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TITOLO DELLA TESI:

Signal Transduction Modeling applied to Environmental-induced Switching in VEGF Biological Pathways

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To Mum, Dad and Andre

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PREFACE AND AIM OF THE WORK

Biologists have always been interested in understanding the basic features of biological processes and in ascertaining all aspects of the involved components. Years of careful experimental analysis have revealed that the cell is a complex system, whose activities are regulated by intracellular signal transduction implicating intricate circuits of multifunctional interactions which occur in a non-linear fashion. The behavior of a complex system is often difficult to grasp only through a combination of experimental data and intuition, because the potential of the experiments may often be limited and our reasoning tend to follow simple causal chain.

Recently, a challenging field, Systems Biology, has emerged as an important research discipline that holds promise to better understand the complexity and dynamics of cellular signaling. There are two prevailing interpretations of what Systems Biology is about: a) the integration of data, obtained from experiments at various levels and associated with the "omics family" of technologies, and b) the dynamic interactions of gene products, proteins and cells that determine cellular functions (Verlag, 2004). The first view is more an informatics perspective, developing tools for data integration and fusion, while the second approach deals with simulation and mathematical modeling based on experimental data; the first camp would often motivate their work by referring to a flood of data, while those interested in modelling of pathways are worried about the lack of quantitative, sufficiently rich data sets. This thesis is related to the second above mentioned approach regarding the dynamic modeling of signal transduction events.

So far, no standard for the development of a pathway model has been established, here we propose a suitable approach that consider the following strategy: 1) writing an initial conceptual model based essentially on the biological information derived from literature and containing the working hypotheses; 2) performing focused wet-biology experiments; 3) rewriting the conceptual model on the basis of experimental data readout; 4) translating the model into mathematical equations; 5) validating the mathematical model through the comparison with experimental data.

In this study a major effort was made to gather biological information relating to the signal transduction downstream of the considered growth factor/receptor system. A detailed collection of the best characterized pathways, reported here in the introductory section, has to be considered as a fundamental preliminary step in order to enable the construction of a biological model. In fact, since a quantitative model containing all the

signal transduction components is hard to obtain at the beginning, it is necessary to be able to identify which components play a role and which of them can be omitted. The selection of a small but sufficient number of key components enable the construction of a conceptual as well as a mathematical model that can be further implemented including additional level of complexity (e.g., other components in terms not only of signal proteins but also in terms of interactions and processes).

Despite the level of abstraction of a conceptual model, the resulting mathematical model has a predictive potential. Systems biologists are aware of this not only after the validation of the mathematical model, but especially during the phase of validation. In fact, the first level of prediction is manifested during the simulation studies based on the written system of equations. In this stage, the simulations give dynamics which are predictions of the system behaviour based on the considered reactions. The predicted dynamics can be compared with the experimental data, and if both data match then researchers will gain a useful confirmation of the model and suggest new hypothesis. However, what often happens is that the experimental and simulated data partially or incompletely match. This means that the simulations suggest/predict that the equations are incorrect or are not sufficient to describe the system. In this way the researchers return to the initial steps to modify the model or parameters values. The sequence of validation results in the convergence of mathematical modeling and measured parameters of the natural biological system. Finally, the mathematical model becomes a powerful tool to explore new hypothesis and to answer questions.

Recently, many mathematical models have been developed for signal transduction of growth factors, but Vascular Endothelial Growth Factor (VEGF), an important angiogenic factor, has not yet been studied with this approach. On the one hand, the ability of VEGF to activate multiple signal pathways resulting in different cellular behaviours make this growth factor an interesting subject for a system biology approach. On the other hand, its multifunctionality involves a considerable effort to create a reliable model capable to describe its complex biology.

Here it has been considered the intriguing possibility to study VEGF biology through a simplified model that can represent a basis for future implementation. This dissertation seeks to investigate VEGF signal transduction in Endothelial Cells (ECs). In particular the aim of this thesis is to understand the relationship between EC environment and VEGF-induced signal transduction events, in other words ascertaining determinants which can trigger different cellular responses.

In the future advanced models of VEGF intracellular network could offer an accurate and detailed understanding of the VEGF functions in EC biology and may subsequently provide a better knowledge of angiogenesis in order to define proper therapeutic strategies.

ABSTRACT

In healthy adult humans, most ECs are quiescent; in this condition, the cells are contact inhibited in their growth, protected from apoptosis and in full control of permeability. By contrast, in pathological conditions ECs change their behavior from quiescent to active. The initial response of quiescent ECs to induce angiogenesis is the activation of the permeability pathway, which is followed by EC migration, proliferation and formation of capillary tube network. Strong evidences support the crucial role of VEGF as a major regulator of multiple endothelial functions: survival, hyperpermeability, migration and proliferation.

Here VEGF biology was studied through a modelistic approach. First, it was written a conceptual model based on the available biological information and containing the working hypotheses. It was supposed that in normal conditions VEGF could contribute to maintain the survival of quiescent ECs and it was considered that in pathological state, in addition to the survival effect, VEGF is able to induce hyperpermeability in quiescent ECs and proliferation in cells which are characterized by the absence of mature cell-to-cell junctions. After that, focused wet-biology experiments were performed to rewriting the model in *a posteriori* form supported by quantitative data. Finally, the model was translated into mathematical equations and validated through the comparison with experimental data.

The model offers a quantitative, dynamic representation of early intracellular signals elicited by VEGF in ECs. It describes how VEGF-induced responses are dependent on the growth factor concentration and the presence of mature cell-to-cell junctions. In particular it proves that VEGF can be considered a survival factor for quiescent ECs as well as a potent angiogenic regulator in pathological conditions. The model is suitable for the addition of further levels of complexity to better understand VEGF biology in order to help the design of strategies for therapeutic purpose.

INTRODUCTION

Biological Background

Angiogenesis and Endothelial Cells

Humans are complex multicellular organisms, and all cells require a dependable, finely controlled supply of oxygen, nutrients, and signaling molecules and the removal of carbon dioxide and metabolic end products. The diffusion of oxygen through tissues is limited to 100 to 200 μm; therefore, a highly developed vascular system has evolved to ensure that all cells are within this distance of a supply of oxygen.

Neovascularization, or new blood vessel formation, is divided into two components: vasculogenesis and angiogenesis. Embryonic or classical vasculogenesis is the process of new blood vessel formation from hemangioblasts that differentiate into blood cells and mature ECs (Dvorak, 2005). In the embryo and yolk sac, early blood vessels develop by aggregation of angioblasts into a primitive network of simple endothelial tubes (Coultas et al., 2005). As primitive vessels are remodelled into a functioning circulatory system, they undergo localized proliferation and regression, as well as branching and migration. In contrast, angiogenesis is the process of new blood vessel formation from pre-existing vascular networks by capillary sprouting. During this process, mature ECs divide and are incorporated into new capillaries.

Adult human vascular ECs constitute an estimated 1 kg of tissue and line the vessels of every organ (Cines et al., 1998); these ECs correspond to an estimated surface area of 1000 m^2. In adult humans, most ECs are quiescent; only 1 in every 10,000 ECs is in the cell division cycle at any one time (Hanahan and Folkman, 1996). However, there is an increased rate of EC mitosis and angiogenesis during wound healing and tissue repair, during ovarian corpus luteum formation, and during placental development establishing pregnancy (Ferrara, 2004). Inhibition of angiogenesis represents a potential therapy for disorders with non-physiological angiogenesis including neovascular age-related macular degeneration of the eye, diabetic retinopathy, endometriosis, psoriasis, rheumatoid arthritis, and tumor growth and metastasis (Ferrara, 2004). Deciphering the mechanisms of developmental, physiological, and aberrant angiogenesis has assumed considerable biomedical importance during the past 35 years.

In tumors and elsewhere, angiogenesis is an intricate process that involves interactions between regulatory and effector molecules. Pepper divided classical angiogenesis into a phase of sprouting and a phase of resolution (Pepper, 1997). The phase of sprouting consists of six components: (i) increased vascular permeability and extravascular fibrin deposition, (ii) vessel wall disassembly, (iii) basement membrane degradation, (iv) cell migration and extracellular matrix invasion, (v) EC proliferation, and (vi) capillary lumen formation. The phase of resolution consists of five components: (i) inhibition of EC proliferation, (ii) cessation of cell migration, (iii) basement membrane reconstitution, (iv) junctional complex maturation, and (v) vessel wall assembly including recruitment and differentiation of smooth muscle cells and pericytes, both of which are mural cells (mural, wall).

Angiogenesis, which is regulated by both endogenous activators and inhibitors, is under stringent control (Hanahan and Folkman, 1996). Under most physiological conditions in adults, the action of negative regulators predominates and angiogenesis is quiescent. Under certain pathological conditions, for example, during tumor progression, the vasculature undergoes the so-called angiogenic switch, the action of positive regulators predominates, and angiogenesis is active (Hanahan and Folkman, 1996). VEGF is the most studied regulator of angiogenesis, and it is the central focus of this PhD thesis. Work done by several laboratories over the last 2 decades has elucidated the pivotal role of VEGF in the regulation of normal and abnormal angiogenesis. VEGF potential is due to its ability to regulate most of the steps in angiogenic cascade.

VEGF Family, Isoforms and Relative Receptors

The VEGF gene family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PLGF) (Ferrara, 2004; Hoeben et al., 2004). These glycoproteins belong to a structural superfamily of growth factors which includes PDGF. VEGF-A is mainly involved in angiogenesis while VEGF-C and VEGF-D are involved in lymphangiogenesis. The human VEGF-A gene is organized into eight exons and alternative exon splicing results in at least 5 different isoforms, the more common isoforms consisting of 121, 145, 165, 189, 206 amino acids, (termed VEGF-A$_{121}$, VEGF-A$_{145}$ VEGF-A$_{165}$, VEGF-A$_{189}$, VEGF-A$_{206}$, respectively) (Tischer et al., 1991). Other isoforms have also been reported consisting of 148, 162 and 183 amino acids (termed VEGF-A$_{148}$, VEGF-A$_{162}$, VEGF-A$_{183}$) and a more recently identified variant of

VEGF-A$_{165}$, which is termed VEGF$_{165b}$. VEGF-A$_{165}$ is the most predominant isoform and is also the most potent in terms of stimulating angiogenesis (Neufeld et al., 1999; Neufeld et al., 1996).

VEGF binds to the three receptor tyrosine kinases (RTKs), flt-1 (fms-like tyrosine kinase, VEGFR-1), Flk-1/KDR (fetal liver kinase 1- murine homologue/Kinase insert Domain containing Receptor-human homologue, VEGFR-2) and flt-4 (VEGFR-3). VEGFR-1 and VEGFR-2 are primarily found on the vascular endothelium whereas VEGFR-3 is mostly found on the lymphatic endothelium. These receptors all have an extracellular domain, a single transmembrane region and a consensus tyrosine kinase sequence interrupted by a kinase-insert domain (Shibuya et al., 1990; Terman et al., 1991). More recently neuropilin (NRP-1), originally identified as a receptor for the semaphorin/collapsing family of neuronal guidance mediators, was shown to act as a receptor for VEGF (Soker et al., 1998).

VEGFR-1 is a 180 kD transmembrane protein, which binds VEGF-A, PLGF and VEGF-B (Park et al., 1994). Alternative splicing produces a shorter soluble form (sVEGFR-1) which can act as an inhibitor of VEGF (Tanaka et al., 1997). The affinity of VEGFR-1 for VEGF is ten-fold higher than VEGFR-2 but its tyrosine kinase activity is ten-fold weaker than VEGFR-2. VEGFR-1 can also act as a decoy receptor preventing VEGF binding to the more mitogenic receptor VEGFR-2. In addition to ECs, VEGFR-1 is also expressed by monocytes, osteoblasts, macrophages, pericytes, hematopoietic stem cells, vascular smooth muscle cells and more recently VEGFR-1 was identified on colorectal tumour cells (Barleon et al., 1996; Fan et al., 2005; Ishida et al., 2001; Zachary and Gliki, 2001).

VEGFR-2 was first identified from a human EC cDNA library (Terman et al., 1991). It is a 230 kD glycoprotein and it binds VEGF, VEGF-C and VEGF-D and has a lower affinity for VEGF-A than VEGFR-1. However, using novel highly selective VEGF mutants with substantially increased selectivity for either VEGFR-1 or VEGFR-2, it was demonstrated that VEGFR-2 is the primary mediator of VEGF-A signaling (Gille et al., 2001). In addition to ECs, hematopoietic stem cells, megakaryocytes, retinal progenitor cells and vascular smooth muscle cells express VEGFR-2. More recently, along with VEGFR-1, VEGFR-2 has been identified on some tumour cell lines, non-small cell lung carcinomas (NSCLCs), breast, neuroblastoma and gastric cancer cells (Ishida et al., 2001; Meister et al., 1999; Neufeld et al., 1999; Price et al., 2001; Tian et al., 2001).

VEGFR-3 (flt-4) is a 170 kD glycosylated protein which was first cloned from human erythroleukemia cells and placental cDNA libraries. It binds the full length and mature forms of VEGF-C and is expressed in embryonic ECs but during development its expression on blood vessels decreases and becomes restricted to the lymphatic endothelium in adult tissue (Kaipainen et al., 1995).

Neuropilin-1 (NRP-1), initially identified on neuronal cells, is also expressed in ECs where it acts as an isoform specific receptor for VEGF-A$_{165}$ (Soker et al., 1998). NRP-1 lacks an intracellular tyrosine kinase domain and therefore must act in conjunction with other receptors to mediate VEGF signaling. NRP-1 can associate with both VEGFR-1 (Fuh et al., 2000) and VEGFR-2 to transduce a signal (Whitaker et al., 2001); indeed VEGFR-2 interacts with NRP-1 to form a receptor complex that can enhance binding of VEGF-A$_{165}$ (Soker et al., 2002).

VEGF Multifunctionality: Signaling/Biological Effect Overview

Among VEGF family, VEGF-A (hereafter referred to as VEGF if other isoforms are not specified) plays a central role in angiogenesis. As above mentioned, while in ECs VEGF binds to VEGFR-1 and VEGFR-2, most biologically relevant VEGF signaling are mediated via VEGFR-2 and considerable progress has recently been made towards delineating the signal transduction pathways distal to activation of VEGFR-2.

