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FUNCTIONAL PROPERTIES OF ION CHANNELS AND TRANSPORTERS IN TUMOR VASCULARIZATION

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ABSTRACT

Vascularization is crucial for solid tumor growth and invasion, providing metabolic support and sustaining metastatic dissemination.

It is now accepted that ion channels and transporters play a significant role in driving the cancer growth at all stages. They may represent novel therapeutic, diagnostic, and prognostic targets for anti-cancer therapies. On the other hand, although the expression and role of ion channels and transporters in the vascular endothelium is well recognized and subject of recent reviews, only recently their involvement in tumor vascularization have been recognized.

Here we review the current literature on ion channels and transporters directly involved in angiogenic process. Particular interest will be focused on tumor angiogenesis *in vivo*, as well as in the different steps that drive this process *in vitro*, such as endothelial cell proliferation, migration, adhesion and tubulogenesis.

Moreover, we compare the 'transportome' system of tumor vascular network with the physiological one.

INTRODUCTION

Endothelium is a multifaceted and dynamic interface between blood components and tissues. Endothelial cells (EC) mediate a great number of physiological functions, including control of metabolism, water supply, inflammation and immune response. Consequently, several diseases are causally due to the deregulation of normal EC functions. The importance of vascularization in the tumor progression sparked hopes that manipulating this process could offer therapeutic opportunities [1,2]. Consequently, so far hundreds of thousands of patients benefit of antiangiogenic therapies that use VEGF as major drug target and approved by the US Food and Drug Administration. The anti-VEGF antibody (bevacizumab [Avastin]) is used in combination with chemotherapy, cytokine therapy or radiotherapy for several advanced metastatic cancers. Additionally, four multitargeted pan-VEGF receptor tyrosine kinase inhibitors have been approved: Sunitinib (Sutent), Pazopanib (Votrient) for metastatic Renal Cacer Carcinoma (RCC), Sorafenib (Nexavar) for metastatic RCC, unresectable hepatocellular carcinoma and advanced pancreatic neuroendocrine tumors, and Vandetanib (Zactima) for medullary thyroid cancer [3]. On the other

hand, despite promising results, emerging data indicate that responses to vascular targeting therapy (VTT) are short-lived and resistance develops in the majority of patients. The discovery of new therapeutic targets is therefore necessary to provide a new input to the antiangiogenic therapy.
Being involved in nearly all of the 'hallmarks of cancer' as defined by Hanahan and Weinberg [4], there is an increasing consensus on the idea that ion channels and transporters could play a significant role in driving cancer progression at all stages. Therefore they may be seen as potential novel therapeutic, diagnostic, and prognostic targets for anti-cancer therapies.
Nonetheless, although the expression and role of ionic channels and transporters (collectively indicated as "trasportome") in the vascular endothelium is well recognized and subject of a number of recent reviews [5–8], 'trasportome' entered only recently as major players in tumor vascularization [9,10].
Here we collect and discuss current literature focused on ion channels and transporters directly involved in angiogenic process. Moreover, starting from a critical review of the experimental data obtained so far *in vitro* and *in vivo*, we will try to define the most promising checkpoints at which tumor vascular 'transportome' differs from the physiological one.

VOLTAGE-GATED CHANNELS
Although EC are generally described as non excitable cells, a number of experimental evidences suggest a role for voltage dependent channels (VOCs) in both cultured and freshly isolated EC [10]. On the other hand, the role of VOCs in tumor progression has been largely described and different data point to Na+, K+ and Ca2+ channels as key players, suitable to be specifically potential target in clinical treatments [11].
K+ channels (K_V) attracted most of the work in oncology since the early discovery unveiling their role in the control of cell proliferation [12,13]. Ether-á-go-go-1 (EAG1, KCNH1, K_V10.1) is a CNS-localized voltage-gated K+ channel that is found ectopically expressed in many solid tumors. Most of the interest in K_V10.1 arises from its expression in up to 70% of tumor cell lines and human cancers [13]. Monoclonal antibodies against human EAG1, developed by Stuhmer's and Pardo's groups, might represent a suitable tool in cancer therapy [14]. K_V10.1 expression might offer an advantage to tumors through increased vascularization and resistance to hypoxia: indeed, EAG1 regulates cellular oxygen homeostasis, increasing HIF-1 activity, and thereby VEGF secretion and tumor vascularization [15]; accordingly EAG1 silencing inhibits tumor growth and angiogenesis in osteosarcoma in vivo [16] (Table 1 and Fig.1).
A promising issue is related to other K+ channels, such as human ether-a-gogo related gene-1 (hERG1)-Kv11 [13,17,18]; Pillozzi and coworkers showed that hERG1 channels regulate vegf-a expression and VEGF-A secretion in cancer cells potentially promoting angiogenesis [19]. Moreover, it has been discovered a correlation between the levels of VEGF-A, hERG1 and microvessel density and proliferation-related parameters in two cases of bilateral retinoblastoma patients [20]. Beside the role of K_V10 and K_V11, K_V 1.3 channels are involved in VEGF-mediated HUVEC proliferation: VEGF-mediated hyperpolarization via Margatoxin (MTX)-sensitive K_V channels causes a Ca2+ entry, leading to an increase in NO synthesis, finally resulting in EC growth enhancement [21] (Table 1 and Fig.1). All together, the data point out an important role for K+ channels in the cross talk between cancer cells and tumor endothelium by induction of VEGF release that in turn promotes neovascularization. This particular function of K+ channels makes them clinically interesting as potential targets to promote vascular "normalization" by interfering with VEGF signaling during a critical window of the antiangiogenic treatments (see also the conclusion section).
Voltage-gated Na+ channels (VGSCs) are also expressed in non excitable cells and functionally up-regulated in metastatic tumor cells [22–24]. Recently, a clear relationship between functional expression and biological role of VGSCs in EC has been described [25]. Molecular expression analyses and electrophysiology revealed consistently that the main functional VGSC isoforms in HUVEC are Nav1.5 and Nav1.7. VGSC activity potentiates VEGF-induced ERK1/2 activation by

attenuating membrane depolarization, altering [Ca2+]i kinetics and PKC activity with a consequent increase in cellular proliferation, chemotaxis, and tubulogenesis [25] (Table 1 and Fig.1). Although the question on the specificity of VGSC on VEGF signaling pathway remains to be elucidated, the data unveil an intriguing mechanism for the control of Vm in non-excitable cells by VGSCs in response to physiological stimuli in vitro.

