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Ca^{2+} Signalling in Endothelial Progenitor Cells: A Novel Means to Improve Cell-Based Therapy and Impair Tumour Vascularisation

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Abstract: Endothelial progenitor cells (EPCs) have recently been employed in cell-based therapy (CBT) to promote regeneration of ischemic organs, such as heart and limbs. Furthermore, EPCs may sustain tumour vascularisation and provide an additional target for anticancer therapies. CBT is limited by the paucity of cells harvested from peripheral blood and suffers from several pitfalls, including the low rate of engrafted EPCs, whereas classic antiangiogenic treatments manifest a number of side effects and may induce resistance into the patients. CBT will benefit of a better understanding of the signal transduction pathway(s) which drive(s) EPC proliferation, trafficking, and incorporation into injured tissues. At the same time, this information might outline alternative molecular targets to impair tumor neovascularisation and improve the therapeutic outcome of antiangiogenic strategies. An increase in intracellular Ca^{2+} concentration is the key signal in the regulation of cellular replication, migration, and differentiation. In particular, Ca^{2+} signalling may regulate cell-cycle progression, due to the Ca^{2+}-sensitivity of a number of cycline-dependent kinases, and gene expression, owing to the Ca^{2+}-dependence of several transcription factors. Recent work has outlined the role of the so-called store-operated Ca^{2+} entry in driving EPC proliferation and migration. Unravelling the mechanisms guiding EPC engraftment into neovessels might supply the biological bases required to improve CBT and anticancer treatments. For example, genetic manipulation of the Ca^{2+} signalling machinery could provide a novel approach to increase the extent of limb regeneration or preventing tumour vascularisation by EPCs.

Keywords: Endothelial progenitor cells, cell therapy, tumor vascularization, Ca^{2+} signalling, store operated Ca^{2+} entry, Orai1, Stim1.

1. INTRODUCTION

Cardiovascular pathologies, including myocardial infarction and peripheral artery disease, are a leading cause of death and disability worldwide [1]. Tissue perfusion through stenotic supplying arteries may be restored by vascular regenerative surgery, including angioplasty, deployment of intracoronary stents and coronary artery bypass surgery [2]. These procedures, however, affect the integrity of the endothelial monolayer lining the lumen of blood vessels and, in the long term, may cause thrombi formation and neointimal hyperplasia, the so-called "in-stent restenosis". The subsequent shrinkage of the arterial wall severely limits the beneficial outcome of reconstructive procedures [3]. These hurdles prompted the quest for alternative strategies to restore blood flow by regenerating the vascular network within the ischemic organ [4-6]. The adult bone marrow (BM) is a rich reservoir of progenitor and stem cells, which possess the capability for self-renewal and differentiation into organ-specific cell types [7]. As shown in several animal models, transplantation of these cells may reconstitute organ systems, a therapeutic approach termed cell-based therapy (CBT) [6, 8-10]. In particular, inoculation of BM-derived endothelial progenitor cells (EPCs) has been proven useful in restoring both the functional performance of ischemic myocardium and the blood flow in several preclinical studies [8-10]. The vessel-forming ability of EPCs, however, may be regarded as a coin with 2 faces, as recent evidence indicates that EPC engagement drives the progression of avascular micrometastatic tumors to lethal macrometastatic ones [11]. Tumor growth requires an adequate supply of oxygen and nutrients, which was thought to be solely provided by the sprouting of existing blood vessels close to the primary malignancy [12]. In addition to this well characterized process, tumor vascularization may be supported by circulating EPCs [11, 13, 14]. Therefore, EPC inhibition has been proposed as a novel therapeutic target in the fight against cancer [11, 13-15]. As a consequence, elucidating the molecular signalling pathways driving EPC-dependent vascularization is predicted to open new avenues for CBT and anti-cancer treatments. Due to the multifaceted role of calcium (Ca^{2+}) ions in controlling cell behaviour [16-19], the Ca^{2+} machinery may be considered as a potential target for strategies aiming at ameliorating

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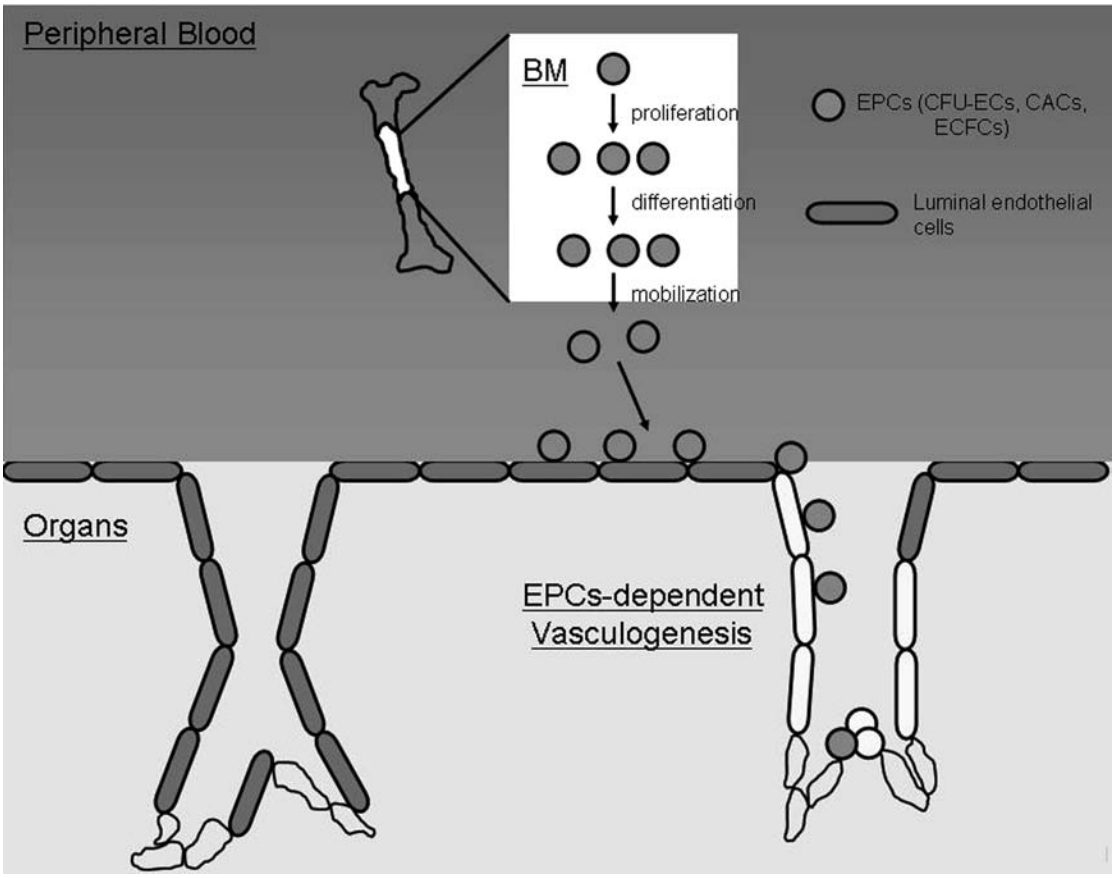

Fig. (1). Endothelial progenitor cells contribute to postnatal vascularisation. Adult bone marrow contains the hemangioblast, the putative precursor of both hematopoietic stem cells and EPCs. Hypoxia induces the liberation of pro-angiogenic factors, such as VEGF and SDF-1α, which stimulate EPCs to proliferate, egress from the osteoblastic niche and migrate towards the hypoxic site, where they may either emit instructive signals (i.e. the paracrine release of growth factors; CFU-ECs and CACs) or provide the building blocks for the nascent vasculature (ECFCs). Adapted from [4].

CBT and impairing tumor neovascularisation [20, 21]. In this review, we will survey current knowledge about the Ca^{2+} toolkit in EPCs and the potential relevance of the so-called store-operated Ca^{2+} entry (SOCE) to therapeutic practice.

2. ENDOTHELIAL PROGENITOR CELLS: DEFINITION, CHARACTERIZATION AND FUNCTIONAL FEATURES

Neovessel formation in adult individuals has long been ascribed to a process, called angiogenesis, in which new capillaries sprouts from pre-existing vessels, whereas vasculogenesis, i.e. the formation of new vessels from circulating endothelial precursors or angioblasts, was thought to occur only in the developing fetus (Fig. **1**) [22-24]. This paradigm was revolutionized by the findings reported in a seminal paper by Asahara and coworkers [25], which described the existence of a population of circulating BM-derived cells with the phenotypic and functional features of putative EPCs. The same study showed that these putative EPCs were able to incorporate into sites of active angiogenesis in an animal model of hind limb ischemia (Fig. **1**), thus opening the way to the concept that, in adult subjects, a *de novo* formation of vessels can be due to circulating EPCs (so-called vasculogenesis) and not only to sprouting from a pre-existing vessel (angiogenesis). In the subsequent years, while it was confirmed that vasculogenesis can occur after birth and can be due to circulating EPCs, 2 main issues emerged from several studies that followed the original Asahara paper [25]: i) vessel remodeling and neoformation in adult subjects involve, besides EPCs, also hematopoietic progenitor and stem cells through a complex network of interactions, and, ii) the definition of the phenotypic and functional characteristics of circulating EPCs as well as the requirements for their identification and enumeration needed further insight and characterization [22, 26-28]. Taking in consideration and summarizing the majority of studies in which the definition of the condition for the *in vitro* growth and identification of EPCs have been investigated, 3 types of putative circulating EPC has been described, whose nature and features depend on the different culture conditions and cell population they emerged from.

a) the Colony forming unit-endothelial cell (CFU-EC, CFU-Hill, CFU-End, Early EPC).

This definition refers to the putative EPC initially isolated by Asahara *et al.* in 1997 [25] although some technical modifications have been brought to the original method. Currently, 5 x 10^6 peripheral blood (PB)- or umbilical cord blood

(UCB)- derived mononuclear cells (MNCs) are plated on fibronectin coated dishes in a standard, commercially available medium containing 20% of foetal bovine serum and vascular endothelial growth factor (VEGF) [29]. After 2 day-culture, the non-adherent fraction is recovered and re-plated in new fibronectin-coated dishes for further 5-9 days. Discrete colonies, consisting of spindle shaped cells emerging from a central cluster of small round cells, appear at the end of the incubation period. The frequency of CFU-ECs in PB oscillates between 50 and 500/ml. These colonies, however, exhibit a blend of surface antigens which is not consistent with a pure endothelial phenotype. Accordingly, some of the cells express the CD34 antigen, as well as the VEGFR-2, the CD31, the Tie-2 and the E-selectin antigens; however, most of them co-express the CD45 antigen, which is a specific marker of the hematopoietic lineage and whose expression does never completely disappear during the culture [29]. Moreover, CFU-ECs do not display a great proliferative potential and cannot be expanded after 1 or 2 passages following their isolation. Nevertheless, the injection of CFU-ECs in various animal models of cardiovascular diseases has a positive impact on the therapeutic outcome, by reducing the extension of necrosis or by favoring revascularization of the infarcted areas [10].

b) the circulating angiogenic cell (CAC).

CAC are obtained by culturing PB-derived MNCs in a medium supplemented with VEGF for 3-4 days. Then, the non-adherent fraction is discarded and culture of the adherent cell population is carried on for few further days (4 to 7). This protocol gives raise to spindle shaped cells, similar to those observed at the edge of the CFU-EC; however, colony formation is never observed. Similar to mature ECs, CACs possess the ability: a) to bind to the endothelial specific lectins *Ulex Europeus* agglutinin-1 (UEA-1) and *Baindeiraea simplicifolia* (BS-1), and 2) to take up the acetylated low-density lipoprotein (acLDL). In addition, they express von Willebrand factor (vWf), CD31, VEGFR-2, VE-cadherin, and Tie-2 [30]. CACs comprise approximately 2% of the whole circulating MNC population, however, this protocol of EPC isolation may be plagued by the presence of numerous platelets co-fractioning with MNCs. Platelets may convey endothelial features to hematopoietic cells (such as CD31 and intake of UEA-1) by attaching to MNCs and transferring them their membrane proteins [28].

c) ECFC: endothelial colony-forming cells (BOEC, late outgrowing EPC).

