### POLITECNICO DI TORINO Repository ISTITUZIONALE

### VEGF-Mediated Signal Transduction in Tumor Angiogenesis

Original

VEGF-Mediated Signal Transduction in Tumor Angiogenesis / Napione, Lucia; Alvaro, Maria; Bussolino, Federico - In: Physiologic and Pathologic Angiogenesis - Signaling Mechanisms and Targeted TherapyELETTRONICO. - [s.I] : InTech, 2017. - ISBN 978-953-51-3024-6. - pp. 227-249 [10.5772/66764]

Availability: This version is available at: 11583/2694245 since: 2018-01-25T19:21:40Z

Publisher: InTech

Published DOI:10.5772/66764

Terms of use:

This article is made available under terms and conditions as specified in the corresponding bibliographic description in the repository

Publisher copyright

(Article begins on next page)





## World's largest Science, Technology & Medicine Open Access book publisher









AUTHORS AMONG **TOP 1%** MOST CITED SCIENTIST





Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Chapter from the book *Physiologic and Pathologic Angiogenesis - Signaling Mechanisms and Targeted Therapy* Downloaded from: http://www.intechopen.com/books/physiologic-and-pathologicangiogenesis-signaling-mechanisms-and-targeted-therapy

> Interested in publishing with InTechOpen? Contact us at book.department@intechopen.com

# VEGF-Mediated Signal Transduction in Tumor

### Angiogenesis

Lucia Napione, Maria Alvaro and Federico Bussolino

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66764

#### Abstract

The vascular endothelial growth factor-A (VEGF) plays a crucial role in tumor angiogenesis. Through its primary receptor VEGFR-2, VEGF exerts the activity of a multitasking cytokine, which is able to stimulate endothelial cell survival, invasion and migration into surrounding tissues, proliferation, as well as vascular permeability and inflammation. The core components of VEGF signaling delineate well-defined intracellular routes. However, the whole scenario is complicated by the fact that cascades of signals converge and branch at many points in VEGF signaling, thus depicting a complex signal transduction network that is also finely regulated by different mechanisms. In this chapter, we present a careful collection of the best-characterized VEGF-induced signal transduction pathways, attempting to offer an overview of the complexity of VEGF signaling in the context of tumor angiogenesis.

Keywords: VEGF, signaling, angiogenesis, endothelial cells

#### 1. Introduction

It has been over four decades that Judah Folkman hypothesized, demonstrated and emphasized the critical importance of angiogenesis in tumor growth [1]. His experimental studies showed that in the absence of vascularization a tumor would grow only to a finite size of few thousand cells, restricted by the inability of oxygen and nutrients to penetrate the tissue beyond the diffusion limits of approximately 1–2 mm. To overcome this passive diffusionlimited size, the tumor must perturb the physiological state of its environment inducing the



© 2017 The Author(s). Licensee InTech. This chapter is distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

so-called angiogenic switch that implicates the transition from quiescent to active endothelium leading to the vascularization of the growing cell mass. The angiogenic switch was initially hypothesized to be triggered by the production and release of a growth factor called TAF (tumor angiogenesis factor) by tumor cells [2]. Indeed, the explosive growth in tumor angiogenesis research identified and characterized a number of angiogenic inducers. Among them, vascular endothelial growth factor (VEGF) is recognized as the major tumor angiogenesis factor [3].

VEGF family consists of five secreted proteins (VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor). In terms of endothelial biology and tumor angiogenesis, VEGF-A (hereafter referred as VEGF)—in particular VEGF-A<sub>165</sub>—is considered to be the most physiologically relevant form. VEGF angiogenic potential is strictly dependent on its multifunctional activity. Indeed, the coordinated arrangement of endothelial cells to form and maintain new vascular tubes requires the induction of vascular permeability, endothelial cell migration, proliferation and survival. These biological responses take place in the endothelium via a complex network of intracellular signal transduction pathways, mainly mediated by VEGF-induced VEGF receptor 2 (VEGFR-2) activation [4].

In this chapter, after a short historical synopsis of Judah Folkman's hypotheses and main discoveries in the field of tumor angiogenesis, we will present a careful collection of the best characterized VEGF-induced signal transduction mechanisms, attempting to offer an overview of the complexity of VEGF signaling. The most intriguing aspect is that cascades of kinases, activity of other enzymes and recruitment of adapter proteins converge and branch at many points in VEGF signaling, emphasizing how linear pathways can integrate to form a complex signal transduction network. If multitasking and integrated signaling go some way toward an understanding of the functional versatility of VEGF, it becomes quite complicated to elucidate how specific information is processed through these pathways and how signaling events are regulated in order to trigger a specific cellular behavior.

# 2. Historical synopsis of Judah Folkman's hypotheses and main discoveries in the field of tumor angiogenesis

Judah Folkman's scientific achievements in angiogenesis research revolutionized biomedical research and clinical drug development. Until the early 1970s and for some years thereafter, the conventional wisdom was that tumor vasculature was an inflammatory reaction to dying tumor cells. In 1971, Folkman articulated several "visionary" hypotheses on tumor angiogenesis which are now widely accepted. His ideas were based not only on his own work, but also on some studies of a small number of investigators [5–7]. As well summarized in a Cancer Research Commentary recently written by Augustin [8], Folkman published his hypothesis article in 1971, (i) predicting that tumors would be restricted to microscopic size in the absence of angiogenesis, (ii) suggesting that tumors secrete diffusible angiogenic molecules, (iii) describing a model of tumor dormancy due to the blocked angiogenesis, (iv) proposing the term anti-angiogenesis for the prevention of new capillary sprouts from being recruited

into a growing tumor, (v) envisaging the future discovery of angiogenesis inhibitors and (vi) proposing the idea that an antibody to a tumor angiogenic factor could be an anticancer drug.

Folkman and collaborators obtained the first evidence of the existence of the avascular and vascular phases of solid tumor growth in 1963, on the basis of experiments in isolated perfused organs [9]. A rabbit's thyroid gland was seeded with cancer cells from mice and perfused with a blood substitute. Tiny tumors formed, but they grew to the same size, then stopped and never became vascularized. When tumors were transplanted into live mice, they rapidly vascularized and grew vigorously. This lab work and some clinical observations (in particular those regarding retinoblastoma in children) help Folkman develop and sustain the hypothesis that tumor growth is angiogenesis dependent.

