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SYSTEMS BIOLOGY OF ION CHANNELS AND TRANSPORTERS IN TUMOR ANGIOGENESIS: AN OMICS VIEW

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Summary

Solid tumors require the formation of new blood vessels to support their growth, invasiveness and metastatic potential. Tumor neovascularization is achieved by vasculogenesis from endothelial precursors and by sprouting angiogenesis from preexisting vessels. The complex sequence of events driving these processes, including endothelial activation, proliferation, migration and differentiation, is associated with fluxes of ions, water and other small molecules mediated by a great pool of ion channels and transporters (ICT). This 'transportome' is regulated by environmental factors as well as intracellular signaling molecules. In turn, ICT play a prominent role in the response to angiogenesis-related stimuli through canonical and 'unconventional' activities: indeed, there is an increasing recognition of the multifunctionality of several ion channels that could also be annotated as receptors, enzymes, scaffolding proteins, mechanical and chemical sensors.

The investigation of ICT structure and function has been far from the experimental oncology for long time and these two domains converged only very recently. Furthermore, the systems biology viewpoint has not received much attention in the biology of cancer transportome. Modulating angiogenesis by interference with membrane transport has a great potential in cancer treatment and the application of an 'omics' logic will hopefully contribute to the overall advancement in the field.

This review is an attempt to apply the systems biology approach to the analysis of ICT involved in tumor angiogenesis, with a particular focus on endothelial transportome diversity.

Introduction

Over the last thirty years, much insight has been gained into the central role of the endothelium in human health and disease (1). Endothelial cells (EC) represent a great evolutionary novelty in vertebrates (2–5): they line the blood vessel and act as a dynamic interface between blood and tissues providing a powerful control system for blood pressure and remodeling of vascular network (6). Since the endothelium can be a target or a causal factor of human disease, its assessment is a valuable tool in clinical investigation (1). The integrated evaluation of endothelial function and dysfunction incorporating, for example, coagulation, inflammatory, and vascular tone properties in normal homeostasis and diseases led some authors to propose the concept of 'endotheliome' (7). This term refers to *endotheli*-al form and function as a whole (*-ome*): a key requirement is to forge a synthesis of the array of endothelial vascular modifications in function of time and to understand how they are required for a given function or dysfunction to occur.

The formation of new blood vessels is required during tissue growth and remodeling in order to provide adequate nutrients and oxygen and overcome the basic surface/volume constraint in biological processes. Neovascularization can occur through different mechanisms such as vasculogenesis from endothelial precursors and sprouting angiogenesis (8). During this process endothelial homeostasis is regulated by pro- and anti-angiogenic factors: the response to these extracellular stimuli depends, among the other proteins, on the plasma-membrane transportome, the great and diversified tool of ion channels and transporters (ICT) expressed in the plasma membrane of EC (9). Neovascularization actually involves activation, proliferation, migration and differentiation of EC and endothelial cell precursors (EPC): all these events are associated with fluxes of ions, water and other small molecules mediated by a great variety of ICT (10,11). The growing interest on the contribution of ICT is clearly revealed by the huge number of very recent reports and reviews focused on this topic and published in a broad range of journals (10,12–17).

The transportome is finely regulated by a large pool of intracellular and extracellular signals, respectively, including signaling/metabolic pathways and soluble pro- and anti-angiogenic peptides, hormones, as well as extracellular matrix components. In turn, they mediate the vascular responsiveness to vasoactive stimuli. Intriguingly, this role is not always played by their canonical activity: indeed, as discussed below, a number of ion channels exhibit non-conductive functions and could also be annotated as receptors, enzymes, scaffolding proteins, mechanical and chemical sensors (18–23).

ICT expression during normal and altered angiogenesis

Globally, the main aims for a systems biology of ICT in altered angiogenesis should be 1) a clear definition of the endothelial transportome and 2) a deep knowledge of its integration with cell signaling and metabolome (the complete set of small molecule metabolites found within a cell compartment) involved in patho-physiological neovascularization: the latter issue is strictly related to protein interactomics (Figure 1).

To address the first goal we need an exhaustive annotation of the entire pool of ICT expressed by EC and EPC, their topological distribution and a description of the great variability due to genetic and epigenetic factors, including tissue microenvironment. The readout of this approach would be particularly attractive for vascular biology due to the great diversity found in normal and tumor-associated vessels and endothelial cells (24,25).

Despite the importance and the increased utility of proteomic tools in medical research for extending basic understanding in vascular biology and for directing the delivery of therapeutic and imaging agents *in vivo*, endothelial proteomics is only at its beginning. In addition, global ontology analyses are required to move beyond a simple list of proteins and to understand better how they interact and function in a given environment, providing a validation of a proteome generated from large-scale mass spectrometry (MS) analysis. This would lead to a better knowledge of the relationship of proteins in a functional network, as well as to the detection of novel functions and pathways in the given tissue (26).

Unfortunately, profiling the plasma membrane proteome (also named 'plasma membranome', e.g. the pool of proteins embedded in plasma membrane lipid bilayer) by the use of standard large-scale methods including MS-based strategies has been limited by problems associated with extraction, solubilization, and identification of intrinsic membrane proteins in cells and tissues. Moreover, ECs form a thin monolayer lining each blood vessel. They constitute a very small fraction of all the cells in the tissue, making it difficult to isolate pure EC plasma membrane fractions for proteomics analysis

using conventional sub-cellular fractionation techniques. Although relatively simple to isolate from tissue and grow in culture, EC require microenvironmental components to maintain their tissue-specific qualities and thus undergo undesired phenotypic changes after isolation. Mass spectrometry (MS) measurements and the use of multiple analytical methods can greatly expand the comprehensiveness of endothelial proteomic profiling. However, the inherent biases and variations in such data limit the quality of a quantitative comparative analysis.