As regarding voltage-gated Ca2+ channels (VGCCs), most of the studies have been conducted on human breast carcinoma cell lines, which actually express VGCCs, mainly of the T-type [26–28]. Nevertheless, the role and expression of VGCCs in endothelium is still debated [3,7,24]. Conflicting data could arise from the use of different cultured EC lines and their well known variable behaviour (see also the conclusion section for a more detailed discussion). In human umbilical vein EC (HUVEC) Angiotensin II stimulates Ca2+ influx via Ca_V and promotes cell migration [30]. On the other hand, Ca_V expressed by VSMCs could play an anti-angiogenic role through indirect effects on EC: nifedipine, a widely used inhibitor of L-type calcium channels, stimulates VEGF production from human coronary smooth muscle cells, an effect abolished by PKC inhibitors and a bradykinin B2 receptor antagonist [31] (Table 1 and Fig.1).

TRANSIENT RECEPTOR POTENTIAL (TRP) PROTEINS AND STIM1-ORAI1 COMPLEX.

Transient Receptor Potential (TRP) channels trigger Ca2+ signals that control intracellular events involved in the initiation and progression of cancer. It is not therefore surprising that the expression and function of some TRP proteins are altered during tumor growth and metastasis [9,32].

TRPs are widely expressed in endothelium and their activity has been related to normal and tumor vascularization [5,6]. TRP -mediated Ca2+ influx can be triggered by the release from intracellular Ca2+ stores giving rise to store-operated Ca2+ entry (SOCE). An alternative route is the store-independent Ca2+ entry (NSOCE) [33].

VEGF mediates NSOCE through TRPC6 channels in human microvascular EC [34,35]. Dominant negative TRPC6 significantly reduces EC number, migration and sprouting [36]. Moreover, TRPC6 promotes both proliferation and tubulogenesis induced by VEGF, but not bFGF, in HUVEC [37]. Phosphatase and tensin homolog (PTEN) regulates cell surface expression of TRPC6, and consequently Ca2+ entry, endothelial permeability, and angiogenesis in human pulmonary EC [38]. Nonetheless, TRPC6 can also exert its proangiogenic role indirectly through its activity on cancer cells being a key mediator hypoxia-mediated notch-driven growth and invasiveness of glioblastoma multiforme (GBM): inhibition of the hypoxia upregulated TRPC6 expression and NFAT activation in glioma cells, markedly reduced the number of branch points in EC grown in conditioned medium harvested from glioma cells, indicating that TRPC6 is essential for the angiogenic potential of glioma cells [39] (Table 1 and Fig.1).

Other groups reported a role of VEGF-mediated SOCE due to TRPC1 in the enhancement of HMVEC and HUVEC permeability [40–42]. Remarkably, TRPC1 is proangiogenic *in vivo*. Knockdown of zebrafish TRPC1 by morfolinos caused severe angiogenic defects in intersegmental vessel sprouting, presumably due to impaired filopodia extension during EC migration [43] (Table 1 and Fig. 1).

This apparently surprising ability of VEGF to couple to different channels responsible for SOCE or NSOCE could simply depend on tissue variability, especially between small capillaries and large vessels. Accordingly, the pattern of TRPC channels expressed in HMVEC and HUVEC is different, TRPC4 being undetectable in HMVEC [36].

Besides TRPC1 and TRPC6, also Orai1 and STIM1, components of the so-called calcium release activated currents (CRAC) channels, concur to the VEGF-mediated SOCE in HUVEC [44,45]. VEGF stimulation promotes STIM1 clustering which in turn activates Orai1 [45]. Moreover, knock-down of Orai1 inhibits VEGF-mediated HUVEC migration, proliferation and tubulogenesis [44–46]. On the other hand, Trebak and coworkers reported recently that the thrombin-induced decrease in EC permeability requires STIM1, but is unrelated to Orai1 and Ca2+ entry across the plasma membrane [47] (Table 1).

Interestingly, STIM1, as well as TRPC1 and TRPC4 knockdown, inhibits tube formation in both HUVEC and EA.hy926 cells, an EC line derived from HUVEC fused with human lung adenocarcinoma cell line A549 [48]. Since Orai1 and TRPC1 can functionally interact at least in some models, the TRP- and Stim/Orai- pathways may give rise to a complex signaling network underlying proangiogenic calcium signals [49].

Since VEGF regulates several activities in EC, the discovery of a specific role for the different channels in selected cell functions, such as migration and proliferation on one side or permeability on the other, could be a more useful molecular target than the broad VEGFR inhibitors (see also Conclusion section).

