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CIRCULATING ENDOTHELIAL PROGENITOR CELLS FROM PATIENTS WITH RENAL CELL CARCINOMA DISPLAY ABERRANT VEGF REGULATION, REDUCED APOPTOSIS AND ALTERED ULTRASTRUCTURE.

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From birth, man carries the weight of gravity on his shoulders. He is bolted to earth. But man has only to sink beneath the surface and he is free.

J. Y. Cousteau

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INTRODUCTION

A BRIEF INTRODUCTION: VASCULOGENESIS AND ANGIOGENESIS

PHYSIOLOGICAL VASCULOGENESIS AND ANGIOGENESIS

The mechanism of blood vessel formation, from the embryo to adult life, has been the subject of a large number of studies which aimed at understanding the fine molecular pathways underlying such process. In the very early stages of embryonic development, nutrients are received by diffusion, and only in a second time a complex network of capillaries and blood vessels develop with the aim of carrying oxygen and nutrients to organs and tissues and removing their catabolic waste (Noden, 1989).

The creation of running capillaries, veins and arteries takes place through two main phases (Figure 1). The first is called vasculogenesis and starts in the yolk sac where mesenchymal cells in the periphery, and hematopoietic stem cells (HSC) in the center, give rise to the so called "blood islands", the well documented first site of hematopoiesis and vascular development during embryogenesis (Ferkowicz and Yoder, 2005). Angioblasts, the precursors of endothelial cells (ECs), and hematopoietic cells emerge near each other in blood islands, and have common surface antigen markers, hence the concept that a common precursor to hematopoietic and endothelial lineage can exists (hemangioblast). The concept of hemangioblast was proposed nearly a century ago based on the close proximity of cells in the yolk sac that give rise to both blood cells and blood vessels. This hypothesis is supported by studies using *in vitro* mouse and human embryonic stem cells cultures, and *in vivo* animal model systems including zebrafish, chick, and mice. During vasculogenesis, angioblasts migrate towards different places where they can differentiate and give rise to endothelial cords and to the primitive vascular network (Carmeliet, 2000). Angioblast recruitment and differentiation is driven by a myriad of cytokines and growth factors, such as vascular endothelial growth factor (VEGF), granulocyte monocyte-colony stimulating factor (GM-CSF) and fibroblast growth factor (FGF) (Ferrara et al., 1996; Swift and Weinstein, 2009).

This primitive vasculature network is remodeled and extended by angiogenesis, the second step of vessel formation, as profusely described by Conway et al., 2001. Angiogenesis can in turn be divided in different steps listed below:

- Vasodilation of existing vessels, mainly in response to nitric oxide (NO) and VEGF;
- Increase in permeability after a modification in the distribution of intracellular adhesion

molecules, such as platelet endothelial cell adhesion molecule (PECAM-1) and vascular endothelial (VE)-cadherine;

- Extravasation of plasma proteins with the creation of a scaffold for ECs, and degradation of surrounding matrix that involves many different matrix metalloproteinases (MMPs) (Nelson et al., 2000);
- Migration of activated and proliferating ECs controlled by a finely tuned balance of activators and inhibitors factors, such as VEGF, angiopoietins, FGF, and their receptors;
- EC differentiation and subsequent interaction with extracellular matrix and periendothelial cells, i.e. pericytes or smooth cells in small and large vessels, respectively; this latter process has been called vascular myogenesis (Conway et al., 2001);
- EC assembly into solid cords and subsequent formation of the lumen through interaction of ECs with both extracellular matrix and existing vessels. Lumen size is tightly regulated by several factors, like VEGF, Angiotensin 1 and multiple integrins (Suri et al., 1998);
- Finally, during arteriogenesis, smooth muscle cells create a thick stabilizing coat around vessels that become viscoelastic and with vasomotor characteristics.

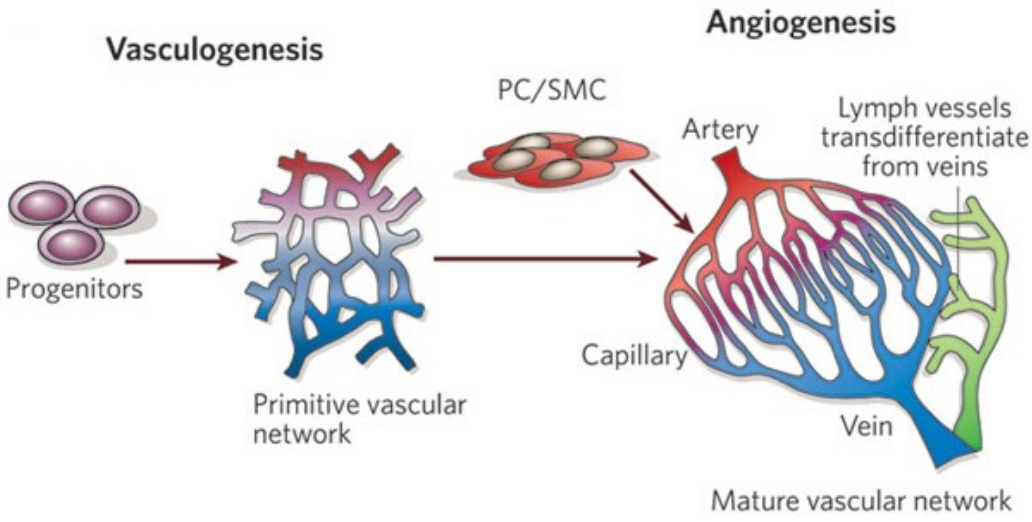


Figure1. Development of the vascular system (PC: pericytes, SMC: smooth muscle cells) (Carmeliet, 2005).

Therefore, the vasculogenic process takes place during embryogenesis, followed by angiogenesis which continues also after birth when it contributes to organs growth. In adult life, most of blood vessels remain in a quiescent state, and ECs are arrested in the G_0 phase of cell cycle. However, vasculogenesis can be resumed in particular physiological conditions, such as ovary cycle, pregnancy, or wound repair, by bone marrow- or arterial wall-derived angioblasts or endothelial progenitor cells (EPCs).

ENDOTHELIAL PROGENITOR CELLS (EPCs)

The dogma that vasculogenesis takes place only during embryonic development has been completely revolutionized in 1997 by Asahara et al. who identified for the first time endothelial progenitor cells (EPCs) in peripheral blood (PB) of adult subjects (Asahara et al., 1997). EPCs have been defined as cells with self-renewal capacity, able to differentiate *in vitro* in mature ECs and generate patent vessels *in vivo* through a process termed postnatal vasculogenesis (Khakoo and Finkel, 2005). EPCs can be isolated from mononucleated cells (MNCs) of bone marrow (BM), peripheral blood (PB), umbilical cord blood (UCB) but also from the wall of large arteries (Yoder, 2012). The seminal paper by Asahara et al. (1997) clearly stated the thesis that a subset of cells circulating in PB function as progenitors of the endothelial lineage, both *in vitro* and *in vivo*. Following the first Asahara's work, an intensive investigation allowed to understand that the cells identified by Asahara were not real EPCs because they rather belong to the hematopoietic lineage, as better described below. Nevertheless, the concept that endothelial precursors circulate in PB of adult individuals was correct, but it took much longer to isolate the cells with the characteristics of true endothelial progenitors.

IDENTIFICATION OF EPCs

As well described by Prater et al. (2007), three methods have been explored to identify true EPCs *in vitro* (Figure 2) (Prater et al., 2007).

Asahara in 1997 at first, and subsequently Hill et al. in 2003, isolated the so called colony-forming unit-endothelial cells (CFU-ECs) or CFU-Hill (Hill et al., 2003). They plated PB-MNC on fibronectin-coated dishes and after 48 hours of culture non-adherent cells were recovered and replated on fibronectin-coated dishes. Colonies of round cells in the centre and spindle-shaped cells in the periphery appeared after 5-9 days of culture. Even though CFU-ECs expressed on their surface antigens specific for endothelial lineage, such as CD31, CD105, CD144, VWF, KDR and UEA-1, they also presented the hematopoietic-specific cell surface antigen CD45 and the antigens specific for monocyte and macrophages lineages, CD14 and CD115 (Rehman et al., 2003; Kalka et al., 2000). Furthermore, they did not show the specific progenitor feature of displaying high proliferative potential, but they stopped growing after 2 or 3 culture passages. Therefore, it was concluded that CFU-ECs were not representative of EPCs but were actually derived from hematopoietic cells (Yoder et al., 2007).

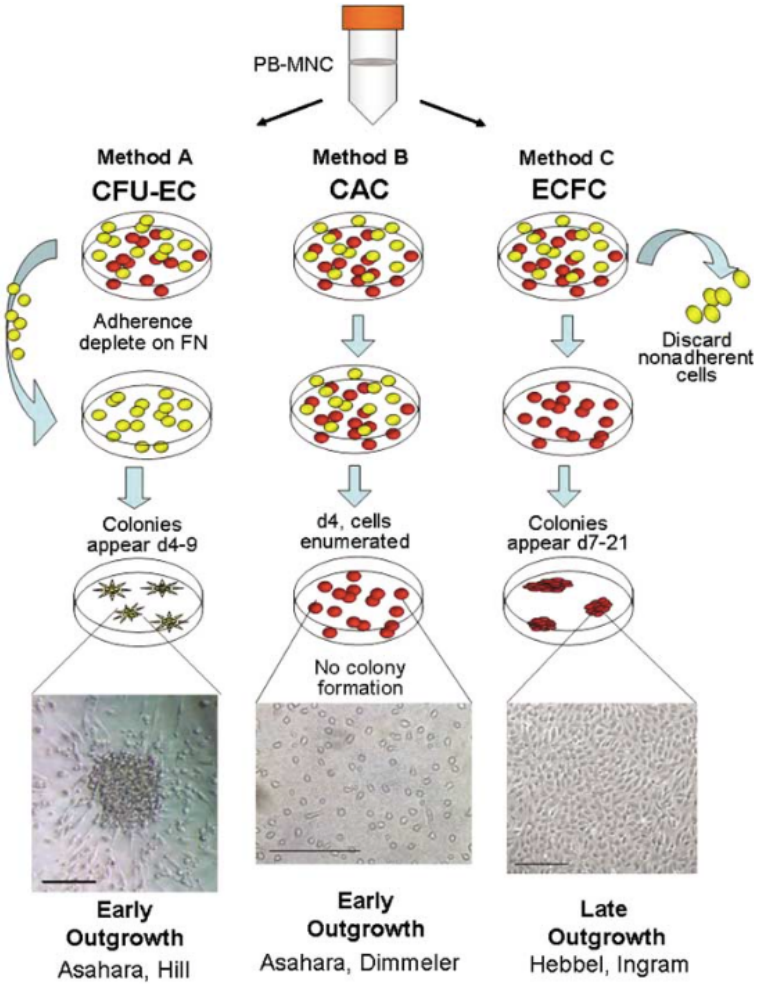


Figure2. Common methods of EPCs culture (Prater et al., 2007).

Another method, introduced for the first time by Asahara and Dimmeler employed PB-MNCs plated on fibronectin-coated plates for 4 days in a supplemented endothelial growth medium; after this period, non adherent cells were discarded and a population of single spindle-shaped cells remained adherent to the culture dish. They called these cells "circulating angiogenic cells" (CACs) for their characteristic of promoting neovascularization in animal models of hind limb ischemia or myocardial infarction. CACs expressed endothelial-specific surface antigens CD31, CD144, VWF, KDR (Dimmeler et al., 2001), but were not able to form colonies, did not exhibit self-renewal properties, and had an intermediate phenotype between hematopoietic and endothelial lineage. An extensive immunophenotypical characterization showed that CACs were hematopoietic by clonal lineage tracking, exactly as it occurred for CFU-Hill (Yoder et al., 2007). Finally, the third method, described by Hebbel and Ingram identified the so called "endothelial colony forming cells" (ECFCs). After plating PB-MNCs or UCB-MNCs in collagen treated plates with endothelial-specific medium, non-adherent cells were discarded by gentle washes after 48 hours of culture and a medium change performed every 48 hours until the first colonies started do appear. It took about

10-21 days if the MNCs source was PB, 7-9 days if MNCs derived from UCB; colonies displayed a cobblestone shape, typical of ECs. ECFCs were also phenotypically indistinguishable from mature ECs, possessed vessel-forming ability *in vivo* and were able to form capillary-like structure in matrigel *in vitro* (Figure 3).

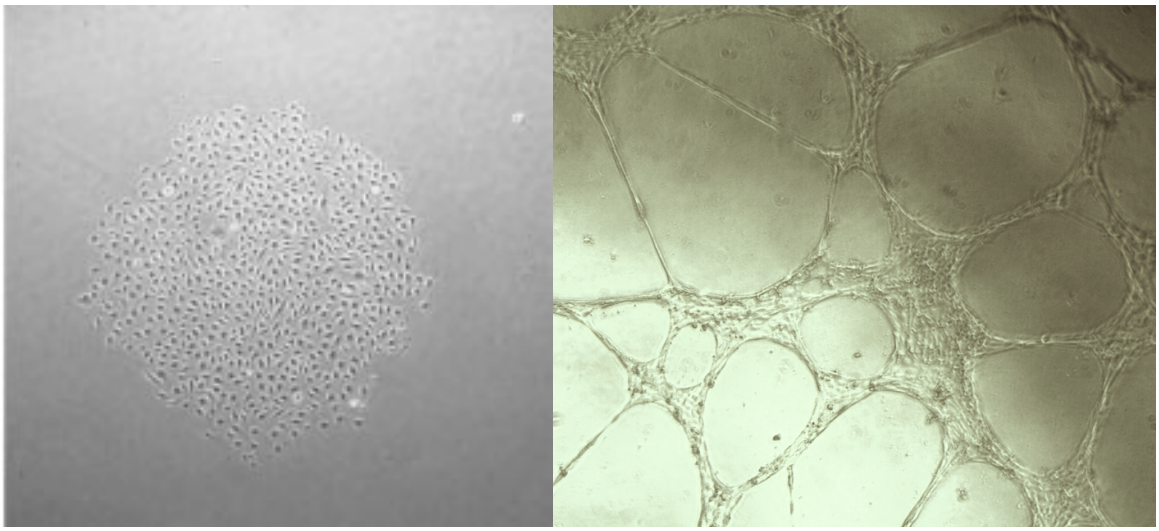


Figure 3. EPCs colony and capillary-like structure in matrigel assay.

ECFCs expressed all the endothelial-specific cell surface antigens, CD105, CD31, CD144, VWF, VEGFR-2, but not the hematopoietic antigens CD14 and CD45, confirming that they did not derive from the hematopoietic lineage. To date, many steps forward have been accomplished on the knowledge on ECFCs and it is clear that ECFCs are rare cells in PB of healthy adult subjects, their concentration being about 0.05-0.2 cell/ml (Prater et al., 2007; Yoder et al., 2007); ECFCs are able to participate to vessel formation in murine models of immunodeficient mouse when they have been injected after suspension in a collagen scaffold (Schechner et al., 2000; Yoder et al., 2007). It can been concluded that because ECFCs have a clonal proliferative capacity, the ability to expand for several passages *ex vivo*, to differentiate in mature ECs, and to form vessel *in vivo*, they display all the characteristics of progenitor cells and have been considered the best EPCs surrogate *in vitro* (Yoder, 2012; Basile and Yoder, 2014).

PHENOTYPICAL AND FUNCTIONAL CHARACTERIZATION OF EPCs

A definitive immuno-phenotypical characterization of EPCs is yet to be described. A big debate, conducted over the last 15 years, concluded that immuno-phenotypical analysis cannot truly identify EPCs in blood circulation. A combination of phenotypical, cultural and functional analyses are necessary to fully characterize these cells. At first, CD34 has been identified as a potential marker of circulating angioblasts, because it is known to be expressed on endothelial cells; however

it is also widely used to identify human hematopoietic stem and progenitor cells. For this reason, CD34 cannot be used as a specific EPC marker. Also KDR (or VEGFR-2), one of VEGF receptors, is expressed on blood, endothelial and cardiac cells, therefore it is not a big discriminating factor among $CD34^+$ cells. CD133 has also been considered a potential EPC marker. Peichev et al. hypothesized that cells positive for CD34, KDR and CD133 were the best EPC representatives and these three markers have been used to identify circulating EPCs in hundreds of papers since 2000 (Peichev et al., 2000). Case at al., however, reported that $CD34^+$, KDR^+ and $CD133^+$ cells are highly enriched in hematopoietic progenitor activity and are not able to give rise to any endothelial colony *in vitro* (Case et al., 2007; Yoder et al., 2007). The debate about EPC identification went on and, in 2012, Mund et al. tried to clarify this confused scenario by carrying out a complex polychromatic flow cytometry analysis of EPCs based on an appropriate panel of optimizing antibodies (Mund et al., 2012). As shown in Figure 4, EPCs have been characterized as CD45 negative cells, positive for CD34, CD31 (an endothelial-specific marker), CD146 (an adhesion molecule expressed by mature ECs) and CD105, or endoglin, a part of TGF beta receptor complex.

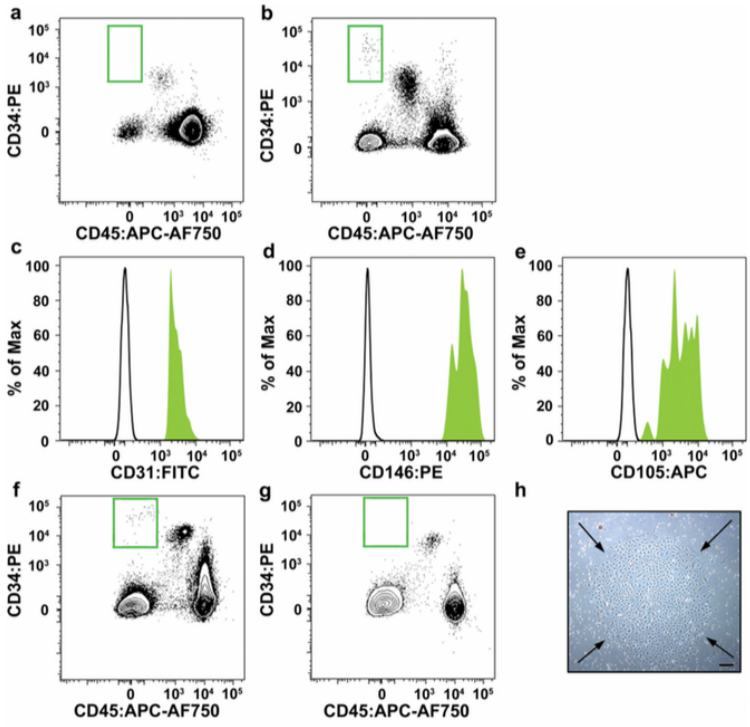


Figure 4. Circulating EPCs identification by polychromatic flow cytometry (Mund et al., 2012).

However, besides phenotypical identification, a colony forming assay has to be used to identify putative EPCs. The method has already been described and is the one used by Hebbel and Ingram that obtained colonies with typical endothelial markers, similar to vascular endothelial cells,

forming blood vessels *in vivo* and differentiating in mature ECs (Ingram et al., 2004). ECFCs have a high proliferative and robust replating potential and high telomerase activity, appearing as being consistent with the original criteria for EPCs identification (Basile and Yoder, 2014). Moreover, ECFCs are able to create capillary-like structure when plated on Matrigel, a commercial mixture protein which mimics the basal membrane of extracellular matrix.

EPC MOBILIZATION

It has been demonstrated that EPCs resides in specific sites of BM, called niches, where cells can stay undifferentiated and quiescent or proceed toward a differentiated state. EPCs, in the niches, interact with microenvironmental cells which, in turn, regulate EPC fate (Papayannopoulou, 2003). EPCs can be released from BM into PB through a process called mobilization. As aforementioned, EPCs are a rare population in PB, and the mechanisms that regulate their number are still unknown, although growth factors such as GM-CSF, granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), stem cell factor (SCF) and TGFβ could have a fundamental role (Pompilio et al., 2009). Both in physiological conditions, such as physical activity or endometrial reconstruction after menstrual cycle, and in pathological conditions, such as myocardial infarction and ischemic attack, EPC frequency in PB can increase (Massa et al., 2009). Although the phenomenon has extensively been studied, a mechanism that explains each phase of mobilization process has not been yet described. Possibly, a central role is played by GM-CSF and G-CSF (both widely used in hematology for the expansion and mobilization of hematopoietic stem cells in BM transplantation), by VEGF, whose administration increase EPC number and functional abilities (Asahara et al., 1999), and by the soluble form of the c-kit ligand, otherwise called stem cell factor (SCF). The latter requires the cleavage by MMP9 to become active and can interact with its c-kit receptor on EPCs, thus triggering the signalling cascade which stimulates EPC exit from BM (Heissig et al., 2002). MMP9 activity can also be increased by another key factor for the mobilization process, namely nitric oxide (NO). The expression of endothelial nitric oxide synthase (eNOS), for example, is enhanced by VEGF in BM stromal cells (Ribatti, 2007). Murohara et al. demonstrated that the impaired neovascularization in mice lacking eNOS was related to a defect in EPC mobilization (Murohara et al., 1998). Thus, within the BM niche, cytokines, chemokines, proteases and adhesion molecules converge in a complicated network of interaction in which every factor has an important role to determine EPC retention or mobilization (Papayannopoulou, 2004). This scenario has further been complicated by a recent model, according to which some cytokines might block EPC interaction with the surrounding stroma thereby enhancing their transendothelial migration. Little is known about this process, but some studies demonstrated that EPCs and murine BM progenitor cells are able to extravasate (Jin et al., 2006). Finally, integrins and their respective

ligands are involved in the EPC mobilization of progenitor cells. Human adult PB-derived EPCs express β2-integrins (Chavakis et al., 2005; Wu et al., 2006). *In vitro* adhesion studies revealed that β2-integrins mediate the adhesion of human EPCs to mature endothelial cell monolayers (Chavakis et al., 2005). Additional studies demonstrated the role of β2 integrins for the homing and neovascularization capacity of EPCs in a murine model of myocardial infarction (Wu et al., 2006). The contribution of α4β1 integrin is more complex. In a recent study, the blockage of α4β1 integrin did not inhibit homing of EPCs to ischemic tissues, but increased mobilization of progenitor cells from the bone marrow and enhanced progenitor cell-mediated neovascularization in the context of ischemia (Qin et al., 2006). Nevertheless, the inhibition of α4β1-integrin significantly blocked adhesion and homing of bone marrow progenitor cells to sites of active tumor neovascularization in xenografted tumor models (Jin et al., 2006). A conceivable explanation for this discrepancy is that different integrins may play distinct context-specific roles (ischemic vs. tumor neovascularization) during EPC mobilization.

EPC RECRUITMENT

Homing is the biological process by which EPCs target their final destination, i.e. ischemic or damaged tissues or growing tumors. Homing to sites of active angiogenesis is finely regulated by a delicate interplay among chemokines, chemokine receptors, adhesion molecules, and proteases (Chavakis et al, 2008). While lymphocyte homing towards inflammation sites is a well known process, the homing of EPCs to sites of ischemia or tumor vascularization is less understood, even though these two processes share some common features. Several studies have, however, sought to elucidate how EPCs reach their target organ. For example, the SDF-1/CXCR4 axis has been shown to play a fundamental role in this context. This axis effectively involves the stromal derived factor-1 (SDF-1) which binds his receptor on the surface of PM, the C-X-C chemochine receptor type 4 (CXCR-4). EPCs express CXCR4 on their surface, and both CXCR4 and SDF-1 expression are up-regulated under specific pathological conditions, such as ischemia (De Falco et al., 2004). Consistently, inhibiting the SDF-1/CXCR4 axis interferes with EPC homing to ischemic myocardium (Abbott et al., 2004); conversely, local SDF-1 overexpression enhances EPC homing and incorporation into ischemic tissues. Thus, it has been hypothesized that a SDF-1 gradient is generated from the ischemic site (more concentrated) to the PB (less concentrated), thereby favoring EPC recruitment to sites of active neovascularization. Sun et al. described a similar mechanism for EPC homing in different types of tumors (Sun et al., 2010). They found that SDF-1 is involved in tumor development by attracting tumor cells to metastatic lesions, by recruiting BM-derived hematopoietic stem cells and EPCs at both primary and secondary deposits, and possibly by favoring the establishment of cancer stem cells within the tumor microenvironment.

Finally, VEGF could also contribute to EPCs recruitment either directly or through an interaction with the SDF-1/CXCR4 axis (Zagzag et al., 2006). Similar to SDF-1, a VEGF gradient is established between the angiogenic site and the BM niche, thereby paving the way for EPC homing to their final destination. Thus, an intricate network of different factors also participates in EPC recruitment, but the molecular mechanisms through which they act are not completely clear yet.

EPC DIFFERENTIATION

As previously described, EPC proliferation, migration and differentiation, are regulated by several growth factors, cytokines, and chemokines, but the fine mechanisms governing these processes are still unclear. VEGF and its receptors play the most prominent role in endothelial differentiation during embryogenesis. It has been demonstrated that fibronectin favors VEGF-induced EPC differentiation with a rather complex mechanism. First, VEGF binds to fibronectin heparin-II domain. Second, this interaction leads to the physical association between VEGFR-2 and $\alpha 5\beta 1$ integrins, thus triggering the intracellular signalling pathways responsible for EPC differentiation (Wijelath et al., 2006). Subsequently, Li et al. and Zhu et al. reported that AMP-activated protein kinase (AMPK) also plays an important role in EPC differentiation by mediating VEGF-induced eNOS activation (Li et al., 2008; Zhu et al., 2011). This observation was supported by subsequent studies focusing on the involvement of the Krüppel-like transcription factor (KLF) family in EPC differentiation. KLF is a central regulator of endothelial proliferation, differentiation and development (McConnell and Yang, 2010). Recent work has demonstrated that KLF2 is activated by VEGF in an AMPK-dependent manner and controls EPC differentiation by inducing the expression of endothelial markers, down-regulating the stem/progenitor marker c-kit, augmenting eNOS expression and improving tube formation capacity (Song et al., 2013).

Finally, shear stress has also been investigated as mediator of EPC differentiation. Shear stress is the tangential force of the flowing blood on the endothelial surface of the blood vessel and is necessary for maintaining cardiovascular homeostasis. In particular, shear stress subserves an important role in angiogenesis, in vascular tone control and in vascular remodeling. Endothelium exposed to shear stress undergoes cell shape changes, alignment and microfilament network remodeling in the direction of flow. Moreover, shear stress causes mature ECs to express and secrete a multitude of anti-atherogenic, anti-thrombotic and anti-inflammatory proteins, and to up-regulate eNOS and NO production. Similarly, shear stress also influences EPC differentiation into mature ECs by promoting adhesion, migration, proliferation, tube formation and expression of mRNAs encoding for endothelial-specific genes, such as VEGFR-1, VEGFR-2, VE-Cadherin and

Tie-2. Finally, shear stress has been demonstrated to induce EPCs differentiation by activating VEGFR-2 and the PI3K/Akt/mTOR pathway (Obi et al., 2012).

In conclusion, several different factors, either biological or mechanical, can influence and regulate EPC differentiation, but the specific pathways underlying the process are still largely unknown.

ANGIOGENESIS AND CANCER

Angiogenesis can restart in adult life, not only in physiological conditions, but also in pathological ones, for example during phlogosis, ischemic diseases and cancer. Since '70s, Judah Folkman has documented that a tumor cannot expand fed only by diffusion, but it needs a vascular network which is necessary for growth, invasion and metastatization (Folkman, 1992). Furthermore, in last decades, a large number of studies focused on the importance of tumor microenvironment (basement membrane, immune cells, capillaries, and extracellular matrix) and on the interactions between cancer cells and normal stromal cells. As an example, cancer associated fibroblasts (CAF), found in tumor microenvironment, have been shown to favor tumor progression by promoting interactions between cancer cells, ECs, perycites, epithelial and inflammatory cells, through the secretion of specific growth factors (Kalluri and Zeisberg, 2006).

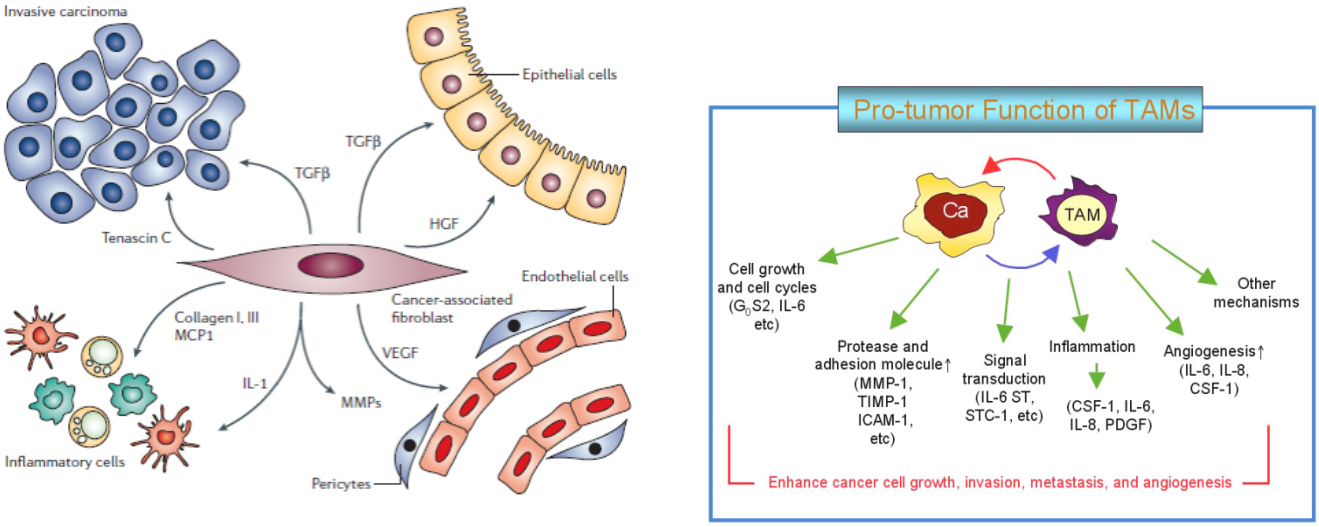


Figure 5. Cancer-associated fibroblast and tumor-associated macrophages: interactions and cytokines release (Shih et al., 2006).