A recent comparative analysis of EC plasma membranome employed four different MS-based strategies involving 2-D and 3-D separation (26). It combines protein pre-fractionation via SDS-PAGE with in-gel digestion to produce peptides separated by one- and two- dimensional nano-HPLC before seamless and continuous MS analysis. Each method used multiple replicate measurements to comprehensively identify proteins, achieving a clear statistical definition of completeness that allows meaningful comparisons. This approach greatly expanded the EC plasma membranome to 1,833 proteins of which nearly 30% are membrane-embedded (26,27). A further unbiased systems analysis unraveled the related pathways and functions including cell surface signaling networks, cytoskeleton organization, adhesion, membrane trafficking, metabolism, mechanotransduction, membrane fusion, and vesicle-mediated transport (28).

Only a few studies incorporate proteomic analysis of freshly isolated ECs from healthy and altered tissues or conditions that mimic key angiogenic variables, such as oxygen tension and shear stress (25).

We should take into account that the endothelium lining blood vessels and surrounding stroma in tumors differs from normal one, but only recently these differences have begun to be characterized at the molecular level (29–31). Since angiogenesis is required for physiological processes, markers that distinguish normal and pathological vasculature are needed in order to selectively deliver antiangiogenic agents to diseased tissues minimizing the potential side effects. A proteomic method has been developed to discover cell surface/secreted proteins, as they represent key antibody therapeutic and biomarker

opportunities (32). It is based on flow cytometric staining of vascular organs with known endothelial markers and their purification by cell sorting. Upon cell surface protein capture and tryptic digestion, the resulting proteolytic peptides are subjected to liquid chromatography – mass spectrometry (LC/MS) to identify the proteins. The comparative analysis revealed differences in the expression in different organs or conditions. Tumor-derived endothelium obtained from the kidney, lung and colon overexpresses more than one hundred of proteins when compared with normal surrounding tissues (32). Interestingly, ATP1B3 was among the more differentially expressed cell surface tumor-specific endothelial markers identified in this study. ATP1B3 belongs to the family of Na^+/K^+-ATPases, integral membrane proteins responsible for establishing and maintaining the electrochemical gradients of Na+ and K+ ions across the plasma membrane. This enzyme is composed of two subunits, a large catalytic α subunit and a smaller β glycoprotein (33). The β subunit regulates, through assembly of α/β heterodimers, the number of Na+/K+ ATPases transported to the plasma membrane. Recent studies indicate that the α- and β-subunits might independently be involved in cellular functions other than ion pumping (34). For example, the β-subunit could play a role in cell-cell adhesion. The amounts of the cell surface β-subunits increase when the cell density becomes higher, whereas the amount of the α–subunit does not change significantly (34). Further studies are needed to unveil the functional outcome of ATP1B3 over-expression on tumor vasculature (32).

Another proteomic work was undertaken on human telomerase-immortalized dermal microvascular endothelial cells (TIME) in order to identify key regulatory events at the protein level during tubular morphogenesis *in vitro* (35). Curiously, one of the VEGF-A-regulated EC proteins was identified as chloride intracellular channel 4 (CLIC4). The CLIC proteins have the unusual ability to translocate from the cytoplasm to various cell membranes and their overexpression promotes plasma membrane localization where they act as functional anion channels. CLIC4 has also been shown to engage in complex formation with cytoskeleton components such as actin, tubulin, and dynamin I. The

ability of CLIC4 to modulate the activity of cell surface ICT in retinal pigment epithelium-photoreceptor adhesion raises the possibility for a similar role in vascular endothelium (36).

Although many compounds have entered clinical trials as modulators of tumor angiogenesis, the conventional anti-angiogenic therapies based on well established molecular targets such as VEGF, integrins and angiopoietin, suppress neovascularization only transiently: they suffer from the so-called 'tumor escape phenomenon', due to compensating pathways that circumvent the initial effect, as well as from 'resistance' to chemotherapy drugs (30,37–40). Intriguingly, the combination of anti-VEGF therapy with conventional chemotherapy has improved survival in cancer patients compared with chemotherapy alone. These seemingly paradoxical results have been explained by a 'normalization' of the abnormal tumor vasculature usually characterized by dilated, tortuous, and hyperpermeable vessels. Vascular normalization leads to an attenuation of hyperpermeability, increased vascular pericyte coverage, a more normal basement membrane, and a resultant reduction in tumor hypoxia and interstitial fluid pressure (41–43). These in turn can lead to an improvement in the metabolic profile of the tumor microenvironment, the delivery and efficacy of exogenously administered therapeutics, the power of radiotherapy and of effector immune cells, and a reduction in number of metastatic cells. An omics approach in models of EC normalization, taking into account also other stromal cells such as fibroblasts and immune cells, could shed more light on the mechanisms underlying this promising effect. The putative contribution of ICT in vascular normalization is unknown but the field is in need of alternative targets, related to the identification of novel and missing angiogenesis annotations and their association with vascularization by the use of statistical analysis and multiple gene expression datasets. GeneHits is a method that combines graph diffusion kernels from PPI and pairwise associations from protein domain occurrence to construct a global angiogenesis protein interaction network, called 'Angiome' (44,45). An initial network of 478 proteins was then extended to 1,233 proteins. The angiome allows one to identify those genes and proteins in the databases that are associated with angiogenesis by comparing the disease- or condition-specific data to the angiome.