TRPV4 is another emerging player in angiogenesis. The availability of high selective antagonists for this channel makes it a promising molecular target for antiangiogenic treatments [50]. TRPV4 is widely expressed in the vascular endothelium where it acts as a mechanosensor during changes in cell morphology, cell swelling and shear stress [50–53]. A study conducted both *in vivo* and in cultured EC reports that both shear stress and agonist-activation of TRPV4 enhance EC proliferation as well as collateral growth after arterial occlusion [54]. Recently, we showed a key role for TRPV4 in tumor-derived EC (TEC) migration (better discussed below) [55] (Table 1 and Fig.1). It is worth noting that the dynamics of a single TRP should be considered in a more integrated framework: for instance, the trafficking to the plasma membrane of TRPV4-TRPC1 heteromeric complex is enhanced by Ca2+ store depletion in HUVEC, resulting in an enhanced Ca2+ influx upon exposure to shear flow [56].

A number of cellular stress factors, including hypoxia, nutrient deprivation, and reactive oxygen species, are important stimuli for angiogenic signaling [57]. TRPM2 promotes macrovascular pulmonary EC permeability in a H2O2-dependent manner. TRPM2 knockdown or overexpression of the TRPM2 short isoform (that acts as dominant negative for TRPM2 long isoform) significantly reduced the H2O2/Ca2+-mediated increase of paracellular permeability and cell death in H5V EC [58,59] (Table 1 and Fig.1). These data open the exciting possibility of targeting TRPM2 for endothelial protection against ROS-induced cell damage. Additionally, the same strategy could be employed for treatment of malignant tumors, because TRPM2 isoforms are expressed in different tumors, and at least one of them may function as a tumor enhancer [60].

Finally, TRPM7, a Ca2+ and Mg2+ permeable channel that regulates Mg2+ homeostasis, is involved in a number of vascular disorders such as hypertension and dysfunction of endothelial and smooth muscle cells [61]. A notable structural feature of TRPM7 is the presence of a kinase domain at its C-terminus, making TRPM7 unique amongst ion channels, and allowing its involvement both in cellular Mg2+ homeostasis and broad signaling [62]. TRPM7 acts negatively on HUVEC proliferation and migration, whereas its functions on HMEC seem to be different [63–65] (Table 1 and Fig.1). Once again, more studies are required to better understand the variability of the effects induced by TRPM7 silencing in vascular endothelium.

In addition to the canonical angiogenesis, tumor vascularization may be supported by bone marrow (BM)-derived endothelial progenitor cells (EPCs) incorporating within sprouting neovessels. This feature hinted at EPC inhibition as a novel therapeutic target to pursue along with anti-angiogenic treatments [1,57]. Suppression of Orai1 in EPC prevents SOCE and tubule formation [45,66]. Moreover, EPCs isolated from RCC patients (RCC-EPCs) display an increased SOCE, which correlates with Orai1, Stim1, and TRPC1 overexpression as compared to EPCs from healthy patients: genetic suppression of Stim1, Orai1, and TRPC1 affects SOCE in RCC-EPCs [67]. TRPC1 regulates proliferation and migration of EPCs isolated from rats bone marrow [68] (see also Table 1 and Fig.1).

NICOTINIC RECEPTORS

nAChR are homo- or hetero-pentameric ion channels activated by endogenous acetylcholine or exogenous agonists like nicotine [69]. EC express most of the known mammalian nAChR subunits

[70–72]. In particular α7 nAChR mediates the main effects of nicotine on EC, such as proliferation, survival, migration, tube formation, and intracellular signaling (calcium and NO signals, phosphorylation events and gene transcription). Interestingly, α9 and α7 nAChRs exert opposing effects on nicotine-induced cell proliferation and survival [72–74].

Exposure to nicotine up-regulates α7-nAChR and pharmacological inhibition of α7-nAChR by Mecamylamine or α-Bungarotoxin significantly and reversibly reduces EC tubulogenesis *in vitro*. Even more importantly, pharmacological inhibitors or genetic disruption of α7-nAChR significantly suppress neo-angiogenesis in inflammation, ischemia, and neoplasia in several models. The angiogenic effect of nAChR is exerted through MAPK, PI3K/Akt, and NF-kB pathway; however, since nAChR-mediated angiogenesis is only partially inhibited in α7-nAChR-deficient mouse, other nAChR isoforms are presumably involved [72]. Nicotine triggers neo-angiogenesis in breast, colon and lung tumor cells implanted in chick chorioallantoic membranes and promotes b-FGF release through the recruitment of nicotinic receptor, □v□3 integrin, and MAPK pathway [75–77]. The ability of nicotine to promote late EPCs proliferation, migration, adhesion, and tubulogenesis strongly suggests that its role is not restricted to mature EC [78] (Table 1 and Fig. 1)

VOLUME-REGULATED ANION CHANNELS

Resting normal EC expresses volume-regulated anion channels (VRACs), mainly permeable to chloride ions and activated by osmotic cell swelling and shear stress. Endothelial VRACs are open in resting conditions and contribute to the maintenance of the resting potential in non-stimulated cells, in addition to K+ channels [10].

VRAC blockers (Mibefradil, NPPB, Tamoxifen, and Clomiphene) inhibit tube formation of rat and human microvascular EC and are strongly antiangiogenic *in vivo* [79] (Table 1). Although the mechanism of VRACs involvement in angiogenesis has not been clarified yet, one possible explanation is that VRAC activation could lead to an increase of the driving force for Ca2+ entry into the cell, thus affecting the intracellular Ca2+ concentration.

WATER CHANNELS

Aquaporins (AQPs) allow passive water flow in response to local osmotic gradients. They contribute to epithelial secretion and absorption, and cell volume regulation. Ectopic AQP expression is associated with several human cancers [12,80]. A number of reports point to AQP, mainly AQP1, involvement in cell motility and tumor vascularization [81–83]: its expression in tumor cells and their vasculature is variable being dependent not only on the origin of the tumor, but also on its location in the host animal. This observation strenghtens the strong inductive role of the microenvironment on tumor features.