In the decade following the 1971 report, research in the tumor angiogenesis field attracted little scientific interest, but Folkman and his team preserved in their investigations, providing convincing evidences for dependence of tumor growth on neovascularization [10–14]. This was achieved in particular thanks to the development and use of bioassays devoted to angiogenesis research, such as the model of eye transplant and chick embryo chorioallantoic membrane. Of note, one of the major steps in allowing scientific appreciation of the role of tumor angiogenesis and demonstrating angiogenesis in vitro was developing methods for passage of endothelial cells. In 1979, Folkman's laboratory reported long-term passage of endothelial cells [15], and the following year, they demonstrated angiogenesis in vitro using endothelial cell cultures exposed to tumor conditioned media [16].

After the developments of the late 1970s, many other scientists entered the field of angiogenesis and Folkman's skeptics became his competitors [17]. In particular, the 1980s were an intense period of hunting for the hypothesized TAF [8]. In 1983, Dvorak and collaborators reported the isolation of a tumor-derived factor that they called "vascular permeability factor" (VPF) on the basis of its capability to induce blood vessel leakage [18]. However, at that time, VPF was not completely purified and therefore not fully identified. In 1989, Ferrara purified a novel angiogenic protein that he termed "vascular endothelial growth factor" (VEGF) on the basis of its observed growth-promoting activity toward only vascular endothelial cells [19]. Around the same time, Folkman's laboratory isolated an angiogenic protein that resulted to be identical to that purified by Ferrara [20]. By 1990, it was realized that VEGF and VPF were in fact the same protein. There is no doubt that it was the discovery of VEGF to set in motion a revolution in the field of angiogenic research.

From 1980 to 2005, Folkman's laboratory reported the discovery of eleven angiogenesis inhibitors, eight of them are endogenous angiogenesis inhibitors [21]. Effort persisted in this area and new anti-angiogenic molecules are continuously being developed. They essentially fall into two distinct types: (i) antibody directed toward angiogenic factors such as VEGF, for example, Avastin (Bevacizumab, Genentech) and (ii) small molecules inhibiting cellular signaling by targeting multiple receptor tyrosine kinases among them VEGFR-2, for example, Sutent (Sunitinib, Pfizer) and Nexavar (Sorafenib, Bayer and Onyx Pharmaceuticals) [17].

Targeting VEGF and VEGFR-2 offers benefit to patients with at least some types of cancer and provides proof of principle that attacking the vasculature is a valid approach to cancer

therapy [22]. At present, however, despite important results, the overall clinical benefits of anti-VEGF/VEGFR-2 therapy are still relatively modest: not all cancer patients respond to anti-VEGF treatments, and when they do increased survival may only be measured in weeks or months [17]. This is realistically due to a number of different and not yet fully clarified reasons, which open discussion going beyond the topic of this chapter. Here, we will only mention, as reported by Van Epps in 2005, that one of Folkman's hopes for the future was that anti-angiogenesis therapy could be initiated—based on diagnostic biomarkers—even before the tumor reveals its location in the body, thus stopping cancer before it really gets started [23, 24].

#### 3. VEGF-mediated signal transduction

In vivo angiogenic response to VEGF is mainly mediated via activation of VEGFR-2, expressed primarily in endothelial cells. VEGFR-2 activation initiates several intricate signaling paths, which eventually lead to different endothelial responses: cell survival, proliferation, migration, invasion into the surrounding tissue, vascular permeability and vascular inflammation [25]. These responses involve (i) a number of pivotal effectors such as phosphoinositide 3 kinase (PI3K), phospholipase C $\gamma$  (PLC $\gamma$ ), SRC, focal adhesion kinase (FAK) and Rho family of GTPases; (ii) several multifunctional docking proteins and adaptors; and (iii) VEGFR-2 partners such as Neuropilin 1 (NRP1), integrins and vascular endothelial (VE)-cadherin. It is apparent that these proteins orchestrate a complex signaling network leading to the integration of the different VEGF-induced endothelial responses that allows tumor angiogenesis to take place. **Figure 1** illustrates, in a simplified manner, VEGF-mediated signal transduction, showing signal core components along with the main well-defined intracellular routes leading to different endothelial responses.

#### 3.1. VEGFR-2 activation

VEGFR-2 is a tyrosine kinase receptor (RTK). Binding of VEGF to VEGFR-2 promotes receptor dimerization, allowing trans/autophosphorylation of intracellular tyrosine residues. Among the 19 tyrosine residues present in the intracellular domain of VEGFR-2, there are five major phosphorylation sites: Y951, Y1054, Y1059, Y1175 and Y1214. The Y1054 and Y1059 are located in the kinase domain activation loop, and their phosphorylation is critical for receptor catalytic activity [26]. Y951 is located in the kinase insert domain, and its phosphorylation serves as a binding site for T cell-specific adaptor (TSAD) also known as VEGFR-2-associated protein (VRAP) [27, 28]. The Y1175 and Y1214 are located in the carboxy-terminal domain. Phosphorylation of Y1175 creates a binding site for PLC $\gamma$  [29], p85 subunit of PI3K [30], the adaptor proteins SHB [31] and SCK [32]. This residue is well-recognized as a critical mediator of VEGFR-2 signaling. Phosphorylated Y1214 has been described to bind the adaptor protein NCK [33].

VEGFR-2 activation and downstream signaling are modulated by different mechanisms. The main of them involve (i) receptor interaction with NRP1, specific integrins and VE-cadherin



Figure 1. Signal transduction mediated by VEGF/VEGFR-2. Core signaling pathways involved in VEGF-induced (a) cell survival and proliferation and (b) vascular permeability and cell migration. See text for details.

(see Section 3.7) and (ii) the activity of the protein tyrosine phosphatases (PTPs) such as vascular endothelial PTP (VEPTP), SRC homology 2 domain PTP (SHP2) and PTP1B [34]. VEGFR-2 activation may also be influenced by the presence of heparin sulfate glycoproteins that modulate VEGF-VEGFR-2 binding and signaling amplitude [35, 36]. Indeed, a change in ligand-receptor affinity may highly influence RTK signaling. We recently reported that

VEGF-VEGFR2 affinity may vary from low to high based on the endothelial cell density state that we also reported to influence the number of total and surface VEGFR-2 [37]. In particular, by combining wet-lab experiments, theoretical insights and mathematical modeling, we found that ligand-receptor affinity is reduced in long-confluent compare to sparse endothelial cells, which recapitulate in vitro the condition of quiescent and angiogenic endothelium in vivo.