Examples may include identification of specific angiogenesis-associated genes that are up- or downregulated or mutated in disease conditions. Some of them may then be considered as putative targets for therapeutic approaches that, ultimately, will need to be experimentally validated. The human interactome was recently integrated with known angiogenesis-annotated proteins to identify a set of 202 angiogenesis-associated proteins in different cell lines (46). The expression of several proteins turned out to be highly perturbed during angiogenesis. Upon exposure to VEGF-A, some proteins were upregulated, such as HIF-1α, APP, HIV-1 tat interactive protein 2, and MEF2C, whereas endoglin, liprin β1 and HIF-2α resulted downregulated. The analysis showed differential regulation of HIF-1α and HIF-2α (46). 'Angiome' database doesn't include yet information about ICT, whose implementation will hopefully cover this lack.

Despite the fact that these high-throughput and large-scale approaches reveal very powerful and increasingly robust, they are still poorly employed in the field of membrane transport. Most of the available information on the differential expression pattern of transportome is actually obtained from small-scale hypothesis-driven works. Two well described examples involve some members of the Transient Receptor Potential (TRP) channels and Orai1 that form calcium-permeable channels. Among the 30 mammalian TRP channels, at least 19 are expressed in vascular ECs and are suggested to participate in a wide range of vascular functions, including control of vascular tone, permeability, mechanosensing, vascular remodeling and angiogenesis (47). However, in spite of the large body of data available, the functional role of many endothelial TRP channels in normal and neoplastic angiogenesis is still poorly understood. Transient receptor potential vanilloid 4 (TRPV4) is a calcium channel involved in EC migratory potential and is overexpressed in human breast-carcinoma derived EC (BTEC) compared to healthy EC (48). Canonical transient receptor potential channel 1 (TRPC1), as well as another calcium channel, Orai1, is upregulated in endothelial progenitor cells (EPC) isolated from renal carcinoma patients compared with EPC from healthy patients (14,49,50). Both these cases will be largely discussed in the following chapter (12,13,15–17). Growing evidence supports the

evidence that cancer cells may drive endothelial changes by exploiting their high flexibility. In particular, tumoral microenvironment has the ability to greatly influence the drug-resistance of ECs through molecular mechanisms still poorly understood. A recent paper postulated that microvessels (MV) derived from MCF-7 adriamycin-resistant cells (MCF-7/ADM) communicate with and influence the resistance of HMECs acting as a 'cargo' in cell-cell communication. MCF-7/ADM cell-derived MVs transfer TRPC5 to HMECs, inducing the expression of P-glycoprotein (P-gp) by activation of the transcription factor NFATc3 (nuclear factor of activated T cell isoform c3) (51). The TRPC5-dependent upregulation of P-gp is suggested to be tighly associated with Ca2+ entry induced by functional TRPC5 ion channel, but only further investigations will provide more mechanistic details on this intriguing process: blocking TRPC5 could contribute to overcome drug-resistance in cancer.

The altered expression panel of tumor-associated ICT does not involve only calcium channels, as suggested by the aberrant level of potassium channels encoded by the human ether-a-go-go related gene (Kv11.1, or hERG1) that regulate vascularization in some human cancers (52–54).

Another very interesting issue is related to the membrane permeability for water. Among the membrane fluxes that play a universal and conserved role in cell physiopathology, water permeation is highly relevant being a direct regulator of intracellular osmoticity and volume. Aquaporins (AQPs) are the integral plasma membrane proteins involved in water transport in many fluid-transporting tissues. Cancer cells express AQPs and a positive correlation exists between histological tumor grade and the AQP expression (55,56). In particular, AQP-1 plays a crucial role in tumor angiogenesis and regulates EC migration (57–61). Deletion of AQP-1 in genetically modified mice reduces tumor angiogenesis (57). In AQP-1 knockout mice, implanted tumors grow slowly and are less vascularized than in wild-type mice, indicating that cancer neovascularization and growth are promoted by endogenous AQP-1. On the other hand, the evidence that these mice develop normally with no detectable vascular defects strengthens the idea that normal and tumor angiogenesis are governed in different ways. Furthermore, microarray analysis has revealed that the AQP-1 gene is upregulated by estrogen and plays a crucial role

in estrogen-induced tubulogenesis of HUVEC possibly contributing to vascular permeability increase seen in response to the hormone mediated by the upregulation of VEGF and its receptors (60,62–64).

Interactomics of vascular ICT

Databases

A global map of protein-protein interactions (PPI) in cellular systems provides key insights into how an organism works as a whole. Indeed, human diseases are rarely the consequence of an isolated abnormality in a particular gene but are usually the outcome of complex perturbations of the underlying molecular network. This has led to systematic studies of interactome networks, whose structure is governed by key graph theoretical laws, where the probability of observing a protein with a small number of interactions is higher than the probability of observing a protein with many interactions (65).