Interestigly, AQP1 is upregulated in human brain tumors: little or no AQP1 expression is found in normal human brain microvessel endothelium, consistently with its general low permeability. On the other hand, AQP1 expression in the vasculature increases with the progression from normal brain to low-grade to high-grade astrocytoma [84].

Verkman and coworkers provided direct evidence for AQP1 role in angiogenesis *in vivo* by implanting melanoma cells in AQP1 null mice and syngenic mice lacking AQP1 [85]. In both cases the authors observed a markedly lower density of microvessels and the presence of islands of viable tumor cells surrounded by necrotic tissue compared to control mice. Functional analyses on mouse aortic EC isolated from AQP1 null mice and wild type mice revealed an impaired migration, invasiveness and capability to form capillary-like structures in matrigel [85]. On the other hand, RNA interference experiments performed by intratumoral injections of AQP1 siRNAs in a mouse model of melanoma suggest that AQP1 inhibition can humper tumor growth significantly lowering microvessel density [86]. AQP1 is also overexpressed in both human and rodent chronic liver disease. Its overexpression during cirrhosis is localized to the altered neovasculature. AQP1 promotes angiogenesis, fibrosis, and portal hypertension through mechanisms dependent on osmotically sensitive microRNAs, as revealed on human and mouse hepatic EC [87]. Finally, microvessel overexpression of AQP1 is associated with bone marrow angiogenesis in patients with active multiple myeloma [88] (tab 1 and Fig.1).

CARRIERS

Beside the role of ion channels, extensive evidence points out the involvement of carriers and transporters in tumor progression [89,90]. We will focus on sodium-proton exchanger and sodium-calcium exchanger, the best studied so far for their involvement in tumor progression and vascularization (Table 1 and Fig.1).

Sodium-proton exchanger (NHE). It is well recognised that pathological elevations of pHi can concur to some functional features of malignant cells [91]. All tumors share an altered regulation of hydrogen ion dynamics and tumor progression correlates with the peculiar acid-base balance in cancer cells: an extracellular acid microenvironment (pHe) linked to an alkaline intracellular pH (pHi). Indeed, tumor cells have alkaline pHi values in the range of 7.12-7.7 *vs* 6.99-7.05 of normal cells, while producing acidic pHe values of 6.2-6.9 *vs* 7.3-7.4 of normal cells. This reversed pH gradient across the cell membrane increases with tumor progression. Since NHE is a universal and conserved regulator of cellular proton balance, it received great attention. Through its action the inwardly directed Na+ gradient can drive the uphill extrusion of protons that drives pHi alkalinization and pHe acidification [91].

The highly hypoxic tumor microenvironment hyperactivates NHE1 and, since specific NHE1 inhibitors (Cariporide) are available, some authors propose them for innovative combination trials with antiangiogenic drugs. Low concentrations of Cariporide can lead to a decrease in pHi and down-regulation of VEGF. Moreover, exposure to cariporide inhibits HUVEC proliferation and migration promoted by conditional medium from K562 leukemia cells. *In vivo* experiments directly confirmed that inhibition of NHE1 by Cariporide could affect tumor growth and angiogenesis. Tumor regression is thus presumably a result of the decreased microvessel density, which causes insufficient oxygen and nutrients supply [92]. Blocking NHE1 reduces VEGF release from the tumor cells suggesting that, in addition to being stimulated by hypoxia, VEGF production and angiogenesis are linked to acidic pHe and to the NHE1-dependent changes in pH [93]. Systemic Amiloride perfusion also reduced neovascularization experimentally induced in an animal model, probably through inhibition of NHE1 [94].

Sodium-calcium exchanger (NCX). Sodium influx mediated by non-selective cation channels can drive to its accumulation beneath the plasma membrane. This event may increase [Ca2+]i by locally inverting (3Na+ out : 1 Ca2+ in) the operation mode of NCX [95].

An intriguing example has been described in HUVEC, in which a coupling between NCX and voltage-dependent sodium channels (VGSCs) occurs. As previously stated, VGSC activity promotes VEGF-induced proliferation, chemotaxis, and tubular differentiation and decreases adhesion to substrate [25]. Moreover, Ca2+ inflow through reverse mode NCX is required for PKC activation and targeting to the plasma membrane, as well as for VEGF-induced ERK1/2 phosphorylation and downstream EC functions in angiogenesis [96].

CA2+ SIGNALS, ION CURRENTS AND CHANNELS IN TUMOR-DERIVED ENDOTHELIAL CELLS.

As previously stated, a possible reason for the failure of the antiangiogenic therapies may be the high instability of EC within the tumor. It is now well established that normal and altered EC are highly heterogeneous in structure and function, due to genetic modifications and the variability of the local microenvironment [97–100]. The basic properties of EC obtained from different human tumors (tumor-derived EC, TEC) have been investigated only recently by a limited number of groups [101–103]. Breast tumor vessels display differential expression of over 1000 genes when compared with normal vessels, as revealed by gene array analysis [104]. Affymetrix microarray analysis of laser-captured CD31-positive blood vessels identified 63 genes that are upregulated significantly (5–72 fold) in angiogenic blood vessels associated with human invasive ductal carcinoma of the breast as compared with blood vessels in normal human breast [105].

On the other hand, TEC have been isolated and cultured from human kidney and breast carcinomas on the basis of membrane markers and exhibit altered genotype, gene expression, phenotype, and function. They are often aneuploid and display chromosomal instability. In addition, TEC avoid senescence, a process typical of normal EC, and display enhanced proliferation, motility, and ability to organize into capillary-like tubules [101,106,107]. Moreover, EC from human breast cancer are significantly more radiosensitive than their normal counterparts from the same patients [108]. A recent report compared the characteristics of two types of human TEC from high-metastatic (HM) and low-metastatic (LM) tumors: HM-TEC showed higher proliferative and invasive activity than LM-TEC [109].

