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5802 *Current Medicinal Chemistry,* **2012,** *19,* **5802-5818**

Store-Dependent Ca^{2+} Entry in Endothelial Progenitor Cells As a Perspective Tool to Enhance Cell-Based Therapy and Adverse Tumour Vascularization

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Abstract: Endothelial progenitor cells (EPCs) have recently been employed in cell-based therapy (CBT) to promote neovascularization and regeneration of ischemic organs, such as heart and limbs. Furthermore, EPCs may be recruited from bone marrow by growing tumors to drive the angiogenic switch through physical engrafting into the lumen of nascent vessels or paracrine release of pro-angiogenic factors. CBT is hampered by the paucity of EPCs harvested from peripheral blood and suffered from several pitfalls, including the differentiation outcome of transplanted cells and low percentage of engrafted cells. Therefore, CBT will benefit from a better understanding of the signal transduction pathway(s) which govern(s) EPC homing, proliferation and incorporation into injured tissues. At the same time, this information might outline alternative molecular targets to combat tumoral neovascularization. We have recently found that store-operated Ca^{2+} entry, a Ca^{2+}-permeable membrane pathway that is activated upon depletion of the inositol-1,4,5-trisphosphate-sensitive Ca^{2+} pool, is recruited by vascular endothelial growth factor to support proliferation and tubulogenesis in human circulating endothelial colony forming cells (ECFCs). ECFCs are a subgroup of EPCs that circulate in the peripheral blood of adult individuals and are able to proliferate and differentiate into endothelial cells and form capillary networks *in vitro* and contribute to neovessel formation *in vivo*. The present review will discuss the relevance of SOCE to ECFC-based cell therapy and will address the pharmacological inhibition of store-dependent Ca^{2+} channels as a promising target for anti-angiogenic treatments.

Keywords: Endothelial progrenitor cells, store-operated calcium entry, calcium signalling, neovascularisation, cardiovascular diseases, cell-based therapy, tumour, antiangiogenic drugs, Stim1, Orai1.

INTRODUCTION

Three major mechanisms underlie the formation and physical remodeling of blood vessels: 1) vasculogenesis, which refers to the *in situ* organization of endothelial progenitor cells (EPCs) that migrate to sites of vascularization, differentiate into endothelial cells (ECs), and coalesce to form the initial vascular plexus; 2) angiogenesis, which denotes the budding of new capillary branches from pre-existing blood vessels; and 3) arteriogenesis, which consists of the remodeling of an existing artery through an increase in luminal diameter, thus resulting in an augmented blood flow [1, 2]. It has long been thought that vasculogenesis occurs only during the early stage of organogenesis in embryonic development, whereas angiogenesis and arteriogenesis support the expansion of the vascular network both in the embryo and the adult organism [3]. This dogma has been revolutioned by the finding of EPCs in adult peripheral blood and bone marrow, and in cord blood [4-6]. Multiple series of experiments have confirmed that circulating EPCs may contribute both to postnatal growth of neovessels and to recovery from endothelial injury [1, 5]. These findings concur with the notion that bone marrow-derived EPCs home to ischemic sites and re-establish the local vascular network in a variety of different contexts, such as graft re-endothelization, wound healing, hindlimb ischemia and myocardial infarction [4, 7, 8]. Accordingly, a large number of preclinical studies conducted on animal models of ischemic disease have shown that *ex-vivo* expanded EPCs injected into athymic nude (immunodeficient) mice may ameliorate the functional performance of infarcted myocardium and restore vascular perfusion in occluded arteries [8-10]. EPCs are mobilized from bone marrow niches by a number of cytokines, such as vascular endothelial growth factor (VEGF) or stromal cell derived factor-1α (SDF-1α), which are synthesized and released to circulation by the hypoxic tissue [11]. As a consequence, EPC transplantation might be a proper way to induce tissue regeneration, and therapeutic strategies exploiting bone marrow-derived cells to treat cardiovascular pathologies, such as myocardial infarction and ischemic diseases, have rapidly been established [12-14]. Furthermore, emerging evidence indicates that EPCs may egress from bone marrow in response to the hypoxic microenvironment of growing tumors and contribute to the neovessels that sustain cancer development and metastatization [15, 16]. Therefore, EPC inhibition has been proposed as a novel means to adverse tumoral neovascularization [15, 17-19]. Despite their huge potential for therapeutic application, the signal transduction pathways regulating EPC proliferation, migration, tubulogenesis, and differentiation are still unclear. Unravelling the molecular mechanisms driving EPC incorporation within neovessels has been predicted to enhance the positive outcome of both cell-based therapy (CBT) and anti-angiogenic treatments [4, 13, 19, 20]. A number of recent studies have outlined the crucial role served by the so-called "store-operated Ca^{2+} entry" (SOCE) in the regulation of EPC proliferation, migration, and tubulogenesis [21-25]. The present review will first survey our current knowledge on the procedures to isolate EPCs from both peripheral and cord blood, on their employment in CBT, and on their participation to tumoral neovascularization. We will then outline the molecular nature and the contribution provided by SOCE to VEGF-induced pro-angiogenic Ca^{2+} signals in EPCs. We will finally address two key issues in the therapeutic employment of SOCE in clinical practice: 1) the design of plasmids and vectors committed to enhance Ca^{2+} entry in *ex vivo* transfected EPCs (in the context of CBT) and 2) the chemical structure and the mechanisms of action of the most popular small molecule inhibitors of SOCE (in the context of anti-cancer treatments).

ENDOTHELIAL PROGENITOR CELLS: DEFINITION, FUNCTION, AND THERAPEUTIC APPLICATIONS

At present, the term EPC fails to refer to a unique cell type with definable features, but encompasses a mix of cell populations, including cells of hematopoietic (progenitors and monocytes) or endothelial (endothelial colony forming cells, see below) origin, that exhibit pro-angiogenic activity [5, 6]. Such ambiguity in EPC definition depends on the lack of a specific cell surface marker or a distinct gene expression profile in the various bone marrow-derived cells and progenitors that have hitherto been employed in pre-

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1875-533X/12 $58.00+.00

clinical studies. We refer the reader to a number of recent reviews for an exhaustive description of the most popular protocols for isolation and identification of EPCs from human mononuclear cells (MNCs) [5-7]. Here, we will briefly recall that three different methods which are currently employed to harvest cells endowed with pro-angiogenic activity both *in vitro* and *in vivo* Fig. (**1**). First, EPCs may emerge after 4-7 days as spindle-shaped cells from MNCs seeded onto fibronectin-, collagen-, or gelatin-coated dishes in the presence of endothelial growth factors and foetal calf serum (FCS) Fig. (**1**). These cells, which are those originally depicted as endothelial progenitors by Asahara and co-workers [26], are referred to as colony forming units-ECs (CFU-ECs). It has, however, been established that CFU-ECs express several myeloid antigens, differentiate into phagocytic macrophages that ingest AcLDL and possess esterase activity, do not give raise to secondary EC colonies, and do not generate vascular networks *in vivo* (in immunodeficient mice) [27, 28]. Second, EPCs may be selected by culturing peripheral MNCs in a growth medium containing angiogenic cytokines for 4-7 days, upon which non-adherent cells are discarded and adherent cells which may enhance angiogenesis *in vivo* remain Fig. (**1**). These cells have been termed circulating angiogenic cells or CACs and display a number of endothelial markers, including CD31, von Willebrand factor (vWF), VE-cadherin and Tie-2/TEK, bind *Bandeiraea simplicifolia* (BS-1) and UEA-1 lectins, and have the potential to take-up acetylated low-density liproprotein/acLDL. Unfortunately, platelets may co-fractionate with MNCs and transfer a number of platelet antigens, including proteins which were regarded as endothelial specific, to any cell attached to the culture matrix. It is, therefore, not surprising that CACs are not able to physically incorporate within neovessels, although they may promote angiogenesis *in vivo* and rescue blood flow to ischemic tissues [5-7]. The third strategy to isolate putative EPCs consists of plating peripheral MNCs on fibronectin- or collagen-coated dishes in the presence of a commercially available endothelial selective medium Fig. (**1**). After 48 hour culture, non adherent cells are discarded and cultures are carried out for the next 20-30 days. This protocol leads to the outgrowth of endothelial colony forming cell (ECFC) colonies with a cobblestone morphology typical of mature endothelium that emerge at day 14 (cord blood-derived cells) or at day 21 (peripheral blood-derived cells) [5-7, 28]. Unlike CFU-ECs, ECFCs do not present monocyte/macrophage markers, may undergo up to 30 population doublings without senescence and replate into secondary and tertiary colonies, organize into capillary-like structures *in vitro*, and form perfused vessels *in vivo* (in immunodeficient mice) [28]. These features hint at ECFCs as the sole bone marrow-derived cell population endowed with a truly vasculogenic potential. The injection of bone marrow cells not belonging to the endothelial lineage, such as CFU-ECs and CACs, might explain the modest benefit of CBT in human patients suffering from either acute and chronic myocardial infarction [14, 20] or peripheral artery disease [13]. It has, however, been recently proposed that ECFCs are actually derived from resident endothelium rather than bone marrow [29]. Although ECFCs are rare in circulating human blood, with a frequency of approximately 1 colony/10^7 MNCs, they are considerably enriched in cord blood [28]. Moreover, cord blood-derived ECFCs display a much higher proliferation potential (up to 100 population doublings) and a greater telomerase activity than their peripheral counterparts [28]. The recent discovery of ECFCs prevented them from being probed in clinical trials on human subjects. A number of pre-clinical studies carried out on rodent models demonstrated that human ECFCs may form *de novo* vessels and establish connections with the systemic circulation of the host animal when implanted in matrix scaffolds in nude mice [5-7, 28, 30-33]. In this view, it must be noticed that cord blood-derived ECFCs form longer lasting vessels, as compared to peripheral blood-isolated cells, and that the rate of neovascularization is sensitive to several factors, such as cell passage and presence of perivascular cells [31, 32]. The feasibility of utilizing ECFCs in tissue engineering has been provided in a recent investigation, which exploited rat ECFCs to enhance fracture repair and bone regeneration by inducing neovessel formation at the site of injury [34].

Mounting evidence indicates that EPC-dependent vascularization may also support tumor growth and metastatization Fig. (**2A**) [15, 17, 35]. This model concurs with the increased release of VEGF and SDF-1α into circulation by hypoxic tumors cells and the well known notion that EPC levels augment in peripheral blood of cancer patients [36-38]. Furthermore, an acute elevation in circulating levels of EPCs may be induced by either chemotherapeutic drugs [39] or vascular disrupting agents (VDA) [39, 40]. This EPC spike may, in turn, lead to tumor relapse and resistance to pharmacological treatments [41]. The controversy surrounding the definition of EPCs makes it difficult to definitively assess their contribution to tumoral neovessels [42, 43]. It has been suggested that truly vasculogenic EPCs may work in concert with bone marrow-derived hematopoietic progenitor cells and resident mesenchymal stem cells to provide the building blocks of the primary vessel lumen and to stimulate surroundings ECs to undergo angiogenesis by paracrine signaling [44]. That bone marrow-derived GFP^+ EPCs integrate into the vasculature of xenograft tumors has been assessed by identifying luminal cells positive for the following markers: 1) GFP, which validates bone marrow derivation; 2) isolectin IB4, which ensures luminal incorporation; 3) CD31, which confirms endothelial origin; and 4) negative for CD11b, an antigen that marks the hematopoietic lineage [15, 17, 18, 35]. These data have been corroborated by the detection of 0.5-12% sex-mismatched ECs into secondary tumors arising in human patients previously transplanted with bone marrow [45]. Moreover, renal cellular carcinoma (RCC) developing within a kidney allograft may display Y-positive chromosome vessels within a Y-negative tumor [46]. A key question in the field regards the low rate (10-20%) of EPC incorporation into tumoral vasculature. This paradoxical feature might be explained by the findings of Nolan and co-workers [18], who found that luminally engrafted EPCs (GFP^+, $IB4^+$, $CD31^+$, $CD11b^-$) were diluted away (up to 1%) by locally recruited ECs at late stages of tumor development. Consistent with the supporting role of EPCs in tumoral perfusion, selective targeting of EPC engrafting may lead to tumor regression. For instance, elimination of the VDA-elicited EPC spike through a VEGF-R2 monoclonal antibody or via short hairpin-RNA (shRNA) mediated ablation of Id1, a transcription factor that promotes EPC egression from bone marrow, causes tumor shrinkage [17, 40]. Therefore, EPC targeting has been proposed as a novel therapeutic approach to purse either alone or in concert with other anti-cancer strategies, such as VDA and chemotherapy drugs [15].