The third (in time order) type of EPCs that was successfully isolated *in vitro* by cell culture is termed "endothelial-colony forming cells" (ECFC). In this method, MNCs, harvested from either PB or UCB, are plated onto collagen-coated Petri dishes in an endothelial growth medium containing 5% of fetal calf serum and supplements, including VEGF, basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), hydrocortisone, ascorbic acid and heparin. After 48 hours incubation, non-adherent cells are discarded and cultures are carried on for up to 4 weeks, with a tri-weekly medium change. ECFC-derived colonies appear from the adherent cell population 10-20 days (if PB is the source of MNCs) or 5-7 days (if CB is used) after plating. Colonies are composed of a monolayer of cells indistinguishable from mature endothelial cells (ECs), with a typical cobblestone appearance. They express all the typical surface proteins of the endothelial lineage (CD105, CD31, CD144, vWf, VEGFR-2) but not the CD45 and CD14 antigens, thus ruling out the possibility that ECFC derive from the hematopoietic lineage. Moreover, they display a great proliferative potential and possess *de novo* vessel forming capacity *in vivo* [29, 31]. Their concentration in adult PB is estimated to be of about 0.05-0.2 cells/ml [29, 32]. Further characterization of the ECFCs allowed the identification, by means of limiting dilution analysis and serial replating, of a hierarchy of ECFCs endowed with different proliferative potential similar to the hierarchic organization of the hematopoietic progenitor and stem cells [31]. Finally, ECFCs participated to *de novo* vessel formation in an immunodeficient mouse in which they were transplanted after being suspended into a collagen scaffold [29, 33]. In the transplanted scaffold, human ECFCs assembled in neovessels which formed anastomoses with the host murine vasculature and permitted blood flow. Interestingly, CFU-ECs were not able, in the same experimental model, to form any vessel [29]. These *in vivo* data, along with the low frequency in the PB, the high proliferative capacity and ability to expand *ex vivo*, have led to believe that ECFCs retain the characteristic of true progenitor cells and that they display features consistent with real EPCs [26-28]. The pro-angiogenic features displayed in preclinical settings by both CFU-ECs and CACs, which do not engraft within perfused vessels, may be explained by their complex interaction with the local cell population [5, 29]. The hypoxic environment of growing tumors or damaged tissues results in the activation of the hypoxia-inducible factor-1 (HIF-1), a transcription factor controlling the expression of a variety of growth factors and cytokines, such as VEGF and stromal derived factor-1α (SDF-1α) [22, 24]. The consequent release of VEGF and SDF-1α to circulation establishes a concentration gradient which paves the way for the recruitment of hematopoietic cells, including CFU-ECs and CACs, to the injured vasculature. Herein, they do not become integral part of the endothelial intima, rather, they stimulate resident ECs to undergo angiogenesis and attract circulating ECFCs by the paracrine secretion of a host of signalling molecules, including VEGF and SDF-1α[5]. ECFCs will, in turn, integrate into the network of forming tubules and contribute to remodelling of the vascular network [27, 29].

3. THE POTENTIAL ROLE OF ENDOTHELIAL PROGENITOR CELLS IN REGENERATIVE MEDICINE

A large body of studies on the potential contribution of circulating EPCs to neovascularisation in both physiological and pathological conditions has been carried out since their initial description. A low level of circulating EPCs predicts an increased risk for the occurrence of cardiovascular diseases [10, 34]. However, in patients suffering from coronary disease there is a less clear relationship with levels of circulating EPCs. With regard to acute myocardial infarction (AMI), at least 5 different studies [35-39] using distinct approaches for EPC detection, found that early phases of AMI are characterized by a significant increase in circulating

EPCs and, in 2 of them [35, 39], elevated progenitor cell counts were predictor of favourable improvement of ventricular function. On the contrary, in 2 studies conducted on patients affected by chronic coronary disease (CAD), EPCs (assessed as $CD34^+/VEGFR\text{-}2^+$ cells) were reduced as compared to age-matched healthy subjects [40, 41]. Conversely, other investigations either found no changes between patients with stable angina and healthy subjects (in this study EPCs were assessed as $CD34^+/CD133^+/VEGFR\text{-}2^+$ cells) [36] or reported that circulating EPCs, measured as late EPCs in culture, proportionally increase with the severity of CAD [42]. Parallel work has established that BM-derived EPCs play a pivotal role in restoring blood perfusion to infarcted myocardium [38], ischemic limbs [43], and burned tissues [44]. Consistently, preclinical and clinical studies have demonstrated that: 1) *ex vivo* expanded human EPCs increased capillary density and reduced the rate of limb loss when implanted into murine models of hind limb ischemia [9, 45]; 2) exogenously administered EPCs increased the extent of neovascularisation and improved the left ventricular ejection fraction (LVEF) when injected intravenously into rats with myocardial ischemia [9, 10]; 3) infusion of autologous EPCs restored endothelial functions and improved vascularisation in patients suffering from peripheral artery disease and limb ischemia, respectively [45, 46]; 4) transplantation of PB-derived EPCs in patients who underwent AMI or suffered from chronic myocardial ischemia led to an improvement in LVEF, a reduction in infarct size, and an increase in capillary density [9-10]. Therefore, EPCs-based cell therapy has been introduced as an alternative strategy to treat severe ischemic diseases [5, 8-10, 45, 46]. However, infusion of such EPCs in human patients was never shown to be as efficient as studies in animal models predicted [9, 28]. For example, the LVEF was either unaffected or exhibited only a 5% increase, the minimum changed required for long-term survival, upon intracoronary injection of EPCs (sorted as $CD133^+$ cells) into infarcted myocardium [6]. Central to the therapeutic outcome of autologous cell-based strategies are:

1) The nature of the cells delivered to rescue blood perfusion to the ischemic tissue [22, 26, 28]. As noted above, the term EPC may include cells that belong to the hematopoietic, rather than to the endothelial lineage, and do not physically integrate into neovessels *in situ*. In this context, both PB- and UCB-derived ECFCs possess all the characteristics of a true endothelial progenitor and are emerging as the most suitable cell population for vascular engineering strategies [22, 26-28].

2) The correct trafficking of injected EPCs to reconstructuring area [24, 47, 48].

3) The *ex vivo* expansion of EPCs, whose quantity in steady state PB may be too limited to exert any beneficial effect *in vivo* [47, 48]. The latter issue might be solved by treating the donor with granulocyte- and granulocyte colony-stimulating factor (G-CSF and GM-CSF), that stimulate EPC mobilization at pharmacological doses and increase the number of circulating cells available for autologous transfusion. Unfortunately, these cytokines may also recruit inflammatory cells, including mast cells, and neutrophils, which might adverse the protective effect of EPCs homing to the hypoxic site [49]. The regenerative potential of EPCs is further decreased in presence of cardiovascular risk factors, such as diabetes, smoking, ageing and hypercholesterolemia [10, 48]. For example, in type II diabetes, EPCs display reduced proliferation and adhesion properties, and fail in forming capillary-like structures *in vitro* [50]. This feature dramatically limits the employment of autologous EPCs to treat cardiovascular diseases in patients burdened with comorbidity [10, 48].

4) The low rate of engraftment and persistence of EPCs within the ischemic tissue: as a consequence, up to 90% of exogenous cells may be lost after the injection and fail in participating to the regeneration process [51].

5) The differentiation fate of transplanted cells, which do not necessarily differentiate into the desired cell type or acquire an unwanted phenotype, thus causing lethal consequences on the host organism. For example, the intramyocardial injection of unsorted BM cells into infarcted mice may result in severe calcification and/or ossification [6, 9]. The incapability of controlling the outcome of the differentiation process may also impair the benefits of CBT in promoting neovascularisation of ischemic limbs. Accordingly, intravenous infusion of spleen-derived EPCs into atherosclerotic mice may augment atherosclerotic lesion size and reduce plaque stability, likely due to EPC differentiation in smooth muscle cells rather than mature ECs [9].

As mentioned above, several cytokines, including VEGF, SDF-1α, epidermal growth factor (EGF), erythropoietin (EPO), G-CSF and GM-CSF, may recruit EPCs to ischemic sites [24, 52]. These factors serve also as mitogens, by promoting the local proliferation of EPCs attracted by the hypoxic tissue [47, 53]. In particular, the signal transduction pathways activated by VEGF and SDF-1α into EPCs are still unclear, although they may both activate the phosphoinositide 3-kinase (PI3K)/Akt pathway [24, 52]. In the light of the pitfalls described above, enhancing the signalling activity which governs the angiogenic response to VEGF and SDF-1α might constitute an efficient means to optimize CBT.

4. EVIDENCE ABOUT THE CONTRIBUTION OF ENDOTHELIAL PROGENITOR CELLS TO TUMORAL NEOVASCULARISATION

Besides cardiovascular diseases, cancer represents the other field in which EPCs have been investigated. This is not surprising when considering that angiogenesis and vasculogenesis play a pivotal role in tumour growth and progression. In fact, both the growth of a tumour and its process of metastasis can occur only if new vessel formation occurs [54]. The initial demonstration for EPCs to contribute to tumor neovascularisation dates back to the beginning of this century and was soon followed by other works, aimed at demonstrating that they are involved not only in primary tumor growth, but also in the formation of what has been termed the pre-metastatic niche (Fig. **2**) [11, 14, 55, 56]. Similar to injured tissues, growing tumors secrete VEGF, SDF-1α, bFGF, and GM-CSF into the circulation, thereby switching the BM niche from a quiescent state to a highly pro-angiogenic and pro-tumorigenic environment. This, in turn,

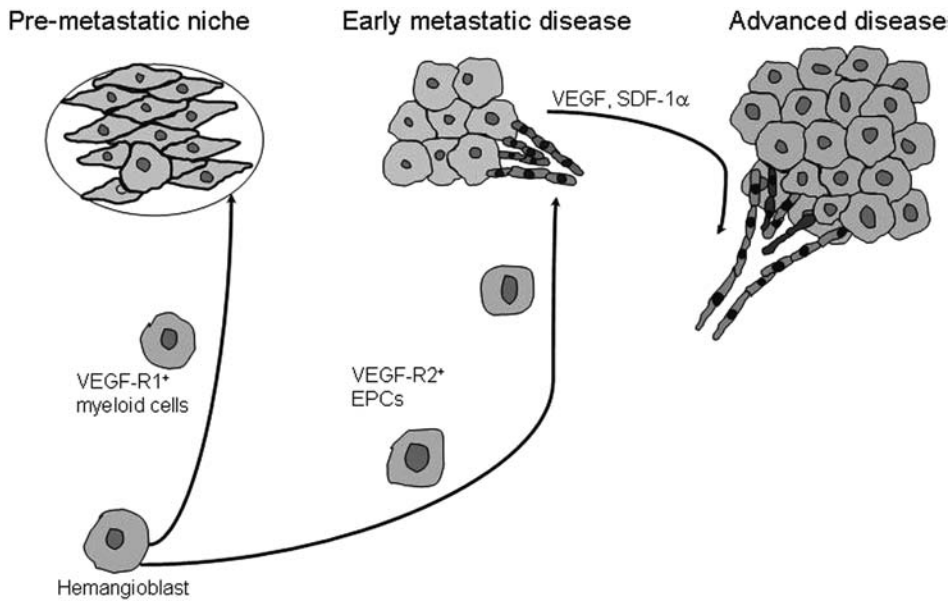

Fig. (2). Endothelial progenitor cells may engraft within tumor vasculature. The hypoxic environment of a growing tumor leads to the SDF1-α- and VEGF-mediated mobilization of bone marrow-derived of VEGF-$R1^+$ myeloid cells, which establish the pre-metastatic niche at the primary tumor site and prepare the organ for metastasis [57]. The VEGF-$R1^+$ cell clusters, in turn, direct tumor cell recruitment, adherence and growth and recruit VEGF-$R2^+$ EPCs. Herein, the latter contribute to enlarging the vascular network and promote further tumor growth [57].