Tumor-derived blood vessels are capillary structures and therefore TEC can be truly considered as altered microvascular EC. They can be compared to 'physiological' microvascular EC and thus the best choice would be the use to human microvascular EC obtained from the same 'healthy' tissue of TEC. Unfortunately, it is often very difficult to isolate and maintain in culture microvascular EC from all human healthy tissues: therefore dermal human microvascular EC (HMEC) are often used as a physiological counterpart. Conversely, macrovascular EC, such as HUVEC, are a less suitable choice, due to their features highly divergent from microvascular endothelium.

In the last years, our group provided substantial evidence that TEC-mediated (mainly Breast cancer-derived TEC, BTEC, and more recently renal-TEC, RTEC) intrcellular signaling pathways linked to Ca2+ signals are quite different from that observed in normal human microvascular EC (Fig. 2). We investigated in detail the differential effects of intracellular Ca2+ signaling regulated by the complex and networking pathways involving arachidonic acid (AA), Nitric Oxide (NO) and Hydrogen Sulfide (H2S), key-intracellular messengers triggered by proangiogenic factors (VEGF, bFGF) in vascular EC [6]. Low micromolar AA concentrations trigger NO release and protein kinase A (PKA)-dependent Ca2+ entry which in turn stimulate BTEC migration and tubulogenesis *in vitro* [110,111]. AA-dependent Ca2+ signals are intriguingly related to the tubule maturation stage, being downregulated in the late phases of the process [55,112]. On the other hand, AA failed to induce any pro-migratory effect in HMEC, with consistent significantly smaller Ca2+ signals compared with BTEC [113] (Fig. 2).

Notably, both the tubulogenic and promigratory effects induced by AA are highly sensitive to carboxyamidotriazole (0.1 μM CAI), a well known inhibitor of agonist-activated Ca2+ entry [112,114]. CAI affects proliferation, invasion, metastasis, and neovascularization both *in vitro* and *in vivo*. Combined to other compounds, it reduces the growth of cholangiocarcinoma, melanoma, colorectal, lung, pancreatic, ovarian and breast cancer [6]. Since CAI is effective from 1 μM on normal EC, the higher sensitivity of BTEC to this compound could be suitable to increase the efficacy of antiangiogenic agents and to reduce their secondary effects in combination therapies. Higher doses of CAI exert antiangiogenic activity in different systems such as mouse presenting ischemic retinopathy, rat aortic ring culture, or chorioallantoic membrane [112].

H2S is a recently discovered gasotransmitter [115,116] involved in angiogenesis regulation, particularly via VEGF signaling [117]. H2S activates Ca2+-permeable non-selective channels in a subpopulation of BTEC and the following Ca2+ is enhanced in BTEC compared to HMEC. Remarkably H2S mediates tumor proangiogenic signaling triggered by VEGF: B-TEC pretreated with DL-propargylglycine, an inhibitor of the H2S-producing enzyme cystathionine γ-lyase, showed drastically reduced migration and Ca2+i signals induced by VEGF.[117] (Fig. 2). H2S donors also activate ATP-dependent K+ (KATP) channels both in normal EC and in BTEC [117–120]. This evidence is of particular interest since during ischemic/hypoxic conditions, typical of the initial phases of cancer progression, KATP channels act as ATP sensors.

We recently provided strong evidences about the role of TRPV4 channels in promoting AA-mediated TEC migration: TRPV4 channels, are upregulated in BTEC and RTEC as compared with dermal HMEC and normal kidney glomerular EC [55]. AA-activated TRPV4 is essential for BTEC

migration: loss of TRPV4 expression results in decreased Ca2+ responses to the TRPV4-specific agonist 4α-phorbol 12,13-didecanoate and in complete inhibition of AA-induced BTEC migration. The mechanism by which AA regulates TRPV4 was also revealed in BTEC. AA induces actin remodeling, which triggers TRPV4 recruitment in the plasma membrane: the consequent Ca2+ entry finally leads to BTEC migration [55].
However, as previously stated, TRPV4 is ubiquitary in healthy vascular endothelium and plays a physiological role both in large arteries and microvessels: these relevant activities require careful consideration of its therapeutic potential. On the other hand, an overexpression on TEC could be exploited for a tumor targeted therapy based on lower inhibiting doses of TRPV4 antagonists which could selectively affect TEC and not normal EC.