Among inflammatory cells, macrophages can be found as a major component of the leukocytic infiltrate. It has been demonstrated that they are also present with an active role in growing tumors, in which they are referred to as tumor associated macrophages (TAM) (Figure 5). Similarly to CAF, TAM can interact with cancer cells and stimulate the transcription of genes important for the regulation of cell cycle, adhesion to extracellular matrix, signal transduction, inflammation and angiogenesis. All together, the interactions between CAF, TAM, cancer cells and other cells belonging to tumor microenvironment result in cancer progression, invasion and metastatization (Shih et al., 2006). The latter process, however, cannot take place in the absence of a supporting vascular network. The transition from *in situ* carcinoma to a highly vascularized tumor has indeed been termed "angiogenic switch" and allows cancer cells to spread to distant sites thereby causing

patients death. The angiogenic switch also allows tumor perfusion which brings nutrients, oxygen and several growth factors, proteinases and cytokines that exert a paracrine effect on tumor cells, while at the same time removing their catabolic waste. Accordingly, some human carcinomas can persist *in situ* for months to years, but they are not able to growth more than 1-2 mm^3 without connecting to peripheral circulation because of the physical constraints imposed by nutrient diffusion rates. Under these conditions, tumor size is shaped by a delicate balance between cell proliferation and apoptosis. In growing tumors, however, microscopic areas of intense angiogenesis can flank hypo-vascularized areas (Ribatti et al., 2007). When the angiogenic switch occurs, a thick capillary network surrounds and penetrates the tumor to sustain cancer growth and metastatization. The angiogenic switch is activated when the balance between pro- and anti-angiogenic factors tips in favor of the former.

Tumor vasculature is completely different from normal vasculature, both in structure and function; the chaotic vessels organization leads to highly vascularized regions in close proximity of vessel-poor areas. Neoplastic vasculature is tortuous, irregular, and lumens can vary from abnormally wide to really thin. Every layer of the vessel wall is abnormal and also ECs do not display the classical cobblestone shape and often lose their interconnections. Basal membrane thickness and composition are irregular and fewer hypocontractile mural cells cover tumor vessels. All these abnormalities result in an irregular perfusion that impairs nutrients, oxygen and drugs delivery. In order to improve vascularization, and consequently nutrients and oxygen supply to the growing tumor, vasculogenesis and angiogenesis are reactivated, thereby giving rise to the angiogenic switch. In the tumoral site, the angiogenic switch consists in the formation of an intricate network of tumor-associated vessels that ensure the access to peripheral circulation and sustains cancer growth and metastatization (Hanahan and Folkman, 1996). In a simplified largely accepted model, neoangiogenesis takes place when VEGF stimulates ECs to synthesize and secrete MMPs which, in turn, are able to break the basal membrane of capillaries to which ECs belong. Thereby, ECs tear off the vascular wall and can migrate towards the extracellular matrix where they can proliferate and give rise to new vessels that supply the growing tumor with blood, nutrients, and oxygen. During this process, a number of accessory cells like TAM, CAF, mural cells and pericytes are recruited to secrete growth factors and cytokines that participate to produce new basal membrane and extracellular matrix. The whole process is regulated by inducer and inhibitor factors (Bergers and Benjamin, 2003). Two fundamental examples of these regulators are, respectively, VEGF and TSP-1. VEGF expression is enhanced by tissue-hypoxia and by some oncogenes, such as Ras and Myc. VEGF role in angiogenesis and VEGF as potential target of antineoplastic therapies will be discussed in next paragraphs. Conversely, TSP-1 has an important inhibitory effect on neovessel formation and on angiogenic switch. It binds its receptor on the ECs and triggers a signalling

pathway that inhibits angiogenesis. Besides VEGF and TSP-1, in the last few years, dozens of angiogenesis regulators have been identified. They are involved in controlling not only tumor, but also physiological angiogenesis during tissue remodeling and wound repair; such inhibitors may act as intrinsic barriers to induction of angiogenesis in incipient neoplasia (Hanahan and Weinberg, 2011). The up-regulation of VEGF as well as other pro-angiogenic growth factors is solicited by the decrease in oxygen tension (pO_2) that features growing tumors. The drop in pO_2 is sensed by the hypoxia-inducible factor-1α (HIF-1α), a transcription factor controlling the expression of genes encoding for several cytokines and growth factors, such as VEGF, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insuline-like growth factor-2 (IGF2). They can be released into blood circulation to stimulate adjacent capillaries to sprout towards avascular neoplasia and also to promote BM niche to switch from a quiescent status to a pro-angiogenic one (Moccia and Poletto, 2014). Once the angiogenic process is activated, the sequence of biological events can slightly differ among different types of tumor, stating that not every type of neoplasia depends on enhanced angiogenesis. Some tumors, indeed, as pancreas adenocarcinoma, show no strict dependency on angiogenesis as opposed to dependency on the secretion of cytokines and growth factors by stromal cells, especially in later stages. Conversely, many other tumors, such as kidney, breast, and colon cancer, are hyper-vascularized and show an abnormally large number of blood vessels attached to them. In conclusion, the angiogenic switch is essential for the first steps of the tumor growth but then, a complex regulation system, which can involve both tumoral and stromal cells, co-operating for neoplastic vascularization, can take over tumor progression.

EVIDENCES IN FAVOUR TO EPC CONTRIBUTION TO TUMOR VASCULATURE

The capability of cancer cells to intravasate into peripheral circulation and disseminate to remote organs is exacerbated by the well known chaotic disorganization of tumor vessels. These are featured by a number of morphological, cellular, and molecular abnormalities that all together concur to shape a dilated, tortuous, and hyperpermeable microvascular network inside the neoplasm (Dudley, 2012; Moccia and Poletto, 2014). The remarkable heterogeneity in tumor endothelium is dictated by three main mechanisms, which are not mutually exclusive, but may interact to produce aberrant endothelial cells: 1) the cross-talk with the surrounding microenvironment - featured by hypoxia, low pH, disorganized basement membrane, elevated interstitial fluid pressure, enrichment in growth factors and cytokines - which may impart both genetic and epigenetic modifications to tumor endothelial cells (TECs); 2) the vascular bed of origin, which conveys a site-specific epigenetic footprint to endothelial cells sprouting towards the neoplastic lesion; and 3) the contribution of additional mechanisms to tumor vascularization, including vasculogenic mimicry,

intussusceptive angiogenesis, vessel co-option, recruitment and engraftment of haematopoietic and endothelial progenitor cells (Dudley, 2012; Aird, 2012). A growing number of studies have now established that the concerted interaction between local endothelial cells and circulating EPCs is key to the angiogenic switch in tumor growth and metastatic progression (Moccia and Poletto, 2014; Gao et al., 2009; Nolan et al., 2007). The angiogenic switch is turned on when the delicate balance between pro- and anti-angiogenic factors is tipped in favour of neovessel formation by tumor microenvironment. This process is regulated by HIF-1α, activated under the hypoxic conditions of a growing tumor thus driving the expression of VEGF, EGF, bFGF, and SDF-1α (Carmeliet and Jain, 2011; Moccia et al, 2012). Once released into circulation, these soluble factors promote local angiogenesis by stimulating nearby endothelial cells to sprout towards the neoplasm; at the same time, they mobilize BMDCs and target them to the nascent vasculature (Gao et al., 2009; Moccia and Poletto, 2014; Coghlin and Murray, 2010). Indeed, a remarkable pro-angiogenic activity has been acknowledged to several populations of BM-derived haematopoietic cells, such as $CXCR4^+$ $VEGFR1^+$ hemangiocytes, Tie2-expressing monocytes, $CD45^+/CD11b^+$ myeloid cells, $F4/80^+$ $CD11b^+$ tumor-associated macrophages (TAMs), $GR1^+$ $CD11b^+$ "myeloid-derived suppressor cells" (MDSCs), and infiltrating neutrophils and mast cells (Gao et al., 2009; Patenaude et al., 2010). These cells sustain tumor growth perivascularly by paracrine liberation of growth factors and cytokines, but do not incorporate within vessel lumen. Conversely, EPCs may provide the building blocks for neovessel formation as well as secrete instructive signals for neighbouring endothelial cells (Gao et al., 2009; Moccia, Dragoni et al., 2014; Moccia et al., 2014). The earlier demonstration that EPCs are involved in tumor angiogenesis was provided by Lyden and coworkers, who found that BM transplantation rescued growth and metastatization of two distinct syngenic tumor models (B6RV2 lymphoma and Lewis lung carcinoma or LLC) xenografted in the angiogenic defective $Id1^{+/+}$ $Id3^{-/-}$ mutant mice (Lyden et al., 2001). Tumor vascularization under these conditions was associated to the recruitment of $VEGFR\text{-}1^+$ myeloid cells and $VEGFR\text{-}2^+$ EPCs from reconstituted BM. The same authors documented that the engraftment of β-galactosidase-positive (lacZ) BM from Rose-26 mice, which express lacZ in all tissues, recapitulated angiogenesis in $Id1^{+/+}$ $Id3^{-/-}$ mice implanted with B6RV2 tumors. Importantly, histological examination disclosed $lacZ^+$ vessels in tumors inoculated for at least 14 days, while 90% of $lacZ^+$ vessels were also stained for von Willebrand factor (vWF) a typical endothelial marker; this latter feature confirmed that BM-derived cells were intraluminally incorporated within tumor vasculature (Lyden et al., 2001). Subsequently, fluorescence *in situ* hybridization (FISH) of sex chromosomes in individuals who developed cancer after BM transplantation with donors of the opposite sex, detected BM-derived endothelial cells throughout tumor vasculature, their percentage

ranging from 1% to 12% depending on the malignancy (Peters et al., 2005). More recently, Nolan and coworkers used BMDCs isolated from GFP^+ mice and injected into lethally irradiated syngenic wild-type recipient to investigate EPC contribution to tumor angiogenesis (Nolan et al., 2007). Reconstituted animals were xenografted with three distinct tumor types, i.e. LLC, B6RV2 and melanoma, and then examined at various stages of tumor development by using endothelial (VE-cadherin, CD31, endoglin, and VCAM), haematopoietic (CD11b, CD45RB, CD41) and progenitor (CD133) markers. GFP expression, in turn, ensured BM origin of vessel cells. These authors first found that BM-derived GFP^+ cells were recruited at the periphery of LLC at the early stages of tumor growth (days 4-6) prior to the sprouting of endothelial cells from nearby capillaries. These cells were identified as EPCs based on their morphological and phenotypic characterization. Accordingly, they presented with low levels of CD31 and uniformly expressed VE-cadherin, which is restricted at adherens junctions between two adjacent cells in mature endothelium. Additionally, these cells were endowed with the progenitor cell marker, CD133, and lacked all the haematopoietic antigens for which they were probed. When LLC tumors were inspected at later stages (6-8 days), they showed chimeric vessels comprising both non-BM-derived cells and BM-derived GFP^+ $CD31^+$ VE-$cadherin^+$ cells with all the features of a typical mature endothelial cells: i.e. spindle-line morphology, high surface expression of CD31, VE-cadherin staining at the intercellular adherens junctions, and absence of haematopoietic antigens. Importantly, high resolution stereo-confocal microscopy confirmed that GFP^+ cells did not occupy a perivascular location, while optical sectioning of multiple z-stacks (30 μm resolution) displayed that BM-derived endothelial cells possess a single nucleus and that CD31 and GFP signals derive from the same individual cell. This proved that tumor endothelial cells could actually originate from BM-mobilized EPCs. Intriguingly, flow cytometric analysis revealed that the percentage of BM-derived EPCs (GFP^+ VE-$cadherin^+$ CD31low $CD11b^-$) decreases from 25%-35% in the early phase of tumor development (4-6 days) to 6-8% at later stages (6-8 days), while the fraction of local non-BM-derived endothelial cells (GFP^- VE-$cadherin^+$ CD31low $CD11b^-$) increased to 65%-75% at days 10-14 (Nolan et al., 2007). Finally, luminal incorporation of BM-derived endothelial cells was probed by the systemic administration of fluorescent isolectin IB4 and flow cytometric analysis of isolectin $IB4^+$ $CD31^+$ GFP^+ $CD11b^-$ cells: this manoeuvre permitted to conclude that, at day 6, 31% of luminally incorporated endothelial cells derived from BM. The same results were found in the other tumor types investigated in this study, namely B6RV2 lymphoma and melanoma, and in a transgenic breast cancer mouse model (MMTV-PyMT) (Nolan et al., 2007). In this model, the polyoma middle T antigen (PyMT) oncogene is expressed under the control of the mouse mammary tumor virus promoter (MMTV) to achieve the neoplastic transformation of mammary epithelium

(Fantozzi and Christofori, 2006). Thus, EPCs play a crucial role during the initial steps of tumor vascularization. The MMTV-PyMT transgenic mice were further exploited to assess EPC contribution to the dynamics of vessel assembly that turns dormant micrometastases into letal macrometastases (Gao et al., 2008). By using the same procedure described in their seminal paper (Nolan et al., 2007), Gao and coworkers focussed on the angiogenic switch in lung metastases that spontaneously develop in this breast cancer model. They found that micrometastases formed by week 12 were poorly vascularised, as shown by the lack of $CD31^+$ vessels. Nevertheless, macrometastases that appeared at week 16, were positive to CD31 staining and displayed luminally incorporated BM-derived GFP^+ $CD31^+$ endothelial cells in about 11% of neovessels (Nolan et al., 2007). This means that EPCs home to micrometastatic foci and contribute to neovessel formation, thereby sustaining the macrometastatic transition. The low percentage of EPC engrafting suggested that, apart from a structural role, they drive the angiogenic switch in a paracrine manner. The same findings were obtained by analyzing lung metastases in LLC xenograft mice; by using this model, the authors further found that many BM-derived GFP^+ cells are recruited to micrometastases, but confocal microscopy analysis revealed that only endothelial cells (GFP^+ $CD31^+$ Isolectin $IB4^+$) integrated into neovessels. Conversely, HSCs adopted a perivascular location (Nolan, 2007). Importantly, the peripheral region of the lesion is the initial target of BM-derived EPCs (GFP^+ VE-$cadherin^+$ CD31dim $CD11b^-$), as detected by fluorescence-activated cell sorting (FACS) of the lungs bearing micrometastases (Nolan et al., 2007). More recently, Id1 was identified as a putative EPC marker in BM-derived GFP^+ VE-$cadherin^+$ EPCs, where it drives the egression into peripheral circulation (Nair et al., 2014), but not in other BM-derived GFP^+ myeloid cells (Mellick et al., 2010). This feature might explain the defective angiogenic process observed in $Id1^{-/-}$ mutants (see also below). The use of Id1 promoted to drive GFP expression enabled Mellick and coworkers to selectively track EPC homing from BM to LLC tumors in the xenograft murine model employed in their previous work (Nolan et al., 2007; Gao et al., 2008). Again, high resolution microscopy revealed that BM-derived $Id^{1+/GFP+}$ VE-$cadherin^+$ CD31low EPCs targeted the periphery of early nonvascularized tumors (days 6-8). Luminally incorporated GFP^+ $CD31^+$ Isolectin $IB4^+$ $CD11b^-$ mature EPCs were then detected in about 9% of tumor neovessels within later tumors (days 8-12). Along with many other parallel studies, these reports reinforced the concept that EPCs sustain the angiogenic switch in primary tumors and micro-to-macrometastatic transition at secondary lesions. An alternative approach consisted in assessing the engraftment and contribution of exogenous EPCs to tumor development in human xenograft models, including those for renal cellular carcinoma (RCC), hepatocellular carcinoma, and LLC (Asahara et al., 1999; H. Zhu et al., 2012; Ahn et al., 2010; Yu et al., 2014). Unlike the previous investigations, however, these studies failed to validate the endothelial phenotype of the injected cells, by relying on rather unspecific markers (i.e. CD133,

CD34, VEGFR-2) that also feature HSCs. More recently, human ECFCs, which are regarded as truly endothelial precursors, were probed for their ability to specifically home to sites of tumor angiogenesis in mice bearing an array of distinct cancer types. For instance, upon intravenous injection into lethally irradiated mice, DiI-labelled ECFCs target LLC lung, but not kidney or liver, metastases; herein, they are mainly found at the periphery, rather than in the centre, and integrate within neovessels, albeit most of them adopt a perivascular location (Wei et al., 2007). Subsequently, Bieback and coworkers evaluated the extent of ECFC recruitment to rat C6 glioma xenograft, which is the most suitable model for the study of glioblastoma multiforme. By using the dorsal skinfold chamber model associated to intravital multi-fuorescence videomicroscopy, they found that DiI-stained ECFCs strongly interacted (adhesion and extravasation) with tumor vasculature, while human umbilical vein endothelial cells (HUVECs) and $CD34^+$ MNCs were much less active (Bieback et al., 2013). These preliminary findings lend strong support to the tenet that ECFCs represent the most suitable subtype to unveil the molecular mechanisms driving EPC-based tumor neovascularization (Moccia Dragoni et al., 2014; Moccia Berra-Romani,et al., 2014; Moccia et al, 2012; Yoder and Ingram, 2009).

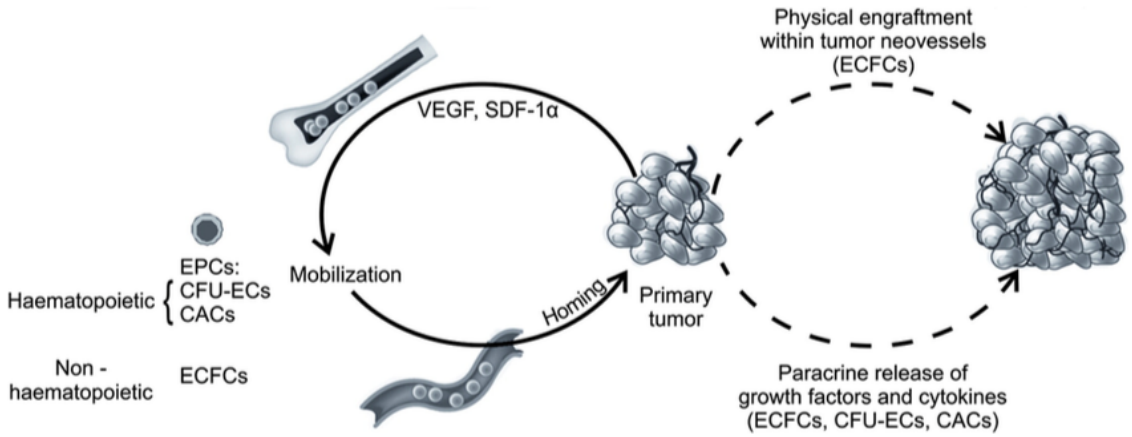


Figure 6. Mechanism of tumor vascularization by EPCs (Moccia and Poletto, 2014).

HOW TO SOLVE THE CONTROVERSY ABOUT EPC CONTRIBUTION TO TUMOR VASCULARIZATION?

Despite the undoubted evidence in favour of EPC involvement in tumor growth and metastatization, several authors questioned their participation to the angiogenic switch (Patenaude et al., 2010; Yoder and Ingram, 2009). This is why several studies failed to evidence a measurable amount of luminally incorporated EPCs within tumor vessels. For instance, De Palma et al. transduced BMDCs with lentiviral vectors expressing the GFP gene under the control of the specific endothelial Tie2 promoter, which was followed by BM implantation into several subcutaneous

tumor model (De Palma et al., 2003). High resolution microscopic inspection detected only rare GFP^+ $CD31^+$ endothelial cells in tumor cells, which were instead abundant of GFP^+ $CD45^+$ $CD11b^+$ $CD31^-$ monocytes and pericyte progenitors, with a preferential perivascular location (De Palma et al., 2003). Subsequently, Göthert et al. (Göthert et al., 2004) generated an endothelial-specific inducible transgenic model to assess the BM origin of tumor endothelium. The basic helix-loop-helix transcription factor stem cell leukemia (SCL) is crucial to both haematopoiesis and vasculogenesis: several enhancers have been identified within the murine SCL locus that direct reporter gene expression in either endothelial cells (5' promoter) or early HSCs (3' promoter). In this study, the 5′ endothelial enhancer was exploited to obtain endothelial-specific expression of the tamoxifen-inducible recombinase $Cre\text{-}ER^T$ (endothelial-SCL-$Cre\text{-}ER^T$). These mice were then intercrossed with Cre reporter strains (R26R) in which lacZ or enhanced yellow fluorescent protein (EYFP) were expressed following Cre-dependent recombination. Tamoxifen administration enabled to detect endothelial lacZ staining in newly formed LLC and B6RV2 vasculature in these animals. However, when wild type mice were subjected to BM transplantation from endothelial-SCL-Cre-ER(T); R26R littermates before tumor inoculation, no lacZ staining appeared in neovessels on tamoxifen administration. These data supported the notion that local, but not BM-derived, endothelial cells support cancer growth (Göthert et al., 2004). Other studies also could not demonstrate EPC incorporation in the endothelial layer of several primary and metastatic tumors (Wickersheim et al., 2009; Machein et al., 2003; Purhonen et al., 2008). An additional proof of evidence that has been carried against EPC contribution to the angiogenic switch is their rarity in some tumor models, which display only 1-2% of EPCs-derived neovessels. The passionate debate arose about this issue might be easily reconciled when taking a few key considerations in account. As recalled by Gao et al. (Gao et al., 2009) and in (Yoder and Ingram, 2009), EPCs are recruited to tumor periphery prior to vasculature formation (Nolan et al., 2007; Gao et al., 2008), acquire an endothelial phenotype and are luminally engrafted into a subset of neovessels in early tumors. At later stages, these chimeric structures are diluted/replaced by local host-derived sprouting vessels, which would explain the low involvement described in already developed tumors by other investigators (De Palma et al., 2003; Göthert et al., 2004; Purhonen et al., 2008; Duda et al., 2006). Accordingly, De Palma et al. (De Palma et al., 2003) assessed EPC incorporation at week 4, while Göthert et al. (Göthert et al., 2004) at 14 days post-implantation, which are fully compatible with the data described in (Nolan et al., 2007) and (Gao et al., 2008). EPC recruitment preceding the engagement of host vasculature suggests that these cells play a key role in the stimulation of non-BM-derived endothelial cells sprouting from nearby capillaries. Nevertheless, by using two distinct

transgenic mosue models of *de novo* tumorigenesis, i.e. Rip-Tag5 that develops pancreatic islet cancer and Alb-Tag that develops liver cancer, Spring et al. (Spring et al., 2005) could not identify BM-derived GFP^+ endothelial cells in pre-neoplastic lesions, while up to 30% of tumor-associated vessels were green at more advanced stages. This report highlight another feature that should be borne in mind when discussing the role served by EPC during tumor development, i.e. the stage specific engagement of EPC might depend on tumor type (Gao et al., 2009). More in general, the overall EPC contribution to the angiogenic switch could be tumor-type dependent. This hypothesis is corroborated by the work carried out on mice heterozygous for the tumor suppressor Pten ($Pten^{+/-}$), that exhibit a wide array of malignancies, such as uterine carcinomas (UC), pheochromocytomas, lymph hyperplasia, and prostate interepithelial neoplasias. Ruzrinova et al. (Ruzinova et al., 2003) discovered that $Pten^{+/-}$ spontaneous lymph hyperplasia lacked BM-derived EPCs, identified as $lacZ^+$ VEGFR-2^+ cells in animals transplanted with BM from Rosa 26 mice expressing lacZ in all their tissues, while these cells were easily detectable on 15%-20% of UC neovessels. Finally, it is worth of recalling that the field has long been flawed by the wrong interpretation of the term EPC (Moccia et al., 2014; Yoder and Ingram, 2009; Basile and Yoder, 2014). As already mentioned throughout this work, several studies claimed to evaluate EPC frequency in cancer patients or to evaluate their contribution to tumor growth and development in xenograft models (Asahara et al., 1999; Jung et al., 2012; H. Zhu et al., 2012); yet, these reports identified EPCs based on the selection of a panel of surface antigens that is inadequate and totally unreliable to detect truly endothelial progenitors (Gao et al., 2009; Moccia et al., 2014; Moccia et al., 2014; Yoder, 2012). These cells were more likely to belong to the haematopoietic lineage that may certainly sustain the angiogenic switch in a paracrine manner, but does not provide structural support to tumor vasculature. On the other hand, BM-derived EPCs are essential for cancer development and metastatization as evident in Id knock-out (KO) mice that fail in mobilizing these progenitors from the stem cell niche (Lyden et al., 2001; Li et al., 2004). These xenograft models either display rapid tumor regression (Lyden et al., 2001; Mellick et al., 2010) or a significantly delayed cancer progression (Li et al., 2004). More recently, Gao et al. (Gao et al., 2008) found that acute and conditional short hairpin RNA (shRNA)-mediated genetic ablation of Id1 in BM-derived EPCs do not decrease the number of micro-metastatic lesions, but prevent the macro-metastatic transformation in LLC xenografts (Gao et al., 2008). Subsequently, Mellick et al. (Mellick et al., 2010) used Id1 proximal promoter (pr/p) to drive the expression of the suicide gene herpes simplex virus (HSV)-thymidine kinase (tk) in BM-derived EPCs; this manoeuvre led to a notable reduction in EPC frequency and impaired tumor (LLC and B6RV2) growth and vascularization. By using the same strategy, Plummer et al. (Plummer et al., 2013) genetically suppressed the expression of the

miRNA-processing enzyme, Dicer, in BM, thereby achieving the decrease in circulating EPCs, tumor (LLC and breast cancer) size and vessel density. Therefore, there is ample experimental evidence to conclude that truly endothelial precursors play a crucial role in cancer development and metastatization.

VEGF AS TARGET FOR ANTI-ANGIOGENIC TREATMENTS

VEGF stimulates angiogenesis and EPC mobilization by binding to VEGFR-2, also called KDR in humans and Flk-1 in mice, thus triggering a complex intracellular signalling cascade (Potente et al., 2011). The downstream mediators of VEGFR-2 include, but are not limited to: 1) phospholipase Cγ (PLCγ), which may either lead to an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) or engage the protein kinase C (PKC/Ras/MEK/MAPK cascade, 2) cytosolic PLA2, which is activated upon extracellular signal-related kinase (ERK)-mediated phosphorylation and controls PGI_2 production; 3) phosphoinositide 3-kinase (PI3K), which in turn recruit the protein kinase B (PKB)/Akt pathway via an increase in phosphatidylinositol-3,4,5-trisphosphate (PIP_3) to activate endothelial nitric oxide (NO) synthase (eNOS) and inhibit B-cell lymphoma 2 (Bcl-2)-associated death promoter homologue (BAD) and Caspase 9; 4) p21-activated protein kinase-2 (PAK-2), which is responsible for the subsequent activation of both Cdc42 and p38 MAPK; and 5) focal adhesion kinase (FAK) and its substrate paxillin. It is, however, worth of noting that most of these studies have been carried out on normal ECs, while the signalling pathways downstream VEGFR-2 in EPCs are less clear. Nevertheless, its tyrosine kinase activity is druggable and VEGF quickly became the main target of a myriad of anti-angiogenic therapies over the last few years. US Food and Drug Administration (FDA) has approved for clinical use some of these anti-angiogenic anti-VEGF drugs. The anti-VEGF monoclonal antibody Bevacizumab (Avastin) has been approved for therapy in combination with chemotherapy for several different types of tumors as non-squamous non-small cell lung cancer, colorectal cancer and metastatic breast cancer. Another class of FDA approved drugs is represented by pan-VEGFR tyrosine kinase inhibitors (TKIs), such as Sunitinib (Sutent), Pazopanib (Votrient) for metastatic RCC, Sorafenib (Nexavar) for metastatic RCC and unresectable hepatocellular and Vandetanib (Zactima) for medullary thyroid cancer. In general, VEGF blockade prolongs progression-free survival and overall survival of cancer patients in the range of weeks to months. Several mechanisms could be used by VEGFR inhibitors to reach a clinical benefit: at first, these drugs could block vascular sprouting and BM-derived cells homing, thus inhibiting tumor vessel expansion. Second, they can produce a regression of pre-existing tumor vessels and deprive ECs of VEGF to survive, thereby sensitizing ECs to chemotherapies and irradiation. Third, a normalization of abnormal tumor vessel restoring a physiological vasculature can be another

mechanism (Goel et al., 2011). About the third point, preclinical studies have shown that anti-VEGF therapy changes tumor vasculature towards a more "mature" or "normal" phenotype. This "vascular normalization" is characterized by attenuation of hyperpermeability, increased vascular pericyte coverage, a more normal basement membrane, and a resultant reduction in tumor hypoxia and interstitial fluid pressure. All these factors, in turn, can lead to an improvement of different factors as the metabolic profile of the tumor microenvironment, the delivery and efficacy of exogenously administered therapies, the efficacy of radiotherapy, and the reduction in number of metastatic cells shed by tumors into circulation in mice. A lot is known about this mechanism but it needs surely future studies (Goel et al., 2011). It has to be taken in mind that VEGFR-inhibitors act not only on the structure and function of tumor vasculature and ECs, but also on every kind of cells expressing VEGFR-2. EPCs express VEGFR-2 on their surface, which allows them to engraft within nascent vessels, proliferate and differentiate in mature ECs. Studies on xenograft models of LLC or B6RV2 tumors demonstrated that VEGFR-2 inhibition through a neutralizing antibody or a specific short hairpin RNA blocks tumor vascularization and growth; these results led to the suggestion that targeting VEGFR-2 could prevent EPCs stimulation in cancer patients (Lyden et al., 2001; Mellick et al., 2010). Despite the encouraging result of preclinical studies, the oncological clinical practice revealed some unexpected limits of VEGFR inhibitors which depend on the poor knowledge about the fine mechanisms regulating tumor neoangiogenesis. Drugs had not the expected effects on progression-free survival which showed only a modest increase, and on overall survival. Furthermore, some patients are intrinsically refractory or develop resistance; therefore, a lot of research group started studying the possible mechanisms underlying patient resistant to drugs. Some of the resistance mechanisms to anti-VEGFR drugs are described below.

