et al. [193]
	Palpable tumor	Positive	Carboxyfluorescein diacetate succinimidyl ester-labeled CFU-ECs	DiI staining	IHC	

Abbreviations: c.a.: carotid artery; DKK1: Dickkopf 1; FACS: fluorescent activated cell sorting; IF: immunofluorescence; IHC: immunohistochemistry; ortho: orthotropic injection; RAGE: receptor for advanced glycated end products; s.c.: subcutaneous injection.
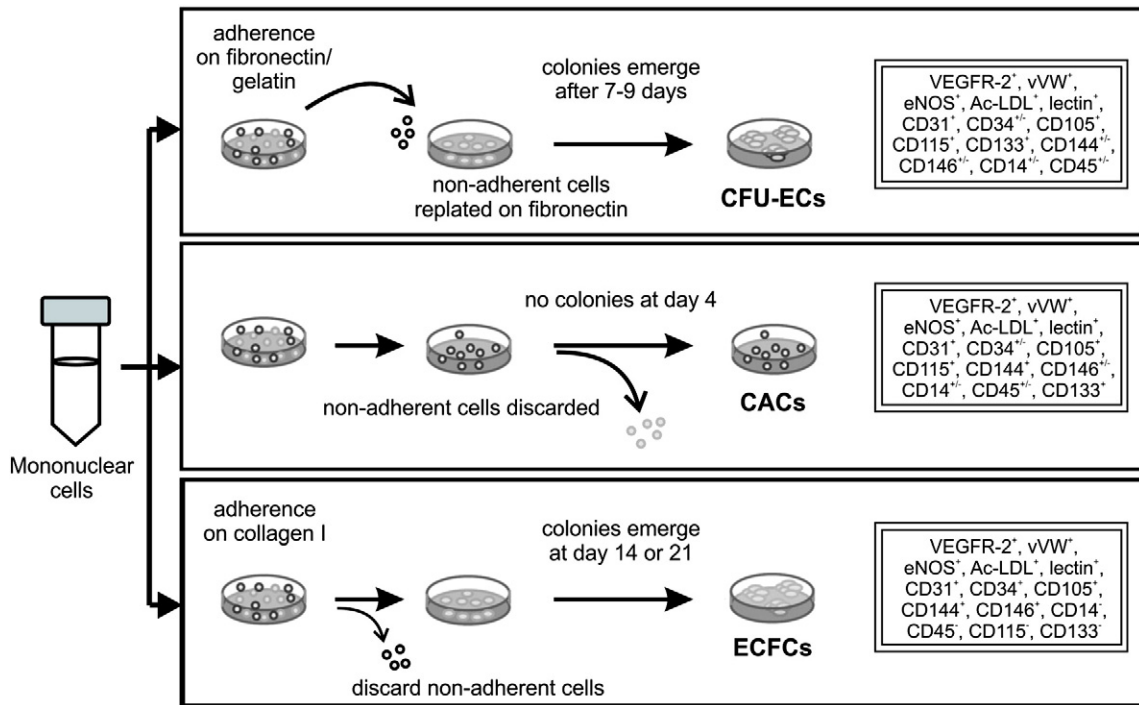
Fig. 1. Isolation and identification of distinct endothelial progenitor cell subtypes. This figure depicts the most common protocols currently employed to isolate EPCs from either peripheral and umbilical cord blood. CFU-ECs (colony forming unit-endothelial cells) require a 5–9 day process which starts with the seeding of 5×10^6 mononuclear cells (MNCs) on fibronectin-coated dishes in a commercially available medium enriched with 20% fetal bovine serum (FBS) and VEGF. Non-adherent cells are removed after 24 h and replated on new fibronectin-coated dished for further 5–9 days before giving raise to a visible EPC colony. Circulating angiogenic cells (CACs) are obtained from the adherent fraction of MNCs after 4 days of culture in endothelial conditions and do not display any significant colony forming ability. The culture of endothelial colony forming cells (ECFCs) requires the seeding of MNCs onto collagen-coated culture dishes in a commercially available endothelial growth medium (EGM-2, Lonza) for 48 h. At this time, non-adherent cells are discarded and ECFC-derived colonies with cob-blestone morphology appear after 10–20 days when MNCs are harvested from peripheral blood. For each EPC subgroup, the panel of antigens for which they are positive (+), partially positive (+/−), or negative has been added on the right. The centrifuge tube on the left side contains the fraction of mononuclear cells from which each EPC subtype is derived.

by the paracrine secretion of growth factors and cytokines; and 2) the lack of a combination of markers and receptors selective for truly endo-thelial EPCs [15,36,38]. The hematopoietic EPCs comprise both colony forming unit-endothelial cells (CFU-ECs) and circulating angiogenic cells (CACs), while the non-hematopoietic EPCs consist of the so-called endothelial colony forming cells (ECFCs) (Fig. 1) [15,36,37]. CFU-ECs and CACs may be recruited towards the growing tumor, but they do not directly incorporate within neovessels: these cells display a peri-vascular location and support malignant growth in a paracrine manner (Fig. 2). Conversely, ECFCs possess an impressive clonogenic potential, are committed to differentiate into mature endothelial cells, form capillary-like structures in vitro and patent vessels in vivo [15,36, 37]. Therefore, they are now regarded as bona fide EPCs that may phys-ically engraft within cancer vasculature (Fig. 2) [37]. Only an extensive gene expression profiling combined with a rigorous ultrastructural ex-amination and an array of functional assays permits to distinguish be-tween CFU-ECs, CACs and ECFCs [37,39]. As illustrated above, only a

few groups have established the integration of bone marrow-derived cells endowed with endothelial markers, but lacking hematopoietic an-tigens, into tumor vasculature [23,32–35]. However, recent studies have confirmed that human ECFCs, which truly belong to the endothelial lin-eage, may be recruited to sites of tumor angiogenesis upon their direct injection into the bloodstream of several murine models of human ma-lignancies. More specifically, DiI-labeled ECFCs actively home to subcu-taneously implanted glioma [40] and breast cancer [41] xenografts as well as to LLC metastases [42]. It turns out that ECFC represents the most suitable subset to investigate the molecular mechanisms driving EPC incorporation into tumor vasculature and, consequently, to identify the most effective target(s) to adverse metastatic progression.

2.3. Is VEGFR-2 the most suitable target for anti-angiogenic treatments?

VEGF has long been known to stimulate angiogenesis and promote endothelial growth/survival by the activation of its cognate receptor, VEGFR-2 (also termed KDR in humans or Flk-1 in mice), which pos-sesses an intrinsic and druggable tyrosine kinase activity [43]. There-fore, the inhibition of VEGF signaling by either humanized monoclonal anti-VEGF antibodies (Bevacizumab [Avastin]) or multi-targeted pan-VEGF receptor tyrosine kinase inhibitors (Sunitinib [Sutent], Pazopanib [Votrient], Sorafenib [Nexavar], and Vandetanib [Zactima]), has been approved by the US Food and Drug Administration and introduced in clinical practice to treat a growing number of advanced metastatic can-cers, such as renal cellular carcinoma (RCC), breast cancer, non-squamous non-small cell lung cancer, colorectal cancer, and recurrent glioblastoma, either in conjunction with chemotherapeutics or as monotherapy [17,43]. In principle, this approach is further recommend-ed by the notion that EPCs require a functional VEGFR-2 to proliferate, engraft within nascent vessels, and differentiate into mature endothelial

Table 2
Functional properties of the three better characterized endothelial progenitor cell populations.

	CFU-ECs	CACs	ECFCs
Clonal proliferative potential	−	−	+
Replating ability	−	−	+
In vitro tube formation (Matrigel scaffold)	+/−	+/−	+
Neovessel formation in vivo	−	−	+
Homing to ischemic sites in vivo	+	+	+
Homing to primary tumors in vivo	+	ND	+
Paracrine stimulation of angiogenesis	+	+	+

Abbreviations: CFU-ECs: colony forming unit-endothelial cells; CACs: circulating angio-genic cells; ECFCs: endothelial colony forming cells.
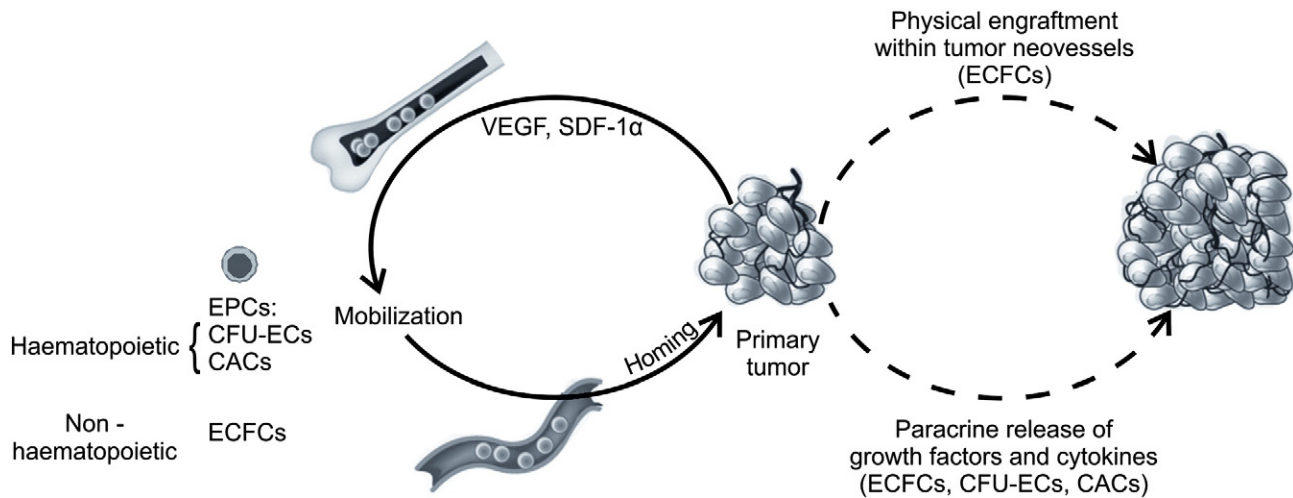
Fig. 2. The mechanism of tumor vascularization by tumor endothelial progenitor cells. Cancer-released growth factors (such as VEGF) and cytokines (such as SDF-1α) stimulate EPC egression from bone marrow, thereby increasing their circulating levels and homing to growing tumor. Herein, ECFCs, which truly belong to the endothelial lineage and therefore non-hematopoietic, may either directly incorporate within tumor neovessels or stimulate local angiogenesis through the paracrine release of further growth factors. Conversely, CFU-ECs and CACs, which derive from hematopoietic precursors, may only accelerate tumor growth and metastizazion in a paracrine-manner.

cells [12,15,26]. A number of studies conducted on xenograft tumor models seemingly confirmed the feasibility of targeting VEGFR-2 to prevent EPC stimulation in cancer patients. EPC-mediated vascularization and growth of either LLC or B6RV2 tumors were blocked by inhibiting VEGFR-2 through the delivery of a neutralizing antibody (DC101) [31] or of a specific short hairpin RNA (shRNA) [33]. Moreover, the EPC spike in systemic circulation induced by chemotherapeutics (paclitaxel, 5-FU, and docetaxel) and vascular disrupting agents (VDAs; combretastin-A4 phosphate, OXi-4503, onbrabulin), that compromise the already established tumor vasculature without interfering with bone marrow activation, was abolished by several drugs targeting VEGFR-2, such as DC101 [44,45] and sunitinib [46]. Despite the initial enthusiasm raised by pre-clinical studies conducted on established cancer models, oncological practice revealed all the unexpected limits of anti-angiogenic medicine, which depend on our poor comprehension of the intrinsic mechanisms of vascular growth [17,43]. Anti-VEGF inhibitors, such as bevacizumab, sorafenib and sunitinib, caused only a modest increase in progression-free survival (PFS) of cancer patients, while they did not significantly improve their overall survival (OS), either when administered alone or in combination with standard chemotherapy and VDA [20,43,47]. This is why resistance shortly arises in the majority of patients, while others are intrinsically refractory and do not display any response to the treatment [17,20]. A number of explanations have been offered to shed light on the therapeutic failure of VEGF blockers and this issue has recently spurred an intense debate [43,47]. The most important issue to bear in mind when analyzing the scarce efficacy of VEGF pathway inhibitors is the tremendous impact that tumor microenvironment may exert on its own vasculature. First, neoplastic transformation is accompanied by the HIF-1α-mediated release of alternative growth factors, including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), placental growth factor (PlGF), and angiopoietin-1, which may replace VEGF in the cellular stroma and render the inhibition of its cognate receptor ineffective [43,47]. Second, anti-angiogenic drugs have been developed on the basis of a wrong rationale, i.e. that cancer endothelium maintains the same features as non-transformed one, including low mutations rate, which makes it genetically stable and less prone to acquire drug resistance.-Unfortunately, it is now evident that TECs display morphological, cytological and functional differences as compared to normal endothelial cells, including chromosomic aberrations, higher proliferative potential and resistance to pro-apoptotic stimulation, and lower sensitivity to cytotoxic drugs [21]. Third, different cancer types have long been thought to require the same process, i.e. VEGF-mediated angiogenesis,

to be vascularized. This notion is, however, false as intussusceptive angiogenesis and vasculogenic mimicry are utilized by melanoma and gliobastoma to gain access to the host vascular system, while vessel cooption is indispensable to nourish lung adenocarcinoma and cutaneous melanoma [48]. These alternative mechanisms of tumor vessel growth do not necessarily require VEGF and are, therefore, insensitive to classic anti-VEGF inhibitors [43,48]. EPC-mediated vascularization may certainly be included among these alternative modes of tumor expansion and metastatization. Their early mobilization from bone marrow does not only sustain the angiogenic switch that promotes metastatic progression (see above); a delayed EPC burst is induced by cytotoxic drugs and VDAs, thereby inducing the well known phenomenon of acquired resistance that leads to patient death despite for continuous treatment [49,50]. Most, if not all, the studies assessing the role of VEGFR-2 in EPC proliferation and recruitment to tumor site, have been conducted on mice xenografted with human cancers and injected with EPCs obtained by normal, i.e. healthy, donors. As VEGF signaling is functional in these cells [13,14,51–53], it is not surprising that blocking VEGFR-2 prevents EPC activation and causes tumor shrinkage in pre-clinical settings. Given the impact of tumor microenvironment on resident endothelium, we endeavored to assess whether and how the pro-angiogenic Ca^{2+} signaling toolkit is deranged in ECFCs harvested from peripheral blood of naïve patients suffering from RCC. The rationale behind our investigations was that only tumor EPCs could be reliably employed to ascertain whether VEGFR-2 is a suitable target to suppress EPC engagement in these patients. Moreover, distinct components of the endothelial Ca^{2+} machinery can be selectively enlisted by different growth factors to stimulate proliferation and tube formation [11,25]; therefore, we reasoned that such investigation could introduce novel signaling pathways, e.g. store-operated Ca^{2+} entry (SOCE) or Transient Receptor Potential (TRP) channels, as alternative therapeutic tools in anti-angiogenic therapy.