#### 3.2. PLC<sub>Y</sub> signaling

Activated VEGFR-2 directly recruits PLC $\gamma$ , which is in turn phosphorylated [38, 39]. Phosphorylated PLC $\gamma$  hydrolyzes the membrane phospholipid phosphatidylinositol-4,5-biphosphate (PIP2), generating diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). This latter mobilizes Ca<sup>2+</sup> from the endoplasmic reticulum, thus leading to an increase in intracellular Ca<sup>2+</sup>. IP3-mediated Ca<sup>2+</sup> increase supports DAG-induced activation of PKC, from which it is triggered the RAF1-MEK-ERK1/2 mitogen-activated protein (MAP) kinase cascade, that is, the best characterized pathway propagated downstream to VEGF/VEGFR-2/PLC $\gamma$  axis resulting, in particular, in endothelial cell proliferation trough the ERK1/2-dependent regulation of gene transcription.

Most RTKs are known to utilize the classical GRB2-SOS-RAS activation of the RAF1-MEK-ERK1/2 cascade. Conflicting results exist in the literature with respect to the interaction of VEGFR-2 with SHC or GRB2, which recruit the RAS activating nucleotide-exchange factor SOS to the receptor, and the significance of the classical RAS-mediated MAP kinase cascade downstream to VEGFR-2 is unclear [32, 40]. Indeed, it is well-accepted that, after VEGF stimulation, most of the MAP kinase activation is mediated via the PLC $\gamma$ -activated PKC as first reported by Takahashi and coworkers [39, 41]. These authors described the pathway as RAS-independent; however, considering the same pathway, Shu and colleagues reported the involvement of sphingosine kinase (SPK), found to link PKC to RAS activation in a manner independent of RAS nucleotide-exchange factor [42].

VEGF-induced activation of PKC also results in activation of protein kinase D (PKD), found to influence ERK1/2 activation and cell proliferation [43]. In response to VEGF, gene repressive action of histone deacetylases (HDAC) 5 and 7 in endothelial cells is overcome by the PLC $\gamma$ -PKC-PKD pathway-dependent HDAC 5 and 7 phosphorylation and nuclear export, resulting in the regulation of gene transcription, cell proliferation and migration [44, 45]. Moreover, VEGF-induced PKC-dependent activation of PKD has also been reported to induce HSP27 phosphorylation and mediate cell migration without involving the p38 MAP kinase (p38 MAPK)/MAP kinase-activated protein kinase 2 signaling cascade [46].

As mentioned above, PLC $\gamma$  activation induced an increase in intracellular Ca<sup>2+</sup>. Ca<sup>2+</sup> signaling is crucial in VEGF/VEGFR-2 signal transduction, not only for PLC $\gamma$ /PKC-mediated pathways but also for activation of other molecular players such as, in particular, the endothelial nitric oxide synthase (eNOS) and the nuclear factor of activated T-cell (NFAT) family of transcription factors. eNOS plays a crucial role in the control of vascular homeostasis and permeability; its activity is regulated by a complex combination of protein–protein interactions and signal transduction cascades involving Ca<sup>2+</sup> mobilization and phosphorylation events [47]. VEGF stimulates both Ca<sup>2+</sup> and phosphorylation-dependent regulation of eNOS activity. VEGF stimulation in endothelial cells first leads to the Ca<sup>2+</sup>-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, resulting in potentiation of its activity [48]. NFAT proteinsextensively studied in the immune system - are functional in several cell types, including cancer cells, endothelial cells and infiltrating immune cells [49]. The multiple functions attributed to NFAT include cell growth, survival, invasion and angiogenesis. These transcription factors are activated through a Ca2+- and calmodulin-activated calcineurin-dependent mechanism. In endothelial cells, VEGF stimulates receptor-mediated activation of PLCy leading to an increase in intracellular Ca<sup>2+</sup>, calcineurin activation and NFAT nuclear translocation that in turn leads to the transactivation of genes that are essential for angiogenesis [49, 50], such as COX-2 resulting in synthesis of PGE2, a mediator of endothelial cell migration and tube formation [51]. Moreover, in endothelial cells, VEGF-mediated NFAT activation induces a gene repertoire that includes an inherent inflammatory component, similar to that of interleukin 1 [52]. In addition, it has been recently reported that endothelial cells decode VEGF-mediated  $Ca^{2+}$  signaling patterns to produce distinct functional responses: cell proliferation and cell migration involving NFAT and myosin light chain kinase, respectively [53].

VEGFR-2/PLC $\gamma$  signaling also involves TRPC channels, which are Ca<sup>2+</sup>-permeable nonselective cation channels. Indeed, VEGF induces Ca<sup>2+</sup> elevation through both Ca<sup>2+</sup> release from intracellular stores and extracellular Ca<sup>2+</sup> entry. In particular, VEGFR-2/PLC $\gamma$  axis activates TRPC3 and TRPC6 in a DAG-dependent manner [54]. TRPC6 has been reported to be required for VEGF-mediated Ca<sup>2+</sup> increase and the subsequent signaling that lead to processes associated with angiogenesis, such as cell migration, proliferation and tube formation [55, 56].

Overall, PLC $\gamma$  appears as a regulator of a number of pathways, leading to cell proliferation and migration and contributing to vascular permeability. Thus, a better understanding of its downregulation may be of interest in further elucidating the regulation of VEGF-induced signaling, as well as in the development of new anti-angiogenic therapies. Indeed, very little is known about the regulation of PLC $\gamma$  activity. Singh and colleagues have been reported that VEGF-induced PLC $\gamma$  ubiquitination inhibits its tyrosine phosphorylation, thus providing a negative feedback to prevent sustained PLC $\gamma$  stimulation [57]. In addition, we have previously reported the essential role of PTPs on VEGF-induced PLC $\gamma$  activation, suggesting the existence of at least one—not yet characterized—PTP directly targeting PLC $\gamma$  and counteracting receptor-mediated signal [37].