VEGF induces the dimerization of VEGFR-2 that leads to receptor autophosphorylation and activation. Autophosphorylation occurs in *trans:* one kinase of the dimmer catalyzes the phosphorylation of tyrosine residues in the second, and the second catalyzes the phosphorylation of tyrosine residues in the first. Among the 19 tyrosine residues present in the intracellular domain of VEGFR-2, seven putative phosphorylation sites have been described in some detail to date: Tyr801, 951, 996, 1054, 1059, 1175 and 1214. A comprehensive study of receptor activity based on in vitro phosphorylation of immunoprecipitated VEGFR- 2, on receptor mutagenesis and on in vivo mapping with phosphorylation site-specific antibodies identified Tyr951, 1054, 1059, 1175 and 1214 as the most prominent phosphorylation sites and Tyr1305, 1309 and 1319 as minor sites, while Tyr801 and 996 phosphorylation was not detected in this study (Matsumoto et al., 2005). In regard of the seven autophosphorylation sites: Tyt801 is located in the juxtamembrane domain, Tyr951 and Tyr996 are in the kinase insert, Tyr1054 and Tyr1059 are in the kinase domain, and Tyr1175 and Tyr1214 are in the C-terminal tail. Autophosphorylation of tyrosine residues Tyr1054 and 1059 within

the activation loop of the kinase domain stimulates catalytic activity; Tyr801 located in the juxtamembrane domain may be phosphorylated following dimerization of VEGFR-2 and maintain the receptor in an active conformation (Hubbard, 2004); autophosphorylation of tyrosine residues at other locations generates docking sites for modular Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains that recognize phosphotyrosine in sequence-specific contexts.
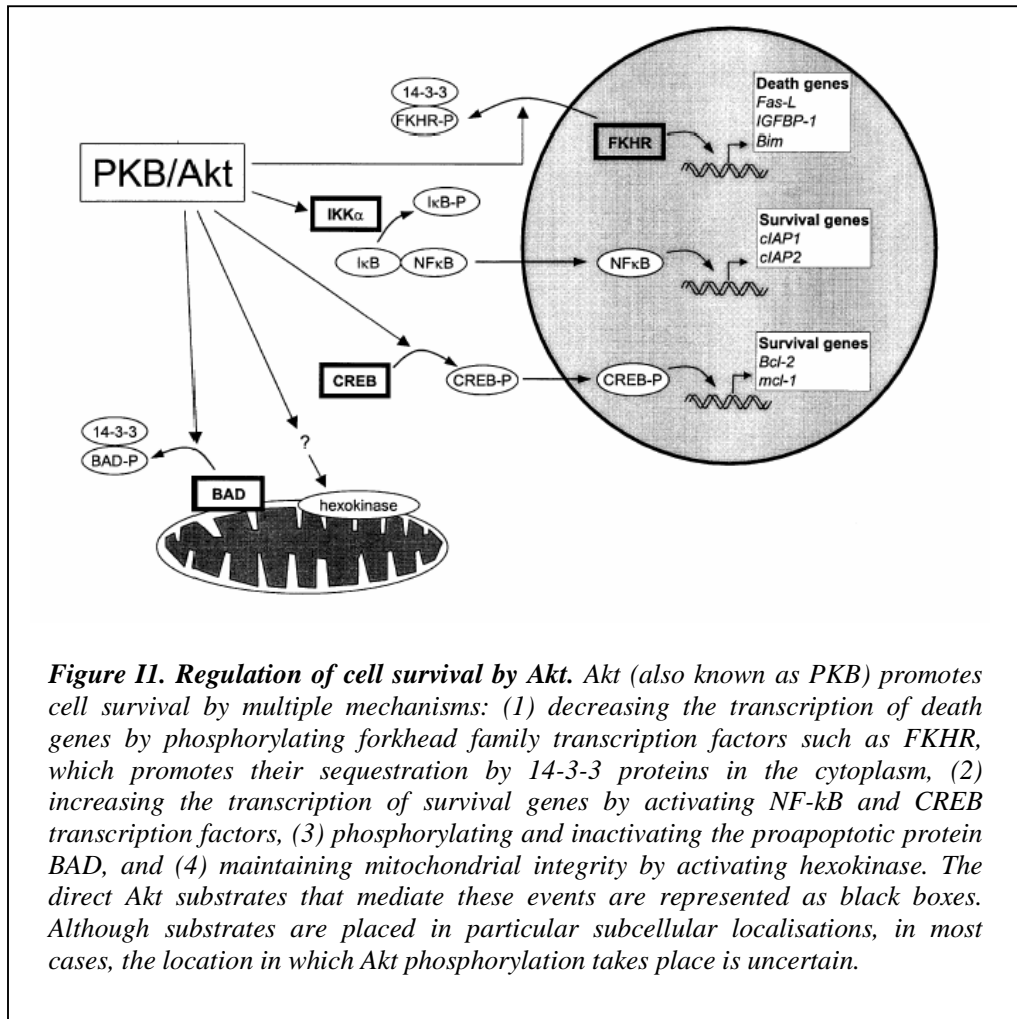
Although there are some evidences for the direct recruitment of certain signal proteins to a specific phosphotyrosine residue, the function of most of these sites has not been defined. Tyr1175 is clearly the most important site implicated in activation of many pathways via PLCγ (Takahashi et al., 2001). Indeed, most studies on VEGF signaling agree that receptor recruitment of PLCγ upon phosphorylation of Tyr1175 is essential for stimulation of mitogenesis.

VEGF was initially recognized as a factor which increased vascular permeability, and it is now apparent that this cytokine cause a complex and integrated networks of signaling pathways which stimulate survival, proliferation and migration of ECs in addition to enhanced vascular permeability. The following sections represent a careful collection of the best characterized VEGF-induced signal transduction mechanisms, reported here in detail in order to achieve an overview of the complexity of VEGF signal transduction. As above mentioned gathering detailed biological information is the first step of biological model design, allowing the creation of a conceptual schema characterized by the simplest possible representation of the biology of interest. The following sections are divided on the basis of a single cellular behaviour effect, although it will be evident that common signal proteins and their activities are involved in more than one cellular readout.

VEGF and Survival

Several reports have implicated VEGF as a major survival factor for ECs, both *in vitro* and *in vivo* (Benjamin et al., 1999; Gerber et al., 1998a; Gerber et al., 1998b; Yuan et al., 1996). *In vitro*, VEGF prevents apoptosis induced by serum starvation. Using an *in vitro* model system of serum-starved human ECs isolated from umbilical veins (HUVECs), Gerber and coworkers first reported that such activity is mediated by the PI3K-Akt pathway (Gerber et al., 1998b). In the same *in vitro* model, VEGF also induced expression of the anti-apoptotic proteins Bcl-2 and A1 (Gerber et al., 1998a). In addition, Tran and colleagues demonstrated that VEGF can also mediate the

induction/upregulation of members belonging to a family of antiapoptotic proteins, namely the Inhibitors of Apoptosis (IAP); in particular VEGF was reported to upregulate the antiapoptotic proteins XIAP and survivin HUVECs; moreover VEGF-dependent upregulation of survivin could be prevented by cell cycle arrest in the G1 and S phases (Tran et al., 1999). See Figure I1 for an illustration of multiple mechanisms regarding the regulation of cell survival by Akt.

Figure I1. Regulation of cell survival by Akt. *Akt (also known as PKB) promotes cell survival by multiple mechanisms: (1) decreasing the transcription of death genes by phosphorylating forkhead family transcription factors such as FKHR, which promotes their sequestration by 14-3-3 proteins in the cytoplasm, (2) increasing the transcription of survival genes by activating NF-kB and CREB transcription factors, (3) phosphorylating and inactivating the proapoptotic protein BAD, and (4) maintaining mitochondrial integrity by activating hexokinase. The direct Akt substrates that mediate these events are represented as black boxes. Although substrates are placed in particular subcellular localisations, in most cases, the location in which Akt phosphorylation takes place is uncertain.*

In general, PI3K is considered as one of the most important regulatory proteins, being involved in a number of diverse signaling pathways and controlling the main functions of the cell. PI3K is a heterodimer of two subunits, catalytic and regulatory, with molecular weights of 110 kD (p110) and 85 kD (p85), respectively. It is supposed that due to the ability of the regulatory p85 subunit to interact with both the catalytic p110 subunit and RTKs directed membrane targeting of p110 occurs, initiating complex formation between the enzyme and its phospholipidsubstrate phosphatidylinositol-4,5-

bisphosphate (PIP2). Following its recruitment in the plasma membrane, PI-3K phosphorylates PIP2 on the 3-OH group generating the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3). This recruits Akt to the membrane where it is activated trough phosphorylation. For an illustration of the activation mechanism of PI3K-Akt see Figure I2.
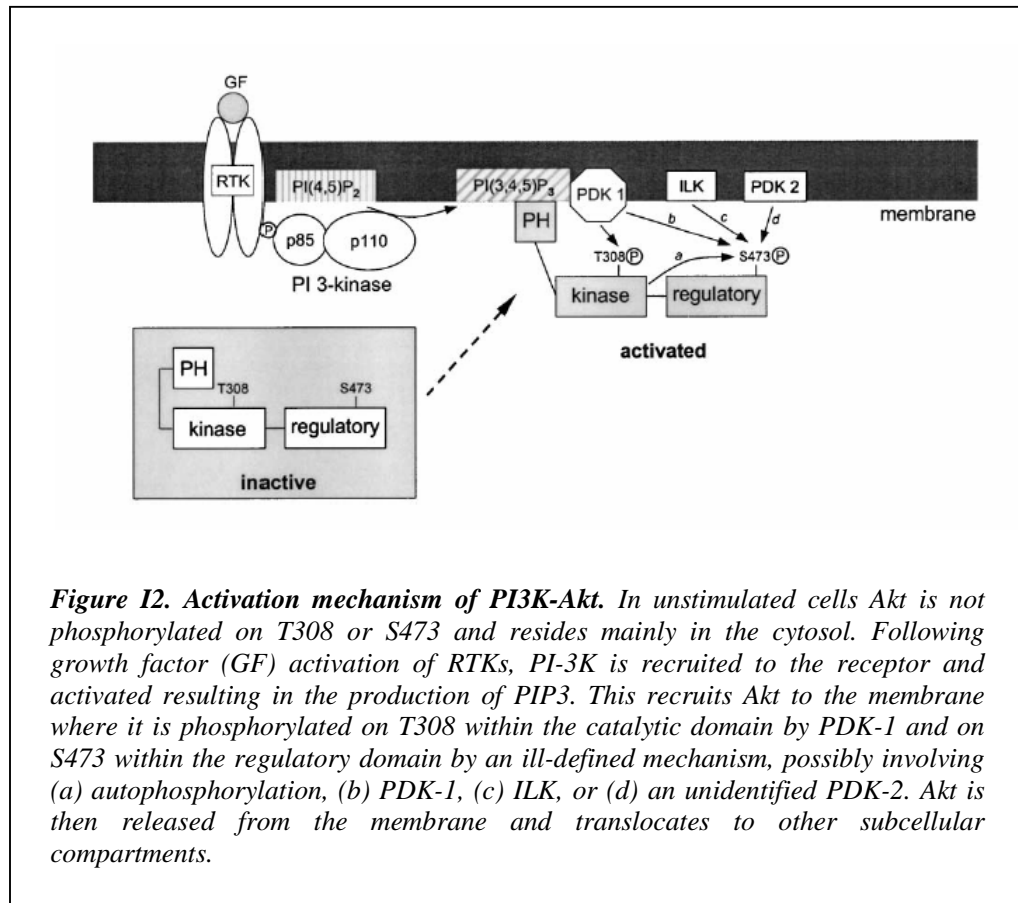

Figure I2. Activation mechanism of PI3K-Akt. *In unstimulated cells Akt is not phosphorylated on T308 or S473 and resides mainly in the cytosol. Following growth factor (GF) activation of RTKs, PI-3K is recruited to the receptor and activated resulting in the production of PIP3. This recruits Akt to the membrane where it is phosphorylated on T308 within the catalytic domain by PDK-1 and on S473 within the regulatory domain by an ill-defined mechanism, possibly involving (a) autophosphorylation, (b) PDK-1, (c) ILK, or (d) an unidentified PDK-2. Akt is then released from the membrane and translocates to other subcellular compartments.*

Despite the amount of data sustaining a role for the PI3K-Akt pathway in VEGF signaling, the mechanisms coupling VEGFR-2 to this pathway remain unclear. Several report showed coimmunoprecipitation of VEGFR-2 and p85, but there are no evidences for a direct association (Cunningham et al., 1995; Guo et al., 1995; Thakker et al., 1999). It is likely that adaptor proteins may be responsible for the coupling of VEGFR-2 to PI3K. In facts, it was reported that adapter proteins such as VEGF receptor associated protein (VRAP/TSAd) and Shb promote PI3K activation downstream of VEGFR-2 (Holmqvist et al., 2004; Matsumoto et al., 2005; Wu et al., 2000b). However, none of these proteins display a single consensus motif required to bind p85 SH2 domains, implying that their participation in PI3K activation is an indirect consequence of their

ability to induce the formation of large signaling complexes. Recently Dance and coworkers suggested that Gab1 is most likely the primary mediator of PI3K activation downstream of VEGFR-2; in particular, they showed a VEGF-induced physical association of endogenous Gab1 with p85 in combination with PI3K activity, in addition they reported a direct association between Gab1 and p85, and the requirement of Gab1 interaction with both the adaptor protein Grb2 and PIP3 for Gab1 recruitment by VEGFR-2 (Dance et al., 2006).

A strong body of evidences indicates that survival signal in ECs may be highly influenced by the presence of cell-to-cell junctions. Carmeliet and colleagues demonstrated that VE-cadherin (VEC), an endothelial cell-specific adherence junction (AJ) component, is required for the survival signal of VEGF (Carmeliet et al., 1999). When polyomavirus middle-T antigen-immortalized $VEC^{+/+}$ and $VEC^{\delta C/\delta C}$ (a mutant VEC gene lacking the β-catenin-binding cytoplasmic tail) ECs were cultured in the absence of serum, $VEC^{\delta C/\delta C}$ ECs exhibited increased apoptosis; interestingly, in a PI3K dependent manner, VEGF rescued survival of $VEC^{+/+}$ but not of $VEC^{\delta C/\delta C}$ ECs. Indeed, immunofluorescent labelling showed that VEGFR-2 was present at intercellular junctions in $VEC^{+/+}$ cells but not in mutant endothelial cells, and coimmunoprecipitation experiments revealed an association between VEC, β-catenin, PI3K and VEGFR-2 in $VEC^{+/+}$ but not in $VEC^{\delta C/\delta C}$ cells. Furthermore, VEGF increased phospho-Akt levels in $VEC^{+/+}$ but not in $VEC^{\delta C/\delta C}$ cells and phospho-Akt levels were also increased by VEGF in confluent HUVECs treated with control but not with VEC antibodies. Thus, when ECs present mature cell-to-cell junction, a multicomponent complex comprising VEC, β-catenin, VEGFR-2, and PI3K appears to be required for the endothelial survival function of VEGF through activation of Akt. Disruption of this complex by truncation of VEC (which abolishes association with β-catenin) or by VEC antibodies (which prevents clustering of VEC) renders ECs refractory to the VEGF survival signal.