3. Ca^{2+} signaling in endothelial progenitor cells

An intricate network of signaling pathways is set in motion upon VEGF binding to its cognate receptor, VEGFR-2, to stimulate endothelial cell growth/survival and promote tumor vascularization in vivo. The downstream mediators of VEGFR-2 include, but are not limited to: 1) phospholipase Cγ (PLCγ), which may either lead to an increase in $[Ca^{2+}]_i$ or engage the protein kinase C (PKC)/Ras/MEK/MAPK cascade, 2) cytosolic PLA2, which is activated upon extracellular signal-regulated kinase (ERK)-mediated phosphorylation and controls

PGI$_2$ production; 3) phosphoinositide 3-kinase (PI3K), which in turn recruit the protein kinase B (PKB)/Akt pathway via an increase in phosphatidylinositol-3,4,5-trisphosphate (PIP$_3$) to activate endothelial nitric oxide (NO) synthase (eNOS) and inhibit B-cell lymphoma 2 (Bcl-2)-associated death promoter homologue (BAD) and Caspase 9; 4) p21-activated protein kinase-2 (PAK-2), which is responsible for the subsequent activation of both Cdc42 and p38 MAPK; and 5) focal adhesion kinase (FAK) and its substrate paxillin [54,55]. In particular, VEGF utilizes Ca^{2+} signaling to promote endothelial cell proliferation and tubulogenesis both in vitro and in vivo [11,56–59]. The pro-angiogenic Ca^{2+} response occurs downstream of PLCγ activation and may adopt distinct patterns depending on the vascular bed of origin. As widely described elsewhere both by us [11] and by other authors [1], PLCγ cleaves the phospholipid precursor, phosphatidylinositol 4,5-bisphosphate (PIP$_2$), into two intracellular second messengers, i.e. inositol 1,4,5-trisphosphate (InsP$_3$) and diacylglycerol (DAG). These two signaling intermediates activates two distinct modes of [Ca^{2+}]$_i$ elevations in micro- vs. macro-vascular endothelial cells. DAG remains tethered to the plasma membrane to directly gate two members of the non-selective cation family of canonical TRP channels (TRPC), namely TRPC3 and TRPC6, in human microvascular endothelial cells [11,25,60]. TRPC3 and TRPC6, in turn, mediate a monotonic increase in intracellular Ca^{2+} levels which do not require further Ca^{2+} release from the endogenous Ca^{2+} pool. Conversely, in human umbilical vein endothelial cells (HUVECs), InsP$_3$ rapidly diffuses across peripheral cytosol to InsP$_3$ receptors (InsP$_3$Rs), which are embedded in the membrane enveloping the endoplasmic reticulum (ER), the most abundant endothelial Ca^{2+} reservoir. InsP$_3$Rs function as Ca^{2+}-permeable channels to release luminally stored Ca^{2+} upon InsP$_3$ binding, thereby causing a rapid increase in [Ca^{2+}]$_i$. The consequent emptying of the ER Ca^{2+} pool signals the opening of store-operated Ca^{2+} channels (SOCs) in the plasma membrane [11,26]. The ensuing influx of Ca^{2+} shapes a plateau phase of intermediate magnitude between pre-stimulation Ca^{2+} levels and the InsP$_3$-dependent initial peak that persists as long as VEGF is presented to the cells [56,57]. Store-operated Ca^{2+} entry (SOCE) is a ubiquitous mechanism that, in addition to replenishing the endogenous Ca^{2+} pool, delivers the bolus of Ca^{2+} necessary for the recruitment of a plethora of endothelial Ca^{2+}-sensitive decoders, including eNOS, nuclear factor of activated T-cells (NFAT), nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB), activating protein-1 (AP-1), calpain and myosin light chain kinase (MLCK) [11,26,61–64]. We have widely illustrated elsewhere the rather heterogeneous structure of this pathway in macrovascular endothelial cells [11,15,20,26]. Briefly, endothelial SOCE may be initiated by Stromal Interaction Molecule-1 (Stim1), which senses the drop in ER Ca^{2+} concentration and rapidly (40–60 s) oligomerizes into clustered puncta that approach as close as 20–30 nm to the plasma membrane. Herein, Stim1 binds to and gates two structurally different types of endothelial SOCs, depending on both the species and the vascular bed. Accordingly, Stim1 exclusively establishes a physical interaction with Orai1, i.e. the pore-forming subunit of the Ca^{2+} release-activated Ca^{2+} (CRAC) channel, in HUVECs [56,57]. Conversely, TRPC1 and TRPC4 provide the core components of plasmalemmal SOCs in mouse aorta [65] and rodent pulmonary artery [66,67]. In particular, in rat pulmonary artery endothelial cells (PAECs), the fall in intraluminal Ca^{2+} induces Stim1 to relocate into sub-plasmalemmal puncta in a TRPC4-dependent manner, thereby promoting the association of TRPC4 with TRPC1 [67]. This mechanism recognizes a privileged role for Orai1, which constitutively interacts with TRPC4 in un-stimulated cells, increases the probability of TRPC1/TRPC4 activation by Ca^{2+} depletion and confers Ca^{2+}-selectivity to the complex [66].

VEGF is not the only growth factor to utilize Ca^{2+} signals to exert a mitogenic effect on mature endothelial cells. Insulin-like growth factor-1 (IGF-1) and bFGF trigger a massive entry of Ca^{2+} from the extracellular milieu in bovine aortic endothelial cells (BAECs), which is required for them to proliferate [68,69]. Subsequent studies have led to

the conclusion that arachidonic acid (AA) mediates bFGF-evoked Ca^{2+} inflow by activating TRP Vanilloid 4 (TRPV4) [70]. TRPV4-mediated Ca^{2+} influx might promote endothelial proliferation and vascular remodeling by enlisting a variety of Ca^{2+}-dependent transcription factors, such as NFAT, myocyte enhancer factor 2C (MEF2C), Kv channel interacting protein 3, calsenilin (KCNIP3/CSEN/DREAM), and cytoplasmic, calcineurin-dependent 1 (NFATc1) [71,72]. EGF, in turn, elicits cytosolic and nuclear Ca^{2+} oscillations in rat microvascular endothelial cells [73], while platelet-derived growth factor (PDGF) evokes irregular fluctuations of [Ca^{2+}]$_i$ in porcine aortic endothelial cells [74]. This spiking response relies on the rhythmic Ca^{2+} discharge from InsP$_3$Rs and is maintained by the Stim1-mediated activation of Orai1 [59,73–75]. Unfortunately, the physiological outcome of EGF- and PDFG-induced intracellular Ca^{2+} spikes has not been probed in these studies; however, endothelial cells often use the information encoded in the oscillatory pattern to finely tune the Ca^{2+}-sensitive decoders that promote their pro-angiogenic behavior [76–81]. In addition to mitogens, the chemoattractant SDF-1α may induce endothelial migration in vitro and recruit bone marrow-derived HSCs to the target vasculature in vivo through an increase in [Ca^{2+}]$_i$ [12,82]. The signaling pathway downstream of its G-protein coupled receptor, CXCR4, is still elusive, but it may involve both InsP$_3$-gated Ca^{2+} release [12] and store-dependent Ca^{2+} inflow [82]. Therefore, there is ample evidence in literature to indicate that VEGF, as well as other growth factors and cytokines, stimulate angiogenesis by activating a different blend of Ca^{2+}-permeable channels depending on the species and the vascular tree under examination. This was the background upon which we undertook the examination of the components of the Ca^{2+} machinery selected by VEGF in normal vs. tumor human ECFCs.

3.1. VEGF evokes pro-angiogenic Ca^{2+} oscillations in normal ECFCs

Among the different subsets of EPCs utilized to induce the angiogenic switch in solid cancers, we focussed on ECFCs, or late outgrowth EPCs, for four main reasons. First, they are the only EPC population possessing all the features of a true endothelial progenitor and do display any known hematopoietic marker. Second, unlike CFU-ECs and CACs, they are capable of forming capillary-like structures in vitro, to originate patent vessels and to anastomose with host vasculature in vivo [36,37]. Third, they home to sites of malignant growth and physically engraft within nascent vessels, thereby enhancing tumor size and vascularization [40,41]. Fourth, they do require VEGF to proliferate, assembly into bidimensional tubulary networks, and differentiate into mature endothelial cells [51–53]. Our Ca^{2+} imaging recordings disclosed that 10 ng/ml VEGF, which is quite close to the dose (25 ng/ml) employed by Ingram and coworkers to stimulate ECFC expansion in the seminal study that led to their identification [83], triggered asynchronous oscillations in [Ca^{2+}]$_i$ in neighboring cells from the same coverslip [13,84]. There were no two cells generating synchronous elevations in [Ca^{2+}]$_i$ in the ECFC monolayer. Such heterogeneity is the hallmark of mitogen-induced Ca^{2+} fluctuations in mature endothelial cells as well as other non-excitable cell types [73,74,76]. Statistical analysis revealed the stochastic nature of VEGF-induced intracellular Ca^{2+} oscillations, as the standard deviation (SD) of their period was of the same order as the average value [13,85,86]. We have discussed elsewhere the molecular underpinnings of the variability in the Ca^{2+} response to VEGF in healthy ECFCs [15,26]. Briefly, this diversity, that is retained by umbilical cord blood-derived ECFCs [14], may be engendered by at least two different sources. First, VEGF binding to VEGFR-2 obeys to a stochastic regime in vascular endothelium, which is key to determine the angiogenic phenotype, i.e. proliferative (tip cells) vs. migratory (stalk cell), adopted by the target cell [43,87]. Second, it is likely that the molecular nature and the spatial arrangement of Ca^{2+} release and entry sites dictates the timing of spike generation for each single ECFC [85,86,88]. Normal ECFCs possess all the three known isotypes of InsP$_3$Rs, namely InsP$_3$R-1, InsP$_3$R-2, and InsP$_3$R-3, the pattern of expression of their

transcripts being InsP$_3$R-3 > InsP$_3$R-2 > InsP$_3$R-1 [13]. Conversely, they are devoid of functional ryanodine receptors [89], whose expression and biological meaning in mature endothelium have been largely debated [11]. Additionally, ECFCs are insensitive to H$_2$S stimulation, which elicits robust Ca^{2+} signals in normal endothelial cells [60,90,91] and mediates VEGF-induced Ca^{2+} entry in breast cancer-derived TECs [60]. VEGF-induced Ca^{2+} oscillations were suppressed by U73122, which selectively blocks PLCγ activity, but not by its inactive analogue, U73343; they were further hindered by 2-aminoethoxydiphenyl borate (2-APB), which selectively abrogates Ca^{2+} release from InsP$_3$Rs under our experimental conditions, i.e. in the absence of Ca^{2+} in the bathing solution (0Ca^{2+}) [13]. The Ca^{2+} transients developed regardless of the absence of extracellular Ca^{2+}, which suggests that they are primarily driven by Ca^{2+} recycling across the ER membrane; however, they persisted only for a short time in Ca^{2+}-free saline and resumed only on Ca^{2+} restitution to the cells [13]. Therefore, InsP$_3$-dependent Ca^{2+} release is sufficient to initiate and support the irregular episodes of intracellular Ca^{2+} mobilization in ECFCs, but Ca^{2+} entry across the plasma membrane is necessary to maintain the oscillations over time. So, which is the membrane pathway conducting extracellular Ca^{2+} into these endothelial precursors? Unlike mature endothelial cells, human circulating ECFCs lack TRPC3 and TRPC6 [13,14], which mediate VEGF-evoked Ca^{2+} entry in a DAG-dependent manner. Conversely, they express all the putative components of SOCE, i.e. Stim1–2, Orai1-3, TRPC1 and TRPC4 and exhibit a massive Ca^{2+} influx in response to ER Ca^{2+} store depletion. Therefore, ECFCs rely on a store-dependent mechanism rather than on a DAG-sensitive conductance to sustain prolonged intracellular Ca^{2+} signals. Consistent with this notion, 1-oleoyl-2-acetyl-sn-glycerol (OAG), a membrane permeable analogue of DAG, does not evoke any detectable Ca^{2+} inrush [14]. This feature rules out the possibility that TRPC1 forms a second messenger-operated channel gated by DAG in human ECFCs, as reported in human prostate cancer cells [92]. Therefore, SOCE provides the molecular gateway for Ca^{2+} inflow in response to agonist stimulation in endothelial precursors [57,89,93]. Parallel work conducted both by us and by Prof. Beech's lab demonstrated that SOCE is mediated by Stim1, Orai1 and TPC1 in endothelial committed progenitors. RNA interference selectively targeting either Stim1 or Orai1 abolished store-dependent Ca^{2+} influx in these cells [57,93]. Likewise, SOCE was abrogated by the expression of a dominant negative mutant Orai1 (R91W), whereas InsP$_3$-evoked emptying of the ER Ca^{2+} pool induced eYFP-GFP tagged Stim1 to cluster into peripheral puncta [57]. Finally, short hairpin RNA-mediated knockdown of TRPC1 dramatically decreased the magnitude of store-dependent Ca^{2+} entry in human ECFCs [93]. These molecular data are supported by the pharmacological profile of SOCE, that is sensitive to a wide spectrum of rather specific inhibitors of both Orai1 and TRPC1 in these cells, including low micromolar doses of the trivalent cations La^{3+} and Gd^{3+}, 2-APB and YM-58483/BTP2 [20,26]. The question then arises as to whether these two channel proteins assemble into a super-molecular ternary complex with Stim1, as illustrated in megakaryocytes [94] and mouse submandibular gland cells [95], or constitute two distinct Ca^{2+}-permeable routes, each recruited by Stim1 [26]. Suppression of either Orai1 or TRPC1 expression reduced SOCE amplitude to the same extent [93], thereby suggesting that both of them contribute to the pore forming channel in human ECFCs. The molecular and pharmacological characterization of the most important Ca^{2+}-permeable route in endothelial committed progenitors enabled us to ascertain its participation to VEGF signaling. One 1-to-4 Ca^{2+} transient arose when the cells were challenged with VEGF in the presence of BTP2, whose effect on Ca^{2+} fluctuations thus mimicked the removal of extracellular Ca^{2+} [13]. In addition, VEGF-evoked Ca^{2+} influx was nearly absent in normal ECFCs transfected with a siRNA directed against Stim1 or Orai1 [57], which is consistent for SOCE requirement to maintain the spiking response to VEGF [13]. Thus, the molecular machinery that translates the extracellular input carried by VEGF into a specific intracellular output, i.e. a defined biological response, involves both InsP$_3$Rs and SOCs in normal ECFCs.

VEGF-elicited Ca^{2+} oscillations drive proliferation and tube formation in healthy ECFCs. The pro-angiogenic activity of VEGF disappeared when the cells were pre-treated with BAPTA, a strong membrane-permeable Ca^{2+} buffer, or BTP2 to hinder the ensuing elevation in [Ca^{2+}]$_i$ [13]. Similarly, siRNA-mediated genetic ablation of Orai1 prevented ECFC tubulogenesis [57]. Therefore, VEGF utilizes intracellular Ca^{2+} signals to promote endothelial proliferation and remodeling not only in mature endothelial cells, but also in their progenitors. The Ca^{2+}-sensitive decoder translating the irregular fluctuations in [Ca^{2+}]$_i$ into a biologically meaningful message is represented by NF-κB. The NF-κB signaling system includes about a dozen different dimmers comprising five homologous subunits, i.e. p50, p52, c-Rel, RelA/p65 and RelB [96]. The cytosolic-nuclear localization of NF-κB is intricately tuned and, in turn, regulates several key steps of the angiogenic process, including endothelial cell proliferation, survival motility, substrate adhesion, interaction with the extracellular matrix and capillary morphogenesis [97]. The heterodimer formed by p65 with p50 or p52 is sequestered in the cytosol by the association with I$_{κB}$, an inhibitory protein which masks the nuclear localisation sequence of the complex. A burst of intracellular Ca^{2+} oscillations recruits Ca^{2+}/calmodulin (CaM)-dependent protein kinase IV (CaMKIV) to activate the enzyme IκB kinase (IKK). IKK, in turn, hyperphosphorylates IκB on two specific NH$_2$-terminal serines and targets the inhibitory protein to site-specific ubiquitination and eventual degradation by the proteasome [98]. As a consequence, NF-κB dimers are released from inhibition and freed into the nucleus, where they turn on the transcriptional program controlling EPC fate [99,100]. Consistent with this model, the pharmacological extinction of VEGF-induced Ca^{2+} oscillations with either BAPTA or BTP2 hindered VEGF-induced I$_{κB}$ phosphorylation in normal ECFCs; as expected, the same effect was exerted by thymoquinone, which selectively interferes with NF-κB activation [13]. The final observation that thymoquinone abrogated ECFC expansion and tubule formation confirmed that NF-κB serves as the Ca^{2+}-dependent decoder of VEGF-evoked Ca^{2+} transients [13]. It has long been thought that NF-κB stimulation by a spiking signal in endothelial cells is controlled by the interspike interval (ISI). The transcriptional activity of endothelial NF-κB reaches its peak within a frequency range of Ca^{2+} spikes ranging from 0–5.2 mHz to 1.7–11.7 mHz [101]. This property does not fully apply to VEGF-challenged ECFCs, which generate irregular Ca^{2+} transients. However, resting cells do not exhibit any detectable elevation in [Ca^{2+}]$_i$, so that the informative content of the irregular Ca^{2+} spikes is obvious as compared to silent ECFCs. Recent mathematical computation has unveiled that any deviation from the pattern of the Ca^{2+} spikes in un-stimulated cells conveys a robust (i.e. high signal-to-noise ratio) message to their downstream effectors, thereby triggering a biological response [85,88]. The same group has further demonstrated that the cell-to-cell heterogeneity in the Ca^{2+} train notwithstanding, stochastic oscillations exhibit a linear correlation between the standard deviation of ISI and the average period of the spikes. It turns out that, under different experimental conditions (e.g. agonist dose, extent of cytosolic Ca^{2+} buffering, cell type), frequency encoding is realized by moving up and down this relation [102]. Interestingly, when ECFCs are exposed to 10 ng/ml VEGF, the interval between two consecutive Ca^{2+} transients in most cells ranges between 200 s and 800 s [13], which is adequate to induce the nuclear translocation of NF-κB [101]. Conversely, when VEGF concentration is raised to 50 ng/ml, the ISI may be lengthened to 1500 s, which is less efficient to stimulate NF-κB. An alternative, albeit not mutually exclusive, mechanism whereby irregular Ca^{2+} fluctuations are decoded involves the spatial distribution of the underlying Ca^{2+} toolkit. Local Ca^{2+} microdomains may be generated around the cytosolic mouth of Ca^{2+}-permeable channels to convey information to specific Ca^{2+}-sensitive decoders located in their immediate surroundings. The seminal example has been provided by Parekh's group [103], who demonstrated that leukotriene C4-induced Ca^{2+} oscillations induced gene expression and mast cell activation only when they were patterned by Orai1, while were inefficient if exclusively contributed by InsP$_3$Rs. The randomness of VEGF-induced Ca^{2+} spikes in ECFCs might be translated

into a biologically meaningful code by the selective activation of either InsP$_3$Rs or store-dependent channels (e.g. Orai1 and TRPC1) or both. These preliminary studies paved the way for the subsequent examination of the Ca^{2+} response to VEGF in tumor ECFCs. Once established that VEGFR-2 is capable of delivering a pro-angiogenic Ca^{2+} signal to their normal counterparts, we sough to elucidate whether this pathway was altered in cancer patients-derived cells and whether it could truly serve as an optimal target in oncology.