AN OVERVIEW ON THE FUNCTIONS ACCOMPLISHED BY STORE-OPERATED CA^{2+} ENTRY IN MATURE ENDOTHELIAL CELLS

Hormones- and growth factors-induced intracellular Ca^{2+} dynamics are a key component of the signaling network controlling EC behaviour. In resting, i.e. non-stimulated cells, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is very low (around 100 nM) due to the activity of various active mechanisms that remove Ca^{2+} out of the cytosol, such as the plasma membrane Ca^{2+}-ATPase (PMCA), the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), and the Na^+/Ca^{2+} exchanger (NCX) [47, 48]. ECs utilize two pathways to generate cytosolic Ca^{2+} signals upon extracellular stimulation: Ca^{2+} release from the endoplasmic reticulum (ER), which houses the largest intracellular Ca^{2+} reservoir (~500 μM), and Ca^{2+} inflow across the plasma membrane (PM) Fig. (**3**). The extracellular milieu, which contains an enormous amount of Ca^{2+} (up to 1.8 mM) as compared to $[Ca^{2+}]_i$, represents a virtually infinite source of Ca^{2+} for EC signaling [48-50]. Ca^{2+} mobilization from ER is consequent to the activation of various isoforms of phospholipase C (PLC), including PLCβ and PLCγ, which cleave phosphatidylinositol-4,5-bisphosphate into inositol-1,4,5-trisphosphate ($InsP_3$) and diacyl-

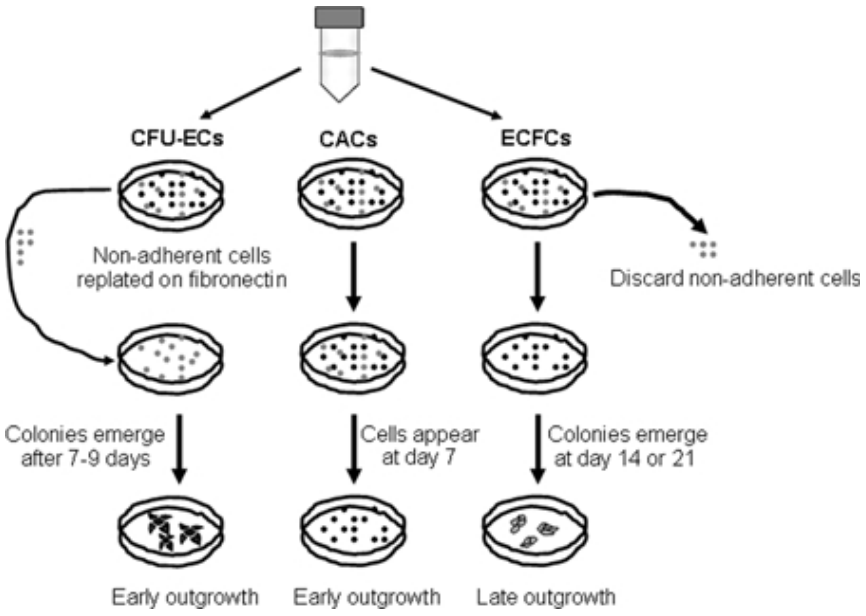


Fig. (1). Isolation and identification of different endothelial progenitor cells populations. Culture of colony forming unit-endothelial cells (CFU-ECs) requires the seeding of 5 x 10^6 peripheral blood- or umbilical cord blood-derived mononuclear cells on fibronectin coated dishes in a standard, commercially available medium containing 20% of foetal bovine serum and vascular endothelial growth factor (VEGF). After 24 hours, the non-adherent fraction is removed and re-plated in new fibronectin-coated tissue culture surfaces for further 5-9 days. Discrete colonies of spindle shaped cells emerge from a central cluster of small round cells at the end of the incubation period. Circulating angiogenic cells (CACs) represent the fraction of peripheral blood-derived MNCs plated on a fibronectin-coated Petri dish that remain adherent to the support after 4 days of culture under "endothelial" differentiation conditions. CAC cultures do not exhibit colony forming properties. Endothelial colony forming cells (ECFCs) are obtained by plating MNCs, harvested from either peripheral blood or umbilical cord blood, onto collagen-coated culture dishes in aa commercially available endothelial growth medium (EGM-2, Lonza). After 48 hours incubation, non-adherent cells are discarded and ECFC-derived colonies with cobblestone morphology appear after 10-20 days (if MNCs are derived from peripheral blood) or 5-7 days (if CB is employed). Redrawn after Fig. (**1**) in [195].

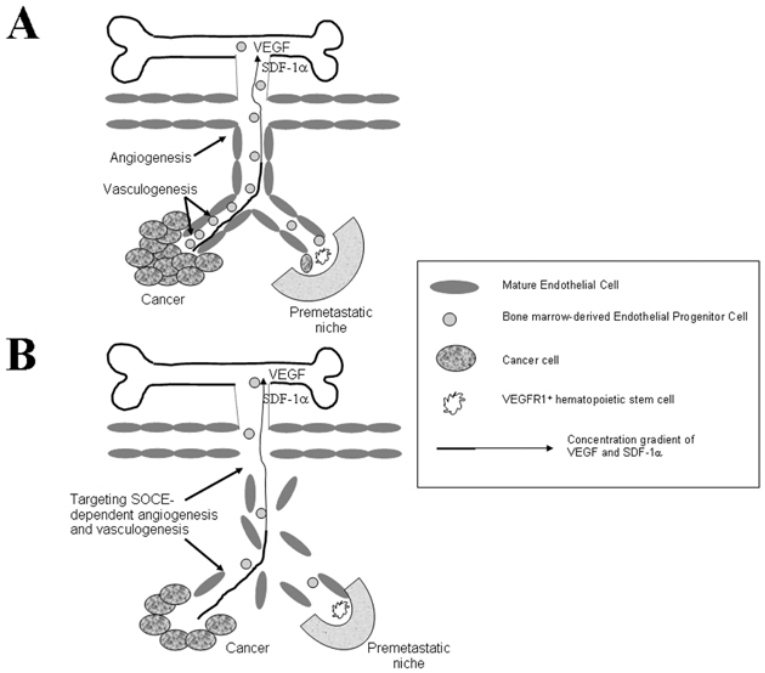


Fig. (2). Targeting store-operated Ca^{2+} entry as a perspective strategy to impair tumor neovascularization. Tumor neovascularization involves the participation of at least two different cell types belonging to the endothelial lineage, both of which are recruited by the concentration gradient of SDF1-α- and VEGF secreted into circulation by cancer cells: 1) local endothelial cells (ECs), which sprout from adjacent capillaries according to the classic process of angiogenesis and 2) endothelial progenitor cells (EPCs) which are mobilized from bone marrow and home to tumor site as described in the text. Moreover, primary tumors may stimulate the formation of the "pre-metastatic niche" in distant organs, which is essential for the incorporation of tumor cells spreading from the original site, by first recruiting bone marrow-derived $VEGFR1^+$ hematopoietic stem cells and, subsequently, EPCs. Store-operated Ca^{2+} entry may drive proliferation, migration, tubulogenesis, and differentiation in both mature ECs and EPCs. Pharmacological blockade of such a crucial membrane pathway might, therefore, lead to tumor shrinkage and suppress metastasis dissemination the throughout the organism.

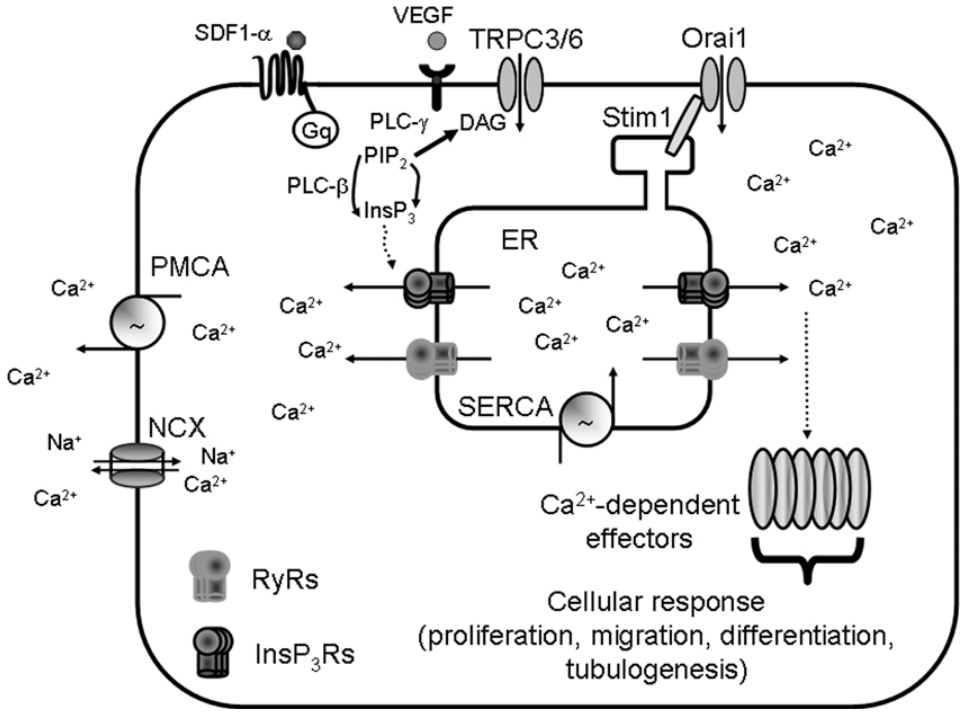


Fig. (3). The basic elements of the Ca^{2+} toolkit in endothelial cells and endothelial progenitor cells. Extracellular ligands, such as SDF-1α and VEGF, may cause an elevation in $[Ca^{2+}]_i$ upon binding to their selective receptors on the PM. The following activation of PLC-β (by GPCRs, such as CXCR-4, which binds to SDF-1α) or PLC-γ (by TRKs, such as KDR, which binds to VEGF) cleaves PIP_2 to produce $InsP_3$ and DAG. $InsP_3$, in turn, releases Ca^{2+} from the ER pool by activating $InsP_3Rs$, while DAG may evoke Ca^{2+} influx by gating TRPC3 and TRPC6. TRPC3 and TRPC6, however, are not present in ECFCs. The $InsP_3$-dependent fall 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 recover to baseline levels through the concerted action of the mitochondrial uniporter, SERCA and PMCA pumps, as well as through NCX. The signaling function of Ca^{2+} is accomplished by a number of Ca^{2+}-sensitive decoders (calmodulin, calmodulin-dependent kinases, calcineurin) and downstream targets. 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.