#### 3.3. PI3K signaling

VEGF/VEGFR-2 axis activates PI3K in different ways, including direct or indirect (i.e., through adaptor and scaffold proteins) binding of PI3K to the receptor, and the involvement of SRC (see Sections 3.4 and 3.6). The activated PI3K converts the plasma membrane lipid PIP2 to phosphatidylinositol-3,4,5-trisphosphate (PIP3), and signaling proteins with pleckstrin homology (PH) domains accumulate at site of PI3K activation by directly binding to PIP3 [58]. Among these signaling proteins of particular interest are the serine-threonine kinases

AKT and phosphoinositide-dependent kinase 1 (PDK1). Phosphorylation of AKT by PDK1 and PDK2 allows the full activation of AKT, resulting in the phosphorylation of a number of proteins [59]. Other PH domain-containing proteins that are activated by PIP3 include GDP-GTP exchange factors for the small GTPase RAC [58, 60].

PI3K/AKT pathway is considered the main mechanism by which VEGF induces endothelial cell survival. AKT mediates both short- and long-term cell survival effects by inhibiting (through direct phosphorylation) pro-apoptotic proteins such as BAD, caspase 9 and forkhead transcription factors, and by upregulating anti-apoptotic proteins such as BCL-2 [61, 62]. In 1998, Gerber and coworkers were the first to report that VEGF regulates endothelial cell survival through the PI3K/AKT signal transduction pathway and that VEGF also induces expression of the anti-apoptotic proteins BCL-2 and A1 in endothelial cells [63, 64]. In the following year, Tran and colleagues reported a marked induction of the IAP family anti-apoptotic proteins survivin and XIAP—which inhibit caspase 3, 7 and 9—by VEGF in endothelial cells [65].

Although crucial for its function in cell survival, AKT is not simply a regulator of cell survival but a multifunctional protein playing a pivotal role in both cancer cells and endothelial cells. In addition to cell survival, AKT activation has been linked to tumor angiogenesis via several other biological processes, including vascular permeability, cell proliferation, synthesis and release of matrix metalloproteinases and induction of VEGF production by cancer cells [59]. Beyond the pro-survival effect and considering the VEGF/VEGFR-2 signal transduction in the endothelium, the main contribution exerted by AKT resides in the direct phosphorylation of eNOS, thus contributing to the control of endothelial permeability [48, 66].

#### 3.4. SRC and FAK

The SRC family of protein tyrosine kinases (SFKs) plays key roles in regulating signal transduction by a diverse set of cell surface receptors in the context of a variety of cellular environments [67]. Endothelial SFKs includes SRC, YES and FYN. Many of the VEGF-mediated pathways involve SRC activity. Moreover, SRC is intimately involved in the modulation of the activity of FAK through direct phosphorylation (see below). VEGFR-2 activates SRC according to different mechanisms: (i) by direct binding to Y951 in the receptor [27], (ii) through the adaptor protein TSAD [68] or (iii) involving the scaffold protein GAB1 and GAB2 [69].

VEGF-induced SRC activity is involved in the activation of different signaling proteins, such as PI3K, FAK and eNOS. Holmqvist and colleagues reported that activated VEGFR-2 recruits SHB and SRC and that this latter phosphorylates SHB, which allows the subsequent activation of PI3K and phosphorylation of FAK at Y576 [31]. Duval and coworkers reported that VEGF induces phosphorylation of VEGFR-2-associated HSP90, which is dependent on receptor internalization and on SRC kinase activation; furthermore, they demonstrated that SRC directly phosphorylates HSP90 and that this event is essential for VEGF-stimulated eNOS association to HSP90 and thus NO release from endothelial cells [70]. In response to VEGF, Src may also activate members of the MAP kinase cascade such as RAF1 [71] and b-RAF [72]; this latter is activated in a manner dependent of SRC-mediated phosphorylation of the scaffold protein IQ motif-containing GTPase-activating protein 1 (IQGAP1).

In parallel with the effect exerted on intracellular signaling proteins, SRC plays a pivotal role in the disorganization of cadherin-dependent cell-cell contacts and in integrin-VEGFR-2 cross-activation. In response to VEGF, SRC phosphorylates VE-cadherin in adherens junctions, allowing endothelial cell migration and inducing vascular permeability [73–76]. Mahabeleshwar and coworkers reported that there is an intimate and coordinated relationship between VEGFR-2 and  $\alpha v\beta 3$  integrin involving SRC activity [77]. In particular, they demonstrated that (i) adhesion- and growth factor-induced  $\beta 3$  integrin tyrosine phosphorylation is critical for interaction between VEGFR-2 and  $\beta 3$  integrin, and (iii) SRC mediates growth factor-induced  $\beta 3$  integrin-dependent cellular adhesion, directional migration of endothelial cells and initiation of angiogenic programming in endothelial cells.

As mentioned above, SRC is involved in the modulation of FAK activity through direct phosphorylation. The non-receptor tyrosine kinase FAK-well recognized as an important regulator of cell migration—is localized in focal adhesions, established as a consequence of integrin ligation to the extracellular matrix. Upon integrin-dependent cell adhesion, phosphorylation of FAK and its catalytic activity are stimulated. FAK possesses six tyrosyl residues (i. e., Y397, Y407, Y576, Y577, Y861 and Y925) that are differentially phosphorylated by diverse agonists and that are implicated in transmitting different signals and effects. Y397 is an autophosphorylation site that recruits SH2 domain-containing proteins, including members of SKFs, PLCy and the p85 subunit of PI3K [78]. It appears that SRC is first recruited to Y397 and then involved in transphosphorylation of other tyrosyl residues within FAK, such as Y576 and Y577; this confers maximal activation of FAK and signaling in response to adhesion [79, 80]. In particular, upon VEGF stimulation, Y576 and Y861 are both phosphorylated in a SRCdependent manner [31, 81], while Y407 is phosphorylated in a SRC-independent manner that involves the recruitment of HSP90 to the receptor, followed by the activation of RHOA and that of RHO activated kinase (ROCK) [81]. This results in phosphorylation of FAK on S732 that allows FAK-related kinase PYK2-mediated phosphorylation of FAK on Y407, promoting cell migration [82].