3.2. Rationale for studying the Ca^{2+} machinery is ECFCs derived from RCC patients

It has long been established that neoplastic transformation is accompanied by a remodeling of the Ca^{2+} toolkit, which may not be key to malignant initiation, but contributes to establish some of the ten hallmarks of cancer [8,9,104], including enhanced proliferation, survival, angiogenesis and invasion. The derangement of the Ca^{2+} machinery is not limited to neoplastic cells, but may also be extended to tumor microenvironment [105]. Accordingly, TECs harvested from breast carcinoma up-regulate TRPV4, which governs the remodeling of actin cytoskeleton that drives endothelial cell migration during the angiogenic switch [70]. Moreover, normal HMECs may be reprogrammed by adriamycin-resistant human breast cancer cells (MCF-7/ADM) to express TRPC5, which is otherwise absent in normal cells [106]. Therefore, we wondered whether tumor ECFCs, which do not belong to the neoplastic clone and are isolated from the mononuclear fraction of peripheral blood [37], could somehow be influenced by the malignant conditions of the donor. The urgency of this study was further prompted by the simultaneous investigation of Ca^{2+} dynamics in ECFCs isolated from other two sources, i.e. umbilical cord blood (UCB) and peripheral blood of individuals affected by primary myelofibrosis (PMF). We found that the Ca^{2+} toolkit may be assembled according to rather different modes in endothelial precursors depending on the superimposed patho-physiological background. For instance, TRPC3, which is absent in circulating ECFCs, appears on the plasma membrane of their UCB counterparts to mediate the influx of Ca^{2+} that triggers the oscillatory response to VEGF [13,89]. A more dramatic dysregulation of the Ca^{2+} machinery occurs in ECFCs derived from PMF patients (PMF-ECFCs), who suffer from fibrotic bone marrow, splenomegaly, cytopenias, and multiple disease-related symptoms that reduce quality and length of life [107]. PMF-ECFCs display a higher ER Ca^{2+} load, which is associated to the over-expression of all InsP$_3$R transcripts as well as of Stim1, Orai1, Orai3, TRPC1 and TRPC4 proteins [108]. It is, therefore, not surprising that SOCE magnitude is significantly enhanced, albeit two separate routes may be discriminated based on their pharmacological profile: one stimulated by passive store depletion and Gd^{3+}-resistant, the other one recruited by the InsP$_3$-dependent Ca^{2+} store and Gd^{3+}-sensitive [108]. A remarkable feature of these cells, that has not been found in both peripheral blood- and UCB-derived ECFCs [14,89], is their relative insensitivity to SOCE inhibitors. BTP-2 and La^{3+} barely affected the proliferation rate, while Gd^{3+} did not block cell growth [108]. This finding suggested that VEGF may exploit signaling pathways other than an increase in [Ca^{2+}]$_i$ to control ECFC replication in a pathological context; in addition, it reawakened the interest towards the earlier notion that proliferative diseases, such as cancer, may turn off the requirement for extracellular Ca^{2+} to drive cell cycle progression into mitosis [109]. This concept does not rule out the contribution of Ca^{2+}-permeable channels, which may serve as scaffold proteins to orchestrate the proliferative process independently on their Ca^{2+}-conducting properties, as recently shown for Orai1 and Orai3 in some cell lines, such as HEK293 and HeLa cells [110]. However, it might have profound therapeutic implications as the in situ disruption of their encoding genes is far less feasible than pharmacological abrogation of Ca^{2+} fluxes. Therefore, we felt the urgency to examine the remodeling, if any, of Ca^{2+} signaling in ECFCs isolated from metastatic RCC patients (RCC-ECFCs). First, RCC is the most common type of kidney

cancer in the adults, and several strong evidences hint at EPC participation to the angiogenic switch in primary tumor [20,29,111–115]. Second, metastatic RCC is the only human cancer where the standard care for treatment is represented by anti-angiogenic drugs, such as bevacizumab, sunitinib, and sorafenib [20,116]. As described in a previous paragraph, however, clinical practice revealed that a fraction of the patients are intrinsically refractory towards this treatment, while those who undergo an initial remission display a later tumor relapse and ultimately die [20,116]. However, TECs cultured from human RCC do respond to VEGF stimulation, which renders them sensitive to bevacizumab, sunitinib and sorafenib [117]. It turns out that an additional component of tumor vasculature must be refractory to this growth factor [20]. All the studies described above concurred to demonstrate that EPCs require a functional VEGFR-2 to sustain the malignant transformation, but they drew this conclusion by exploiting normal, rather than tumor, cells. Third, RCC has recently been associated to an important derangement of Ca^{2+} machinery, Orai1 and TRPC6 expression being enhanced in primary tumor samples as compared to normal renal tissues [118,119]. Conversely, TRPC4 is down-regulated in two different human kidney carcinoma cell lines [120]. These alterations may play a central role in the neoplastic transformation of normal kidney. While TRPC6 up-regulation favors the transition through G2/M phase [118], and Orai1 regulates RCC cell proliferation and migration [119], the loss of TRPC4 leads to a diminished secretion of the endogenous inhibitor thrombospondin-1, thereby promoting the angiogenic switch [120]. Thus, understanding whether RCC-ECFCs develop Ca^{2+}-handling abnormalities might permit the accomplishment of two goals. It could unveil alterations in the pro-angiogenic signaling pathways initiated by VEGF, thereby providing a solid cellular and molecular rationale for the failure of anti-VEGF therapies; and, at the same time, it could highlight unforeseen targets, i.e. Ca^{2+}-permeable ion channels and/or Ca^{2+} transporters, to devise alternative treatments.

3.3. The Ca^{2+} machinery is deranged in tumor ECFCs: consequences for VEGF-induced Ca^{2+} oscillations

Our work was carried out on ECFCs isolated from naïve metastatic RCC patients, i.e. these individuals were newly diagnosed and yet to undergo the anti-angiogenic treatment. There was no difference in the basal Ca^{2+} concentration, as measured by the resting Fura-2 ratio, between control and RCC cells; in addition, there was no unsolicited Ca^{2+} activity in tumor ECFCs [93], while spontaneous oscillations in [Ca^{2+}]$_i$ arise in murine carcinoma stem cells [121,122]. Nevertheless, ER Ca^{2+} load is significantly reduced in RCC-ECFCs, as confirmed by a variety of experimental approaches. First, we measured the magnitude of cytosolic Ca^{2+} mobilization consequent to SERCA inhibition in the absence of extracellular Ca^{2+}, which is an established procedure to evaluate intraluminal Ca^{2+} levels (Fig. 3A–B). Second, we transfected the cells with a plasmid encoding the Ca^{2+}-sensitive luminescent protein aequorin specifically targeted to the mitochondrial matrix, which faithfully senses InsP$_3$-dependent Ca^{2+} discharge. Consistent with the previous data, InsP$_3$ triggers a lower increase in cytosolic (Fig. 3C–D) and intra-mitochondrial (Fig. 3G–H) Ca^{2+} concentration in RCC-ECFCs as related to normal cells, thereby confirming that ER Ca^{2+} dynamics are altered in the former [93]. This feature may help to disclose the potential impact of ECFCs on tumor vasculature. It is well known that a reduction in intraluminal Ca^{2+} levels favors cancer cell survival and evasion of programmed cell death [123,124]. Chronic underfilling of the ER and, consequently, of mitochondrial Ca^{2+} levels reduce the execution of the intrinsic apoptosis pathway [123,124], that culminates in the opening of permeability transition pore (PTP) and permits release crucial components of the apoptosome, including cytochrome c, apoptosis-inducing factor, and SMAC/DIABLO [125]. Moreover, this fall in ER Ca^{2+} has been associated to the over-expression of P-glycoprotein (P-gp), a ABCB1 member of ABC transporter family that drives the efflux of many chemotherapeutic agents out of
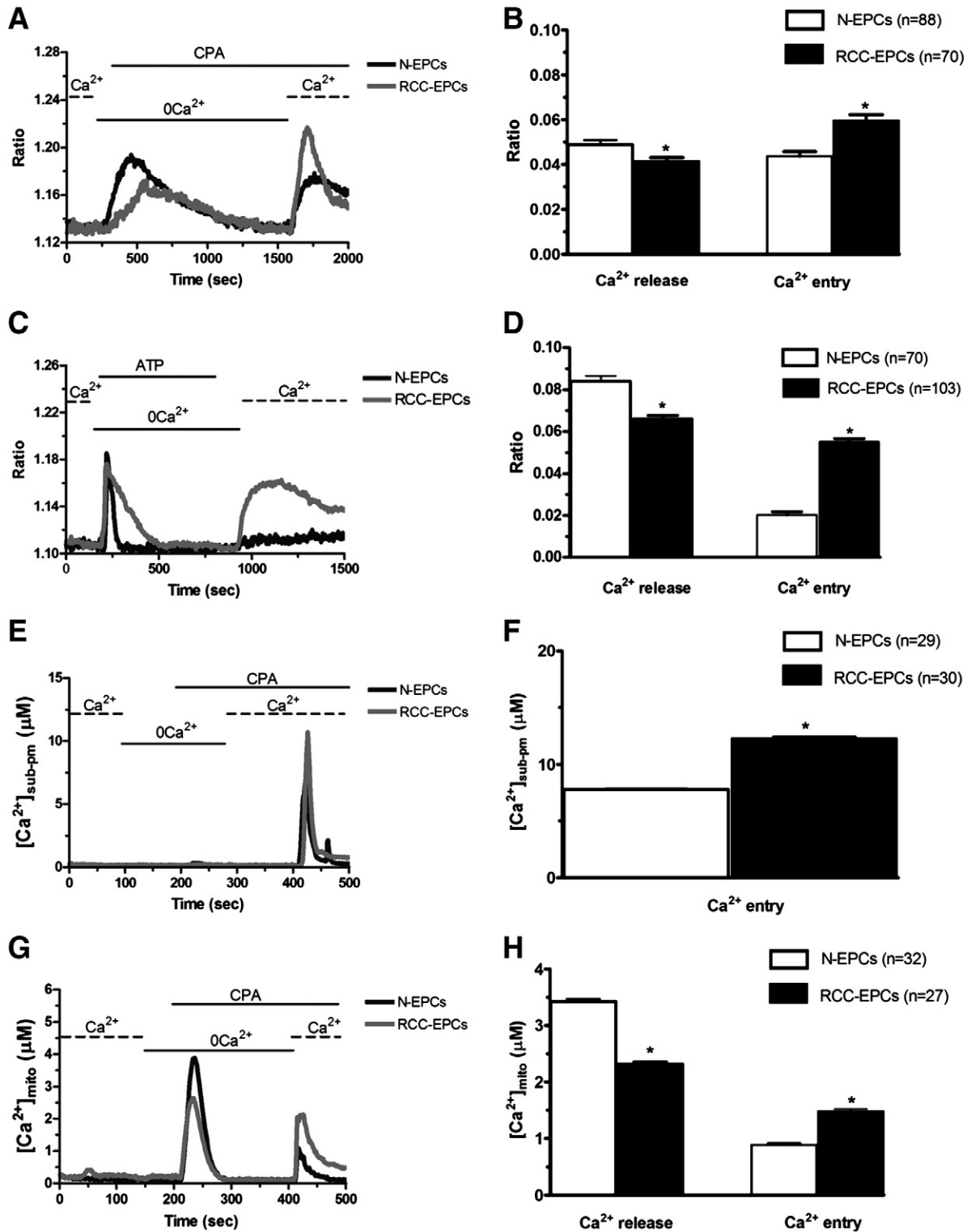
Fig. 3. Remodeling of the Ca^{2+} toolkit in endothelial progenitor cells isolated from patients suffering from renal cellular carcinoma (RCC-EPCs). Both normal EPCs (N-EPCs) and RCC-derived cells with loaded with the Ca^{2+} sensitive dye, Fura-2/AM (4 μM), and subsequently exposed to the "Ca^{2+} add-back" protocol to monitor both ER Ca^{2+} levels and SOCE. Stimulating the cells with either cyclopiazonic acid (CPA; 10 μM) (A–B) or the $InsP_3$-producing autacoid, ATP (100 μM) (C–D), revealed that RCC-EPCs display a reduction in intraluminal Ca^{2+} and higher SOCE as compared to N-EPCs. Likewise, when the cells were transfected with plasma membrane-targeted aequorin, the magnitude of CPA-induced Ca^{2+} inflow was significantly larger in RCC-EPCs (E–F). Finally, when EPCs were transfected with mitochondrial-targeted aequorin, CPA-induced changes in intra-mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mito}$) reflected those recorded in Fura-2/AM-loaded cells (G–H). In Panels B, D, F, H, the asterisk indicates $p < 0.05$ and the data displayed are the average ± SE of pooled data. The EPC population utilized in these sets of experiments belongs to the ECFC subtype.
Extracted from [93].

transformed cells [126]. Finally, reduced ER Ca^{2+} mobilization favors the nuclear translocation of the human telomerase reverse transcriptase (hTERT), which functions as catalytic component of telomerase, thereby

adversing telomeric shortening and cellular senescence in A549 human lung adenocarcinoma cells [127]. The interaction between endogenously liberated Ca^{2+} and hTERT is likely to be mediated by the

Ca^{2+}-binding protein S1000A8 [128]. Pioneering work conducted in Prof.Prevarskaya's laboratory has shown that the drop in the intracellular Ca^{2+} pool is caused by a reduction in both Sarco-Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA) 2B and calreticulin levels in neuroendocrine-differentiated prostate cancer cells [124]. While SERCA pumps are the only mechanism responsible for Ca^{2+} sequestering into ER lumen, calreticulin, as well as calnexin, glucose-related protein (GRP)-78, and GRP-94, are Ca^{2+} binding chaperones that maintain ER Ca^{2+} levels [11]. These findings have subsequently been confirmed in small and non-small cell lung cancer cell lines [129]. Our unpublished observations indicate that there is no difference in the expression of SERCA2B, calreticulin and calnexin between RCC-ECFCs and their healthy counterparts. However, the rate of [Ca^{2+}]$_i$ recovery to the baseline is significantly slower in RCC-ECFCs stimulated with ATP as compared to control cells [93]. Under these conditions, all Ca^{2+}-removing systems, i.e. mitochondria, SERCA, plasma membrane Na$^+$/Ca^{2+} exchanger (NCX) and plasma membrane Ca^{2+}-ATPase (PMCA), are active and may clear cytosolic Ca^{2+}. Conversely, the decay to resting levels is unaltered when the [Ca^{2+}]$_i$ is elevated by CPA, i.e. when SERCA is inhibited and Ca^{2+} is mainly extruded across the plasma membrane by both PMCA and NCX. These data strongly suggests that the delayed removal of cytosolic Ca^{2+} observed in the presence of ATP, but not CPA, is due to the impairment of SERCA activity [130]. This feature might nicely explain the reduced intracellular Ca^{2+} reservoir in RCC-ECFCs, albeit it remains to be experimentally probed. Moreover, future studies will have to ascertain whether Bax Inhibitor-1 (BX-1), a proto-oncogene that controls ER Ca^{2+} permeability, is up-regulated in RCC-ECFCs, thus lowering steady-state intraluminal Ca^{2+} levels [131]. Supporting the evidence that these cells are less prone to release endogenous Ca^{2+} is the down-regulation of InsP$_3$R2 and InsP$_3$R3 transcripts, while InsP$_3$R1 is not even present [93]. This finding is somehow in contrast with the notion that InsP$_3$Rs are generally more abundant in malignant cells [8,131], but we should recall that ECFCs do not derive from neoplastic clones and that InsP$_3$R1 expression is repressed in a colorectal cancer cell line [132]. The reduction in ER Ca^{2+} content associated to the loss of all InsP$_3$R isoforms might be an adaptive mechanism devised by tumor ECFCs to dismantle pro-death and stimulate pro-survival decisions in tumor stroma [133]. This might dramatically increase the probability that RCC-ECFCs successfully home to and are retained within the hostile microenvironment of the growing neoplasm, thereby favoring the angiogenic switch. Consistently, our preliminary data revealed that RCC-ECFCs are more resistant to serum starvation- and rapamycin-induced apoptosis as compared to normal cells (Poletto, Rosti and Moccia, unpublished observations).