glycerol (DAG) Fig. (**3**). Whereas DAG may activate both protein kinase C and a number of plasmalemmal non-selective cation channels, $InsP_3$ binds to and stimulates the so-called $InsP_3$ receptors ($InsP_3Rs$) to release luminally stored Ca^{2+} into the cytosol Fig. (**3**). The following drop in ER Ca^{2+} levels functions as a primary message that is returned to the PM to activate the SOCE pathway [49-51]. SOCE, in turn, replenishes the emptied Ca^{2+} pool and sustains long-lasting intracellular Ca^{2+} signals by engaging a variety of Ca^{2+}-sensitive downstream decoders. Although Ca^{2+} influx may be mediated by other membrane routes, such as members of the transient receptor potential (TRP) family of cation channels [reviewed in 52], SOCE provides the main pathway for agonist-evoked Ca^{2+} entry in ECs [24, 51, 53, 54]. More specifically, store-dependent Ca^{2+} influx is involved in the sprouting of new capillary vessels by driving several key steps of the angiogenic process. For instance, SOCE triggers the genetic programme leading to EC replication by enlisting the Ca^{2+}-sensitive transcription factors, nuclear factor of activated T-cells (NFAT), nuclear factor kappaB (NF-κB), and activating protein-1 (AP-1) [24, 51, 55-57]. These transcription factors are recruited at the G_0/G_1 transition to regulate cyclin-dependent kinase 4 (cdc4) and cdc2 by inducing the expression of D- and E-type cyclins, respectively [58, 59]. Cyclin D-cdc4 and cyclin E-cdc2 complexes, in turn, ensure phosphorylation and inactivation of the retinoblastoma protein (pRb), which leads to entry into S phase and to the massive transcription of genes required for the subsequent cell cycle phases [60, 61]. Moreover, SOCE contributes to the morphological and adherence changes accompanying EC re-organization into patent capillary-like structures by activating a number of Ca^{2+}-dependent effectors, such as calpain and myosin light chain kinase (MLCK) [24, 53, 62, 63]. The complex sequence of events responsible for EC polarization, leading edge extension and attachment, contraction, and trailing end detachment may indeed be suppressed by either pharmacological or genetic blockade of SOCE [24, 53, 63]. A number of recent studies have demonstrated that store-dependent Ca^{2+} influx governs cell proliferation, motility and organization into a tubular network *in vitro* in both human and rodent EPCs. These novel findings shed bright light on the molecular targets that might be exploited to improve the therapeutic outcome of both CBT and anti-cancer treatments.

STORE-OPERATED CA^{2+} ENTRY IN ENDOTHELIAL COLONY FORMING CELLS IS MEDIATED BY STIM1 AND ORAI1 AND CONTROLS CELL PROLIFERATION AND TUBULOGENESIS

Intracellular Ca^{2+} signaling has long been known to drive key steps of the angiogenic process, such as EC proliferation, motility, remodeling and assembly in tubulary-like structures with patent lumen [48, 63-65]. SOCE emerged among all the components of the Ca^{2+} toolkit not only by its capability of repleting the ER Ca^{2+} store after the $InsP_3$-dependent Ca^{2+} release, thus maintaining the long-lasting oscillations in $[Ca^{2+}]_i$ elicited by growth factors in vascular endothelium [57, 66-70]. SOCE is selectively coupled to a plethora of Ca^{2+}-sensitive effectors that translate the submembranal Ca^{2+} microdomain into an angiogenic signal [24, 51, 53, 54, 71]. Any reduction in the ER Ca^{2+} content, whether the consequence of $InsP_3R$ activation or not, can serve as a stimulus for SOCE activation [69, 71, 72]. Accordingly, SOCE may be pharma-

cologically induced by using selective inhibitors of SERCA activity, such as thapsigargin and cyclopiazonic acid (CPA). Under resting conditions, Ca^{2+} continually leaks out of the ER through leakage channels and is, then, sequestered back into ER lumen by SERCA pumps. Thapsigargin and CPA target this cycle by preventing SERCA-mediated Ca^{2+} reuptake, thus resulting in the depletion of the ER Ca^{2+} pool and full SOCE activation [73]. In the view of the relevant role served by SOCE in vascular endothelium, it was worth investigating whether EPCs possess the SOCE pathway as well. Our group has examined for the first time the expression and the functional role played by agonist-evoked store-dependent Ca^{2+} influx in both peripheral blood- and umbilical cord blood-derived ECFCs (PB-ECFCs and UCB-ECFC, respectively). As illustrated above, these cells represent true vasculogenic EPCs that are able of differentiating into mature ECs and forming *de novo* vessels *in vivo* [5-7, 28]. Electrophysiological recordings revealed that human PB-ECFCs have a resting membrane potential of about -50/-20 mV, which is similar to that recorded in mature ECs [74] and is mainly set by an inwardly rectifying membrane conductance [75; Lodola, Tanzi and Moccia, unpublished observations). The expression of voltage-gated Ca^{2+} channels in vascular endothelium is highly debated [76], however, no voltage-dependent Ca^{2+} entry is detectable in either PB-ECFCs [25] or UCB-ECFCs (unpublished observations from Dragoni, Tanzi and Moccia). Furthermore, PB-ECFCs are not endowed with TRP canonical 3 (TRPC3), TRPC6, and TRPC7, which mediate DAG-operated Ca^{2+} inflow in mature endothelium [77-82], whereas only TRPC3 is present in UCB-ECFCs [25]. Consistent with these findings, 1-oleoyl-2-acetyl-sn-glycerol (OAG), a membrane-permeant analog of DAG, elicits Ca^{2+} entry in UCB-ECFCs, but not PB-ECFCs (unpublished observations from Dragoni, Tanzi and Moccia). Conversely, both types of cells express a SOCE pathway that is activated upon either pharmacological (i.e., CPA or thapsigargin) or physiological (i.e., the endothelial autacoid ATP) emptying of the $InsP_3$-sensitive Ca^{2+} reservoir [25]. Subsequent work has unveiled that, in ECFCs, SOCE is mediated by the physical coupling between the ER Ca^{2+}-sensor, Stromal Interaction Molecule-1 (Stim1), and the Ca^{2+}-permeable channel, Orai1 [24] Fig. (**3**). Orai1 is a plasmalemmal protein which functions as pore-forming subunit of store-operated channels in an increasing number of cell types [71, 72], including T and mast cells [83], smooth muscle cells [84], platelets [85], and ECs [51]. The Orai family of calcium entry channels includes three members (Orai1-3) and bares little resemblance to other ion channel families. In particular, Orai1 is predicted to have four transmembrane (TM1-4) spanning regions with both NH_2 and COOH tails directed to the cytoplasm [71, 72]. As more extensively illustrated elsewhere in this Issue, the molecular architecture of Orai1 comprises a negatively charged glutamate residue in TM1, E106, as a Ca^{2+}-binding site in the ion channel pore, as shown by site-directed mutagenesis and cystein scanning mutagenesis of Orai1 TM domains for pore lining residues [83, 86]. The permeation pathway is also contributed by an additional acidic residue in TM3, E190, as revealed by the decrease in Ca^{2+} selectivity observed upon E190Q replacement [83, 86]. Critical to Orai1 engagement by Stim1 are a putative coiled-coil region in the COOH-terminal and a proline- and arginine-rich NH_2-terminal sequence of the pore-forming subunit [87]. Consistently, an arginine-to-tryptophan (R91W) single amino acid substitution in Orai1 just prior to TM1 results in a loss of SOCE activity [83, 86, 87]. Orai1 seems to be present as a dimer in the PM, however, functional channels consist of tetramers formed by Stim1-mediated dimerization of Orai dimers [83, 86]. Stim1, in turn, is an ER-resident type I single transmembrane protein with an NH_2-tail facing the ER lumen and a cytosolic COOH-terminal. It contains two NH_2-terminal EF hand domains (a canonical and a hidden one) followed by a sterile α-motif (SAM) domain, the TM region, two coiled-coil sequences comprising an ezrin-radixin-moesin (ERM) domain and at the COOH-terminus a proline-rich and a lysine-rich domain. The Stim-Orai Activating Region (SOAR) domain, which is key to Orai1 activation, includes the second coiled-coil motif and the following approximately 55 amino acids [83, 86, 87]. Under resting conditions, i.e. when ER Ca^{2+} stores are full, Stim1 is uniformly distributed throughout the ER membrane. When ER Ca^{2+} concentration falls below a threshold level due to $InsP_3Rs$-dependent Ca^{2+} release, Ca^{2+} dissociates from the canonical EF domain, thereby causing the unfolding of the adjacent EF-SAM domains and multimerization of Stim1. Stim1 multimers rapidly redistribute to peripheral ER sites in close proximity to PM, where they aggregate into multiple *puncta*, bind to and trigger Orai1 tetramerization [83, 86, 87]. The opening of the channel pore and consequent Ca^{2+} flood into the cytosol are then promoted by the physical interaction between the SOAR domain of Stim1 and the both the NH_2- and COOH-termini of Orai1 [83, 86, 87]. ECFCs, isolated from either PB or UCB, express all the three Orai1 isoforms and the two known Stim paralogues (Stim1-2) [24, 25]. The molecular make-up of SOCE in these cells was unveiled by recent experiments conducted on genetically modified cells: transfecting ECFCs with a small interference RNA (siRNA) directed against Orai1 or expression of a dominant negative (DN) mutant Orai1 (R91W) did inhibit thapsigargin-evoked SOCE [24]. Furthermore, Ca^{2+} entry in response to depletion of the ER Ca^{2+} store was suppressed by knocking down endogenous Stim1 by RNA interference [24]. Finally, $InsP_3$-dependent Ca^{2+} release induced eYFP-GFP tagged Stim1 to re-arrange into near PM *puncta* [24]. The pharmacological profile of SOCE is also consistent with the molecular data. CPA-induced Ca^{2+} entry in human ECFCs is, indeed, sensitive to YM-58483/BTP-2 (4-methyl-4'-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]-1,2,3-thiadiazole-5-carboxanilide), which has been shown to abolish Orai1-mediated Ca^{2+} entry in the immunitary system [88, 89] (see also below). The electrophysiological properties of Orai1-mediated Ca^{2+} currents have been extensively characterized in the cells of the peripheral immune systems and has been termed Ca^{2+} Release-Activated Ca^{2+} current (I_{CRAC}). The biophysical fingerprints of I_{CRAC} encompass the following features: 1) a prominent inward rectification at negative potentials (the current approaches the zero current level at potentials >+60 mV); 2) a high Ca^{2+} selectivity, which is manifested by a Ca^{2+}:Na^+ permeability ratio (P_{Ca}/P_{Na}) of about 100:1 and a reversal potential (E_{rev}) >+60 mV; 3) a low single channel conductance, estimated to be as low as 2 fS by stationary noise analysis [90, 91]. However, Li and coworkers failed in recording any I_{CRAC}-like current in response to depletion of the $InsP_3$-sensitive Ca^{2+} pool [24]. These findings are not surprising as it has long been known that the current density of the endothelial I_{CRAC} is 6-to-10 times lower than that reported in RBL cells [51, 76, 90, 92]. As a consequence, store-dependent Ca^{2+} currents may fall below the resolution limit of a whole-cell patch-clamp recording system [51, 76, 90, 92]. Notably, SOCE may be recruited by stimulation of some, but not all, types of G-Protein Coupled Receptors (GPCRs) in PB-ECFCs. For instance, SOCE underlies the long-lasting plateau phase following the initial Ca^{2+} mobilization induced by ATP-sensitive P_{2Y} receptors. Conversely, bradykinin, which elicits an $InsP_3$-dependent Ca^{2+} discharge upon binding to B2 receptors, fails to activate any detectable Ca^{2+} entry [25]. This feature led us to hypothesize that ECFCs express two different $InsP_3$-governed Ca^{2+} stores: one sub-plasmalemmal compartment which is selectively devoted to gate SOCE and a cytosolic Ca^{2+} pool which is responsible for the bulk increase in $[Ca^{2+}]_i$ induced by extracellular stimulation. According to this model, that has been supported by convincing experimental data in a number of cell types [see 90, for a recent review], only ATP engages the Ca^{2+} storage site which lies in close apposition to the PM in ECFCs [25]. In this view, it has been shown that $InsP_3R$-2 and $InsP_3R$-3, but not $InsP_3R1$, drive SOCE activation in avian B cells [93], while $InsP_3R$-1 is the main isoform linked to Stim1 translocation in H4IIE liver cells [94]. ECFCs express all the three known $InsP_3R$ subtypes: future work will have to assess which of them is selectively coupled to SOCE in these cells.