promotes the mobilization of both hematopoietic and endothelial progenitors, which are then recruited to primary tumors or metastatic lesions (Fig. **2**) [57]. Indeed, it should be pointed out that EPCs co-operate with hematopoietic progenitor cells and mature myeloid cells, in particular $Tie2^+$ monocytes, to support cancer progression and metastasis [11, 57]. The first evidence about EPC contribution to tumor vasculature was provided by Lyden and coworkers [56], who utilized immunodeficient mice engrafted with BM cells expressing the β-galactosidase gene (lacZ) under the control of endothelial-specific promoters. The histological inspection of subcutaneously injected Lewis lung carcinoma cells (LLCs) revealed lacZ-positive cells in tumor vessels [56]. Consistently, sex chromosome fluorescence *in situ* hybridization (FISH) analysis of secondary tumors arising in subjects previously transplanted with BM cells from a sex-mismatched donor revealed that tumor ECs are donor-derived [58]. Finally, a number of studies evaluated the incorporation of BM-derived GFP^+ EPCs into tumoral vasculature by assessing luminal cells positive for the following markers: 1) GFP, which determines bone marrow derivation; 2) isolectin IB4, which ensures luminal incorporation; 3) CD31, which confirms endothelial origin; and 4) CD11b, which rules out any hematopoietic contamination [11, 13, 14]. This rigorous examination of vessel-incorporated BM-derived ECs has not been systematically carried out in all the studies aiming at assessing EPC integration into tumoral endothelium [13, 59, 60]. The wide variation in the methods of tissue sampling, as well as the number of cells transplanted and the animal models examined, might explain the controversy on the extent of EPC contribution to tumor vasculature, which may be extremely variable (from 0% to more than 90%) [13, 59, 60]. Moreover, EPC involvement in carcinogenesis has been shown to depend on type, stage and location of tumour [13, 59, 60]. The injection of a monoclonal antibody against VEGF-R2 (see below) to impair EPC mobilization in mice implanted with a B6RV2 lymphoma inhibited angiogenesis and severely affected tumor growth [56]. Moreover, the conditional suppression of Id1, a transcription factor controlling EPC egression from BM, in mice transplanted with Lewis Lung Carcinoma cells did not prevent primary tumor growth, but dramatically curtailed the total number of metastasis [11]. These observations led clinicians to regard EPCs as the most promising target to affect metastasis progression and prevent death in cancer patients, who are usually diagnosed when the angiogenic switch has already occurred and chemotherapy and vascular disruptive agents (VDA) fail to improve the outcome [13, 59]. This notion was reinforced by the finding that cytotoxic drugs and VDA may promote a further EPC spike into the peripheral circulation, thereby resulting in tumor relapse and resistance to long term treatments [13, 15]. Studies conducted on animal models suggested that targeting tumoral angiogenesis by inhibition of VEGF signalling might prove to be an efficient means to adverse cancer growth and metastasis in human subjects. Patients treated with either humanized anti-VEGF neutralizing monoclonal antibodies (i.e. bevacizumab and avastin) or tyrosine kinase inhibitors (i.e. sorafenib and sunitinib) displayed clinical benefit in a number of randomized trials conducted on a number of cancer types [61]. This strategy should also prevent EPC mobilization and recruitment to tumor site. Unfortunately, the efficacy of anti-VEGF therapies is short-lived and resistance arises in the majority of patients [62]. Unravelling the molecular underpinnings of EPC proliferation, tubulogenesis, and differentiation might shed light on novel pharmacological targets, possibly downstream the signalling pathway exploited by VEGF, to successfully impair tumoral neovascularisation.

5. THE CA^{2+} SIGNALLING MACHINERY IN ENDOTHELIAL CELLS: FOCUS ON $INSP_3$-DEPENDENT CA^{2+} RELEASE AND STORE-OPERATED CA^{2+} ENTRY

Intracellular Ca^{2+} signals may regulate a wide array of cellular functions, ranging from short-term responses, such as exocytosis, muscle contraction, and cytoskeleton rearrangement, to longer-term regulation of proliferation, migration, gene expression and apoptosis [3, 16-19]. In resting i.e. non-stimulated cells, the $[Ca^{2+}]_i$ is very low (around 100 nM) due to the activity of various mechanisms that extrude Ca^{2+} out of the cytosol [16]. More specifically, the plasma membrane Ca^{2+}-ATPase (PMCA) and the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) remove Ca^{2+} by direct ATP hydrolysis (Fig. **3**), while the Na^+/Ca^{2+} exchanger (NCX) clears cytosolic Ca^{2+} by exploiting the Na^+ gradient across the PM (Fig. **3**) [3, 16]. Cytoplasmic increases in Ca^{2+} levels within stimulated cells are due to the release of the ion from intracellular stores and show a typical spike-like time course: this event is often, but not necessarily, followed by Ca^{2+} entry from the extracellular medium due to the activation of Ca^{2+} channels in the PM (Fig. **3**) [16-19] Alternatively, the concerted interplay between Ca^{2+} release and Ca^{2+} entry may lead to repetitive Ca^{2+} oscillations that persist for as long as the agonist is presented to the cells, but without the toxic effects associated to a long-term sustained increase in $[Ca^{2+}]_i$ [63-66]. The exhaustive description of the molecular machinery responsible for intracellular Ca^{2+} signalling in ECs is beyond the scope of this article and are covered by recent and comprehensive reviews [21, 67]. Herein, we will briefly recall that the endoplasmic reticulum (ER) houses the largest intracellular Ca^{2+} reservoir in ECs amounting to approximately 75% of the total cellular storage capacity (Fig. **3**), the remaining 25% being loaded within the mitochondrial matrix [67]. The endothelial ER consists of tubulo-vesicular structures which are interconnected at various sites to form a widespread network within the cell. Ca^{2+} is captured into ER lumen, where it attains a concentration of about 100-500 μM, by high-affinity ($K_m \approx 1$ μM) SERCA pumps (Fig. **3**) [18, 67, 68]. An increase in $[Ca^{2+}]_i$ up to 1 μM is the key signal to activate vascular ECs following recruitment of either $G_{q/11}$ protein-coupled receptors (GPCRs) or tyrosine-kinase linked receptors (TRKs) (Fig. **3**) [3, 21, 67]. Accordingly, ligand binding to either GPCRs or TKRs results in the activation of various isoforms of the PM-bound phospholipase C (PLC). More specifically, the β isoform of PLC (PLCβ) is engaged by GPCRs, while TKRs recruit PLC-γ (Fig. **3**) [16-19]. PLC, in turn, cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2), a minor phospholipid component of cell membranes, into inositol-1,4,5-trisphosphate ($InsP_3$) and

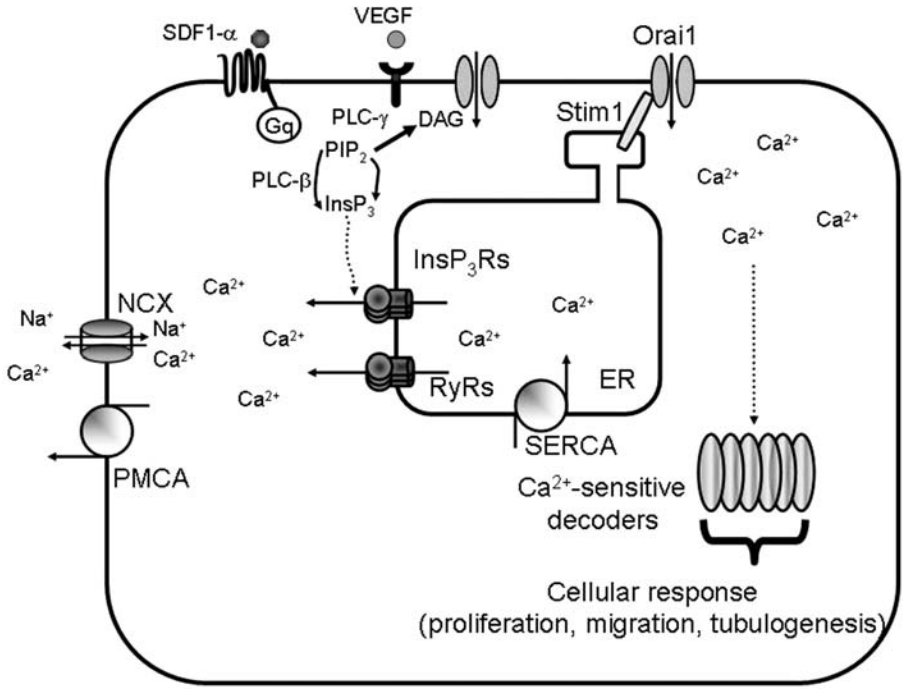

Fig. (3). Overview of the basic elements of the Ca^{2+} signalling machinery in endothelial cells. An increase in $[Ca^{2+}]_i$ is the physiological response to a variety of extracellular ligands, such as SDF-1α and VEGF, which bind to their specific plasmalemmal receptors. The following activation of PLC-β (by GPCRs) or PLC-γ (by TRKs) cleaves PIP_2 to yield $InsP_3$ and DAG. $InsP_3$, in turn, releases Ca^{2+} from the ER reservoir *via* the recruitment of $InsP_3Rs$, while DAG may induce Ca^{2+} entry by gating the TRPC3 and TRPC6 non-selective cation channels. The $InsP_3$-dependent drop in ER Ca^{2+} levels causes the opening of an alternative Ca^{2+}-permeable route on the PM, namely Orai1, by inducing Stim1 activation and clustering in well defined *puncta* beneath the plasmalemma. Ca^{2+} signals return to pre-stimulated levels through the concerted action of the mitochondrial uniporter, SERCA and PMCA pumps, as well as through NCX. The signalling function of Ca^{2+} is carried out by a number of Ca^{2+}-sensitive decoders (calmodulin, calmodulin-dependent kinases, calcineurin) and downstream targets. GPCR, G-protein coupled receptor; TKRs, tyrosine kinase-receptors; PLC, phospholipase C; PIP_2, phosphatidylinositol-4,5-bisphosphate; $InsP_3$, inositol-1,4,5-trisphosphate; DAG, diacylglycerol; $InsP_3Rs$, $InsP_3$ receptors; RyRs, ryanodine receptors; NCX, Na^+–Ca^{2+} exchanger; PMCA, plasma membrane Ca^{2+} ATPase; SERCA, Sarco-Endoplasmic Reticulum Ca^{2+}- ATPase.