Recently, through gene profile analysis, Spagnuolo and coworkers identified a member of growth arrest-specific (Gas) gene, Gas1, as a gene markedly up-regualated by VEC and VEGF in ECs; interestingly, in this report, Gas1 upregulation has been correlated with cell survival effect (Spagnuolo et al., 2004). In particular, they showed that in HUVECs VEC clustering *per se* induced Gas1 overexpression and VEGF also upregulated Gas1 both in confluent and sparse cells, moreover VEGF-induced upregulation of Gas1 was augmented more so in confluent cells than in sparse cells,

suggesting an additive effect; furthermore they demonstrated that activation of PI3K was needed for Gas1 synthesis but not for its activity in cell survival.

Many studies have demonstrated that integrins, a family of cell adhesion receptors, as important transducers of ECM signals, maintain EC survival. The integrins are heterodimer complex of two transmembrane subunits, α and β, which represent a link between extracellular matrix (ECM) adhesion proteins and the intracellular machinery (both signal proteins and cytoskeleton components). If the integrins cannot interact with the ECM, the ECs will no longer receive the survival signal from the ECM and will rapidly undergo apoptosis. In facts, several studies have demonstrated that integrin-mediated cell adhesion can prevent apoptosis (Brooks et al., 1995; Frisch and Francis, 1994; Meredith et al., 1993). Brooks and associates examined a SCID mouse/human chimeric model with transplanted full thickness human skin containing $\alpha v\beta 3$-negative human breast tumor cells; intravenous administration of a monoclonal antibody against $\alpha v\beta 3$ either prevented tumor growth or markedly reduced tumor cell proliferation by causing increased apoptosis of angiogenic blood vessels (Brooks et al., 1995). Stromblad and associates further elucidated this apoptotic pathway; these investigators showed that *in vitro* the integrin $\alpha v\beta 3$ suppressed p53 activity, blocked $p21^{WAF1=CIP1}$ expression, and increased the bcl-2/bax ratio, thereby promoting cell survival and suppression of the bax cell death pathway (Stromblad et al., 1996). Given integrin $\alpha v\beta 3$ as an important survival system for nascent blood vessels during angiogenesis, it is of interest the finding that in HUVECs VEGFR-2 associates selectively with $\alpha v\beta 3$ and that VEGFR-2 activity is enhanced by endothelial adhesion to the $\alpha v\beta 3$ ligand, vitronectin (Soldi et al., 1999).

The non-receptor protein tyrosine kinase, focal adhesion kinase (FAK), plays a key role in integrin dependent signaling (Zachary and Rozengurt, 1992) and is strongly implicated in the maintenance of survival signals in several adherent cell types including ECs (Abedi and Zachary, 1997; Ilic et al., 1998). Consistent with a role for FAK in VEGF survival signaling, VEGF increases tyrosine phosphorylation and focal adhesion association of FAK and the FAK-associated protein paxillin in HUVECs (Abedi and Zachary, 1997; Ilic et al., 1998; Rousseau et al., 2000; Wu et al., 2000a).

In the contest of FAK involvement in VEGF-induced cell survival, Abu-Ghazaleh and coworkers identified Tyr861 and 397 as major sites of FAK phosphorylation in HUVECs and demonstrated that VEGF promotes a selective and Src-dependent FAK phosphorylation at Tyr861 leading to an antiapoptotic effect (Abu-Ghazaleh et al., 2001). Furthermore they suggested that VEGF may signal independently through the
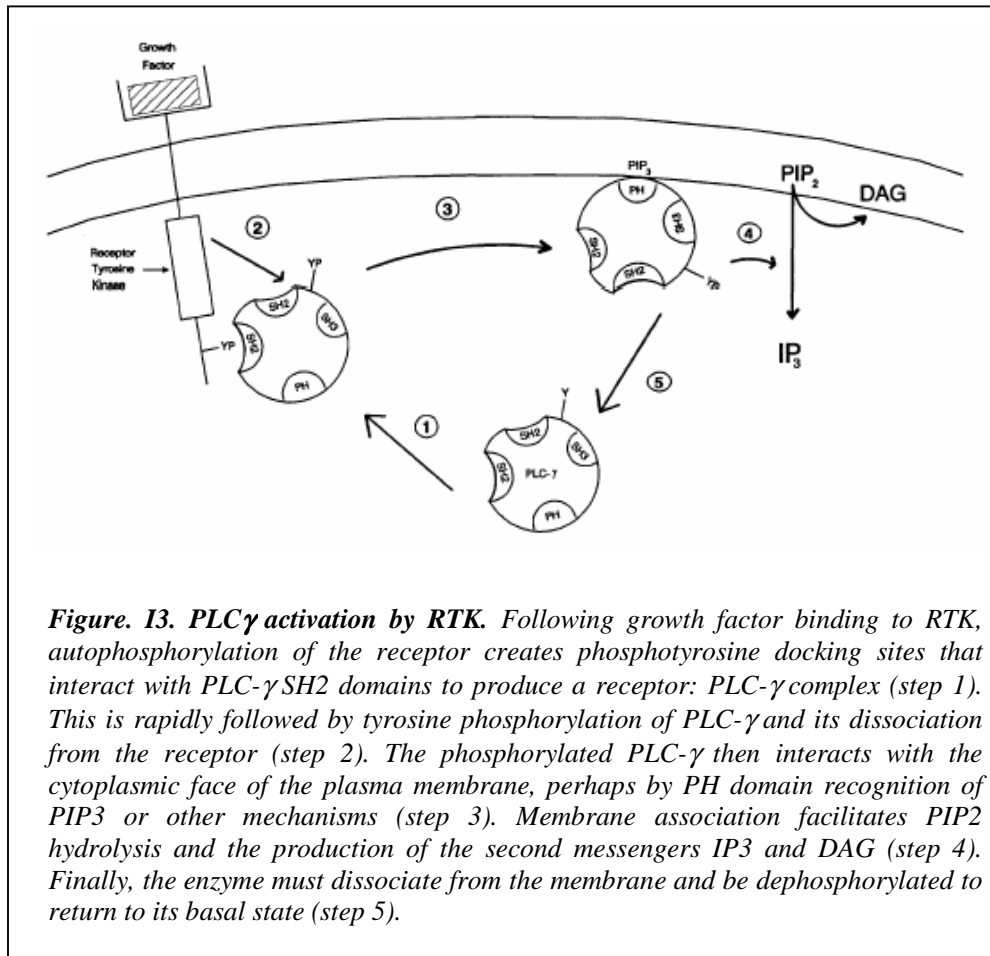
Src-FAK and PI3K-Akt pathway in order to trigger cell survival. Indeed, their results indicate that, whereas a major effect of PP2 (the specific inhibitor of Src) is inhibition of FAK Tyr861 phosphorylation, decreased FAK expression and increased FAK cleavage are likely to be important in apoptosis mediated through the inhibition of PI3K. Interestingly, the finding that PI3K inhibition was markedly apoptotic and induced FAK cleavage in the absence of VEGF strongly suggests that the maintenance of basal PI3K-Akt activity is an important survival mechanism in HUVECs. This work suggests that inhibition of the Src-FAK and PI3K-Akt pathways has distinct consequences for cell survival: in the case of Src-FAK, inhibition leads to FAK dephosphorylation and features of early apoptosis, whereas PI3K-Akt inhibition causes FAK instability and full-blown programmed cell death.

Recently 3 orthologues of the forkhead transcription factor DAF-16 (FKHR/FOXO1, FKHRL1/FOXO3, and AFX/FOXO4) were identified in mammalian cells and shown to be substrates for Akt; subsequent studies uncovered a critical role for the forkhead family of transcription factors in coupling extracellular signals to downstream changes in gene expression leading to survival effects. Abid et al. examined whether VEGF is coupled to PI3K/Akt/forkhead in ECs (Abid et al., 2004). They showed for the first time that VEGF regulates the phosphorylation, subcellular localization, and transcriptional activity of endogenous forkhead transcription factors in ECs through a PI3K/Akt-dependent mechanism. In particular they reported the following findings: incubation of HUVECs or human coronary artery ECs (HCAECs) with VEGF results in the phosphorylation of endogenous FKHR at Ser256 and AFX at Ser193; VEGF induces PI3K-dependent phosphorylation of FKHR at Thr24 (tested in HCAECs); in HCAECs VEGF stimulation results in the FKHR, FKHRL1, and AFX exclusion from the nucleus (PI3K-dependent exclusion from the nucleus was tested and confirmed for FKHR in HCAECs; a phosphorylation-resistant form of FKHRL1 was constitutively localized to the nucleus in HCAECs); in cotransfection studies in HUVECs, VEGF reduced the transactivation potential of FKHRL1 in a PI3K-dependent manner.

VEGF and Proliferation

Most of the representative RTKs, such as EGF receptor, are known to utilize Ras activation pathway toward MAP kinase activation; for such Ras activation, Shc-Grb2-Sos or Grb2-Sos pathway is directly involved in this system. However, VEGFR-2 was

not found to utilize the Ras-activation pathway, or only very little of it, if any, and most of the MAP kinase activation was mediated via the PLCγ-PKC pathway. Takahashi and colleagues reported that in HUVECs PLCγ is directly bound to the autophosphorylated VEGFR-2, then tyrosine phosphorylated and activated; the activated PLCγ catalyzes PIP2 into IP3 and diacylglycerol (DAG), which in turn activates PKC, particularly the PKCβ form, that activated Raf-1 to MAP kinase cascade (Takahashi and Shibuya, 1997; Takahashi et al., 1999). See Figure I3 for an illustration of PLCγ activation by RTK. Activated Raf-1 phosphorylates and stimulates MAP kinase kinase, or MEK, which in turn phosphorylates and stimulates MAP kinase, or ERK. Activation of ERK stimulates downstream signaling, including activation of ternary control factor, c-Fos, and Elk-1. These events promote transcription and activation of AP-1 promoter, which in turn initiates DNA synthesis and proliferation (Whitmarsh and Davis, 1996).

Figure. I3. PLCγ activation by RTK. *Following growth factor binding to RTK, autophosphorylation of the receptor creates phosphotyrosine docking sites that interact with PLC-γ SH2 domains to produce a receptor: PLC-γ complex (step 1). This is rapidly followed by tyrosine phosphorylation of PLC-γ and its dissociation from the receptor (step 2). The phosphorylated PLC-γ then interacts with the cytoplasmic face of the plasma membrane, perhaps by PH domain recognition of PIP3 or other mechanisms (step 3). Membrane association facilitates PIP2 hydrolysis and the production of the second messengers IP3 and DAG (step 4). Finally, the enzyme must dissociate from the membrane and be dephosphorylated to return to its basal state (step 5).*

Although most studies on VEGF signaling agree that receptor recruitment of PLCγ is essential for stimulation of mitogenesis, it is possible that VEGF can trigger

cell proliferation through different pathways: the majority are PLCγ dependent, whereas some are sensitive to Ras (Meadows et al., 2001).

Shu and associates extended the results of previous studies by demonstrating that sphingosine kinase (SPK) links PKC to Ras activation (Shu et al., 2002). They showed that VEGF induced SPK activity in HUVECs and T24 bladder tumour cell line; VEGF stimulation of T24 cells caused a slow and sustained accumulation of Ras-GTP (also verified in HUVECs) and ERK1 activation; purified PKC phosphorylated and activated recombinant SPK1; PKC and SPK inhibitors prevented Ras-GTP accumulation in HUVECs and T24 cells; small interfering RNA (siRNA) that targets SPK1 blocked VEGF-induced accumulation of Ras-GTP and ERK activation in T24 cells; VEGF stimulation of ERK phosphorylation was unaffected by dominant-negative Ras-N17 in T24 cells; Raf kinase inhibition blocked VEGF-stimulated accumulation of phospho-ERK1. They suggested that VEGF induces DNA synthesis in a pathway which sequentially involves PKC, SPK, Ras, Raf, and ERK1. Of interest the authors proposed that membrane-associated sphingosine in cells attenuates the basal Ras activity by stimulating Ras-GAP activity and that VEGF stimulation results in the conversion of sphingosine to sphingosine-1-phosphate (S1P) such that S1P levels are sufficient to displace sphingosine from GAP; overall, this would decrease GAP activity and increase the level of activated Ras-GTP without involving Ras-GEFs. This could be a possible explanation for the discrepancy over whether Ras is or is not involved in VEGF signaling. In facts some studies have relied on the use of dominant-negative RasN17 in concluding that Ras is not involved in VEGF signaling events; however, RasN17 does not directly block Ras function but rather the GEFs that can activate Ras.

Hood and coworkers minimized the importance of PKC in the VEGF-induced activation of Raf-1 in favour of a critical role of cGMP-dependent protein kinase (PKG) (Hood and Granger, 1998). They started by considering that VEGF induces nitric oxide (NO) production through NO synthase (NOS) and that NOS inhibitors block proliferation induced by VEGF in addition to hyperpermeability (see the section "VEGF and Permeability"); then they examined the mechanisms by which NO and its downstream signals (NO activates soluble guanylate cyclase ensuing conversion of GTP to cGMP, which in turn activates PKG) mediate the VEGF-induced proliferative response in HUVECs. They showed that both NOS and PKG inhibitors prevented Raf-1 activation by VEGF; moreover the PKG activator, 8-pCPT-cGMPs, stimulated both Raf-1 kinase activity and endothelial proliferation, and finally a recombinant catalytically active PKG phosphorylated and activated Raf-1 in a reconstituted system.

These findings suggest a role for the system NO/cGMP/PKG in VEGF-induced MAP kinase cascade which appears PKC independent.

Phosphatidylinositol-3 kinase (PI3K) has also been implicated in mitogenic signaling by KDR, based on treatment of cells with specific inhibitors (Thakker et al., 1999; Yu and Sato, 1999); this lipid kinase regulates the S6 kinase/Akt pathway which has been shown in many cell types to stimulate cell growth. However, conflicting results suggest that PI 3-kinase is not required for VEGFR-2-mediated mitogenesis (Meadows et al., 2001; Takahashi et al., 1999).

In the context of cell proliferation, an interesting aspect to consider is that ECs are contact inhibited in their growth and loose the capacity to respond to growth factors when they reach confluence; in particular VEGF-induced cell proliferation seems to be influenced by cell confluency. Investigations of this mechanism were mainly carried out using syngenic immortalized EC lines differing for expression of VEC only (VEC-null and VEC positive). Confluent VEC-positive cells behaved like wild-type endothelium (Caveda et al., 1996; Vinals and Pouyssegur, 1999), showing a markedly lower DNA synthesis upon VEGF stimulation; in contrast, VEC-null cells were highly responsive to VEGF (Grazia Lampugnani et al., 2003). Indeed it has been reported that in confluent ECs upon VEGF stimulation VEGFR-2 forms a complex with VEC which requires the presence of β-catenin and results in the reduction of VEGFR-2 tyrosine phosphorylation and, consequently, in the attenuation of ERK activation; this effect was attributed to the phosphatase DEP that, binding β-catenin and p120, may associate with the VEC-receptor complex and dephosphorylate the Tyr residue of the receptor responsible for PLC docking (Grazia Lampugnani et al., 2003). In 2006 Lampugnani and associates described another aspect of the mechanism through which VEC expression and clustering inhibits VEGFR-2 proliferative signaling. They found that in condition in which VEC is not clustered at adherens junctions, as in sparse cell, VEGFR-2 is endocytosed to an higher extent in intracellular compartment, from where it maintains its signal activity (PLCγ and ERK activation); VEC could therefore reduce receptor activity by inhibiting VEGFR-2 internalization and promoting its inactivation at the cell surface (Lampugnani et al., 2006).