Similar to mature endothelium (see above), store-dependent Ca^{2+} influx is crucial to ECFC physiology. Both the genetic suppression of Orai1 by RNA interference and the pharmacological blockade of SOCE with BTP-2 affect ECFC proliferation and organization into tubulary-like structures [24, 25]. These data were corroborated by the observation that BAPTA, a membrane permeable buffer of intracellular Ca^{2+}, prevents ECFC from reaching the confluence after three days in culture and from assembling into a capillary network when plated into Matrigel plugs (Bonetti and Rosti, unpublished observations). Therefore, the molecular components of store-operated channels, namely Stim1 and Orai1, have been put forth as novel molecular targets to enhance the regenerative outcome of cell based therapy and to adverse tumoral neovascularization [21, 25].

STORE-OPERATED CA^{2+} ENTRY SUSTAINS VEGF-INDUCED OSCILLATIONS IN $[CA^{2+}]_I$ IN ENDOTHELIAL COLONY FORMING CELLS

The signaling cascade leading to SOCE activation and ECFC proliferation is triggered by the $InsP_3$-dependent depletion of the ER Ca^{2+} pool. After an ischemic insult, bone marrow resident cells are exposed to a myriad of growth factors and cytokines released into circulation by hypoxic tissues, such as VEGF, which stimulates EPC proliferation, resistance to apoptosis and differentiation into mature ECs, and SDF-1α, which acts as a chemoattractive factor towards the injury site [20]. The human VEGF (also referred to as VEGFA) is organized as eight exons separated by seven introns. Alternative exon splicing leads to the generation of four different isoforms ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$) having, respectively, 121, 165, 189 and 206 amino acids after cleavage of the signal sequence. $VEGF_{165}$ is the predominant isoform and displays properties similar to those of native VEGF [95]. The biological effects of VEGF are mainly mediated by two tyrosine kinase receptors, namely VEGFR-1 (Flt-1) and VEGFR-2 (also knows as kinase domain region (KDR) or Flk-1). Despite the fact that VEGF binds to VEGFR-1 with ~ 10-fold higher affinity than VEGFR-2, it is mainly the latter that mediates VEGF signaling in both ECs and EPCs [95-97]. VEGF stimulates mature endothelium to undergo angiogenesis by triggering an intricate network of intracellular signaling cascades that is initiated by VEGFR-2 dimerization and autophosphorylation [95, 98]. More specifically, phosphorylation on Y1175 provides the docking site for the COOH-terminal Src homology-2 domain (SH2) of PLC-γ, whose phosphorylation leads to the synthesis of both $InsP_3$ and DAG [95, 98]. The ensuing increase in $[Ca^{2+}]_i$ drives VEGF-induced human EC proliferation, migration, and angiogenesis and may be mediated by two distinct mechanisms depending on the vascular bed under examination. For instance, VEGF may cause a monotonic increase in intracellular Ca^{2+} levels due to the AA-mediated opening of TRP vanilloid 4 (TRPV4) [65, 99] or to the DAG-dependent activation of TRPC3 and TRPC6 [77-79]. Alternatively, VEGF elicits a rapid Ca^{2+} peak, due to the $InsP_3$-dependent Ca^{2+} release, that is followed by a long-lasting plateau phase sustained by SOCE [51, 53, 54]. We have recently shown that PB-ECFCs display discrete repetitive discharges of luminally stored Ca^{2+} in response to VEGF Fig. (**4A**) [21]. VEGF-induced Ca^{2+} oscillations are not synchronized between adjoining cells, occur randomly during cell stimulation and run down upon removal of the growth factor. The amplitude of the first Ca^{2+} spike increases by raising VEGF concentration from 1 ng/ml to 50 ng/ml, whereas the lag time between stimulus and onset of the response decreases [21]. Conversely, the frequency of VEGF-elicited Ca^{2+} transients (i.e. the mean number of intracellular Ca^{2+} waves recorded over 1 hour recording) reaches a peak of about 9 oscillations per 1 hour. The initiation of the spiking response to VEGF requires the activation of the PLC-γ/$InsP_3$ pathway by VEGF-R2, however, intracellular Ca^{2+} waves rapidly cease in absence of Ca^{2+} influx to replenish the intracellular stores Fig. (**4C**) [21]. Orai1-mediated Ca^{2+} entry fulfils the role to support VEGF-induced Ca^{2+} oscillations overtime, as PB-ECFCs stimulated in presence of BTP-2 display only 1-4 Ca^{2+} spikes Fig. (**4D**) [21]. In agreement with this observation, VEGF-dependent Ca^{2+} entry is dramatically reduced when the cells are transfected with siRNAs targeting either Orai1 or Stim1 [24]. Agonist-induced oscillations in $[Ca^{2+}]_i$ are shaped by the interplay between $InsP_3$-dependent Ca^{2+} release and SOCE activation in a variety of cell types, as recently reviewed [69, 71, 72]. According to this model, Ca^{2+} oscillations arise from the periodical release and re-uptake of Ca^{2+} from the $InsP_3$-sensitive intracellular Ca^{2+} pool. The discharge of Ca^{2+} stores by $InsP_3$, in turn, activates store-dependent Ca^{2+} influx, that is sequestered by SERCA into ER lumen to maintain the stores filled in preparation for the next cyclical opening of $InsP_3Rs$ [69, 71, 72]. The large heterogeneity in the oscillatory response to VEGF may be explained by both the variability in VEGF-R2 expression in ECFC cultures (60%) and the spatial arrangement of the Ca^{2+} machinery, as suggested by the statistical analysis of VEGF-induced Ca^{2+} waves. As discussed in [21], the Ca^{2+} transients elicited by VEGF arise randomly and the standard deviation of the interspike interval (ISI) is of the same order of average ISI. As demonstrated by mathematical modeling [100, 101], this feature is nicely explained by the spatial arrangement of $InsP_3Rs$ in clusters spaced from each other by several microns. The local elevation in $InsP_3$ levels induced by PLC-γ primes all the channels in the cluster for stimulation by Ca^{2+} and triggers a local Ca^{2+} release. Ca^{2+} is, however, a poorly diffusible messenger and cannot promote further Ca^{2+} discharge from adjoining sites. The globalization of the Ca^{2+} signal requires a supercritical number of $InsP_3R$ clusters to open at the same time: the Ca^{2+} burst is thus large enough to synchronize all the $InsP_3$-bound $InsP_3Rs$ via the CICR mechanism and induce a global Ca^{2+} spike. This process may not be deterministically predicted, but occurs randomly and explains the variation in the period during a spike train [100-102]. As PLC-γ phosphorylation by VEGFR-2 is inversely correlated to VEGF concentration [103], this model is also consistent with the shorter duration of the oscillatory response observed at higher ligand doses [21]. Pioneering studies conducted on a number of $InsP_3R$ knockout cells revealed that $InsP_3R$-2 serves as the most suitable isoform to drive agonist-induced oscillations in $[Ca^{2+}]_i$ due to its sharpest dependence on ambient Ca^{2+} and higher affinity to $InsP_3$ [104-106]. Mathematical modeling has, subsequently, reinforced this notion by demonstrating that $InsP_3R$-3, which exhibits the lowest sensitivity to both $InsP_3$ and Ca^{2+}, primes adjoining $InsP_3R$-2 to adopt the oscillatory regime and periodically release Ca^{2+} with the aid of $InsP_3R$-1 [102, 107]. It is worth of noting that VEGF triggers intracellular Ca^{2+} oscillations in truly endothelial progenitors, but not in mature ECs. This feature might be explained when considering that 1) TRPC3 and TRPC6 are absent in human ECFCs [25]; 2) TRPV4 is expressed in human ECFCs, but Ca^{2+} imaging recordings suggest that it is insensitive to low doses of AA (manuscript in preparation from our lab); 3) the spatial arrangement of VEGFR-2, PLC-γ, $InsP_3Rs$, Stim1, Orai1 and SERCA, whose interplay shape both the biphasic Ca^{2+} signal in mature ECs [51, 53, 54] and the long lasting Ca^{2+} oscillations in human ECFCs, might be different in fully differentiated *vs.* immature cells.

The oscillations in $[Ca^{2+}]_i$ evoked by VEGF drive ECFC proliferation and tubulogenesis. Accordingly, impairing the Ca^{2+} burst with both BAPTA and BTP-2 prevented cell growth and suppressed the formation of a bidimensional tubular network in Matrigel [21]. This pharmacological observation was supported by data obtained from cells transduced with Orai1 siRNA: the extent of *in vitro* tubulogenesis was significantly reduced as compared to cells engineered with control vectors [24]. Repetitive Ca^{2+} spikes have long been known to regulate cell fate by inducing the nuclear translocation of the Ca^{2+}-sensitive transcription factors, NFAT and NF-κB [57, 71, 108, 109]. NF-κB, in particular, regulates the expression of many genes controlling several key steps of the angiogenic process, such