While RCC-ECFCs liberates less Ca^{2+} in response to InsP$_3$-dependent store depletion, they undergo a dramatic up-regulation of SOCE (Fig. 3A–H) due to the higher expression of Stim1, Orai1, and TRPC1 [93]. Both the pharmacological profile and the molecular make-up of SOCs are not different as compared to control cells, and this feature enabled us to assess whether and how they impact on the angiogenic activity of RCC-ECFCs. The inhibition of SOCE with BTP2 and low micromolar doses of lanthanides suppressed both cell proliferation and tube formation when these cells were seeded in the presence of EGM-2, an endothelial growth medium enriched with VEGF, bFGF, insulin-like growth factor (IGF) and EGF [93]. Thus, RCC-ECFCs differ from PMF-ECFCs, which replicate quite well despite for the blockade of InsP$_3$-dependent SOCs [108]. No study has hitherto analyzed the expression and the role, if any, of Stim1, Orai1 and/or TRPC1 in TECs, while it is now established that these proteins are grossly altered in neoplastic cells [134–136]. In this perspective, it is intriguing that Stim2 and Orai3 levels are not changed in RCC-ECFCs [93], while they are dysregulated in both PMF-ECFCs [108] and several cancer types [137,138]. The larger magnitude of SOCE in RCC-ECFCs correlates with the higher abundance of circulating EPCs in RCC patients. There is no published information yet about RCC-ECFC frequency in peripheral blood of these individuals, but it is conceivable that they are significantly more numerous than in healthy individuals. One explanation is likely to be provided

by their higher resistance to apoptosis, an intrinsic property that may enhance their levels in circulation as well as within the tumor. But does the up-regulation of Stim1, Orai1 and TRPC1 has the potential to favor RCC-ECFC proliferation within the bone marrow, egression in systemic vasculature and engraftment within the tumor? As anticipated in a previous paragraph, it has long been thought that latent micrometastases stimulate and recruit circulating EPCs through the over-production of growth factors and cytokines, such as VEGF and SDF-1α. VEGF is a major component of the commercial EGM-2 medium routinely employed to elicit ECFC expansion and tubulogenesis. Consequently, we turned to monitor VEGF-induced Ca^{2+} oscillations in RCC-ECFCs by expecting an aberrant and spectacular increase in their amplitude, frequency and/or duration. Surprisingly, we found that VEGF does not elicit any detectable increase in [Ca^{2+}]$_i$ in these cells, despite the fact that the same batch and dose of agonist trigger beautiful Ca^{2+} spikes in control ECFCs (Fig. 4) [93]. Enhancing VEGF concentration up to 100 ng/ml did not render RCC-ECFCs more sensitive and not even a single transient arose (unpublished observations from our lab). Thus, VEGF does not elicit pro-angiogenic Ca^{2+} oscillations in RCC-ECFCs, albeit they express normal levels of VEGFR-2 [93]. NF-κB transcriptional activity may, however, be recruited by signaling pathways other than Ca^{2+}, such as protein kinase C (PKC), mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinases (ERK), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), glycogen synthase kinase-3β (GSK3β) and casein kinase II (CKII) [139,140]. Nevertheless, our preliminary data disclosed that VEGF-induced, NF-κB-mediated gene expression (VCAM-1, ICAM-1, E-selectin) is robust in healthy endothelial precursors, but is absent in RCC-ECFCs (Poletto, Rosti, and Moccia, unpublished data). These data strongly suggests that VEGFR-2 does not properly function in these cells: this feature might well be explained by the ER Ca^{2+} underfilling and/or the down-regulation of InsP$_3$Rs described above. The ER is not a homogenous organelle, but it exhibits tremendous plasticity and heterogeneity in the distribution of its Ca^{2+}-handling/transporting proteins [141]. The intricate tubular network of the ER is organized into modular Ca^{2+} signaling units, which operate independently or in synchrony, thus generating either local or global Ca^{2+} elevations [141]. The reduction in luminal Ca^{2+} levels might be stronger in the ER sub-compartment supporting the InsP$_3$-evoked Ca^{2+} response to VEGF that gives raise to the oscillations, thus preventing the onset of the pro-angiogenic signal. Similarly, InsP$_3$ synthesized upon VEGFR-2 activation might fail to bind to its target receptors, as they are deficient in RCC-ECFCs, and, consequently, to trigger the spiking activity. Future work is mandatory to confirm that VEGFR-2 does not stimulate RCC-ECFCs. Moreover, it will be necessary to
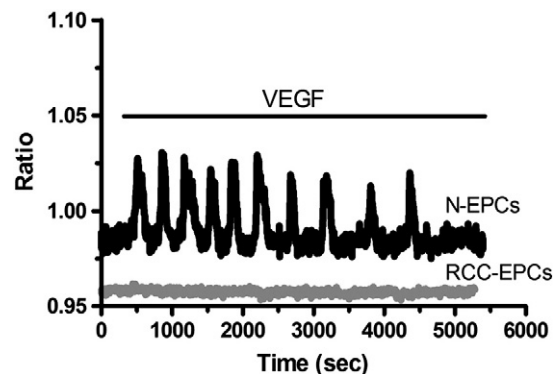

Fig. 4. VEGF does not elicit any detectable increase in [Ca^{2+}]$_i$ in endothelial progenitor cells isolated from patients suffering from renal cellular carcinoma (RCC-EPCs). VEGF (10 ng/ml) triggers intracellular Ca^{2+} oscillations in N- but not RCC-EPCs. The black tracing is representative of the Ca^{2+} spikes elicited by VEGF in N-EPCs, while the gray tracing refers to RCC-EPCs, which are insensitive VEGF and do not display any detectable increase in [Ca^{2+}]$_i$ upon VEGF stimulation. The EPC population utilized in these sets of experiments belongs to the ECFC subtype.
Extracted from [93].

examine whether this receptor is actually able to bind to VEGF and undergo auto-phosphorylation in these cells. Indeed, the blockade of VEGFR-2 signaling by the preferential expression of a modulatory/ancillary protein in either RCC-ECFCs or neighboring stromal cells should not be ruled out. For instance, VEGFR-1 has long been known to trap VEGF with higher affinity than VEGFR-2, thereby serving as negative regulator of angiogenesis [55]. Moreover, the expression of delta-like ligand 4 (DLL4) in human glioblastoma cells activates Notch signaling in adjacent TECs, thereby down-regulating VEGFR-2 expression and inducing tumor resistance to bevacizumab [142].

This finding might have remarkable pathological and therapeutic implications. According to the data generated by RCC-ECFCs, targeting VEGF might not be sufficient to block EPC mobilization in neoplastic patients. These cells are indispensable for inducing the angiogenic switch in solid malignancies. However, if they display a silent VEGFR-2, either humanized anti-VEGF monoclonal antibodies (bevacizumab) or tyrosine kinase inhibitors (sunitinib and sorafenib) will not prevent them from homing to and integrating within tumor vessels. This is why tumor ECFCs are not able to sense VEGF. This feature could shed light on the refractoriness of human kidney cancer to current therapies. Indeed, TECs are certainly sensitive to anti-VEGF drugs, as shown by their sensitivity to bevacizumab, sunitinib and sorafenib [117]. We speculate that, in real patients and not in xenografted murine models, tumor endothelium might initially stop proliferating and undergo apoptosis in response to the anti-angiogenic treatment. However, subsequent tumor hypoxia will stimulate the bone marrow to release EPCs, which home to the shrinked vasculature, and proliferate and restore blood supply in spite of anti-VEGF inhibitors. Several issues remain to be addressed. First, are the other EPC subsets, i.e. CFU-ECs and CACs, equally insensitive to VEGF in subjects affected by RCC? Second, does this paradigm apply to other cancer types? It will be important to assess whether the Ca^{2+} toolkit and VEGFR-2 signaling are dys-regulated in other malignancies. Third, which is the physiological inducer of SOCE in RCC-ECFCs? The answer to this question is likely to unveil the ideal target for anti-angiogenesis therapy in RCC patients. Accordingly, SOCE is operative and sustains RCC-ECFC proliferation and tubulogenesis in the presence of EGM-2; this medium is enriched with growth factors other than VEGF, such as bFGF and EGF, which all trigger pro-angiogenic Ca^{2+} signals in mature endothelial cells. Thus, another ligand is responsible for SOCE activation in RCC-ECFCs: uncovering its (their) identity(ies) is predicted to reveal the mitogen(s) responsible for their mobilization in vivo and to suggest alternative therapeutic treatments. In this view, growing attention has been paid to bFGF, whose plasma levels exhibits a dramatic increase before the metastatic progression in RCC patients [20]. Consistently, several pharmacological companies are developing an original approach to target bFGF signaling by exploiting either anti-bFGF or anti-bFGF receptor agents, albeit they are still far from entering the clinical practice [49]. The combination between VEGF and bFGF inhibition might, therefore, overcome patient refractoriness to anti-angiogenic treatment. However, the finding that Orai1- and TRPC1-mediated Ca^{2+} inflow is central to RCC-ECFC activation sheds light on an additional target for human kidney cancer treatment that has the advantage to circumvent the limitation imposed by drug resistance.

3.4. Orai1 and TRPC1 as alternative targets to prevent EPC activation and tumor growth

A growing body of evidence has been accumulated to substantiate the proposal that SOCE may be successfully inhibited to adverse tumor growth and metastatization [9,20,25,134]. For instance, Stim1- and Orai1-mediated Ca^{2+} inflow drives cell cycle progression and metastatic behavior in many human cancers, including those of breast and uterine cervix [59], glia [136], lungs [143], colon [144], nasopharyngeal and esophageal tracts [145,146], and epidermis [138]; likewise, TRPC1 is implicated in proliferation and motility of a widespread number of

neoplastic cells, such as those of human breast carcinoma [147,148], glioblastoma multiforme [149], non-small lung cell carcinoma [150, 151], nasopharyngeal carcinoma [152], and ovarian carcinoma [153]. The observation that these proteins are frequently over-expressed in cancer cells is a strong indicator of the central role they serve during neoplastic transformation [8]. Therefore, targeting store-dependent Ca^{2+} influx might reliably induce tumor regression and eradicate disseminated metastases in human patients [8,9,20,25,134], as suggested by pre-clinical studies on xenografted animals [59,146,154,155]. We speculate that SOCE constitutes an intriguing target to develop alternative anti-angiogenic treatments of RCC as well as of other highly vascularized solid malignancies. Unlike VEGFR-2, Orai1 and TRPC1 do sustain human RCC-ECFC growth and tube formation in vitro and there is no reason to believe it should be different in real patients. Moreover, this route is gated following the $InsP_3$-evoked depletion of endogenous Ca^{2+} reserves, thus representing the putative endpoint of the myriad of pro-angiogenic pathways activated during RCC development [20]. As mentioned above, the metastatic transition of human kidney cancer is preceded by a cytokine storm released in peripheral circulation: serum levels of bFGF, HGF and interleukin-6 (IL-6) are significantly higher as compared to healthy individuals [156]. In particular, bFGF and HGF are virtually capable of activating PLCγ to catalyze $InsP_3$ synthesis, which might ultimately lead to SOCE opening. Consistent with this hypothesis, HGF-induced CFU-EC proliferation and migration are partly regulated by Stim1 and TRPC1, as discussed in [12] and [26]. Consequently, the pharmacological blockage of SOCE in mRCC patients provides one hitherto unmet therapeutic advantage: with one single shot (i.e. a specific inhibitor of Ca^{2+} entry), it affects the signaling mechanism onto which all the growth factors and cytokines responsible for tumor resistance and escape from the therapy converge [20]. In addition, SOCE underlies the activation of bone marrow-derived hematopoietic cells that support EPCs during tumor vascularization (see references in [12] and [20]), such as $CD45^+/CD11$ myeloid cells, dendritic cells, mast cells, and neutrophils. When considering the impact of SOCE on healthy endothelial cells [11,56,57], this pathway is likely to stimulate TECs as well. Conversely, TRPV4, which controls the early steps of TEC-dependent angiogenesis [25,70], does not activate ECFCs at least when isolated from healthy donors [157]. Finally, it has recently been shown that Stim1 and Orai1 constitute a native pathway that is critical for RCC cell proliferation and migration [119]. Therefore, targeting SOCE is envisaged to affect all the cellular components of tumor microenvironment even without any adjuvant (e.g. chemo-, immuno-, or radio-therapy) administration [20]. Once again, a number of cumbersome roadblocks separate this hypothesis from its pre-clinical testing. First, we have to examine the efficacy of SOCE inhibitors on hematopoietic progenitors isolated from RCC patients. As evident from the discrepancies between animal models and oncologic reality, cells derived from healthy subjects do not truly reflect the biology of their malignant counterparts. Second, selective non-small molecule blockers of Orai1 and TRPC1 are yet to be commercially available. The unspecific action exerted by 2-APB on membrane conductances other than Orai1 and TRPC1 has widely been illustrated [24,26]. BTP2, in turn, is a selective SOCE antagonist in ECFCs [26,93], but not in lymphocytes [158]. Synta Pharmaceuticals has recently synthesized Synta 66 (3-fluoropyridine-4-carboxylic acid (2′,5′-dimethoxybiphenyl-4-yl)amide) (WO 2005/009954), which hinders Orai1-mediated Ca^{2+} inflow with no reported action on TRPC or TRPV channels [57,159]. Similarly, the novel pyrazole derivative Pyr6 has been designed to discriminate between Orai1- and TRPC3-mediated Ca^{2+} inflow [160]. However, the characterization of these intriguing compounds is at an early stage and they have not been probed on xenograft models of human cancers yet. Moreover, a recent study showed that Stim1-dependent Ca^{2+} inflow controls antitumor immunity by $CD8^+$ T cells and is essential to adverse the engraftment of melanoma and colon carcinoma cells [161]. It turns out that targeting SOCE might favor, rather than contrast, the infiltration and spreading of tumor cells. Nevertheless, blocking Orai1 repressed

forkhead box protein 3 (FoxP3) expression in the highly suppressive, therapy-resistant inducible T regulatory cells (iTreg) [162], which promote tumor progression by down-regulating effector T cells [163]. Evaluating the consequences of Orai1 and TRPC1 inhibition on the immune response against cancer development is, therefore, mandatory before introducing this approach into a pre-clinical setting. It is, however, suggestive that blocking SOCE did not cause any evident untoward effects in two different models of human cancer xenografts [146,154]. Chen and coworkers first investigated the impact of SK&F96365, a non selective SOCE inhibitor, on 4T1 mouse mammary tumor cells metastatization in BALB/c mice. Twenty days after their implantation in the mammary gland, SK&F96365 repressed the dissemination of metastatic lesions to the lungs "with no signs of overt toxicity" on the animals, which well tolerated the treatment [154]. More recently, Zhu et al. assessed the effect of SK&F96365 on NCr nu/nu nude mice subcutaneously inoculated with KYSE-150 cells, a malignant esophageal squamous cell carcinoma (ESCC) cell line. The application of this drug for up to two weeks significantly retarded tumor growth and "appeared to be safe" for the animals "since no obvious abnormality … was observed" [146]. Finally, we have recently demonstrated that the pharmacological inhibition of SOCE in metastatic RCC cells isolated from patients resistant to anti-angiogenic treatments does not dampen cell proliferation [164]. This feature, which could be ascribed to patient's reaction to the therapy, suggests that future studies should be conducted in vivo to assess whether anti-VEGF drugs impact on subject sensitivity to SOC blockers.