#### 3.5. Rho family GTPases

The RHO family GTPases—shuttling between inactive GDP-bound and active GTP-bound forms—include RHO, RAC and CDC42, which are known to regulate primarily the reorganization of actin cytoskeletal systems such as actin stress fibers and focal adhesions, lamellipodia and filopodia, respectively [83]. A growing body of evidence indicates a crucial role for VEGF-induced RHO GTPases activity in endothelial cell during the processes involved in angiogenesis such as, in particular, cell migration and vascular permeability [84]. VEGF/ VEGFR-2 axis stimulates the activities of RHO [81, 85], RAC [86, 87] and CDC42 [88]. As reported in Section 3.4, VEGF-induced RHO activity stimulates FAK activation promoting cell migration [81]. VEGF-induced RAC activation has been linked to both endothelial permeability and cell migration. Gavard and Gutkind reported that RAC takes part in a signaling pathway by which VEGF stimulation promotes the rapid endocytosis of VE-cadherin, thereby disrupting the endothelial barrier function [86]. In particular, they demonstrated that VEGFR-2 activates RAC through the SRC-dependent phosphorylation of the nucleotide-exchange factor VAV2 and that RAC activation, in turn, promotes the p21-activated kinase (PAK)mediated phosphorylation of VE-cadherin resulting in the recruitment of beta-arrestin 2 to phosphorylated VE-cadherin, thereby promoting its internalization into clathrin-coated vesicles and the consequent disassembly of intercellular junctions. To complement this study, Garrett and coworkers reported that VEGF-induced SRC-dependent VAV2 phosphorylation and downstream activation of RAC1 are also responsible for endothelial cell migration and wound closure [87]. The involvement of CDC42 in VEGF signaling has been reported in particular by Lamalice and colleagues [33, 88]. They proposed a model according to which, upon VEGFR-2 activation phosphorylated Y1214 within the receptor recruits the adaptor protein NCK that becomes phosphorylated providing a recruitment site for FYN that is also phosphorylated and required for the phosphorylation of NCK and that of the p21-activated protein kinase PAK-2, an effector of CDC42; then, this early molecular complex containing VEGFR-2·NCK·FYN·PAK-2 triggers the sequential activation of CDC42 and p38 MAPK leading to actin polymerization, stress fiber formation and endothelial cell migration.

#### 3.6. Key docking, adaptor and scaffold proteins

As it emerges from what above described, it is evident that, beyond a number of kinases and other enzymes, VEGFR-2 signaling involves several key docking, adaptor and scaffold proteins including SHB, TSAD, NCK, IQGAP1 and GAB1. In the following, we briefly summarize their main involvement downstream to VEGF/VEGFR-2 axis.

The adapter protein SHB contains at least four different domains responsible for protein-protein interactions (i.e., the proline-rich motifs in its N-terminus, the phospho-tyrosine binding (PTB) domain, potential tyrosine phosphorylation sites and the C-terminal SH2 domain) and has been shown to operate downstream of several RTKs exerting versatile effects on a number of signaling pathways [89]. SHB binds to phosphorylate Y1175 in VEGFR-2 and is phosphorylated by SRC; this allows the subsequent activation of PI3K and phosphorylation of FAK at Y576 in the kinase domain, regulating the migratory response in endothelial cells [31]. Along this pathway, it is possible that SHB is required for VEGF-mediated activation of FAK by allowing SRC to phosphorylate Y576. Indeed, it has been demonstrated that the PTB domain of SHB can bind directly to FAK and regulate its activity in response to FGFR-1 activation in endothelial cells [90].

TSAD is an adaptor protein containing a SH2 domain, tyrosines in protein binding motifs and a proline-rich domain allowing SH3-dependent interactions. TSAD interacts with and modulates the activity of some SFKs such as LCK and SRC [68, 91], and has been found to control actin polymerization events in both T cells and endothelial cells [27, 92]. TSAD binds to phosphorylate Y951 in VEGFR-2 via its SH2 domain and to SRC via its proline-rich domain. TSAD has been reported as an important docking mechanism for SRC to VEGFR-2, involved in the regulation of cell migration, endothelial cell junctions and vascular permeability, but not cell proliferation [27, 68]. NCK is an adapter protein consisting of one SH2 domain and three SH3 domains. A main function of NCK is to link receptor and receptor-associated tyrosine kinases with proteins that directly or indirectly regulate remodeling and reorganization of the actin cytoskeleton [93]. According to the model proposed by Lamalice and colleagues, upon phosphorylation on Y1214 in VEGFR-2, NCK is recruited to the receptor thus allowing the formation of a molecular complex containing VEGFR-2·NCK·FYN·PAK-2 that convey the sequential activation of CDC42 and p38 MAPK leading to actin polymerization, stress fiber formation and endothelial cell migration [33]. Moreover, it has been reported that NCK participates with PAK in the signaling pathway by which VEGF stimulates the assembly of focal adhesions [94].

The multidomain scaffold protein IQGAP1 binds to several structural and signaling proteins. For example, interactions of the IQGAP1 calponin homology domain (CHD) with F-actin and the GAP-related domain (GRD) with small GTPases regulate the cytoskeleton to promote actin binding or polymerization that regulates cell migration, stability of cell-cell contacts and cytokinesis; moreover, IQGAPs also scaffold molecules form signaling complexes, such as components of the MAP kinase cascade, thus promoting their activity [95]. Along VEGF signal transduction, IQGAP1 becomes phosphorylated by SRC and activates b-RAF, contributing to cell proliferation [72]. Moreover, IQGAP1 has been implicated in regulation of cell migration and cell-cell contacts [96, 97]. In particular, Yamaoka-Tojo and colleagues suggested that IQGAP1 may function as a scaffold to link VEGFR-2 to the adherens junctions through binding to VEGFR-2 and VE-cadherin/ $\beta$ -catenin complex, thereby dissociating  $\alpha$ -catenin from the adherens junctional complex and contributing to VEGF-stimulated loss of cell-cell contacts in endothelial cell [97].

GAB1 is the prototype of a subfamily of large multiadapter proteins sharing an N-terminal PH domain, two proline-rich regions involved in constitutive binding to GRB2 and multiple tyrosine phosphorylation sites [98]. Downstream to VEGF/VEGFR-2 axis, GAB1—recruited through an amplification loop involving PIP3 and its PH domain—has been proposed to be a primary actor in coupling VEGFR-2 to PI3K/AKT [98]. In response to VEGF, it has been reported that GAB1 is phosphorylated and associates not only with PI3K, but also with GRB2, SHP2, SHC and PLC $\gamma$  influencing the signaling downstream to VEGFR-2 and, in particular, cell migration and capillary formation [99].

#### 3.7. Neuropilin 1, integrins and Vascular Endothelial Cadherin

VEGF signaling is complicated by the fact that VEGFR-2 interact with additional cellular proteins such as, in particular, NRP1, specific integrins and VE-cadherin [100]. This modulates the signal strength induced by VEGFR-2 on the basis of the extracellular cues arising from the soluble ligand, cell-substratum and cell-cell interactions.