CONCLUSIONS

Since the seminal hypothesis proposed by Judas Folkman in ’70, interference with tumor vascularization is considered a key therapeutic opportunity in cancer treatment [2].
Unfortunately, despite promising results, vascular targeted therapy (VTT) appear short lived and resistance develops in the majority of patients [121]. The relative inefficacy of VTT maybe due to several reasons.
More suitable preclinical cancer models are needed in oncological practice. As previously stated, vessels in cancer significantly differ from normal vasculature and the instability of EC within the tumor is a relevant feature. To this purpose, the use of TEC seems a more appropriate model compared to the normal EC. We expect that more detailed studies on the “transportome” in tumor vascularization using the aforementioned models (beside the EC models already in use) will give new input in unveiling the differences in signaling, transcriptome profiles, and vascular ‘‘ZIP codes’’ and will likely prove to be important for understanding the conversion of normal endothelial cells into tumor-associated endothelial cells. As a preliminary example, overexpression of TRPV4 in TEC [55] could be useful for selectively targeted therapy using lower doses of channel antagonists which affect TEC reducing seondary undesired effects on normal EC.
Another high priority challenge is the research of novel molecular anti-vascular targets (related or unrelated to VEGF signaling). The evaluation of their clinical potential, in particular as combination therapy with current VEGF (receptor) inhibitors, is likely to expand the antiangiogenic armamentarium. In particular it could be useful to narrow the field of action for VEGF-mediated targeted therapy. In this context, the recent interest on human ‘transportome’ involvement in tumor vascularization is a promising field, since several members are activated downstream the recruitment of VEGF receptors. For example, whereas the interference with the bulk VEGF signaling alters the activity of a moltitude of different cells and functions, targeting TRPC6 or Orai1 may only affect EC migration and proliferation [36,37,39,45,66], while TRPC1 and STIM1 may selectively influence vascular permeability [40–42,47].
It is worth noting that channels and transporters are widely distributed and ubiquitous. This feature has to be carefully taken in account when considering them as clinical targets. This problem could be overcome by directed targeted therapies taking advantage from nano-biomedicine: for example, nanoparticle functionalization with peptide cyclic RGD for angiogenesis-specific targeting [122] together with a specific channel modulator could be successfully employed.
On the other hand, the ubiquitous expression of the channels could be used as a positive feature, due to the redundancy of the signaling pathways which regulates the different hallmarks of cancer: in other words, the use of specific channels to selective co-target different key steps of carcinogenesis beside tumor vascularization, could result in more effective and long lasting therapies. For example, TRPC6 channels targeting could affect VEGF release from tumor cells as well as EC migration and tumor vascularization [36,37,39].
Another important issue is the therapeutic potential of sustained vessel normalization to suppress metastasis and enhance chemotherapy. Indeed, several preclinical studies have revealed that the

high levels of VEGF in tumors induce vessel abnormalities. It is reasonable to postulate that these vessel abnormalities could be decreased by lowering VEGF signaling. VEGF-targeted therapy induces characteristic features of vessel normalization, including reduced number and size of immature tumor vessels and increased pericyte coverage, together with decreased permeability, oedema and interstitial fluid pressure [123]. Interfering with K+ channels, such as EAG1 and hERG1, TRPC6 channels or NHE exchanger on tumor cells could be useful to promote vascular "normalization" by interfering with VEGF signaling during a critical window of the antiangiogenic treatments .

Finally, even if big efforts have been produced in the last years in order to characterize and study the involvement of transportome in cancer cell biology, and in particular in tumor vascularization, the field is relatively novel. The scientific interest on this topic is largely increasing as pointed out by PubMed search. The research on transportome and cancer is expected to expand even more in the next decade, and we believe that the oncogenic roles of channels, as well as the molecular mechanisms responsible for their regulation, will be largely unveiled.

FIGURE LEGEND

Table 1

Ion Channels and carriers involved in the different phases of angiogenesis. HMEC, human microvascular EC; HPAEC, human pulmonary artery EC; HUVEC, human umbilical vein EC; EA.hy926, EC line derived from HUVEC fused with human lung adenocarcinoma cell line A549; PAEC, porcine aortic endothelial cells; BTEC, tumor derived EC from breast carcinoma; H5VEC, heart endothelioma (H5V) EC; MAEC, mouse aortic EC; EPC, endothelial progenitor cells; RCC-EPC, EPC isolated from renal carcinoma patients; Numbers in parenthesis indicate the respective reference number.

Figure 1

Schematic representation of channels/transporters role in the different key steps of tumor vascularization. The mechanisms are presented in representative EC, SMC, EPC and tumors without any tissue specification. EC, endothelial cells; EPC, endothelial progenitor cells; VSM, vascular smooth muscle cells; MAPK, mitogen-activated protein kinase; PI3K, Phosphatidylinositide 3-kinases; AKT, protein kinase B; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; bFGF, basic Fibriblast Growth Factor; VEGF, Vascular Endothelium Growth Factor; VEGFR, VEGF Receptor; NFAT, Nuclear factor of activated T-cells; PAR, protease-activated receptors; PTEN, Phosphatase and tensin homolog; PKC, protein kinase C.

Figure 2

A. Schematic representation of the differences between normal endothelial cells (EC) and tumor derived endothelial cells (TEC) in terms of Ca2+-related intracellular signaling pathways. Arachidonic Acid (AA), Nitric Oxide (NO) and Hydrogen sulfide (H2S)-promoted Ca2+i signals are significantly upregulated in TEC compared with EC. These differences are at least in part due to TRPV4 overexpression and consequent TEC migration. ***B.*** Schematic representation of the signal transduction pathway involved in proangiogenic Ca2+ signals in TEC: *(1)* AA-mediated actin-remodeling promotes TRPV4 vesicles to traffic and insert in the plasma membrane; as a consequence, more functional channels allow Ca2+ entry required for TEC migration. *(2)* Activation of endothelial NO synthase (eNOS) mediated by AA-mediated protein kinase A (PKA) promotes NO release and consequent Ca2+ entry via unknown channels. *(3)* VEGF promotes promigratory Ca2+ signals mediated by H2S via cystathionine γ-lyase (CSE).