- VEGF-independent vessel growth: tumors can release alternative growth factors including FGF, epidermal growth factor (EGF), and placental growth factors (PlGF) which can replace VEGF functions on cellular stroma and cause a resistance to VEGFR-inhibitors;
- Stromal cells: all cells belonging to tumor stroma, including TAMs and CAFs, can produce other proangiogenic factors and recruit BM-derived cells;
- ECs instability: it is clear that tumor ECs acquire morphological, functional and genetic differences as compared to normal endothelium. They can display chromosomic aberrations, higher proliferative potential, apoptosis resistance and lower sensitivity to cytotoxic drugs. Chromosomic alterations can also involve VEGFR-2 and, therefore, blocking its responsiveness to treatment;
- Vascular independence: tumor cells are able to survive in the hypoxic tumor environment, with a consequent reduced vascular dependence that impairs the anti-angiogenic response. Moreover,

HIF-1α is able to render ECs resistant to irradiation. Some tumors also have a hypovascular stroma, but they can metastasize through the lymphatic vessels and the process cannot be blocked by anti-angiogenic treatments.

- Sprouting-independent vessel growth: some tumors use alternative mechanisms for vessel development, as vasculogenic mimicry, intussusceptive angiogenesis and vessel cooption;

These alternative mechanisms of tumor vessel growth do not necessarily require VEGF and are, therefore, insensitive to classic anti-VEGF inhibitors (Potente et al., 2011; Vasudev and Reynolds, 2014). EPC-mediated vascularization may certainly be included among these alternative modes of tumor expansion and metastatisation. Not only does their earlier mobilization from bone marrow sustain the angiogenic switch that promotes metastatic progression (see above); a delayed burst of EPC is induced by cytotoxic drugs and VDAs, thereby inducing the well known phenomenon of acquired resistance that leads to patient death despite for continuous treatment (Moccia and Poletto, 2014). Most, if not all, the studies assessing the role of VEGFR-2 in EPC proliferation and recruitment to tumor site, have been conducted on mice xenografted with human cancers and injected with EPCs obtained by normal, i.e. healthy donors. As VEGF signalling is functional in these cells (Dragoni et al., 2011; Dragoni et al., 2013; Turtoi et al., 2012; D. Yu et al., 2014), it is not surprising that blocking VEGFR-2 prevents EPC activation and causes tumor shrinkage in these pre-clinical settings. It is conceivable that only EPCs deriving from oncological patients should be employed to understand whether VEGFR-2 is a suitable target to suppress EPC recruitment in these patients (Moccia and Poletto, 2014).

RENAL CELL CARCINOMA (RCC): A PERFECT MODEL FOR STUDYING ANGIOGENESIS AND EPCs

Renal cell carcinoma accounts for the 2.1% of all solid neoplasms in the adults with 271.000 new cases and 116.000 deaths estimated in 2008. Male sex is more frequently involved with a 1.5:1 ratio (167.000 new cases in men *vs*. 103.000 new cases in women); the age standardized risk for this neoplasm is 3.9 cases every 100.000 people-year, with a cumulative risk of 0.5 (Ferlay et al., 2010). Despite being still considered a rare tumor, kidney cancer is showing constantly increasing incidence, 2-3% increase every 10 years (in the US the incidence has grown of 126% from 1950 up to our days) (Mathew et al., 2002). Epidemiologic meta-analysis of case-control studies has shown correlation with possible risk factors, among all tobacco consumption and obesity (Dhote et al., 2004). As far as we know, kidney cancer is a neoplasm highly dependent on angiogenesis: our knowledge on its cancerogenesis (despite we are not referring to a single entity, as we will discuss briefly) derives from genetic studies on hereditary syndromes in which RCC is a clinical

component. Patients affected by von Hippel-Lindau syndrome, who carry a mutation on von Hippel-Lindau (VHL) oncosuppressor gene, are more likely to develop emangioblastomas, tumors of the yalk sack, cranio-spinal emangioblastomas and clear cell renal cell carcinomas (the latter in 35-45% of the cases) (Walther et al., 1995); some partial gene deletions show higher correlations to tumor development than complete gene deletions (Maranchie et al., 2004), enforcing the evidence VHL inactivation plays a crucial role in cancer development (despite the molecular picture of this tumor has been recently shown to be much more intricate as we tend to consider it, by the evidence of intra-tumor genetic heterogeneity) (Gerlinger et al., 2012). Malignant kidney cancer is a heterogeneous class of diseases which can be distinguished histologically according to the 2004 World Health Organization classification (H. Sobin, 2003); the major histotypes represented have slightly different pathogenesis and clinical behaviour. The most common is represented by clear cell RCC, in the 70% of the cases (Eble et al., 2004), where the correlation with VHL alterations is striking, followed by papillary RCC (15 to 30% of the cases), where a second, highly relevant, cellular pathway is involved (represented by the hepatocyte growth factor – scatter factor (HGF/SF) / c-Met axis), and chromophobe RCC, which has a less clinical malignancy than the others (Eble et al., 2004). A last less frequent feature to be considered, is represented by the sarcomatoid variant, which is not a proper RCC histotype by its own, but it is a histological component which complicates 1% to 8% cases of RCC, usually worsening the prognosis (Cangiano et al., 1999). RCC originates from malignant transformation of renal tubule cells. Inactivation of the VHL oncosuppressor gene, which encodes for a ubiquitin ligase responsible for the ubiquitinization of HIF-1α and HIF-2α, leads to their accumulation while they are actively overexpressed thanks to the hypoxic environment created by the tumor growth, promoting definitively a stimulus on cell survival, angiogenesis and metabolism (Lonser et al., 2003; Linehan, 2007; Cheng et al., 2009).

Hypoxia is highly relevant in the oncogenesis of human neoplasms as is a putative mechanism for clonal selection. HIF family enhances the transcription of many receptor proteins such as c-Met and growth factors such as TGF-α, VEGF, PDGF-β, and CXCL12 (Lonser et al., 2003). VEGF and PDGF-β induction are likely to be involved in the extremely pronounced vascularization of RCC; VEGF among both is an active player in the angiogenic switch, and correlates to increased risk of metastatization and resistance to therapies (Ferrara, 2005). VEGF and PDGF-β favour the migration and proliferation of endothelial cells as well as create an autocrine/paracrine loop responsible for self manteinance of tumoral cells, being their receptors expressed on the tumoral cell surface, (Ferrara, 2005; Badalian et al., 2007; Rivet et al. 2008). RCC carcinoma has been considered an orphan disease up to the last decade, as the only treatment available was represented by inteleukin-2 and interferon-γ (IFN-γ), whose efficacy has always remained under debate. The natural history of

this neoplasm started slightly to change with the introduction of new molecularly targeted agents, represented either by monoclonal antibodies or by small molecules tyrosine kinases. The majority of compounds used nowadays in the treatment of the metastatic disease act against the VEGF/VEGFR pathway, and the drugs represented in this group are Sorafenib, Sunitinib, Pazopanib, Axitinib and Bevacizumab. Sorafenib, a small tyrosine kinase inhibitor (TKI), has been the first compound registered for therapy in metastatic RCC (mRCC) and showed a significant improvement of PFS in actively treated patients as opposed to the placebo treated control group (24 weeks vs 6 weeks) (Escudier et al., 2007). Sunitinib is a second TKI which showed improvement in PFS in the treated population in the registration study, as compared to an IFN-γ treated group (Motzer et al., 2007). Pazopanib and Axitinb are, instead, relatively recently developed compounds, which have been registered for usage in mRCC after their respective registrative studies (Sternberg et al., 2010; Rini et al., 2011). VEGF receptor is not the only possible therapeutic target: its ligand, VEGF itself, can be blocked by a further compound which acts on this pathway, Bevacizumab, a monoclonal antibody used in association with IFN-γ, registered soon after Sorafenib (Escudier et al., 2007; Rini et al., 2008). The therapeutic arena is occupied by two other small molecule inhibitors: Everolimus and Temsirolimus, which are acting on a different cellular pathway, the mammalian target of rapamycin (mTOR) (Amato et al., 2009; Hudes et al., 2007).

From a quite recent era of no therapeutic options available, many compounds are nowadays available: the reality of everyday clinical use shows that none of those guarantees stable therapeutic success and the way in which they should be used (sequence, patient sub-group selection, possible combination) is still not clear and under investigation.

The clinical world of RCC, despite an improvement in therapy, faces constantly the lack of new strategies to achieve longer and stable results. One emerging cellular mechanism, which is starting to be explored, is the role of EPCs in RCC oncogenesis and their intimate connection to angiogenesis. EPC is an interesting cellular type, since many evidences showed a possible involvement in kidney cancer tumorigenesis: their ratio to CEC is increased in VHL patients who developed RCC and in patients with sporadic RCC, differently from VHL patients who did not develop the tumor (Bhatt et al., 2011); EPCs are possible biomarker in RCC patients as they correlate with VEGF (Yang et al., 2012) and possibly promote angiogenesis from RCC adjacent tissues where they are abundant (Zhao et al., 2013; P. Yu et al., 2014). Moreover, the level of peripheral EPCs positively correlates with RCC prognosis, the mean frequency being statistically higher in stage III-IV RCC patients as compared to those with stage I-II (Yang et al., 2012). Second, the number of circulating EPCs augments upon surgical removal of the primary tumor if the disease relapses in the form of metastases in distant organs (Hernandez-Yanez et al., 2012). Third, BM-derived EPCs may engraft within tumor vasculature as demonstrated by Hill, who found

that RCC developing within a kidney allograft manifest Y-positive chromosome vessels within a Y-negative tumor (Hill, 2010). Consistent with this finding, TECs harvested from human RCC tumors sub-cutaneously implanted into nude mice expressed the mouse stem cell antigen (Sca)-1 (Matsuda et al., 2010). In addition, the expression of CD133, a surface antigen that features BM-derived progenitors, was far more abundant in mouse TECs isolated from OSRC-2 (a well estabilished human RCC cell line) cell xenografts in immunodeficent mice than in normal endothelium (Akino et al., 2009). Altogether, these findings support the notion that RCC vascularization is sustained by endothelial committed cells released from the osteoblastic niche. Their putative role as biomarkers, as their possible involvement in cancerogenesis, make EPCs a very likely cell lineage to be explored by further investigations aiming at searching for new therapeutic options in the treatment of RCC.

PRO-ANGIOGENIC CALCIUM SIGNALLING

Calcium (Ca^{2+}) is the most widespread signalling molecule in both excitable and non-excitable cells across the phylogenic tree (Berridge et al., 2003). Unlike other intracellular messengers, Ca^{2+} is neither synthesized or metabolised, but is delivered to specific Ca^{2+}-dependent decoders upon cell stimulation by chemical (e.g. hormones, transmitters, growth factors, and cytokines) and physical (e.g. shear stress, pulsatile stretch and osmotic swelling) stimuli. Each cell disposes of a unique pathway (or "toolkit") of Ca^{2+}-handling proteins that enables it to generate intracellular Ca^{2+} signals of a particular amplitude, duration, frequency, and spatial location (Berridgeet al., 2003). Consequently, the Ca^{2+} signature produced by each cell in response to a precise stimulus is able to control a multitude of cellular processes, ranging from fertilisation to programmed cell death (Sammels et al., 2010). For instance, transient (in the μsec range) elevations in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) activate myocardial contraction, neurotransmitter release, and focal adhesion dynamics (Bers, 2008; Brini et al., 2014; Chen et al., 2013), while prolonged (in the minutes-to-hours or days range) oscillations in $[Ca^{2+}]_i$ control proliferation, differentiation, and gene expression (Dragoni et al., 2011). Ca^{2+} signals may be delivered as global Ca^{2+} spikes or may be restricted to nano- or micro-scoping domains emanating from the cytosolic mouth of Ca^{2+}-releasing channels (Parekh, 2008). This local mode of signalling has the advantage to target Ca^{2+} precisely to a specific Ca^{2+}-dependent effector among the many that are dispersed throughout the cell (Berridge et al., 2003; Lam and Galione, 2013). Alternatively, Ca^{2+} waves may be transferred to adjacent cells by either paracrine or gap-junctional communication to coordinate multicellular processes, such as vascular tone regulation (Altaany et al., 2014) and wound repair (Moccia and Poletto, 2014). Evolution has developed a number of conserved Ca^{2+}-binding sequences that transmit Ca^{2+} sensitivity to the proteins they belong to. The combination between the spatio-temporal of the Ca^{2+} signal with the differences in Ca^{2+} interaction kinetics among the known Ca^{2+}-binding proteins allows this ubiquitous messenger to finely regulate highly specific intracellular processes (Clapham, 2007). The best characterized Ca^{2+}-binding domain is the EF-hand motif, that is found in a wide array of proteins accomplishing cellular functions as diverse as contraction (e.g. troponin C), apoptosis and disassembly of cell adhesions (e.g. calpain, calcium and integrin-binding protein 1, and S100 proteins), gene transcription (e.g. neuronal Ca^{2+} sensor proteins (NCS) and S100 proteins) and intracellular Ca^{2+} modulation (e.g. Stim1 and Ca^{2+}-binding proteins (CaBPs)). Another widely diffused Ca^{2+}-binding sequence is represented by the C2 domain, which gives Ca^{2+}-sensitivity to protein kinase C (PKC), phospholipase A2 (PLA2) and phospholipase C (PLC), thereby regulating the exocytosis of secretory vesicles at synaptic terminals (Carafoli et al., 2001). In addition to directly activating the Ca^{2+}-sensitive effectors, local Ca^{2+} signals may exert their influence event at

considerable distance (e.g. into the nucleus) by recruiting intermediary Ca^{2+} sensors, such as the promiscuous EF-hand-containing protein calmodulin (CaM) (Parekh, 2010). CaM, in turn, establishes a close correlation between the spatial-temporal pattern of the Ca^{2+} signal and the ensuing physiological response. The most important CaM-regulated effectors are Ca^{2+}/CaM-dependent kinases (CaMKI, CaMKII and CaMKIV), the phosphatase calcineurin, and the eNOS (Carafoli et al., 2001). Moreover, CaM may relocate into the nucleus, where it activates the cAMP response element binding protein (CREB), a transcription factor with an established role in neuronal plasticity and long-term memory consolidation in the brain. Alternatively, CaM may stimulate calcineurin to dephosphorylate the nuclear localization signals (NLS) of NFAT, thereby promoting its migration into the nucleus, where it binds to the cis-regulatory elements of its target genes. Finally, CaMKIV may phosphorylate the inhibitory protein, I_{kB}, which retains nuclear-factor kappa B (NF-κB) into the cytosol by masking its NLS. As a consequence, I_{kB} is targeted for site-specific ubiquitination and proteolitic degradation, whereas NF-κB translocates into the nucleus (Alonso and García-Sancho, 2011).

PRO-ANGIOGENIC Ca^{2+} SIGNALS IN MATURE ENDOTHELIAL CELLS

Endothelial cells are not an exception to the widespread dependence on intracellular Ca^{2+} signalling. It has long been known that an increase in $[Ca^{2+}]_i$ plays a key role in the intricate network of signal transduction pathways exploited by ECs to maintain cardiovascular homeostasis (Mocciaet al, 2012). Being located at the interface between the vessel wall and circulating blood, they perceive a multitude of extracellular signals, conveyed both the chemical messengers (such as growth factors, cytokines, and autacoids) and mechanical forces (such as pulsatile stretch, shear stress, and changes in local osmotic pressure), through an armamentarium of membrane-bound receptors, each of which is capable of triggering an increase in $[Ca^{2+}]_i$. The spatio-temporal profile of the Ca^{2+} signal enables the stimulated endothelial cell to select the most suitable response to extracellular inputs, i.e. production of vasoactive mediators, such as nitric oxide (NO), prostacyclin (PGI_2), hydrogen sulphide (H_2S), endothelial-dependent hyperpolarizing (EDHF) and contracting (EDCF) factors, biosynthesis of von Willebrand factor and tissue plasminogen activator, control of intercellular permeability, nuclear factor of activated T-cells (NFAT) and NF-kB activation, gene expression, cell proliferation, angiogenesis, and wound repair (Munaron and Fiorio Pla, 2009; Mancardi et al., 2011; Moccia et al., 2012;Moccia et al., 2006; Piscopo et al., 2007). It has long been known that Ca^{2+} signals regulate angiogenesis by controlling all the key steps of vessel remodelling, including endothelial proliferation, permeability, motility, and interaction with the extracellular matrix. In particular, VEGF utilizes Ca^{2+} signalling to promote endothelial cell

proliferation and tubulogenesis both *in vitro* and *in vivo* (Moccia et al., 2012; Abdullaev et al. 2008). The pro-angiogenic Ca^{2+} response occurs downstream of PLCγ activation and may adopt distinct patterns depending on the vascular bed of origin. PLCγ cleaves the phospholipid precursor, phosphatidylinositol 4,5-bisphosphate (PIP_2), into two intracellular second messengers, i.e. inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). These two signalling intermediates activates two distinct modes of $[Ca^{2+}]_i$ elevations in micro- *vs.* macro-vascular endothelial cells. DAG remains tethered to the plasma membrane to directly gate two members of the non-selective cation family of canonical TRP channels (TRPC), namely TRPC3 and TRPC6, in human microvascular endothelial cells (Moccia et al., 2012; Fiorio Pla and Gkika, 2013; Pupo et al., 2011). TRPC3 and TRPC6, in turn, mediate a monotonic increase in intracellular Ca^{2+} levels which do not require further Ca^{2+} release from the endogenous Ca^{2+} pool. Conversely, in human umbilical vein endothelial cells (HUVECs), $InsP_3$ rapidly diffuses across peripheral cytosol to $InsP_3$ receptors ($InsP_3Rs$), which are embedded in the membrane enveloping the endoplasmic reticulum (ER), the most abundant endothelial Ca^{2+} reservoir. $InsP_3Rs$ function as Ca^{2+}-permeable channels to release lumenally stored Ca^{2+} upon $InsP_3$ binding, thereby causing a rapid increase in $[Ca^{2+}]_i$. The consequent emptying of the ER Ca^{2+} pool signals the opening of store-operated Ca^{2+} channels (SOCs) in the plasma membrane (Moccia et al., 2012; Moccia, Dragoni et al., 2012). The ensuing influx of Ca^{2+} shapes a plateau phase of intermediate magnitude between pre-stimulation Ca^{2+} levels and the $InsP_3$-dependent initial peak that persists as long as VEGF is presented to the cells (Abdullaev et al., 2008; Li et al., 2011). Store-operated Ca^{2+} entry (SOCE) is a ubiquitous mechanism that, in addition to replenishing the endogenous Ca^{2+} pool, delivers the bolus of Ca^{2+} necessary for the recruitment of a plethora of endothelial Ca^{2+}-sensitive decoders, including eNOS, nuclear factor of activated T-cells (NFAT), nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB), activating protein-1 (AP-1), calpain and myosin light chain kinase (MLCK) (Mocciaet al., 2012; Moccia, Dragoni et al., 2012; Holmes et al., 2010; Rinneet al., 2009; Berra-Romani et al., 2013). Briefly, endothelial SOCE may be initiated by Stromal Interaction Molecule-1 (Stim1), which senses the drop in ER Ca^{2+} concentration and rapidly (40-60 sec) oligomerises into clustered *puncta* that approach as close as 20-30 nm to the plasma membrane. Herein, Stim1 binds to and gates two structurally different types of endothelial SOCs, depending both on the species and the vascular bed. Accordingly, Stim1 exclusively establishes a physical interaction with Orai1, i.e. the pore-forming subunit of the Ca^{2+} release-activated Ca^{2+} (CRAC) channel, in HUVECs (Abdullaev et al., 2008; Li et al., 2011). Conversely, TRPC1 and TRPC4 provide the core components of plasmalemmal SOCs in mouse aorta (Freichel et al., 2001) and rodent pulmonary artery (Cioffi et al., 2012). In particular, in rat pulmonary artery endothelial cells (PAECs), the fall

in intralumenal Ca^{2+} induces Stim1 to relocate into sub-plasmalemmal *puncta* in a TRPC4-dependent manner, thereby promoting the association of TRPC4 with TRPC1 (Sundivakkam et al., 2012). This mechanism recognizes a privileged role for Orai1, which constitutively interacts with TRPC4 in un-stimulated cells, increases the probability of TRPC1/TRPC4 activation by Ca^{2+} depletion and confers Ca^{2+}-selectivity to the complex (Cioffi et al., 2012).

VEGF is not the only growth factor to utilize Ca^{2+} signals to exert a mitogenic effect on mature endothelial cells. Insulin-like growth factor-1 (IGF-1) and bFGF trigger a massive entry of Ca^{2+} from the extracellular milieu in bovine aortic endothelial cells (BAECs), which is required for them to proliferate (Munaron and Fiorio Pla, 2000; Antoniotti et al., 2003). Subsequent studies have led to the conclusion that arachidonic acid (AA) mediates bFGF-evoked Ca^{2+} inflow by activating TRP Vanilloid 4 (TRPV4) (A Fiorio Pla et al., 2012). TRPV4-mediated Ca^{2+} influx might promote endothelial proliferation and vascular remodelling by enlisting a variety of Ca^{2+}-dependent transcription factors, such as NFAT, myocyte enhancer factor 2C (MEF2C), Kv channel interacting protein 3, calsenilin (KCNIP3/CSEN/DREAM), and cytoplasmic, calcineurin-dependent 1 (NFATc1) (Troidl et al., 2009; Troidl et al., 2010). EGF, in turn, elicits cytosolic and nuclear Ca^{2+} oscillations in rat microvascular endothelial cells (Moccia et al., 2003), while platelet-derived growth factor (PDGF) evokes irregular fluctuations of $[Ca^{2+}]_i$ in porcine aortic endothelial cells (Ridefelt et al., 1995). This spiking response relies on the rhythmic Ca^{2+} discharge from $InsP_3Rs$ and is maintained by the Stim1-mediated activation of Orai1 (Chen et al., 2011; Moccia et al., 2003; Ridefelt et al., 1995). Unfortunately, the physiological outcome of EGF- and PDFG-induced intracellular Ca^{2+} spikes has not been probed in these studies; however, endothelial cells often use the information encoded in the oscillatory pattern to finely tune their Ca^{2+}-sensitive decoders that promote their pro-angiogenic behaviour (Pal et al., 2006; Scharbrodt et al., 2009; Berra-Romani et al., 2012; De Bock et al., 2011; Zhu et al., 2008). In addition to mitogens, the chemo-attractant SDF-1α may induce endothelial migration *in vitro* and homing of bone marrow-derived HSCs to the target vasculature *in vivo* through an increase in $[Ca^{2+}]_i$ (Moccia, Dragoni et al. 2012; Seidel et al., 2007). The signalling pathway downstream of its G-protein coupled receptor, CXCR4, is still elusive, but it may involve both $InsP_3$-gated Ca^{2+} release (Moccia et al., 2012) and store-dependent Ca^{2+} inflow (Seidel et al., 2007).

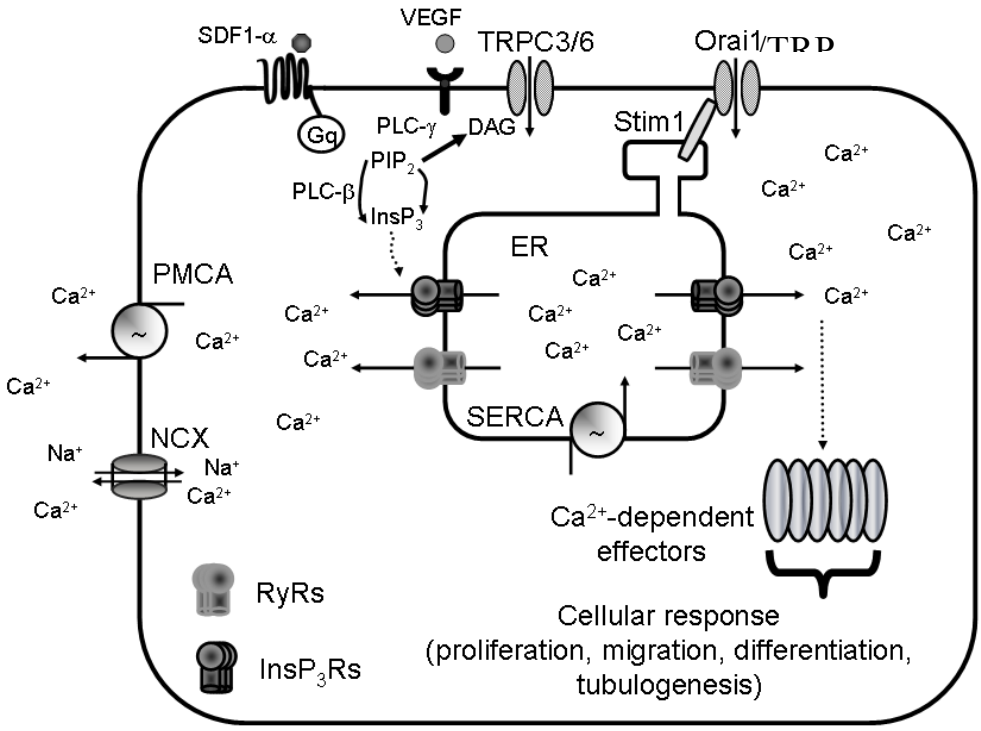


Figure 7. The Ca^{2+}-toolkit in mature ECs (Moccia et al., 2012).

VEGF EVOKES PRO-ANGIOGENIC Ca^{2+} OSCILLATIONS IN NORMAL ECFCs

Among the different subsets of EPCs utilized to induce the angiogenic switch in solid cancers, we focussed on ECFCs, or late outgrowth EPCs, for four main reasons. First, they are the only EPC population possessing all the features of a true endothelial progenitor and do display any known haematopoietic marker. Second, unlike CFU-ECs and CACs, they are capable of forming capillary-like structures *in vitro*, to originate patent vessels and to anastomose with host vasculature *in vivo* (Yoder, 2012; Basile and Yoder, 2014). Third, they home to sites of malignant growth and physically engraft within nascent vessels, thereby enhancing tumor size and vascularization (Bieback et al., 2013; Smadja et al., 2010). Fourth, they do require VEGF to proliferate, assembly into bidimensional tubulary networks, and differentiate into mature endothelial cells (Turtoi et al., 2012; Song et al., 2013; D. Yu et al., 2014). Our Ca^{2+} imaging recordings disclosed that 10 ng/ml VEGF, which is quite close to the dose (25 ng/ml) employed by Ingram and coworkers to stimulate ECFC expansion in the seminal study that led to their identification (Ingram et al., 2004), triggered asynchronous oscillations in $[Ca^{2+}]_i$ in neighbouring cells from the same coverslip (Dragoni et al., 2011). There were no two synchronous cells generating coordinated elevations in $[Ca^{2+}]_i$ in the ECFC monolayer. Such heterogeneity is the hallmark of mitogens-induced Ca^{2+} fluctuations in mature endothelial cells as well as other non-excitable cell types (Moccia et al., 2003; Ridefelt et al., 1995; Pal et al., 2006). Statistical analysis revealed the stochastic nature of VEGF-induced

intracellular Ca^{2+} oscillations, as the standard deviation (SD) of their period was of the same order as the average value (Dragoni et al., 2011; Skupin and Falcke, 2007; Skupin et al., 2008). We have discussed elsewhere the molecular underpinnings of the variability in the Ca^{2+} response to VEGF in healthy ECFCs (Moccia, Lodola et al. 2014; Moccia et al. 2012). Briefly, this diversity, that is retained by umbilical cord blood-derived ECFCs (Dragoni et al., 2013), may be engendered by at least two different sources. First, VEGF binding to VEGFR-2 obeys to a stochastic regime in vascular endothelium, which is key to determine the angiogenic phenotype, i.e. proliferative (tip cells) *vs.* migratory (stalk cell), adopted by the target cell (Potente et al., 2011; Mac Gabhan et al., 2005). Second, it is likely that the molecular nature and the spatial arrangement of Ca^{2+} release and entry sites dictates the timing of spike generation for each single ECFC (Skupin and Falcke, 2007; Skupin et al., 2008; Thurley et al., 2012). Normal ECFCs possess all the three known isotypes of $InsP_3Rs$, namely $InsP_3R$-1, $InsP_3R$-2, and $InsP_3R$-3, the pattern of expression of their transcripts being $InsP_3R$-3>$InsP_3R$-2>$InsP_3R$-1 (Dragoni et al. 2011). Conversely, they are devoid of functional ryanodine receptors (Sánchez-Hernández et al., 2010), whose expression and biological meaning in mature endothelium have been largely debated (Moccia et al., 2012) . Additionally, ECFCs are insensitive to H_2S stimulation, which elicits robust Ca^{2+} signals in normal endothelial cells (Pupo et al., 2011; Moccia et al., 2011; Munaron et al., 2013) and mediates VEGF-induced Ca^{2+} entry in breast cancer-derived TEC (Pupo et al., 2011). VEGF-induced Ca^{2+} oscillations were suppressed by U73122, which selectively blocks PLCγ activity, but not by its close inactive analogue, U73343; and by 2-aminoethoxydiphenyl borate (2-APB), which selectively abrogates Ca^{2+} release from $InsP_3Rs$ under our experimental conditions, i.e. in the absence of Ca^{2+} in the bathing solution ($0Ca^{2+}$) (Dragoni et al., 2011). The Ca^{2+} transients developed regardless of the presence of extracellular Ca^{2+}, which suggests that they are primarily driven by Ca^{2+} recycling across the ER membrane; however, they persisted only for a short time in Ca^{2+}-free saline and resumed only on Ca^{2+} restitution to the cells (Dragoni et al., 2011). Therefore, $InsP_3$-dependent Ca^{2+} release is sufficient to initiate and support the irregular episodes of intracellular Ca^{2+} mobilization in ECFCs, but Ca^{2+} entry across the plasma membrane is necessary to maintain the oscillations over time. So, which is the membrane pathway conducting extracellular Ca^{2+} into these endothelial precursors? Unlike mature endothelial cells, human circulating ECFCs lack TRPC3 and TRPC6 (Dragoni et al., 2011; Dragoni et al., 2013), which mediate VEGF-evoked Ca^{2+} entry in a DAG-dependent manner. Conversely, they express all the putative components of SOCE, i.e. Stim1-2, Orai1-3, TRPC1 and TRPC4 and exhibit a massive Ca^{2+} influx in response to ER Ca^{2+} store depletion. Therefore, ECFCs rely on a store-dependent mechanism rather than on a DAG-sensitive conductance to sustain prolonged intracellular Ca^{2+} signals. Consistent with this notion, 1-oleoyl-2-

acetyl-sn-glycerol (OAG), a membrane permeable analogue of DAG, does not evoke any detectable Ca^{2+} inrush (Dragoni et al., 2013). This feature rules out the possibility that TRPC1 forms a second messenger-operated channel gated by DAG in human ECFCs, as reported in human prostate cancer cells (Sydorenko et al., 2003). Therefore, SOCE provides the molecular gateway for Ca^{2+} inflow in response to agonist stimulation in endothelial precursors (Yu et al., 2010; Sánchez-Hernández et al., 2010; Lodola et al., 2012). Parallel work conducted both by us and Prof. Beech's lab demonstrated that SOCE is mediated by Stim1, Orai1 and TPC1 in endothelial committed progenitors. RNA interference selectively targeting either Stim1 or Orai1 abolished store-dependent Ca^{2+} influx in these cells (J. Li et al., 2011; Lodola et al., 2012). Likewise, SOCE was abrogated by the expression of a dominant negative mutant Orai1 (R91W), whereas $InsP_3$-evoked emptying of the ER Ca^{2+} pool induced eYFP-GFP tagged Stim1 to cluster into peripheral *puncta* (Li et al., 2011). Finally, short hairpin RNA-mediated knockdown of TRPC1 dramatically decreased the magnitude of store-dependent Ca^{2+} entry in human ECFCs (Lodola et al., 2012). These molecular data are supported by the pharmacological profile of SOCE, that is sensitive to a wide spectrum of rather specific inhibitors of both Orai1 and TRPC1 in these cells, including low micromolar doses of the trivalent cations La^{3+} and Gd^{3+}, 2-APB and YM-58483/BTP2 (Moccia, Dragoni et al. 2014; Moccia, Dragoni et al. 2012). The question the arises as to whether these two channel proteins assembly into a super-molecular ternary complex with Stim1, as illustrated in megakaryocytes (Di Buduo et al., 2014) and mouse submandibular gland cells (Ong et al., 2007), of constitute two distinct Ca^{2+}-permable routes, each recruited by Stim1 (Moccia, Dragoni et al. 2012). Suppression of either Orai1 or TRPC1 expression reduced SOCE amplitude to the same extent (Lodola et al., 2012), thereby suggesting that both of them contribute to the pore forming channel in human ECFCs. The molecular and pharmacological characterisation of the most important Ca^{2+}-permeable route in endothelial committed progenitors enabled us to ascertain its participation to VEGF signalling. One 1-to-4 Ca^{2+} transients arose when the cells were challenged with VEGF in the presence of BTP2, whose effect on Ca^{2+} fluctuations thus mimicked the removal of extracellular Ca^{2+} (Dragoni et al., 2011). In addition, VEGF-evoked Ca^{2+} influx was nearly absent in normal ECFCs transfected with a siRNA directed against Stim1 or Orai1 (Li et al., 2011), which is consistent for SOCE requirement to maintain the spiking response to VEGF (Dragoni et al., 2011). Thus, the molecular machinery that translates the extracellular input carried by VEGF into a specific intracellular output, i.e. a defined biological response, involves both $InsP_3Rs$ and SOCs in normal ECFCs. The well known signalling role served by Orai1 and TRPC1 in mature endothelium suggests that SOCE does more than replenishing depleted Ca^{2+} stores during the oscillatory response to VEGF in endothelial precursors.