Several public databases allow interactive investigation of interactome, including String-db, Genehits, Uniprot, Biogrid, DIP, IntAct, Reactome, Pathwayscommons, Pathway Interaction Database, and Unihi (65). A repository of well-validated high-quality PPI can be used in both large- and small-scale studies to generate and validate a wide range of functional hypotheses. The pattern of binary interactions can be obtained by literature-curation (LC) and high-throughput experiments (HT) (66). LC refers to systematically collecting interaction data from small-scale studies while HT experiments produce large-scale interaction maps. Because most LC data are generated by hypothesis-driven experiments, it is much easier to infer biological function from those studies as compared to HT experiments (66).

Despite the quality of this approach being widely questioned, the reproducibility of large-scale protein interaction results has much improved. Moreover, common data standards and coordinated curation practices between the databases that collect the interactions have made these valuable results available to a wide community of researchers (65). Nonetheless, it is not easy to reconcile information from

different independent and not-coordinated sources. For this reason, a number of them joined in International Molecular Exchange consortium (IMEx) to provide a unique standard curation practice and data presentation (67).

Interaction databases may lead to different predictions whose accuracy can be improved by incorporating datasets on organ- and cell type-specific gene expression, and by obtaining additional independent experimental evidence (68). A recent survey evaluated the characteristics of six interaction databases, incorporated tissue-specific gene expression information and then investigated if the most popular proteins of scientific literature are involved in good quality interactions (68). The databases result comparable in terms of node connectivity (i.e. proteins with few interaction partners also have few interaction partners in other databases), but may differ in the identity of interaction partners. Moreover, the incorporation of tissue-specific expression information significantly alters the interaction landscape and many of the most intensively studied proteins are engaged in interactions associated with low confidence scores.

An advanced systems biology of vascular ICT in angiogenesis aims at investigating the network of physical and biochemical PPI at two levels: the multimeric ICT assembly and, more broadly, PPI occurring between membrane transportome and proteins canonically associated with other functions such as cell signaling and metabolism. This second level would help to explain how channels and transporters integrate and coordinate intracellular pathways as well as how ICT are regulated by signaling proteins, lipids, and gasotransmitters (69–75). Accordingly, defective interactions of protein partners with ICT represent alternative mechanisms of membrane channelopathies (76). Molecular interactions actually create local microenvironments that can modify the functional properties of ICT. The assemblies built around a transporter or channel, called 'transportsomes' and 'channelosomes', can be considered as functional units even if they are difficult to purify and reconstitute, posing a significant challenge in the investigation of their functional role (77,78). A special issue on 'Channels' provides an overview of the nature and function of transportsomes and channelosomes (77). Successful studies

focusing on PPI could potentially lead to new drug discovery strategies targeting ion channel complexes (79).

As already mentioned, the interactome dynamics is strictly related to the physiological or altered cellular targeting of ICT. Indeed, the actual functions and biological effects of ion-transport-related proteins critically depend upon their intracellular distribution and, consequently, their interaction with a specific pattern of other proteins (specific interactome). The term 'interaction' refers to direct physical protein-protein (first-order) binding detectable by yeast-two-hybrid and protein complementation assays, as well as to indirect interaction through the mediation of other components, including scaffolding proteins, detectable by affinity purification followed by mass spectrometry (65). In some cases PPI are associated with post-translational modifications that drive cell signaling, such as phosphorylation, nitrosylation, sulphydration, redox reactions, and palmitylation.

According to this well-established knowledge, a more integrative view of ICT is now required, that should be investigated as components of the global vascular proteome involved in angiogenesis. The application of such a systems biology approach, that is experiencing its initial stage in this particular field, will hopefully open new exciting directions in both basic and biomedical biology.

Angiogenesis-related channelosomes

Among the channels that play remarkable roles in tumor vascularization, the aforementioned Transient Receptor Potential (TRP) proteins are the best known and their functions are regulated through interaction with many cellular proteins (70,80,81). The TRIP (Transient receptor potential channel-Interacting Protein) manually curated database has been recently developed, that aims to offer comprehensive information in the field (78,82,83). According to the broad considerations discussed in the previous chapter on interactome databases, great attention should be devoted in order to extract suitable unbiased information. Indeed, not all TRPs and their interactors are simultaneously expressed in a cell; PPI may occur only in a specific biological microenvironment; a TRP-binding protein concurrently interacts with multiple TRPs in a given cell at a specific condition; moreover, TRP-interacting proteins may competitively bind to the same region of a TRP channel. Given that interactions can dynamically modify the activities and subcellular locations of TRP, cell type- or cell context-specific PPI data are required to reveal the physiological relevance of TRP interactome in a more integrated viewpoint. Such data can be harvested by omics experimental approach under cell type- or cell context-specific conditions or computational integration of TRP channel PPI data with gene expression profile data.

TRP channels assemble into homomeric or heteromeric tetramers (84). The latter complexes are formed among the isotypes within or across subfamilies, suggesting a high-order complexity of TRP channel regulation in a physiologic context. Though not fully characterized, some evidence shows that new biophysical properties are created by heteromerization between TRP channel isotypes, including TRPC1-TRPC3, TRPC1-TRPC4, TRPC4-TRPC5, TRPC1-TRPC5, TRPC1-TRPP1, TRPC1-TRPV4, TRPV4-TRPP1, TRPV5-TRPV6, TRPML1-TRPML2, and TRPML1-TRPML3 (84,85). TRPC1 interacts physically with TRPV4 to form a complex that mediates flow-induced and store-operated calcium entry (SOCE) in primary mouse aortic and human HUVEC (86,87). Endothelial

SOCE involves signalplexes embodying Orai1, Stim1 and TRPC1 and regulates critical vascular processes, remodeling and angiogenesis (85,88).