NRP1 is a transmembrane receptor for VEGF and the neuronal guidance cue SEMA3A, with essential roles in both vascular and neuronal development, as well as in pathological angiogenesis [101, 102]. The precise mechanism of VEGF-VEGFR-2-NRP1 interaction and the functional consequences of this molecular complex are still being explored. The most widely accepted model of NRP1 function in angiogenesis postulates that it forms a VEGF-dependent

complex with VEGFR-2 to enhance the activation of a wide variety of intracellular signal transduction pathways, including those that involve ERK1/2, AKT, SRC and p38 MAPK [103]. Moreover, it has been reported that NRP1 promotes VEGFR-2 trafficking through RAB11 vesicles thereby specifying signal output [104].

Integrins link intracellular signaling pathways induced by soluble factor to output elicited by cellular interactions with extracellular matrix. Specific integrins, particularly integrin  $\beta$ 1 and  $\beta$ 3, act as important partners for VEGFR-2. An intimate and coordinated relationship between VEGFR-2 and  $\alpha v\beta$ 3 has been reported by Mahabeleshwar and coworkers (see Section 3.4) [77]. In particular, the relationship between VEGFR-2 and  $\beta$ 3 integrin appears to be synergistic, because VEGFR-2 activation induces  $\beta$ 3 integrin tyrosine phosphorylation, which, in turn, is crucial for maximum phosphorylation of VEGFR-2 [77]. Exposure of endothelial cells to matrix-bound VEGF promotes VEGFR-2-integrin  $\beta$ 1 complex formation, redistribution to focal adhesion, prolonged activation of VEGFR-2 with differential phosphorylation of Y1214 and extended activation kinetics of p38 MAPK [105].

VE-cadherin is involved in the formation of adherens junctions in endothelial cells and plays a crucial role in VEGF signaling. In resting endothelial cells, VE-cadherin complexes with VEGFR-2 at cell-cell contacts and attenuates VEGFR-2 phosphorylation through the phosphatase DEP1 [106]. Upon VEGF stimulation, VEC is phosphorylated and in turn internalized, thereby disrupting the endothelial barrier function (see Sections 3.4 and 3.5).

#### 3.8. VEGFR-2 internalization

It is well known that VEGFR-2 undergoes internalization and trafficking upon VEGF stimulation. Emerging evidences suggest that VEGFR-2 internalization and trafficking are tightly controlled processes that influence the sensitivity of endothelial cells to VEGF and the signaling propagated downstream to the receptor. Although the mechanisms regulating VEGFR-2 internalization and trafficking and their exerted effects on signaling are still not fully understood, it is apparent that a pivotal role is played by VEGFR-2 interaction with specific protein partners such as, in particular but not only, VE-cadherin and NRP1.

In endothelial cells, VEGFR-2 is located in different subcellular pools, including receptors diffusely distributed in the plasma membrane, engaged in cell-cell junctions through the interaction with VE-cadherin and associated with various intracellular compartments. Resting endothelial cells have two surface pools of VEGFR-2: a stable pool that is complexed with VE-cadherin, and a flux pool that is constantly cycling between the surface and the endocytic compartment in a VEGF independent manner [107, 108]. VE-cadherin prevents internalization of VEGFR-2 by physical interaction and recruitment of the DEP-1 phosphatase [106]. VEGF stimulation results in clathrin-dependent internalization of VEGFR-2. The clathrincoated vesicles fuse with early endosomes and then proceed through a series of steps that can either direct their recycling back to the plasma membrane via the fast (RAB4) or slow (RAB11) recycling pathways or target them for degradation into lysosome via the RAB7 pathway [109]. Ballmer-Hofer and colleagues reported that when complexed with NRP1, internalized VEGFR-2 is recycled through RAB4 and RAB11 positive vesicles; while in the absence of NRP1, internalized VEGFR-2 bypassed RAB11 vesicles and rapidly accumulated in RAB7 vesicles indicative of receptor degradation [104]. They also showed that VEGFR-2 is dephosphorylated before entry into the RAB11 compartment and then targeted to the plasma membrane where it presumably initiates a new round of ligand binding and receptor activation thereby prolonging VEGF signaling to downstream targets [104]. Furthermore, there are also evidences that when activated VEGFR-2—not yet dephosphorylated—is trapped inside endosomes, it is still capable of stimulating some downstream signaling proteins such as those belonging to the MAP kinase cascade [110].

# 4. Synopsis of VEGF-induced endothelial responses and their role in tumor angiogenesis

VEGF/VEGFR-2 signal transduction leads to six major endothelial responses: cell survival, invasion and migration into the surrounding tissue, proliferation, vascular permeability and vascular inflammation. These endothelial responses are tightly integrated to allow tumor angiogenesis to progress successfully.

Vascular permeability-crucial for normal tissue homeostasis-is a prerequisite for VEGFinduced angiogenesis. Endothelial permeability is mediated by the so-called transcellular and paracellular pathways, that is, solutes and cells can pass through (transcellular) or between (paracellular) endothelial cells. Transcellular passage requires cell fenestration and/or a complex system of transport vesicles that includes organelles called vesiculo-vacuolar organelles, while the paracellular pathway depends on the coordinated opening and closure of endothelial cell-to-cell junctions, combined with cell retraction. VEGF is involved in both transcellular and paracellular permeability. VEGF/VEGFR-2 axis induces increased endothelial permeability mainly through SRC-mediated signaling of VE-cadherin internalization (see Sections 3.4 and 3.5) that disrupts the endothelial barrier function, and by activation of PLC $\gamma$  that mediates an increase in intracellular Ca<sup>2+</sup> resulting in Ca<sup>2+</sup>-calmodulin-dependent regulation of eNOS (see Section 3.2). Activation of eNOS-also achieved by AKT-mediated phosphorylation-promotes an increase in vascular permeability by NO production that is followed by vasodilatation. VEGF-induced vascular permeability contributes to the dissemination of extracellular proteases and the deposition of a fibrin gel provisional stroma that changes the extracellular matrix of normal tissues from anti- to pro-angiogenic and stromagenic, favoring and supporting inward migration of endothelial cells and the growth of new endothelial sprouts [111]. In addition, the reduced vessel integrity may promote leukocyte extravasation and facilitate exit of metastasis from the primary tumors.