VEGF and Permeability

Endothelial permeability is mediated by the so called transcellular and paracellular pathways – that is, solutes and cells can pass through (transcellular) or between

(paracellular) ECs. Transcellular passage requires either cell fenestration (the appearance of specialized pore-like fenestrae which can control cellular permeability to water and solutes) or a complex system of transport vesicles, that includes organelles called vesiculo-vacuolar organelles (VVOs) (Dvorak et al., 1996; Feng et al., 1999; Roberts and Palade, 1995). The paracellular pathway, by contrast, is mediated by the coordinated opening and closure of endothelial cell-to-cell junctions; this function must be tightly regulated to maintain endothelial integrity and to prevent exposure of the subendothelial matrix of blood vessels, a highly thrombogenic event (Dejana, 2004; Vestweber, 2007).

VEGF increases permeability in intact vessels as well as in cultured monolayers, implicating VEGF as an important mediator regulating endothelial permeability. A huge amount of data demonstrated the involvement of VEGF in both transcellular and paracellular permeability; moreover intracellular pathways activated in VEGF-induced hyperpermeability appear to include the activation of a large number of signal molecules common to VEGF-induced pathways of cell survival, migration and proliferation.

VEGF upon binding to its receptor activates PLCγ, which in turn produces IP3 and DAG (see the section "VEGF and Proliferation" for further details); generation of these two second messengers increases intracellular Ca^{2+} by increasing Ca^{2+} release from intracellular stores (IP3-dependent pathway), as well as Ca^{2+} influx through store-independent TRP channels (DAG-dependent pathway) (Bates and Curry, 1997; Jho et al., 2005; Pocock et al., 2004). Jho and coworkers demonstrated that in HUVECs TRPC1-induced Ca^{2+} entry is involved in VEGF permeability response by activating the coupling of IP3R to TRPC1 channel; this interaction seems to be regualated by RhoA as demonstrated upon activation of this GTPase with thrombin in human pulmonary arterial ECs (HPAEC) and human microvessel ECs (HMEC) (Mehta et al., 2003). VEGF-induced intracellular calcium increase has been associated with permeability increase occurs through the development of transvascular pathways for fluid and solute flux. This is likely to occur through Ca^{2+} activation of endothelial NOS (eNOS) and consequential NO production which in turn activates guanylyl cyclase and cGMP production (Wu et al., 1996). The link between cGMP and increased permeability is not known, but it may rest on increased vesicle fusion to form transcellular pathways through VVOs, fenestrations, and ultimately transcellular gaps (Bates and Harper, 2002).

Strong evidences outline eNOS as a key protein in the VEGF-induced permeability pathway. In addition of intracellular Ca^{2+} level, there are other critical determinants of eNOS activity such as nytrosylation, acetylation, and phosphorylation. In particular, signal proteins downstream of RTKs can affect eNOS activity through phosphorylation in different regulatory loci: phosphorylations at Ser1117, Ser635, and Ser617 are stimulatory while phosphorylations at Thr495 and Ser116 are inhibitory.

VEGF stimulates both Ca2+ and phosphorylation-dependent regulation of eNOS activity. Brouet and associates documented that early VEGF stimulation in HUVECs first leads to the Ca^{2+}-calmodulin disruption of the caveolin-eNOS complex and promotes the association between eNOS and the chaperon protein hsp90; eNOS-bound hsp90 can then recruit VEGF-activated Akt to the complex, which in turn can phosphorylate eNOS; moreover, through further experiments in transfected COS, they identified Ser1177 as the critical residue for the hsp90-dependent Akt-mediated activation of eNOS (Brouet et al., 2001). In addition Kou et al. demonstrated that in bovine aortic ECs (BAECs) VEGF promotes the dephosphorylation of eNOS at Ser116 which helps coordinate eNOS activation (Kou et al., 2002).

The signaling transduced by VEGF-VEGFR-2 system is strictly linked to VEC, which links to the actin cytoskeleton to provide both mechanical stability and signal transduction. The reciprocal influence between VEGFR-2 and VEC assumes a critical meaning in the context of VEGF-induced paracellular permeability which, as above mentioned, is governed by the opening and closure of endothelial cell-to-cell junctions. In resting ECs, VEGFR-2 forms a complex with VEC at the site of cell junctions (Grazia Lampugnani et al., 2003); in this condition VEGF induces the tyrosine phosphorylation of VEC and its binding partners which is associated with weak junctions and impaired barrier function (Esser et al., 1998). The tyrosine kinase Src is probably implicated in the mechanism, because it associates directly with VEC, and VEGF-induced phosphorylation of VEC is inhibited in Src-deficient mice or in wild-type mice treated with Src inhibitors (Weis and Cheresh, 2005). Moreover it was found that VEGF disrupts endothelial-barrier function by activating Src, which in turn phosphorylates VAV2, a guanine-nucleotide-exchange factor for the GTPase Rac (Gavard and Gutkind, 2006); activated Rac induces the phosphorylation of VEC at Ser665, which induce the recruitment to VEC of β-arrestin-2 promoting clatrin-dependent internalization of VEC.

In condition of increased permeability, ECs retract and cause intracellular gaps to open; this process is probably mediated by the contraction of actomyosin that is

anchored to cell junctions (Dudek and Garcia, 2001). The actin-myosin interaction is mainly governed by the phoshorylation status of the regulatory myosin light chain (MLC). On the one hand, Ca^{2+}-calmodulin dependent MLC kinase (MLCK) directly phosphorylates MLC, resulting in actomyosin contraction; on the other hand, myosin-associated protein phosphate dephosphorylates MLC leading to cell relaxation. An increase in cytosolic Ca^{2+} has been established as the initial pivotal signal that precedes the opening of EC junctions: increased cytosolic Ca^{2+} promotes MLC phosphorylation resulting in actomyosin contraction.

There are also evidences, obtained by both *in vivo* and *in vitro* assays , that ERK can contribute to the VEGF-induced permeability through a phosphorylation-dependent regulation of MLC. Klemke and coworkers showed the ability of Erk1/2 to directly phosphorylate MLCK leading to the phosphorylation of MLC (Klemke et al., 1997). Of interest, Wu and associates proposed a model in postcapillary venules in which, upon VEGF stimulation, the signaling derived both from PKC (activated by DAG) and PKG (activated by NOS/cGMP) promotes Raf activation and consequential ERK activation triggering increased permeability (Wu et al., 2005).

In addition to MLC phosphorylation, evidences indicates a role for actin polymerization in endothelial contraction and increased permeability response (Mehta and Malik, 2006). Because of the complexity of the linkages of actin with AJs, actin polymerization occurring at specific sites may influence the function of cell junctions, thereby affecting endothelial permeability. Moreover, actin polymerization is required for stress fiber formation, and hence EC contraction. In this context, it has been suggested the involvement of p38 MAPK in the VEGF-induced permeability (Lal et al., 2001). Rousseau and colleagues showed that in HUVECs VEGF induces p38 activation which appears to be responsible for induced actin polymerization and stress fiber formation; this mechanism is probably dependent on the activation of MAPK activated protein kinase 2/3 (by p38) leading to HSP27 phosphorylation which is associated with increased actin polymeryzation (Rousseau et al., 1997).

VEGF and Migration

Degradation of the basement membrane is necessary for endothelial cell migration and invasion and is an important step in angiogenesis. VEGF induces a variety of enzymes and proteins important in the degradation process, including matrix-degrading metalloproteinases, metalloproteinase interstitial collagenase, and serine proteases such

as urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (TTPA) (Choong and Nadesapillai, 2003; Zachary and Gliki, 2001). Activation of these various compounds leads to a prodegradative environment that facilitates migration and sprouting of ECs. Other studies have shown that VEGF promotes expression of the uPA receptor (uPAR) on vascular ECs (Ferrara and Davis-Smyth, 1997). Considering that the PA-plasmin system, in particular the interaction of uPA with uPAR, is an important element in the chain of cellular processes that mediate cellular invasion including proteolysis and tissue remodeling, these findings are consistent with the proangiogenic activities of VEGF. Furthermore, it has been shown that uPA itself leads to increased production of a variety of different angiogenic factors including VEGF, suggesting that an autocrine regulatory loop may exist.

The intracellular mechanisms by which VEGF leads to increased EC migration are not entirely clear, but appears to involve FAK-associated signaling leading to focal adhesion turnover and actin filament organization, as well as p38 MAPK-induced actin reorganization, and finally members of the Rho family small monomeric GTPases.

A large body of evidences indicates that FAK-associated signaling is critical for regulating focal adhesion turnover, actin filament organization, and cell migration. VEGF induces tyrosine phosphorylation of FAK and paxillin and promotes recruitment of FAK to new focal adhesions in HUVECs (Abedi and Zachary, 1997).

In 1997 Rousseau and associates found that in HUVECs VEGF increases cell migration and induces a marked reorganization of the microfilament network that is characterized by the formation of stress fibers and the recruitment of vinculin to focal adhesions; VEGF also stimulates p38, which results in activation of MAP kinase activated protein kinase-2/3 and phosphorylation of the F-actin polymerization modulator, heat shock protein 27 (HSP27); moreover inhibition of p38 activity by the specific inhibitor SB203580 leads to an inhibition of HSP27 phosphorylation, actin reorganization and cell migration (Rousseau et al., 1997). The results indicate that the p38 pathway conveys the VEGF signal to microfilaments inducing rearrangements of the actin cytoskeleton that regulate cell migration.

In 2000 Rousseau and coworkers showed that upon VEGF stimulation in HUVECs, activation of p38 triggers actin polymerization whereas FAK, which becomes phosphorylated independently of p38, initiates assembly of focal adhesions; both processes contributes to the formation of stress fibers; moreover geldanamycin, an inhibitor of HSP90, blocks tyrosine phosphorylation of FAK, assembly of focal adhesions, actin reorganization, and cell migration, all of which were reversed by

overexpressing HSP90 (Rousseau et al., 2000). They conclude that VEGFR2 mediates the physiological effect of VEGF on cell migration and that two independent pathways downstream of VEGFR2 regulate actin-based motility; one pathway involves p38 and leads to enhanced actin polymerization activity, while the other involves HSP90 as a permissive signal transduction factor implicated in FAK phosphorylation and assembly of focal adhesions.

In addition to FAK and p38, it has been proposed that NO may also play an important role in VEGF-induced EC migration. In facts, there are evidences that suggest a permissive role of NO in EC migration promoted by VEGF. Noiri and colleagues demonstrated that VEGF, shown to stimulate NO release from HUVECs, induces HUVEC migration, whereas inhibition of NOS with NG-nitro Larginine methyl ester (L-NAME) or antisense oligonucleotides targeting eNOS suppresses VEGF-stimulated migration (Noiri et al., 1998). Furthermore, Goligorsky and coworkers suggested an important interaction of NO with focal adhesions; they established the phenotype of HUVECs subjected to increased concentrations of NO as that of a cell with the increased turnover of focal adhesions, reversibly reduced tractional forces exerted through focal adhesions on the matrix, and propensity for migration; HUVECs with the suppressed activity of endothelial NOS, in contrast, exhibit a gradual decrease in the expression of phosphorylated 125FAK, inability to recruit it to focal adhesions, increased ability to spread, and decreased ability to migrate (Goligorsky et al., 1999).

The Rho family of the small GTPase superfamily has been shown to play an important role in migration; RhoA, Rac1, and CDC42 are the most extensively studied members; RhoA primarily induces the formation of stress fibers, whereas Rac1 and CDC42 promote the formation of lamellipodia and filopodia, respectively. Since it was reported theat VEGF induces actin-based mobility, several research groups studied whether the Rho family proteins might be involved in this response.

Zeng and associates showed that in HUVECs VEGF activates RhoA and Rac1 through a G proteins Gq/11, Gβγ, and PLC dependent mechanism which was not influenced by PI3K and intracellular Ca^{2+} mobilization; dominant negative RhoAN19 almost completely inhibits VEGF-stimulated HUVEC migration, dominant negative Rac1N17 inhibits VEGF-stimulated HUVEC migration by ~50%, whereas CDC42N17 has no effect; moreover the RhoA activation is partially inhibited by Rac1N17, but RhoAN19 has no effect on Rac1 activation; finally, Gq/11 and Gβγ subunits are required for VEGF-stimulated HUVEC migration (Zeng et al., 2002). Their model for VEGF-induced RhoA activation is that VEGF-stimulated VEGFR-2 activates Gq/11

family protein and releases Gβγ subunits, which than activates RhoA that, in combination with Rac1, promotes cell migration.

Lamalice and colleagues showed that in HUVECs the VEGF signaling that leads to the activation of p38 (leading to the formation of stress fibers) requires the autophosphorylation site Tyr1214 of VEGFR-2, and involves the activation of CDC42; they also found that the activation of RhoA is also required for the formation of stress fibers, but independent of p38 (Lamalice et al., 2004). Data bank searching revealed that Tyr1214 is an environment that can bind Src-related protein, moreover inhibiting Src activity could also inhibit p38. Their working model is the following: VEGF binds to VEGFR-2 on ECs, which stimulates autophosphorylation on cytoplasmic tyrosine residues of the receptor, notably Tyr1214; phosphorylation of Tyr1214 then triggers, through yet unknown proteins (Src?), the activation of Cdc42 and then the p38 module (MAP3K, MAP2K and p38); in turn, activation of p38 triggers the activation of MAPKAP kinase2/3 and phosphorylation of HSP27 initiating actin remodeling and actin-based motility. The p21-activated protein kinase PAK-1 is an effector of the GTP-bound Cdc42 that possibly links activated Cdc42 to the p38 module.

Work by Garret and associates showed the ability of VEGF to activate RhoA, Rac1 and CDC42, but in particular it was focused on the mechanism that trigger Rac1 activation (Garrett et al., 2007). This study identified the exchange factor Vav2 as mediating VEGF signaling to Rac1; using a nucleotide-free G15ARac1 mutant, it was showed that Vav2 interacts with Rac1 after VEGF stimulation and this association correlates with a robust increase in Rac1 activity; additionally, it was observed that VEGF stimulates tyrosine phosphorylation of Vav2, which is prevented by inhibiting either VEGFR-2 signaling or Src kinase activity; moreover, knockdown of Vav2 not only inhibits the increase in Rac1 activity in response to VEGF stimulation, but also inhibits VEGF stimulated migration. Furthermore, in immunoprecipitation studies, it was observed that Vav2 may not direct associate with VEGFR-2, thus other intermediate proteins may link VEGF signaling to Vav2-Rac1 activity; in fact it has been indicated that Src may phosphorylate Vav2 downstream of VEGF signaling (Eliceiri et al., 1999; Servitja et al., 2003).