Functional SOCE is also present in human circulating endothelial progenitor cells (EPCs), a sub-population of mononuclear cells that is recruited from either bone marrow or arterial wall to replace damaged/senescent EC and recapitulate the vascular network of lesioned organs. They act by either stimulating local angiogenesis *via* paracrine signaling or by physically engrafting within neovessels. SOCE controls proliferation and *in vitro* tubulogenesis in EPCs isolated from both peripheral and umbilical cord blood of healthy donors (N-EPC). Notably, SOCE is upregulated, mitogenic and protubulogenic for EPCs isolated from peripheral blood of patients affected by renal cellular carcinoma (RCC-EPC) (14,49,50,89). The enhanced SOCE in these cells is associated with the over-expression of Stim1, Orai1, and TRPC1 (49,50,89). N-EPCs possess TRPV4, which plays a master signaling role in mature healthy and tumor-derived endothelium, by controlling both vascular remodeling and arterial pressure (90). TRPV4 mediates cell migration of BTEC (but not HMEC) *via* arachidonic acid-activated actin remodeling (91). The role of TRPV4 in EPC is unknown, but it is not involved in the control of cell proliferation (90). Surprisingly, both N-EPC and RCC-EPC lack TRPC3, TRPC5 and TRPC6 that play critical functions in mature endothelium (11,12,16,17). Endothelial colony forming cells isolated from patients with primary myelofibrosis (PMF-ECFCs) undergo a distinctive remodeling of the Ca2+ machinery: Stim1, TRPC1, TRPC4, Orai3 and, perhaps, Orai2 proteins are up-regulated and, unlike N- and RCC-ECFC, the InsP3-dependent SOCE does not drive PMF-ECFC proliferation (92).

An overview of the current state and future directions of TRP channel network biology is reported in (78).

Interactions between ICT and cytoskeleton

Some ion channels and transporters bind to members of the large family of proteins that constitute the cytoskeleton (21,93). This sub-network underlies the endothelial mechanosensitivity and transduction during sprouting angiogenesis (21,94,95).

TRP channels. TRP channels selectively associate with the cytoskeleton (96). Many of them include ankyrin repeat domains, 33-residue motifs consisting of pairs of antiparallel α-helices connected by β-hairpin motifs, that link proteins, such as InsP3R or NHE, to the cytoskeleton. The occurrence of such domains is differently pronounced within the TRP family but their function is still poorly understood (70). The relationship between TRP proteins and cytoskeleton is bidirectional, with a role of ion fluxes in the rearrangement of the cytoskeleton structures and, in turn, the activity of the cytoskeleton and associated proteins in supporting the appropriate TRP targeting, PPI and channel gating (96).

Furthermore, TRP channels are part of macromolecular complexes including different signal transduction proteins involved in a variety of cell functions (77). A relevant example is the role of the scaffolding proteins belonging to the families of Homer and INAD that regulate TRPC channel gating (96). Intriguingly, TRP themselves can act as scaffolding proteins. Recent analysis of the function of TRPC4 in vascular EC of divergent phenotype revealed a novel aspect of TRPC signaling, extending the current concept of TRPC regulation by a phenotype-dependent switch between Ca2+ transport and a potential intracellular scaffold function of the TRPC protein (97). TRPC4 contains six transmembrane domains and its cytosolic C terminus includes several binding domains that tether TRPC4 to the membrane skeleton. A functional mammalian INAD homologous protein was identified by the characterization of peptide sequences of NHE Regulatory Factor (NHERF) and the subsequent identification of TRPC4 and TRPC5 as potential interaction partners (98). NHERF was originally isolated as a factor required for inhibition of NHE type 3 (NHE3) mediated by PKA (99). The protein has two PDZ domains, the first one recognizing peptides carrying T-R-L at the C-terminus. The

sequences of the mammalian TRPC4 and TRPC5 also end with the T-R-L motif. The activation mode of the TRPC4/5 complex is still an issue of controversy. Some groups proposed a store-depletion mechanism, while other laboratories reported the activation of TRPC4/5 to be dependent on receptor/G-protein/PLC activation, but independent of store depletion (100). PDZ1 of NHERF1 binds to TRPC4 and TRPC5 heterologously expressed proteins (98). The interaction of two partners with the same PDZ domain suggests a model in which NHERF forms a homodimer via PDZ2 and PDZ1 domains, bringing TRP channels (TRPC4, TRPC5) in proximity of PLC (98). Like INAD, which controls function, stability, and plasma membrane expression of Drosophila TRP, NHERF regulates TRPC4 targeting.

A model of TRPC4 embedded in its signaling scaffold has been proposed in pulmonary artery EC (PAEC) (101): TRPC4 interacts directly with protein 4.1 through its protein 4.1 binding domain, as well as with NHERF, linked to actin through ezrin-radixin-moesin (ERM) proteins. NHERF is a key scaffolding protein in that it can also bind directly to PLC and G-protein–coupled receptors through its PDZ domains. The signaling assembly is localized in cholesterol-rich caveolae containing caveolin-1, a binding partner for different ion channels (see below) (101). In the same cells TRPC4/Orai1 coupling controls TRPC1/4 activation and channel permeability, including Ca2+ selectivity, and the ensuing endothelial cell barrier function (102,103). It is worth noting that, although Orai1 expression is usually associated with its canonical function as a Ca2+ channel, growing evidence suggests that Orai1 and Orai3 proteins may be more important than Ca2+ influx to control proliferation of different cell lines (104). Intriguingly, thrombin-mediated decrease of transendothelial electric resistance (TER), a marker of endothelial barrier disruption, requires Stim1 independently of Orai1 and Ca2+ entry across the plasma membrane in HUVECs and HMVECs. The regulation of endothelial barrier function may not be due to Stim1-mediated interaction with TRPC channels. Rather, Stim1 seems to be required for RhoA activation, MLC phosphorylation, and formation of actin stress fibers (105). Other TRPC4 interactors are the large molecular weight immunophilins FKBP51 and FKBP52 that respectively