diacylglycerol (DAG) (Fig. **1**) [16-19]. $InsP_3$ diffuses within the cytoplasm and binds to Ca^{2+}-permeable $InsP_3$ receptors ($InsP_3Rs$) located in the ER membranes, thus causing a short-lasting Ca^{2+} efflux (Fig. **3**) [16-19]. ECs possess all the 3 known $InsP_3R$ isoforms i.e. $InsP_3R$-1, $InsP_3R$-2, and $InsP_3R$-3, the pattern of expression being $InsP_3R$-3>$InsP_3R$-2>$InsP_3R$-1 [69]. $InsP_3Rs$ may form either homo- or hetero-tetramers, and exhibit a biphasic dependence on cytosolic Ca^{2+}: $InsP_3$ induces $InsP_3R$ opening when the surrounding Ca^{2+} ranges between 50 and 200 nM, whereas higher Ca^{2+} levels inhibit Ca^{2+} release at saturating $InsP_3$ concentrations [70]. The drop in the $InsP_3$-dependent Ca^{2+} pool signals the activation of a plasmalemmal Ca^{2+}-permeable pathway, which has therefore been termed store-dependent Ca^{2+} entry (Fig. **3**) [71-74]. In addition to refilling the ER Ca^{2+} pool, store-operated Ca^{2+} channels (SOCs) control a variety of endothelial processes, such as nitric oxide (NO) synthesis, cell proliferation and tubulogenesis, gene transcription and luminal permeability [71, 74-77]. Store-operated Ca^{2+} entry (SOCE) is activated by the $InsP_3$-store depletion due to the physical coupling between the ER Ca^{2+}-sensor, Stromal Interaction Molecule-1 (Stim1), and Orai1, which serves as pore subunit of the Ca^{2+}-permeable channel on PM [78-80]. Stim1 possesses an intraluminal region of approximately 22 kDa after cleavage of its signal sequence, a single transmembrane TM segment, and a cytosolic domain of about 51 kDa [78-80]. The ER-luminal portion contains an EF-hand domain (EF1), which serves as ER Ca^{2+}-sensor due to the presence of a binding site located between an E and an F helix that are aligned like a thumb and a finger, and a sterile α-motif (SAM) domain. A hidden, non-canonical EF-hand domain (EF2), so called due its surprising lack of affinity for Ca^{2+}, is also present between EF1 and SAM. The cytosolic domain comprises 2 or 3 coiled-coil regions, which overlap with an ezrin-radixin-moesin (ERM) motif, a serine/proline-rich (S/P) sequence and a polybasic lysine rich (KKK) domain [80]. In addition, the ERM domain of Stim1 presents crucial Orai-activating regions, which have been termed Orai-activating small fragment (OASF), CRAC-activating domain (CAD; see below for definition of CRAC), or Stim-Orai1 activating region (SOAR), and include the second coiled-coil domain and the following approximately 55 amino acids [80]. When ER Ca^{2+} concentration falls below a threshold level due to $InsP_3Rs$-dependent Ca^{2+} release, Ca^{2+} dissociates from EF1, thereby causing the unfolding of the adjacent EF-SAM domains and multimerization of Stim1 [80]. The Stim oligomers rapidly redistribute to peripheral ER sites, termed *puncta*, in close proximity to PM, bind to and activate Orai1 [80]. Orai1, in turn, is a 33 kDa PM protein with a tetraspanning PM topology and cytosolic NH_2- and COOH-tails [80]. Orai1 exists as a dimer in unstimulated cells, but forms tetramers after the $InsP_3$-dependent store emptying [80]. Ca^{2+} influx is then gated by the direct, physical interaction between the COOH-terminal coiled-coil regions of Stim1 and an NH_2-terminal domain proximal of the first TM α-helix of Orai1 and a COOH-terminal coiled-coil domain of the channel protein [80]. Two negatively charged glutamate residues in the first and third TM domain of Orai1, namely E106 and E190, are supposed to act as Ca^{2+}-binding sites in the ion channel pore [80]. Other SOCE-related proteins are Stim2, Orai2, and Orai3, which may contribute to store-dependent Ca^{2+} influx in heterologous expression systems, although their contribution to SOCs in naïve cells is yet to be elucidated [78-80]. Stim2 might, however, regulate basal Ca^{2+} entry in human umbilical vein ECs (HUVECs) by virtue of its higher sensitivity to ER Ca^{2+} levels [81]. Stim1- and Orai1-mediated Ca^{2+} entry is gated by a current displaying biophysical features similar to those of the Ca^{2+} release activated Ca^{2+} (CRAC) current (I_{CRAC}) recorded in hematopoietic cells i.e. strong inward rectification, reversal potential (E_{rev}) >+60 mV, permeability to Ca^{2+}, but not to Na^+ and K^+ under physiological conditions, and a single-channel conductance in the order of fS [71, 82]. An alternative pathway for SOCE has been reported in a number of ECs from various vascular beds, where a Ca^{2+}-selective SOCE has not been attributed Orai1/Stim1, but to a TRPC1/TRPC3/TRPC4 complex [83]. These are members of the Canonical Transient Receptor Potential (TRPC) family of non-selective cation channels, which consists of 7 isoforms (TRPC1-TRPC7). TRPC channels resemble voltage-gated Na^+, K^+, and Ca^{2+} channels in that they have six putative TM domains, cytosolic NH_2 and COOH tails, and a pore domain between TM5 and TM6 [84, 85]. However, the paucity of positively charged arginines in TM4 helices indicates the lack of voltage sensitivity of TRPC channels [84]. The NH_2-terminal contains 4 ankyrin-like repeats, while the protein:protein interaction sites on the COOH-terminal include a conserved EWKFAR sequence (TRP signature motif), a highly conserved proline rich motif, a calmodulin/$InsP_3R$ binding domain and a predicted coiled-coil region [85]. TRPC channels may be subdivided into 4 families: TRPC1, TRPC2 (which is a pseudogene in humans), TRPC3/6/7, which share 70-80% amino-acid identity, and TRPC4/5, which share 65% homology [84]. Similar to voltage-gated channels, functional TRPC channels may assemble as either homo- or hetero-multimers of four TRPC subunits; in particular, it has been suggested that the heteromultimerization process may confer Ca^{2+} selectivity on the TRPC complex [84, 86]. Whereas TRPC3/6/7 may be activated by DAG in ECs from all the vascular districts analyzed (Fig. **3**) [86], a recent study has suggested that in human lung ECs SOCs are comprised of one TRPC1, one TRPC3, and two TRPC4 [83]. According to this model, the extent of store depletion is conveyed to PM by protein 4.1, which links the cytoskeletal protein, spectrin, to TRPC4. The latter, in turn, contributes pore-forming subunits to the store-dependent channel along with TRPC1 [83]. Alternatively, TRPC1 may be informed about the ER Ca^{2+} content by the caveolin 1-mediated direct binding to $InsP_3R$-3 [87]. It should further be pointed out that Stim1 itself may bind to TRPC1, TRPC4, and TRPC5 and determine their function as SOCs in a heterologous expression system [84, 88].

6. CA^{2+} STIMULATES CELL PROLIFERATION, TUBULOGENESIS, AND DIFFERENTIATION

It has long been known that intracellular Ca^{2+} signals are crucial to EC proliferation, adhesion, spreading, migration, and tubulogenesis [3, 19-21, 89]. This feature is supported by the observation that a variety of growth factors, including VEGF, bFGF, and EGF [63, 90, 91], as well as SDF-1α [92], elicit a Ca^{2+} responses in mature ECs. For instance, VEGF

may induce either a monotonic elevation in $[Ca^{2+}]_i$ [90, 91] or a Ca^{2+} spike followed by a plateau phase of lower amplitude [73], while EGF triggers repetitive Ca^{2+} oscillations [63]. Consistently, either genetic ablation or pharmacological blockade of SOCE prevents EC growth by causing cell arrest at S and G2/M phases [71, 73, 74, 93]. Store-dependent Ca^{2+} influx is translated into a proliferative signal by the downstream targets of the Ca^{2+}/calmodulin (CaM) pathway, such as calcineurin and CaM kinase II (CaMKII), which regulate the activation and the assembly of cyclin-dependent kinases (cdc) and their activating partners i.e. cyclins (Fig. **4**) [94, 95]. In particular, calcineurin may drive the expression of both cyclin A and cdc2, which, in turn, form a complex responsible for entry into and progression through the S phase (Fig. **4**) [95, 96], while CaMKII triggers the exit from M-phase by stimulating cyclin B degradation and cdc2 inactivation (Fig. **4**) [97, 98]. Moreover, SOCE induces the expression of cyclin D1 [99], cyclin D2 [100], and cyclin D3 [101] which may all assemble with cdc4 to ensure progression through G1 [98]. Finally, SOCE may regulate the cell cycle through the recruitment of Ca^{2+}-sensitive transcription factors, such as 1) cAMP-responsive element binding protein (CREB) [102], which binds to the cyclin D1 promoter and promotes its transcription (Fig. **4**) [95]; 2) nuclear factor of activated T-cells (NFAT) [75], which favours the G0/G1 transition by inducing the expression of the immediate early gene, Myc, stimulates progression through G1 by increasing cyclin D1 and cyclin D2 levels, and drives the entry into S phase by enlisting the E2F/Rb pathway (Fig. **4**) [95, 98]; and 3) nuclear factor *k*B (NF-*k*B), which is involved in the transcription of cyclin D1 (Fig. **4**) [103, 104]. Orai1-dependent Ca^{2+} microdomains couple to gene transcription by specifically engaging NFAT and NF-kB during the course of an oscillatory change in $[Ca^{2+}]_i$ [78, 105]. This notion also applies to mature ECs, where a train of repetitive Ca^{2+} spikes provides the most suitable stimulus to induce the nuclear translocation of both transcription factors [66, 106]. Moreover, intracellular Ca^{2+} oscillations orchestrate the cyclic changes in cell shape and adherence that accompany EC movement [107, 108]. High-frequency Ca^{2+} microdomains, the so-called "Ca^{2+} flickers", drive the leading edge of a migrating fibroblast, and probably of an EC, towards a chemoattractant [109]. Similarly, in ECs, Ca^{2+} oscillations at the trailing end trigger focal adhesion (FA) disassembly and generate traction force by engaging calcineurin and the Ca^{2+}-sensitive neutral protease calpain. The latter, in turn, has the potential to cleave several FA proteins, such as integrins, talin, vinculin, and FA kinase [64, 89, 108, 110, 111]. Furthermore, repetitive Ca^{2+} spikes may induce retraction of the rear end by stimulating the myosin light chain (MLC) kinase to phosphorylate myosin II and promote actin disassembly [108, 111]. Once again, SOCE provides the fuel to drive the cytoskeletal re-arrangement responsible for EC movement [72, 74, 77], albeit this process may be also regulated by non-store dependent Ca^{2+} permeable pathways [20, 21]. It is, therefore, not surprising that blockade of Stim1- and Orai1-mediated SOCE prevents all the steps involved in endothelial cell tubulogenesis, such as cell spreading and adherence to the substratum, motility, and formation of a patent lumen, both *in vitro* [72, 74, 89] and *in situ* [21, 89, 108]. When considering the pivotal role played by intracellular Ca^{2+} dynamics in controlling cell processes as vital as proliferation, migration, and gene expression, it is not surprising that Ca^{2+} signals may drive also cell differentiation. Oscillations in $[Ca^{2+}]_i$ have been recorded in a variety of progenitor and stem cells, such as serum-stimulated primary rat marrow stromal cells [112], human preadipocytes [113], and human cardiac fibroblasts [114]. Moreover, spontaneous repetitive Ca^{2+} spikes may arise in human mesenchymal stem cells [115], adult mice neural stem cells [116], and human cardiac progenitor cells [117]. In more detail, spontaneous Ca^{2+} spikes have been suggested to engage the specific cellular programs which stimulate a number of progenitor and stem cells to undergo differentiation. Such a process requires the expression of genes that specify cellular fate and down-regulation of genes that hold the cell in a pluripotent state [118]. For instance, spontaneous Ca^{2+} transients drive the acquisition of an adipocytic phenotype by human mesenchymal stem cells (hMSCs) by inducing the nuclear translocation of NFAT [115]. Similarly, RANKL (receptor activator of NF-kappaB ligand) induces osteoclastogenesis by activating NFATc1 *via* robust intracellular Ca^{2+} bursts [119]. In addition, spontaneous Ca^{2+} signals specify neuronal morphology and dictate neurotransmitter choice in mice neuronal progenitor cells [120], whereas the onset of Ca^{2+} spiking is a pre-requisite for adult liver stem cells to acquire a myocardial phenotype [121]. Conversely, the loss of spontaneous Ca^{2+} oscillations facilitates osteodifferentiation of hMSCs [122]. It is, therefore, evident that unravelling the Ca^{2+} signalling toolkit which regulates proliferation, migration, tube formation, and, possibly, differentiation of EPCs would not only gain valuable insights into the biology of such cells. Manipulating intracellular Ca^{2+} signals might enable pharmacologists with a powerful double-edged sword. On the one hand, they might enhance the rate and/or extent of repair of damaged tissue upon delivery of genetically transformed cells. On the other hand, they might selectively target the Ca^{2+} machinery responsible for EPC-dependent vasculogenesis to adverse tumour neovascularisation.