3.5. A few hypotheses to comprehend the derangement of the Ca^{2+} machinery in tumor ECFCs

The derangement of the Ca^{2+} toolkit in RCC-ECFCs does not only provide one of the first mechanistic explanations of the failure of anti-VEGF therapies. It sheds new light on the molecular mechanisms underpinning neoplastic transformation. Somatic and germline variants in lung cancer and germline mutations in colon cancer of ATP2A2 gene (encoding for SERCA2) have been reported [165], while germline mutations of the ATP2A2 and ATP2A3 (encoding for SERCA3) genes might serve as susceptibility alleles in head and neck squamous cell carcinoma [166,167]. However, it is generally accepted that the remodeling of Ca^{2+} transport proteins in cancer cells is associated to either epigenetic changes or post-translational modifications in the properties of pre-existing signaling components [8,104]. As aforementioned, ECFCs undergo distinct alterations of the Ca^{2+} toolkit depending on the pathophysiological background of the donor [14,93,108]; this feature hints at tumor microenvironment as a key instigator of the changes in intracellular Ca^{2+} homeostasis. Cancer is an ecosystem operating inside living organisms and comprising not only transformed cells, but also their neighboring partners, including fibroblasts, adipocytes, endothelial cells and bone marrow-derived hematopoietic and endothelial precursors [27,168]. Cancer cells utilize multiple autocrine and paracrine signaling pathways to coordinate a sophisticated network of interactions with stromal cells. This molecular cross-talk reprograms tumor associated-host cells to facilitate and sustain disease progression and metastases, at the same time protecting the malignant milieu against aggressive pharmacologic and radiotherapeutic interventions [27,168]. Among the mechanisms of phenotypic heterogeneity mentioned above, endothelial cells might undergo epigenetic modifications induced by the transfer of both bioactive factors (membrane receptors and channels, protein and lipids) and genetic information (oncogenes, mRNAs, and miRNAs) or through apoptotic bodies and/or exosomes detached from cancer cells [106,169–175]. The consequent reprogramming of the transcriptional activity hijack normal endothelial functions to facilitate the angiogenic switch, thereby favoring tumor growth and metastatization. For instance, renal cancer stem cells liberate microvesicles (MVs) containing mRNAs and miRNAs that confer an activated phenotype to normal endothelial cells, stimulating their proliferation and vessel

formation after in vivo inoculation in non-obese diabetic severe combined immunodeficiency (NOD/SCID) mice [170]. Moreover, tumor-conditioned endothelial cells silence angiogenesis-suppressing genes in association with promoter histone modifications, but not DNA methylation [173], which invokes the key role of histone deacetylase (HDAC) in the epigenetic regulation of tumor angiogenesis. From the point of view of Ca^{2+} signaling, the co-culture of bone marrow-derived human mesenchymal stem cells (MSCs) with several epithelial cancer lines caused a reduction in basal Ca^{2+} influx in MSCs that was coupled with an increased expression of hematopoietic makers [176]. Furthermore, MCF-7/ADM cells are capable of transferring TRPC5 to HMECs, thereby providing them with the source of Ca^{2+} necessary to activate NFAT and induce P-gp expression [106]. This chain of events underlies the acquired resistance of HMECs to the chemotherapeutic agent adriamycin both in vitro [106] and in vivo [177]. Consistently, TRPC5-containing extracellular vesicles are far more abundant in peripheral blood from breast cancer patients who underwent chemotherapy, but did not show complete regression, as compared to naïve ones [178]. This novel mode of communication between tumor and resident endothelial cells might lead to TRPV4 up-regulation in B-TECs as well [70]. It is now evident that a malignancy does not only influence the immediate surroundings of the primary tumor compartment: it exerts long-range systemic effects by releasing soluble factors and exosomes in circulation that reach the bone marrow and, herein, educate hematopoietic progenitor cells to support tumor growth and dissemination [179,180]. This paradigm also applies to EPCs that may be reprogrammed by neoplastic cells to over-express miR-10b and miR-196b, which sustain angiogenesis-mediated LLC growth in xenografted animals [34]. Therefore, we speculate that the components of the Ca^{2+} toolkit are plastically selected and shuffled by ECFCs depending on the instructive signals emanating from cancer cells. This hypothesis is supported by the observation that ECFCs isolated from PMF and RCC patients exhibit clearly distinct Ca^{2+} signatures in terms of InsP$_3$R, Stim, Orai and TRPC expression [93,108]. Likewise, our preliminary data reveal that ECFCs harvested from naïve mammary carcinoma patients further differ from normal, PMF- and RCC-ECFCs (manuscript in preparation from our group). Just like TECs retain their original site-specific epigenetic fingerprint, endothelial precursors might differentially be conditioned by tumor-derived moieties that are peculiar for each malignancy [179,180]. The elucidation of the signals by which growing tumors reprogram the Ca^{2+} toolkit of bone marrow-derived ECFCs will not only shed more light on the complex biology of neoplastic transformation: it will likely emerge as a new avenue of research in the endless quest for novel anti-angiogenic strategies.

4. Conclusions

The investigation of the intracellular Ca^{2+} dynamics in tumor ECFCs has been instigated by the curiosity to challenge the hypothesis that tumor microenvironment may condition the host organism event at remote distances from the primary lesion site. Our initial results that are corroborated by the subsequent findings obtained on different models of proliferative diseases, support the notion that ECFCs, which are part of the non-hematopoietic EPCs, undergo a dramatic remodeling of their Ca^{2+} machinery. Thus, the dys-regulation of Ca^{2+} handling/transporting proteins that has long been reported in neoplastic cells has now to be extended to tumor microenvironment, which is consistent with the data provided by tumor and tumor-conditioned endothelial cells [70,106]. It will be mandatory to confirm whether the Ca^{2+} signature of circulating ECFCs is typical of a given malignancy or not. Novel flow cytometry-based assays are currently under development to accelerate the expansion of ECFC clones from peripheral blood; when these assays will be available, it might be possible to utilize the pattern of InsP$_3$Rs, Stim, Orai or TRPC protein expression as a prognostic marker of a specific cancer type. The road ahead is still long to walk: additionally, it will be necessary to find the correlations, if any, between

tumor grade and stage and channel expression by circulating ECFCs. Needless to say, we will have to evaluate also whether and how all the other components of the Ca^{2+} machinery (e.g. SERCA, PMCA, NCX, TRPV and TRPM) vary in tumor ECFCs. The therapeutic implications of the alterations in intracellular Ca^{2+} homeostasis were rather unexpected. The failure of VEGF to trigger the oscillatory signal and its downstream transcriptional response in RCC-ECFCs might aid to understanding the well known inefficacy of anti-angiogenic drugs in kidney cancer patients. This treatment does not prove to be as efficient as in preclinical models also in other life-threatening cancer types, such as mammary carcinoma. As recognized by several authors, anti-VEGF therapy has been devised by utilizing normal endothelial cell lines, such as HUVECs and HMECs, rather than tumor-conditioned or patients-derived cells. The availability of modern technologies to harvest endothelial cells from human tumor vasculature and truly endothelial progenitors from peripheral circulation of neoplastic subjects is predicted to amend this therapeutically relevant mistake. Albeit the drop in ER Ca^{2+} levels might be responsible for VEGF failure, and therefore for patient resistance to anti-VEGF inhibitors, Ca^{2+} signaling offers a novel battery of promising, and easily accessible from the blood stream, targets. Stim1, Orai1 and TRPC1 control cell proliferation not only in ECFCs but also in the other cellular components of tumor microenvironment, including resident endothelial cells, fibroblasts, bone marrow-derived hematopoietic progenitors and cancer cells. It will be indispensable to ascertain whether this ubiquitous mechanism of Ca^{2+} entry is also operative in stromal cells to enable one single bullet (i.e. a specific SOCE inhibitor) to target all the cellular actors of human malignancies and circumvent refractory mechanisms.

Acknowledgements

References

[1] M.J. Berridge, M.D. Bootman, H.L. Roderick, Calcium signalling: dynamics, homeostasis and remodelling, Nat. Rev. Mol. Cell Biol. 4 (2003) 517–529.
[2] D.E. Clapham, Calcium signaling, Cell 131 (2007) 1047–1058.
[3] L. Santella, D. Lim, F. Moccia, Calcium and fertilization: the beginning of life, Trends Biochem. Sci. 29 (2004) 400–408.
[4] Z. Altaany, F. Moccia, L. Munaron, D. Mancardi, R. Wang, Hydrogen sulfide and endothelial dysfunction: relationship with nitric oxide, Curr. Med. Chem. 21 (2014) 3646–3661.
[5] F. Moccia, F. Tanzi, L. Munaron, Endothelial remodelling and intracellular calcium machinery, Curr. Mol. Med. 14 (2014) 457–480.
[6] M.J. Berridge, Dysregulation of neural calcium signaling in Alzheimer disease, bipolar disorder and schizophrenia, Prion 7 (2013) 2–13.
[7] M.J. Berridge, Calcium signalling remodelling and disease, Biochem. Soc. Trans. 40 (2012) 297–309.
[8] G.R. Monteith, D. McAndrew, H.M. Faddy, S.J. Roberts-Thomson, Calcium and cancer: targeting Ca^{2+} transport, Nat. Rev. Cancer 7 (2007) 519–530.
[9] N. Prevarskaya, R. Skryma, Y. Shuba, Calcium in tumour metastasis: new roles for known actors, Nat. Rev. Cancer 11 (2011) 609–618.
[10] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell 144 (2011) 646–674.
[11] F. Moccia, R. Berra-Romani, F. Tanzi, Update on vascular endothelial Ca^{2+} signalling: a tale of ion channels, pumps and transporters, World J. Biol. Chem. 3 (2012) 127–158.
[12] F. Moccia, E. Bonetti, S. Dragoni, J. Fontana, F. Lodola, R.B. Romani, U. Laforenza, V. Rosti, F. Tanzi, Hematopoietic progenitor and stem cells circulate by surfing on intracellular Ca^{2+} waves: a novel target for cell-based therapy and anti-cancer treatment? Curr. Signal Transduct. Ther. 7 (2012) 161–176.
[13] S. Dragoni, U. Laforenza, E. Bonetti, F. Lodola, C. Bottino, R. Berra-Romani, G. Carlo Bongio, M.P. Cinelli, G. Guerra, P. Pedrazzoli, V. Rosti, F. Tanzi, F. Moccia, Vascular endothelial growth factor stimulates endothelial colony forming cells proliferation

[14] and tubulogenesis by inducing oscillations in intracellular Ca^{2+} concentration, Stem Cells 29 (2011) 1898–1907.
[15] S. Dragoni, U. Laforenza, E. Bonetti, F. Lodola, C. Bottino, G. Guerra, A. Borghesi, M. Stronati, V. Rosti, F. Tanzi, F. Moccia, Canonical transient receptor potential 3 channel triggers vascular endothelial growth factor-induced intracellular Ca^{2+} oscillations in endothelial progenitor cells isolated from umbilical cord blood, Stem Cells Dev. 22 (2013) 2561–2580.
[16] F. Moccia, F. Lodola, S. Dragoni, E. Bonetti, C. Bottino, G. Guerra, U. Laforenza, V. Rosti, F. Tanzi, Ca^{2+} signalling in endothelial progenitor cells: a novel means to improve cell-based therapy and impair tumour vascularisation, Curr. Vasc. Pharmacol. 12 (2014) 87–105.
[17] K. De Bock, M. Mazzone, P. Carmeliet, Antiangiogenic therapy, hypoxia, and metastasis: risky liaisons, or not? Nat. Rev. Clin. Oncol. 8 (2011) 393–404.
[18] P. Carmeliet, R.K. Jain, Molecular mechanisms and clinical applications of angiogenesis, Nature 473 (2011) 298–307.
[19] H. Peinado, S. Lavotshkin, D. Lyden, The secreted factors responsible for pre-metastatic niche formation: old sayings and new thoughts, Semin. Cancer Biol. 21 (2011) 139–146.
[20] A.C. Dudley, Tumor endothelial cells, Cold Spring Harb. Perspect. Med. 2 (2012) a006536.
[21] F. Moccia, S. Dragoni, V. Poletto, V. Rosti, F. Tanzi, C. Ganini, C. Porta, Orai1 and Transient Receptor Potential channels as novel molecular targets to impair tumor neovascularisation in renal cell carcinoma and other malignancies, Anti Cancer Agents Med. Chem. 14 (2014) 296–312.
[22] W.C. Aird, Endothelial cell heterogeneity, Cold Spring Harb. Perspect. Med. 2 (2012) a006429.
[23] D.C. Gao, D. Nolan, K. McDonnell, L. Vahdat, R. Benezra, N. Altorki, V. Mittal, Bone marrow-derived endothelial progenitor cells contribute to the angiogenic switch in tumor growth and metastatic progression, Biochim. Biophys. Acta 1796 (2009) 33–40.
[24] D.J. Nolan, A. Ciarrocchi, A.S. Mellick, J.S. Jaggi, K. Bambino, S. Gupta, E. Heikamp, M.R. McDevitt, D.A. Scheinberg, R. Benezra, V. Mittal, Bone marrow-derived endothelial progenitor cells are a major determinant of nascent tumor neovascularization, Genes Dev. 21 (2007) 1546–1558.
[25] L. Munaron, T. Genova, D. Avanzato, S. Antoniotti, A. Fiorio Pla, Targeting calcium channels to block tumor vascularization, Recent Pat. Anticancer Drug Discov. 8 (2013) 27–37.
[26] A. Fiorio Pla, D. Gkika, Emerging role of TRP channels in cell migration: from tumor vascularization to metastasis, Front. Physiol. 4 (2013) 311.
[27] F. Moccia, S. Dragoni, F. Lodola, E. Bonetti, C. Bottino, G. Guerra, U. Laforenza, V. Rosti, F. Tanzi, Store-dependent Ca^{2+} entry in endothelial progenitor cells as a perspective tool to enhance cell-based therapy and adverse tumour vascularization, Curr. Med. Chem. 19 (2012) 5802–5818.
[28] C. Coghlin, G.I. Murray, Current and emerging concepts in tumour metastasis, J. Pathol. 222 (2010) 1–15.
[29] A. Patenaude, J. Parker, A. Karsan, Involvement of endothelial progenitor cells in tumor vascularization, Microvasc. Res. 79 (2010) 217–223.
[30] P.A. Hill, Recipient origin of vasculature in renal cell carcinoma in a kidney allograft, Pathology 42 (2010) 479–480.
[31] B.A. Peters, L.A. Diaz, K. Polyak, L. Meszler, K. Romans, E.C. Guinan, J.H. Antin, D. Myerson, S.R. Hamilton, B. Vogelstein, K.W. Kinzler, C. Lengauer, Contribution of bone marrow-derived endothelial cells to human tumor vasculature, Nat. Med. 11 (2005) 261–262.
[32] D. Lyden, K. Hattori, S. Dias, C. Costa, P. Blaikie, L. Butros, A. Chadburn, B. Heissig, W. Marks, L. Witte, Y. Wu, D. Hicklin, Z. Zhu, N.R. Hackett, R.G. Crystal, M.A. Moore, K.A. Hajjar, K. Manova, R. Benezra, S. Rafii, Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth, Nat. Med. 7 (2001) 1194–1201.
[33] D.C. Gao, D.J. Nolan, A.S. Mellick, K. Bambino, K. McDonnell, V. Mittal, Endothelial progenitor cells control the angiogenic switch in mouse lung metastasis, Science 319 (2008) 195–198.
[34] A.S. Mellick, P.N. Plummer, D.J. Nolan, D. Gao, K. Bambino, M. Hahn, R. Catena, V. Turner, K. McDonnell, R. Benezra, R. Brink, A. Swarbrick, V. Mittal, Using the transcription factor inhibitor of DNA binding 1 to selectively target endothelial progenitor cells offers novel strategies to inhibit tumor angiogenesis and growth, Cancer Res. 70 (2010) 7273–7282.
[35] P.N. Plummer, R. Freeman, R.J. Taft, J. Vider, M. Sax, B.A. Umer, D. Gao, C. Johns, J.S. Mattick, S.D. Wilton, V. Ferro, N.A. McMillan, A. Swarbrick, V. Mittal, A.S. Mellick, MicroRNAs regulate tumor angiogenesis modulated by endothelial progenitor cells, Cancer Res. 73 (2013) 341–352.
[36] M.B. Ruzinova, R.A. Schoer, W. Gerald, J.E. Egan, P.P. Pandolfi, S. Rafii, K. Manova, V. Mittal, R. Benezra, Effect of angiogenesis inhibition by Id loss and the contribution of bone-marrow-derived endothelial cells in spontaneous murine tumors, Cancer Cell 4 (2003) 277–289.
[37] M.C. Yoder, Human endothelial progenitor cells, Cold Spring Harb. Perspect. Med. 2 (2012) a006692.
[38] D.P. Basile, M.C. Yoder, Circulating and tissue resident endothelial progenitor cells, J. Cell. Physiol. 229 (2014) 10–16.
[39] T. Asahara, A. Kawamoto, H. Masuda, Concise review: circulating endothelial progenitor cells for vascular medicine, Stem Cells 29 (2011) 1650–1655.
[40] R.J. Medina, C.L. O'Neill, M. Sweeney, J. Guduric-Fuchs, T.A. Gardiner, D.A. Simpson, A.W. Stitt, Molecular analysis of endothelial progenitor cell (EPC) subtypes reveals two distinct cell populations with different identities, BMC Med. Genomics 3 (2010) 18.
[41] K. Bieback, S. Vinci, S. Elvers-Hornung, A. Bartol, T. Gloe, M. Czabanka, H. Klüter, H. Augustin, P. Vajkoczy, Recruitment of human cord blood-derived endothelial