A recent paper by Tan and associates demonstrated the essential role of Rac in EC function and vascular development using a conditional Cre/Flox approach, enabling the deletion of Rac1 gene in ECs (Tan et al., 2008). In particular, in regard to VEGF signaling, they showed that Rac1 deletion in primary ECs impairs the formation of

lamellipodial structures and focal adhesions in response to VEGF (and also upon S1P stimulation).

Of interest strong evidences suggest that the activation of some Rho family members can be regulated by PI3K. Eriksson and colleagues demonstrated that in PAE/VEGFR-2 VEGF-induced Rac1 activation was prevented by PI3K inhibitor (Eriksson et al., 2003). Their data are consistent with the model according to which PI3K, through SH2 domains in its regulatory subunit p85, binds to phosphotyrosine residues in VEGFR-2 and becomes activated by tyrosine phosphorylation; then lipid products of PI3K may bind to and activate Rac GTPase by stimulating GDP dissociation (Missy et al., 1998).

Considerations on VEGF Multifunctionality

The preceding discussion has tried to highlight the multifunctionality and integration of VEGF signal transduction mechanisms. It is apparent that cascades of kinases, activity of other enzymes and recruitment of adapter proteins converge and branch at many points in functional VEGF signaling, emphasizing how linear pathways can integrate to form signal transduction networks.

If multi-tasking and integrated signalling go some way towards an understanding of the functional versatility of VEGF, it become quite complicated to highlight how specific information is processed through these pathways and how cellular decisions are regulated in order to trigger a specific cellular behaviour. At this point the following important considerations arise spontaneously:

- Specific hidden differences intrinsic to one or more signaling pathways triggered by VEGF may help to understand the flow of information for a specific cellular behaviour. In this context, even if a lot of common signal proteins are involved in more than one VEGF-induced pathway, different cellular conditions, such as the presence or absence of mature cell junction, as well as the amount of growth factor, may make the difference.

- Does VEGF multifunctionality have a meaning? From a Systems biology point of view, multifuntionality attitude may be translate in "complex system behaviour". If we consider the total complement of signaling cascades triggered by VEGF, we conclude that it is unique; therefore we can speculate on peculiar properties arising from the VEGF-complex system. A way to find out complex system properties may

be to start writing a model to describe the simplest possible representation of the biology of interest studying a small, but sufficient number of components, and then use this initial model as a basis for further implementation adding additional levels of complexity where needed.

So what does the future hold for VEGFR research? We can say that the well-established multifunctionality of VEGF makes its signal transduction cascade an interesting subject of study using a System Biology approach. "Equipped" with high quality reagents, using proper quantitative techniques, employing *in vitro* conditions that mimic *in vivo* situations, using new explorative techniques based on mathematical modeling, it will be possible to better understand how VEGF signal transduction works and, probably, identify determinants regulating EC behaviour.

Modeling of Signal Transduction Pathways

Signaling pathways enable cells to sense changes in their environment, to integrate external with internal signals, and to respond to them by changes in specific protein activation, transcriptional activity, or other regulatory measures. The proper functioning of these pathways is crucial for survival, but also for cell behaviour under varying conditions. At first glance, signaling can be seen as a linear connection between input elements (e.g., growth factors) and output elements (e.g., signal protein activities); a closer inspection reveals that signaling pathways interact with each other, forming a network. Years of careful experimental analysis have revealed that signaling molecules are organized into complex networks of biochemical reactions exquisitely regulated in time and space to provide a cell with high-fidelity information about the environment.

Biologists usually make use of mental models in order to capture the biology of interest. Qualitative biological information derived from literature, important considerations and working hypothesis can be contained in conceptual models which represent a helpful starting point for optimal design of focused experiments and for the following rewriting of the model on the based of experimental data readout.

In order to better understand cell behaviour, researchers have adopted computational modeling approaches, ranging from models that emphasize some key features of signaling pathways to detailed models that describe the dynamics of specific

pathways. A mathematical model is a formal representation of physical or biological phenomena, describing the interactions and dynamics of the system in a precise and quantitative language as expressed by the resulting equations (Potter and Tobin, 2007). Modeling can be described as equation-based reasoning. The model captures the behavior of the system by incorporating the details of how each biological entity interacts with the other entities and how each entity changes over time or space. A signal transduction mathematical model is first a correct description of the biology of interest and then it may become a versatile tool for exploring biological questions of interest, testing and generating hypotheses, designing new experiments, and predicting the effects of perturbations.

The Modeling Process

Although so far, no standard for the development of pathway models has been established, a suitable approach embarks the strategy based on the following consecutive steps: 1) gathering biological information; 2) drawing an *a priori* conceptual model; 3) performing focused experiments; 4) writing the *a posteriori* conceptual model based on experimental data readout; 5) translating the *a posteriori* conceptual model into a mathematical model; 6) calibrating the mathematical model; and 7) using the model.

The first thing to do is to ascertain the need and purpose for a model. The particular needs for the model and the questions to be addressed are important factors in determining the type and complexity of model to build. In general, the aim is to describe the simplest possible representation of the biology, adding layers of complexity where needed to capture specific behaviors of interest. The most basic decision in model building concerns the model components: which molecules and interactions play a role and which of them will be left out? Omitting certain processes from the models is based on the assumption that they have only a minor influence on the event under study, that their values remain constant in the experimental setup, or that they simply cannot be described with the currently available means. Simplicity is a judgment call because oversimplification may drastically change the behavior of the system. However, introducing more complexity increases the number of parameters and hence the need for more data.

The available biological knowledge and qualitative experimental data can be used to determine the general structure of the model, whereas quantitative data are necessary for calibrating model parameters.

Once all available biological information have been gathered and the modeling objectives have been determined, an *a priori* conceptual model can be written on the basis of the known, supposed, and plausible (i.e., guessed) mechanisms of the biology in combination with the formulated working hypotheses. This conceptual model usually is a schematic description that represents a simplified form of the known biology, including only the components essential to capture the behavior of the phenomena of interest. Relevant biological and chemical species are included as model variables, and the interactions of these species are mapped conceptually.

After the design of the conceptual form, it is possible to perform focused wet biology experiments in order to test the working hypotheses. It is important to take into account that quantitative data generation is necessary in order to obtain measurements suitable for mathematical modeling. If the model is to capture changes over time, it is crucial to have time-course data that show the dynamic behavior of the system. It is also desirable to have simultaneous measurements in time of multiple different species, so that all data are not centered on a single model variable. It is almost impossible to have "too much" data for model calibration. Without enough data to sufficiently describe the interactions of multiple species in the system, model development can become difficult or intractable. In receptor-mediated signaling, relevant data may come from experiments such as evaluation of the activities of downstream signal proteins involved, binding assays, and other measurements.

Once obtained data, it is possible to use them in order to rewriting the conceptual model in *a posteriori* form. It is important to note that an optimal experimental strategy may provide results useful not only to answer to the formulated biological questions but also to add further information that was not included in the *a priori* conceptual model.

The *a posteriori* conceptual model can then be translated into a mathematical model that captures the dynamics of the biology. The first step in describing the quantitative signaling process is writing a system of the reaction equations and the second step is to compute numerical solutions to the model equations. Most models are systems of ordinary differential equations (ODEs) which are based on mass-action-law. In general, systems of nonlinear differential equations do not have analytic solutions that can be derived by hand. Limiting a model to a form that can be solved analytically can result in severe limitations on the complexity of the model, as well as the ability to

accurately represent the known biology. On the other hand, solving a system of differential equations numerically is a straightforward process, and there are many efficient algorithms and software packages freely available to solve these types of equations, even for extremely large systems.

Once the system of equations is developed, the model parameters must be determined. To this end, it is necessary to estimate model parameters by comparing model simulations to known behaviors derived from experimental data. In this context it is important to consider that there are often constraints that have to be applied to the parameters themselves; for example, in many physical systems the parameters and variables are restricted to non-negative values and often are constrained to values within a fixed range; other constraints may arise from well-characterized behaviors of the system under certain conditions. A set of model parameters can be determined that gives the "best" match between the model simulations, the corresponding data, and the expected behaviors.

A properly calibrated model is able to describe the biology of interest and may represent a powerful tool to answer questions. One major advantage of a mathematical model over more traditional experimental models is the capability to explore a large variety of scenarios relatively quickly. In the ideal scenario, the mathematical and experimental protocols would be used together iteratively to achieve maximal effectiveness from each of them. Experimental data are used to calibrate and validate the mathematical model, which in turn can help design future experiments that continue to inform the model. In addition, a mathematical model derived from a conceptual model characterized by a simple level of abstraction, can be considered as the basis for further implementation. This fact is of particular importance if we consider the existence of peculiar proprieties arising from complex systems behaviour.

Finally, it is important to note that a mathematical model is only an approximation of the biology, and its accuracy is a function of that approximation as well as the experimental data that were used to inform and calibrate the model.

Quantitative Data Generation for Modeling

By combining experimental data with mathematical modeling of signal transduction networks, researchers aim to better understand intracellular signals which regulate cellular behaviour and to predict perturbation-sensitive targets. So far, the majority of

the analysis of signaling pathways by mathematical models has been performed in the framework of simulations. For example, in the case of the MAP kinases, three different models were investigated by simulations (Asthagiri and Lauffenburger, 2000; Bhalla et al., 2002; Huang and Ferrell, 1996). As outlined by Swameye and coworkers (Swameye et al., 2003), a comparison of the first two models (Blüthgen N. & Herzel, 2001) revealed that these models exhibit different properties, thus emphasizing the necessity for experimental data to enable a decision between the different models. In this type of study, the structure of the equations is based on assumptions about the underlying biology, and the parameters are taken from published data often obtained under different conditions or even from different organisms. This poses a fundamental problem: it is difficult to decide whether the results of the simulations reflect the underlying biology or the specific choice of the parameters. Therefore, quantitative time-resolved measurements for estimation of the dynamical parameters are critical to achieve models of high quality.

The major limitation at present is the lack of reliable quantitative data-set. Techniques that quantitatively and selectively measure biochemical reactions and protein modifications within the cell must be used to test and validate models in order to capture the characteristic dynamic behavior of systems.

For the analysis of components (proteins) in biological systems, one of the most widely used techniques is immunoblotting, which is based on the separation of components according to the molecular weight using gel-electrophoresis and transfer to a membrane followed by a detection process; the presence of proteins and/or their modifications in complex mixtures (cell extracts) is examined by immunoblotting using specific antibodies in combination with chemiluminescence detection. It is important to consider that data generated by standard immunoblotting are primarily qualitative and not suitable for mathematical modeling. However, it is possible to advance the established technique of immunoblotting to more accurate and quantitative procedures using methods allowing the shift from qualitative data acquisition to quantitative measurements. Quantitative immunoblotting data must be characterized by a significative experimental error reduction.

A suitable approach in order to obtain quantitative immunoblotting data of high quality is based essentially on the use of appropriate normalization criteria and signal acquisition by a Charged Coupling Device (CCD)-camera detection.

Normalisation procedure is based on the use of signal acquisition data obtained from selected control proteins to correct immunoblotting data. Normalizers

(endogenous proteins) are used in the case of total cellular extract analysis. For certain signaling components, prepurification by immunoprecipitation is required prior to immunoblotting; in this case calibrator (purified proteins of a different molecular weight than the protein of interest added to cell lysates prior to immunoprecipitation) are employed to control both the blotting and the additional steps involved in the procedure. It is assumed that calibrators and normalizers possess a constant concentration, consequently fluctuations occur only as measurement errors. Therefore, the blotting error can be estimated using the calibrator or normalizer signal. Based on this blotting error estimate, the protein of interest can be normalized by division. However, since the blotting error is a local property of the gel, normalizers and calibrators are required with a similar molecular weight as the protein of interest. If the molecular weight of the normalizer/calibrator is different it does not reflect the blotting error for the protein of interest.

Traditionally, chemiluminescent detection is achieved through X-ray film following by densitometric analysis. This process resulted in a restricted linear dynamic range which in turn limits scanning densitometry. The use of a CCD-camera for signal detection allows both an increase in linear dynamic range and immediate quantitative data analysis.

Another aspect that is important to notice is the fact that signaling pathways have been primarily studied in the context of propagatable cell lines. However, as such cell lines have lost restrictive growth control mechanism, it is of great importance to analyze the behavior of signaling pathways in primary cells. Mammalian cells grow either in suspension or attached to a support. Suspension cells are primarily cells of hematopoietic origin and are particularly suited for biochemical studies on cell populations with high temporal resolution because they permit bulk stimulation and rapid sampling. For biochemical studies in adherent cells, separate stimulations are required for each time-point, potentially resulting a higher sample-to-sample variation.

On the whole these considerations converge in emphasizing the need for an appropriate experimental procedures allowing the generation of quantitative data suitable for mathematical modeling.

RESULTS

A Priori Conceptual Model

The available biological knowledge has been used to create an *a priori* conceptual model which deals with the following basics considerations:

(i)   In healthy adult humans, most ECs are quiescent; in this condition, the cells are contact inhibited in their growth, protected from apoptosis and in full control of permeability. By contrast, in pathological conditions – e.g., when ECs are exposed to angiogenic stimuli – their behaviour changes from quiescent to active; in this context the initial response of quiescent ECs to induce angiogenesis is the activation of the permeability pathway, which is followed by EC migration, proliferation and formation of capillary tube network.

(ii)   VEGF exerts a major role in EC biology which is due to its ability to induce multiple signaling pathways such as survival, permeability, migration and proliferation of ECs.

(iii)   Normal human serum contains a low level of VEGF, while in pathological states it increases; in particular VEGF concentrations above 0.5 ng/ml have been reported in serum of patients with various malignancies (Kraft et al., 1999). Moreover *in vitro* the permeability effect of VEGF has been showed for growth factor concentration above 1-2 ng/ml (Kevil et al., 1998; Lal et al., 2001).

(iv)   Several reports have implicated VEGF as a major survival factor for ECs; however the survival effect has been reported during *in vivo* angiogenesis and *in vitro* serum starvation of cells upon the stimulation with high concentrations of growth factor. This raises questions as to whether VEGF exerts its survival effect only during angiogenic process or if it is able to maintain EC survival even under normal conditions.

In this study it has been hypothesized that in normal conditions, characterized by a low/non angiogenic concentration of VEGF – which can be assumed ≤ 0.5 ng/ml – VEGF could be one of the factors able to maintain the survival of quiescent ECs, without exert any effect on the permeability pathway. By contrast, in pathological conditions, it has been considered that, in addition to the survival effect, a high/angiogenic concentration of VEGF – for example 30 ng/ml – is able to induce first

hyperpermeability in quiescent ECs and then, after cell migration, the proliferative effect in cells that are characterized by the absence of mature cell-to-cell junctions.