7. EVIDENCE ABOUT THE ROLE OF CA^{2+} SIGNALLING IN ENDOTHELIAL PROGENITOR CELLS: THE ROLE OF STORE-OPERATED CA^{2+} ENTRY

The first evidence about the role played by Ca^{2+} signalling in EPCs came from experiments conducted on $CD34^+$ progenitor cells assayed for their sensitivity to SDF-1α. This cytokine is crucial in initiating progenitor and stem cell mobilization from BM and establishes the gradient which tracks the way up to the engraftment site [123]. SDF-1α may bind to CXCR4 receptor, a seven transmembrane GPCR that recruits a network of signal transduction pathways, including the PLCβ3/PLCγ1/protein kinase C (PKC) cascade [123]. PLC activation implies $InsP_3$ production and, consequently, Ca^{2+} release from intracellular ER stores [16]. Accordingly, SDF-1α may induce a transient increase in $[Ca^{2+}]_i$ in $CD34^+$ enriched hematopoietic progenitor cells derived from both BM and PB [124-127]. Subsequent work has unveiled SDF-1α-elicited Ca^{2+} entry in PB-derived $CD133^+$ progenitors: this signal was abrogated by YM-58483/BTP-2 [123], one of most powerful and selective blocker of Orai1-mediated Ca^{2+} entry [78]. This result suggested that SOCs may open following ER Ca^{2+} stores depletion by $InsP_3$ in BM-derived progenitor cells [124]. Importantly,

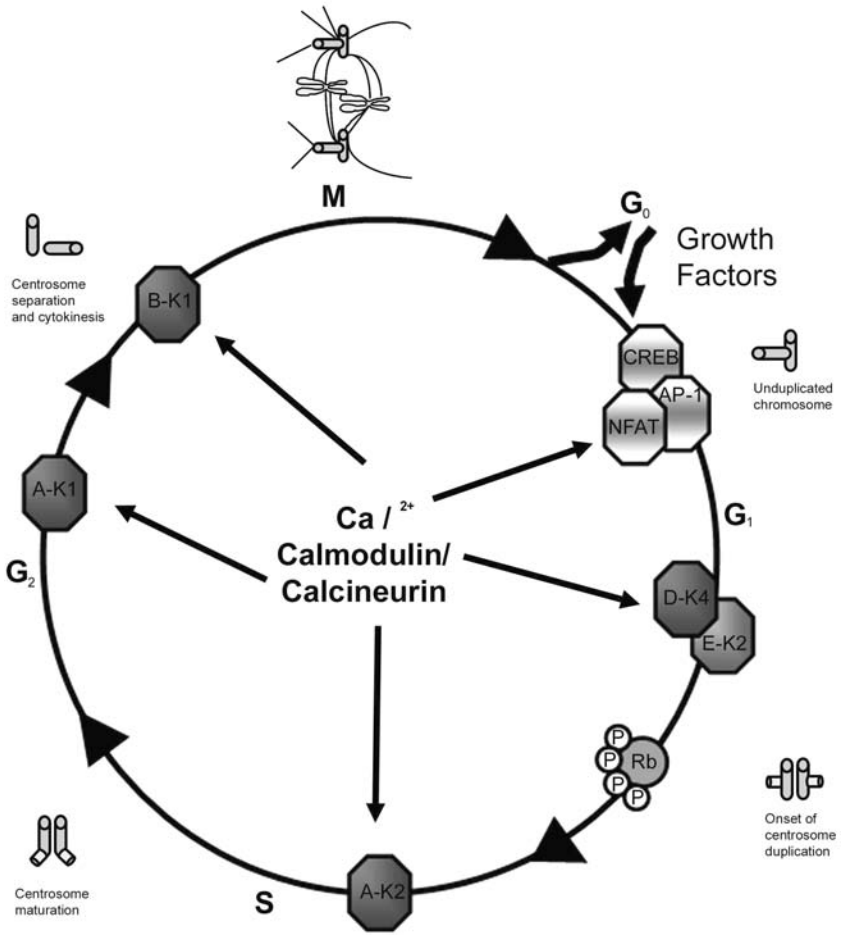

Fig. (4). Ca^{2+} and the cell cycle. The cell cycle comprises two distinct events: interphase, which in turn consists of G_1, S, and G_2, and mitosis. Each cell cycle transition is governed by specific cyclin-dependent kinases (cdk). While cdk protein levels remain stable during the cycle, cyclin levels oscillate and, in this way, they periodically activate cdks. The Ca^{2+}-dependence of the cell cycle mainly relies on the regulation of cyclin levels. Ca^{2+} regulates the progression through G_1 by binding to and activating CaM, which in turn engages CaMKII and calcineurin. Their concerted action results in the activation and/or expression of the transcription factors required to re-enter the cell cycle, including the nuclear factor of activated T-cells (NFAT), the nuclear factor-κB, the cAMP-responsive element binding protein (CREB), and members of the activating protein 1 (AP-1) family (c-fos, c-jun, and c-myc). These transcription factors regulate the expression of cyclin D1, which complexes cdk4 (D–K4), and cyclin E, which binds to and stimulates cdk2 (E-K2). Both D-K4 and E-K2 converge on the phosphorylation and consequent inactivation of the retinoblastoma protein (Rb). This event, in turn, induces the transcription of the E2F-regulated genes, whose products (cyclin A, cyclin E and Cdc25) govern G_1/S transition and DNA synthesis. In particular, when cells enter the S phase, cyclin A/cdk2 (A-K2) becomes activated and regulates the progression throughout the S phase itself. Later in the cell cycle, the A-K1 (cyclin A-cdk1) complex and Cdc25 are involved in the progression through G2, whereas the B-K1 (cyclin B-cdk1) complex governs the entry into mitosis. Adapted from [95].

BTP-2 affected SDF-1α-stimulated motility of $CD133^+$ cells in a 3-dimensional collagen matrix [134]. In agreement with these data, CXCR4 over-expression enhanced Ca^{2+} fluxes and cellular migration towards the BM of $CD34^+$ cells transplanted into immunodeficient mice [125]. Collectively, these reports indicate that SOCE provides the fuel to the molecular engine driving the migrational response of BM-derived progenitor cells *in vitro* as well as *in vivo*. These earlier studies, however, focus on hematopoietic and progenitor stem cells, rather than true EPCs. The first mechanistic link between intracellular Ca^{2+} signalling and tissue neovascularisation by endothelial committed progenitor cells was found by Maeng and coworkers [126], who investigated the role of the insulin-like growth factor 2 (IGF2)/IGF2 receptor (IGF2R) system in ECFC homing. As extensively described in Paragraph 2, ECFCs truly belong to the endothelial lineage and are able of forming perfused vessels *in vivo*. A drop in O_2 tension induces CB-derived ECFCs to secrete IGF2, which in turn autocrinally acts on IGF2R to promote chemotactic motility and adhesion to the extracellular matrix. When the cells were intravenously injected into a murine model of hindlimb ischemia, the IGF2/IGF2R system stimulated exogenous cells to lodge into the injured area and rescue blood perfusion. Remarkably, IGF2-induced cell migration and adhesion were inhibited by either U73122, a widely employed PLC blocker, or BAPTA, an intracellular Ca^{2+} buffer. These data hinted at a role for Ca^{2+} signalling in cell homing *in vivo*. Consistently, silencing PLCβ2 with small interference RNA (siRNA) reversed ECFC adhesion to extracellular matrix [126]. These results clearly indicate a relevant role for cal-

cium ions in promoting the formation of new blood vessels by ECFCs, but did not address the primary source of the underlying Ca^{2+} signal. Our group has recently demonstrated that circulating human ECFCs express all the known $InsP_3R$ isoforms, namely $InsP_3R1$, $InsP_3R2$, and $InsP_3R3$, as well as Orai1, Stim1, TRPC1, and TRPC4 [127]. Notably, ECFCs do not possess TRPC3 and TRPC6, which may mediate VEGF-dependent Ca^{2+} entry and angiogenesis in mature ECs [20, 90]. Similarly, voltage-dependent Ca^{2+} inflow and RyRs-dependent Ca^{2+} release have not been detected in human ECFCs [127]. In agreement with these data, SOCE provides the main pathway for Ca^{2+} inrush into human ECFCs upon depletion of the $InsP_3$-sensitive Ca^{2+} reservoir [74, 127]. The genetic ablation of both Stim1 and Orai1 proteins by siRNA technology suppressed SOCE in these cells, whereas the external application of an anti-TRPC1 blocking antibody did not exert any statistically significant effect [74]. Similarly, the expression of a dominant negative (DN) mutant Orai1 (R91W) abrogated SOCE in ECFCs, while $InsP_3$-dependent Ca^{2+} release induced eYFP-GFP tagged Stim1 to re-arrange into near PM *puncta* [74]. These experiments clearly indicate that store-dependent Ca^{2+} inflow is underlain by the physical interaction between Stim1 and Orai1 in human ECFCs. Pharmacological blockade of SOCE with BTP-2 inhibited ECFC proliferation when the cells were bathed in an endothelial differentiation medium enriched in growth factors, such as VEGF, bFGF, and EGF [127]. In line with these findings, silencing Orai1 impaired ECFC tubulogenesis [74]. These data were supported by the finding that BAPTA, a membrane permeant buffer of intracellular Ca^{2+}, prevents ECFC from reaching the confluence after three days in culture and from organizing into bi-dimensional structures when plated into Matrigel plugs (Bonetti and Rosti, unpublished observations). It, therefore, appears that Stim1- and Ora1-mediated SOCE plays a key role in promoting ECFC growth and tubulogenesis, a feature which renders this signalling pathway promising to implement the outcome of CBT.

The contribution of SOCE to *in vitro* vasculogenesis was also assessed in rat BM-derived CACs. In these cells, SOCE was abrogated by siRNA-mediated knockdown of both Stim1 and TRPC1 [128-130]. These findings concur with a model according to which TRPC1 may be gated by an electrostatic interaction between the positively charged amino acids in the Stim1 polybasic tail (KKK) and the negatively charged ones in the COOH-terminus of TRPC1 [131], as anticipated in Paragraph 5. This hypothesis is supported by the evidence that store depletion caused Stim1 and TRPC1 to form a molecular complex in rat BM-derived CACs [128]. The genetic suppression of either protein significantly suppressed CAC proliferation, migration, and tubulogenesis [128-130]. More specifically, knockdown of TRPC1 caused an arrest in the G1 phase by inducing the up-regulation of 9 cell cycle-related genes, including Ak1, Brca2, Camk2b, p21, Ddit3, Inha, Slfn1, Mdm2, Prm1, and the down-regulation of other 4 genes, such as Bcl2, Mki67, Pmp22, and Ppp2r3a [129]. In particular, Slfn1, which is a member of the Schlafen protein family and a negative regulator of cell growth by causing cell cycle arrest in G1, mediates the anti-proliferative effect of TRPC1 suppression on rat BM-derived CACs. Similarly, the pharmacological inhibition of SOCE with BTP-2 suppressed rat BM-derived CAC proliferation and tubulogenesis [130]. Although these studies did not address Orai1 involvement in SOCE, and were performed on murine, rather than human cells, they highlight notable differences in the molecular make-up of SOCE between ECFCs and CACs, which are routinely included in the same term i.e. EPC. When considering the local nature of intracellular Ca^{2+} signalling, and the specific coupling between a given plasmalemmal Ca^{2+}-permeable channel and its downstream Ca^{2+}-sensitive decoders, future studies will have to assess: 1) whether the ability of ECFCs, but not CACs, to participate in neo-angiogenesis is attributable to Ca^{2+}-dependent effectors which reside in close proximity to Orai1, but not TRPC1; 2) the molecular composition and functional role played by SOCE in human CFU-ECs, which stimulate angiogenesis *in vivo*, but do not differentiate in the ECs which subsequently build up the vasculature; 3) whether the animal species influences the molecular make-up and the downstream signalling pathways of SOCE in a given cell population. For example, it would be interesting to investigate whether store-dependent Ca^{2+} influx is gated by the interaction between Stim1 and Orai1 also in murine or rat ECFCs. Disclosing this issue would be useful to safely translate the results obtained from animal models to clinical practice, such as CBT and anti-tumoral treatments.