VEGF-elicited Ca^{2+} oscillations drive proliferation and tube formation in healthy ECFCs. The pro-angiogenic activity of VEGF disappeared when the cells were pre-treated with BAPTA, a strong membrane-permeable Ca^{2+} buffer, or BTP2 to hinder the ensuing elevation in $[Ca^{2+}]_i$ (Dragoni et al., 2011). Similarly, siRNA-mediated genetic ablation of Orai1 prevented ECFC tubulogenesis (J. Li et al., 2011). Therefore, VEGF utilizes intracellular Ca^{2+} signals to promote endothelial proliferation and remodelling not only in mature endothelial cells, but also in their progenitors. The Ca^{2+}-sensitive decoder translating the irregular fluctuations in $[Ca^{2+}]_i$ into a biologically meaningful message is represented by NF-κB. The NF-κB signalling system includes about a dozen different dimmers comprising five homologous subunits, i.e. p50, p52, c-Rel, RelA/p65 and RelB (Hoffmann et al.,2006). The cytosolic-nuclear localization of NF-κB is intricately tuned and regulates several key steps of the angiogenic process, including endothelial cell proliferation, survival motility, substrate adhesion, interaction with the extracellular matrix and capillary morphogenesis (Minami and Aird, 2005). The heterodimer formed by p65 with p50 or p52 is sequestered in the cytosol by the association with I_{kB}, an inhibitory protein which masks the nuclear localisation sequence of the complex. A burst of intracellular Ca^{2+} oscillations recruits Ca^{2+}/calmodulin (CaM)-dependent protein kinase IV (CaMKIV) to activate the enzyme $I_{\kappa B}$ kinase (IKK). IKK, in turn, hyperphosphorylates $I_{κB}$ on two specific NH_2-terminal serines and target the inhibitory protein to site-specific ubiquitination and eventual degradation by the proteasome (Mellström et al., 2008). As a consequence, NF-κB dimers are released from inhibition and freed into the nucleus, where they turn on the transcriptional programme controlling EPC fate (Su et al., 2013; Li et al., 2012). Consistent with this model, the pharmacological extinction of VEGF-induced Ca^{2+} oscillations with either BAPTA or BTP2 hindered VEGF-induced I_{kB} phosphorylation in normal ECFCs; as expected, the same effect was exerted by thymoquinone, which selectively interfered with NF-κB activation (Dragoni et al., 2011). The final observation that thymoquinone abrogated ECFC expansion and tubule formation confirmed that NF-κB served as the Ca^{2+}-dependent decoder of VEGF-evoked Ca^{2+} transients (Dragoni et al., 2011). It has long been thought that NF-κB stimulation by a spiking signal in endothelial cells is controlled by the interspike interval (ISI). The transcriptional activity of endothelial NF-κB reaches its peak within a frequency range of Ca^{2+} spikes ranging from 0-5.2 mHz to 1.7-11.7 mHz (Smedler and Uhlén, 2014). This property does not fully apply to VEGF-challenged ECFCs, which generate irregular Ca^{2+} transients. However, resting cells do not exhibit any detectable elevation in $[Ca^{2+}]_i$, so that the informative content of the irregular Ca^{2+} spikes is obvious as compared to silent ECFCs. Recent mathematical computation has unveiled that any deviation from the pattern of Ca^{2+} spikes in un-stimulated cells conveys a

robust (i.e. high signal-to-noise ratio) message to their downstream effectors, thereby triggering a biological response (Skupin and Falcke, 2007; Thurley et al., 2012). The same group has further demonstrated that the cell-to-cell heterogeneity in the Ca^{2+} train notwithstanding, stochastic oscillations exhibit a linear correlation between the standard deviation of ISI and the average period of the spikes. It turns out that, under different experimental conditions (e.g. agonist dose, extent of cytosolic Ca^{2+} buffering, cell type), frequency encoding is realized by moving up and down this relation (Skupin et al., 2010). Interestingly, when ECFCs are exposed to 10 ng/ml VEGF, the interval between two consecutive Ca^{2+} transients in most cells ranges between 200 sec and 800 sec (Dragoni et al., 2011), which is adequate to induce the nuclear translocation of NF-κB (Smedler and Uhlén, 2014). Conversely, when VEGF concentration is raised to 50 ng/ml, the ISI may be lengthened to 1500 sec, which is less efficient to stimulate NF-κB. An alternative, albeit not mutually exclusive, mechanism whereby irregular Ca^{2+} fluctuations are decoded involves the spatial distribution of the underlying Ca^{2+} toolkit. Local Ca^{2+} microdomains may be generated around the cytosolic mouth of Ca^{2+}-permeable channels to convey information to specific Ca^{2+}-sensitive decoders located in their immediates surroundings. The seminal example has been provided by Parekh's group (Parekh, 2009), who demonstrated that leukotriene C4-induced Ca^{2+} oscillations induced gene expression and mast cell activation only when they were patterned by Orai1, while were inefficient if exclusively contributed by $InsP_3Rs$. The randomness of VEGF-induced Ca^{2+} spikes in ECFCs might be translated into a biologically meaningful code by the selective activation of either $InsP_3Rs$ or store-dependent channels (e.g. Orai1 and TRPC1) or both. These preliminary studies paved the way for the subsequent examination of the Ca^{2+} response to VEGF in tumor ECFCs. Once established that VEGFR-2 is capable of delivering a pro-angiogenic Ca^{2+} signal to their normal counterparts, we sough to elucidate whether this pathway was altered in cancer patients-derived cells and whether it could serve as an alternative target in oncology.

RATIONALE FOR STUDYING THE Ca^{2+} MACHINERY IN ECFCs DERIVED FROM RCC PATIENTS

It has long been established that neoplastic transformation is accompanied by a remodelling of the Ca^{2+} toolkit, which may not be key to malignant initiation, but may contribute to establish some of the ten hallmarks of cancer (Monteith et al., 2007; Prevarskayaet al., 2011; Roderick and Cook, 2008), including enhanced proliferation, survival, angiogenesis and invasion. The derangement of the Ca^{2+} machinery is not limited to neoplastic cells, but may also be extended to tumor microenvironment (Nielsen et al., 2014). Accordingly, TECs harvested from breast carcinoma up-regulate TRPV4, which governs the rearrangement of actin cytoskeleton driving endothelial cell

migration during the angiogenic switch (A Fiorio Pla et al., 2012). Moreover, normal HMECs may be reprogrammed by adriamycin-resistant human breast cancer cells (MCF-7/ADM) to express TRPC5, which is otherwise absent in normal cells (Dong et al., 2014). Therefore, we wondered whether tumor ECFCs, which do not belong to the neoplastic clone and are isolated from the mononuclear fraction of peripheral blood (Basile and Yoder, 2014), could somehow be influenced by the malignant conditions of the donor. The urgency of this study was further prompted by the simultaneous investigation of Ca^{2+} dynamics in ECFCs isolated from other two sources, i.e. umbilical cord blood (UCB) and peripheral blood of individuals affected by primary myelofibrosis (PMF). We found that the Ca^{2+} toolkit may be assembled according to rather different modes in endothelial precursors depending on the superimposed patho-physiological background. For instance, TRPC3, which is absent in circulating ECFCs, appears on the plasma membrane of their UCB counterparts to mediate the influx of Ca^{2+} that triggers the oscillatory response to VEGF (Dragoni et al., 2011; Sánchez-Hernández et al., 2010). A more dramatic dysregulation of the Ca^{2+} machinery occurs in ECFCs derived from PMF patients (PMF-ECFCs), who suffer from fibrotic bone marrow, splenomegaly, cytopenias, and multiple disease-related symptoms that reduce quality and length of life (Barosi et al., 2012). PMF-ECFCs display a higher ER Ca^{2+} load, which is associated to the over-expression of all $InsP_3R$ transcripts as well as of Stim1, Orai1, Orai3, TRPC1 and TRPC4 proteins (Dragoni et al., 2014). It is, therefore, not surprising that SOCE magnitude is significantly enhanced, albeit two separate routes may be discriminated based on their pharmacological profile: one stimulated by passive store depletion and Gd^{3+}-resistant, the other one recruited by the $InsP_3$-dependent Ca^{2+} store and Gd^{3+}-sensitive (Dragoni et al., 2014). A remarkable feature of these cells, that distinguishes them from both peripheral blood- and UCB-derived ECFCs (Dragoni et al., 2013; Sánchez-Hernández et al., 2010), is their relative insensitivity to SOCE inhibitors. BTP-2 and La^{3+} barely affected the proliferation rate, while Gd^{3+} was inefficient (Dragoni et al., 2014). This finding suggested that VEGF may exploit signalling pathways other than an increase in $[Ca^{2+}]_i$ to control ECFC replication in a pathological context; in addition, it reawakened the interest towards the earlier notion that proliferative diseases, such as cancer, may turn off the requirement for extracellular Ca^{2+} to drive cell cycle progression into mitosis (Jaffe, 2005). This concept does not rule out the contribution of Ca^{2+}-permeable channels, which may serve as scaffold proteins to orchestrate the proliferative process independently on their Ca^{2+}-conducting properties, as recently shown for Orai1 and Orai3 in several cell lines (Borowiec et al., 2014). However, it might have profound therapeutic implications as the *in situ* disruption of their encoding genes is far less feasible than pharmacological abrogation of Ca^{2+} fluxes. Therefore, we felt the urgency to examine the remodelling, if any, of Ca^{2+} signalling in ECFCs isolated from

metastatic RCC patients (RCC-ECFCs). First, RCC is the most common type of kidney cancer in the adults, and several strong evidences hint at EPC participation to the angiogenic switch in primary tumor (Moccia et al., 2014; Hill, 2010; Bhatt et al., 2011; Yang et al., 2012; Akino et al., 2009). Second, metastatic RCC is the only human cancer where the standard care for treatment is represented by anti-angiogenic drugs, such as bevacizumab, sunitinib, and sorafenib (F Moccia et al., 2014; Escudier et al., 2012). As described in a previous paragraph, however, clinical practice revealed that a fraction of the patients is intrinsically refractory towards this treatment, while those who undergo an initial remission display a later tumor relapse and ultimately die (Moccia et al., 2014; Escudier et al., 2012). However, TECs cultured from human RCC do respond to VEGF stimulation, which renders them sensitive to bevacizumab, sunitinib and sorafenib (Bussolati et al., 2003). It turns out that an additional component of tumor vasculature must be refractory to this growth factor (F Moccia et al., 2014). All the studies described above concurred to demonstrate that EPCs require a functional VEGFR-2 to sustain the malignant transformation, but they drew this conclusion by exploiting normal, rather than tumor, cells. Third, RCC has recently been associated to an important derangement of Ca^{2+} machinery, Orai1 and TRPC6 expression being enhanced in primary tumor samples as compared to normal renal tissues (J. Song et al., 2013; Kim et al., 2014). Conversely, TRPC4 is down-regulated in two different human kidney carcinoma cell lines as related to normal human epithelial renal cells (Veliceasa et al., 2007). These alterations may play a central role in the neoplastic transformation of normal kidney. While TRPC6 up-regulation favours the transition through G2/M phase (J. Song et al., 2013), and Orai1 regulates RCC cell proliferation and migration (Kim et al., 2014), the loss of TRPC4 leads to a diminished secretion of the endogenous inhibitor thrombospondin-1, thereby promoting the angiogenic switch (Veliceasa et al., 2007). Thus, understanding whether RCC-ECFCs develop Ca^{2+}-handling abnormalities might permit the accomplishment of two goals. It could unveil alterations in the pro-angiogenic signalling pathways initiated by VEGF, thereby providing a solid cellular and molecular rationale for the failure of anti-VEGF therapies; and, at the same time, it could highlight unforeseen targets, i.e. Ca^{2+}-permeable ion channels and/or Ca^{2+} transporters, to devise alternative treatments.

THE Ca^{2+} SIGNALLING TOOLKIT IS REARRANGED IN ECFCs ISOLATED FROM PATIENTS SUFFERING FROM RCC

Based on the rationale above, we analyzed the Ca^{2+} machinery in ECFCs isolated from naïve RCC patients (RCC-ECFCs), before they underwent to anti-angiogenic treatments. We found that the ER Ca^{2+} content is dramatically lower in RCC-derived cells as compared to control cells, albeit the resting Ca^{2+} levels are not different (Lodola et al., 2012). In addition, RCC-ECFCs lack $InsP_3R1$

transcripts, $InsP_3R2$ and $InsP_3R3$ being less abundant than in normal ECFCs. These cells are, therefore, less prone to release Ca^{2+} from intracellular stores. This feature gains particular relevance when recalling that a decreased ER Ca^{2+} load may protect tumor cells from mitochondrial Ca^{2+} overload, thereby preventing the permeabilization of the outer mitochondrial membrane and inhibiting the release of apoptogenic factors (Prevarskaya et al., 2004; Prevarskaya et al., 2014). In this view, TECs isolated from several types of solid tumors, including RCC and breast cancer, are extremely resistant to apoptosis induced by serum starvation and by vincristine, a well known chemotherapeutic (Bussolati et al., 2010). Since renal TECs include bone marrow-derived cells, it has been speculated that this pro-survival phenotype is, at least partially, linked to the reduction in the intracellular Ca^{2+} reservoir detected in EPCs (Moccia et al. 2014; Moccia and Poletto 2014). This therapeutically relevant feature, however, remains to be experimentally probed. Moreover, these experiments were performed by measuring the amplitude of the peak fluorescence emitted by the Ca^{2+}-fluorophore Fura-2/AM when the cells were bathed in the absence of extracellular Ca^{2+} and challenged with cyclopiazonic acid (CPA). CPA is a selective inhibitor of SERCA activity that blocks Ca^{2+} re-uptake into the ER and unmasks an endogenous Ca^{2+} leak across ER membrane (Dragoni, Laforenza et al., 2014; Lodola et al., 2012). Consequently, CPA induces an increase in $[Ca^{2+}]_i$ that is supposed to reflect $[Ca^{2+}]_{ER}$ (Bergner et al., 2009; Lodola et al., 2012). Nevertheless, CPA-induced elevation in $[Ca^{2+}]_i$ might not provide the most accurate estimation of ER Ca^{2+} content as this organelle is rather uneven and CPA-sensitive pumps might reside in leakage channels-free sub-regions. Furthermore, the lower increase in $[Ca^{2+}]_i$ elicited by CPA could depend on the activation of ER-independent sequestration mechanisms, such as mitochondria, as discussed in (Vanden Abeele et al., 2002) and (Vanoverberghe et al., 2004). Thus, direct measurement of ER Ca^{2+} levels is mandatory to confirm that RCC-ECFCs undergo a drop in their intraluminal Ca^{2+} concentration. Conversely, SOCE is up-regulated in RCC-ECFCs due to the over-expression of Stim1, Orai1, and TRPC1 mRNAs and proteins, and has an important role in their angiogenic behaviour (Lodola et al., 2012). Accordingly, both cell proliferation and tubulogenesis are inhibited by BTP-2, low micromolar doses of La^{3+} and the anti-angiogenic agent CAI (Lodola et al., 2012). The blocking effect exerted by CAI is particularly intriguing, as it has been employed as cytostatic agent of the disease in Phase II clinical trials of subjects with advances RCC and refractory to immunotherapy (Dutcher et al., 2005; Stadler et al., 2005). Conversely, RCC are insensitive to OAG (unpublished observations from our group) and do not present the transcripts encoding for DAG-sensitive channels, i.e. TRPC3 and TRPC6 (Lodola et al., 2012). Another puzzling feature of Ca^{2+} signalling in RCC-ECFCs is represented by their lack of responsiveness to VEGF, despite the fact that VEGFR-2 expression is unaltered in these cells (Lodola et al., 2012). Accordingly, RCC-ECFCs do not manifest any detectable elevation in $[Ca^{2+}]_i$ when exposed to VEGF. It is conceivable

that either the decrease in ER Ca^{2+} content and/or the down-regulation of $InsP_3Rs$ render these cells less prone to release Ca^{2+} in response to VEGF. This result has at a least two patho-physiological implications. First, the physiological activator of SOCE must be a growth factor other than VEGF, albeit we cannot rule out the possibility that a VEGF-induced sub-membranal Ca^{2+} elevation occurs and is missed by our Ca^{2+} imaging system. Second, all VEGF targeting therapies might fail in interfering with ECFC mobilization from bone marrow in RCC patients. Indeed, if VEGFR-2 is expressed by RCC-ECFCs, but does not activate any pro-angiogenic Ca^{2+} signal, either VEGF monoclonal antibodies (bevacizumab) or TKR inhibitors (sunitinib and sorafenib) will not have any impact on SOCE activation and recruitment of its downstream Ca^{2+}-sensitive machinery. However, VEGF could exert its pro-angiogenic effect by inducing the nuclear translocation of NF-κB through alternative pathways NF-κB transcriptional activity may, however, be recruited by signalling pathways other than Ca^{2+}, such as protein kinase C (PKC), mitogen-activated protein kinases (MAPK)/ extracellular signal-regulated kinases (ERK), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), glycogensynthase kinase-3β (GSK3β) and casein kinase II (CKII). Thus, further investigation is required to assess whether VEGF does stimulate RCC-ECFCs.

AIM OF THE WORK

The aim of this project is to initially carry out the phenotypical and functional characterization of ECFCs isolated from healthy subjects and from patients affected by RCC. Although EPCs have been extensively studied in last few years, they are always subjects of debate about their phenotypical identification and their role in physiological and pathological processes. We focus on the differences, if any, in frequency, proliferation rate, *in vitro* tubulogenesis and apoptosis between healthy and tumor-derived ECFCs. Next, we assess whether and how VEGF-induced Ca^{2+}-dependent protein expression in altered in RCC-ECFCs, which fail to generate pro-angiogenic Ca^{2+} oscillations in response to this growth factor. Since our data point at a decrease in ER Ca^{2+} concentration as a major responsible for the lower sensitivity of RCC-ECFCs to pro-apoptotic insults and to VEGF stimulation, we finally focus on the molecular mechanisms responsible for the drop in ER Ca^{2+} levels.

MATHERIALS AND METHODS

ECFC ISOLATION AND CULTIVATION

PB samples (about 40 mL) were obtained from healthy human volunteers and from RCC patients from Structure of Medical Oncology, Policlinico San Matteo Foundation, Pavia. The demographic characteristics of patients and healthy donors are summarized in Table 1. The Institutional Review Board at "Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo Foundation" in Pavia approved all protocols and an informed consent was signed by patients and HDs. The study was conducted according to the principles of the Declaration of Helsinki.

	Number	Age [range]	Male/Famele	Chemotherapy	Metastasis
Patients	27	50 [38-67]	15-12	NO	NO
HD	34	39 [27-54]	19-15	N.A.	N.A.

Table 1. Demographic characteristics of patients and healthy donors (HD) involved in the study (N.A.: not applicable)**.**

To isolate ECFCs, MNCs were separated from PB by density gradient centrifugation on lymphocyte separation medium for 30 min at 400g and washed twice in EBM-2 with 2% FCS. After counting in a Burker chamber, a median of $36x10^6$ MNCs (range 18-66) were plated on 30 mm collagen-coated culture dishes (Becton Dickinson (BD) Biosciences, Franklin Lakes, New Jersey) in the presence of the endothelial cell growth medium EGM-2 MV Bullet Kit (Lonza, Basel Switzerland) containing endothelial basal medium (EBM-2), 5% foetal bovine serum, recombinant human (rh) EGF, rhVEGF, rhFGF-B, rhIGF-1, ascorbic acid and heparin, and maintained at 37°C in 5% CO_2 and humidified atmosphere. Discard of non-adherent cells was performed after 2 days; thereafter medium was changed three times a week. The outgrowth of ECFCs from adherent MNCs was characterized by the formation of a cluster of cobblestone-appearing cells after between 8 and 21 days of culture (Sánchez-Hernández et al., 2010). Once colonies were detached by trypsinization (trypsine/EDTA, Sigma Aldrich, St Louis, Missouri), monolayers of endothelial cells were obtained in the following passages.

IMMUNOPHENOTYPICAL ANALYSIS

Cells were trypsinized, recovered and incubated with the primary or isotopic control antibody for twenty minutes at 4°C. Cells were then washed and analysed by a fluorescence-activated cell sorter

(FACS; BD Biosciences). The following antibodies were used (all provided by BD Bioscience unless otherwise indicated): FITC-conjugated anti-CD34, FITC-conjugated anti-CD31, PE-conjugated anti-CD45; FITC-conjugated anti-CD105; FITC-conjugated anti-CD144; PE-conjugated anti-CD146; PerCP-conjugated anti VEGFR-2, and FITC-conjugated anti-CD133. All the antibodies have been obtained from BD Biosciences. Cells ($2x10^5$) were acquired by flow cytometer (FACSCanto, Becton Dickinson), and analysed by CellQuest software (BD Biosciences).

PROLIFERATION ASSAY

$1,5x10^4$ ECFCs (1st passage) were plated in 5 collagen treated wells (30mm) in EGM-2 medium. Cultures were incubated at 37°C (in 5% CO_2 and humidified atmosphere). The day after, cells were recovered by trypsinization and counted with a Burker chamber. This passage was performed for other 3 times in the three following days, about at the same hour, with the remaining cells.

***IN VITRO* TUBULOGENESIS ASSAY**

In vitro tubulogenesis assay was performed using Matrigel™ (BD Biosciences, Franklin Lakes, New Jersey). The composition of the substrate is laminin, collagen IV, entactine and proteoglycan isolated from a mouse sarcoma (Engelbreth-Holm-Swarm), a tumor embedded by proteins of extracellular matrix. At room temperature, Matrigel™ polymerizes and produces a biologically active scaffold which mimics the mammalian cells basal membrane. From 5 to $10x10^3$ ECFCs were resuspended in 200 µl EGM-2 and incubated onto a thin layer of Matrigel™ at 37°C in 5% CO_2 in 96 wells plate. Between 4 and 36 hours from the seeding, ECFCs-derived cells form a capillary-like tridimensional structure which reproduces the *in vivo* vessels network and was observed by visual observation using an inverted microscope. At least 3 different sets of cultures were performed per every experimental point.

APOPTOSIS ASSAY

For apoptosis investigation, the *In Situ* Cell Death Detection Kit, AP (Roche, Life Science, Basel, Switzerland) which is based on the detection of single- and double-stranded DNA breaks that occur at the early stages of apoptosis, was used. RCC-ECFCs and N-ECFCs were plated in Nunc™ Lab-Tek™ II Chamber Slide™ System (Thermo Scientific, Waltham, Massachussets), at a density of $3x10^3$ cells per well, in 300 µl EGM-2 medium. The day after, cells were treated with the apoptosis inducers 1 µM thapsigargin (Sigma Aldrich, St. Louis, Missouri) or 5 µg/ml rapamycin (Sigma Aldrich, St. Louis, Missouri). After 24 and 48 hours of treatment, the cells were fixed by paraformaldehyde (4%) for 1 hour at room temperature and permeabilized (0.1% Triton X-100,

0.1% sodium citrate) for 2 minutes on ice. Controls experiments were conducted with cells from the same batch, cultured in EGM-2 only. Subsequently, the cells were incubated with the TUNEL reaction mixture that contains deoxynucleotidyl-transferase (TdT) and fluorescein dUTP.

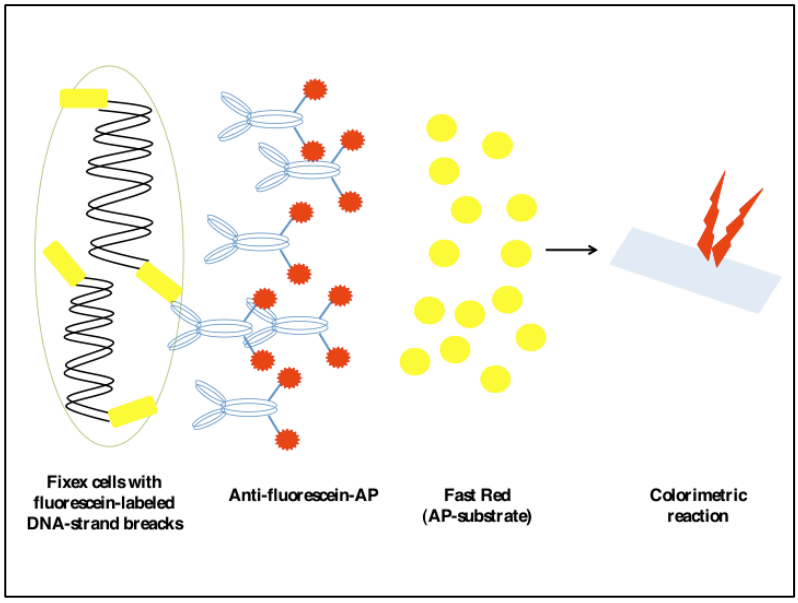


Figure 8. Tunel assay (AP: alkaline phosphatase).

During this incubation period, TdT catalyzes the addition of fluorescein-dUTP at free 3'-OH groups in single- and double-stranded DNA. After washing, the label incorporated at the damaged sites of the DNA is marked by an anti-fluorescein antibody conjugated with the reporter enzyme alkaline phosphatase (AP). After washing to remove unbound enzyme conjugate, samples can be analysed in a drop of PBS under a fluorescent microscope. Otherwise, the cells could be incubated with converter-AP provided by the kit and then treated with Fast Red (Roche), the AP substrate which precipitates when interacts with AP and produces a colorimetric reaction which can be analysed under optical microscope. ECFCs with red nuclei will be apoptotic cells.

IMMUNOCYTOCHEMISTRY

Twenty-four hours before treatment with VEGF and the specific Ca^{2+} inhibitors, $6x10^4$ RCC-ECFCs and N-ECFCs were plated onto 13 mm coverslips in 24-well plates. The cells were treated as described in the previous paragraph. ECFCs-derived cells were fixed in 4% formaldehyde in PBS for 15 minutes at room temperature, permeabilized for 7 min in PBS with 0.1% Triton X-100 and blocked for 30 min in 2% gelatine. Then, primary (incubated for 1 hour at 37°C) and secondary (incubated for 1 hour at room temperature) antibodies were applied in PBS with 2% gelatin. The primary anti-p65 (NF-kB subunit) antibody specific for immunocytochemistry (Santa Cruz) was

used at 1:50 dilution, whereas AlexaFluor 488 secondary antibody from Life Sciences (Milan, Italy) was used at 1:200. After washing (3 times for 5 minutes each), nuclei were stained with 40,6-diamidino-2-phenylindole dihy-drochloride (DAPI) for 15 minutes at RT. Fluorescence images were acquired using a Leica epifluorescent microscope equipped with S Fluor X40/1.3 objective using MetaMorph software.