VEGF-induced vascular permeability goes in parallel with vascular inflammation. Although VEGF is not an inflammatory cytokine, VEGF-induced gene transcription—in particular through NFAT activation—includes a conspicuous inflammatory component (see Section 3.2). This could conceivably promote attraction of inflammatory cells that may contribute to the angiogenic response. Indeed, it is well established that tumor angiogenesis is linked to inflammation. On the one hand, tumor cells can be killed by the immune system; on the other hand, tumor can use leukocytes to supports its expansion.

Endothelial cell invasion into the surrounding tissue is made possible by means of the release of MMPs, which degrade the basal membrane and the extracellular matrix and allow the migration of endothelial cells to form capillary sprouts. Endothelial cells express different MMPs, and it has been reported that their expression is induced by VEGF and regulated by Ets transcription factors [112, 113]. Signaling pathways, such as MAP kinase cascade, PI3K/AKT axis, and Ca<sup>2+</sup>-specific signals, converge on the Ets transcription factors, controlling their activity.

VEGF-induced endothelial cell migration and proliferation are tightly regulated and coordinated spatio-temporal behaviors, which—in parallel with sustained cell survival—enable angiogenic sprouting and capillary lumen formation, necessary to create the new vessels devoted to support tumor growth and metastasis spread. VEGF-induced endothelial survival and proliferation are stimulated primarily via PI3K/AKT and PLCγ/ERK pathways, respectively, involving—as above described—several signaling intermediates. VEGF-induced endothelial cell migration appears to be regulated by a larger number of pathways, including the involvement of PLCγ, PI3K, RHO GTPases, SRC and FAK activities.

#### 5. Conclusions

In this chapter, we attempted in particular to describe the best characterized signal transduction events downstream to VEGF/VEGFR-2 axis involved in tumor angiogenesis. Multiple VEGF-induced signaling pathways take part in the promotion of different biological responses in endothelial cells. Although it is possible to recognize distinct patterns along VEGF-induced signaling, they are intricate, characterized by the involvement of a number of enzymes and adaptor/scaffold proteins-whose activity converges and branches at many point-and by the presence of VEGFR-2 molecular partners influencing endothelial cell sensitivity to VEGF and receptor signal output. This depicts a complex signal network induced by VEGF, where the apparent redundancy in operating signaling pathways is likely to reflect a need for a fine-tuning and a differential control of the biological effects in response to VEGF [25]. Although past decade has seen an important advancement in our understanding of VEGF signaling, there is still a lack of insight in many aspects of VEGF/VEGFR-2 signal transduction, in particular for what concerns its fine regulation. A further elucidation of the multifaced VEGF signaling network in the context of endothelial biology is crucial for developing new potential anti-angiogenic therapies. In parallel with current therapies that directly target VEGF and VEGFR-2, agents able to influence key molecular player - proximal, median or distal to VEGFR-2-could be of clinical interest.

#### Acknowledgement

Publication costs have been supported by "Associazione Italiana per la Ricerca sul Cancro" (AIRC), project code 12182.

#### Author details

Lucia Napione<sup>1,2,\*</sup>, Maria Alvaro<sup>1,2</sup> and Federico Bussolino<sup>1,2</sup>

\*Address all correspondence to: lucia.napione@ircc.it

- 1 Laboratory of Vascular Oncology, Candiolo Cancer Institute IRCCS, Candiolo, Italy
- 2 Department of Oncology, University of Torino, Candiolo, Italy

#### References

- Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med. 1971; 285(21):1182–6.
- [2] Folkman J. Proceedings: tumor angiogenesis factor. Cancer Res. 1974;34(8):2109–13.
- [3] Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003;9(6):669–76.
- [4] Koch S, Claesson-Welsh L. Signal transduction by vascular endothelial growth factor receptors. Cold Spring Harb Perspect Med. 2012;2(7):a006502.
- [5] Algire G, Chalkley H. Vascular reactions of normal and malignant tissues in vivo. I. Vascular reactions of mice to wounds and to normal and neoplastic implants. J Natl Cancer Inst. 1945. p. 73–85.
- [6] Greenblatt M, Shubi P. Tumor angiogenesis: transfilter diffusion studies in the hamster by the transparent chamber technique. J Natl Cancer Inst. 1968;41(1):111–24.
- [7] Warren BA, Shubik P. The growth of the blood supply to melanoma transplants in the hamster cheek pouch. Lab Invest. 1966;15(2):464–78.
- [8] Augustin HG. Commentary on Folkman: "How is blood vessel growth regulated in normal and neoplastic tissue?". Cancer Res. 2016;76(10):2854–6.
- [9] Ribatti D. Judah Folkman, a pioneer in the study of angiogenesis. Angiogenesis. 2008; 11(1):3–10.
- [10] Gimbrone MA, Leapman SB, Cotran RS, Folkman J. Tumor dormancy in vivo by prevention of neovascularization. J Exp Med. 1972;136(2):261–76.
- [11] Gimbrone MA, Leapman SB, Cotran RS, Folkman J. Tumor angiogenesis: iris neovascularization at a distance from experimental intraocular tumors. J Natl Cancer Inst. 1973;50(1):219–28.
- [12] Gimbrone MA, Cotran RS, Leapman SB, Folkman J. Tumor growth and neovascularization: an experimental model using the rabbit cornea. J Natl Cancer Inst. 1974;52(2):413–27.