8. STORE-OPERATED CA^{2+} ENTRY SUSTAINS VEGF-INDUCED CA^{2+} OSCILLATIONS IN ENDOTHELIAL COLONY FORMING CELLS

As mentioned in the previous Paragraph, SOCE is activated by an $InsP_3$-dependent drop in ER Ca^{2+} content to drive ECFC proliferation and tubulogenesis. These functional assays were carried out by bathing the cells in a commercially available medium (i.e. EGM-2, supplied by Lonza) enriched in a variety of growth factors, including VEGF. It has long been known that VEGF is released into circulation by hypoxic tissues to induce EPC mobilization and recruitment into sites of active neovascularisation [23, 24, 132]. VEGF is a homodimeric, heparin-binding glycoprotein that may interact with two TKRs, VEGFR-1 (Flt-1) and VEGFR-2 (KDR or Flk-1) [132]. VEGF-binding induces the receptors to dimerize and, thus, activate the signal transduction cascade that regulates EPC fate [132]. Although EPC express both VEGFR-1 and VEGFR-2 [23, 132], only the latter is crucial for delivering cellular signals for mitosis, egression from BM, and acquisition of a mature endothelial phenotype [23, 132]. However, the molecular pathways whereby VEGF impact on EPCs are still unclear. VEGFR-2 exerts its pro-angiogenic effects on mature ECs by stimulating a number of signalling cascades, including the Raf-Mek-Erk and the PI3K/Akt pathways [132, 133]. In addition, ligand-dependent VEGFR-2 dimerization induces the autophosphorylation of specific intracellular tyrosine residues which are recognized by the Src homology-2 (SH2) domain of several intracellular proteins, including PLC-γ [132, 133]. VEGF induce mature ECs to undergo angiogenesis by recruiting either Stim1 and Orai1 [71-73] or the DAG-sensitive channels, TRPC3 and TRPC6 [19-21]. We have recently examined the signalling pathways recruited by VEGF in human circulating ECFCs. This study was conducted by removing the cells from EGM-2 and bathing them in a physiological salt solution for 1 h before re-exposition to VEGF.

We found that VEGF stimulates human ECFCs to generate asynchronous Ca^{2+} oscillations (Figs. **5A-5B**), whose latency, amplitude, and frequency are correlated to the growth factor dose. In more detail, the amplitude of the Ca^{2+} spikes increased and the latency of the Ca^{2+} response decreased as VEGF dose was raised from 1 to 50 ng/ml. Conversely, the mean number of Ca^{2+} spikes over a defined period of time (1 h) reached a peak at 10 ng/ml VEGF [134]. VEGF-induced Ca^{2+} spikes are shaped by the interplay between $InsP_3$-dependent Ca^{2+} release and SOCE. VEGF binding to VEGFR-2 engages the PLC-γ/$InsP_3$ signalling pathway, which, in turn, promotes the periodic release of Ca^{2+} from ER. The Ca^{2+} transients, however, rapidly run down in the absence of extracellular Ca^{2+} or in the presence of BTP-2 (Figs. **5C-5D**). Therefore, SOCE is required to refill the intracellular Ca^{2+} pool and maintain the oscillatory signal over time [134]. These results have been corroborated by the evidence that Orai1 mediates VEGF-elicited Ca^{2+} influx in ECFCs [74] and sustains intracellular Ca^{2+} oscillations in a variety of cell types [65, 79, 105]. The statistical analysis of VEGF-induced Ca^{2+} waves confirmed that Ca^{2+} transients arise randomly and that standard deviation of the interspike interval (ISI) is of the same order of average ISI. As demonstrated by the mathematical modelling described in [135, 136], this feature is nicely explained by the spatial distribution of $InsP_3$Rs within ER membrane. $InsP_3$R clusters are randomly scattered the ER, so that $InsP_3$ produced by PLC-γ sensitizes all the channels in the cluster for stimulation by Ca^{2+} and triggers a local Ca^{2+} release. Ca^{2+} liberated at 1 elementary site would be unable to ignite Ca^{2+} mobilization from an adjoining site, as Ca^{2+} is a poorly diffusible messenger. If, by chance, a supercritical number of $InsP_3$R clusters simultaneously open, the CICR synchronizes all the unitary Ca^{2+} events and generate a global Ca^{2+} spike [105, 135, 136]. This mechanism would contribute to explain the cell-to-cell variability observed in VEGF-stimulated ECFCs. Suppressing VEGF-induced Ca^{2+} oscillations with either BAPTA, a membrane-permeable buffer of intracellular Ca^{2+}, or BTP-2 prevented ECFC proliferation and tubulogenesis [134]. The same effect was obtained by siRNA-mediated down-regulation of Orai1 [74]. The mechanistic link between repetitive Ca^{2+} spikes and the pro-vasculogenic action of VEGF is provided by NF-κB. In resting cells, NF-κB is retained in the cytosol by the complex with the inhibitory protein, I_{kB}, which masks its nuclear localization signals (NLS) [137]. Oscillations in $[Ca^{2+}]_i$ promote a phosphorylation cascade which is mediated by Ca^{2+}/CaM-dependent protein kinases and leads I_{kB} to site-specific ubiquitination and subsequent degradation [66, 106, 137]. Consequently, NF-κB is released from inhibition and translocates into the nucleus, where it activates the transcriptional programme responsible for cell survival and proliferation [137]. VEGF stimulates I_{kB} phosphorylation in human ECFCs, a reaction which is prevented by BAPTA, BTP-2, and thymoquinone, a specific inhibitor of NF-κB. Thymoquinone, in turn, abolishes ECFC proliferation and tubulogenesis, which hints at NF-κB as a downstream effector of VEGF-dependent Ca^{2+} oscillations [134]. Earlier work has suggested that periodic Ca^{2+} signals encode information in a `digital' manner, whereby the increasing strength of an extracellular stimulus results in an increasing frequency, but not amplitude, of intracellular Ca^{2+} spiking [65, 105, 138]. In this view, high- and low-frequency Ca^{2+} transients were shown to activate two different transcription factors, such as NFAT and NF-κB, respectively [138]. The stochastic nature of VEGF-induced Ca^{2+} oscillations in ECFCs, however, indicate that they cannot be regarded as a simple digital read-out of cell stimulation [139]. Remarkably, recent studies revealed that the sub-cellular spatial profile of the Ca^{2+} transient might be crucial in recruiting specific Ca^{2+}-dependent targets by repetitive Ca^{2+} waves. For instance, Ca^{2+} microdomains arising within a few nanometers of Orai1, rather than $InsP_3$Rs-dependent global Ca^{2+} oscillations, selectively engage the transcriptional programme responsible for mast cell activation by leukotriene C4 [65, 140]. Future work is required to address this issue and to gain more insights into the involvement of VEGF-induced Ca^{2+} oscillations into ECFC differentiation. In this regard, the oscillatory Ca^{2+} response does not desensitize as long as VEGF is exposed to the cells [134]. When considering that EPC take 3-4 weeks to acquire a mature phenotype in the presence of VEGF [141], it is conceivable that in the short term i.e. 12-72 hours, intracellular Ca^{2+} oscillations promote ECFC proliferation and tubulogenesis, whereas in the long term i.e. 3-4 weeks, they promote cell differentiation.

9. TARGETING CA^{2+}-PERMEABLE CHANNELS AS A NOVEL THERAPEUTIC APPROACH TO ENHANCE ENDOTHELIAL PROGENITOR CELL-BASED THERAPY

The role served by calcium ions in modulating EPC-dependent vasculogenesis hints at the Ca^{2+} handling machinery as an alternative option to overcome the limits of CBT and improve its therapeutic outcome. While a growing number of selective drugs may inhibit the diverse array of Ca^{2+}-related proteins i.e. channels and pumps, pharmacological activators of such pathways are far less known [142; see also Paragraph 11]. In addition, these compounds usually lack the selectivity required to target specific isoforms of Ca^{2+}-permeable pathways [142]. There is, however, general agreement that molecular engineering of stem cells (the so-called "gene therapy") to be injected into patients affected by severe cardiovascular diseases, such as myocardial ischemia and critical limb ischemia, may significantly augment the percentage of cells engrafting within the target tissue [8, 47, 48, 52]. This, in turn, would favour neovessel formation in the injured areas, thus improving tissue regeneration and restoration of the damaged functions. We refer the reader to a number of recent reviews for an exhaustive description of the most popular strategies currently adopted for *ex vivo* transfection of stem cells [51, 143]. Our poor knowledge of stem cells biology limited the amount of therapeutic gene targets to exploit for EPCs-based treatment of cardiovascular pathologies. For instance, EPCs engineered to overexpress VEGF displayed higher proliferative and adhesion capabilities *in vitro* than non-transduced cells [144]. In addition, when these cells were injected into a murine model of hind limb ischemia, both the rate of neovascularisation and the recovery of blood flow were significantly improved as compared with controls [144]. Similarly, transplantation of VEGF gene-transduced EPCs accelerated organization and recanalization of venous thrombi by increasing capillary density at this location [145]. A remarkable feature of com-

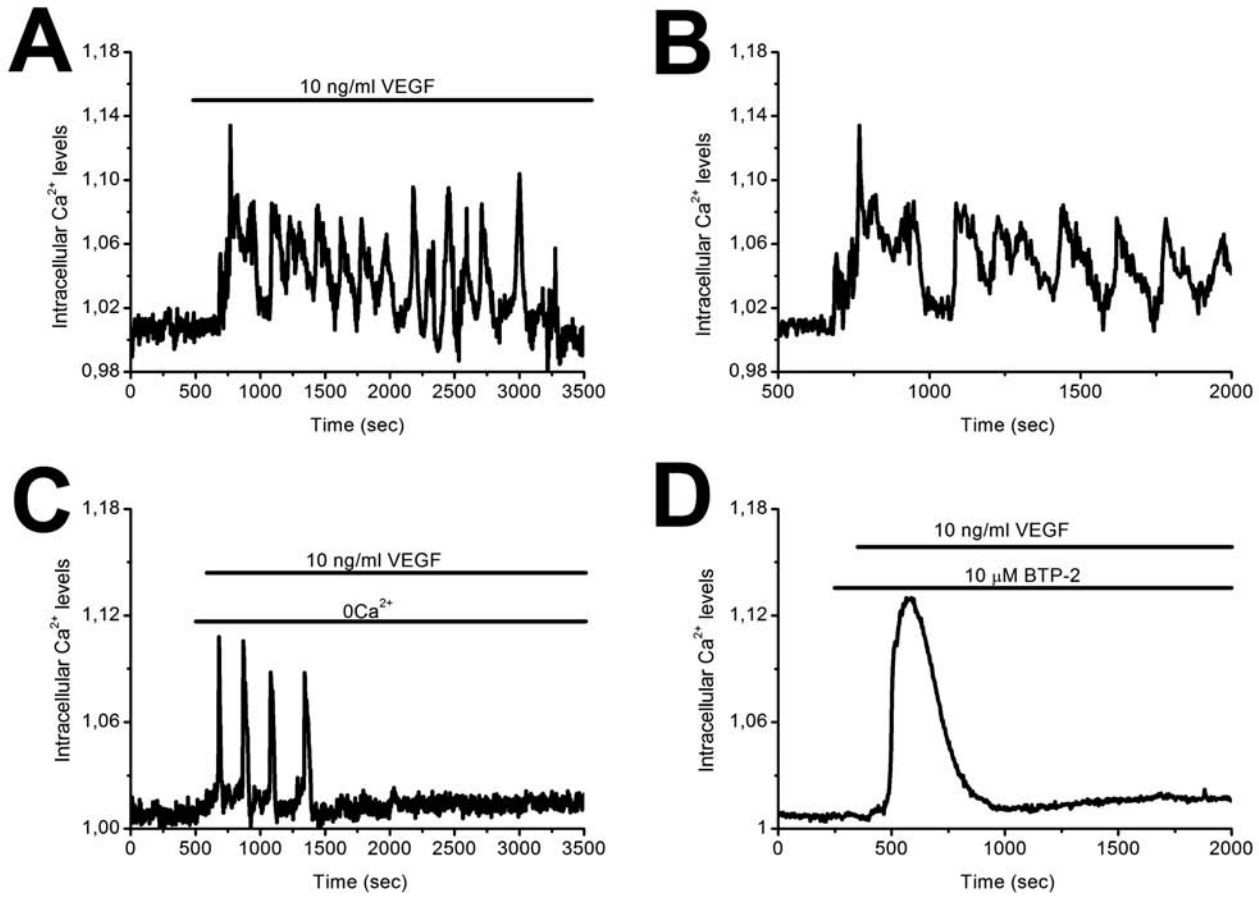

Fig. (5). VEGF induces oscillations in $[Ca^{2+}]_i$ in human circulating endothelial colony forming cells. A, repetitive Ca^{2+} spikes elicited by VEGF (10 ng/ml) in an ECFC loaded with the Ca^{2+}-fluorochrome, Fura-2, as described in [134]. **B**, expanded section of the tracing depicted in panel A. VEGF-elicited intracellular Ca^{2+} oscillations rapidly subside in absence of extracellular Ca^{2+} ($0Ca^{2+}$) (**C**) or in presence of BTP-2 (20 μM) (**D**), a selective inhibitor of store-dependent Ca^{2+} influx.