Model components were chosen in order to describe the simplest possible representation of the biology of interest. VEGF, VEGFR-2, Akt, PLCγ were introduced as the players; among them VEGF concentrations were the inputs, while the activities (measured as phoshorylation level) of both Akt and PLCγ were selected as outputs. In particular Akt signal was considered as the indicator of the survival pathway, while PLCγ signal was assumed to be able to provide information about both the permeability and the proliferative pathways.

The conceptual model, can be pictured in a very simple manner starting from the normal condition of quiescent ECs and then visualizing the effect of an angiogenic concentration of VEGF during the time. See Figure R1 for an illustration of the *a priori* conceptual model.
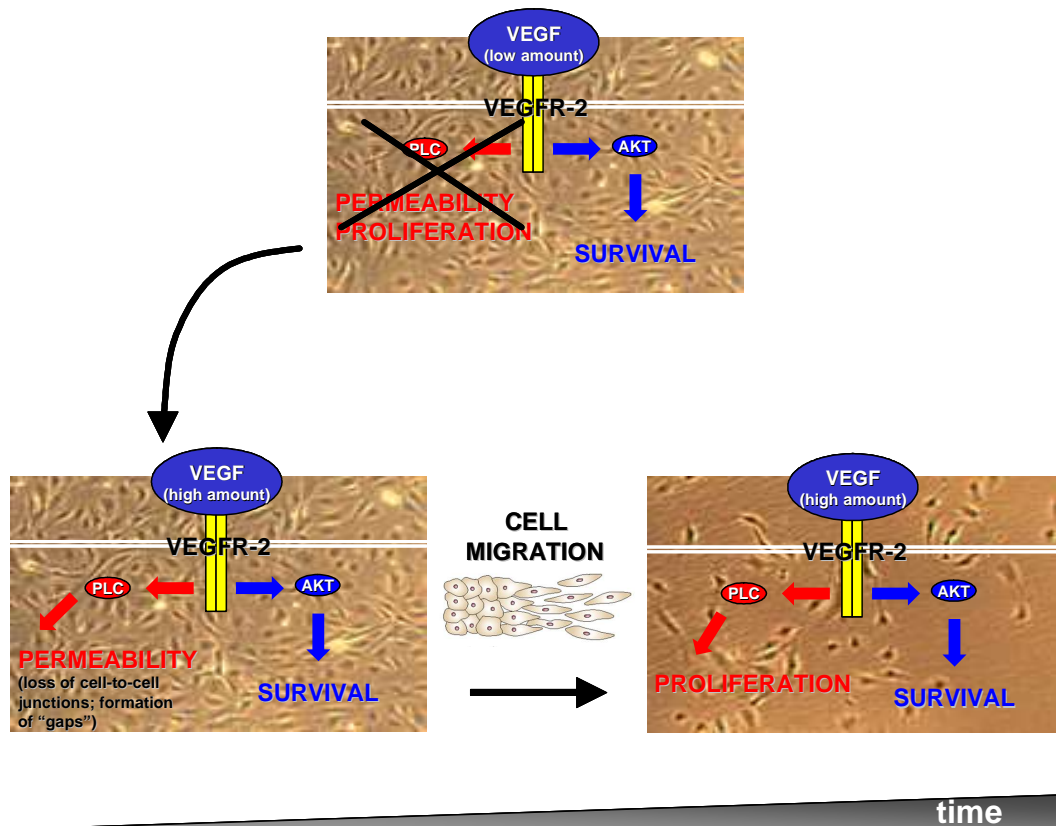

Figure R1. Illustration of the a priori conceptual model.

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Wet Biology Experimental Analysis

To explored experimentally the *a priori* conceptual model long-confluent and sparse ECs were used as *in vitro* settings for the normal condition of quiescent endothelium and for the early and late pathological/angiogenic state of ECs. This choice was motivated by the fact that long-confluent cells are organized in a tightly dense monolayer with mature cell-to-cell junctions which is comparable with the physiological state of quiescent endothelium *in vivo*, while sparse cells are characterized by the absence of cell-to-cell contacts that is the condition similar to the *in vivo* phenotype of ECs during angiogenesis after cell migration (for further details see the section "Material and Methods"). To test the *a priori* conceptual model, the following inputs were used: 0.5 and 30 ng/ml of VEGF, which were assumed as a low/non angiogenic and a high/angiogenic concentration, respectively.

A non angiogenic concentration of VEGF contributes to quiescent EC survival

First it was studied the effect of a low concentration of VEGF on the Akt-dependent survival pathway in long-confluent cell after serum starvation. For this purpose it was evaluated the activation level of Akt and the phosphorylation of its effector FOXO4 upon VEGF treatment after serum starvation, comparing them with the signal derived from the same proteins in the presence of full medium. As showed in Figure R2 (panel a and b), the concentration of 0.5 ng/nl of VEGF was sufficient to induce Akt activation which reached a maximal phosphorylation signal, similar to that of cell in full medium, around 10-20 minutes of stimulation; moreover the same low amount of growth factor was able to induce FOXO4 phosphorylation (Figure R2, panel c and d).

In order to exclude the possibility that the low concentration of VEGF used could promote hyperpermeability in addition to maintain ECs survival, it was evaluated the activation of PLCγ (in terms of its phosphorylation) as an indicator of the activation of the permeability pathway. It was found that in long-confluent cells the concentration of 0.5 ng/ml is not sufficient to induce the phoshorylation of PLCγ (Figure R2, panel e and f). The absence of pro-permeability signal was also confirmed through cell-monolayer permeability assays *in vitro* (data not shown).

Overall these data indicate that a concentration of 0.5 ng/ml of VEGF represents a non angiogenic amount of growth factor that is able to contribute to quiescent ECs survival without exert any effect of hyperpermeability.
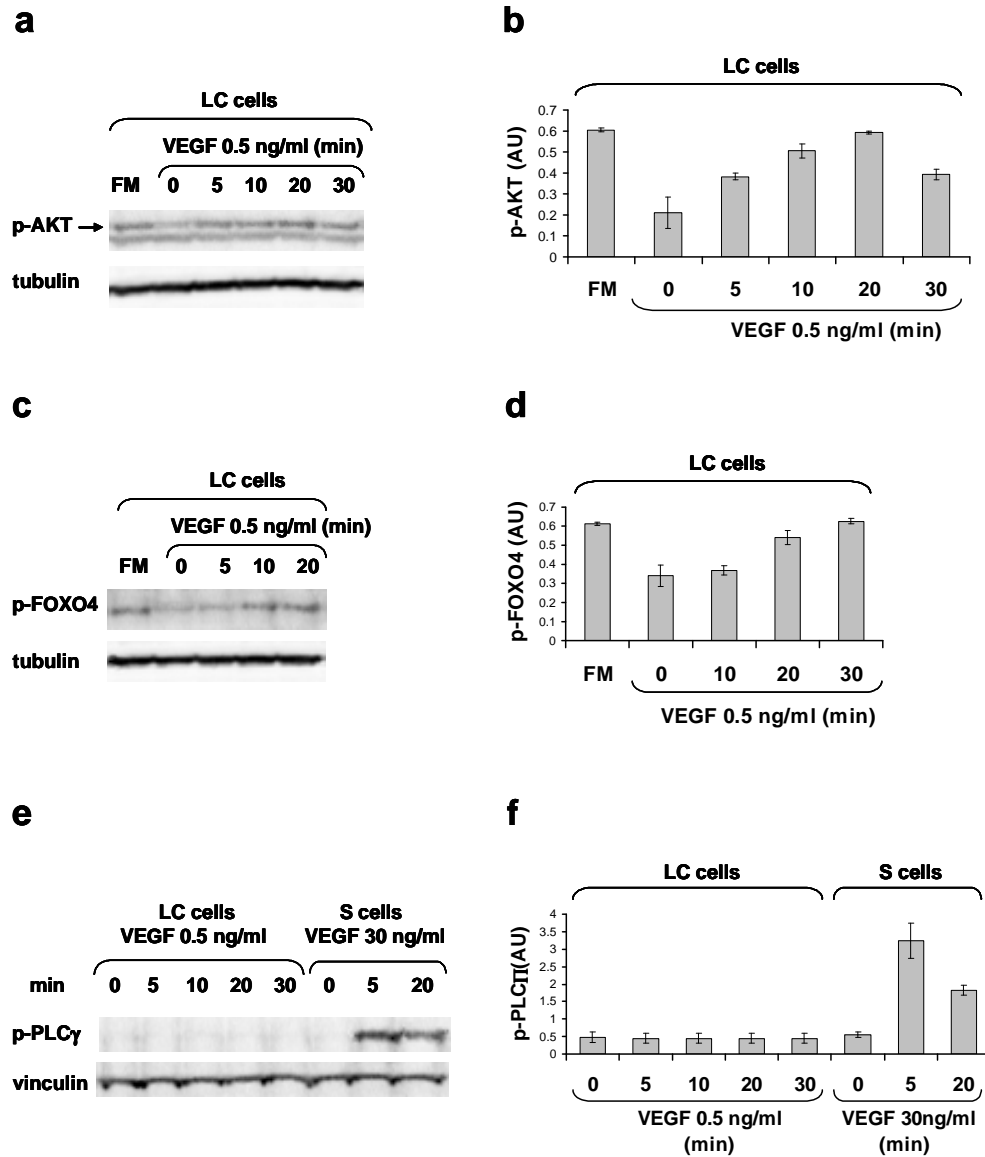
Figure R2. A low amount of VEGF promotes Akt activation without effecting PLCγ phosphorylation. *(**a**, **c**, and **e**) long-confluent (LC) cells were incubated with serum-free medium for 3 hours and then stimulated with 0.5 ng/ml of VEGF for the indicated time. Long-confluent cells exposed to the full medium (FM) (**a**) and serum starvated sparse (S) cells treated with 30 ng/ml of VEGF for 5 and 20 minutes (**e**) were used as positive controls. Whole cell lysates were prepared, separated though SDS-PAGE, and analysed by Western blotting using antibodies which recognize phosphorylated AKT, phophorylated FOXO4, and phosphorylated PLCγ; membranes were reprobed for total tubulin and total vinculin. **b**, **d**, and **f** show the data analysis of **a**, **c**, and **e** respectively; protein phosphorylation levels were normalized as described in the sections "Material and Methods" and expressed in arbitrary unit (AU); values shown are means ± SD of three independent experiments.*

An angiogenic concentration of VEGF is able to induce mechanisms which are additional to quiescent EC survival signalling

To study the behaviour of our model components during angiogenesis both in an initial condition where the permeability pathway is supposed to be active and in a later stage in which cells are considered to have a proliferative phenotype, it was introduced the comparison between long-confluent and sparse cells treated with a high concentration of VEGF after serum starvation.

As expected, the concentration of 30 ng/ml of VEGF was able to induce PLCγ phosphorylation both in long-confluent and sparse cells; moreover signal intensities and kinetics were similar in both conditions; in particular the maximal activation level of PLCγ corresponded to 5 minutes of stimulation (Figure R3, panel a and b). Interestingly, the comparison of the activation level of Akt in these conditions revealed an higher AKT phosphorylation in long-confluent than in sparse cells (Figure R3, panel c and d).

Data relative to PLCγ, in combination with the notion that PLCγ activation could be considered as an indicator of both cell-monolayer permeability and cell proliferation, suggest that an angiogenic concentration of VEGF is able to transduce downstream signals which could start from the activation of the same effector and could lead to a different cell behaviour depending on the cell confluence condition. This suggestion can be supported by evidences previously reported showing the following findings: (i) high concentration of VEGF induces hyperpermeability in confluent monolayer of ECs (Lal et al., 2001); (ii) confluent ECs show a markedly lower DNA synthesis upon stimulation with high concentration of VEGF than sparse cells (Grazia Lampugnani et al., 2003).
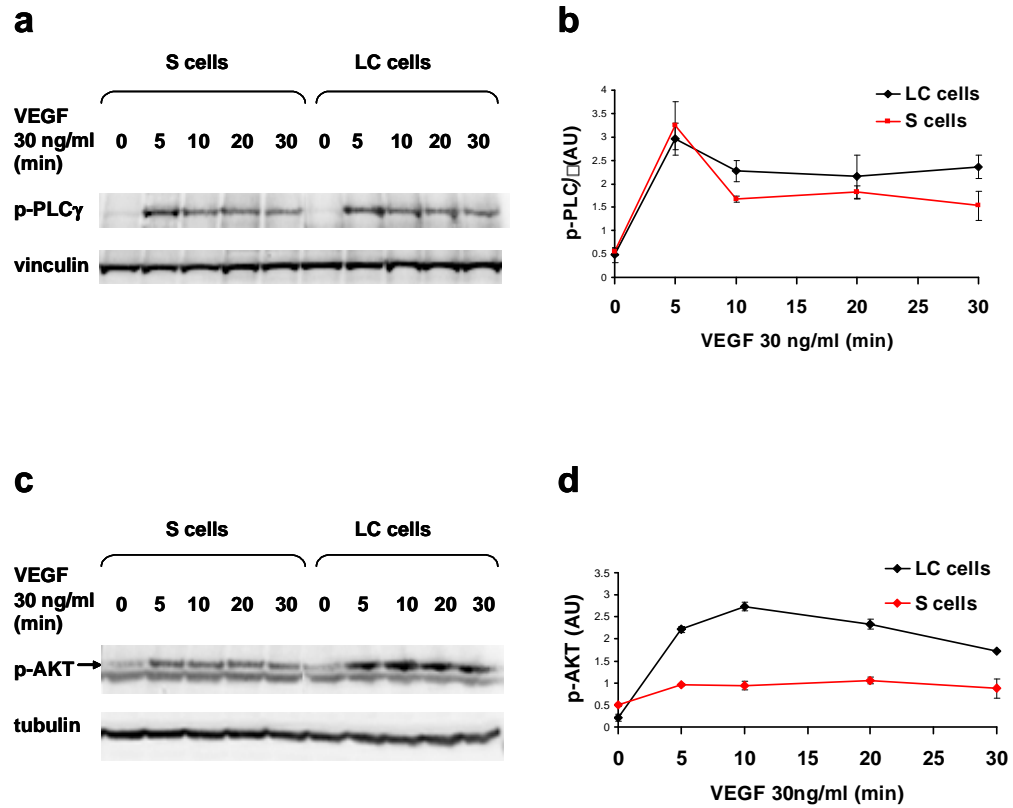
Figure R3. An high amount of VEGF induces both a similar PLCγ phosphorylation signal and a different activation of Akt on the basis of cell confluence. (a and c) long-confluent (LC) and sparse (S) cells were incubated with serum-free medium for 3 hours and then stimulated with 30 ng/ml of VEGF for the indicated time. Whole cell lysates were prepared, separated though SDS-PAGE, and analysed by Western blotting using antibodies which recognize phosphorylated Akt and phosphorylated PLCγ; membranes were reprobed for total tubulin and total vinculin. b and d show the data analysis of a and c respectively; protein phosphorylation levels were normalized as described in the section "Material and Methods" and expressed in arbitrary unit (AU); values shown are means ± SD of three independent experiments.