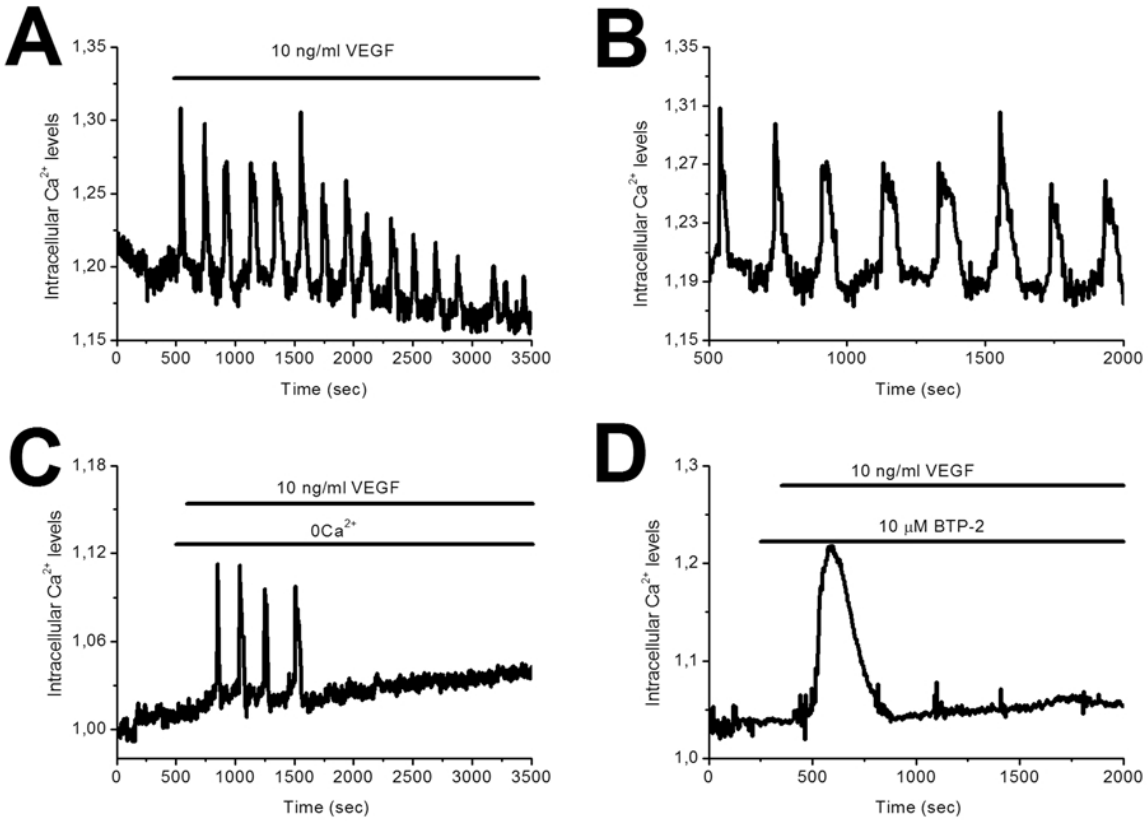


Fig. (4). VEGF triggers repetitive oscillations in $[Ca^{2+}]_i$ in human circulating endothelial colony forming cells. A, repetitive Ca^{2+} transients induced by VEGF (10 ng/ml) in an ECFC loaded with the Ca^{2+}-fluorochrome, Fura-2, as described in [134]. **B**, expanded section of the tracing depicted in panel A. VEGF-elicited intracellular Ca^{2+} oscillations rapidly cease upon removal of extracellular Ca^{2+} ($0Ca^{2+}$) (**C**) or in presence of BTP-2 (10 μM) (**D**), a selective inhibitor of store-dependent Ca^{2+} influx.

as endothelial proliferation and migration, cell cycle progression, degradation of the membrane basement, and inhibition of apoptosis [110-112]. In un-stimulated cells, NF-κB dimers reside within the cytoplasm due to the association with an inhibitory subunit, I_KB, which masks the nuclear localization signal (NLS) on NF-κB itself [112]. Intracellular Ca^{2+} oscillations may lead to I_KB phosphorylation, thereby targeting it for ubiquitination and subsequent proteolysis [57, 71, 108, 109]. As a consequence, free NF-κB dimers then translocate into the nucleus, where they bind to *k*B sites within promoters/enhancers of targets genes and regulate the transcription of these genes [112]. VEGF-induced I_KB phosphorylation in human ECFCs is dramatically affected by blocking the Ca^{2+} train with either BAPTA or BTP-2 [21]. Moreover, thymoquinone, a rather selective NF-κB inhibitor, prevents both I_KB phosphorylation and ECFC proliferation and tubulogenesis [21]. It is, therefore, conceivable that NF-κB provides a mechanistic link between the spiking response to VEGF and its physiological outcome. Future studies will have to assess the recruitment of other Ca^{2+}-sensitive transcription factors, such as NFAT, calcineurin-dependent 1 (NFATc1), Kv channel interacting protein 3, calsenilin (KCNIP3/CSEN/DREAM), and myocyte enhancer factor 2C (MEF2C) in VEGF-stimulated ECFCs [113, 114]. Moreover, it will also be worth of assessing the Ca^{2+} source responsible for the nuclear translocation of NF-κB in the presence of VEGF. A number of recent studies have unveiled that gene expression during Ca^{2+} oscillations is selectively driven by the Ca^{2+} microdomains arising near open Orai1 channels rather than $InsP_3Rs$. This specificity is due to the physical coupling between Orai1 proteins and the downstream Ca^{2+}-sensitive decoders [71, 115, 116]. For instance, Ca^{2+} oscillations evoked by leukotriene C4 (LC4) in mast cells in presence of extracellular Ca^{2+} are similar to those occurring in absence of Ca^{2+} entry when PMCA is blocked by the trivalent cation, La^{3+}. However, c-fos gene expression takes place only when Orai1-mediated Ca^{2+} influx accompanies LC4-induced Ca^{2+} transients [71, 115].

STORE-OPERATED CA^{2+} ENTRY IN CFU-ECS BELONGING TO THE MYELOID LINEAGE

The molecular nature and functional role of SOCE have also been investigated in rat BM-derived EPCs belonging to the myeloid lineage, namely CFU-ECs. As discussed above, these cells emerge after 4-7 days as spindle-shaped cells from MNCs seeded onto fibronectin-coated culture dishes and promote vasculogenesis by paracrine signaling [5-7]. In other words, CFU-ECs enhance vasculogenesis by stimulating local ECs to undergo angiogenesis upon the release of several growth factors and cytokines, such as VEGF and SDF-1α. These cells may not be regarded as truly committed endothelial progenitors and do no engraft within neovessels *in vivo* [117]. In these cells, silencing of Stim1 by adenoviral expression of a selective siRNA reduced both cell proliferation and migration by the inhibition of store-dependent Ca^{2+} entry. The angiogenic phenotype was, in turn, rescued by re-expression of Stim1 in silenced cells [22]. The same group reported that Stim1 may gate Ca^{2+} entry via the selective coupling to TRPC1. Accordingly, Stim1 may co-immunoprecipitate TRPC1 and siRNA-mediated ablation of TRPC1 dramatically affects SOCE, proliferation and migration in rat BM-derived CFU-ECs [23]. More specifically, the genetic suppression of TRPC1 cause the arrest in the G1 phase of the cell cycle due to the up- and down-regulation, respectively, of 9 and 4 genes. Indeed, silencing TRPC1 caused the over-expression of Ak1, Brca2, Camk2b, p21, Ddit3, Inha, Slfn1, Mdm2, Prm1, and induced a decrease in the mRNA levels of Bcl2, Mki67, Pmp22, and

Ppp2r3a [23]. The Ca^{2+}-sensitive decoder of TRPC1-mediated Ca^{2+} entry in rat BM-derived EPCs might be schlafen-1 (Slfn1), as both G1 arrest and CFU-EC proliferation were partially rescued by a Slfn1-blocking peptide [23]. These results are not surprising when considering that TRPC1 may be gated by a plethora of signaling mechanisms downstream of PLC, including direct binding to Stim1. Similar to other members of the TRPC sub-family, the basic structure of TRPC1 channels consists of six TM domains (TM1-TM6) with the NH_2- and COOH-termini located in the cytoplasm. Between TM5 and TM6, there is a re-entrant pore loop constituted by a short hydrophobic stretch lining the channel pore. TRPC1 contains a number of calmodulin/$InsP_3R$ binding domains (CIRB) at both its NH_2- and COOH-tails, 3 to 4 NH_2-terminal ankyrin repeats, a homologous sequence of ~25 amino acids immediately COOH-terminal to TM6, which has been termed TRP domain and consists of a highly conserved 6 amino acids TRP box (EWKFAR), and a predicted coil:coil region [118, 119]. Moreover, TRPC1 may assemble with a variety of membrane-associated proteins, such as caveolin-1, homer1, β-tubulin, and RhoA [see 118, for a recent and comprehensive review]. TRPC1 may be informed about the Ca^{2+} content of ER lumen by an electrostatic interaction between the negatively charged amino acids at its COOH-terminus and the positively charged ones in the Stim1 polybasic tail (KKK) [118]. This mechanism might explain the contribution of Stim1 and TRPC1 to SOCE in rat BM-derived CFU-ECs. Future studies will have, however, to assess the involvement of Orai1 in this signaling cascade. Indeed, the interaction between Stim1 and TRPC1 upon depletion of the $InsP_3$ -sensitive Ca^{2+} pool might occur only in the context of a ternary complex formed by both proteins and Orai1 [120]. Nevertheless, the crucial role served by TRPC1 in rat CFU-ECs is at variance with the result obtained in human ECFCs, where TRPC1 does not contribute to SOCE [24]. This discrepancy might be explained by their different lineage, as the former display a monocytic/hematopoietic phenotype, whereas the latter are endothelial committed cells. In addition, the molecular nature of SOCE was investigated in rat, rather than human, CFU-ECs, which might provide additional variability between the two cell types. These issues should be kept in mind when translating into clinics the observations provided by rodent models (see below).

STORE-OPERATED CA^{2+} ENTRY AS A PUTATIVE TARGET OF REGENERATIVE MEDICINE

The potential of EPCs for regenerating ischemic tissues and rescuing local blood perfusion may be hampered by a variety of pitfalls which mitigate the therapeutic outcome of CBT in human patients. As mentioned above, the choice of the most suitable cell population is not the only limiting factor when approaching myocardial ischemia and peripheral artery disease, such as critical hindlimb ischemia and intermittent claudication, with vascular engineering strategies [4, 20, 121-123]. Additional hurdles that negatively impact on the clinical benefits of EPC injection into ischemic patients are the following: 1) the fundamental scarcity of EPCs in PB Fig. (**1**), combined with a decrease in both their colony forming capacity and migratory activity in the presence of cardiovascular risk factors, including ageing, smoke, hypercholesterolemia, hyperhomocysteinemia, and diabetes [20, 123, 124]; 2) the low rate of EPC trafficking, engraftment, and survival within the target tissue [20, 122]; 3) the trans-differentiation of BM-derived cells into undesidered lineages, as suggested by the calcifications occurring in the infarcted heart of rats transplanted with unfractioned MNCs [20, 125]. A number of strategies have been put forth to overcome these severe limitations, including genetic modifications of autologous EPCs prior to their inoculation Fig. (**5**) [4, 20, 121-123]. In particular, enhancing the signal transduction pathways driving the angiogenic response has been shown to restore blood flow even at subtherapeutic doses, i.e. 30 times less than that required in previous experiments, of EPCs. For instance, the injection of heterologous EPCs transduced with adenovirus containing VEGF gene improved the extent of neovascularization, partially recovered local perfusion and reduced the percentage of auto-amputation in a rodent model of hindlimb ischemia [4, 126, 127]. Similarly, transplantation of VEGF-gene-transfected EPCs accelerated thrombi recanalization in a rat model of chronic vein thrombosis [128]. Finally, the intraventricular injection of CB-derived $CD133^+/CD34^+$ progenitor cells transduced with a bicistronic vector over-expressing both VEGF and Platelet Derived Growth Factor (PDGF) resulted in the revascularization of the damaged area, which was associated to an improvement in cardiac function and a better exercise capacity, in a rat model of acute myocardial infarction [129]. These observations suggest that the signaling pathway engaged by VEGF to stimulate EPC-dependent vasculogenesis might offer an alternative means to improve the outcome of CBT. As a consequence, we propose that SOCE in ECFCs represents one of the most suitable molecular targets to enhance the regenerative potential of vascular engineering strategies. First, both BM- and CB-derived ECFCs are able to form patent vessels *in vivo* and to anostomose with the host circulation when implanted into immunodeficient mice by means of a collagen scaffold [30-33, 130]. Second, Orai1- and Stim1-mediated SOCE sustain the oscillatory Ca^{2+} signal whereby VEGF stimulates ECFCs to undergo mitosis and tubulogenesis though the nuclear translocation of NF-κB [21, 24]. Third, the genetic ablation of Orai1 impairs the organization into a tubular network when ECFCs are plated into Matrigel plugs [24]. Fourth, a number of recent evidences suggest that Orai1, rather than $InsP_3Rs$, selectively triggers gene expression during agonist-induced intracellular Ca^{2+} oscillations [71, 115]. Fifth, preliminary experiments carried out in our laboratory have shown that SDF-1α elicits a long-lasting Orai1-mediated Ca^{2+} influx upon emptying of the $InsP_3$-sensitive Ca^{2+} stores. It is, therefore, conceivable that engineering ECFCs with Orai1 and Stim1 prior to their autologous injection might enhance their regenerative potential and overcome the limits imposed by their paucity in PB [122, 127].