bining gene- and cell-based therapies concerns the number of cells employed to repair the damaged tissue. Indeed, the dose of VEGF-overexpressing EPCs required to induce neovascularisation was 30 times lower than that employed in previous trials carried out with wild type cells [144, 145]. In this light, the Ca^{2+} signalling machinery exploited by VEGF to stimulate ECFC proliferation, tubulogenesis, and differentiation could open a new avenue in CBT by providing a novel battery of molecular targets to enhance EPC-dependent neovascularisation in clinical settings. First, ECFC represent the most suitable cell population to achieve the therapeutic goal of rescuing blood perfusion in ischemic tissues [22, 26, 28]. Second, the following evidence hints at SOCE as a suitable signalling pathway to enhance the regenerative potential of ECFCs: 1) store-dependent Ca^{2+} influx maintains VEGF-elicited Ca^{2+} oscillations, which, in turn, drive ECFC proliferation and tubulogenesis by inducing the nuclear translocation of NF-κB [134]; 2) the genetic ablation of Orai1 severely impairs the formation of an organized tubular network when ECFCs are plated into Matrigel [74]; and 3) Orai1 proteins, rather than $InsP_3Rs$, are selectively coupled to gene transcription during repetitive Ca^{2+} spikes [65, 105, 140, 141]. Future work should aim at assessing whether transducing the cells with plasmids encoding for either Stim1, the physiological activator of Orai1, and Orai1 itself will improve the therapeutic outcome of either therapeutic or subtherapeutic doses of EPCs. Caution will, however, be required when transfecting EPCs with the molecular machinery responsible for SOCE. As a consequence of the tight stoichiometric coupling between Stim1 and Orai1 (~2Stim1:1Orai1) [146], the over-expression of Orai1 alone leads to a significant reduction in both SOCE amplitude [147, 148] and cell proliferation [147], while expression of Stim1 alone caused only a modest elevation in the peak of the Ca^{2+} current [148]. As reviewed in [149], the requirement of more than one ER Ca^{2+}-sensor (i.e. Stim1) to bind to and activate Orai1 turns out in a dilution of the coupling process and, therefore, in a decrease of SOCE when the channel protein (i.e. Orai1 itself) is up-regulated. On the other hand, a substantial (i.e. 25-to-50 fold) increase in Ca^{2+} influx is brought about by the co-expression of both Orai1 together with Stim1 [148, 149]. Genetic manipulation of SOCE might thus overcome the therapeutic limit imposed by the low number of ECFCs that are harvested from PB prior of *ex vivo* expansion and subsequent autologous injection [8]. In addition to enhance VEGF signalling, it is conceivable that the up-regulation of SOCE in *ex vivo* expanded EPCs will favour their engraftment within the target organ under the guidance of the SDF-1α

gradient. This notion is supported by the observation that SOCs drive the migration of $CD133^+$ progenitors [124] and that SDF-1α elicits a BTP-2-sensitive entry of Ca^{2+} in human ECFCs (unpublished observations by our group). Finally, genetic modification of the Ca^{2+} machinery might aid in improving the outcome of EPC-based therapy when the recipient is burdened with comorbidities. The lower regenerative potential of such cells in presence of the risk factors described in Paragraph 2 might be overcome by over-expressing the Ca^{2+} pathways, such as SOCE, tailored to selectively guide proliferation or motility. In this context, it would be interesting to compare both the amplitude and the molecular make-up of SOCE between control ECFCs and ECFCs isolated from PB of patients affected by cardiovascular risk factors, such as those described in Paragraph 2. Indeed, compelling evidence indicates that SOCs may be down-regulated by coronary atherosclerosis in porcine vessel wall cells [150], whereas SOCE is significantly increased in aortic smooth muscle cells isolated from hypercholesterolemic mice [151] and in bovine aortic ECs exposed to high glucose levels [152]. The feasibility of manipulating VEGF-induced Ca^{2+} oscillations to enhance ECFC lodging into ischemic organs has been further underscored by recent work conducted on $c\text{-}Kit^+$ human cardiac progenitor cells. The induction of repetitive Ca^{2+} transients by stimulating cyclic Ca^{2+} release from $InsP_3Rs$ before injection into infarcted mice heart enhanced their engraftment, expansion and formation of a myocyte progeny [117]. Although these results have not been translated into clinics yet, they do highlight the possibility to target the Ca^{2+} signalling toolkit to address the pitfalls which harm EPC-based therapy. As warned above, the components of the Ca^{2+} machinery underlying EPC proliferation, tubulogenesis, and differentiation might differ in cells isolated and cultured according to different protocols (see Paragraph 7). As a consequence, the molecular results obtained from circulating human ECFCs cannot be straightforwardly translated to $CD133^+$ progenitors or $CD34^+$-enriched cells.

10. CA^{2+} CHANNELS AS NOVEL MOLECULAR TARGETS TO TREAT TUMORAL NEOVASCULARISATION

Due to its multifaceted role in the control of angiogenesis, the Ca^{2+} signalling toolkit is a potential molecular target in novel therapies devoted to impair tumoral vascularization [19-21]. Accordingly, carboxyamidotriazole (CAI), a non-selective synthetic Ca^{2+} influx blocker, has been successfully employed in antiangiogenic therapy and it is currently under phase I and phase III clinical trials for the treatment of solid tumors [142]. The main drawback in targeting Ca^{2+} channels is related to the housekeeping roles of this ion in normal tissues potentially leading to harmful side effects. Clinical experience, however, already exploited blockers of voltage-gated Ca^{2+} channels in hypertension treatment, while CAI has only been shown to cause fatigue and vomiting, which are acceptable undesired effects for an oncologic drug [142]. The design of novel anti-cancer treatments based on the blockade of pro-angiogenic Ca^{2+} signals has hitherto focussed on Ca^{2+} channels expressed by mature ECs [19-21, 108]. CAI has been shown to abrogate VEGF-induced SOCE in HUVECs [73] and AA-evoked Ca^{2+} entry in breast tumor-derived ECs (B-TECs) [20]. These observations provide the molecular and cellular bases of the reported anti-angiogenic effect of such a drug. Tumor vascularization, however, may also be supported by BM-derived EPCs, which participate in primary tumor growth and drive the metastatic switch (see Paragraph 6). The finding that Stim1- and Orai1-mediated Ca^{2+} entry is well placed within the signalling cascade governing ECFC growth and tubulogenesis highlighted a potential, novel molecular target to combat tumor neovascularisation. We recently focussed on the molecular nature and the role played by SOCE in ECFCs harvested from PB of patients suffering from renal cellular carcinoma (RCC), a highly angiogenic cancer which is responsible for 20.000 deaths per year in Europe [153]. A number of evidences suggest that RCC vascularisation might be contributed by circulating EPCs. First, EPC levels are higher in RCC patients [154] and in human RCC xenograft models [155]. Second, RCC developing within a kidney allograft may display Y-positive chromosome vessels within a Y-negative tumor [156]. Unlike other solid cancers, however, toxicities as severe as neuropathies and gastrointestinal disorders have been reported during Phase II studies of CAI in patients with advanced RCC and refractory to immunotherapy [157]. We have shown that SOCE is up-regulated in ECFCs isolated from PB of RCC patients (RCC-ECFCs) as compared to control cells (N-ECFCs) (manuscript submitted). The higher amplitude of Ca^{2+} entry in RCC-ECFCs was associated to the over-expression of Stim1 and Orai1 i.e. the molecular underpinnings of SOCE, at both mRNA and protein level. Conversely, the magnitude of the intracellular Ca^{2+} response to the $InsP_3$-producing agonist, ATP, decreased because of the down-regulation of $InsP_3R$-2 and $InsP_3R$-3 and the loss of $InsP_3R$-1 (manuscript submitted). Similar to N-ECFCs, SOCE was selectively inhibited by BTP-2, whereas CAI suppressed also the $InsP_3$-dependent Ca^{2+} release (manuscript submitted). This feature is not surprising, as CAI is a non-specific drug which may impair both $InsP_3$ synthesis [73] and Ca^{2+} efflux through $InsP_3Rs$ [158]. It is, therefore, conceivable that CAI targets signalling pathway other than SOCE in patients affected by RCC, which would explain the severe side-effects described above [157]. RCC-ECFC proliferation and tubulogenesis were dampened by BAPTA and BTP-2 [134]: this feature gains particular relevance when considering that oncogenesis reduces the dependence of cell growth on extracellular Ca^{2+} entry in some [159], but not all [108], types of tumour. These novel observations on the provasculogenic effect of Ca^{2+} influx in ECFCs isolated from PB of tumoral patients might shed a bright light on alternative strategies to adverse tumor growth and metastasis. In addition to TRPC3 and TRPC6, which are expressed in mature ECs [20], but not in ECFCs [134], Orai1 and Stim1 provide pharmacologists and clinicians with promising molecular targets in the fight against cancer. Accordingly, SOCE drives VEGF-induced proliferation and tube formation in both ECs and ECFCs [71, 74, 127, 134]. This feature renders it possible to affect the two main processes underlying tumor vascularisation i.e. local angiogenesis and EPC recruitment, by affecting one single signalling pathway. A number of SOCE inhibitors [160] are available for pre-clinical studies aiming at assessing whether it is therapeutically feasible to prevent the angiogenic response to VEGF without the hur-

dles associated to anti-VEGF neutralizing antibodies and tyrosine kinase inhibitors (see Paragraph 4). These promising drugs, in the perspective of alternative anti-angiogenic strategies, will be described in the next Section. It is worth of noting here that intratumoral polymer implants have been recently developed to release a variety of drugs for the locoregional therapy of cancer and might be employed to release the agents able to affect Ca^{2+} entry pathways in both ECs and EPCs [161]. In addition, the local administration of siRNA(s) targeting a gene encoding for a membrane channel responsible for cell engraftment into the tumor site might be devised [see 162, for strategies and vectors in the field of anti-angiogenesis cancer gene therapy]. Due to their contribution to proliferation and tubulogenesis in both ECs and ECFCs, Orai1 and Stim1 might prove to be optimal targets for this kind of approach. Consistently, it has recently been shown that the local administration of lentiviral particles encoding short hairpin RNA (shRNA) targeting either Orai1 or Stim1 significantly reduced neointima formation at 14 days after balloon injury of rat carotids [163]. The same result was obtained by employing siRNA directed against Stim1 [164]. A few caveats should, however, be bore in mind with this kind of approach. First, the contribution of EPCs to tumor vascularisation has been clearly demonstrated only for a few murine models of solid cancer (LLC, B6RV2 lymphoma, melanoma, and breast cancer). Future work is required to assess whether and which EPCs sustain the neovessel growth in human malignancies [13]. The possibility that this process occurs in some, but not all, tumor types should also be considered. Second, although Orai1 and Stim1 stand out as promising targets for developing novel anti-angiogenic drugs, the molecular machinery responsible for EPC proliferation, migration, tubulogenesis, and differentiation might be altered in cells isolated from patients affected by different types of cancer. The molecular mechanisms underlying the rearrangement of the Ca^{2+} toolkit in RCC-ECFCs are far from being elucidated. It might be speculated that ECFCs remodel their repertoire of Ca^{2+} transporters in response to instructive signals released from tumor microenvironment [165]. This hypothesis is supported by the finding that normal ECs may undergo genetic and epigenetic modifications by receiving DNA directly from the tumor through apoptotic bodies, or following mRNA and microRNA transfer by microvesicles [165]. This novel mode of communication is likely to underpin TRPV4 overexpression in B-TECs [166]. The signals whereby a growing tumor may reprogram the gene expression profiles of sprouting ECs and, perhaps, circulating ECFCs, is a promising avenue of research in the quest for novel anti-angiogenic strategies.