TREATMENT WITH VEGF AND INHIBITORS OF Ca^{2+} SIGNALLING

N-ECFCs and RCC-ECFCs were plated in 25 cm^2 flasks or in 30 mm wells depending on the cell number required for the planned experiments. When cells reached about 90% of confluence in EGM2, the growth medium was removed and cells were left for 6 hours in a medium without serum and without growth factors (EBM2, Lonza, Basel Switzerland) at 37°C in 5% CO_2 and humidified atmosphere. Then, VEGF (Promega, Milano, Italy) at 10 ng/ml was added to the medium. For qRT-PCR analysis of VEGF-induced transcripts, cells were challenged with VEGF for 2, 4, and 6 hours. For western blot analysis of VEGF-induced protein expression, the cells were stimulated with VEGF for 4 hours. When the effect of specific Ca^{2+} inhibitors (BTP-2, BAPTA and thymoquinone) had to be tested, after the initial 6 hours of washing in EBM2, cells were further treated for 30 minutes with 20 μM BTP-2 (Calbiochem, La Jolla, Canada), 30 μM BAPTA (Sigma Chemical Co., St. Louis, Missouri) and 25 μM thymoquinone (Sigma Chemical Co.). Thereafter, VEGF was added and tested at the conditions described above.

RNA ISOLATION AND REAL TIME RT-PCR (qRT-PCR)

N-ECFCs and RCC-ECFCs cells were challenged with VEGF for 2, 4, and 6 hours. After this treatment, cells were detached from 30 mm wells by enzymatic reaction of 200 μl of QIAzol Lysis Reagent (QIAGEN, Duesseldorf, Germany). Total RNA was extracted from cells using the QIAzol Lysis Reagent (QIAGEN) and miRNeasy Mini Kit (QIAGEN). Single cDNA was synthesized from RNA (1 μg) using oligo(dT), random hexamers, and M-MLV Reverse Transcriptase in iScript cDNA Syntesis Kit (BIO-RAD, Hercules, Canada). Reverse transcription was always performed in the presence or absence (negative control) of the reverse transcriptase enzyme. qRT-PCR was performed in triplicate using 1 μg cDNA and TaqMan Assay for gene expression for Real-Time PCR (Applied Biosystem|Life Technologies, Waltham, Massachusetts). The genes that were analyzed and the corresponding primers are listed in Table 2.

Gene	Primers sequences	Size (bp)	Accession number
BCL2	Hs00608023_m1 TaqMan gene expression Assay	81	NM_000633.2
MMP9	Hs00234579_m1 TaqMan gene expression Assay	54	NM_004994.2
ICAM1	Hs00164932_m1 TaqMan gene expression Assay	87	NM_000201.2
E-SELE	Hs00950401_m1 TaqMan gene expression Assay	104	NM_000450.2
VCAM1	Hs01003372_m1 TaqMan gene expression Assay	62	NM_080682.2
CCND1	Hs00765553_m1 TaqMan gene expression Assay	57	NM_053056.2
MYC	Hs00153408_m1 TaqMan gene expression Assay	107	NM_002467.4
SERCA2b	Forward 5': AATGTGTAACGCCCTCAACA Reverse 5': GCAGGCTGCACACACTCTT	282	NM_170665.3
SERCA3	Forward 5': GTGGACCAGTCCATCCTGAC Reverse 5': GCTTTGCCCGATGTGATATT	134	NM_005173.3
PMCA1a	Forward 5': CCAAACACAGATGGATGTAGTGA Reverse 5': GAAAACACTACATGTGTAGGGGTAGA	135	NM_001001323.1
PMCA1b	Forward 5': CCAAACACAGATTCGAGTGG Reverse 5': AAGGGGGATATGAGGCTCTG	142	NM_001682.2
PMCA4a	Forward 5': ACCGTATCCAGACTCAGATCG Reverse 5': TGTTGACCCATGTTCTGTCG	91	NM_001001396.2
PMCA4b	Forward 5': TCCAGACTCAGATCAAAGTGG Reverse 5': GCTGTGGACTTTTGGTT	89	NM_001684.4
NCX1.3	Forward 5': GTGCAGTTTCTCCCTTGTGC Reverse 5': TTGTCATCATATTCGTCTGTTATTG	90	NM_001112802.1
NCX1.7	Forward 5': GTGCAGTTTCTCCCTTGTGC Reverse 5': GCATGAACCTTCCTGAAGACA	102	NM_001112801.1
YWHAZ	Forward 5': ACTTTTGGTACATTGTGGCTTCAA Reverse 5': CCGCCAGGACAAACCAGTAT	94	NM_003406.3
GAPDH	Forward 5': CGGATTTGGTCGTATTGG Reverse 5': GGTGGAATCATATTGGAACA	130	NM_002046.3

Table 2. Primer sequences/ID TaqMan Gene expression assay used for real-time reverse transcription-polymerase chain reaction (bp: base pair).

Sso Advanced Universal Probes Supermix and Sso Fast EvaGreen Supermix (both from BIO-RAD) were used according to the manufacturer instructions, and qRT-PCR performed using CFX 96 Real-Time system, C1000 Thermal Cycler (BIO-RAD). The conditions of the reaction were: initial polymerase activation and DNA denaturation at 95°C for 30 seconds; 40 cycles of denaturation at 95°C for 5 seconds; annealing and extension at 60°C for 5 seconds. The qRT-PCR reactions were normalized using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and tyrosine 3-

monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) as housekeeping genes. Melting curves were generated to detect the melting temperatures of specific products immediately after the PCR run. The triplicate threshold cycles (Ct) values for each sample were averaged resulting in mean Ct values for both the gene of interest and the housekeeping genes. Relative mRNA levels were determined by comparative quantitation and the results expressed as fold change by CFX manager software 3.0 (BIO-RAD).

CELL LYSIS, PROTEINS EXTRACTION AND IMMUNOBLOTTING

Protein expression was tested in N-ECFCs and RCC-ECFCs stimulated with VEGF for 4 hours. When the effect of BTP-2, BAPTA and thymoquinone had to be tested, cells were further treated for 30 minutes with the inhibitors Thereafter, VEGF was added and cells stimulated for 4 hours.

For protein extraction, radio-immuno precipitation assay (RIPA) buffer was previously prepared with the following composition: NaCl 150 mM, 1% triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), Tris(hydroxymethyl)amino-methane 50mM pH 8.0. All the reagents were purchased from Sigma Aldrich. Lysis buffer was prepared with 10 ml of RIPA buffer and 1 tablet of protease inhibitor cocktail, cOmplete Mini (Roche Life Science, Basel, Switzerland). Each step of this protocol was performed on ice. N-ECFCs and RCC-ECFCs were harvested from 25 cm^2 culture flasks and washed twice with cold PBS 1X. 50 µl of lysis buffer was added to each flask and adherent cells were scraped by a plastic rubber policeman. Cell suspension was then transferred into a microcentrifuge tube, maintained in agitation for 30 minutes at 4° C and then sonicated on ice for 3 minutes with Sonorex RK100 (Bandelin, Berlin, Germany). Cell lysates were centrifuged at 14000g for 15 minutes at 4° C, and the supernatant containing the ECFCs total proteins homogenized. Protein contents from each sample was determined by the Bradford's method with DC Protein Assay reagent Package (BIO-RAD) using bovine serum albumin (BSA) as standard. The homogenates were solubilized in Laemmli buffer (Sánchez-Hernández et al., 2010) and 20 µg proteins were separated on 4-15% polyacrylamide mini protean Tris/Glycine/SDS (TGX) precast gel for electrophoresis (BIO-RAD) at a fixed voltage of 180V for 45 minutes. The proteins were then transferred to the Trans Blot turbo mini PVDF Transfer Membranes (BIO-RAD) by Trans Blot Turbo transfer system (BIO-RAD) using pre-set programs from the machine. After 1 hour blocking with Tris buffered saline (TBS) containing 7% powder milk (Sigma Aldrich) and 0.1% Tween (blocking solution), the membranes were incubated over night at room temperature with the affinity purified antibodies listed in Table 3, diluted in TBS and 0.1% Tween containing 1% BSA. Control

experiment were performed using an anti-tubulin antibody.

Antibody	Source	Protein MW (kDa)	Diluition
Anti MMP9 (Santa Cruz Biotechnology)	Mouse	92	1:200
Anti VCAM-1 (Santa Cruz Biotechnology)	Mouse	110	1:300
Anti E-selectin (Santa Cruz Biotechnology)	Mouse	115	1:800
Anti Bak (Santa Cruz Biotechnology)	Rabbit	30	1:1000
Anti Bcl-2 (Santa Cruz Biotechnology)	Mouse	26	1:300
Anti-Calreticulin (Sigma Aldrich)	Rabbit	55	1:1000
Anti-Calnexin (Sigma Aldrich)	Rabbit	90	1:1000
Anti-phospho-VEGFR-2 (Sigma Aldrich)	Rabbit	230	1:500
Anti-tubulin (Sigma Aldrich)	Mouse	50	1:1000

Table 3. Primary antibodies used for the proteins of interest, source, approximative molecular weight (MW) and respective diluitions.

The membranes were washed and incubated for 1 h with anti-mouse and anti-rabbit IgG peroxidase-conjugated secondary antibodies produced in goat (1:50000 in blocking solution) (Sigma Aldrich). The bands were detected with clarity western ECL substrate (BIO-RAD). Precision Plus Protein™ WesternC™ Standard (BIO-RAD) was used to estimate the molecular weights. The Precision Plus Protein™ WesternC™ Standard was used to accurately estimate the molecular weight and as a positive control for the immunoblot. Blots were developed in a dark room through the exposure of the photographic film Hyperfilm ECL (GE Healthcare Life Sciences, Milano, Italy) to ECL treated membranes. Bands were detected with the development in Canestream Kodak processing chemicals for autoradiography films, developer solution and Canestream Kodak processing chemicals for autoradiography films, fixer solution (Sigma Aldrich). Densitometric analysis of the bands was performed by Image-j computer program and the results were expressed as a percentage of the protein/tubulin densitometric ratio.

Ca^{2+} MEASUREMENTS USING LENTIVIRAL AEQUORIN-BASED PROBES

ER and mitochondrial Ca^{2+} levels in the lumen of the endoplasmic reticulum (ER) were monitored using luminescent Ca^{2+} sensor aequorin (AEQ). We opted for use of lentiviral AEQ vectors as lentiviral transduction assures robust expression of the probe in ECFCs (Lodola et al., 2012). Generation of lentiviral vector pLV-er-EGFP-AEQmut (pLV-erAEQ) was described previously

(Lazzari et al., 2011). For generation of pLV-mit-EYFP-AEQmut (pLV-mitAEQ) mitEYFP cassette was amplified form pEYFP-mito vector (Clontech) using following primers (5' to 3'):

forward CCTCTAGAATGTCCGTCCTGACGCCG;

reverse CGAAGCTTTGACTTGTACAGCTCGTCCAT;

disrupting stop codon and generating restriction sites for XbaI (at 5') and HindIII (at 3') enzymes. HA1-AEQmut cassette was liberated from pCDNA1-er-AEQmut (Montero et al., 1995) using HindIII and EcoRI enzymes. mitEYFP and HA1-AEQmut fragments were first subcloned into pBSK+ vector (digested with XbaI/EcoRI) in a three-part ligation reaction, after which mitEYFP-HA1-AEQmut entire cassette was liberated by XbaI/XhoI enzymes and cloned in XbaI/SalI digested with pRRLsin.PPTs.hCMV.GFPpre (Follenzi and Naldini, 2002) (pLV) lentiviral vector generating pLV-mitEYFP-AEQmut vector referred as to pLV-mitAEQ.

Lentiviral particles were produced as described previously with minor modifications (Lodola et al., 2012). Briefly, 2x10e6 HEK293T cells were transfected with pMDLg/pRRE, pMD2.VSVG, pRSV-Rev and either pLV-erAEQ or pLV-mitAEQ in 100 mm petri dishes using Lipofectamine 2000 (Life Technologies, Milan, Italy). 48-72 h after transfection culture medium was collected and filtered through 0.45 μm PES filter (Millipore Corporation, Bedford, MA, USA). Viral particles were precipitated by PEG solution (8% PEG8000, 100 mM NaCl, 0.4 mM TrisHCl, pH 7.2) for 24 h at 4°C and pelleted by centrifugation (1500 xg, 30 min, 4°C). Pellet was resuspended in PBS at 1/100 of the initial medium volume, aliquoted and stored at -80°C. Viral particles were titred by infecting ECFCs by serial dilutions and examination of EGFP or EYFP fluorescence. The dilutions resulting 70–90% of infected cells were used for AEQ experiments.

For AEQ Ca^{2+} measurement, RCC-ECFCs and N-ECFCs were spotted onto fibronectin-coated 13 mm coverslips in a 24 well plate (Costar) at a density 2–3x10e3 cells per spot. 24 hours after plating the cells were infected with erAEQ and mitAEQ expressing lentivirus for 48–72 hours. The erAEQ was reconstituted in modified Krebs–Ringer buffer (KRB, 135 mM NaCl, 5 mM KCl, 0.4 mM KH2PO4, 1 mM MgSO4, 5.5 mM glucose, 20 mM HEPES (pH 7.4) supplemented with 600 μM EGTA, 5 μM coelenterazine n and 3 μM ionomycin (all reagents from Sigma) for 1 h at 4°C. After reconstitution, the cells were washed 3 times with KRB containing 600 μM EGTA and 2% BSA, followed by 3 washes with KRB containing 600 μM EGTA after which the coverslips were transferred into perfusion chamber of a custom built aequorinometer (CAIRN research, UK). The cells were initially perfused with KRB supplemented with 100 μM EGTA and, after baseline recording, the perfusion solution was switched to KRB containing 2 mM Ca^{2+}. Recording continued

until the $[Ca^{2+}]_{ER}$ did reach the steady-state level, after that ATP (100 μM) was added to perfusate. The mitAEQ was reconstituted in DMEM containing 1% FBS and 5 μM coelenterazine at 37°C for 1 hour after which the cells were used for experiment. At the end of each experiment the remaining AEQ pool was discharged by perfusing distilled water containing 100 mM Ca^{2+} and 100 μM digitonin and the luminescent signals were calibrated off-line into $[Ca^{2+}]$ values using an algorithm developed by Brini et al. (Brini et al., 2005).

ELECTRON MICROSCOPY

ECFCs were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde (Sigma Aldrich) in 0.1 M sodium phosphate buffer, pH 7.4. After fixation, cells were postfixed in 2% osmium (OsO4) for 1 hour at room temperature, dehydrated and embedded in LR White (Sigma Aldrich). Polimerization was performed for 24 hours at 60°C. Ultrathin sections were cut with a Reichert (Depew, New York) OM-U3 ultramicrotome. The sections were stained with uranyl acetate and lead citrate (Sigma Aldrich), examined and photographed at 3000X, 7.000X or 20.000X magnifications on a Zeiss (Jena, Germany) EM900 (80kV, objective diaphragm 30μm) electron microscope.

STATISTICS

All data are expressed as mean ±SE. The significance of the differences of the means was evaluated with Student's t-test. Differences were assessed by the Student t-test for unpaired values.

All statistical tests were carried out with GraphPad Prism 4.

RESULTS

THE FREQUENCY OF CIRCULATING RCC-ECFCs IS HIGHER COMPARED TO N-ECFCs

The first set of experiments was aimed at ascertaining the phenotypical and functional differences between ECFCs isolated from patients with RCC and ECFCs from healthy donors. We focused on frequency in PB, growth potential, tubulogenic rate, apoptosis resistance and pro-angiogenic response to VEGF. In order to assess whether there was a difference in frequency between N-ECFCs and RCC-ECFCs, the number of ECFC colonies for 10^7 MNCs plated *in vitro* was evaluated. As shown in Table 4, RCC-ECFC frequency was significantly higher as compared to N-ECFCs.

N-ECFCs (n=34)	RCC-ECFCs (n=27)
0.28*	**0.51***

Table 4. Median value with standard deviation (SD) of ECFCs number for 10^7 MNC plated (p-value=0.027). *=ECFCs frequency for 10^7 MNCs plated.

The frequency median value in patients affected by RCC was 0.51 (with a range 0-7.1) whereas in healthy subjects was 0.28 (with a range 0-1.87) colonies for 10^7 MNCs plated; the p-value was 0.027 (obtained with a Student's t-test) which demonstrates the statistical significance of the frequency difference. Moreover, the observed significance was maintained also when patients and healthy subjects were divided for sex and the number of ECFCs colonies counted and the median values compared. Therefore, it could be concluded that the frequency of circulating ECFCs was higher in patients with RCC compared with healthy subjects, which is consistent with their involvement in tumor vascularization.

RCC-ECFCs AND N-ECFCs SHOW THE SAME IMMUNOPHENOTYPIC PROFILE

In order to study the immunophenotypic profile of RCC-ECFCs and N-ECFCs and to understand whether they express different markers on their surface, early (P1-P2), intermediate (P3-P4), and late passages (>P6) of culturing cells were assessed by cytofluorimetric analysis and, in selected cases, also by immunocytochemistry reaction on cytocentrifuged cells (Figure 9). The aim was to

understand whether *in vitro* passages could modify the classical ECFC phenotype. Both ECFCs from RCC patients and from healthy subjects had the same pattern of expression of surface proteins which was similar to that described by Ingram et al. (Ingram et al., 2004); the pattern was maintained during cell culture passages. In Table 5 are listed the median values of surface proteins expression typical of endothelial (CD31, CD105, CD144 or VE-Caderin, CD146 an vWf) and hematopoietic lineage (CD14, CD45). It is evident that N-ECFCs and RCC-ECFCs express all the endothelial-specific but not the hematopoietic-specific markers. There was no significant ($p<0.05$) difference in the expression levels of the common endothelial-specific markers between RCC-ECFCs and N-ECFCs.

	ECFC P2 (n=3)		**ECFC P4 (n=3)**		**ECFC P6 (n=2)**	
	normal	RCC	normal	RCC	normal	RCC
VE-cad	98.70%	99.10%	99.30%	99.20%	98.80%	97.90%
CD31	99.20%	98.40%	97.10%	98.30%	97.90%	97.20%
CD105	88.70%	94.90%	92.90%	90.10%	97.10%	98.20%
CD146	98.90%	99.10%	99.20%	98.20%	97.90%	98.90%
vWf	98.70%	99.10%	91.80%	95.40%	90.90%	92.10%
CD45	1.80%	2.20%	0.90%	1.10%	0.70%	0.50%
CD14	0.80%	1.10%	2.10%	1.90%	0.80%	0.70%
VEGFR-2	50.3%	49.8%	43%	47.5%	48.5%	51.6%

Table 5. Percentage of expression of the surface antigens probed for ECFC characterization.

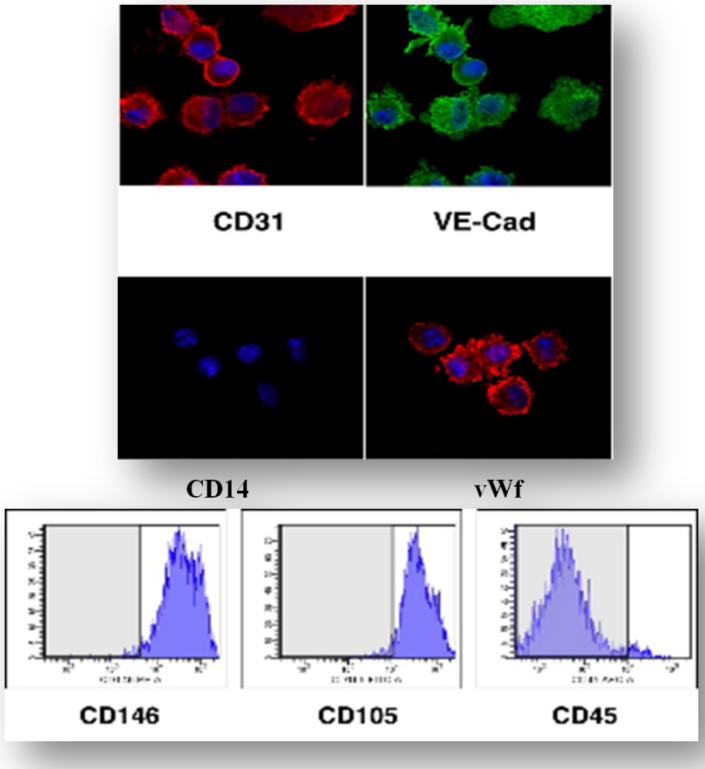


Figure 9. Examples of cytofluorimetric and immunocytochemistry analyses conducted for the immunophenotypic characterization of ECFCs.

THE GROWTH CURVES OF RCC-ECFCs AND N-ECFCs ARE OVERLAPPING

Next, we assessed whether RCC-ECFCs and N-ECFCs had a different growth potential. Therefore, growth curves were evaluated. $2x10^4$ ECFCs were plated in 5 collagen treated wells and from the next day for 5 days, cells were recovered by trypsinization and counted. The number of RCC-ECFCs and N-ECFCs detached every 24 hours were compared and shown in Figure 10.

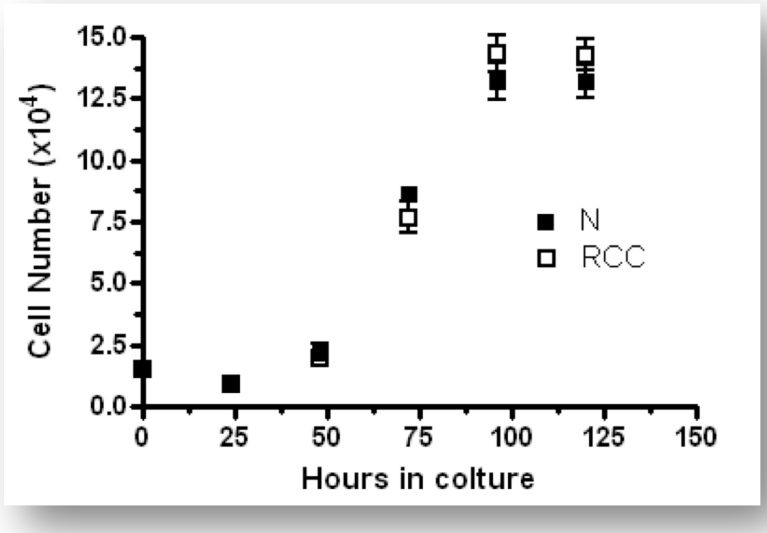


Figure 10. Growth curves of RCC-ECFCs (n=10), represented by white squares and N-ECFCs (n=9), black squares.

It was observed that RCC-ECFCs and N-ECFCs growth curves were overlapping, clearly indicating that there was not a significant difference in their growth potential *in vitro*.

***IN VITRO* TUBULOGENESIS IS NOT DIFFERENT IN RCC-ECFCs AND N-ECFCs**

In order to understand whether N-ECFCs and RCC-ECFCs display differences in their tubulogenic capacity *in vitro*, Matrigel assay was performed as previously described (see Materials and methods). The development of a tridimensional capillary-like structure was analysed from 4 to 48 hours after cell seeding. As shown in Figure 13 (above panel), there was no significant ($p<0.05$) difference in tube length between N-ECFCs and RCC-ECFCs. They did not present any alteration either in the tubules formation timing or in the degradation of the same structures during the observation period. In Figure 11 (lower panel), two capillary-like structures, representative of ECFCs isolated from patients and from healthy subjects, are shown.

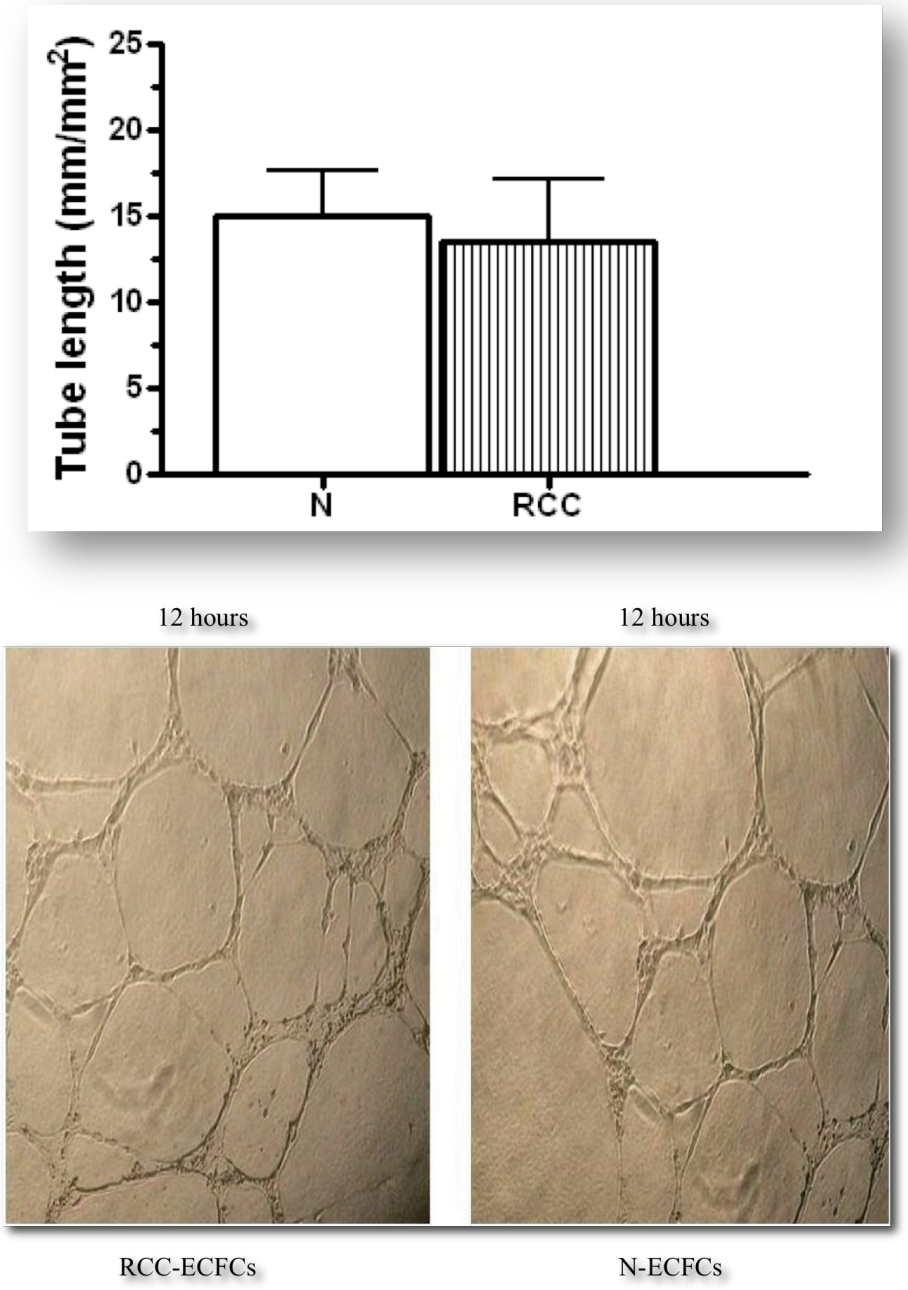


Figure 11. ***In vitro*** **tubulogenesis.** Above, tube length histogram, measured in mm/mm^2 in N-ECFCs (n=8) and in RCC-ECFCs (n=7). N=N-ECFCs and RCC=RCC-ECFCs. Below, a representative example of two Matrigel assays. Capillary-like structures *in vitro* from a healthy subject (N-ECFCs), and from a patient with RCC (RCC-ECFCs) after 12 hours of culture.

RCC-ECFCs ARE MORE RESISTANT TO RAPAMYCIN-INDUCED APOPTOSIS AS COMPARED TO N-ECFCs

It has long been known that carcinogenesis is associated to an acquired resistance to pro-apoptotic stimulation (Prevarskaya et al. 2014; Prevarskaya et al., 2004). ECFCs do not belong to the neoplastic clone, as shown in Piaggio et al. (2009) and discussed in Moccia and Poletto (2014), yet

a recent study provided the evidence that endothelial cells may be reprogrammed to develop resistance to adriamycin-induced apoptosis in a Ca^{2+}-dependent manner (Dong et al., 2014; Piaggio et al., 2009; Francesco Moccia and Poletto, 2014). Therefore, we evaluated the impact of well-known pro-apoptotic stimuli on RCC- and N-ECFCs. We found that the percentage of apoptotic cells induced by rapamycin (5 μg/ml) was significantly ($p<0.05$) lower in RCC-ECFCs as compared to N-ECFCs at both 24 h (Figure 12 (a)) and 48 h (Figure 12 (b)). Notably, RCC-ECFCs were less prone to undergo apoptosis even under control conditions at 24 h (Figure 12 (a)).

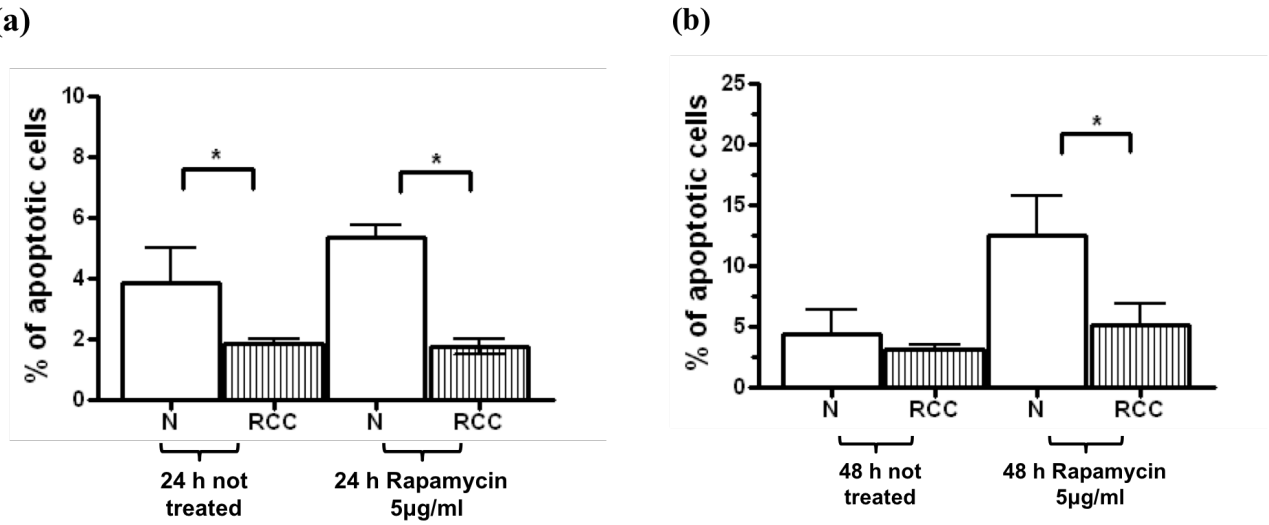


Figure 12. Resistance to rapamycin-induced apoptosis. Control cells (not treated) and cells treated with rapamycin (5 μg/ml) for 24 hours (a) and for 48 hours (b), were analysed by Tunel assay. The number of apoptotic cells was registered and reported in histograms.