colony-forming cells to sites of tumor angiogenesis, Cytotherapy 15 (2013) 726–739.

[41] D.M. Smadja, C. d'Audigier, L.B. Weiswald, C. Badoual, V. Dangles-Marie, L. Mauge, S. Evrard, I. Laurendeau, F. Lallemand, S. Germain, F. Grelac, B. Dizier, M. Vidaud, I. Bièche, P. Gaussem, The Wnt antagonist Dickkopf-1 increases endothelial progenitor cell angiogenic potential, Arterioscler. Thromb. Vasc. Biol. 30 (2010) 2544–2552.

[42] J. Wei, G. Jarmy, J. Genuneit, K.M. Debatin, C. Beltinger, Human blood late out-growth endothelial cells for gene therapy of cancer: determinants of efficacy, Gene Ther. 14 (2007) 344–356.

[43] M. Potente, H. Gerhardt, P. Carmeliet, Basic and therapeutic aspects of angiogenesis, Cell 146 (2011) 873–887.

[44] Y. Shaked, E. Henke, J.M. Roodhart, P. Mancuso, M.H. Langenberg, M. Colleoni, L.G. Daenen, S. Man, P. Xu, U. Emmenegger, T. Tang, Z. Zhu, L. Witte, R.M. Strieter, F. Bertolini, E.E. Voest, R. Benezra, R.S. Kerbel, Rapid chemotherapy-induced acute en-dothelial progenitor cell mobilization: implications for antiangiogenic drugs as chemosensitizing agents, Cancer Cell 14 (2008) 263–273.

[45] Y. Shaked, A. Ciarrocchi, M. Franco, C.R. Lee, S. Man, A.M. Cheung, D.J. Hicklin, D. Chaplin, F.S. Foster, R. Benezra, R.S. Kerbel, Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors, Science 313 (2006) 1785–1787.

[46] M. Taylor, F. Billiot, V. Marty, V. Rouffiac, P. Cohen, E. Tournay, P. Opolon, F. Louache, G. Vassal, C. Laplace-Builhé, P. Vielh, J.C. Soria, F. Farace, Reversing resis-tance to vascular-disrupting agents by blocking late mobilization of circulating en-dothelial progenitor cells, Cancer Discov. 2 (2012) 434–449.

[47] G. Bergers, D. Hanahan, Modes of resistance to anti-angiogenic therapy, Nat. Rev. Cancer 8 (2008) 592–603.

[48] N.S. Vasudev, A.R. Reynolds, Anti-angiogenic therapy for cancer: current progress, unresolved questions and future directions, Angiogenesis 17 (2014) 471–494.

[49] F. Bertolini, P. Marighetti, I. Martin-Padura, P. Mancuso, D.D. Hu-Lowe, Y. Shaked, A. D'Onofrio, Anti-VEGF and beyond: shaping a new generation of anti-angiogenic therapies for cancer, Drug Discov. Today 16 (2011) 1052–1060.

[50] L.M. Ellis, D.J. Hicklin, Pathways mediating resistance to vascular endothelial growth factor-targeted therapy, Clin. Cancer Res. 14 (2008) 6371–6375.

[51] A. Turtoi, D. Mottet, N. Matheus, B. Dumont, P. Peixoto, V. Hennequière, C. Deroanne, A. Colige, E. De Pauw, A. Bellahcène, V. Castronovo, The angiogenesis suppressor gene AKAP12 is under the epigenetic control of HDAC7 in endothelial cells, Angiogenesis 15 (2012) 543–554.

[52] Y. Song, X. Li, D. Wang, C. Fu, Z. Zhu, M.H. Zou, Y. Zhu, Transcription factor Krüppel-like factor 2 plays a vital role in endothelial colony forming cells differentiation, Cardiovasc. Res. 99 (2013) 514–524.

[53] D. Yu, W. Chen, J. Ren, T. Zhang, K. Yang, G. Wu, H. Liu, VEGF-PKD1-HDAC7 signal-ing promotes endothelial progenitor cell migration and tube formation, Microvasc. Res. 91 (2014) 66–72.

[54] N. Ferrara, H.P. Gerber, J. LeCouter, The biology of VEGF and its receptors, Nat. Med. 9 (2003) 669–676.

[55] S. Koch, L. Claesson-Welsh, Signal transduction by vascular endothelial growth fac-tor receptors, Cold Spring Harb. Perspect. Med. 2 (2012) a006502.

[56] I.F. Abdullaev, J.M. Bisaillon, M. Potier, J.C. Gonzalez, R.K. Motiani, M. Trebak, Stim1 and Orai1 mediate CRAC currents and store-operated calcium entry important for endothelial cell proliferation, Circ. Res. 103 (2008) 1289–1299.

[57] J. Li, R.M. Cubbon, L.A. Wilson, M.S. Amer, L. McKeown, B. Hou, Y. Majeed, S. Tumova, V.A.L. Seymour, H. Taylor, M. Stacey, D. O'Regan, R. Foster, K.E. Porter, M.T. Kearney, D.J. Beech, Orai1 and CRAC channel dependence of VEGF-activated Ca^{2+} entry and endothelial tube formation, Circ. Res. 108 (2011) 1190–1198.

[58] P.C. Yu, S.Y. Gu, J.W. Bu, J.L. Du, TRPC1 is essential for in vivo angiogenesis in zebrafish, Circ. Res. 106 (2010) 1221–1232.

[59] Y.-F. Chen, W.-T. Chiu, Y.-T. Chen, P.-Y. Lin, H.-J. Huang, C.-Y. Chou, H.-C. Chang, M.-J. Tang, M.-R. Shen, Calcium store sensor stromal-interaction molecule 1-dependent signaling plays an important role in cervical cancer growth, migration, and angiogenesis, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 15225–15230.

[60] E. Pupo, A.F. Pla, D. Avanzato, F. Moccia, J.-E.A. Cruz, F. Tanzi, A. Merlino, D. Mancardi, L. Munaron, Hydrogen sulfide promotes calcium signals and migration in tumor-derived endothelial cells, Free Radic. Biol. Med. 51 (2011) 1765–1773.

[61] K. Holmes, E. Chapman, V. See, M.J. Cross, VEGF stimulates RCAN1.4 expression in endothelial cells via a pathway requiring Ca^{2+}/calcineurin and protein kinase C-delta, PLoS One 5 (2010) e11435.

[62] A. Rinne, K. Banach, L.A. Blatter, Regulation of nuclear factor of activated T cells (NFAT) in vascular endothelial cells, J. Mol. Cell. Cardiol. 47 (2009) 400–410.

[63] R. Berra-Romani, J.E. Avelino-Cruz, A. Raqeeb, A. Della Corte, M. Cinelli, S. Montagnani, G. Guerra, F. Moccia, F. Tanzi, Ca^{2+}-dependent nitric oxide release in the injured endothelium of excised rat aorta: a promising mechanism applying in vascular prosthetic devices in aging patients, BMC Surg. 13 (Suppl. 2) (2013) S40.

[64] C. Tiruppathi, G.U. Ahmmed, S.M. Vogel, A.B. Malik, Ca^{2+} signaling, TRP channels, and endothelial permeability, Microcirculation 13 (2006) 693–708.

[65] M. Freichel, S.H. Suh, A. Pfeifer, U. Schweig, C. Trost, P. Weissgerber, M. Biel, S. Philipp, D. Freise, G. Droogmans, F. Hofmann, V. Flockerzi, B. Nilius, Lack of an en-dothelial store-operated Ca^{2+} current impairs agonist-dependent vasorelaxation in TRP4$^{-/-}$ mice, Nat. Cell Biol. 3 (2001) 121–127.

[66] D.L. Cioffi, S. Wu, H. Chen, M. Alexeyev, C.M. St Croix, B.R. Pitt, S. Uhlig, T. Stevens, Orai1 determines calcium selectivity of an endogenous TRPC heterotetramer chan-nel, Circ. Res. 110 (2012) 1435–1444.

[67] P.C. Sundivakkam, M. Freichel, V. Singh, J.P. Yuan, S.M. Vogel, V. Flockerzi, A.B. Malik, C. Tiruppathi, The Ca^{2+} sensor stromal interaction molecule 1 (STIM1) is necessary and sufficient for the store-operated Ca^{2+} entry function of transient re-ceptor potential canonical (TRPC) 1 and 4 channels in endothelial cells, Mol. Pharmacol. 81 (2012) 510–526.

[68] L. Munaron, A. Fiorio Pla, Calcium influx induced by activation of tyrosine kinase receptors in cultured bovine aortic endothelial cells, J. Cell. Physiol. 185 (2000) 454–463.

[69] S. Antoniotti, A.F. Pla, S. Pregnolato, A. Mottola, D. Lovisolo, L. Munaron, Control of endothelial cell proliferation by calcium influx and arachidonic acid metabolism: a pharmacological approach, J. Cell. Physiol. 197 (2003) 370–378.

[70] A.F. Pla, H.L. Ong, K.T. Cheng, A. Brossa, B. Bussolati, T. Lockwich, B. Paria, L. Munaron, I.S. Ambudkar, TRPV4 mediates tumor-derived endothelial cell migration via arachi-donic acid-activated actin remodeling, Oncogene 31 (2012) 200–212.

[71] C. Troidl, K. Troidl, W. Schierling, W.-J. Cai, H. Nef, H. Moellmann, S. Kostin, S. Schimanski, L. Hammer, A. Elsaesser, T. Schmitz-Rixen, W. Schaper, Trpv4 induces collateral vessel growth during regeneration of the arterial circulation, J. Cell. Mol. Med. 13 (2009) 2613–2621.

[72] C. Troidl, H. Nef, S. Voss, A. Schilp, S. Kostin, K. Troidl, S. Szardien, A. Rolf, T. Schmitz-Rixen, W. Schaper, C.W. Hamm, A. Elsaesser, H. Moellmann, Calcium-dependent signalling is essential during collateral growth in the pig hind limb–is-chemia model, J. Mol. Cell. Cardiol. 49 (2010) 142–151.

[73] F. Moccia, R. Berra-Romani, S. Tritto, S. Signorelli, V. Taglietti, F. Tanzi, Epidermal growth factor induces intracellular Ca^{2+} oscillations in microvascular endothelial cells, J. Cell. Physiol. 194 (2003) 139–150.

[74] P. Ridefelt, K. Yokote, L. Claesson-Welsh, A. Siegbahn, PDGF-BB triggered cytoplas-mic calcium responses in cells with endogenous or stably transfected PDGF beta-receptors, Growth Factors 12 (1995) 191–201.

[75] J.M. Bisaillon, R.K. Motiani, J.C. Gonzalez-Cobos, M. Potier, K.E. Halligan, W.F. Alzawahra, M. Barroso, H.A. Singer, D. Jourd'heuil, M. Trebak, Essential role for STIM1/Orai1-mediated calcium influx in PDGF-induced smooth muscle migration, Am. J. Physiol. Cell Physiol. 298 (2010) C993–C1005.

[76] S. Pal, J. Wu, J.K. Murray, S.H. Gellman, M.A. Wozniak, P.J. Keely, M.E. Boyer, T.M. Gomez, S.M. Hasso, J.F. Fallon, E.H. Bresnick, An antiangiogenic neurokinin-B/ thromboxane A2 regulatory axis, J. Cell Biol. 174 (2006) 1047–1058.

[77] W. Scharbrodt, Y. Abdallah, S.A. Kasseckert, D. Gligorievski, H.M. Piper, D.K. Boker, W. Deinsberger, M.F. Oertel, Cytosolic Ca^{2+} oscillations in human cerebrovascular endothelial cells after subarachnoid hemorrhage, J. Cereb. Blood Flow Metab. 29 (2009) 57–65.

[78] R. Berra-Romani, A. Raqeeb, J. Torres-Jácome, A. Guzman-Silva, G. Guerra, F. Tanzi, F. Moccia, The mechanism of injury-induced intracellular calcium concentration oscillations in the endothelium of excised rat aorta, J. Vasc. Res. 49 (2012) 65–76.

[79] M. De Bock, N. Culot, N. Wang, M. Bol, E. Decrock, E. De Vuyst, A. da Costa, I. Dauwe, M. Vinken, A.M. Simon, V. Rogiers, G. De Ley, W.H. Evans, G. Bultynck, G. Dupont, R. Cecchelli, L. Leybaert, Connexin channels provide a target to manipulate brain endothelial calcium dynamics and blood–brain barrier permeability, J. Cereb. Blood Flow Metab. 31 (2011) 1942–1957.

[80] L. Zhu, S. Song, Y. Pi, Y. Yu, W. She, H. Ye, Y. Su, Q. Hu, Cumulated Ca^{2+} spike dura-tion underlies Ca^{2+} oscillation frequency-regulated NF kappa B transcriptional ac-tivity, J. Cell Sci. 124 (2011) 2591–2601.

[81] L.P. Zhu, Y.G. Luo, T.X. Chen, F.R. Chen, T. Wang, Q. Hu, Ca^{2+} oscillation frequency regulates agonist-stimulated gene expression in vascular endothelial cells, J. Cell Sci. 121 (2008) 2511–2518.

[82] J. Seidel, B. Niggemann, M. Punzel, J. Fischer, K.S. Zänker, T. Dittmar, The neuro-transmitter GABA is a potent inhibitor of the stromal cell-derived factor-1alpha in-duced migration of adult CD133+ hematopoietic stem and progenitor cells, Stem Cells Dev. 16 (2007) 827–836.

[83] D.A. Ingram, L.E. Mead, H. Tanaka, V. Meade, A. Fenoglio, K. Mortell, K. Pollok, M.J. Ferkowicz, D. Gilley, M.C. Yoder, Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood, Blood 104 (2004) 2752–2760.