11. PHARMACOLOGY OF STORE-OPERATED CA^{2+} ENTRY: PERSPECTIVES FOR NOVEL DRUGS FOR CHEMOTHERAPY

SOCE provides a membrane pathway which is easily and directly accessible *via* the bloodstream by either pharmacological modulators or specific antibodies [20]. A variety of molecules that are able to interfere with and suppress Orai1-mediated Ca^{2+} entry were discovered long before the identification of Orai1 gene and protein. These drugs may vary both in their molecular structure and in the mechanism of action and might provide either the rationale or the molecular template for designing more powerful and selective anti-angiogenic strategies. As aforementioned, indeed, no selective activator of Orai1 has hitherto been described that might be exploited to boost EPC-based therapy *in vivo*. Briefly, the most popular inhibitors currently employed to study the functional role of SOCE include cations (lanthanides and divalent transition ions), putative channel blockers (SK&F 96365, tetrandrine, 2-aminoethyl diphenylborinate or 2-APB), 3,5-bistrifluoromethyl pyrazole derivatives (BTP-1, BTP-2 and BTP-3), and mechanism-based inhibitors (nocodazole, ML-9, 2-APB, and U73122, which prevents PLC activation), non-steroidal anti-inflammatory drugs (acetylsalicylic-acid, mefenamic acid and sulindac sulfide), and CAI [167-170]. Other drugs, which have been extensively used in past to inhibit store-dependent Ca^{2+} channels, are P450 inhibitors (econazole, miconazole, clotrimazole and ketoconazole), cyclooxygenase blockers (niflumic acid, flufenamic acid and tenidap), and lipoxygenase inhibitors (nordihydroguaiaretic acid and eicosatetraynoic acid) [168, 169].

The simplest and most reliable SOCE blockers in vascular endothelium are the trivalent lanthanides La^{3+} and Gd^{3+} when used at low micromolar concentrations (1-10 μM) [71, 168, 171, 172]. Accordingly, these doses of La^{3+} and Gd^{3+} are able to selectively suppress Orai1-mediated Ca^{2+} entry, whereas they also interfere with TRPC channels at concentrations ≥50 μM [168]. In addition, these trivalent metals may prevent PMCA activity when employed in the low millimolar range [68, 168]. La^{3+} and Gd^{3+} exert a fast block at a site which lies close to, or in, the permeation pathway, thereby competing with permeating ions i.e. Ca^{2+}, and preventing their entry into the cytoplasm [173]. Trivalent cations have successfully been employed to suppress Orai1-mediated Ca^{2+} entry in both mature ECs [71] and immature EPCs [submitted manuscript], however, they are not useful for functional assays, such as the evaluation of proliferation rate and tubulogenesis, as they readily precipitate with multivalent anions and serum proteins contained in the growing media [173].

Another pharmacological agent that is widely exploited for probing store-dependent Ca^{2+} influx is 2-aminoethoxydiphenylborane (2-APB). This drug, also named bis-phenyl(2-aminoethoxy)borate or diphenylboric acid 2-aminoethyl ester, was initially introduced as a membrane permeable inhibitor of $InsP_3Rs$ before being identified among the most powerful SOCE blockers. 2-APB is a synthetic heterocyclic ring which is comprised of an ethanolamine chain, a boron-oxygen core (BOC) and 2 phenyl rings [168, 173]. At low concentrations (1-5 μM), 2-APB potentiates I_{CRAC} up to 5-fold in Jurkat lymphocytes, whereas, at higher concentrations, it first enhances I_{CRAC} but then the inhibitory effect dominates [168, 169, 173]. Such dose-dependent effect has been observed in other hematopoietic cells as well as in mature endothelium and circulating ECFCs. The mechanism(s) whereby 2-APB activates Orai1-mediated Ca^{2+} influx is yet to be elucidated. Conversely, its blocking action may be accomplished by 2 distinct mechanisms: 1) 2-APB may either directly block Orai1 channels from the extracellular, but not the cytosolic side, or oppose to Stim1 recruitment by Stim1, and, 2) 2-APB decouples Orai1-Stim1 proteins by reversing Stim1 accumulation beneath the plasma membrane. The

main drawback related to the employment of 2-APB to dissect SOCE-dependent signalling is its lack of specificity, whereas 2-APB has long been known to interfere with $InsP_3Rs$, SERCA pump, mitochondrial Na^+/Ca^{2+} exchanger, TRPM7, and several members of the Vanilloid subfamily of TRP channels (TRPV), such as TRPV1, TRPV2, and TRPV4 [168, 169, 173]. Much promise in the search for novel and selective SOCE inhibitors is based on 2 structural isomers of 2-APB, namely DPB162-AE and DPB163-AE (where the DPB stands for diphenylborinate). These compounds, which are formed by 2-APB dimers (with four phenyl rings) connected *via* 2 different linker chains, prevent store-dependent Ca^{2+} inflow with 100-fold more potency than 2-APB by interfering with Stim1 clustering [174]. Future work will have to be devoted to assess their inhibitory effect on both SOCE and SOCE-dependent signalling pathways in both mature ECs and immature EPCs.

The pyrazole derivative BTP-2, also termed YM-58483, is a recently synthesized inhibitor of SOCE which has been successfully utilized in both hematopoietic cells and other cell types [168-170, 173], including rat aortic ECs [175] and human EPCs. BTP-2 was originally found to act as an immunosuppressant by inhibiting both I_{CRAC} and I_{CRAC}-triggered signalling pathways in human Jurkat T cells with an IC_{50} ~100 nM and ~10 nM, respectively [78]. These experiments were conducted by adopting a chronic exposure to the drug, a feature which might explain the slow time-dependent blockade observed at extremely low doses [78]. Indeed, the subsequent electrophysiological analysis of I_{CRAC} inhibition by BTP-2 revealed that the IC_{50} was approximately 10-fold higher (2.2 μM) upon acute application of the compound [176]. This finding may be attributed to its rapid binding to an external site on Orai1 protein when the drug is applied at low micromolar concentrations (1-10 μM) [176]. Alternatively, the discrepancy between the results provided by the 2 different (chronic *vs.* acute) treatments might be due to the activation of the non-selective cation channel TRP melastatin 4 (TRPM4), which results in membrane depolarization and might, therefore, reduce SOCE by decreasing the driving force for Ca^{2+} entry [78, 176]. Indeed, BTP-2 may stimulate TRPM4 with an IC_{50} similar to that observed for Orai1 inhibition [176]. Whereas this mechanism might occur in mature ECs, it is unlikely in EPCs for the following reasons: 1) SOCE is not affected by 2 μM BTP-2 [127, 134], which may fully activate TRPM4 and depolarize the cells; 2) 20 μM BTP-2 still inhibits SOCE in EPCs exposed to CPA in absence of extracellular Na^+ to prevent the positive shift in the membrane potential caused by TRPM4. Along with TRPM4, BTP-2 might target Ca^{2+}-permeable channels other than Orai1, such as TRPC3 and TRPC5, in mature ECs when administrated at 1-10 μM [177]. These channels are, however, absent in human ECFCs, as recently demonstrated by our Group [127]. It thus appears that BTP-2 shall not be used as a selective drug to combat tumor neovascularisation, but it might serve as a starting point for the development of novel small-molecule inhibitors of SOCE. In this context, a new compound has been synthesized based on BTP-2 structure, namely Synta 66 (3-fluoro-pyridine-4-carboxylic acid (2',5'-dimethoxy-biphenyl-4-yl)-amide, GSK1349571A, patent ref. no. WO2005/009954 and US2004/02379; Synta Pharmaceuticals). Synta 66 prevents SOCE with an IC_{50} value of 3 μM in gut T cells, while it was found to ineffective towards a large panel of additional ion channels, including voltage-dependent Na^+ channels, VGCCs, and inward rectifier K^+ channels, TRPC1, 5, and 6 [178, 179]. Interestingly, Synta 66 blocks Orai1-dependent Ca^{2+} inflow with an IC_{50} of approximately 25 nM in both HUVECs and circulating ECFCs [74]. As a consequence, it abrogated *in vitro* tubulogenesis and *in vivo* angiogenesis [74]. As recently outlined, it is not yet clear whether this compound directly targets Orai1 or prevents the coupling between the emptied stores and the Ca^{2+} channel. Nevertheless, these recent findings hint at pyrazole derivates as exciting and promising molecular templates for the synthesis of alternative drugs to be safely utilized in anti-cancer strategies. It should, however, be pointed out that additional drugs, that have already been introduced in the healthcare system, are worth of being further probed as perspective anti-angiogenic drugs. Accordingly, salicylate, the major aspirin metabolite, and non-steroidal anti-inflammatory drugs (NSAIDs), which are commonly prescribed for the treatment of pain, inflammation and fever, have repeatedly been shown to block SOCE in a variety of cell types, including Jurkat and human colon cancer cells and rat aortic smooth muscle cells [180, 181]. They act so by preventing mitochondrial Ca^{2+} uptake, which is essential to prevent the Ca^{2+}-dependent inactivation of Orai1 channels [78, 180]. Substantial preclinical evidence demonstrated that NSAIDs have anti-angiogenic properties by virtue of their ability to inhibit cyclooxigenase-2 (COX-2) and interfere with human colorectal cancer vascularisation [181]. It would, therefore, be interesting to test whether these drugs may prevent tumor angiogenesis by interfering with SOCE in both ECs and EPCs.

12. CONCLUSION

EPCs are a double-edged sword. EPCs are mobilized from BM to support the cellular reconstruction of damaged tissues, such as ischemic limbs and infarcted myocardium. The same chemical stimulus i.e. hypoxia, that stimulates EPCs to restore lost cardiovascular functions, recruits these cells within tumoral neovasculature and turns them into a life threatening weapon. Cell-based therapy is rapidly emerging as a powerful approach to treating cardiovascular diseases by exploiting the regenerative properties of these cells. The hurdles associated to CBT, such as the low number of EPCs in PB and the cell population to inoculate, might be addressed by carefully harnessing the Ca^{2+} handling machinery in ECFCs. Deciphering the components of the Ca^{2+} toolkit utilized by these cells to proliferate, move along the chemoattractants gradient, and acquire a mature phenotype will outline novel genes, such as Stim1 and Orai1, to engineer in order to optimize the efficiency of stem cells transplantation. On the other hand, the Ca^{2+} signalling toolkit might be usefully exploited to shed bright light on the dark side of EPCs. Interfering with EPC recruitment by tumor neovascularisation is nowadays recognized as one of the most promising strategies to pursue in concert with VDA, chemotherapy or antiangiogenic treatments. The finding that SOCE controls ECFC proliferation and tubulogenesis highlights a novel, hitherto non-considered molecular target to design novel antiangiogenic strategies. Due to the scant information available on the ion channels and transporters that shape Ca^{2+}

signals in EPCs, however, a large amount of work is yet to be done before clinical benefits may be obtained by harnessing the Ca^{2+} armamentarium of these cells.

CONFLICT OF INTEREST

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

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