Then, we evaluated the expression of the anti-apoptotic protein Bcl-2 and of the pro-apoptotic protein Bak in order to get some hint at the molecular mechanisms of RCC-ECFCs' apoptosis resistance. Surprisingly, both Bak and Bcl-2 were normally expressed in the two cell types (Figure 13)

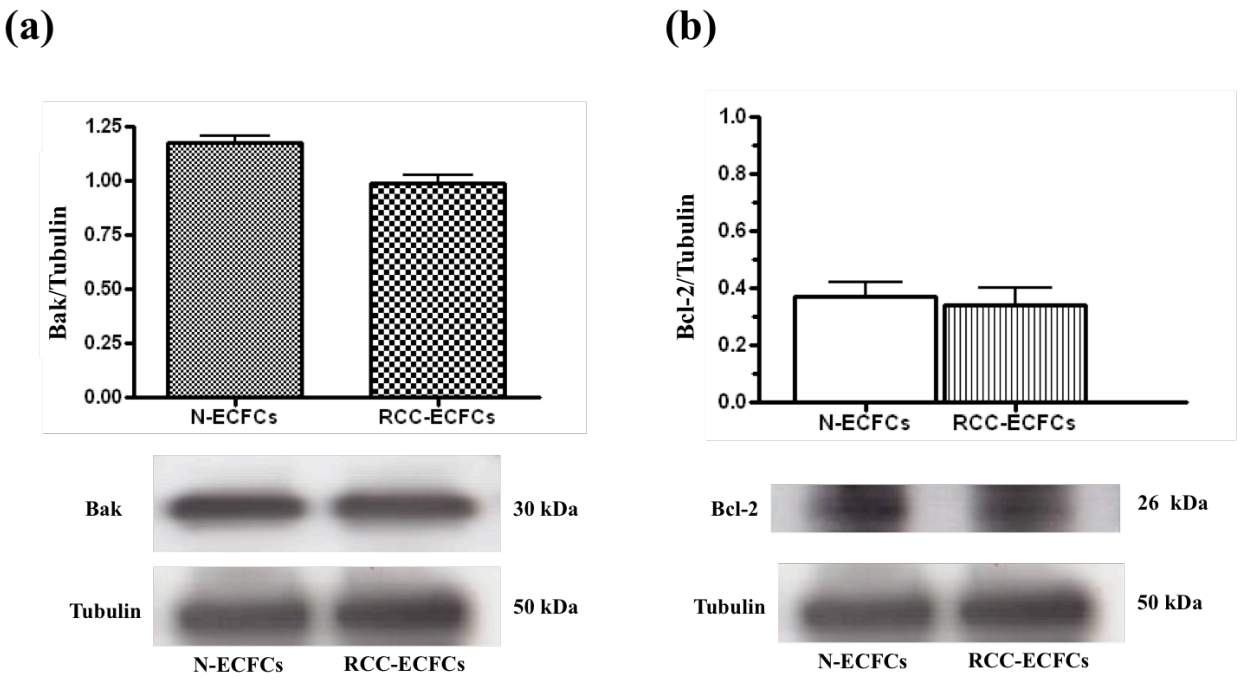


Figure 13. Expression of Bak (a) and Bcl-2 (b) proteins in ECFCs isolated from healthy subjects and from RCC patients. Densitometry in the upper panels and western blots in lower panels representative of three separate experiments were shown. Major bands of the expected molecular weights for BAK and Bcl-2 were observed. Each bar in the upper panel represents the mean±SD of the densitometric analysis of three different experiments. Proteins bands derive from the same experiment, thus tubulin bands are common to the two proteins studied.

Overall, these data demonstrate that RCC-ECFCs were more resistant to apoptosis as compared to their healthy counterparts. Nevertheless, this pro-survival feature was not due to an anti-apoptotic oncoprotein dependent mechanism.

VEGF INDUCES THE NUCLEAR TRANSLOCATION OF NF-kB (p65) IN N-ECFCs IN A Ca^{2+}-DEPENDENT MANNER

Finally, we evaluated whether VEGF-induced, NF-kB-mediated protein expression changes between N-ECFCs and RCC-ECFCs. Previously, our laboratory has demonstrated that VEGF causes I_{kB} phosphorylation (Dragoni et al., 2011), but did not assess whether this actually induces the nuclear translocation of NF-kB. We thus exploited immunocytochemistry to determine the localization of RelA/p65, a major subunit of NF-kB.

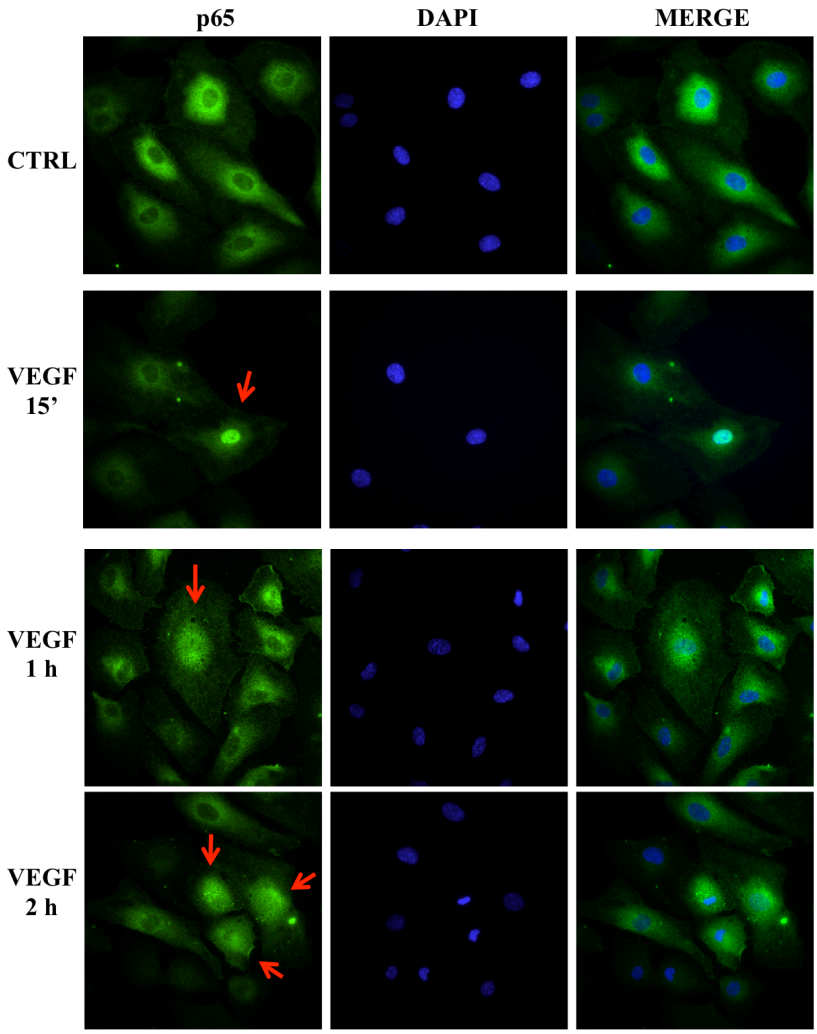


Figure 14. Nuclear NF-kB translocation after VEGF stimulation. Immunocytochemistry assay of p65 in N-ECFCs untreated (CTRL) and treated with VEGF (10 ng/ml) for 15 minutes, 1 and 2 hours. The arrows indicate the presence of fluorescent signal in nucleus of cells treated with VEGF. The first column shows the fluorescent p65 signal, the second column the nuclei coloured by DAPI and the third one the merge.

Under resting condition, p65 protein-dependent fluorescence was predominantly located in the cytoplasm (Figure 14, CTRL). However, VEGF (10 ng/ml) caused the rapid translocation of p65 into the nucleus, which is fully concurrent with the findings on IkB phosphorylation reported by Dragoni et al. (2011). The fluorescent signal associated to p65 protein was evident in the nucleus already at 15 m after VEGF stimulation and persisted as long as 2 hours. In order to assess whether VEGF-induced Ca^{2+} oscillations drive the nuclear translocation of p65, we pre-treated the cells with BAPTA (30 μM), which is a membrane-permeable buffer of intracellular Ca^{2+}, and BTP-2 (20 μM), which is a selective inhibitor of SOCE and dramatically curtails the Ca^{2+} train (Dragoni et al., 2011).

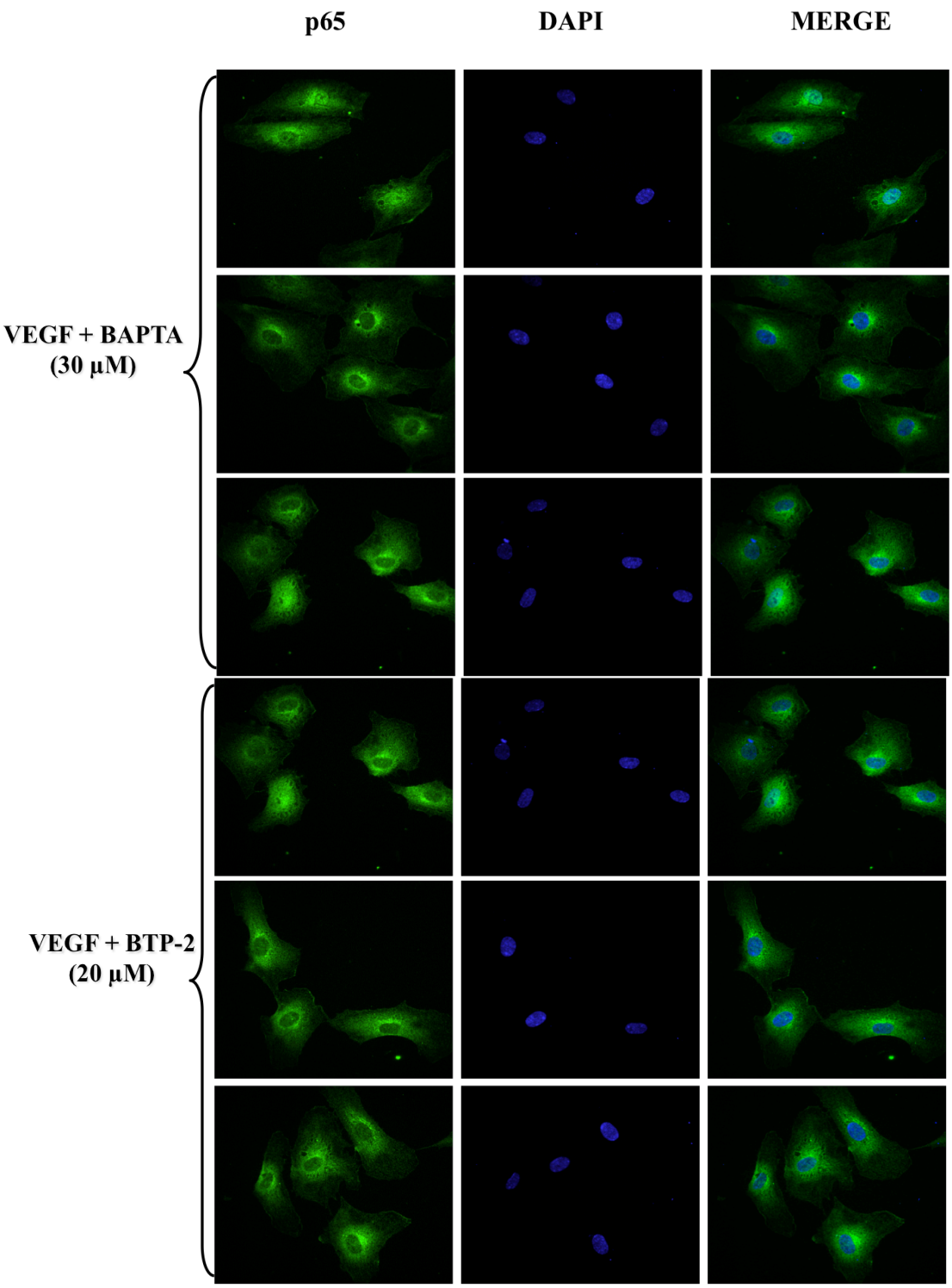


Figure 15. Nuclear NF-kB translocation after VEGF and inhibitors stimulation. Immunocytochemistry assay of p65 in N-ECFCs treated with VEGF+BAPTA (30µM) and VEGF+BTP-2 (20µM) The first column shows the fluorescent p65 signal, the second column the nuclei coloured by DAPI and the third one the merge.

As shown in Figure 15, both BAPTA and BTP-2 fully prevented the nuclear translocation of NF-kB, thereby confirming that NF-kB translocates into the nucleus in N-ECFCs challenged with VEGF (Dragoni et al., 2011).

VEGF INDUCES GENE AND PROTEIN EXPRESSION IN N-ECFCs

The next step was to assess whether VEGF-induced, NF-kB-mediated protein synthesis actually occurs in N-ECFCs. We evaluated the expression of a number of VEGF-dependent genes involved in key cellular processes, such as proliferation, apoptosis, adhesion, and survival, by qRT-PCR. The genes analysed were: B-cell lymphoma 2 gene (BCL2), involved in apoptosis; VCAM1, ICAM and E-SELE involved in cell adhesion; MMP9 involved in the breakdown of extracellular matrix; cyclin D1 (CCND1) and C-MYC involved in cell cycle regulation. N-ECFCs were treated with VEGF (10 ng/ml), and cDNA subsequently amplified with primers specific for the selected genes (see Materials and methods). As shown in Figure 16, VEGF induced a significant increase in E-SELE and VCAM1 expression after 2 and 4 hours of incubation; however, after 6 hours, the expression dramatically decreased at unstimulated levels. The genes BCL2, ICAM1 and CCND1 showed the maximum value of fold change after 2 hours of VEGF stimulation. After this timing, gene expression levels returned to basal values or lower. Moreover, VEGF seemed not to promote MMP9 gene expression although a trend toward an increase was observed with a maximum at 4 hours of VEGF stimulation. Finally, C-MYC expression was not influenced in VEGF-stimulated N-ECFCs. Three genes (E-SELE, VCAM1 and MMP9), which showed the highest increase in their transcript levels, were then selected to confirm their expression at protein level through western blot analysis. We showed that VEGF (10 ng/ml) significantly ($p<0.05$) enhanced the expression of E-SELE, VCAM1 and MMP9 in N-ECFCs at 4 hours after the beginning of the stimulation (Figure 17).

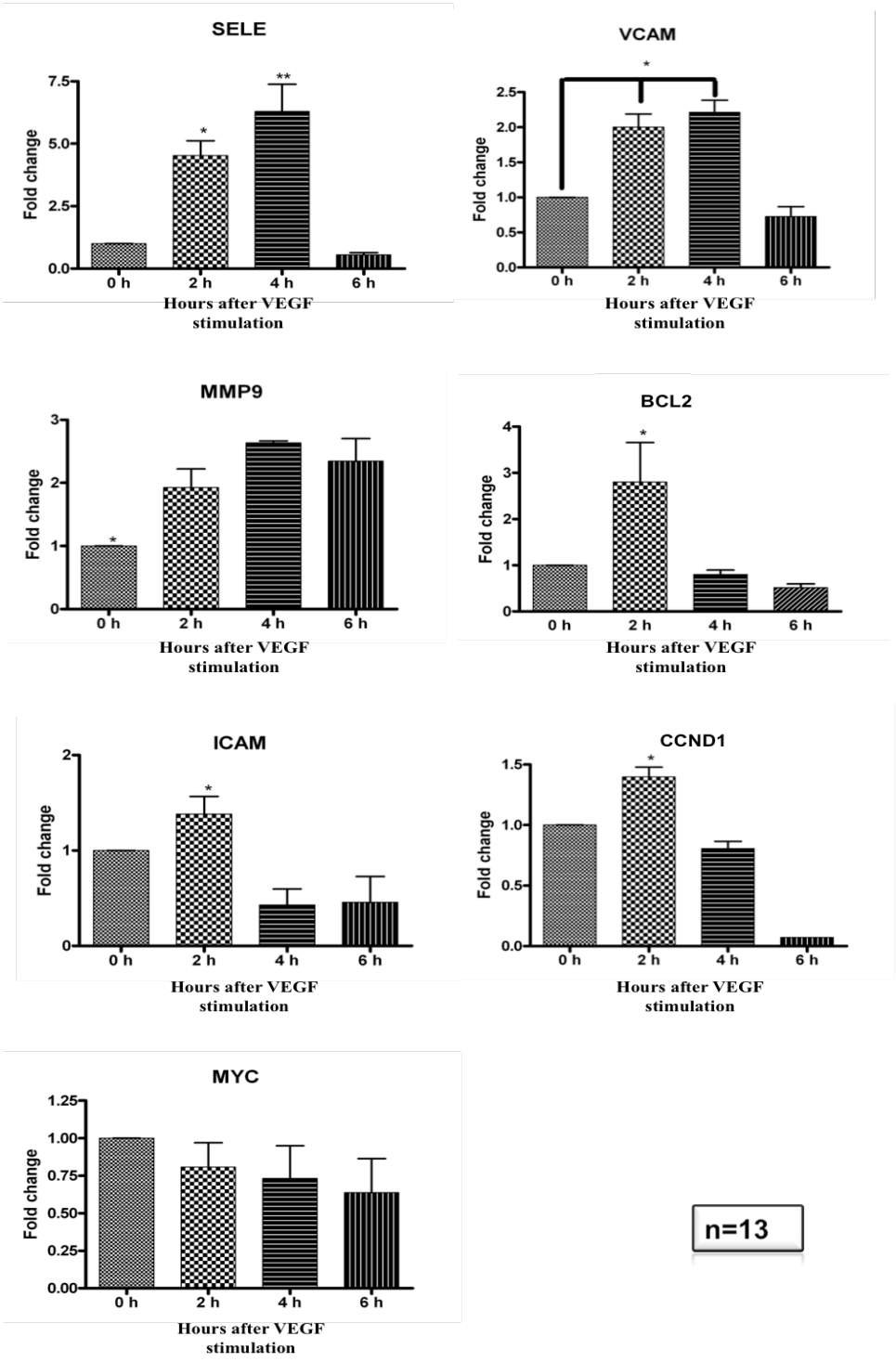


Figure 16. Expression of selected genes transcripts in N-ECFCs after VEGF cell treatment. Gene expression experiments using relative quantification (relative to controls which assume values of 1) in N-ECFCs stimulated with VEGF (10 ng/ml) for 2, 4 and 6 hours (x axis). 0 hours (0 h) corresponds to untreated controls. On y axis are represented the average values and standard deviation of fold change values. In these sets of experiments ECFCs from 13 healthy subjects have been analysed.

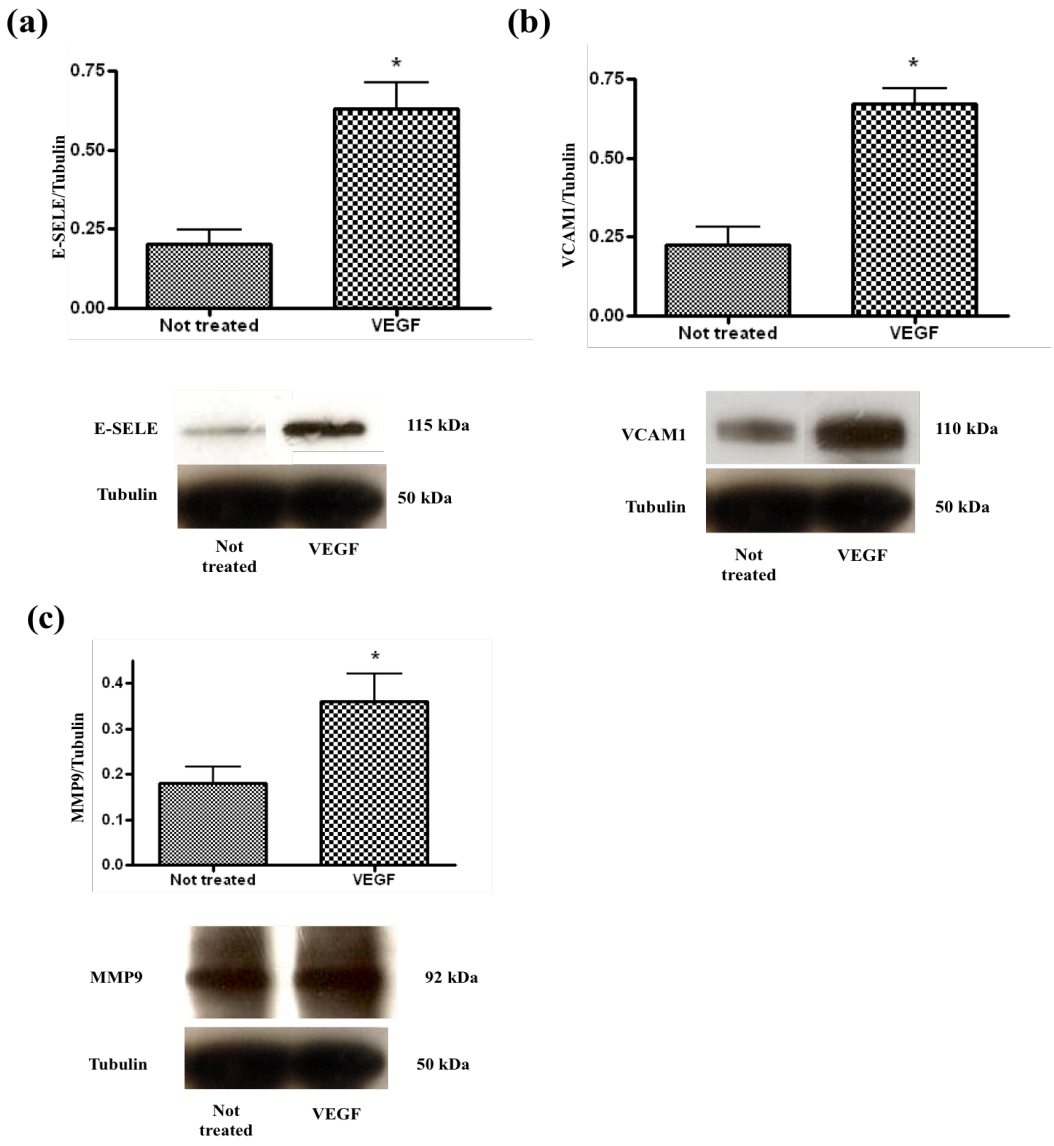
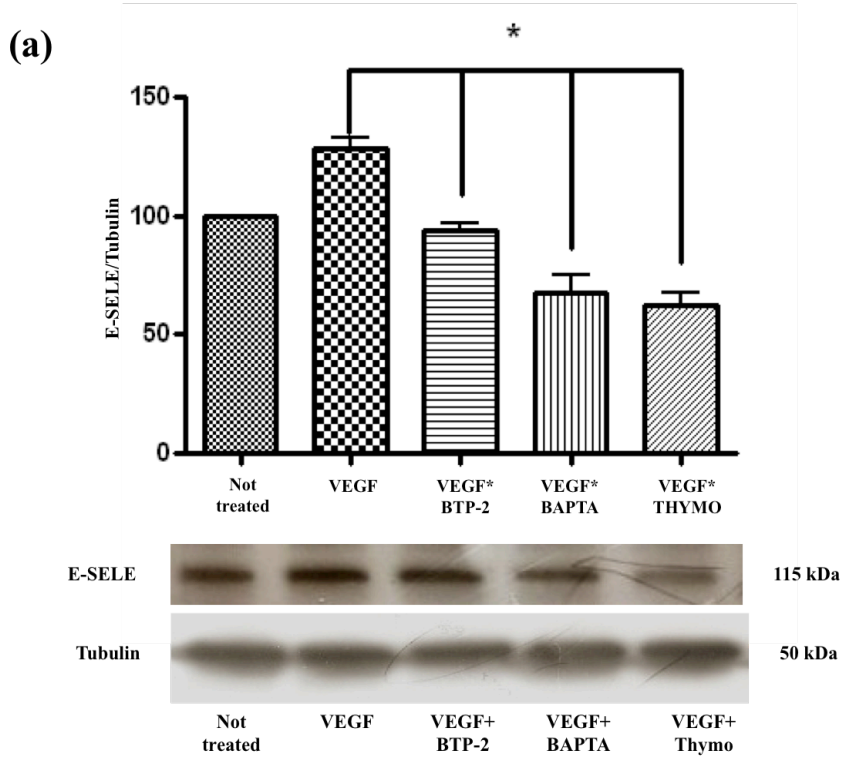
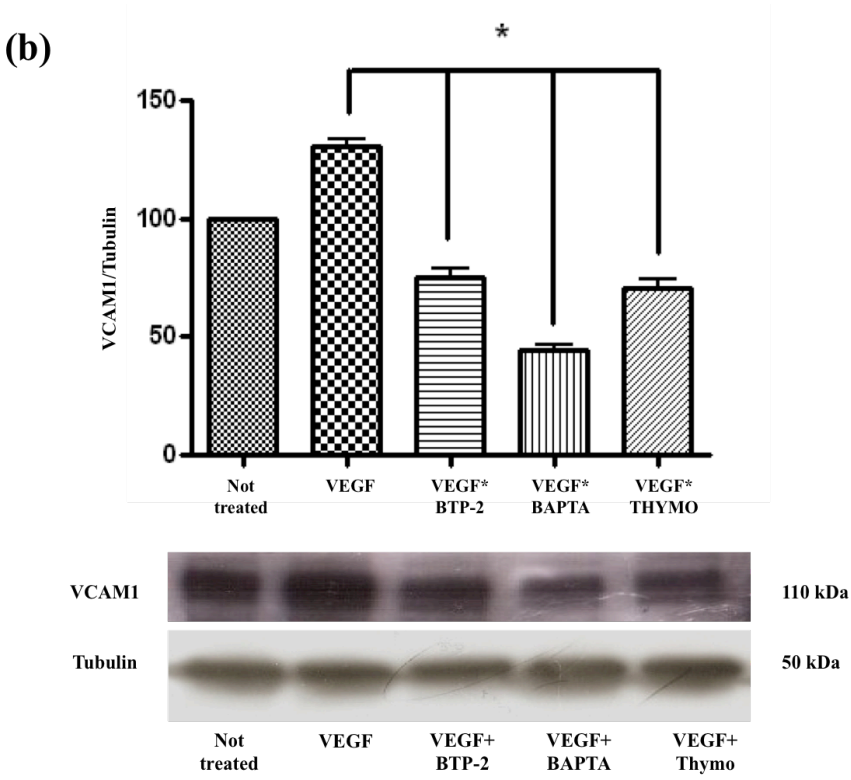


Figure 17. Expression of E-SELE, VCAM1 and MMP9 proteins in N-ECFCs after VEGF treatment. E-SELE (a), VCAM1 (b) and MMP9 (c) protein expression was analyzed in N-ECFCs under control conditions (not treated) and after treatment with VEGF (10 ng/ml). Densitometry in the upper panels and western blots in lower panels representative of four separate experiments were shown. Major bands of the expected molecular weights for E-SELE, VCAM1 and MMP9 were observed. Each bar in the upper panel represents the mean±SD of the densitometric analysis of four different experiments. The asterisk indicates $p<0.05$ (Student's t-test). Protein bands derive from the same experiment, thus tubulin bands are common to the three proteins studied.

In agreement with our previous data, VEGF-induced protein expression was dramatically reduced by BTP-2 (20 μM), BAPTA (30 μM), and thymoquine (25 μM), a selective NF-kB blocker (Figure 18).