[84] D.M. Potenza, G. Guerra, D. Avanzato, V. Poletto, S. Pareek, D. Guido, A. Gallanti, V. Rosti, L. Munaron, F. Tanzi, F. Moccia, Hydrogen sulphide triggers VEGF-induced in-tracellular Ca^{2+} signals in human endothelial cells but not in their immature pro-genitors, Cell Calcium 56 (2014) 225–234.

[85] A. Skupin, M. Falcke, Statistical properties and information content of calcium os-cillations, Genome Inform. 18 (2007) 44–53.

[86] A. Skupin, H. Kettenmann, U. Winkler, M. Wartenberg, H. Sauer, S.C. Tovey, C.W. Taylor, M. Falcke, How does intracellular Ca^{2+} oscillate: by chance or by the clock? Biophys. J. 94 (2008) 2404–2411.

[87] F. Mac Gabhann, M.T. Yang, A.S. Popel, Monte Carlo simulations of VEGF binding to cell surface receptors in vitro, Biochim. Biophys. Acta 1746 (2005) 95–107.

[88] K. Thurley, A. Skupin, R. Thul, M. Falcke, Fundamental properties of Ca^{2+} signals, Biochim. Biophys. Acta 1820 (2012) 1185–1194.

[89] Y. Sánchez-Hernández, U. Laforenza, E. Bonetti, J. Fontana, S. Dragoni, M. Russo, J.E. Avelino-Cruz, S. Schinelli, D. Testa, G. Guerra, V. Rosti, F. Tanzi, F. Moccia, Store-operated Ca^{2+} entry is expressed in human endothelial progenitor cells, Stem Cells Dev. 19 (2010) 1967–1981.

[90] F. Moccia, G. Bertoni, A.F. Pla, S. Dragoni, E. Pupo, A. Merlino, D. Mancardi, L. Munaron, F. Tanzi, Hydrogen sulfide regulates intracellular Ca^{2+} concentration in endothelial cells from excised rat aorta, Curr. Pharm. Biotechnol. 12 (2011) 1416–1426.

[91] L. Munaron, D. Avanzato, F. Moccia, D. Mancardi, Hydrogen sulfide as a regulator of calcium channels, Cell Calcium 53 (2013) 77–84.

[92] V. Sydorenko, Y. Shuba, S. Thebault, M. Roudbaraki, G. Lepage, N. Prevarskaya, R. Skryma, Receptor-coupled, DAG-gated Ca^{2+}-permeable cationic channels in LNCaP human prostate cancer epithelial cells, J. Physiol. 548 (2003) 823–836.

[93] F. Lodola, U. Laforenza, E. Bonetti, D. Lim, S. Dragoni, C. Bottino, H.L. Ong, G. Guerra, C. Ganini, M. Massa, M. Manzoni, I.S. Ambudkar, A.A. Genazzani, V. Rosti, P. Pedrazzoli, F. Tanzi, F. Moccia, C. Porta, Store-operated Ca^{2+} entry is remodelled and controls in vitro angiogenesis in endothelial progenitor cells isolated from tu-moral patients, PLoS One 7 (2012) e42541.

[94] C.A. Di Buduo, F. Moccia, M. Battiston, L. De Marco, M. Mazzucato, R. Moratti, F. Tanzi, A. Balduini, The importance of calcium in the regulation of megakaryocyte function, Haematologica 99 (2014) 769–778.

[95] H.L. Ong, K.T. Cheng, X. Liu, B.C. Bandyopadhyay, B.C. Paria, J. Soboloff, B. Pani, Y. Gwack, S. Srikanth, B.B. Singh, D.L. Gill, I.S. Ambudkar, Dynamic assembly of TRPC1–STIM1–Orai1 ternary complex is involved in store-operated calcium influx. Evidence for similarities in store-operated and calcium release-activated calcium channel components, J. Biol. Chem. 282 (2007) 9105–9116.

[96] A. Hoffmann, G. Natoli, G. Ghosh, Transcriptional regulation via the NF-kappaB signaling module, Oncogene 25 (2006) 6706–6716.

[97] T. Minami, W.C. Aird, Endothelial cell gene regulation, Trends Cardiovasc. Med. 15 (2005) 174–184.

[98] B. Mellstrom, M. Savignac, R. Gomez-Villafuertes, J.R. Naranjo, Ca^{2+}-operated transcriptional networks: molecular mechanisms and in vivo models, Physiol. Rev. 88 (2008) 421–449.

[99] Y. Su, L. Gao, L. Teng, Y. Wang, J. Cui, S. Peng, S. Fu, Id1 enhances human ovarian cancer endothelial progenitor cell angiogenesis via PI3K/Akt and NF-κB/MMP-2 signaling pathways, J. Transl. Med. 11 (2013) 132.

[100] W. Li, H. Wang, C.Y. Kuang, J.K. Zhu, Y. Yu, Z.X. Qin, J. Liu, L. Huang, An essential role for the Id1/PI3K/Akt/NFkB/survivin signalling pathway in promoting the proliferation of endothelial progenitor cells in vitro, Mol. Cell. Biochem. 363 (2012) 135–145.

[101] E. Smedler, P. Uhlén, Frequency decoding of calcium oscillations, Biochim. Biophys. Acta 1840 (2014) 964–969.

[102] A. Skupin, H. Kettenmann, M. Falcke, Calcium signals driven by single channel noise, PLoS Comput. Biol. 6 (2010).

[103] A.B. Parekh, Local Ca^{2+} influx through CRAC channels activates temporally and spatially distinct cellular responses, Acta Physiol. (Oxf) 195 (2009) 29–35.

[104] H.L. Roderick, S.J. Cook, Ca^{2+} signalling checkpoints in cancer: remodelling Ca^{2+} for cancer cell proliferation and survival, Nat. Rev. Cancer 8 (2008) 361–375.

[105] N. Nielsen, O. Lindemann, A. Schwab, TRP channels and STIM/ORAI proteins: sensors and effectors of cancer and stroma cell migration, Br. J. Pharmacol. (2014), http://dx.doi.org/10.1111/bph.12721 (Epub ahead of print).

[106] Y. Dong, Q. Pan, L. Jiang, Z. Chen, F. Zhang, Y. Liu, H. Xing, M. Shi, J. Li, X. Li, Y. Zhu, Y. Chen, I.C. Bruce, J. Jin, X. Ma, Tumor endothelial expression of P-glycoprotein upon microvesicular transfer of TrpC5 derived from adriamycin-resistant breast cancer cells, Biochem. Biophys. Res. Commun. 446 (2014) 85–90.

[107] G. Barosi, L. Lupo, V. Rosti, Management of myeloproliferative neoplasms: from academic guidelines to clinical practice, Curr. Hematol. Malig. Rep. 7 (2012) 50–56.

[108] S. Dragoni, U. Laforenza, E. Bonetti, M. Reforgiato, V. Poletto, F. Lodola, C. Bottino, D. Guido, A. Rappa, S. Pareek, M. Tomasello, M.R. Guarrera, M.P. Cinelli, A. Aronica, G. Guerra, G. Barosi, F. Tanzi, V. Rosti, F. Moccia, Enhanced expression of Stim, Orai, and TRPC transcripts and proteins in endothelial progenitor cells isolated from patients with primary myelofibrosis, PLoS One 9 (2014) e91099.

[109] L.F. Jaffe, A calcium-based theory of carcinogenesis, Adv. Cancer Res. 94 (2005) 231–263.

[110] A.S. Borowiec, G. Bidaux, R. Tacine, P. Dubar, N. Pigat, P. Delcourt, O. Mignen, T. Capiod, Are Orai1 and Orai3 channels more important than calcium influx for cell proliferation? Biochim. Biophys. Acta 1843 (2014) 464–472.

[111] R.S. Bhatt, A.J. Zurita, A. O'Neill, A. Norden-Zfoni, L. Zhang, H.K. Wu, P.Y. Wen, D. George, V.P. Sukhatme, M.B. Atkins, J.V. Heymach, Increased mobilisation of circulating endothelial progenitors in von Hippel–Lindau disease and renal cell carcinoma, Br. J. Cancer 105 (2011) 112–117.

[112] B. Yang, W. Gu, B. Peng, Y. Xu, M. Liu, J. Che, J. Geng, J. Zheng, High level of circulating endothelial progenitor cells positively correlates with serum vascular endothelial growth factor in patients with renal cell carcinoma, J. Urol. 188 (2012) 2055–2061.

[113] M. Hernandez-Yanez, J.V. Heymach, A.J. Zurita, Circulating biomarkers in advanced renal cell carcinoma: clinical applications, Curr. Oncol. Rep. 14 (2012) 221–229.

[114] K. Matsuda, N. Ohga, Y. Hida, C. Muraki, K. Tsuchiya, T. Kurosu, T. Akino, S.-C. Shih, Y. Totsuka, M. Klagsbrun, M. Shindoh, K. Hida, Isolated tumor endothelial cells maintain specific character during long-term culture, Biochem. Biophys. Res. Commun. 394 (2010) 947–954.

[115] T. Akino, K. Hida, Y. Hida, K. Tsuchiya, D. Freedman, C. Muraki, N. Ohga, K. Matsuda, K. Akiyama, T. Harabayashi, N. Shinohara, K. Nonomura, M. Klagsbrun, M. Shindoh, Cytogenetic abnormalities of tumor-associated endothelial cells in human malignant tumors, Am. J. Pathol. 175 (2009) 2657–2667.

[116] B. Escudier, C. Szczylik, C. Porta, M. Gore, Treatment selection in metastatic renal cell carcinoma: expert consensus, Nat. Rev. Clin. Oncol. 9 (2012) 327–337.

[117] B. Bussolati, I. Deambrosis, S. Russo, M.C. Deregibus, G. Camussi, Altered angiogenesis and survival in human tumor-derived endothelial cells, FASEB J. 17 (2003) 1159–1161.

[118] J. Song, Y. Wang, X. Li, Y. Shen, M. Yin, Y. Guo, L. Diao, Y. Liu, D. Yue, Critical role of TRPC6 channels in the development of human renal cell carcinoma, Mol. Biol. Rep. 40 (2013) 5115–5122.

[119] J.H. Kim, S. Lkhagvadorj, M.R. Lee, K.H. Hwang, H.C. Chung, J.H. Jung, S.K. Cha, M. Eom, Orai1 and STIM1 are critical for cell migration and proliferation of clear cell renal cell carcinoma, Biochem. Biophys. Res. Commun. 448 (2014) 76–82.

[120] D. Veliceasa, M. Ivanovic, F.T. Hoepfner, P. Thumbikat, O.V. Volpert, N.D. Smith, Transient potential receptor channel 4 controls thrombospondin-1 secretion and angiogenesis in renal cell carcinoma, FEBS J. 274 (2007) 6365–6377.

[121] R.R. Resende, J.L. da Costa, A.H. Kihara, A. Adhikari, E. Lorençon, Intracellular Ca^{2+} regulation during neuronal differentiation of murine embryonal carcinoma and mesenchymal stem cells, Stem Cells Dev. 19 (2010) 379–394.

[122] R.R. Resende, A. Adhikari, J.L. da Costa, E. Lorençon, M.S. Ladeira, S. Guatimosim, A.H. Kihara, L.O. Ladeira, Influence of spontaneous calcium events on cell-cycle

[123] V.y. Lehen'kyi, G. Shapovalov, R. Skryma, N. Prevarskaya, Ion channnels and transporters in cancer. 5. Ion channels in control of cancer and cell apoptosis, Am. J. Physiol. Cell Physiol. 301 (2011) C1281–C1289.

[124] N. Prevarskaya, R. Skryma, Y. Shuba, Ca^{2+} homeostasis in apoptotic resistance of prostate cancer cells, Biochem. Biophys. Res. Commun. 322 (2004) 1326–1335.

[125] M. Patron, A. Raffaello, V. Granatiero, A. Tosatto, G. Merli, D. De Stefani, L. Wright, G. Pallafacchina, A. Terrin, C. Mammucari, R. Rizzuto, The mitochondrial calcium uniporter (MCU): molecular identity and physiological roles, J. Biol. Chem. 288 (2013) 10750–10758.

[126] L. Gibalová, J. Sedlák, M. Labudová, M. Barancík, A. Reháková, A. Breier, Z. Sulová, Multidrug resistant P-glycoprotein positive L1210/VCR cells are also cross-resistant to cisplatin via a mechanism distinct from P-glycoprotein-mediated drug efflux activity, Gen. Physiol. Biophys. 28 (2009) 391–403.

[127] C.H. Liao, Y.M. Hsiao, G.T. Sheu, J.T. Chang, P.H. Wang, M.F. Wu, G.J. Shieh, C.P. Hsu, J.L. Ko, Nuclear translocation of telomerase reverse transcriptase and calcium signaling in repression of telomerase activity in human lung cancer cells by fungal immunomodulatory protein from Ganoderma tsugae, Biochem. Pharmacol. 74 (2007) 1541–1554.

[128] S. Rosenberger, I.S. Thorey, S. Werner, P. Boukamp, A novel regulator of telomerase. S100A8 mediates differentiation-dependent and calcium-induced inhibition of telomerase activity in the human epidermal keratinocyte line HaCaT, J. Biol. Chem. 282 (2007) 6126–6135.

[129] A. Bergner, J. Kellner, A. Tufman, R.M. Huber, Endoplasmic reticulum Ca^{2+}-homeostasis is altered in small and non-small cell lung cancer cell lines, J. Exp. Clin. Cancer Res. 28 (2009) 25.

[130] F. Moccia, R. Berra-Romani, S. Baruffi, S. Spaggiari, S. Signorelli, L. Castelli, J. Magistretti, V. Taglietti, F. Tanzi, Ca^{2+} uptake by the endoplasmic reticulum Ca^{2+}-ATPase in rat microvascular endothelial cells, Biochem. J. 364 (2002) 235–244.

[131] H. Akl, G. Bultynck, Altered Ca^{2+} signaling in cancer cells: proto-oncogenes and tumor suppressors targeting IP3 receptors, Biochim. Biophys. Acta 1835 (2013) 180–193.

[132] C. Pierro, S.J. Cook, T.C. Foets, M.D. Bootman, H.L. Roderick, Oncogenic K-Ras suppresses IP3-dependent Ca^{2+} release through remodelling of the isoform composition of IP3Rs and ER luminal Ca^{2+} levels in colorectal cancer cell lines, J. Cell Sci. 127 (2014) 1607–1619.

[133] H. Ivanova, T. Vervliet, L. Missiaen, J.B. Parys, H. De Smedt, G. Bultynck, Inositol 1,4,5-trisphosphate receptor-isoform diversity in cell death and survival, Biochim. Biophys. Acta 1843 (2014) 2164–2183.

[134] Y.F. Chen, Y.T. Chen, W.T. Chiu, M.R. Shen, Remodeling of calcium signaling in tumor progression, J. Biomed. Sci. 20 (2013) 23.

[135] G.R. Monteith, F.M. Davis, S.J. Roberts-Thomson, Calcium channels and pumps in cancer: changes and consequences, J. Biol. Chem. 287 (2012) 31666–31673.

[136] R.K. Motiani, M.C. Hyzinski-Garcia, X. Zhang, M.M. Henkel, I.F. Abdullaev, Y.-H. Kuo, K. Matrougui, A.A. Mongin, M. Trebak, STIM1 and Orai1 mediate CRAC channel activity and are essential for human glioblastoma invasion, Pflugers Arch. 465 (2013) 1249–1260.

[137] R.K. Motiani, X. Zhang, K.E. Harmon, R.S. Keller, K. Matrougui, J.A. Bennett, M. Trebak, Orai3 is an estrogen receptor α-regulated Ca^{2+} channel that promotes tumorigenesis, FASEB J. 27 (2013) 63–75.

[138] H. Stanisz, S. Saul, C.S. Müller, R. Kappl, B.A. Niemeyer, T. Vogt, M. Hoth, A. Roesch, I. Bogeski, Inverse regulation of melanoma growth and migration by Orai1/STIM2-dependent calcium entry, Pigment Cell Melanoma Res. 27 (2014) 442–453.

[139] S. Mincheva-Tasheva, R.M. Soler, NF-κB signaling pathways: role in nervous system physiology and pathology, Neuroscientist 19 (2013) 175–194.

[140] B. Hoesel, J.A. Schmid, The complexity of NF-κB signaling in inflammation and cancer, Mol. Cancer 12 (2013) 86.

[141] M.J. Berridge, The endoplasmic reticulum: a multifunctional signaling organelle, Cell Calcium 32 (2002) 235–249.