inhibit and enhance TRPC4-related SOCE in PAECs (106). As discussed below, TRPC4 is critically involved in the regulation of EC permeability by Thrombin (107). In HMEC TRPC4 is regulated by cell-cell contact formation and interacts with β-catenin, and the related Ca^{2+} entry acts in a specific endothelial state during the transition from a proliferating to a quiescent phenotype. Hence TRPC4 may adopt divergent functions in endothelial Ca^{2+} homeostasis and emerges as a potential key player in endothelial phenotype switching and tuning of cellular growth factor signaling (108).

Cyclic mechanical strain produced by pulsatile blood flow regulates the orientation of EC lining blood vessels and contributes to the control of angiogenesis. It has been reported that flow-induced stretching activates mechanosensitive TRPV4 that, in turn, stimulates PI3K-dependent activation and binding of additional integrin receptors, which promotes cytoskeleton remodeling and cell reorientation. Inhibition of integrin activation using blocking antibodies and knockdown of TRPV4 channels with specific siRNA suppress strain-induced capillary cell reorientation (109). A direct interaction between TRPV4, α2 integrin and the Src tyrosine kinase Lyn has been reported in sensory neurons (110), raising the possibility that TRPV4 could reside in a common mechanosignaling complex with extracellular matrix (ECM) receptors also in endothelium.

Other interactors of integrins are potassium channels. In particular, hERG1 are frequently found aberrantly expressed in tumors (see above) and control different aspects of the neoplastic cell physiology: they trigger and modulate intracellular signaling cascades through the assembly of multiprotein membrane complexes which also recruit integrin subunits and receptors for growth factors or chemokines (52,53,111). Therefore, hERG1 may be a key component of the 'functional hubs' that control angiogenesis in cancer. The microenvironment, through the functional interplay between integrins and hERG1, regulates angiogenesis and tumor progression possibly contributing to VEGF resistance (13,52,111,112). A signaling pathway that sustains angiogenesis and progression in colorectal cancer cell lines has been proposed in which β1 integrins and hERG1 channels form a functional plasma membrane complex able to recruit and activate PI3K and Akt. This event in turn increases the

Hypoxia Inducible Factor (HIF)-dependent transcription of VEGF-A and other tumor progression genes. Similarly, VEGFR-1 (FLT-1), β1 integrin, and hERG form a macromolecular signaling complex in acute myeloid leukemia (113).

Aquaporins. As mentioned before, water fluxes mediated by Aquaporins are involved in tumor angiogenesis. Presumably AQP1, which is specifically and strongly expressed in most EC of the microvasculature outside the brain (114), seems to act not only as a water channel but it contributes to cell migration (57,115). In two different cell lines expressing AQP1, the human melanoma cell line WM115 and the human microvascular cell line HMEC, the knock down of AQP1 promotes a re-organization of F-actin and affects the cell shape (115). In non-silenced cells F-actin is preferentially polarized at the leading edge of the plasma membrane and the cells produce tubules *in vitro* (115). In contrast, AQP1-silenced cells loose significantly their tubulogenic potential and decrease their ability to migrate (115). Actually, the polarization at the leading edge of migrating cells has been demonstrated for several transporters involved in migration, including Na+/H+ and Cl-/HCO3- exchangers and the Na+/HCO3- co-transporter, and cell migration involves the transient formation of lamellipodia and plasma membrane ruffles at the leading edge of the cell, suggesting that a rapid local changes in ion fluxes and cell volume are accompanied by rapid transmembrane water movement (116). Thereby, the actin cleavage and ion uptake at the tip of lamellipodium might create local osmotic gradients that drive the influx of water across the plasma membrane. Saadoun et al. postulated that water entry increases local hydrostatic pressure causing the polarization of AQP1 (57). The intracellular mechanisms triggered by AQP1 are not well known, but it has been recently shown that Lin-7 co-immunoprecipitates with AQP1 and interacts with β-catenin through Lin-7, affecting the organization of cytoskeleton (115). Intriguingly, the lack of AQP1 leads the Lin-7/ β-catenin complex to proteolytic degradation (115). Altogether, these data suggest that the physiological role of AQP1 goes beyond its water transport function. This protein rather emerges as a critical scaffold for a plasma membrane

associated multi-protein complex critical for endothelial cytoskeleton build-up, adhesion and motility (115).

Other TRP interactors.

A number of interacting proteins bind to different TRP members, such as of caveolin 1, calmodulin, Src, and IP3 receptor 3 (IP3R3). This sub-network offers the potential functional link to human diseases. For example, the oncogene Src interacts with nine TRP members, including TRPV1, TRPC6 and TRPV6: all of them are associated with cancer progression and the first two with tumor angiogenesis (12,16,17,78).