(a)
*
150
100
50
0
E-SELE/Tubulin
Not treated
VEGF
VEGF* BTP-2
VEGF* BAPTA
VEGF* THYMO
E-SELE
115 kDa
Tubulin
50 kDa
Not treated
VEGF
VEGF+ BTP-2
VEGF+ BAPTA
VEGF+ Thymo


(b)
*
150
100
50
0
VCAM1/Tubulin
Not treated
VEGF
VEGF* BTP-2
VEGF* BAPTA
VEGF* THYMO
VCAM1
110 kDa
Tubulin
50 kDa
Not treated
VEGF
VEGF+ BTP-2
VEGF+ BAPTA
VEGF+ Thymo

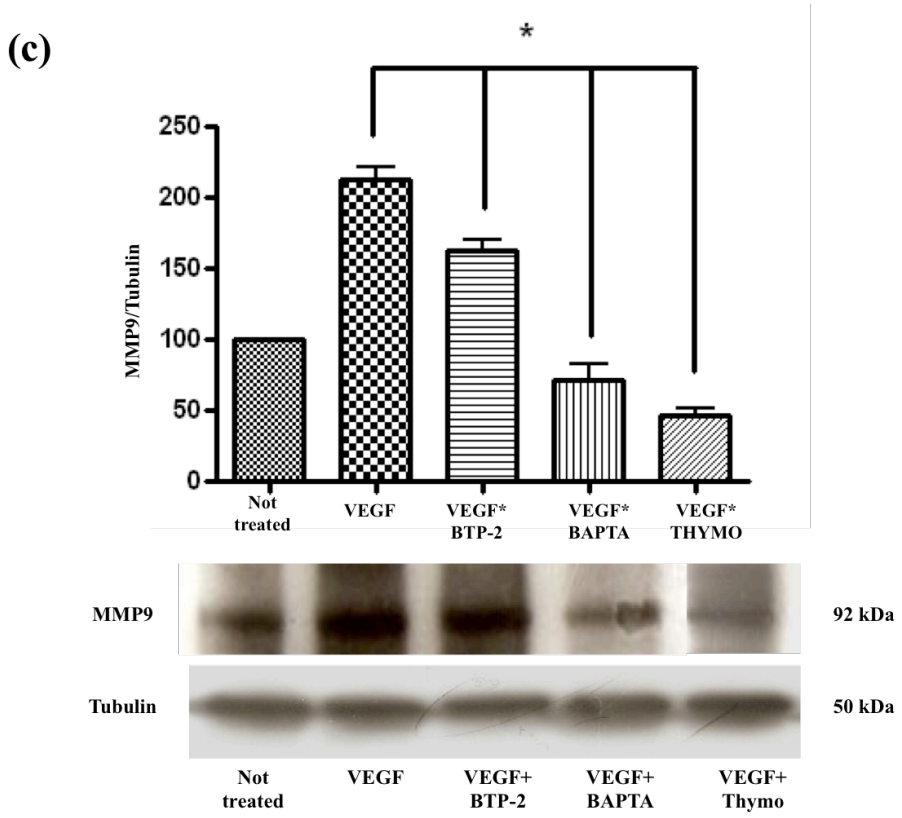


Figure 18. Expression of E-SELE (a), VCAM1 (b) and MMP9 (c) proteins in ECFCs isolated from healthy subjects. Protein expression was analyzed under control conditions (not treated), after treatment with VEGF (10 ng/ml), and after stimulation with specific inhibitors: BTP-2 (20 μM), BAPTA (30 μM) and Thymoquinone (25 μM). Densitometry (upper panels) and western blots (lower panels) representative of four separate experiments were shown; data have been normalized to percentage of expression inhibition. Major bands of the expected molecular weights for E-SELE, VCAM1 and MMP9 were observed. Each bar in the upper panel represents the mean±SD of the densitometric analysis of four different experiments. The asterisk indicates $p<0.05$ (Student's t-test). Proteins bands derive from the same experiment, thus tubulin bands are common to the three proteins studied.

Collectively, these findings demonstrate that NF-kB mediates the VEGF-induced and Ca^{2+}-dependent expression of VCAM1, MMP9 and E-SELE in N-ECFCs

VEGFR-2 IS ACTIVATED BUT VEGF-INDUCED PROTEIN EXPRESSION IS NOT ENHANCED IN RCC-ECFCs.

Once confirmed that VEGF induces NF-kB-dependent protein expression in N-ECFCs, we evaluated VEGF effect on RCC-derived cells. We first ascertained whether VEGF induces protein

expression in RCC-ECFCs as previously demonstrated for N-ECFCs. Therefore, we treated the cells with VEGF (10 ng/ml) and analysed by western blot VCAM1, E-SELE and MMP9 protein expression. Our results clearly show that, unlike for N-ECFCs, VEGF did not induce any protein expression in ECFCs from patients with RCC (Figure 19) despite the fact that VEGFR-2 is normally expressed in these cells.

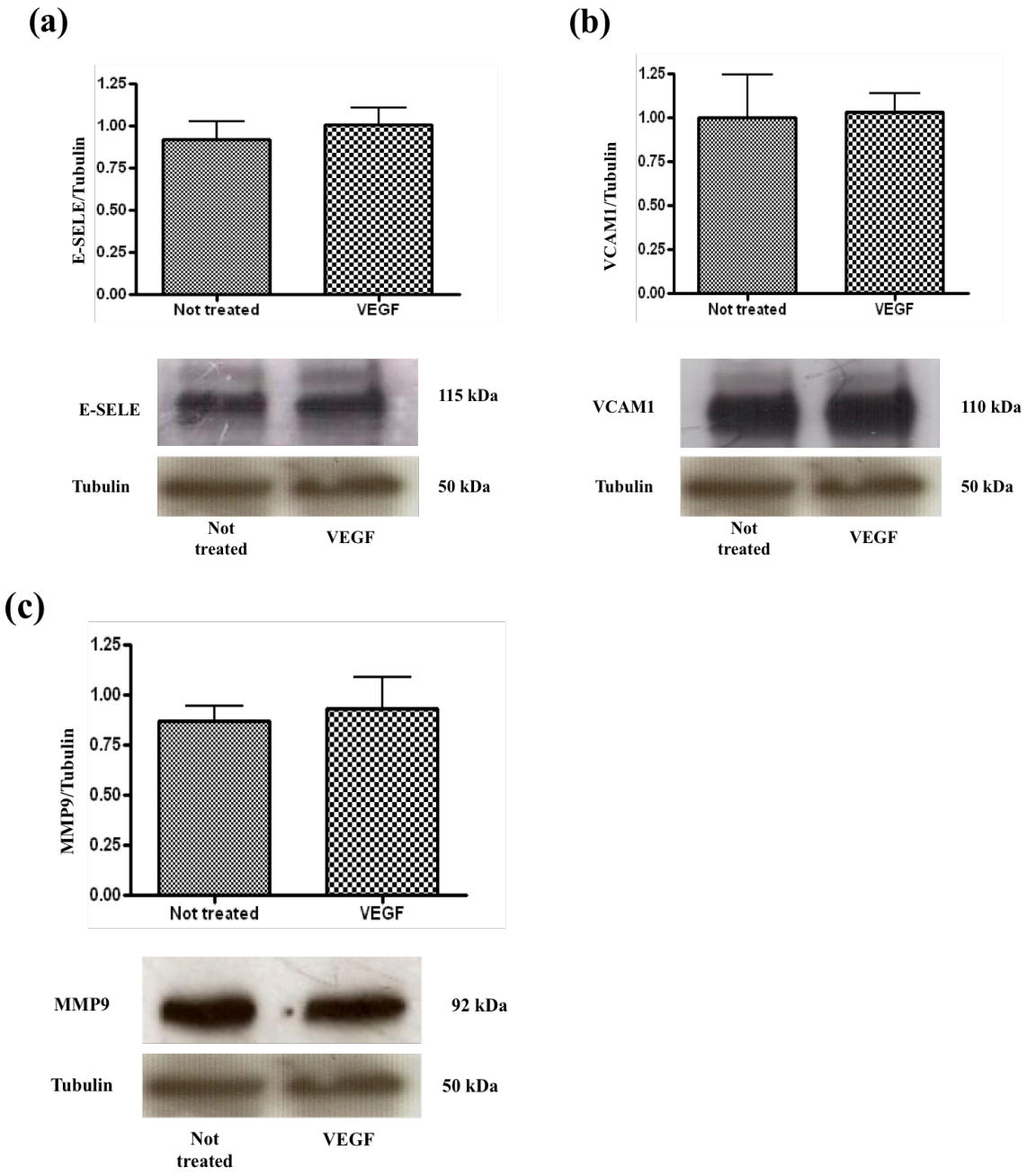


Figure 19. Expression of E-SELE, VCAM1 and MMP9 proteins in RCC-ECFCs after VEGF treatment. E-SELE (a), VCAM1 (b) and MMP9 (c) protein expression was analyzed in RCC-ECFCs under control conditions (not treated) and after treatment with VEGF (10 ng/ml). Densitometry (upper panels) and western blots (lower panels) representative of four separate experiments were shown. Major bands of the expected molecular weights for E-SELE, VCAM1 and MMP9 were observed. Each bar in the upper panel represents the mean±SD of the densitometric analysis of four different experiments. Proteins bands derive from the same experiment, thus tubulin bands are common to the three proteins studied.

The present results and those presented by Lodola et al. (2012) demonstrate that RCC-ECFCs fail to respond to VEGF. Therefore, we wondered whether VEGFR-2 is functional in these cells. The

auto-phosphorylation and consequent activation of VEGFR-2 (VEGFR-2P) were studied by western blot analysis in N- and RCC-ECFCs challenged with VEGF (10 ng/ml). As shown in Figure 20, there was no significant ($p<0.05$) difference in the extent of VEGFR-2 autophosphorylation between N-ECFCs and RCC-ECFCs. These data suggest that the lack of a Ca^{2+}-dependent pro-angiogenic response to VEGF is due to a defect in the Ca^{2+}-signalling machinery downstream of VEGFR-2.

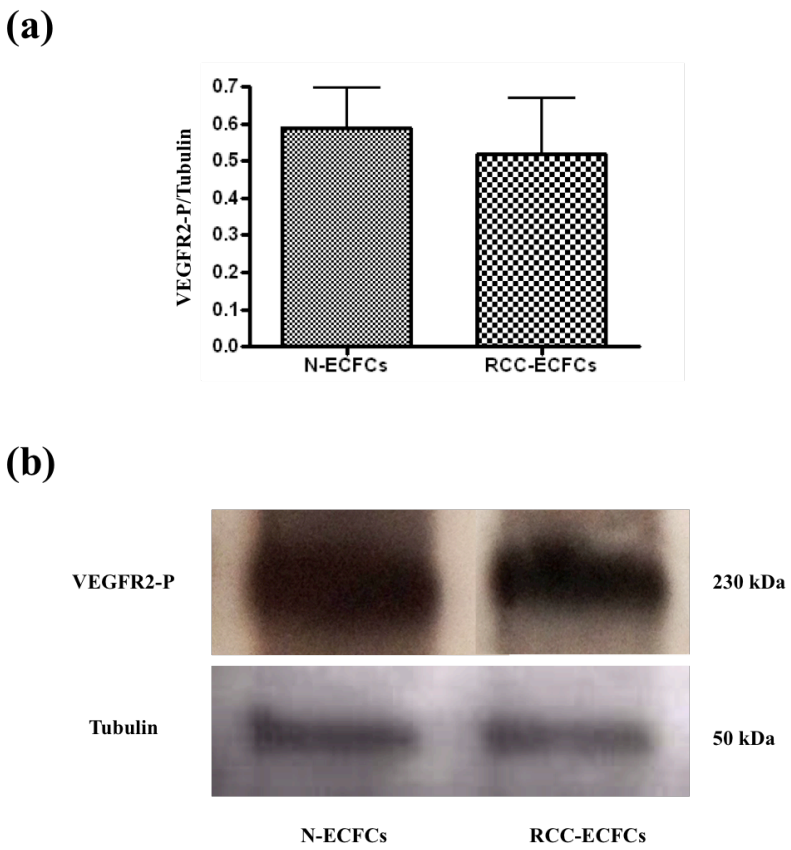


Figure 20. Expression of VEGFR2-P in ECFCs isolated from N-ECFCs and from RCC-ECFCs. Densitometry (a) and western blots (b) representative of three separate experiments were shown. A major band of the expected molecular weights for VEGFR2-P proteins was observed. Each bar in the upper panel represents the mean±SD of the densitometric analysis of three different experiments.

STEADY STATE ER Ca^{2+} LEVELS AND MITOCHONDRIAL Ca^{2+} UPTAKE ARE REDUCED IN RCC-ECFCs

Our phenotypical and functional characterization has revealed that N-ECFCs and RCC-ECFCs differ in two main aspects: RCC-ECFCs are more resistant to apoptosis and are insensitive to VEGF despite the fact that VEGFR-2 is normally expressed and activated. These features hint at the drop in ER Ca^{2+} levels as the major responsible for these very important functional differences between the two cell types. Accordingly, a decrease in ER and, consequently, mitochondrial Ca^{2+} levels is a common means by which neoplastic cells become resistant to pro-apoptotic stimuli (Skryma et al., 2000; Vanden Abeele et al., 2002; Vanoverberghe et al., 2004). Moreover, the reduction in intraluminal Ca^{2+} could prevent RCC-ECFCs from generating a detectable increase in $[Ca^{2+}]_i$ in spite of $InsP_3$ production. The data obtained by our laboratory suggested, but not experimentally confirmed that the ER Ca^{2+} load in RCC-ECFCs is lower as compared to N-ECFC (Lodola et al., 2012). Therefore, we sought to directly evaluate the $[Ca^{2+}]_{ER}$ by expressing ER-targeted Aequorine (AEQ) in both types of cells.

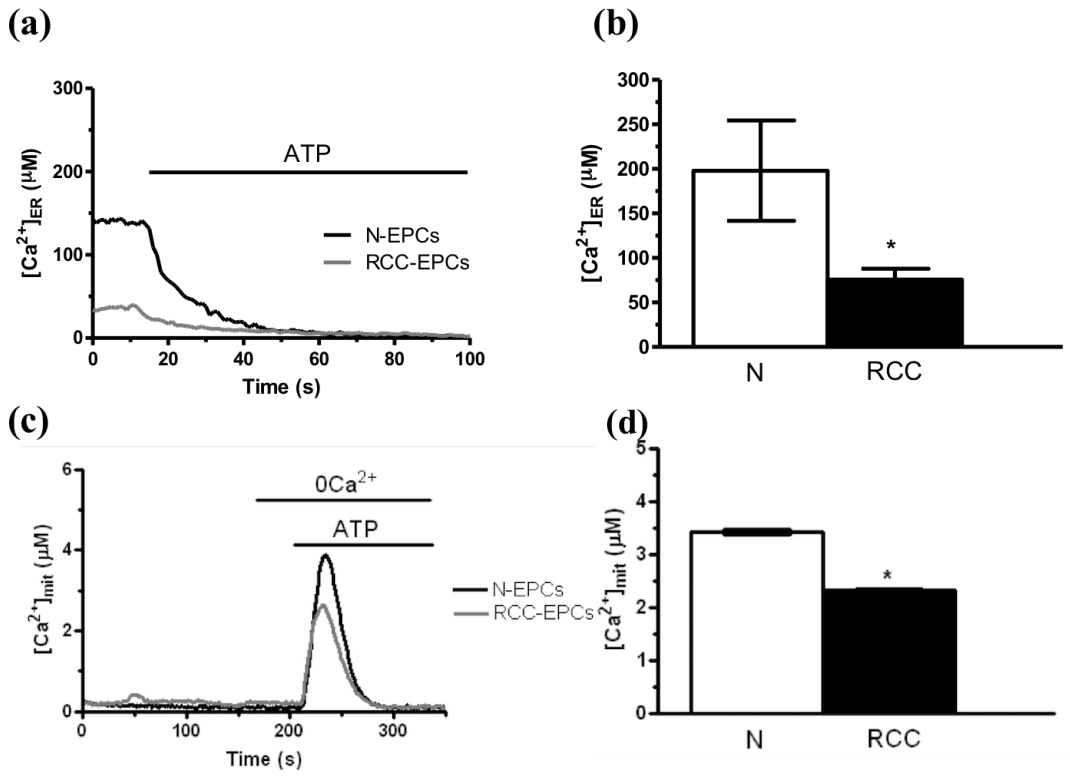


Figure 21. Sub-cellular measurement of Ca^{2+} levels in N-ECFCs and RCC-ECFCs. (a) Steady-state $[Ca^{2+}]_{ER}$ in N-ECFCs is significantly higher as compared to RCC-ECFCs. In order to confirm ER-targeting of the aequorin probe, Ca^{2+} release from ER after ATP (100 μM) treatment was evaluated. (b) Mean±SE of $[Ca^{2+}]_{ER}$ in N-ECFCs and RCC-ECFCs. The asterisk indicates $p<0.05$. (c) ATP-induced, $InsP_3$-dependent increase in $[Ca^{2+}]$ is significantly higher in N-ECFCs as compared to RCC-ECFCs. (d) Mean±SE of $[Ca^{2+}]_{mit}$ in N-ECFCs and RCC-ECFCs. The asterisk indicates $p<0.05$.ATP was administrated at 100 μM. Agonists and extracellular solutions containing different concentrations of Ca^{2+} were administered at the time indicated by the horizontal bars.

We found that $[Ca^{2+}]_{ER}$ was significantly ($p<0.05$) lower in RCC-ECFCs as compared to their normal counterparts (Figure 21 (a) and (b)), while the $InsP_3$-producing autacoid ATP (100 μM), fully depleted intraluminal Ca^{2+} in both cell types (Figure 21(a)). Likewise, the amount of ER Ca^{2+} mobilized by ATP was significantly ($p<0.05$) higher in N-ECFCs as related to tumor ECFCs (Figure 21(a) and (b)). Next, we exploited mitAEQ to evaluate the ATP-induced, $InsP_3$-dependent elevation in mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mit}$) in both N- and RCC-ECFCs (Lodola et al., 2012). $InsP_3$-evoked Ca^{2+} signals were constitutively transferred to the mitochondrial matrix by the ER-mitochondrial shuttle and a reduction in the amount of Ca^{2+} redirected by $InsP_3R$ towards the mitochondria is an established mechanism for apoptosis resistance in cancer cells (Prevarskaya et al., 2014; Prevarskaya et al., 2013; Prevarskaya et al., 2004). In agreement with the reduction in their $[Ca^{2+}]_{ER}$, RCC-ECFCs displayed a lower InsP3-dependent increase in $[Ca^{2+}]_{mit}$ as compared to N-ECFCs (Figure 21 (c) and 21 (d)). Taken together, these data confirmed that the $[Ca^{2+}]_{ER}$ was

remarkably lower in RCC-ECFCs and such dysregulation reflects in a decrease in the amplitude of mitochondrial $InsP_3$-induced Ca^{2+} elevations.

THE ENDOTHELIAL Ca^{2+} TRANSPORTING SYSTEMS AND ER Ca^{2+} BINDING PROTEINS ARE NOT ABERRANTLY EXPRESSED IN RCC-ECFCs

Our previous results strongly suggested that the chronic depletion of ER Ca^{2+} content in RCC-ECFCs was determined by their slower SERCA activity (Lodola et al., 2012). In order to get deeper insights into the mechanisms responsible for the drop in $[Ca^{2+}]_{ER}$ in RCC-ECFCs, we evaluated the mRNA expression of the most widespread endothelial transporter and pump isoforms, i.e. SERCA2B, SERCA3, PMCA1A, PMCA1B, PMCA4B, NCX1.3 and NCX1.7 . We found that both N- and RCC-ECFCs only expressed SERCA2B, PMCA1B and PMCA4B (Figure 22(a)). Furthermore, the comparison of mRNA levels obtained by qRT-PCR did not assess any quantitative difference in the transcript levels of these Ca^{2+}-transporting systems between N- and RCC-ECFCs (Figure 22(b)).

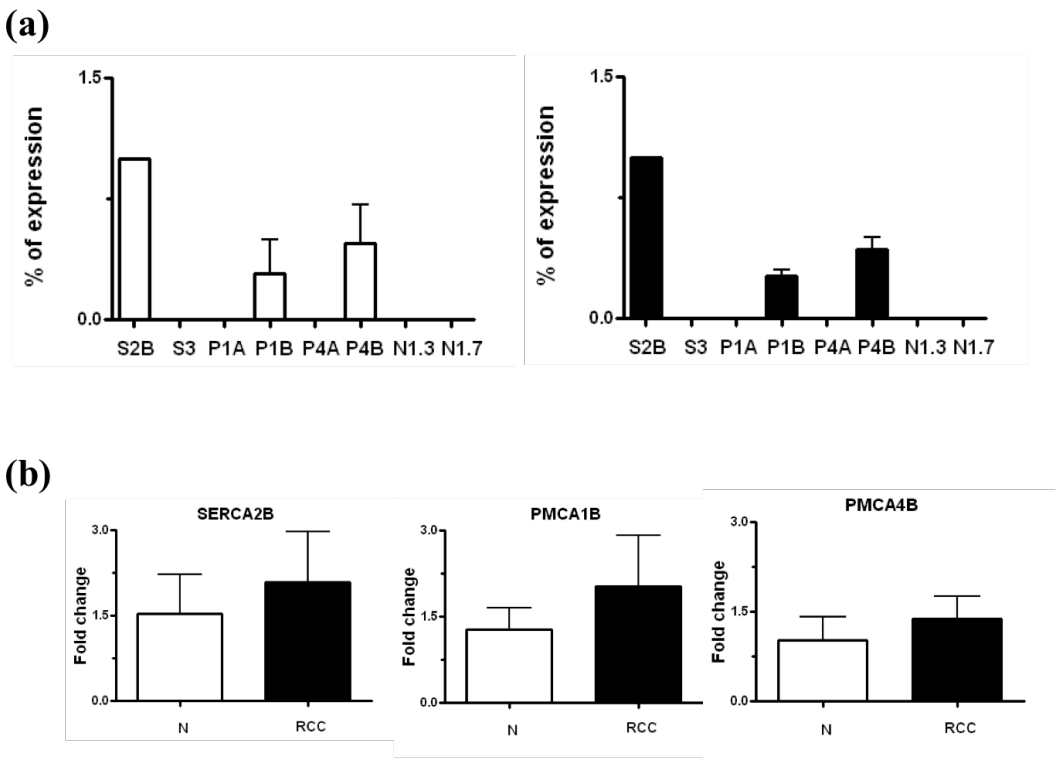


Figure 22. Gene expression analysis of the most widespread Ca^{2+}-clearing mechanisms in N-ECFCs and RCC-ECFCs. (a) Both N-ECFCs (left panel, white bars) and RCC-ECFCs (right panel, black bars) only express SERCA2B, PMCA1B and PMCA4B transcripts. On x axis are reported the genes studied and on y axis the percentage of their expression relative to an housekeeping gene (GAPDH). Each bar represents the mean±SE of three different experiments each from different RNA extracts. (b) Relative fold change in gene expression of the three genes expressed in (a). On y axis are represented the average values and standard errors of fold change values. In these sets of experiments, ECFCs from 3 healthy subjects and 3 patients have been analysed. (S2B=SERCA2B; S3=SERCA3; P1A=PMCA1a; P1B=PMCA1b; P4A=PMCA4a; P4B=PMCA4b; N1.3=NCX1.3; N1.7=NCX1.7)

The next step was to analyse the expression of the two most important ER Ca^{2+}-binding proteins, namely calreticulin and calnexin, in N- and RCC-ECFCs. Nevertheless, both proteins were normally expressed in N- and RCC-ECFCs (Figure 23(a) and (b)). Consequently, the decrease in $[Ca2+]_{ER}$ did not involve either the remodelling of the ER Ca^{2+}-handling proteins (i.e. SERCA), as well as those of the plasma membrane (i.e. PMCA and SERCA), or the down-regulation of the luminal Ca^{2+} binding/storage chaperones, calreticulin and calnexin.

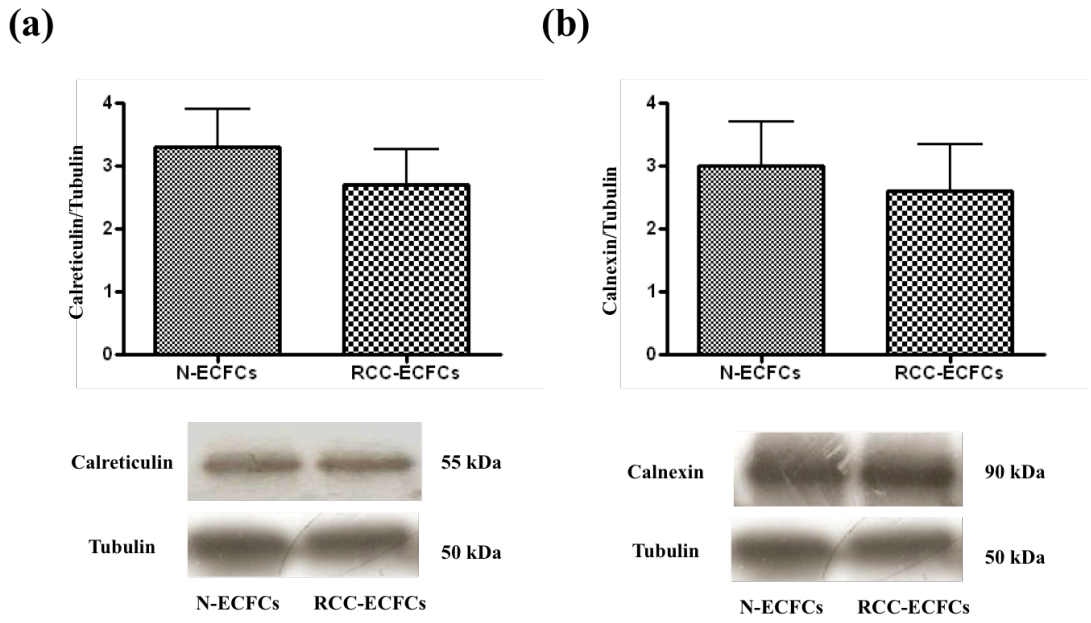


Figure 23. Expression of Calreticulin and Calnexin proteins in N- and RCC-ECFCs. Densitometry (upper panels) and western blots (lower panels) representative of three separate experiments were shown. Major bands of the expected molecular weights for Calreticulin and Calnexin proteins were observed. Each bar in the upper panel represents the mean±SD of the densitometric analysis of three different experiments. Proteins bands derive from the same experiment, thus tubulin bands are common to the two proteins studied.

ULTRASTRUCTURAL REMODELLING OF RCC-ECFCs

The reduction in Ca^{2+}-storage capacity could also involve a long-term rearrangement of ER structure (Sammels et al., 2010), which might collapse and loose its ability to sequester sufficient amount of Ca^{2+}. A thorough ultrastructural analysis of both N- and RCC-ECFCs was, therefore, carried out at electron microscopy level. This investigation disclosed major morphological differences between the two cell types. Cisternae of rough endoplasmic reticulum (rER) are evident in both N- and RCC-ECFCs (Figure 24; Table 6): however, they are closely arranged in the former, while are more widely spaced and occupy a wider area in the latter. Multivesicular bodies can frequently be found between these rER cisternae in tumor-derived ECFCs (Figure 24; Table 6). Likewise, smooth ER (sER) can be found as constituted by vesicles and tubules, although its presence is rather scarce in N-ECFCs (Figure 24; Table 6). Conversely, sER is present in the form of large vesicles occupying a large part of the cytoplasm in RCC-ECFCs. This peculiar shape is most likely due to the fact that he tubules normally present are seemingly enlarged into vesicles. Finally, we evaluated mitochondrial numbers and morphology in both cell types. In control cells, mitochondria are mainly present as short, roundish organelles, showing wide-spaced cristae, while they are more abundant and very frequently elongated and branched in tumor-derived cells,

possibly due, as in other cases, to a fusion or incomplete separation (Figure 24; Table 6). Overall, the ultrastructural remodeling observed in RCC-ECFCs, i.e. ER expansion and increase in number and size of mitochondria, are strikingly different from our expectations, but do confirm that normal and tumor-derived ECFCs profoundly differ from each other.

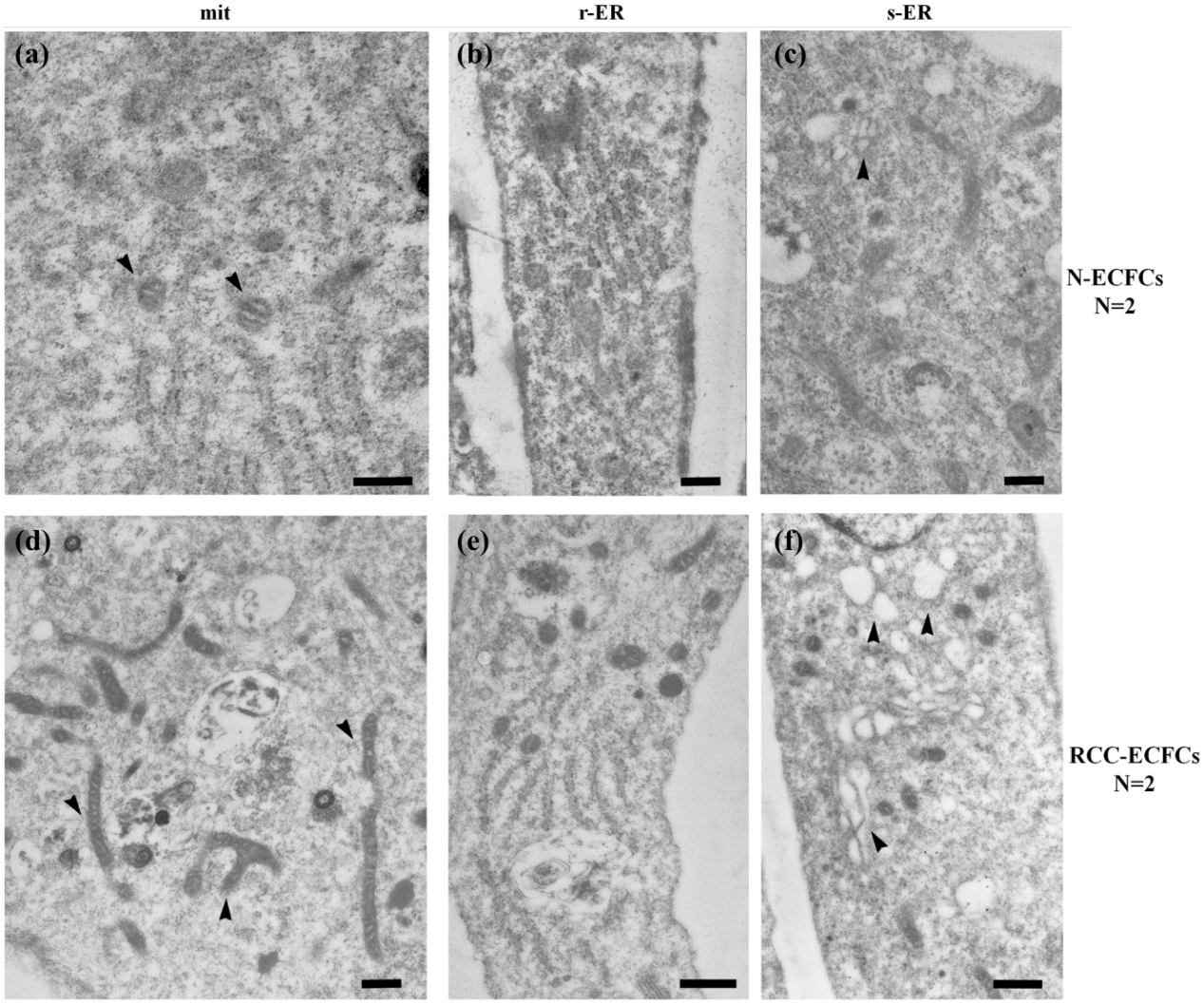


Figure 24. Ultrastructural analysis of ECFCs morphology. Representative images of two RCC-ECFCs and two N-ECFCs samples analyzed. The bars in a-f correspond to 500 nm. (a)-(c) N-ECFCs: Two round mitochondria (arrowheads) display widely separated cristae (a); r-ER cisternae, closely arranged, are evident in the cytoplasm (b); Smooth endoplasmic reticulum is scanty, and present in the forma of small vesicles and tubules (c). (d)-(f) RCC-ECFCs: mitochondria arrowheads are larger, branched and striated by numerous cristae (d); r-ER occupies a wider area, and the cisternae appear to be widely spaced. Note the presence of multivesicular bodies (e); the s-ER can be seen as composed by numerous large vesicles, present on a wider area than in control cells (f)

Cell Type	Structure	Total analysed ER area (μm^2)	Number of mitochondria per cell	Total analysed mitochondria area (μm^2)
N-ECFCs	s-ER	0.937±0.245	4.2±0.4	0.722±0.112
RCC-ECFCs	s-ER	2.564±0.369*	8.4±2.0*	0.993±0.312
N-ECFCs	r-ER	0.277±0.241		
RCC-ECFCs	r-ER	1.241±0.253*		

Table 6. Measurements of ultrastructural analysis of ECFCs morphology. Averages±SE; values shown were obtained from 17 images of N-ECFCs and from 19 images of RCC-ECFCs obtained from two distinct donors and examined at XXXX magnification. The asterisk indicates $p<0.05$.