A *Posteriori* Conceptual Model

Data readout confirmed the working hypotheses contained in the *a priori* conceptual model and added new findings (i.e., the different activation level of Akt in long-confluent than sparse cell). These experimental data in combination with further information derived from literature were used to rewrite the conceptual model.

The proposed *a posteriori* conceptual model is illustrated in Figure R4. Here it was considered that VEGF/VEGFR-2 system is be able to activate both PI3K and PLCγ, but in quiescent ECs VEC inhibits PLCγ through DEP (Grazia Lampugnani et al., 2003), for this reason only PI3K-Akt-dependent survival pathway is active. An angiogenic concentration of VEGF is able to induce the internalization of a significant fraction of VEC (Gavard and Gutkind, 2006), this is supposed to unlock PLCγ inhibition. Moreover in order to explain the higher activation level of Akt in long-confluent cells than in sparse cells, it has been speculate that the VEC fraction remaining in membrane could be responsible for the high level of Akt activation through the multicomponent complex between VEC–VEGFR-2–PI3K which was previously described (Carmeliet et al., 1999).
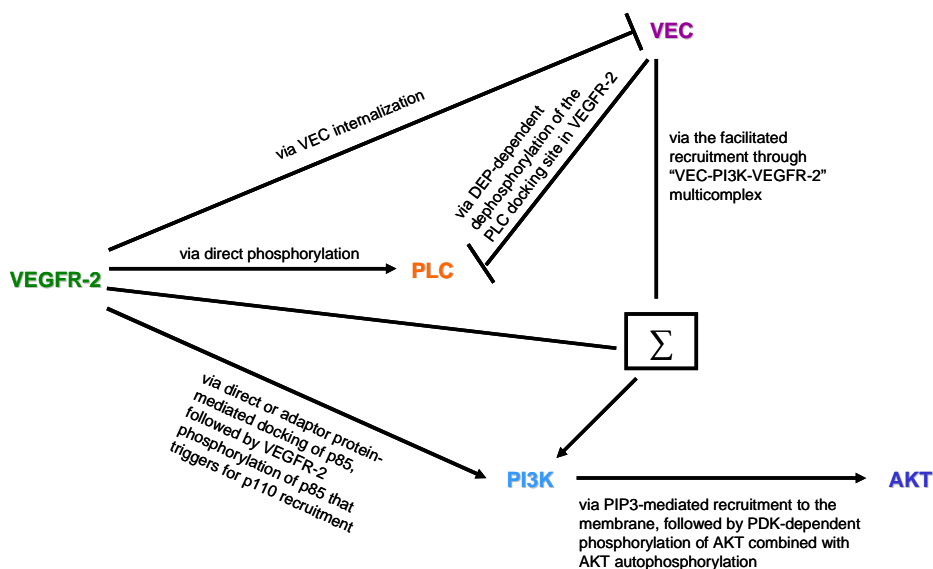

Figure R4. Illustration of the a posteriori conceptual model.

Mathematical Model

The *a posteriori* conceptual model, supported by both experimental data and extra above mentioned information derived from other research groups, has been considered as a visual representation of the key reactions to take into account in the model. Starting from this framework, it was developed a mathematical model based on the following equations:

$$\dot{\gamma} = -\alpha(\gamma - \gamma_0) + B(r\gamma_M - \gamma) - rv\pi$$
$$\dot{k} = -\alpha'(k - k_0) + r(D + Mc)(k_M - k) - z\pi'$$
$$\dot{c} = -\alpha_c(c - c_0 + Ar)$$
$$\dot{\pi} = -r\alpha_\pi(\pi - \pi_M)$$
$$\dot{\pi'} = -r\alpha_{\pi'}(\pi' - \pi'_M)$$

This ODE system represents the variation in time of the selected biochemical species, where:

$\gamma \equiv pPLC\gamma$

$k \equiv pAkt \propto active\ PI3K$

$c \equiv VEC\ (clusterized\ at\ the\ cell\ junction)$

$\pi \equiv active\ phosphatase\ able\ to\ influence\ PLC\gamma\ signal$

$\pi' \equiv active\ phosphatase\ able\ to\ influence\ Akt\ signal$

Briefly, in the mathematical model the behaviour of γ and k was deduced from experimental data, the trend of c (representing membrane VEC at the level of cell-to-cell and here considered as "active" in regard to its capability to interfere with VEGF signaling) was derived from literature, the activity of two different phosphatases, π and π', were introduced in order to explain signal downregulation (see the section "Discussion" for more details about the choice of signal downregulation mechanisms), and finally the input was represented by the concentration of VEGF, r.

The above ODE description is a phenomenological representation of the system; it does not specifically take into account all the reactions involved in terms of kinetic constants, but reproduces the experimental system behaviour considering the dynamic of the main actors involved. Even though not specifically, it implicitly takes into

account all the kinetic constants through its parameters (see the section "Material and Methods" for more details regarding the parameters used).

As shown in Figure R5, model simulations are in good agreement with experimental data offering a description of the system behaviour in normal condition (long-confluent cells exposed to a non angiogenic concentration of VEGF) as well as in pathological states (long-confluent and sparse cells exposed to an angiogenic concentration of VEGF).
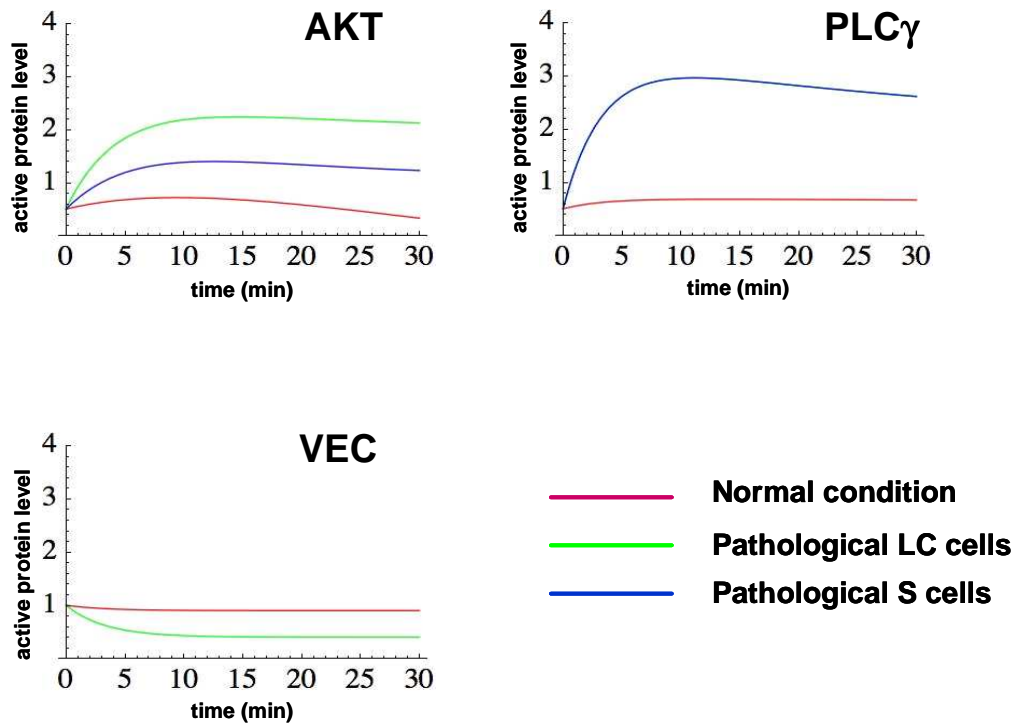

Figure R5. Computational simulation of Akt, PLCγ and VEC behaviors. *Normal condition (LC cells) and pathological states (LC and S cells) were simulated using as input VEGF concentration of 0.5 and 30 ng/ml, respectively. Akt is active in both normal and pathological state: in normal condition its activation is low, while in pathological condition Akt activation is increased and it is higher in LC than in S cells. PLC is not active in normal condition, on the contrary it becomes active in pathological states both in LC and S cells with the same activation level and kinetic (note the overlapping of green and blue curves). VEC remains unchanged in normal condition, while in pathological states of LC cells it decreases (note the absence of VEC behaviour in pathological state of S cells, due to the fact that in these cells VEC is not clusterized in the membrane and not associated with VEGFR-2, therefore it does not influence VEGF signaling).*

DISCUSSION

In this work VEGF biology was studied through a Systems Biology approach in order to develop a mathematical model of signal transduction to better understand the relationship between cellular environment and VEGF-induced signaling. The resulted model offers a quantitative, dynamic representation of early intracellular signals elicited by VEGF in ECs. It describes how VEGF-induced responses are dependent on the growth factor concentration and the presence of mature cell-to-cell junctions. In particular it proves that VEGF can be considered as a survival factor for quiescent ECs as well as a potent angiogenic regulator in pathological conditions. The model represents a basic framework suitable for the addition of further levels of complexity to study VEGF biology in fine details in order to help the design of strategies for therapeutic purpose.

To develop the current model, the following strategy has been used:

- The available biological information, regarding the signaling transduction of VEGF and its effects both *in vivo* and *in vitro*, were collected.

- It was written an *a priori* conceptual model based on the available biological information and containing the working hypotheses. In particular, it was supposed that in normal condition VEGF could contribute to maintain the survival of quiescent ECs and it was considered that in pathological states, in addition to the survival effect, VEGF is able to induce hyperpermeability in quiescent ECs and proliferation in cells which are characterized by the absence of mature cell-to-cell junctions.

- Focused wet-biology experiments were performed using quantitative immunoblotting technique combined with a proper data analysis to generate quantitative data characterized by error reduction and control of experimental reproducibility.

- The a priori conceptual model was rewritten in *a posteriori* form using quantitative data readout in combination with further information derived from the literature and not included in the initial version of the model.

- Finally, the model was translated into mathematical equations and validated through the comparison with experimental data.

The mathematical model is in good agreement with experimental data readout. It reproduces the different behaviour of the system in the normal condition of the endothelium as well as in the pathological state of ECs, showing the different behaviour of key selected proteins and explaining the link between EC environment and VEGF-induced signal transduction.

Mathematical Model Feature

Most models are systems of ODEs based on mass-action-law. This approach requires that the kinetic parameters (rate constants, K_m, V_{max}, and concentrations) are known for each respective reaction. Some of these values are available in the literature, but they often are the results of measures obtained in different type of cells or through various techniques. This poses a fundamental problem: it is difficult to decide whether model outcome reflect the underlying biology or the effect of heterogeneous parameters.

On the contrary, in this dissertation ODE description were used as a phenomenological representation of the system. It does not specifically take into account all the reactions involved (since many of the kinetic constants are unknown), but reproduces the experimental system behaviour considering the dynamic of the main actors involved. Even though not specifically, it implicitly takes into account all the kinetic constants through its parameters.

The Choice of Model Components and Signaling Meaning

The current model includes early signaling events downstream VEGF–VEGFR-2 system, which are strictly link to EC environment. As previously discussed, since a quantitative model containing all the signal transduction components is hard to obtain at the beginning, it is necessary to choose which components play a role and which of them can be omitted. In this work the selection of a small but sufficient numbers of key components has been of primary importance, because it allowed the construction of a conceptual as well as a mathematical model that describes the simplest possible representation of the biology of interest and corresponds to a basic structure for further implementation.

In the context of the proposed model, the inputs are different concentrations of VEGF and the outputs are considered in terms of the activity of selected signal proteins,

but the main assumption − that is supported by information derived from the literature − is that these would feed into downstream effector process leading to a specific cell behaviour.

Akt has been considered as the output for cell survival. Its PI3K-dependent activity induces the expression of prosurvival protein (BCL2 via CREB) as well as the downregulation of proapoptotic proteins (BIM via FOXO), leading to cell survival; moreover Akt may also play additional roles, for example it may contribute to the function of Ca^{2+}-activated eNOS.

PLCγ was assumed to be able to provide information for both permeability and proliferative signaling; this is motivated by the fact that PLCγ represents the early VEGFR-2 effector able to activate the above-mentioned pathways using various downstream proteins. Although it is very risky to clearly separate these two ways of signal, as a first approximation it is possible to say that the route of permeability requires that the signal dependent on PLC preferentially converges on e-NOS, while the the proliferative pathway implicates the PLCγ-PKC dependent MAP kinases cascade activation, leading to DNA synthesis and cell proliferation.

Indeed, from a viewpoint that takes into account the complexity of the system, additional levels of signaling have to be considered. For example, the literature tell us that ERK also plays a role in hyperpermeability, thanks to its ability not only to operate in the nucleo (triggering cell proliferation), but also in the cytoplasm through a phosphorylation-dependent regulation of MLC that contributes to VEGF-induced permeability. Therefore it is very intriguing considering the notion that the cell may use signal effectors that traditionally have been classified as belonging to separate signal paths in order to use their multiple functions tailored to the need to obtain a specific response.

These considerations, in combination with some recent evidences (e.g., those reported an additional PI3K-dependent ERK role in cell survival) determine the choice of the exclusion of ERK, as well as other signal effectors, as model components in this phase of work. In fact, to include extra effector proteins, requires a more detailed study not only in terms of activation but also in terms of spatial distribution. This falls outside the purpose of the current model.

Signal Transmission in the Model

In the proposed model VEGF-VEGFR-2 system is considered to be able to activate both PLCγ and PI3K, but their activations are strictly dependent on the presence of mature cell junctions. In normal condition, characterized by a low amount of growth factor, VEGFR-2 induces only the PI3K-Akt dependent survival pathway without effecting the activation of PLCγ. In pathological conditions, where the concentration of VEGF is increased, the receptor is able to activate both Akt and PLCγ; moreover this happens in quiescent cells as well as in cells characterized by the loss of cell junctions (sparse cells).

The receptor ability to induce Akt signaling and its failure to promote PLCγ activity in normal conditions are described and explained as due to the influence exerted by VEC. This is supported by the biological knowledge derived from literature. In particular, Lampugnani and coworkers proposed that, at the level of cell junctions, VEC binding to VEGFR-2 concentrates the receptor at junctions and makes it available to DEP and possibly other junctional-associated phosphates that would limit its activation (Grazia Lampugnani et al., 2003). Moreover, on the basis of experimental data, they suggested that phosphatase activity associated with the VEC complex may be specific for the VEGFR-2 tyrosine residue involved in PLC-dependent signaling and not others. This would inhibit receptor interaction with PLCγ without affecting PI3K activation. In fact, some authors reported that phosphorylation of Tyr1175 is crucial for PLCγ but not for PI3K binding to VEGFR-2 (Takahashi et al., 2001).