Caveolin-1 is a particular plasma membrane binding partner for a number of ion channels. A work conducted on HMEC and endothelium from CAV-$1^{-/-}$ mice reported that Cav-1 scaffold domain interacts with TRPC1 and IP3R3 to regulate SOCE (117,118). TRPC1 is embedded in a protein complex that can include IP3R, homer, calmodulin, caveolin-1, FKBP25, I-mfa, MxA, GluR1α, bFGFR-1, Gq/11 protein, phospholipase C-β/γ, protein kinase C-α and RhoA (119).

An integrated functional example is given by the role of TRPC1 in the regulation of thrombin-mediated endothelial permeability. Thrombin binds to the endothelial surface protease-activated receptor-1 (PAR-1) triggering a signaling cascade that results in the development of inter-endothelial junctional gaps that finally lead to an increase in endothelial permeability, the hallmark of tissue inflammation (120,121). The formation of these gaps is the result of cell-cell contact alteration: in particular, Ca^{2+} signaling is critical for PKCα activation in mediating disassembly of VE- cadherin junctions (122,123). Endothelial permeability is also decreased through a cell shape change induced by actinomyosin-mediated endothelial contraction: this endothelial 'rounding up' is mediated by Ca2+ entry via TRPC1 mediated by the monomeric GTP-binding protein RhoA (124). In summary, Rho activation signals interaction of IP3R with TRPC1 at the EC plasma membrane, and triggers Ca2+ entry following IP3-dependent store depletion (SOCE) and the resultant increase in endothelial permeability (124). It has been reported a similar causal link between the Gq-mediated increase in

cytosolic Ca2+ via TRPC6 and activation of RhoA, finally leading to the increase in endothelial permeability in response to Thrombin (125). This is an intriguing example involving an unusual scaffolding protein, Phosphatase and tensin homologue (PTEN). PTEN is a dual lipid-protein phosphatase that catalyzes the conversion of PIP3 to PIP2 and inhibits PI3K-Akt-dependent cell proliferation, migration, and tumor vascularization. Nonetheless, PTEN may play a role beyond suppressing PI3K signaling: in HPAEC it serves as a scaffold for TRPC6 through residues 394–403, enabling cell surface expression of the channel (126).

TRPC4-dependent Ca2+ entry is another critical determinant of this process: indeed, both Ca2+ entry and lung microvascular permeability in response to Thrombin are drastically reduced in TRPC4$^{-/-}$ mice (127). In addition to TRPC1, TRPC6 and TRPC4, also the melastatin-family transient receptor potential 2 (TRPM2) channel regulates vascular permeability. TRPM2 is an oxidant-sensitive Ca2+ permeable channel that mediates H2O2-induced Ca2+ entry and endothelial hyperpermeability (128). Oxidants generated by activated EC are also known to induce apoptosis, a pathogenic feature of vascular injury and inflammation from multiple pathogeneses. The proapoptotic signaling mechanism involves reactive oxygen species–induced protein kinase C-α activation resulting in phosphorylation of the short splice variant TRPM2 (TRPM2-S) that allows enhanced TRPM2-mediated gating of Ca2+ and triggers the apoptosis program. Strategies aimed at preventing the uncoupling of TRPM2-S from TRPM2 and subsequent Ca2+ gating during oxidative stress may mitigate endothelial apoptosis and its consequences in mediating vascular injury and inflammation (129). Oxidative stress also increases the expression of TRPM7. This protein has the peculiar dual ability to act as a magnesium/calcium permeable channel and as a kinase through its functional α-kinase domain at the carboxyl terminus. For this reason it has been called, together with TRPM2, 'chanzyme' (130). TRPM7 permeability for Mg2+ is intriguing: in particular, an inhibitory effect of hypomagnesaemia on tumor growth and neoangiogenesis is under investigation (131). TRPM7 channels are expressed and functional in human EC. In HMEC low Mg2+ inhibits proliferation and migration without affecting metalloprotease

production and tridimensional organization: this effect is mediated, at least in part, by the decrease of TRPM7, since its silencing mimics the effects of Mg deficiency, thus suggesting TRPM7 as a possible contributor to the regulation of angiogenesis (132). The opposite effect has been observed in HUVEC: two independent reports have shown that siRNAs transiently silencing TRPM7 stimulate cell proliferation, a behavior which is unique to HUVEC, because in other cell types the same manipulation induces cell cycle arrest. In addition to the significant elevation of TRPM7 in the vasculature of MgL mice (a model of inherited hypomagnesemia), the increase of TRPM7 transcript in HUVEC exposed to shear stress has been described (133). TRPM7 silencing promotes endothelial growth/proliferation and nitric oxide production via the ERK pathway (134). The enzymatic kinase activity of TRPM7 might influence the ERK pathway although it is still not clear whether inhibition of TRPM7 channel also affects its kinase activity.

Another ion channel interactor is the superfamily of G-proteins that are particularly relevant as a physical link to cell signaling (135). An interesting example is provided by the metabotropic glutamate receptor-1 (mGluR1) that may play a key role in regulating EC phenotype during tumor-induced angiogenesis. A loss of mGluR1 expression and activity is associated with an anti-angiogenic phenotype and tumor suppression (136). Despite the lack of information on PPI between mGluR1 and endothelial transportome this interaction seems likely since in Purkinje cells mGluR1 is physically associated and activates TRPC1 (137).