DISCUSSION

Endothelial progenitor cells can be isolated from MNCs of BM, UCB, PB and from large arteries walls (Yoder, 2012). They normally reside in BM niches from where they can be mobilized and circulate in PB to replace damaged/senescent endothelial cells or to restore blood perfusion in ischemic tissues (Massa et al., 2009); circulating EPCs can be recruited to their final destination through an interplay between chemokine and adhesion molecules with mechanisms not yet defined clearly (De Falco et al., 2004). Lyden, in 2001, provided the first evidence of EPCs involvement in tumor vasculature, followed by Gao et al who showed their involvement in the metastatic switch. They demonstrated that EPCs are able to home to micrometastatic foci, thereby promoting the transition to macrometastasis. Despite the strong evidence of EPCs involvement in tumor angiogenesis, many authors denied their role in the angiogenic switch, as they could not provide the evidence of their incorporation into tumor neovessel (De Palma et al., 2003; Göthert et al., 2004). These highly contrasting data could derive from the lack of a final and definite phenotypical characterization of EPCs, but can also be explained by the fact that EPCs could interact in angiogenesis at different stages according to the tumor type (Gao et al., 2009).

Considering the debate on EPCs and their possible involvement in cancer angiogenesis, our group, who has always been focussing on the involvement of EPCs in myeloprolipherative disorders, decided to analyse ECFCs from cancer patients. In collaboration with San Matteo Clinical Oncology Department, we decided to investigate if ECFCs isolated from healthy subjects and from patients affected by RCC could show significant phenotypical or functional differences, although RCC-ECFCs do not belong to malignant clone. Furthermore, we tried to characterize N- and RCC-ECFCs behaviour after stimulation with cytokines such as VEGF, the most important ECFCs clonogenic stimulus.

San Matteo Clinical Oncology Department holds one of the most important national centres for metastatic RCC treatment and could provide us with fresh blood samples of patients with RCC, after proper informed consent has been obtained. RCC is a good model for tumor angiogenesis studies as its strong dependence on angiogenesis has already been shown. The main mutation known to be associated with RCC cancerogenesis is the VHL gene mutation. VHL inactivation leads to the reduction of HIF-1α ubiquitination with the consequent transcription of pro-angiogenic receptor proteins, such as c-Met, and growth factors, such as TGF-a, CXCL-12, PDGF-b and VEGF (Lonser et al., 2003). Among of all these, VEGF plays a crucial role in endothelium homeostasis as well as in tumor neoangiogenesis. RCC therapy for advanced forms (local ones, if early treated, can be totally cured in most cases) has been almost fruitless up to the first years of this century, but it is

not surprising that the first compounds which could influence the natural history of the disease have been principally directed, being either mAbs or TKI, against VEGF or its receptor. Despite a dramatic increase of PFS in patients treated with anti-angiogenic drugs, the OS is not improved much, and long lasting responses are just rare cases. Another striking problem is represented by patients who intrinsically, primary, do not respond to this class of molecules. A better understanding of RCC pathogenesis could lead to the evidence of alternative targetable pathways which could also be directed to the microenvironment rather than just to the cancerous cell itself.

Tumor microenvironment has gathered attention as a possible regulator of carcinogenesis since the beginning of the last decade, as many scientific evidences suggested that the tumor cell does not only interact with the surrounding endothelial cells, but can also be influenced by other surrounding normal stromal and immune cells. Tumor microenvironment has thus been studied as a possible primary actor in cancerogenesis, rather than a secondary player just influenced by tumor development. Many cell populations have been suggested playing roles during tumor growth, including TAM, CAF, myofibroblasts, adypocites and, of course, endothelial cells. We cannot rule out that the trigger mechanism promoting the transformation of a normal epithelium into a malignant phenotype does not primarily lie in the epithelium itself, but rather derives from the surrounding, disturbed, environment (Albini and Sporn, 2007). The microenvironment is thus a critical subject to investigate in the aim of better understanding cancer growth and progression; the aforementioned evidences hint at EPCs as key contributors of tumor, and in particular of RCC, microenvironment. For these reasons, it is clear the growing interest about EPCs both from a biological viewpoint, as contributors to neoangiogenesis, and as potential therapeutic target to impair tumor growth.

The phenotypical and eventually functional identification of EPCs is fundamental: phenotypical characterization alone can already suggest some possible interactions between cell microenvironment and tumor growth, as for the case of M2 type TAM favouring cancer progression as opposed to M1 type TAM, protecting from it (Albini and Sporn, 2007). As far as we know, EPCs contribute to tumor neoangiogenesis, despite being not cancerous cells: therefore we tried to compare the behaviour of ECFCs isolated from healthy donors with ECFCs isolated from RCC patients based on the earlier evidence that their pro-angiogenic Ca^{2+} toolkit is dysregulated (Lodola et al., 2012). To date, most studies addressed the chromosomic, genetic and functional differences arising between normal endothelial cells and TECs (Aird, 2012; Hida et al., 2013; Dudley, 2012). Unfortunately, TECs are only barely compared with normal matched endothelial cells from the same tissue. Moreover, when this is possible, control endothelial cells are obtained from peri-tumoral samples that could be influenced by tumor microenvironment and/or carry some of the genetic aberrations as the neoplasm (Aird, 2012; Dudley, 2012). Conversely, we used as control the

same cell population investigated in tumor patients, i.e. circulating ECFCs, but derived from healthy donors. We initially focussed on the number of circulating EPCs in healthy subjects compared to RCC patients. From our observations, the first difference between N-ECFCs and RCC-ECFCs was their frequency in PB, calculated as number of colonies for 10^7 MNCs plated. RCC-ECFCs were significantly more frequent than N-ECFCs (p-value = 0.027). Besides differences in PB frequency of ECFCs, RCC-ECFCs and N-ECFCs show the same immunophenotypic profile, the one described by Ingram et al. in 2004, the same potential growth, studied by growth curves, and the same ability to develop a tridimensional capillary-like structure in Matrigel assay. This data could support anyway the hypothesis that phenotypically normal ECFCs could cooperate in tumor growth; their enhanced mobilization can be explained by the possibility that the tumor microenvironment secretes growth factors or cytokines specific for ECFCs recruitment from BM. A higher frequency can thus be explained either by a higher mobilization, or by an increase in BM production of these progenitors. This evidence is anyway difficult to obtain as we lack of both RCC patient and healthy donors BM samples. The data obtained are in line with other clinical studies in which EPCs were evaluated under their possible role as cancer biomarker. Yang et al. and Bhatt et al. compared EPC frequency between RCC patients and healthy donors showing higher statistically significant frequencies of EPCs in RCC patients; these data must be considered and compared carefully as the debate about the phenotypical EPCs characterization is still largely open and we find many different representatives of EPCs in PB, which might lead to slightly different conclusions (Yang et al., 2012; Bhatt et al., 2011).

Tumor-derived endothelial cells are less sensitive to pro-apoptotic stimulation as compared to healthy cells. For instance, both RCC- and breast cancer derived TECs are more resistant than normal endothelial cells to serum-starvation and chemotherapeutics, such as temozolomide, doxorubicin and vincristine (Bussolati et al., 2011). The constitutive activation of the phosphoinositide-3-kinase-Akt (PI3K/Akt) pathway has been linked to the higher TEC resistance to apoptosis (Bussolati et al., 2003; Bussolati et al., 2011). Likewise, if stimulated with rapamycin, molecule that is known to induce apoptosis through the inhibition of the mTOR pathway, RCC-ECFCs are more resistant to apoptosis than N-ECFCs; this was evident both at 24 h and 48 h, in a statistically significant fashion on a TUNEL assay. Stating this evidence, we decided to investigate the expression of some proteins whose role in apoptosis is well established and we evaluated the expression of the anti-apoptotic protein Bcl-2 and of its pro-apoptotic counterpart Bak. Western blot analysis on cell lysates showed that there was no significant difference in the protein expression for both Bcl-2 and Bak between N-ECFCs and RCC-ECFCs, suggesting that the pro-survival phenotype of RCC-ECFCs was not due to an oncoprotein dependent mechanism.

Even if RCC-ECFCs do not belong to a malignant clone, and despite them showing the same phenotype and ability to form tubular capillary structure on Matrigel as their healthy counterpart, they differ in frequency and in resistance to apoptotic stimuli. These evidences suggest a potential role of EPCs in tumor development and support the hypothesis that they could also resist better to pro-apoptotic damages such as the ones induced by some anti-angiogenic therapies used in RCC treatment as well as by some classical chemotherapeutic drugs (to which RCC has always shown intrinsic resistance).

RCC-ECFCs do only differ from N-ECFCs in terms of apoptosis resistance. VEGF stimulates N-ECFCs by evoking intracellular Ca^{2+} oscillations that promote the Ca^{2+}-dependent ubiquitination of IkB. This process should in turn promote the nuclear translocation of the pro-angiogenic transcription factor NF-kB (Dragoni et al., 2011). However, the nuclear translocation of NF-kB in N-ECFCs was not assessed in this study. Moreover, preliminary data from our group revealed that VEGF fails to trigger intracellular Ca^{2+} oscillations in RCC-ECFCs (Lodola et al., 2012). Therefore, we then ascertained whether VEGF promotes NF-kB-dependent protein expression in RCC-ECFCs. We found that the nuclear translocation of NF-kB initiated at already at 15 minutes from the beginning of the stimulation and persisted as long as two hours afterwards. Consistent with the data reported in (Dragoni et al., 2011), the nuclear translocation of NF-kB was inhibited by preventing VEGF-induced Ca^{2+} oscillations with BAPTA and BTP-2. These results were further mimicked by thymoquinone, a selective NF-kB inhibitor. NF-kB is thus the link between Ca^{2+} signalling and gene and protein expression after VEGF stimulation. To further confirm this statement, we focussed on VEGF-elicited gene expression and found that VEGF induces the transcription of E-SELE, VCAM1, MMP9, BCL2, ICAM, CCND1, but not of C-MYC. In agreement with mRNA data, VEGF enhanced the expression of E-SELE, VCAM1 and MMP9 proteins. Moreover, VEGF-induced expression of E-SELE, VCAM1, and MMP9 proteins was significantly reduced by BAPTA, BTP-2 and thymoquinone. These experiments demonstrate that VEGF-induced Ca^{2+} oscillations promote protein expression in N-ECFCs through the nuclear translocation of NF-kB. Although Lodola et al. have already shown that RCC-ECFCs do not produce any Ca^{2+} response to VEGF, we evaluated VEGF-induced protein expression in RCC-ECFCs. We further confirmed that RCC-ECFCs are insensitive to VEGF stimulation as western blot analysis revealed that E-SELE, VCAM1 and MMP9 protein expression was not enhanced after 4 hours of stimulation. As VEGFR-2 expression in expressed on RCC-ECFCs surface (Lodola et al., 2012), we studied VEGFR-2 activation through western blot experiments: VEGFR-2 is present and phosphorylated (VEGFR2-P) in both healthy and RCC-derived ECFCs. We thus demonstrated, that although VEGF does not

induce protein expression, VEGFR-2 is expressed and normally activated by this growth factor in RCC-ECFCs. These data led us to speculate that VEGF fails to stimulate pro-angiogenic Ca^{2+} oscillations in RCC-ECFCs as a consequence of the inhibition/down-regulation of a signalling pathway triggered by VEGFR-2.

The comparison of the immunophenotypical and functional properties displayed by N- and RCC-ECFCs, therefore, highlighted two main differences: tumor-derived ECFCs are more resistant to apoptosis and are insensitive to VEGF. Another data only suggested by Lodola et al. (2012), which in part could explain the differences reported in our study between RCC-ECFCs and N-ECFCs, was the Ca^{2+} concentration in ER and mitochondria. RCC-ECFCs present a dramatic rearrangement of their Ca^{2+} toolkit that seemingly leads to the reduction of ER-dependent Ca^{2+} release due to the concomitant drop in $[Ca^{2+}]_{ER}$ and $InsP_3R$ down-regulation (Lodola et al., 2012). Such re-arrangement of the Ca^{2+} machinery might affect RCC-ECFC behavior in two ways. $InsP_3Rs$ redirect Ca^{2+} from ER to mitochondrial matrix through the ER-mitochondrial shuttle. Mitochondria represent a central integration point for the signals regulating cell destiny (Ivanova et al., 2014; Murgia et al., 2009; Sammels et al., 2010). Cellular Ca^{2+} overload, which may be triggered by various initial stimuli, promotes mitochondrial Ca^{2+} uptake. Excessive Ca^{2+} accumulation within the mitochondria is one of the primary causes for mitochondrial permeability transition, which is at least in part mediated by the opening of permeability transition pore (PTP), a multiprotein complex located at the contact sites between the inner and the outer mitochondrial membranes. PTP opening permits the release of mitochondrial apoptogenic factors into the cytoplasm where they in turn activate death-executing caspase cascade. Mitochondrial permeability in general, and PTP complex in particular, is regulated by the members of Bcl-2 family of proteins, of which those preventing apoptogenic factors release, Bcl-2, Bcl-x_L, and Mcl-1 protect against apoptosis, whereas those promoting it, Bak and Bax, act as apoptosis enhancers (Bonora and Pinton, 2014; Giorgi et al., 2012). As a consequence, a reduction in $[Ca^{2+}]_{ER}$ is a biological trick exploited by cancer cells to become less sensitive to apoptosis (Prevarskaya et al., 2014). Pioneering work ideed unveiled that LNCaP (Lymph Node Carcinoma of the Prostate16) prostate cancer epithelial cells undergo a significant Bcl-2 down-regulation, yet they become resistant to apoptosis by virtue of the drop in steady state $[Ca^{2+}]_{ER}$ (Prevarskaya et al., 2004; Vanden Abeele et al., 2002; Vanoverberghe et al., 2004). Similarly, the chronic reduction in intraluminal Ca^{2+} levels results in apoptosis resistance in small cell lung cancer (H1339) and adenocarcinoma lung cancer (HCC) cell lines (Arbabian et al., 2013; Bergner et al., 2009). Thus, if confirmed, a drop in ER Ca^{2+} levels could explain RCC-ECFC resistance to rapamycin-induced apoptosis. Moreover, it could explain the lack of a pro-angiogenic Ca^{2+} response to VEGF: despite the fact that $InsP_3$ is normally synthesized upon VEGFR-2 phosphorylation, the fall in $[Ca^{2+}]_{ER}$ will prevent the onset of the following Ca^{2+} oscillations and the

activation of their down-stream pro-angiogenic machinery (i.e. the nuclear translocation of NF-KB).

We therefore used ER-AEQ and mit-AEQ to confirm that ER Ca^{2+} levels are significantly lower in RCC-ECFCs and that this dysregulation is translated in a decrease in the amplitude of mitochondrial Ca^{2+} uptake. Aequorin-based measurements are indeed an established procedure to measure Ca^{2+} levels within sub-cellular organelles, such as ER, mitochondria, lysosomes and nucleoplasm. The reasons that could explain why $[Ca^{2+}]$ is significantly lower in ER and mitochondria of RCC-ECFCs in comparison with N-ECFCs are still unclear, but several factors may determine the chronic underfilling of ER Ca^{2+} content in RCC-ECFCs. First, a reduction in SERCA expression would dampen Ca^{2+} sequestration and result in a lower $[Ca^{2+}]_{ER}$. For instance, SERCA2b is down-regulated during the transition of prostate cancer to the aggressive androgen-independent phenotype (Vanden Abeele et al., 2002; Vanoverberghe et al., 2004). Similarly, H1339 and HCC cell lines present lower levels of SERCA2 protein (Bergner et al., 2009), whereas SERCA3 is selectively decreased or lost during colon and breast carcinogenesis (Papp et al., 2012). Second, the up-regulation of the Ca^{2+}-transporting systems on the plasma membrane would limit SERCA-dependent Ca^{2+} uptake by the ER. In this view, PMCA1 and PMCA2 transcripts are up-regulated in several breast cancer cell lines (Lee et al., 2006), while PMCA4 is over-expressed in H7-29 colon cancer cells (Aung et al., 2009). Nevertheless, our thorough transcriptomic analysis revealed that: 1) both N- and RCC-ECFCs only express SERCA2B, PMCA1B and PMCA4B; and 2) the expression of these Ca^{2+} pumps is normal in tumor-derived cells. It is herein worth of noting that we could not detect the most widespread endothelial NCX isoforms, i.e. NCX1.3 and 1.7 (Moccia et al.,2012). This feature is consistent with our preliminary results (Dragoni, Tanzi and Moccia, unpublished data), according to which removal of extracellular Na^{2+} does not lead to any increase in $[Ca^{2+}]_i$ in ECFCs, as otherwise predicted by the recruitment of the reverse-mode of NCX (Berra-Romani et al., 2010; Moccia et al., 2002). Third, a decrease in the expression of ER-Ca^{2+} binding proteins would severely compromise ER capability of storing Ca^{2+}, as shown in prostate and lung cancers. Accordingly, calreticulin is down-regulated and causes a decrease in $[Ca^{2+}]_{ER}$ in both androgen-independent prostate cancer cells (Vanden Abeele et al., 2002; Vanoverberghe et al., 2004) and in H1339 and HCC cell lines (Bergner et al., 2009). Conversely, there was no difference in calreticulin and calnexin expression between N- and RCC-ECFCs. Therefore, an alternative mechanism must be invoked to understand the reduction in intraluminal ER Ca^{2+} levels in tumor-derived ECFCs. Our previous study demonstrated that the decay to the baseline of the Ca^{2+} transient induced by ATP in $0Ca^{2+}$ in RCC-ECFCs is significantly slower as compared to healthy cells (Lodola et al., 2012). Under such conditions, Ca^{2+} clearance from the cytosol is mainly accomplished by SERCA activity (Berra-Romani et al., 2010; Moccia et al., 2002). Conversely, the

recovery of CPA-induced increase in $[Ca^{2+}]_i$ to resting Ca^{2+} is not impaired in RCC-ECFCs and is carried out by PMCA and NCX as SERCA is inhibited by this treatment (Lodola et al., 2012). This result led us to conclude that SERCA activity is slower in RCC-ECFCs, a feature which might contribute to explain the fall in $[Ca^{2+}]_{ER}$ reported in the present investigation. SERCA is sensitive to the oxidative stress that features tumor microenvironment and attenuates its Ca^{2+}-pumping rate (Vangheluwe et al., 2005). For instance, both reactive oxygen and nitrogen species (ROS and RNS, respectively) target and inactivate SERCA2, thereby causing the derangement of ER Ca^{2+}-dependent signalling that features heart failure and skeletal muscle fatigue (Vangheluwe et al., 2005). However, the oxidative stress imposed by tumor microenvironment to its cellular constituents may not be the only reason for the inhibition of SERCA activity in RCC-ECFCs. A large gene-profiling study recently conducted by our group has demonstrated that RCC-ECFCs over-express the tetratricopeptide repeat-containing adapter protein TMTC1 (Tancredi, Della Porta, Porta, Rosti, and Moccia, not shown), which binds to and inhibits SERCA2B activity, thus causing a reduction in ER Ca^{2+} levels (Sunryd et al., 2014). Future work will have to assess whether TMTC1 dysregulation affects $[Ca^{2+}]_{ER}$ also in RCC-ECFCs.

The alteration in Ca^{2+}-storage capacity might also depend on the long-term remodeling of ER structure in RCC-ECFCs (Sammels et al., 2010). For instance, the up-regulation of the ER Ca^{2+} pool and Ca^{2+}-dependent hyper-inflammatory response has been associated to ER expansion in cystic fibrosis human airway epithelium (Ribeiro, 2006; Ribeiro and O'Neal, 2012). The same chain of events has been suggested to occur in the inflammatory bowel disease and during plasma-cell differentiation (Sammels et al., 2010). Moreover, ER remodeling may determine the extent of $InsP_3$-releasable Ca^{2+} during oocyte maturation (Lim et al., 2003; Santella et al., 2004). It has long been known that ER develops cortical clusters in maturing oocytes of hamster, mouse and *Xenopus*, thereby leading to an increase in the Ca^{2+} response to $InsP_3$ (Kline, 2000). Surprisingly, our EM analysis revealed that the decrease in $[Ca^{2+}]_{ER}$ is associated to a remarkable ER expansion, rather than collapse, in RCC-ECFCs. Thus, these cells are endowed with a larger ER, but their slower SERCA activity results in a decrease, rather than an increase, in their overall Ca^{2+} levels. This is the first time that such a rearrangement in ER structure has been described in an endothelial cell type under neoplastic conditions. The decline in steady-state $[Ca^{2+}]_{ER}$ may confer a survival advantage by conferring higher resistance to apoptosis, but poses a serious problem for the correct functioning of the ER. Synthesis, folding, and orderly transport of proteins are tightly regulated by the ER-resident and Ca^{2+}-dependent chaperones calreticulin and calnexin. A chronic Ca^{2+} depletion of the ER causes an imbalance between the cellular demand for protein synthesis and the capacity to satisfy such request. This results in the accumulation of misfolded or unfolded proteins, a condition that has been referred to as ER stress (Mekahli et al., 2011; Sammels et al., 2010). As discussed in

Prevarkaya's work, in 2013, tumor cells must cope with the chronic underfilling of the ER and develop adaptive mechanisms to face the reduction in $[Ca^{2+}]_{ER}$ (Prevarskaya et al., 2013). The unfolded protein response (UPR) is the survival strategy that cancer cells activate to reestablish the protein-folding ability of ER (Walter and Ron, 2011; Wang and Kaufman, 2014). One of the hallmarks of UPR is actually represented by ER expansion, which is mediated by the transcription factor X-box binding protein-1 (XBP-1) and alleviates ER stress independently on any increase in ER chaperone levels (Walter and Ron, 2011; Schuck et al., 2009). Therefore, we speculate that ER remodeling is an adaptive mechanism by which RCC-ECFCs withstand the chronic drop in $[Ca^{2+}]_{ER}$ that enable them to evade apoptosis. In this view, it is worth of noting that RCC-ECFCs display many more mitochondria as compared to control cells. It turns out that a lower amount of ER-derived Ca^{2+} could be constitutively transferred in an $InsP_3$-dependent manner to a larger number of mitochondria to fuel cell bioenergetics without reaching the threshold of induction of apoptotis cascade causing resistance to apoptotic stimuli.

Overall, these results clearly demonstrate that RCC-ECFCs display a lower $[Ca^{2+}]_{ER}$ as compared to control cells which reflects into a lower $InsP_3$-dependent increase in $[Ca^{2+}]_{mit}$ and involves a slower SERCA activity. The chronic underfilling of the ER Ca^{2+} pool might underpin both the two key functional differences observed between normal and RCC-derived ECFCs: i.e. their higher resistance to apoptosis and lower sensitivity to VEGF. These findings could be crucial for redirecting anti-angiogenic therapies towards a more effective path. Anti-angiogenic therapies in RCC are likely to fail after a few months after the beginning of the treatment (or to fail immediately in the so called 'primary refractory' patients). This is almost inevitable except for a few, selected and anecdotic cases. In the last few years, research has focused on the molecular mechanisms that eventually lead to resistance to targeted therapies, and many alternative signalling pathways seem to be activated after the selective pressure induced by anti-angiogenic molecules, e.g. c-Met-HGF/SF or bFGF (Porta et al., 2013). Resistance mechanisms have anyway been mostly explored in tumor cells, while very few attempts have been trying to evaluate the role of the microenvironment. From our observations, we now know that tumor-derived ECFCs are more resistant to apoptosis and to VEGF stimulation. This latter feature is not linked to a blockade of VEGFR itself, but to a downstream dysregulation of this pathway. The drop in $[Ca^{2+}]_{ER}$ represents the most likely explanation to understand why ECFCs are less sensitive to apoptosis and to VEGF. These rather unexpected observations lead to the intriguing hypothesis that EPCs play a key role in mounting RCC patients resistance to anti-VEGF therapies.

CONCLUSION

In conclusion, our work provides a wide phenotypical and functional comparison between ECFCs isolated from healthy individuals and from individuals affected by renal cell carcinoma. We found that ECFCs are more frequent in peripheral blood of patients with RCC as expected by their predicted role in neovascularization. RCC-ECFCs truly belong to the endothelial lineage and are able to form capillary-like structures in vitro. Unlike TECs, there is no difference either in the proliferation or tubulogenic rates between normal and tumor-derived ECFCs; however, the latter are more resistant to pro-apoptotic stimulation. Additionally, RCC-ECFCs are not sensitive to VEGF, although VEGFR-2 is normally expressed and activated by VEGF, which in turn induces NF-KB-dependent gene and protein expression in N-ECFCs. We further demonstrated that RCC-ECFCs present a lower ER and mitochondrial Ca^{2+} content as compared to normal cells, albeit the ER undergoes a remarkable expansion. The chronic drop in ER Ca^{2+} levels is seemingly due to a slower SERCA activity and could underpin both the higher resistance to apoptosis and the lower sensitivity to VEGF. The remodeling of the Ca^{2+} toolkit might thus be exploited not only by tumor cells, but also by tumor-associated ECFCs to promote cancer development. Although RCC-ECFCs do not belong to the neoplastic clone (Piaggio et al., 2009), our data clearly show that they differ from the same population in normal individuals. Growing evidence has demonstrated that bone marrow-derived EPCs are reprogrammed by tumor-secreted soluble mediators or exosomes to favor tumor growth, vascularization, and metastatization (Barcellos-Hoff et al., 2013; Moccia and Poletto, 2014; Plummer et al., 2013). These data corroborate the notion that carcinogenesis alters ECFCs, a truly endothelial EPC subtype, by impacting on their intracellular Ca^{2+} toolkit (Dragoni, Turin, et al. 2014; Lodola et al. 2012). The role of EPCs in human tumors is not yet fully elucidated, but we would like to speculate that EPCs engraft within neoangiogenesis sites and constitute one of the factors inducing the resistance to anti-angiogenic treatments. Accordingly, the resistance to apoptosis related to the chronic reduction in $[Ca^{2+}]_{ER}$ is predicted to confer a survival advantage to RCC-ECFC within the hostile microenvironment of the growing tumor, thereby favoring their engraftment in nascent vessels and accelerating RCC expansion and spreading (Moccia and Poletto, 2014). Moreover, the remodelling of the Ca^{2+} machinery in RCC-ECFCs prevents the stimulating effect of VEGF. This feature could have a tremendous impact on the therapeutic efficacy of anti-angiogenic treatments. Endothelial cells isolated from the primary tumor are quite sensitive to VEGF, which is required for them to proliferate, survive to pro-apoptotic insults and form capillary-like structures in vitro (Bussolati et al., 2003). Thus, anti-VEGF drugs will cause significant tumor

shrinkage at the beginning of the therapy by blocking local angiogenesis; this, however, will rapidly lead to hypoxia-induced secretion of further growth factors and cytokines, e.g. VEGF, bFGF, EGF, SDF-1α and angiopoietins, and recruitment of BMDCs to the collapsing tumor. Herein, ECFCs, and perhaps all the other EPC subgroups, will not be affected by the therapeutic inhibition of VEGFR-2, which does not deliver pro-angiogenic signals to these cells. Consequently, ECFCs will be incorporated within tumor vasculature and fuel the formation of new blood vessels, thereby favouring tumor rebound. This adaptive mechanism would enable the tumor to circumvent the anti-angiogenic strategy by reducing its dependence on VEGF. This scenario is fully compatible with the modes of resistance to anti-angiogenic therapies suggested by Bergers and Hanahan (Bergers and Benjamin, 2003), by Carmeliet and coworkers (Loges et al., 2010), and by Ellis and Hicklin (Ellis and Hicklin, 2008). These results further imply that signalling pathways other than VEGF activate RCC-ECFCs; such hypothesis is corroborated by the well known up-regulation of multiple pro-angiogenic factors that may readily substitute for each other in cancer patients. These include the already mentioned bFGF, EGF, angiopoietins, as well as placental growth factor (PGF), osteopontin, granulocyte colony-stimulating factor (G-CSF), and ephrins (Bergers and Hanahan, 2008; Loges et al., 2010). Consistently, in a large fraction of kidney cancer patients who developed resistance to sunitinib, the metastatic progression was preceded by an increase in the circulating levels of bFGF, hepatocyte growth factor (HGF) and interleukin-6 (IL-6) (Porta et al., 2013). Future experiments will have of course to challenge these hypotheses. It will be mandatory to assess whether: 1) ECFC do engraft within RCC neovessels and 2) restoring the $[Ca^{2+}]_{ER}$ in RCC-ECFCs rescues their sensitivity to pro-apoptotic stimulation and VEGF.

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E così anche questo percorso si conclude, non senza prove, non senza difficoltà, ma d'altro canto, se così non fosse stato, quale sarebbe la soddisfazione?

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