Gene transfer consists of the introduction of foreign DNA into target cells to achieve a localised, sustained therapeutic overexpression of the selected gene. Several different approaches have been evaluated to transfer an angiogenic growth factor gene into vascular ECs and EPCs, such as VEGF, bFGF and angiopoietin-1 [121, 131-133]. A number of approaches have been developed to enhance DNA intake by receiving cells, such as the use of cationic phospholipids (liposomes) or cationic polymers, electroporation and exposure to ultrasounds [121, 131]. Nevertheless, a transfection efficiency able to produce clinically significant levels of exogenous protein is difficult to achieve even with the best gene transfer strategies. As a consequence, both plasmid and viral vectors have been used successfully to transduce stem cells and stimulate therapeutic vascularization in both ischemic limbs and infarcted myocardium. The advantages of non-viral vectors include the ease of vector production, the reduced limitation on the promoter cassette size, and the relatively minimal biosafety risks [121, 131-133]. Their limitations include low transfection efficiency and transient effect due to the fact that they are not integrated into the host genome, but are retained into the cytoplasm where they are degradated. Furthermore, they are lost at each cell division. On the other hand, viral vectors are the most suitable means to engineer EPCs, as they offer a relatively high gene transfer efficiency and long-term transgene expression. Their disadvantages include the limited packaging capacity, inconsistencies in bioactivity and purity between vector stocks, and biosafety risks [121, 131-133]. The viral vectors most commonly employed in cardiovascular pathologies are adenoviral vectors, adeno-associated virus vectors, and lentivirus vectors. Adenoviral vectors can transduce both dividing and quiescent cells and display a transfection efficiency that is 1000 folds better than plasmid DNA. However, they do not integrate into host genome and, therefore, the genetic information they carry is gradually lost as the cells divide. Moreover, the antigenicity of residual viral pro-

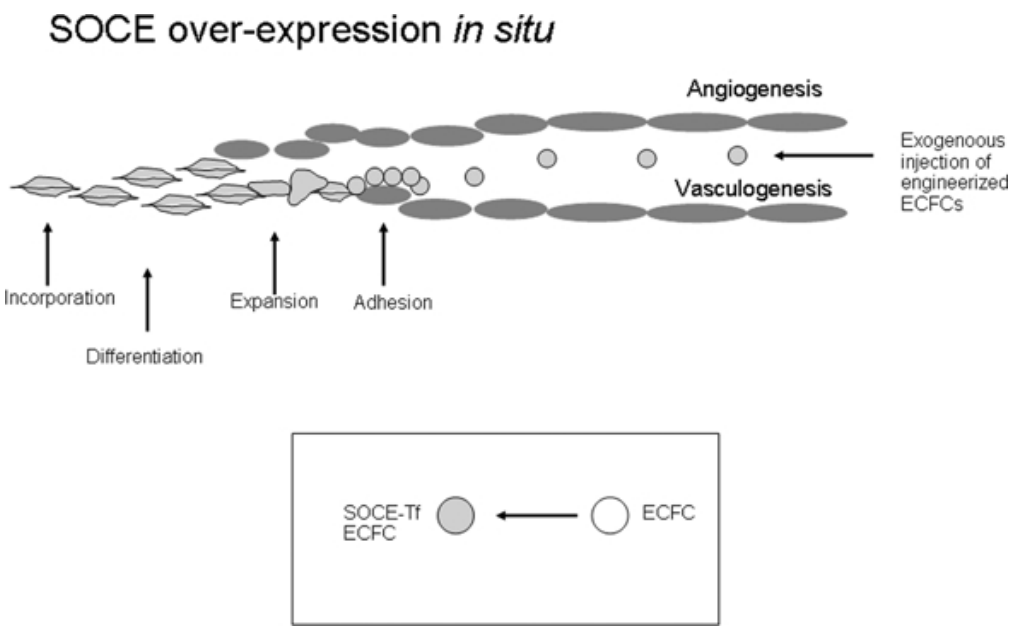


Fig. (5). Over-expression of store-operated Ca^{2+} entry as a putative tool to improve the therapeutic outcome of endothelial colony forming cells-dependent vasculogenesis. EPCs are recruited from bone marrow towards ischemic sites to rescue local blood perfusion. Once reached the target organ, EPCs incorporate into neovessels, proliferate, acquire a mature phenotype and adhere to the luminal surface of the capillary. In the virtue of the key role served by store-operated Ca^{2+} entry in controlling proliferation and tubulogenesis of endothelial colony forming cells, it is conceivable that engineering the cells with Stim1 and/or Orai1 before inoculation will enhance the restoration of blood flow in ischemic tissues. SOCE-Tf ECFC: ECFC transfected with the molecular machinery encoding for store-dependent Ca^{2+} channels.

teins tends to trigger an immune response from the host, although *ex vivo* transfection can limit their immunogenicity [121, 131-133]. These pitfalls notwithstanding, the adenoviruses-mediated transduction of heterologous EPCs has been shown to restore blood perfusion in a number of pre-clinical studies, as described above. In their pioneering work, for instance, Iwaguro and colleagues reported a significant increase in VEGF plasma levels and in the extent of neovascularization at 28 days after cell injection [26; see also below].

Adeno-associated virus vectors (AAVVs) can infect both replicating and non-replicating cells with high efficacy, but do not express any viral protein and are non-pathogenic to human subjects. This feature renders them particularly amenable for combining cell and gene therapy. In addition, AAVVs have the capacity for stable long-term gene expression as compared to adenovirus due to site specific integration into the host cell chromosomes. Their major limitations include a limited packaging capacity of 4.8 kb (but humanOrai1 and Stim1 trancripts are 1.5 kb and 363 bp, respectively), the potential for pre-existing neutralizing antibodies in human populations, the tendency to undergo insertional mutagenesis, and the difficulty with the production of large quantities [121, 131-133].

Retroviruses are RNA viruses that integrate into the host genome thereby providing long-term transgene expression. These vectors can be manipulated *ex vivo* to eliminate infectious gene particles to minimize the risk of systemic infection and patient-to-patient transmission. The main disadvantages of retroviruses are an inability to tranduce non-dividing cells and low-titre vector stocks. It should, however, be kept in mind that EPCs undergo a rapid growth in their expansion medium and might, therefore, be more suitable to retroviral infection as compared to other mitotically quiescent cells, including cardiomyocytes and smooth muscle cells. Similar to AAVVs, retrovirus vectors may undergo insertional mutagenesis which renders them potentially harmful for the recipient, as recently shown in patients suffering from trial of X-linked severe combined immunodeficiency and treated with gene therapy [134]. It has, therefore, been suggested to modify such vectors, for instance by targeting genome integration sites or introducing insulator sequences, to reduce their biosafety risks [121, 131-133]. Pre-clinical studies have, however, successfully exploited retroviral infection to engineer EPCs to over-express VEGF and rescue blood perfusion in a murine model of hindlimb ischemia. Importantly, this procedure significantly increased VEGF plasma levels and capillary density at 28 days post-injection [135].

Lentiviral vectors are a subgroup of retroviruses which are based on the human immunodeficiency virus type-1 (HIV-1). Lentiviruses may transduce both quiescent and proliferating cells via genome integration and are particularly suited to target the cardiovascular system. Improvements of these viral vectors have been based on the deletion of all accessory proteins from the packaging system and on the separation of packaging elements into multiple plasmids: these features are necessary to reduce the pathogenicity of the parental virus, to increase the packaging capacity as compared to AAVVs, and to support long term trans-gene expression [121, 131-133]. A recent study has demonstrated that ECFCs may be transduced with lentiviral vectors to stably express a transgene (green fluorescent protein) under the control of a cytomegalovirus promoter [136].

A comparative investigation has evaluated the efficiency of different viral and non-viral constructs for gene delivery into human CFU-ECs and analyzed their effects on cell survival and toxicity *in vitro*. Adenoviruses provided the higher percentage of cell transduction as compared to other vectors, such as AAVVs, lentiviruses, and liposomes [137]. Notably, adenovirus-transfected cells successfully incorporated within a tubular network when plated in Matrigel plugs [137], a result which supported the *in vivo* observations by Iwaguro and coworkers [126]. As a consequence, it would be interesting to assess whether engineering ECFCs with adenoviral constructs able to over-express either Stim1 or Orai1 (or both, see below) will: 1) increase their rate of proliferation and tubulogenesis *in vitro* and 2) improve the therapeutic outcome of either therapeutic or sub-therapeutic doses of *ex vivo* expanded cells in animal models of myocardial ischemia or peripheral artery disease. Furthermore,

gene modification of the Ca^{2+} handling machinery might recorrect the well known EPC dysfunctions that occur in patients affected by cardiovascular pathologies and hamper autologous cell transplantation. For instance, SOCE is up-regulated in bovine aortic ECs exposed to high glucose levels [138], whereas it is significantly decreased in ECs cultured in the presence of high concentrations of a mixture of fatty acids (oleate and palmitate) [139]. EPCs isolated from either diabetic or obese patients display reduced proliferative potential and migratory function. Future experiments will have to compare the amplitude of SOCE and the levels of Orai1/Stim1 expression in ECFCs isolated from PB of these subjects. A few caveats should, however, be considered when exploiting SOCE to enhance the regenerative outcome of EPC-based cell therapy. First, the contribution of Stim1 and Orai1 to SOCE has been unveiled in circulating human ECFCs, but not in CFU-ECs or in $CD34^+$ HSCs, which have been widely employed in preclinical studies. As described above, for instance, TRPC1 plays a key role in mediating store-dependent Ca^{2+} influx in rat CFU-ECs, but is not activated by depletion of the ER Ca^{2+} pool in human ECFCs. As a consequence, we recommend elucidating the molecular make-up of SOCE in cell types other than ECFCs before harnessing SOCE in CBT. Second, the stringent stoichiometric coupling between Stim1 and Orai1 (≈2Stim1:1Orai1) might affect the positive outcome of genetic engineering strategies aiming to augment SOCE in ECFCs. It has been shown that Orai1 over-expression leads to a decrease, rather than to an increase, in SOCE amplitude owing to a dilution of endogenous Stim1 by the exogenous channel protein [140, 141]. Similarly, Stim1 up-regulation results only in a modest elevation in store-regulated Ca^{2+} inflow [141]. On the other hand, when both proteins are over-expressed, SOCE may augment by 25-to-50 fold without affecting the intracellular Ca^{2+} reservoir [140, 141].