[142] J.L. Li, R.C. Sainson, C.E. Oon, H. Turley, R. Leek, H. Sheldon, E. Bridges, W. Shi, C. Snell, E.T. Bowden, H. Wu, P.S. Chowdhury, A.J. Russell, C.P. Montgomery, R. Poulsom, A.L. Harris, DLL4-Notch signaling mediates tumor resistance to anti-VEGF therapy in vivo, Cancer Res. 71 (2011) 6073–6083.

[143] M.F. Hou, H.C. Kuo, J.H. Li, Y.S. Wang, C.C. Chang, K.C. Chen, W.C. Chen, C.C. Chiu, S. Yang, W.C. Chang, Orai1/CRACM1 overexpression suppresses cell proliferation via attenuation of the store-operated calcium influx-mediated signalling pathway in A549 lung cancer cells, Biochim. Biophys. Acta 1810 (2011) 1278–1284.

[144] J.Y. Wang, B.K. Chen, Y.S. Wang, Y.T. Tsai, W.C. Chen, W.C. Chang, M.F. Hou, Y.C. Wu, Involvement of store-operated calcium signaling in EGF-mediated COX-2 gene activation in cancer cells, Cell. Signal. 24 (2012) 162–169.

[145] J. Zhang, J. Wei, M. Kanada, L. Yan, Z. Zhang, H. Watanabe, S. Terakawa, Inhibition of store-operated Ca^{2+} entry suppresses EGF-induced migration and eliminates extravasation from vasculature in nasopharyngeal carcinoma cell, Cancer Lett. 336 (2013) 390–397.

[146] H. Zhu, H. Zhang, F. Jin, M. Fang, M. Huang, C.S. Yang, T. Chen, L. Fu, Z. Pan, Elevated Orai1 expression mediates tumor-promoting intracellular Ca^{2+} oscillations in human esophageal squamous cell carcinoma, Oncotarget 5 (2014) 3455–3471.

[147] F.M. Davis, A.A. Peters, D.M. Grice, P.J. Cabot, M.-O. Parat, S.J. Roberts-Thomson, G.R. Monteith, Non-stimulated, agonist-stimulated and store-operated Ca^{2+} influx in MDA-MB-468 breast cancer cells and the effect of EGF-induced EMT on calcium entry, PLoS One 7 (2012) e36923.

[148] H. Ouadid-Ahidouch, I. Dhennin-Duthille, M. Gautier, H. Sevestre, A. Ahidouch, TRP channels: diagnostic markers and therapeutic targets for breast cancer? Trends Mol. Med. 19 (2013) 117–124.

[149] V.C. Bomben, K.L. Turner, T.T.C. Barclay, H. Sontheimer, Transient Receptor Potential Canonical channels are essential for chemotactic migration of human malignant gliomas, J. Cell. Physiol. 226 (2011) 1879–1888.

[150] N. Tajeddine, P. Gailly, TRPC1 protein channel is major regulator of epidermal growth factor receptor signaling, J. Biol. Chem. 287 (2012) 16146–16157.

[151] H.N. Jiang, B. Zeng, Y. Zhang, N. Daskoulidou, H. Fan, J.M. Qu, S.Z. Xu, Involvement of TRPC channels in lung cancer cell differentiation and the correlation analysis in human non-small cell lung cancer, PLoS One 8 (2013) e67637.

[152] B. He, F. Liu, J. Ruan, A. Li, J. Chen, R. Li, J. Shen, D. Zheng, R. Luo, Silencing TRPC1 expression inhibits invasion of CNE2 nasopharyngeal tumor cells, Oncol. Rep. 27 (2012) 1548–1554.

[153] B. Zeng, C. Yuan, X. Yang, S.L. Atkin, S.Z. Xu, TRPC channels and their splice variants are essential for promoting human ovarian cancer cell proliferation and tumorigenesis, Curr. Cancer Drug Targets 13 (2013) 103–116.

[154] S. Yang, J.J. Zhang, X.Y. Huang, Orai1 and STIM1 are critical for breast tumor cell migration and metastasis, Cancer Cell 15 (2009) 124–134.

[155] A. Chantôme, M. Potier-Cartereau, L. Clarysse, G. Fromont, S. Marionneau-Lambot, M. Guéguinou, J.C. Pagès, C. Collin, T. Oullier, A. Girault, F. Arbion, J.P. Haelters, P.A. Jaffrès, M. Pinault, P. Besson, V. Joulin, P. Bougnoux, C. Vandier, Pivotal role of the lipid Raft SK3–Orai1 complex in human cancer cell migration and bone metastases, Cancer Res. 73 (2013) 4852–4861.

[156] C. Porta, C. Paglino, I. Imarisio, C. Ganini, L. Sacchi, S. Quaglini, V. Giunta, M. De Amici, Changes in circulating pro-angiogenic cytokines, other than VEGF, before progression to sunitinib therapy in advanced renal cell carcinoma patients, Oncology 84 (2013) 115–122.

[157] S. Dragoni, G. Guerra, A. Fiorio Pla, G. Bertoni, A. Rappa, V. Poletto, C. Bottino, A. Aronica, F. Lodola, M.P. Cinelli, U. Laforenza, V. Rosti, F. Tanzi, L. Munaron, F. Moccia, A functional Transient Receptor Potential Vanilloid 4 (TRPV4) channel is expressed in human endothelial progenitor cells, J. Cell. Physiol. 230 (2015) 95–104.

[158] R. Takezawa, H. Cheng, A. Beck, J. Ishikawa, P. Launay, H. Kubota, J.P. Kinet, A. Fleig, T. Yamada, R. Penner, A pyrazole derivative potently inhibits lymphocyte Ca^{2+} influx and cytokine production by facilitating transient receptor potential melastatin 4 channel activity, Mol. Pharmacol. 69 (2006) 1413–1420.

[159] D.J. Beech, Orai1 calcium channels in the vasculature, Pflugers Arch. 463 (2012) 635–647.

[160] H. Schleifer, B. Doleschal, M. Lichtenegger, R. Oppenrieder, I. Derler, I. Frischauf, T.N. Glasnov, C.O. Kappe, C. Romanin, K. Groschner, Novel pyrazole compounds for pharmacological discrimination between receptor-operated and store-operated Ca^{2+} entry pathways, Br. J. Pharmacol. 167 (2012) 1712–1722.

[161] C. Weidinger, P.J. Shaw, S. Feske, STIM1 and STIM2-mediated Ca^{2+} influx regulates antitumour immunity by $CD8^+$ T cells, EMBO Mol. Med. 5 (2013) 1311–1321.

[162] S. Jin, J. Chin, C. Kitson, J. Woods, R. Majmudar, V. Carvajal, J. Allard, J. Demartino, S. Narula, D.A. Thomas-Karyat, Natural regulatory T cells are resistant to calcium release-activated calcium (CRAC/ORAI) channel inhibition, Int. Immunol. 25 (2013) 497–506.

[163] T.L. Whiteside, P. Schuler, B. Schilling, Induced and natural regulatory T cells in human cancer, Expert. Opin. Biol. Ther. 12 (2012) 1383–1397.

[164] S. Dragoni, I. Turin, U. Laforenza, D.M. Potenza, C. Bottino, T.N. Glasnov, M. Prestia, F. Ferulli, A. Saitta, A. Mosca, G. Guerra, V. Rosti, O. Luinetti, C. Ganini, C. Porta, P. Pedrazzoli, F. Tanzi, D. Montagna, F. Moccia, Store-operated Ca^{2+} entry does not control proliferation in primary cultures of human metastatic renal cellular carcinoma, Biomed. Res. Int. 2014 (2014) 739494.

[165] B. Korosec, D. Glavac, T. Rott, M. Ravnik-Glavac, Alterations in the ATP2A2 gene in correlation with colon and lung cancer, Cancer Genet. Cytogenet. 171 (2006) 105–111.

[166] B. Korosec, D. Glavac, M. Volavsek, M. Ravnik-Glavac, ATP2A3 gene is involved in cancer susceptibility, Cancer Genet. Cytogenet. 188 (2009) 88–94.

[167] B. Korosec, D. Glavac, M. Volavsek, M. Ravnik-Glavac, Alterations in genes encoding sarcoplasmic-endoplasmic reticulum Ca^{2+} pumps in association with head and neck squamous cell carcinoma, Cancer Genet. Cytogenet. 181 (2008) 112–118.

[168] M. Mareel, M.J. Oliveira, I. Madani, Cancer invasion and metastasis: interacting ecosystems, Virchows Arch. 454 (2009) 599–622.

[169] G. Zhuang, X. Wu, Z. Jiang, I. Kasman, J. Yao, Y. Guan, J. Oeh, Z. Modrusan, C. Bais, D. Sampath, N. Ferrara, Tumour-secreted miR-9 promotes endothelial cell migration and angiogenesis by activating the JAK-STAT pathway, EMBO J. 31 (2012) 3513–3523.

[170] C. Grange, M. Tapparo, F. Collino, L. Vitillo, C. Damasco, M.C. Deregibus, C. Tetta, B. Bussolati, G. Camussi, Microvesicles released from human renal cancer stem cells stimulate angiogenesis and formation of lung premetastatic niche, Cancer Res. 71 (2011) 5346–5356.

[171] B.S. Hong, J.H. Cho, H. Kim, E.J. Choi, S. Rho, J. Kim, J.H. Kim, D.S. Choi, Y.K. Kim, D. Hwang, Y.S. Gho, Colorectal cancer cell-derived microvesicles are enriched in cell cycle-related mRNAs that promote proliferation of endothelial cells, BMC Genomics 10 (2009) 556.

[172] B. Bussolati, C. Grange, G. Camussi, Tumor exploits alternative strategies to achieve vascularization, FASEB J. 25 (2011) 2874–2882.

[173] D.M. Hellebrekers, V. Melotte, E. Viré, E. Langenkamp, G. Molema, F. Fuks, J.G. Herman, W. Van Criekinge, A.W. Griffioen, M. van Engeland, Identification of epigenetically silenced genes in tumor endothelial cells, Cancer Res. 67 (2007) 4138–4148.

[174] D.M. Hellebrekers, K. Castermans, E. Viré, R.P. Dings, N.T. Hoebers, K.H. Mayo, M.G. Oude Egbrink, G. Molema, F. Fuks, M. van Engeland, A.W. Griffioen, Epigenetic regulation of tumor endothelial cell anergy: silencing of intercellular adhesion molecule-1 by histone modifications, Cancer Res. 66 (2006) 10770–10777.

[175] D.M. Hellebrekers, A.W. Griffioen, M. van Engeland, Dual targeting of epigenetic therapy in cancer, Biochim. Biophys. Acta 1775 (2007) 76–91.

[176] L. Zhang, A. Tang, Y. Zhou, J. Tang, Z. Luo, C. Jiang, X. Li, J. Xiang, G. Li, Tumor-conditioned mesenchymal stem cells display hematopoietic differentiation and diminished influx of Ca^{2+}, Stem Cells Dev. 21 (2012) 1418–1428.

[177] X. Ma, Y. Cai, D. He, C. Zou, P. Zhang, C.Y. Lo, Z. Xu, F.L. Chan, S. Yu, Y. Chen, R. Zhu, J. Lei, J. Jin, X. Yao, Transient receptor potential channel TRPC5 is essential for P-glycoprotein induction in drug-resistant cancer cells, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 16282–16287.

[178] X. Ma, Z. Chen, D. Hua, D. He, L. Wang, P. Zhang, J. Wang, Y. Cai, C. Gao, X. Zhang, F. Zhang, T. Wang, T. Hong, L. Jin, X. Qi, S. Chen, X. Gu, D. Yang, Q. Pan, Y. Zhu, Y. Chen, D. Chen, L. Jiang, X. Han, Y. Zhang, J. Jin, X. Yao, Essential role for TrpC5-containing extracellular vesicles in breast cancer with chemotherapeutic resistance, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 6389–6394.

[179] H. Peinado, S. Lavotshkin, D. Lyden, The secreted factors responsible for pre-metastatic niche formation: old sayings and new thoughts, Semin. Cancer Biol. 21 (2011) 139–146.

[180] H. Peinado, M. Alečković, S. Lavotshkin, I. Matei, B. Costa-Silva, G. Moreno-Bueno, M. Hergueta-Redondo, C. Williams, G. García-Santos, C. Ghajar, A. Nitadori-Hoshino, C. Hoffman, K. Badal, B.A. Garcia, M.K. Callahan, J. Yuan, V.R. Martins, J. Skog, R.N. Kaplan, M.S. Brady, J.D. Wolchok, P.B. Chapman, Y. Kang, J. Bromberg, D. Lyden, Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET, Nat. Med. 18 (2012) 883–891.

[181] T. Asahara, T. Takahashi, H. Masuda, C. Kalka, D. Chen, H. Iwaguro, Y. Inai, M. Silver, J.M. Isner, VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells, EMBO J. 18 (1999) 3964–3972.

[182] A.M. Davidoff, C.Y. Ng, P. Brown, M.A. Leary, W.W. Spurbeck, J. Zhou, E. Horwitz, E.F. Vanin, A.W. Nienhuis, Bone marrow-derived cells contribute to tumor neovasculature and, when modified to express an angiogenesis inhibitor, can restrict tumor growth in mice, Clin. Cancer Res. 7 (2001) 2870–2879.

[183] M. Reyes, A. Dudek, B. Jahagirdar, L. Koodie, P.H. Marker, C.M. Verfaillie, Origin of endothelial progenitors in human postnatal bone marrow, J. Clin. Invest. 109 (2002) 337–346.

[184] M. De Palma, M.A. Venneri, C. Roca, L. Naldini, Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells, Nat. Med. 9 (2003) 789–795.

[185] M. Garcia-Barros, F. Paris, C. Cordon-Cardo, D. Lyden, S. Rafii, A. Haimovitz-Friedman, Z. Fuks, R. Kolesnick, Tumor response to radiotherapy regulated by endothelial cell apoptosis, Science 300 (2003) 1155–1159.

[186] A. Dwenger, F. Rosenthal, M. Machein, C. Waller, A. Spyridonidis, Transplanted bone marrow cells preferentially home to the vessels of in situ generated murine tumors rather than of normal organs, Stem Cells 22 (2004) 86–92.

[187] I. Rajantie, M. Ilmonen, A. Alminaite, U. Ozerdem, K. Alitalo, P. Salven, Adult bone marrow-derived cells recruited during angiogenesis comprise precursors for periendothelial vascular mural cells, Blood 104 (2004) 2084–2086.

[188] J.R. Göthert, S.E. Gustin, J.A. van Eekelen, U. Schmidt, M.A. Hall, S.M. Jane, A.R. Green, B. Göttgens, D.J. Izon, C.G. Begley, Genetically tagging endothelial cells in vivo: bone marrow-derived cells do not contribute to tumor endothelium, Blood 104 (2004) 1769–1777.

[189] H. Spring, T. Schüler, B. Arnold, G.J. Hämmerling, R. Ganss, Chemokines direct endothelial progenitors into tumor neovessels, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 18111–18116.

[190] D.G. Duda, K.S. Cohen, S.V. Kozin, J.Y. Perentes, D. Fukumura, D.T. Scadden, R.K. Jain, Evidence for incorporation of bone marrow-derived endothelial cells into perfused blood vessels in tumors, Blood 107 (2006) 2774–2776.

[191] S. Purhonen, J. Palm, D. Rossi, N. Kaskenpää, I. Rajantie, S. Ylä-Herttuala, K. Alitalo, I.L. Weissman, P. Salven, Bone marrow-derived circulating endothelial precursors do not contribute to vascular endothelium and are not needed for tumor growth, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 6620–6625.

[192] S.Y. Jung, J.H. Choi, S.M. Kwon, H. Masuda, T. Asahara, Y.M. Lee, Decursin inhibits vasculogenesis in early tumor progression by suppression of endothelial progenitor cell differentiation and function, J. Cell. Biochem. 113 (2012) 1478–1487.

[193] H.R. Zhang, F.L. Chen, C.P. Xu, Y.F. Ping, Q.L. Wang, Z.Q. Liang, J.M. Wang, X.W. Bian, Incorporation of endothelial progenitor cells into the neovasculature of malignant glioma xenograft, J. Neurooncol. 93 (2009) 165–174.