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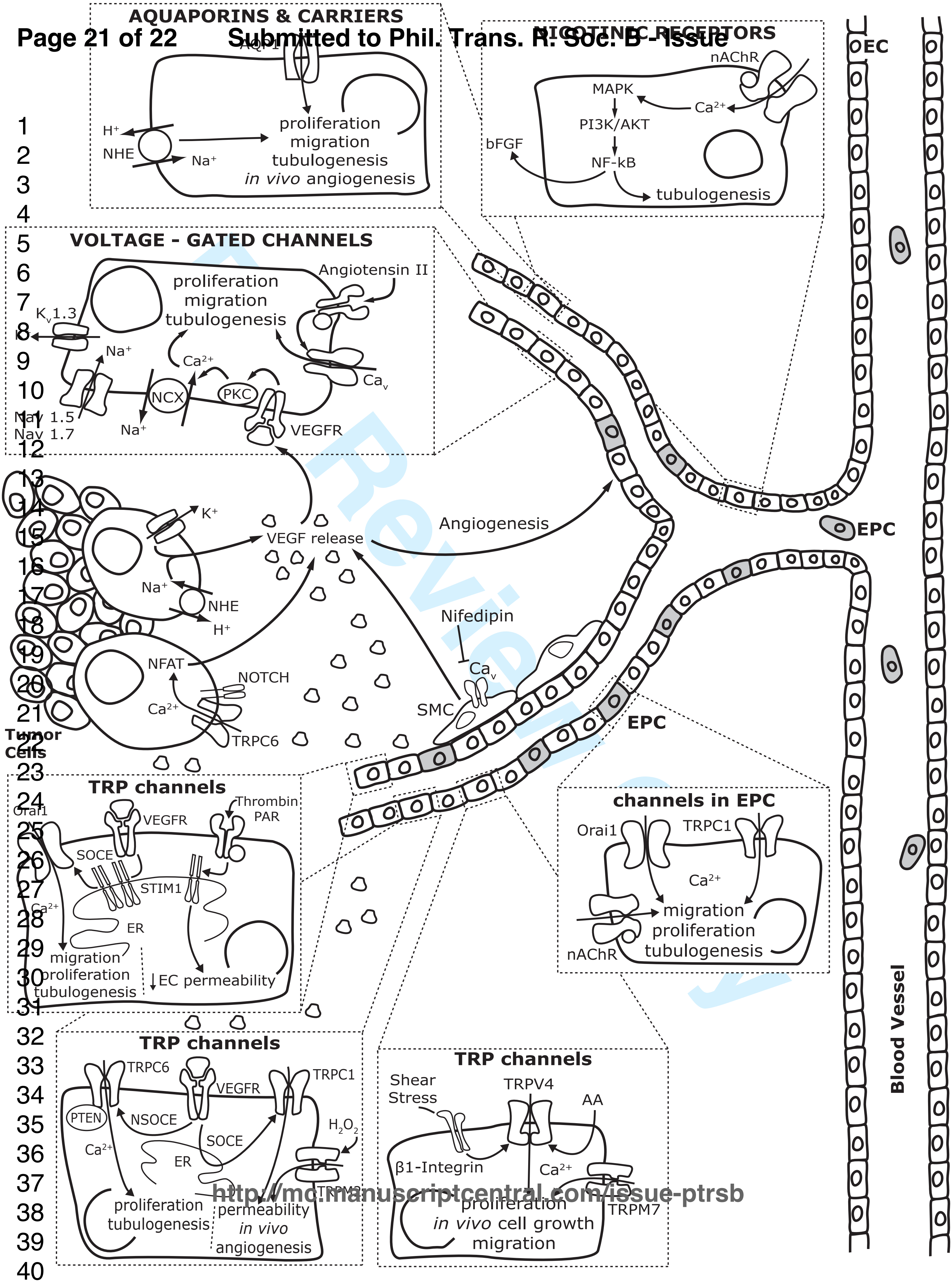
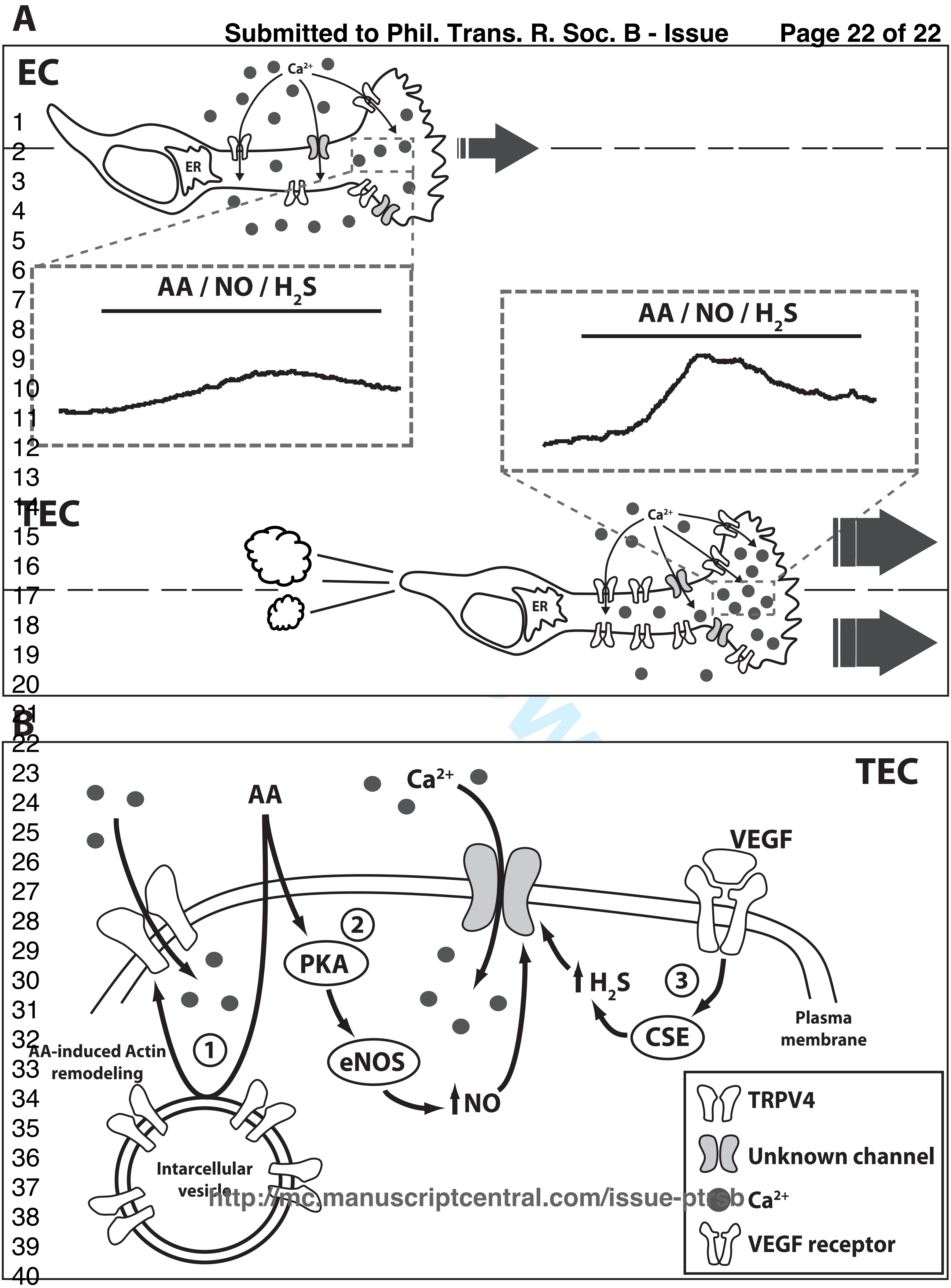
Channles/transporters	TRPC1	TRPC6	TRPC3,4,5	TRPV4	TRPM2	TRPM7	Orai1/Stim1	Nav	Cav	Kv	VRAC	NHE1	NCX	AQP1	nAChR
Migration	RCC-EPC [67] EPC[65]	HMEC [36]		BTEC [55]			RCC-EPC [67] HUVEC [44-46]	HUVEC [25]	HUVEC [30]			HUVEC [92]	HUVEC [25, 97]	MAEC from KO mice [85]	HMEC [73, 74] EPC [78]
Survival and Proliferation	RCC-EPC [67] EPC [45, 68]	HMEC [36] HUVEC [37]		PAECs [54]	H5VEC [58]	HUVEC, HMEC [63-65]	RCC-EPC [67] HUVEC [42]	HUVEC [25]		Kv 1.3-HUVEC [21]		HUVEC [92]	HUVEC [25, 97]	human hepatic sinusoidal ECs [87]	Human pulmonary artery ECs, human retinal microvascular ECs, HUVEC, HMEC [73] EPC [78]
***In vitro* Tube Formation**	RCC-EPC [67] HUVEC, EA.hy926 [48]	HMEC [36, 39]	EA.hy926 [48]				RCC-EPC [67] HUVEC, EPC [45, 48]	HUVEC [25]			Microvascular ECs from the rat adrenal medulla (RAMECs), HMEC [79]	HUVEC [92]	HUVEC [25, 97]	MAEC from KO mice [85]	HUVEC, HMEC [72, 73] EPC [78]
Permeability	HMEC,HUVEC [40-42]	HPAEC [38] Frog mesenteric microvessels [34]			H5VEC [58]		HUVEC [47]								
***In vivo* Angiogenesis**	Zebrafish [43]	CAM [37]		Collateral growth [54]			CAM [43]			HERG-1-Retinoblastoma [20] EAG1-Xenograft in SCID mice and human osteosarcoma [15, 16]	CAM [79]	Xenograft in nude mice [92] Rabbit cornea [94]		Human mammary carcinoma, glioblastoma [83, 84] AQP1 KO mice and C57BL/6 mice [85, 86, 87] Bone marrow angiogenesis in patients with active multiple myeloma [88]	Disc angiogenesis system, hind limb ischemia [72] Breast, colon and lung tumor cells implanted in CAM [75]