STORE-OPERATED CA^{2+} ENTRY AS A NOVEL MOLECULAR TARGET TO ADVERSE TUMOR VASCULARIZATION

It has recently been proposed that blockade of BM-derived EPC mobilization towards the tumor might prevent the angiogenic switch leading to the metastatization process which ultimately ends up with patient death [15, 17, 18, 142]. Unfortunately, the strategies designed to affect EPC recruitment and proliferation are targeted against a limited number of pro-angiogenic effectors, such as VEGF and its related pathways. In particular, the anti-VEGF neutralizing monoclonal antibody, bevacizumab, and the tyrosine kinase inhibitors, sorafenib and sunitinib, showed clinical benefit in randomized trials performed on patients with metastatic colorectal cancer, advanced non-small cell lung cancer, renal cell carcinoma, hepatocellular carcinoma and metastatic breast cancer [143]. However, despite these promising results, emerging data indicate that their impact on patient overall survival and progression-free survival responses is limited to a few months and that tumor subsequently relapses in most (if not all) patients [142, 143]. Moreover, sorafenib and sunitinib are associated with hypertension and cardiotoxicity [143, 145, 146], while bevacizumab is also associated with hypertension and instances of thromboembolism, pulmonary hemorrhage pulmonary edema, and gastrointestinal tract bleeding [145]. There is, therefore, an urgent need for novel targets to affect neoplastic vascularization and prevent untoward off-target effects. Pioneering work separately conducted by Kohn's and Munaron's groups has revealed that the Ca^{2+} machinery in tumor endothelium offers a brand new battery of signaling events that might be harnessed to disrupt tumor neovessels without toxic side effects [63-65, 147-150]. Indeed, endothelial Ca^{2+} dynamics regulate all the steps involved in the angiogenic process, including EC proliferation, migration, adhesion to the extracellular matrix and differentiation [see 64, for a comprehensive review]. This feature is consistent with the well known notion that an elevation in $[Ca^{2+}]_i$ plays a crucial role in the intricate network of signaling pathways recruited by VEGF and other growth factors in vascular ECs [49, 95]. It is worth noting that blockers of voltage-gated Ca^{2+} channels (VGCCs) have long been exploited to treat severe cardiovascular diseases, such as hypertension, chronic stable angina, infarction-induced cardiac failure, and arrhythmia [151-153]. Consistently, a number of Phase I and II clinical trials demonstrated that the oral administration of carboxyamidotriazole (CAI, L651582, NSC 609974), a synthetic non-specific inhibitor of Ca^{2+} entry, causes disease stabilization in ovarian cancer, RCC, melanoma, and gastrointestinal (stomach and pancreas) adenocarcinomas [63, 154]. In most cases, the toxicities associated to CAI treatment were well tolerable by patients and included fatigue, nausea and/or vomiting, hyperglycemia, constipation, anorexia, diarrhea, neutropenia and a mild neuropathy [154]. The cytostatic action of CAI is due to its ability to inhibit angiogenesis both *in vitro* and within cancer metastases *in vivo* by affecting either SOCE or TRPV4-induced Ca^{2+} inflow in ECs [54, 63, 65, 155] (see also below). Three pieces of evidence hint at the Ca^{2+} toolkit of circulating EPCs as a suitable, novel molecular target to adverse tumor neovascularization and spreading to distant sites Fig. (**2B**). First, BM-derived EPCs support the formation of cancer neovessels and drive the metastatic switch. Second, Stim1 and Orai1 sustain ECFC proliferation and tubulogenesis by mediating Ca^{2+} inflow upon depletion of the $InsP_3$-sensitive Ca^{2+} pool [21, 24, 25]. Third, SOCE is a signaling pathway whereby VEGF stimulates ECFCs-dependent angiogenesis *in vitro* [21, 24]. These novel observations on the pro-angiogenic effect of Ca^{2+} influx in ECFCs might shed light on alternative strategies to adverse tumor growth and metastatization. In addition to TRPC3 and TRPC6, which are expressed in mature ECs [see 65], but not in ECFCs [25], Orai1 and Stim1 are emerging as promising molecular targets in the fight against cancer. Accordingly, SOCE drives VEGF-induced proliferation and tube formation in both ECs and ECFCs [21, 24, 25, 51, 53, 54]. This feature renders it possible to affect the two main processes underlying tumor vascularization, i.e. local angiogenesis and EPC recruitment, by affecting one single signaling pathway, i.e. SOCE. In support with this hypothesis, interference with Stim1 expression or blockade of SOCE activity inhibits tumor angiogenesis and growth in several animal models, thus confirming the crucial role of SOCE in tumor development *in vivo* [120]. A few caveats should, however, be borne in mind with this kind of approach. First, the contribution of EPCs to tumor vascularization 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 do sustain the neovessel growth in human malignancies. 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. For instance, other Ca^{2+}-permeable pathways, such as TRPC1, might replace Orai1 in providing the pore-forming subunits of store-dependent membrane routes. Third, Orai1 provides the Ca^{2+} source responsible for the activation of both Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII) and calcineurin A, which, in turn, regulate physiological postnatal cardiac hypertrophy [156] and the immune response accomplished by T cell, B cell, and Fc receptors [83], respectively. Moreover, in skeletal muscle fibres, SOCE contributes to refill the sarcoplasmic reticulum during prolonged stimulation, thereby maintaining normal metabolism and preventing muscle weakness over time [157]. Finally, Orai1-mediated Ca^{2+} influx drives platelet adhesion, activation, and aggregation and thrombus formation [158, 159]. It, therefore, appears that stringent pre-clinical studies will be required to ensure that targeting SOCE in tumoral patients will not exert unacceptable side effects, such as heart failure, immunodeficiency, and myopathies [160].

PHARMACOLOGY OF STORE-OPERATED CA^{2+} ENTRY IN ECFCS: WHERE TO START FROM TO DESIGN ALTERNATIVE ANTI-ANGIOGENIC DRUGS

A number of SOCE inhibitors are available for pre-clinical studies aiming at assessing whether it is therapeutically feasible to impair VEGF-dependent signaling without the pitfalls associated to anti-VEGF neutralizing antibodies and tyrosine kinase inhibitors. A number of recent and comprehensive reviews addressed both the molecular structure and the mechanism of action of the most popular blockers of Stim1- and Orai1-mediated Ca^{2+} entry (Table **1**) [71, 91, 161-163]. Briefly, SOCE inhibitors include cations (lanthanides and divalent transition ions), P450 inhibitors (econazole, miconazole, clotrimazole and ketoconazole), cyclooxygenase inhibitors (niflumic acid, flufenamic acid and tenidap), lipoxygenase inhibitors (nordihydroguaiaretic acid and eicosatetraynoic acid), putative channel blockers (SK&F 96365, tetrandrine, 2-aminoethyl diphenylborinate), 3,5-bistrifluoromethyl pyrazole derivatives (BTP-1, BTP-2 and BTP-3), non-steroidal anti-inflammatory drugs (acetylsalicylic-acid, mefenamic acid, and sulindac sulfide) [164], and mechanism-based inhibitors (nocodazole, ML-9 and 2-APB) [71, 91]. Herein, however, we will mainly focus on the agents that have been shown to suppress SOCE in both mature ECs and EPCs.

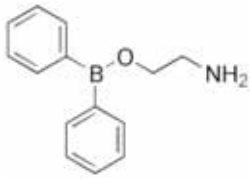
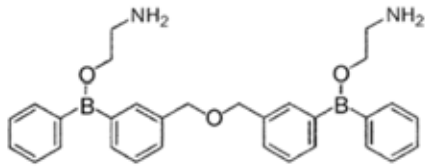
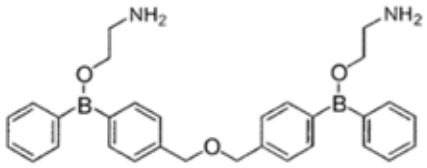
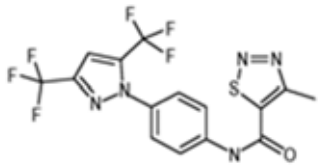
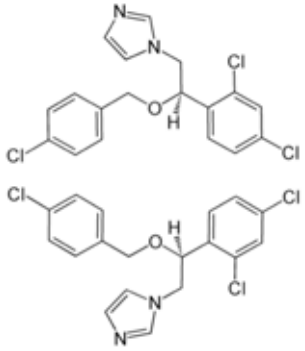
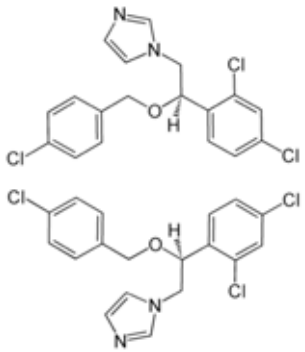
The most widely exploited tools to interfere with SOCE in vascular endothelium are the lanthanides La^{3+} and Gd^{3+} that selectively block this pathway at low micromolar concentrations (1-10 μM) (Table **1**), whereas they also affect TRPC channels at around 100 μM [51, 72, 90, 165, 166]. These trivalent metals directly plug the external face of Orai1 channel by virtue of their large hydrated ionic radius, which is similar to that of Ca^{2+} and precludes their influx into intact cells. As a consequence, La^{3+} and Gd^{3+} block the channel by competing with the permeant ions, i.e. Ca^{2+}, at an anionic site which is probably located at the mouth of the selectivity filter [91, 167, 168]. Albeit trivalent cations suppress SOCE induced by either physiological (i.e. thrombin or VEGF) or pharmacological (i.e. thapsigargin) stimulation in to mature ECs [51, 165], trivalent cations readily precipitate with multivalent anions and serum proteins [91], so that they engender serious technical disadvantages when probed in the presence of cell culture media enriched with growth factors and FBS, such as EGM-2.

2-aminoethyl diphenylborinate (2-APB) is a membrane permeable synthetic heterocyclic ring which consists of an ethanolamine chain, a boron-oxygen core (BOC) and two phenyl rings (Table **1**). 2-APB exerts a biphasic effect on Orai1-mediated Ca^{2+} entry in mature endothelium as well as in many hematopoietic cells: potentiation at low doses (1-5 μM) and full blockade at high concentrations (50-100 μM) [51, 169]. The diphenylboronic moiety is the sole requirement for abrogating Ca^{2+} influx while the ethanolamine group is not necessary for SOCE inhibition [170]. Whereas the molecular underpinnings of 2-APB-dependent Orai1 activation are still unknown [91], its blocking action may be accomplished by two distinct mechanisms. First, 2-APB reverses near plasma membrane punctate Stim1 localization. Second, 2-APB may either directly plug Orai1 channels or prevent Orai1 engagement by Stim1 [171]. 2-APB may also impair Ca^{2+} inflow through Orai2 and Orai3 tetramers, however, these isoforms have not been involved in endothelial SOCE [24, 51, 91]. Unfortunately, 2-APB is a rather non-specific drug being able to affect also $InsP_3$-dependent Ca^{2+} release, SERCA activity and the mitochondrial Na^+/Ca^{2+} exchanger [91, 172]. Moreover, high 2-APB concentrations activate several members of the Vanilloid subfamily of TRP channels (TRPV), such as TRPV1, TRPV2, and TRPV4 [173]. Accordingly, 2-APB (50 μM) suppresses $InsP_3$-evoked Ca^{2+} signals in ECFCs exposed to ATP in absence of extracellular Ca^{2+} [21]. Therefore, this drug may not be considered as a *bona fine* SOCE inhibitor. Further work might be carried out to assess whether two recently discovered structural isomers of 2-APB, namely DPB162-AE and DPB163-AE (where the DPB stands for diphenylborinate), selectively affect SOCE and its downstream signaling cascade in ECFCs. Such analogues consist in 2-APB dimers (with four phenyl rings) connected via two different linker chains. Intriguingly, both compounds impair thapsigargin-evoked Ca^{2+} influx with 100-fold more potency than 2-APB, although only DPB163-AE displayed a bimodal effect on SOCE [174].