Interactions between channels and transporters

The functional/physical coupling of ion channels with transporters seems to contribute to the neovascularization of healthy and tumoral tissues. Some examples involve Na/Ca (NCX) and Na/H (NHE) exchangers, two broadly expressed carriers that regulate intracellular calcium and proton homeostasis respectively (138–140). In transfected HEK cells Na+ entry mediated by TRPC3 enables local Na+ accumulation that drives Ca2+ entry via reverse mode NCX. This functional interaction is probably mediated by a tight physical interaction between TRPC3 and NCX1, suggesting a close spatial proximity between these ion transport systems (141,142). In EC from excised rat aorta the reverse mode NCX mediates, in cooperation with ATP-sensitive potassium channels, K_{ATP}, calcium signals activated by hydrogen sulfide, a gaseous bioactive messenger that has been implicated in tumor angiogenesis (74,141,142). Intriguingly, reverse mode NCX could also be triggered by voltage-dependent sodium channels in endothelium. Voltage-gated Na^+ channels (Na_V) have long been considered as being characteristic of excitable cells: however, different Na_V isoforms have been found in non-excitable cancer cells and their function enhances cancer cell invasiveness (145,146). Nav 1.5 isoform is expressed and functional in HUVEC and mediates multiple angiogenic functions. In particular, it adjusts membrane potential and potentiates VEGF-induced ERK1/2 activation through the PKCα-B-RAF signaling (74,143,144). Accordingly, Ca2+ influx through reverse mode NCX is also required for the activation and targeting of PKCα to the plasma membrane, an essential step for VEGF-induced ERK1/2 phosphorylation and downstream EC functions in angiogenesis (147). Whether the voltage-gated sodium channel contribution to agonist-induced ERK1/2 activation is specific to VEGF and ECs remains to be elucidated.

In addition to NCX, voltage-gated sodium channels can also interact with the Na+/H+ exchanger NHE1. In highly invasive breast cancer cells and high-grade breast cancer biopsies, the overexpression of the $Na_V1.5$ isoform has been associated with extracellular matrix remodeling and the increased probability of developing metastases (145,146,148). The proteolytic activity of invadopodia is

highly dependent on the acidification of the peri-invadopodial extracellular compartment through the localized activity of NHE1 (140,149,150). NHE1 is known to have a substantial role in extracellular acidification and in the invasiveness of cancer cells. In highly invasive breast cancer cells MDA-MB-231, $Na_V1.5$ interacts with and allosterically increases NHE1 activity in a pHi range between 6.4 and 7.0: this interaction is localized in focal ECM degradation sites corresponding to caveolin-1-containing invadopodia (148). In addition, $Na_V1.5$ activity controls Src kinase activity, cortactin phosphorylation and actin cytoskeleton dynamics (148). NHE1 may be directly involved in angiogenesis. HIF-1 overexpression upregulates VEGF, NHE1 and calpains in HUVEC and thus enhances endothelial proliferation, migration, and tube formation (151). Furthermore, suppression of NHE1 reduces calpain-2 expression and activity, finally leading to inhibition of HIF-1-induced angiogenesis. In addition to its canonical function, NHE1 can act as an anchor for actin filaments to control the integrity of the cortical cytoskeleton (152). This occurs through a structural link between NHE1 and the actin-binding proteins ERM. NHE1 and ERM proteins associate directly and colocalize in lamellipodia. Notably, fibroblasts expressing NHE1 with mutations that disrupt ERM binding, but not ion translocation, have impaired organization of focal adhesions and an irregular cell shape. It could be interesting to investigate non-canonical NHE activities in angiogenesis.

Finally, although no data are available so far concerning the potential interaction between ICT and ion pumps as a component of angiogenic signaling, in other tissues some examples have been reported. In the brain and kidney TRPC6 and the Na+/K+ ATPase are part of a functional complex that may be involved in ion transport and homeostasis (153).

Conclusions

The post-genomic era is witnessing an explosive accumulation of biological data. At the same time, several public databases offer the opportunity to access such a wide range of biologically relevant literature on biomolecules and their expression, structure, location and interaction with other biological components. These novelties have attracted research focus towards data analysis and mining, in which the extraction of robust and suitable information from sparse and non-homogeneous sources is currently the main challenge. We often look at data- and hypothesis-driven methods as dichotomic strategies because they are hard to be integrated. Data-driven methods afford the view of the molecular makeup of biological systems and insight into biological phenomena but they are difficult to be combined to perform controlled experiments for testing hypotheses and, thus, to solve specific questions in biology. Therefore, the protein-protein network approach is a promising alternative that allows us to formulate testable hypotheses and to design validating experimental settings. In addition, since physiological and altered functions are regulated across many orders of magnitude in space and time, quantitative computational multi-scale modeling is increasingly adopted by researchers as a valuable descriptive and predictive tool in systems biology (154–158).

Unfortunately, the systems biology viewpoint is still poorly employed in the biology of transportome in cancer. A simple explanation of the delay is that the research focused on membrane transport has been far from the field of experimental oncology for long time. Only very recently these two domains converged and an increasing amount of experimental data is currently available on canonical and non-canonical roles of ion channels in tumor vascularization. Thus, ICT are still under-represented in interactomic databases when compared to other protein families with a longer tradition in oncology (receptors, kinases, phospholipase, transcription factors and others). Consequently, very few human proteins involved in membrane transportare annotated as components of cancer- and angiogenesis-associated signaling pathways, with the partial exception of TRP channels.

More theoretical and experimental effort will be needed to acquire a network perspective of transportome hopefully leading to a significant advancement in basic biology and oncology.

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