The different response of VEGFR-2 signaling in pathological conditions is again dependent on VEC, but in this case it occurs in terms of its reduction at the level of the plasma membrane (quiescent/long-confluent cells) or its absence (sparse cells). Once more this is supported by literature data. Specifically it has been taken as reference the strong evidence suggesting VEGF capability to induce VEC internalization during angiogenesis. In particular, Gavard and colleagues demonstrated that an angiogenic concentration of VEGF results in the rapid internalization of VEC in a clathrin-containing vesicular compartment, and that the expression of a VEC mutant that is persistently internalized provokes the disruption of the cell junctions and enhances EC permeability (Gavard and Gutkind, 2006). On the basis of this notions, in the model was consider the fact VEGF-induced internalization of a significant fraction of VEC could trigger the unlock of the inhibition exerted by VEC-DEP on PLCγ in quiescent ECs exposed to an high concentration of VEGF. In regarding of the activation of PLCγ in

sparse cells, it was sufficient to consider that these cells do not present mature cell junctions containing VEGFR-2–VEC complex and therefore they are not influenced by junctional-associated phosphatases.

It is important to note that in the model the action of VEC-DEP on PLCγ, in long-confluent cells exposed to a high concentration of VEGF, was not explicitly written in the PLCγ's equation; however it is implicitly included in the effect of increasing amount of growth factor. Indeed, PLCγ's equation is actually a critical point of the system: it describes well the experimental data, but does not reflect the assumed underlying biological mechanism. A plausible hypothesis considers that further differences may be present on the basis of cell confluence, leading to different initial conditions. Of interest in 1998, Liu and coworkers showed that VEGFR-2 expression in confluent HUVECs is more than two-fold greater than in sparse cells (Liu and Ellis, 1998). This finding, transposed to the current model, may mean that the basal conditions – in terms of VEGFR-2 ($\propto$VEGF) – are different, resulting in the need to reformulate PLCγ's equation and this could actually require the introduction of VEC dependence to obtain a good agreement with experimental data and consequently a more consistent representation of the natural system.

On the one hand, an angiogenic concentration of VEGF promotes PLCγ activity both in long-confluent and sparse cells, with a similar activation level and kinetics. In this context it is reasonable to attribute a difference in terms of meaning: permeability in long-confluent cells and proliferation in sparse cells. On the other hand, upon the same stimulation, VEGF induces a higher activation of Akt in long-confluent than in sparse cells. Interestingly, it has been showed that in confluent cells VEC in associated with VEGFR-2 in a multicomplex containing PI3K (probably the regulative subunit p85) and that this reinforced VEGFR-2 activation of PI3K. Therefore, here it was supposed that the VEC fraction that remains in the membrane could be responsible for the high level of AKT activation in long-confluent cells through the facilitated recruitment of the catalytic subunit p110 of PI3K to this multicomplex. This speculation has been included in the model as an additive effect. Moreover, regarding the high level of AKT activation in long-confluent cells exposed to an high concentration of VEGF, one can supposed that the meaning could be the need of increased survival signal in order to counter the possible presence of negative signals induced, for example, by the loss of cell junction and/or that enhanced Akt activity is necessary for its additional functions contributing to VEGF-induced permeability pathway (i.e., Akt assisted stimulation of Ca^{2+}-activated eNOS).

In terms of time – as time of angiogenic process – the signal transmission and the model itself can be summarized as follows: quiescent endothelial cells are exposed to a low concentration of VEGF which only help them to survive; then, when the concentration of VEGF increases in pathological conditions, ECs become active and, in addition to the survival pathway, VEGFR-2 is able to induce PLCγ activity and hyperpermebility; this is followed by cell migration resulting in a condition very similar to *in vitro* sparse cells, where PLCγ-dependent proliferative pathway is active.

Signal Downregulation in the Model

In a classical pathway, early effector proteins are recruited to the receptor and in turn they transmit the signal to downstream key proteins which ultimately lead to the activation of the final effector proteins. At the same time, however, while signaling proteins are still being recruited to positively convey the signal into the cell, negative signaling events are initiated that will attenuate the amplitude and duration of the signal. The most obvious of these are the receptor internalization mechanisms and the activation of specific phosphatases.

Internalization of activated receptor is triggered by specific receptor ubiquitination events that in general target the receptor for inactivation and destruction in the lysosome. However strong evidences indicate that internalization is not just a sink through which receptors are degraded. In fact, it has been proposed that internalized receptors can maintain their activity; in particular, recent publications indicate that signaling through growth factor receptors does not occur only at the cell membrane but may continue from intracellular compartments (Di Fiore and De Camilli, 2001; Le Roy and Wrana, 2005; Miaczynska et al., 2004; Sorkin and Von Zastrow, 2002).

Recently, it has been reported that VEGFR-2 internalization and degradation are regulated by ubiquitination through a Cbl-dependent mechanism (Duval et al., 2003) or C-tail serine phosphorylation activated PKC (Singh et al., 2005). Unfortunately, these studies were not conducted in primary ECs and they did not take into account the possible influence by the state of cell confluence (i.e., the presense or absence of mature cell-to-cell junctions). Indeed cadherins may influence growth factor receptor internalization, but the extent to which they do depends on the cadherin or growth factor receptor. In tumor cell lines, N-cadherin forms a complex with FGF receptor 1 that inhibits its internalization and degradation; this causes a sustained FGF signaling and abnormal cell growth (Suyama et al., 2002). In contrast, E-cadherin cointernalizes with

FGF receptor 1, which facilitates its nuclear translocation and signaling activity (Bryant and Stow, 2005; Bryant et al., 2005). Lampugnani and coworkers analyzed the role of VEC on VEGFR-2 internalization and signaling in ECs (Lampugnani et al., 2006). They found that in HUVECs the receptor is internalized more rapidly and efficiently when VEC is not clustered at intercellular contacts (i.e., in sparse cells); moreover internalization does not terminate receptor signaling, which instead continues in endosomes.

In the model developed in this thesis, the downregulation of VEGFR-2 signaling was not considered in terms of rapid receptor degradation, but it was related to the activity of specific phospatases. This choice was essentially motivated by 1) the lack of information relative to the degradation of VEGFR-2 in long-confluent and sparse HUVECs and 2) the consideration that internalized receptor may continue its signaling before being degraded or recycled.

Previous studies suggested that several phosphatases, including SHP-1, SHP-2, HCPTPA, and PTP1B, may regulate VEGFR-2 signaling. SHP-1 is involved in TNFα–mediated prevention of VEGFR-2 phosphorylation (Sugano et al., 2007), whereas SHP-2 inhibits tyrosine phosphorylation of VEGFR2 in ECs when they are cultured only on type I collagen (Mitola et al., 2006). HCPTPA overexpression attenuates VEGFR2 autophosphorylation and reduces EC proliferation and migration (Huang et al., 1999). Overexpression of PTP1B, inhibits VEGF-induced phosphorylation of VEGFR2 and ERK as well as EC proliferation, without effecting VEGF-induced Akt and p38 phosphorylation and EC migration (Nakamura et al., 2008). These findings suggest that different phosphatases may influence different VEGF-induced cell responses; in other words, dephosphorylation of the receptor by phosphatases is highly site-specific as well as the downstream signaling events that derive from the autophosphorylated tyrosines within VEGFR-2. Therefore, in the contest of the model proposed in this dissertation, it was possible to assume the existence of two different phosphatases playing a role in terms of VEGFR-2 signal downregulation: the first phosphatase may preferentially bind to the VEGFR-2–PLCγ complex but not to the VEGFR-2–PI3K complex, and the second phosphatase may do the opposite. These proteins are assumed to have an activity not correlated with the presence of cell junctions.

Although in the model the degradation of the receptor was not considered, the reactions corresponding to the included phosphatases were written in such a way as to make them dependent on the VEGF concentrations and stimulation time. In fact it is reasonable to consider that the phosphatases are sensitive to the different level of target

phosphorylation and that they are equipped with its own kinetic of activation. Therefore, this behaviour is similar to that of a supposed mechanism of receptor degradation. The main difference between the mechanisms of degradation and dephosphorylation consist in the consideration that in the first case the amount of ligand (VEGF) cannot be considered as a constant value over time of stimulation, while in the second case it can be kept invariable. Moreover degradation and dephosphorylation mechanisms may coexist and may be responsible for the downregulation of downstream protein, such as PI3K, Akt and PLCγ. Even if the mathematical model is in good agreement with experimental data, indeed further biological information about the downregulation of VEGFR-2 in long-confluent and sparse HUVECs are need to implement the proposed model. This can be considered as an additional level of complexity to include in the model for a better understanding of VEGF biology.

Conclusions and Perspectives

Conceptual as well as mathematical models of growth factors signaling make a valuable contribution to better understand cell biology. Nonetheless they contain gaps, in particular with respect to the intricate cellular mechanisms that often involved the contribution of many common effector proteins to different transduction pathways. Here the proposed model has been developed in order to deal with the simple possible representation of the biology of interest, characterized by a small but sufficient number of components and processes. While this model does not aim for completeness, it provides a better understanding of VEGF biology in terms of the relationship between EC environment and VEGF-induced signal transduction events.

The presented model can be considered as the basis for future implementation through the insertion of further levels of complexity such as receptor degradation as well as additional effectors proteins. To address this issue, the natural continuation of the current work will have to incorporate more signal modules downstream of VEGF–VEGFR-2 system. This will require a move towards a more integrated, spatially rich description of the system and the adoption of other modeling techniques beyond ODEs. By including more details the generated model may attain relevance and enough predictive power to suggest new experimental or therapeutic interventions.

MATERIAL AND METHODS

Cell Culture and Cell Preparation

Human EC isolated from umbilical veins were routinely cultured, as previously described (Bussolino et al., 1992) on tissue culture vessels coated with gelatine (1%). Cells within 3 *in vitro* passages were used. Taking into account the fact that primary cultures isolated directly from umbilical veins often show undesirable variability from culture to culture, pulls of HUVECs, isolated from different donors, were used.

On the basis of the previously reported effect of cell density on cell-to-cell junction assembly (Lampugnani et al., 1995), long-confluent cells were seeded at a density of 10×10^3 cell/cm^2 allowing the achievement of confluence 48-72 hours before the experiment; the density of long-confluent EC monolayer was monitored several times a day and care was taken to use cultures that had received the last change of medium 48 h before the experiment. Long-confluent cells were characterized by the presence of mature cell-to-cell junctions containing VEC. Sparse cells were seeded at a density of 3.5×10^3 cell/cm^2 24 hours before the experiments; these cells showed the almost total absence of cell-to-cell contact and a low cell density, moreover they were also distinguished from long-confluent cells on the basis of a shorter period relative to the formation of the cell-substrate adhesion.

Reagents and Antibodies

Human recombinant VEGF-A$_{165}$ was from R&D Systems. The following antibodies were used: rabbit anti-phospho-PLCγ (Tyr783) (Cell Signaling); rabbit anti-Akt (Ser473) (Cell Signaling); rabbit anti-FoxO4 (Ser193) (Cell Signaling); mouse anti-tubulin (SIGMA); goat anti-vinculin (Santa Cruz). The following secondary horseradish peroxidase (HRP)-coupled antibodies were utilized: anti-rabbit-HRP (Jackson), anti-mouse HRP (Jackson), anti-goat-HRP (Santa Cruz).

Biochemical Analysis and Quantitavive Data Generation

Long-confluent and sparse cells were serum starvated for 3 hours, stimulated with 0.5 or 30 ng/ml of VEGF for different times as indicated in the specific sections. The medium was removed from the cells, after two washes with ice-cold PBS hot SDS buffer (62.5 mM Tris-HCl pH 6.8, 4,4% SDS, 22,2% glycerol) was added, cells were immediately collected using a cell scraper, and the extract was further boiled for 10 minutes. A total of 30 µg protein was loaded for each lane of 8% polyacrylamide gels, separated by SDS electrophoresis, and transferred to nitrocellulose (GE Healthcare).

The blots were analyzed with the antibodies against phosphorylation sites in target signaling molecules and then reprobed with the antibody anti-total endogenous proteins. The immunoblots were incubated for 1 minute with enhanced chemiluminescent (ECL; PerkinElmer) and immediately placed under the CCD camera (ChemiDoc^{TM} XRS; Bio-Rad) in a dark environment; the CCD chip was exposed to the image over a specific exposure time and a digitized image was acquired.

The data resulting in the determination of an integrated density value per data point were quantified using Quantity One$^{®}$ software and then analysed as follows:

- The signal derided from every samples detected with anti-phospho antibodies was divided by the signal of the corresponding normalizer protein. Vinculin was selected as the normalizer for PLCγ and tubulin as the normalizer for both Akt and FOXO4; the choice was motivated by the fact that these endogenous protein possess a similar molecular weight as the proteins of interest and therefore their signals can be used as blotting error estimate;

- Normalized data relative to time-course experiments were analyzed calculating the weighted mean of the same time point belonging to different experimental sets relative to a specific target.

The plots reported in the section "Results" represents the weighted mean of at least three independent experiments; they show the relative SD, which has been used as an estimate for experimental reproducibility.

Mathematical Modeling

The mathematical model was based on the following system of ODEs:

$$
\begin{aligned}
\dot{\gamma} &= -\alpha(\gamma - \gamma_0) + B(r\gamma_M - \gamma) - rv\pi \\
\dot{k} &= -\alpha'(k - k_0) + r(D + Mc)(k_M - k) - z\pi' \\
\dot{c} &= -\alpha_c(c - c_0 + Ar) \\
\dot{\pi} &= -r\alpha_\pi(\pi - \pi_M) \\
\dot{\pi}' &= -r\alpha_{\pi'}(\pi' - \pi'_M)
\end{aligned}
$$

γ, k, c, π and π' represent the biochemical species necessary to describe the biology of interest. In particular: γ is pPLCγ, k is pAkt (which is assumed to be directly proportional to active PI3K; this is supported by the fact that pAkt is PI3K dependent), c is VEC clusterized at the cell junction, π is the active phosphatase able to influence PLC signaling, and π' is the active phosphatase acting on Akt signaling. r stand for the concentration of VEGF.

In the simulation studies, r has been considered as the input of the system and it was used in the range 0→30; in particular r = 0.5 was applied in the case of normal condition and r = 30 was employed to show the pathological behaviour of the systems.

An educated guessing of both the parameters and the initial conditions, allowed the choice of the set below.
Parameters:
$\{\alpha; B; \gamma_M; v\}$ = {0.1; 0.2; 1.6; 0.03};
$\{\alpha'; D; M; k_M; z\}$ = {0.06; 0.01; 0.01; 9.0; 0.1};
$\{\alpha_c; A\}$ = {0.3; 0.2};
$\{\alpha_\pi; \pi_M\}$ = {0.01; 4.5};
$\{\alpha_{\pi'}; \pi'_M\}$ = {0.05; 2.0};
all these parameters were kept unchanged except for M that was converted to zero in the case of pathological state in S cells.
Initial conditions:
$\{\gamma_0; k_0; c_0; \pi_0; \pi'_0\}$ = {0.5; 0.5; 1; 0.1; 0.3}.

ODEs were solved using the Runge-Kutta method.

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