AQUAPORINS & CARRIERS
AQP-1
H+
NHE
Na+
proliferation
migration
tubulogenesis
in vivo angiogenesis
NICOTINIC RECEPTORS
nAChR
MAPK
Ca2+
PI3K/AKT
bFGF
NF-kB
tubulogenesis
OEC
VOLTAGE - GATED CHANNELS
proliferation
migration
tubulogenesis
Angiotensin II
Kv1.3
K+
Na+
Ca2+
NCX
PKC
Cav
Nav 1.5
Nav 1.7
Na+
VEGFR
K+
VEGF release
Angiogenesis
EPC
Na+
NHE
H+
Nifedipin
Cav
NFAT
NOTCH
Ca2+
SMC
TRPC6
EPC
Tumor Cells
TRP channels
Orai1
VEGFR
Thrombin PAR
SOCE
STIM1
Ca2+
ER
migration
proliferation
tubulogenesis
↓EC permeability
channels in EPC
Orai1
TRPC1
Ca2+
migration
proliferation
tubulogenesis
nAChR
Blood Vessel
TRP channels
TRPC6
VEGFR
TRPC1
PTEN
NSOCE
SOCE
H2O2
Ca2+
ER
TRPM2
proliferation
tubulogenesis
permeability
in vivo angiogenesis
TRP channels
Shear Stress
TRPV4
AA
β1-Integrin
Ca2+
TRPM7
proliferation
in vivo cell growth
migration


A
EC
Ca2+
ER
AA / NO / H2S
AA / NO / H2S
TEC
Ca2+
ER
B
TEC
AA
Ca2+
VEGF
2
PKA
eNOS
NO
H2S
3
CSE
Plasma membrane
1
AA-induced Actin remodeling
Intercellular vesicle
TRPV4
Unknown channel
Ca2+
VEGF receptor