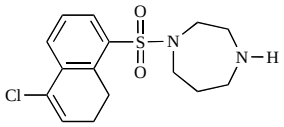
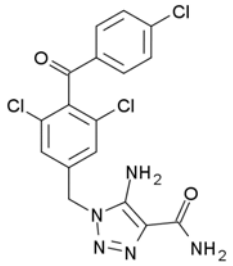
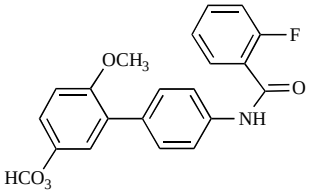
CAI is a synthetic small molecule inhibitor of non-voltage-gated Ca^{2+} channels that has entered Phase I and II clinical trials both as single cytostatic agent and in combination with cytotoxic therapies (http://clinicaltrials.gov/) (Table **1**) [63, 154, 175-178]. CAI has long been shown to block tumor cell proliferation and invasive behaviour at concentrations ranging between 1 and 10 μM [179, 180]. At the same time, pioneering work by Kohn's group revealed that CAI (1-10 μM) inhibits proliferation, motility, and tubulogenesis of HUVECs during *in vitro* angiogenesis [176]. More specifically, CAI affects cell flattening that is a prerequisite for normal and pathological vase formation. CAI also weakens cell adhesion to the substrate, extracellular matrix stimulated migration and collagenase IV production. Finally, CAI impairs the production of metalloproteinase-2 and attenuates collagenolysis, which is crucial to angiogenesis and cancer cell invasion [63, 176, 179, 180]. CAI proved effective at reducing tumor growth and metastatization in several human xenograft cancer progression models [180]. Importantly, CAI was found to inhibit tumor angiogenesis within metastastis *in vivo* by causing reduction in both number of microvessels/mm^2 and microvessel size [155]. The anti-proliferative and anti-angiogenic effect of CAI have been ascribed to its ability to prevent Ca^{2+} influx induced by VEGF ad bFGF in mature endothelium [64]. CAI is, however, a non-specific drug in ECs, as well as most non-excitable cells [49], being capable to blocking both SOCE [54] and AA-elicited Ca^{2+} influx through TRPV4 channels [181]. Moreover, CAI has long been known to impair both $InsP_3$ synthesis [54] and Ca^{2+} efflux through $InsP_3Rs$ [182, 183]. In addition, CAI blocks the mitochondrial Ca^{2+} uniporter [184] and, therefore, cannot be regarded as a *bona fide* inhibitor of SOCE [71, 91]. That CAI may target ion channels and/or transporters other than Orai1-mediated Ca^{2+} entry is interesting in the view of the severe toxicities, such as neuropathies and gastrointestinal disorders, that have been reported during Phase II clinical trials of patients with advanced RCC and refractory to immunotherapy [185, 186]. Similarly, an increase in adverse events and a decrement in the quality of life have been observed in Phase III clinical trials of subjects affected by advances non-small cell lung cancer and treated with CAI [187]. These untoward side-effects, which are absent in patients treated with drugs selectively targeting Ca^{2+}-permeable channels [151], might depend on the unspecific effects of CAI on Ca^{2+} signals.

The pyrazole derivative BTP-2 is a powerful inhibitor of SOCE in hematopoietic cells as well as in other non excitable cells (Table **1**) [89, 188-190], including the endothelium of rat aorta [190, 191]. BTP-2 acts as a powerful immunosuppressant by abating NFAT activation and cytokine production in Jurkat T lymphocytes and by suppressing T cell proliferation [189]. A recent investigation disclosed that the trifluoromethyl groups at positions C3 and C5 of the pyrazole compound are involved in the mechanism of action of the drug, with the trifluoromethyl group at C3 being more critical for its activity [88]. The kinetics of Orai1 channel blockade revealed that BTP-2 rapidly acts through an external binding site when applied at low micromolar concentrations (1-10 μM) [192], although it might also affect SOCE by preventing the cytoskeletal remodeling governed by the actin-binding protein debrin [193]. Similar to 2-APB and other widely employed SOCE inhibitors see (Table **1**), BTP-2 may target ion channels other than Orai1. Accordingly, BTP-2 reduces the open probability of ectopically expressed TRPC3 and TRPC5 when administrated in the same concentration range known to inhibit Orai1 (1-10 μM) [194]. Moreover, BTP-2 in

Table 1. Structure and Mechanism of Action of Widely Employed Inhibitors of Store-Operated Ca^{2+} Entry

Inhibitor Type	Species	Structure	Description	Reference
Direct channel blockers	Lanthanides (La^{3+} and Gd^{3+})		Relatively selective inhibition at 0.1-10 μM	51, 61, 71, 72, 90, 91, 165, 166
	2-APB		Active at concentrations >30 μM; Not specific (inhibitory actions reported on $InsP_3Rs$, SERCA, mitochondrial Na^+/Ca^{2+} exchanger, TRPV1, TRPV2, TRPV4); Activates Orai1 at doses <20 μM	51, 71, 72, 91, 169, 170, 172, 173
	2-APB analogues	DPB162-AE  DPB163-AE 	Enhanced potency/specificity (inhibition observed at 0.6 – 6-6 μM; DPB163-AE stimulates Orai1 at significantly lower doses than 2-APB (10 nM)	91, 174
	BTP-2		IC_{50} value of 10 nM (chronically applied); IC_{50} value of 2.2 μM (acute exposure); Blocks TRPC3 and TRPC5 with an IC_{50} value of 0.3 μM; Activates TRPM4 with an IC_{50} value of 8 nM	21, 25, 71, 72, 91, 88, 89, 188-194
Mechanism-based inhibitors	Econazole		IC_{50} for blockade of SOCE ~ 0.6 μM: acts by causing microtubule depolymerization; Blocks P_{450} cyclooxygenase, non-selective cation channels and cAMP-dependent Cl^- channels over the same concentration range	71
	2-APB		Prevents Stim1 translocation towards plasmalemmal *puncta* (10-100 μM)	91, 171

(Table 1) contd….

Inhibitor Type	Species	Structure	Description	Reference
	ML-9		Well known inhibitor of the myosin light chain kinase; Blocks SOCE with an IC_{50} value of 10 μM: it likely acts by preventing Stim1 translocation towards the plasma membrane	91
Mechanism not known	Carboxy-amidotriazole		IC_{50} value of 0.5 μM: it probably acts by inhibiting mitochondrial depolarization; Blocks also $InsP_3$ synthesis and $InsP_3$-dependent Ca^{2+} release, the mitochondrial Ca^{2+} uniporter, and TRPV4-mediated Ca^{2+} entry over the same concentration range	49, 54, 64, 65, 71, 91, 147, 181
	Synta 66		IC_{50} value of 25.5 nM: unknown mechanism of action; Not probed against TRP channels	24, 71, 91

the low nanomolar range may cause membrane depolarization by activating the non-selective cation channel TRP melastatin 4 (TRPM4), thereby reducing the driving force for Ca^{2+} entry and decreasing SOCE amplitude [188]. We have recently discovered that BTP-2 abrogate cell proliferation and tubulogenesis in ECFCs [21, 25]. As mentioned above, this effect is consequent to the blockade of NF-κB engagement by SOCE [21]. The following pieces of evidence indicate that BTP-2 selectively targets Orai1-mediated Ca^{2+} entry in ECFCs. First, TRPC3 and TRPC5 are not expressed in ECFCs [25]. Second, SOCE as well as ECFC proliferation and tubulogenesis are not affected by 2 μM BTP-2, which is supposed to fully activate TRPM4 and depolarize the cells [21, 25]. These features are particularly intriguing when pursuing the goal to exploit BTP-2 as a template to design novel anti-angiogenic agents to adverse tumor neovascularization. For instance, the oral administration of BTP-2 proved to be successful in the treatment of autoimmune diseases and chronic inflammation in animal models [71], such as 2,4,6-trinitrochlorobenzene-induced contact hypersensitivity in mice, which is paradigmatic of T lymphocyte-mediated delayed-type hypersensitivity reactions [189], and antigen-induced airway eosinophilia in actively sensitized guinea pig [195]. Unfortunately, BTP-2 selectively targets Orai1-dependent Ca^{2+} influx in ECFCs, but not in mature ECs, which also express TRPC3 and TRPC5 [65]. Nevertheless, a new compound has been synthesized based on BTP-2 structure, namely Synta 66 (Table **1**) (3-fluoro-pyridine-4-carboxylic acid (2',5'-dimethoxy-biphenyl-4-yl)-amide, GSK1349571A, patent ref. no. WO2005/009954 and US2004/02379; Synta Pharmaceuticals) [162, 196]. Synta 66 was found to inhibit SOCE with an IC_{50} value of 3 μM in gut T cells and was negatively probed against a large panel of additional ion channels, including voltage-dependent Na^+ channels, VGCCs, and inward rectifier K^+ channels, but not TRP conductances [196]. A recent investigation demonstrated that Synta 66 inhibited Orai1-gated Ca^{2+} influx with a significantly higher potency (IC_{50} of approximately 25 nM) in both human umbilical vein ECs (HUVECs) and in circulating ECFCs [24]. Accordingly, Synta 66 abrogated *in vitro* tube formation and *in vivo* angiogenesis by HUVECs [24]. It, therefore, appears that pyrazole derivates, such as BTP-2 and Synta 66, hold great promise in the quest for novel anti-angiogenic treatments by providing a solid molecular framework for a new generation of small molecule inhibitors [71, 88].

CONCLUSION

The recent elucidation of the molecular underpinnings of SOCE provided pharmacologists with novel and powerful targets, namely Stim1 and Orai1, to design alternative treatments of a number of severe pathologies, including cardiovascular diseases, bowel inflammation, bronchial asthma, primary immunodeficiencies, and solid tumors [71, 91, 197, 198]. Subsequent studies, conducted both by ours and other research groups, have unveiled the key role served by SOCE in driving proliferation, motility and tubulogenesis in BM-derived ECFCs. These are the key steps involved in the vascularization process arising both in damaged organs and within growing tumors. As a consequence, Orai1 and Stim1 represent a sharp double-edged sword for the whole organism as SOCE may either support tissue regeneration or stimulate primary tumor growth and metastatization. Cell-based therapy (CBT) is rapidly emerging as a powerful approach to cure cardiovascular diseases by exploiting the regenerative properties of ECFCs. The hurdles associated to CBT might be addressed by carefully harnessing SOCE in these cells as well as other types of EPC, i.e. CFU-ECs and CACs. The adjuvant cells might be properly engineered to over-express Stim1 and Orai1 before inoculation in order to optimize the efficiency of stem cells transplantation. On the other hand, interfering with EPC recruitment by tumor cells is nowadays recognized as one of the most promising strategies to pursue in concert with VDA, chemotherapy or anti-angiogenic treatments. The finding that SOCE controls ECFC proliferation and tubulogenesis highlights a novel, hitherto non-considered molecular target for anti-angiogenic strategies. Recent advances by pharmaceutical industry have led to development of novel compounds which provide a promising template for the development of more selective inhibitors of SOCE. In addition, humanized antibodies targeted against the extracellular domains of Orai1 might be devised to implement the therapeutic arsenal of compounds that able to interfere with tumor vascularize-

tion. It is, however, important to recall that membrane pathways other than SOCE might be exploited when utilizing EPCs as a therapeutic tool or target. The elucidation of the blend of ion channels controlling EPC proliferation, tubulogenesis, and differentiation is still in its infancy. We predict that future studies will unveil a key role for K^+ channels and several members of the TRP family of non-selective cation channels, such as TRPV4 and TRPC1, which are indispensable in mature endothelium.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

We acknowledge Dr. Luisa Ottobrini, Department of Biomedical Sciences and Technologies, University of Milan, Italy, for critical reading of the manuscript.

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Received: December 14, 2011 **Revised: April 24, 2012** **Accepted: May 28, 2012**