- [13] Knighton D, Ausprunk D, Tapper D, Folkman J. Avascular and vascular phases of tumour growth in the chick embryo. Br J Cancer. 1977;35(3):347–56.
- [14] Brem S, Brem H, Folkman J, Finkelstein D, Patz A. Prolonged tumor dormancy by prevention of neovascularization in the vitreous. Cancer Res. 1976;36(8):2807–12.
- [15] Folkman J, Haudenschild CC, Zetter BR. Long-term culture of capillary endothelial cells. Proc Natl Acad Sci U S A. 1979;76(10):5217–21.
- [16] Folkman J, Haudenschild C. Angiogenesis in vitro. Nature. 1980;288(5791):551-6.
- [17] Stephenson J, Goddard J, Al-Taan O, Dennison A, Morgan B. Tumor Angiogenesis: a growth area – from John Hunter to Judah Folkman and beyond. J Cancer Res: Hindawi Publishing Corporation; 2013. pp. 1–6.
- [18] Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science. 1983;219(4587):983–5.
- [19] Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science. 1989;246(4935):1306–9.
- [20] Rosenthal RA, Megyesi JF, Henzel WJ, Ferrara N, Folkman J. Conditioned medium from mouse sarcoma 180 cells contains vascular endothelial growth factor. Growth Factors. 1990;4(1):53–9.
- [21] Folkman J. Endogenous angiogenesis inhibitors. APMIS. 2004;112(7–8):496–507.
- [22] Sitohy B, Nagy JA, Dvorak HF. Anti-VEGF/VEGFR therapy for cancer: reassessing the target. Cancer Res. 2012;72(8):1909–14.
- [23] Van Epps H. What tumors need: a brief history of angiogenesis. J Exp Med. 2005. p. 1024.
- [24] Folkman J. Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov. 2007;6(4):273–86.
- [25] Claesson-Welsh L, Welsh M. VEGFA and tumour angiogenesis. J Intern Med. 2013;273(2): 114–27.
- [26] Kendall RL, Rutledge RZ, Mao X, Tebben AJ, Hungate RW, Thomas KA. Vascular endothelial growth factor receptor KDR tyrosine kinase activity is increased by autophosphorylation of two activation loop tyrosine residues. J Biol Chem. 1999;274(10):6453–60.
- [27] Matsumoto T, Bohman S, Dixelius J, Berge T, Dimberg A, Magnusson P, et al. VEGF receptor-2 Y951 signaling and a role for the adapter molecule TSAd in tumor angiogenesis. EMBO J. 2005;24(13):2342–53.
- [28] Wu LW, Mayo LD, Dunbar JD, Kessler KM, Ozes ON, Warren RS, et al. VRAP is an adaptor protein that binds KDR, a receptor for vascular endothelial cell growth factor. J Biol Chem. 2000;275(9):6059–62.

- [29] Takahashi T, Yamaguchi S, Chida K, Shibuya M. A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells. EMBO J. 2001;20(11):2768–78.
- [30] Dayanir V, Meyer RD, Lashkari K, Rahimi N. Identification of tyrosine residues in vascular endothelial growth factor receptor-2/FLK-1 involved in activation of phosphatidylinositol 3-kinase and cell proliferation. J Biol Chem. 2001;276(21):17686–92.
- [31] Holmqvist K, Cross MJ, Rolny C, Hägerkvist R, Rahimi N, Matsumoto T, et al. The adaptor protein shb binds to tyrosine 1175 in vascular endothelial growth factor (VEGF) receptor-2 and regulates VEGF-dependent cellular migration. J Biol Chem. 2004;279(21):22267–75.
- [32] Warner AJ, Lopez-Dee J, Knight EL, Feramisco JR, Prigent SA. The Shc-related adaptor protein, Sck, forms a complex with the vascular-endothelial-growth-factor receptor KDR in transfected cells. Biochem J. 2000;347(Pt 2):501–9.
- [33] Lamalice L, Houle F, Huot J. Phosphorylation of Tyr1214 within VEGFR-2 triggers the recruitment of Nck and activation of Fyn leading to SAPK2/p38 activation and endothelial cell migration in response to VEGF. J Biol Chem. 2006;281(45):34009–20.
- [34] Kappert K, Peters KG, Böhmer FD, Ostman A. Tyrosine phosphatases in vessel wall signaling. Cardiovasc Res. 2005;65(3):587–98.
- [35] Gitay-Goren H, Soker S, Vlodavsky I, Neufeld G. The binding of vascular endothelial growth factor to its receptors is dependent on cell surface-associated heparin-like molecules. J Biol Chem. 1992;267(9):6093–8.
- [36] Jakobsson L, Kreuger J, Holmborn K, Lundin L, Eriksson I, Kjellén L, et al. Heparan sulfate in trans potentiates VEGFR-mediated angiogenesis. Dev Cell. 2006;10(5):625–34.
- [37] Napione L, Pavan S, Veglio A, Picco A, Boffetta G, Celani A, et al. Unraveling the influence of endothelial cell density on VEGF-A signaling. Blood. 2012;119(23):5599–607.
- [38] Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling in control of vascular function. Nat Rev Mol Cell Biol. 2006;7(5):359–71.
- [39] Takahashi T, Shibuya M. The 230 kDa mature form of KDR/Flk-1 (VEGF receptor-2) activates the PLC-gamma pathway and partially induces mitotic signals in NIH3T3 fibroblasts. Oncogene. 1997;14(17):2079–89.
- [40] Kroll J, Waltenberger J. The vascular endothelial growth factor receptor KDR activates multiple signal transduction pathways in porcine aortic endothelial cells. J Biol Chem. 1997;272(51):32521–7.
- [41] Takahashi T, Ueno H, Shibuya M. VEGF activates protein kinase C-dependent, but Rasindependent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. Oncogene. 1999;18(13):2221–30.

- [42] Shu X, Wu W, Mosteller RD, Broek D. Sphingosine kinase mediates vascular endothelial growth factor-induced activation of ras and mitogen-activated protein kinases. Mol Cell Biol. 2002;22(22):7758–68.
- [43] Wong C, Jin ZG. Protein kinase C-dependent protein kinase D activation modulates ERK signal pathway and endothelial cell proliferation by vascular endothelial growth factor. J Biol Chem. 2005;280(39):33262–9.
- [44] Ha CH, Wang W, Jhun BS, Wong C, Hausser A, Pfizenmaier K, et al. Protein kinase D-dependent phosphorylation and nuclear export of histone deacetylase 5 mediates vascular endothelial growth factor-induced gene expression and angiogenesis. J Biol Chem. 2008;283(21):14590–9.
- [45] Wang S, Li X, Parra M, Verdin E, Bassel-Duby R, Olson EN. Control of endothelial cell proliferation and migration by VEGF signaling to histone deacetylase 7. Proc Natl Acad Sci U S A. 2008;105(22):7738–43.
- [46] Evans IM, Britton G, Zachary IC. Vascular endothelial growth factor induces heat shock protein (HSP) 27 serine 82 phosphorylation and endothelial tubulogenesis via protein kinase D and independent of p38 kinase. Cell Signal. 2008;20(7):1375–84.
- [47] Shaul PW. Regulation of endothelial nitric oxide synthase: location, location, location. Annu Rev Physiol. 2002;64:749–74.
- [48] Brouet A, Sonveaux P, Dessy C, Balligand JL, Feron O. Hsp90 ensures the transition from the early Ca<sup>2+</sup>-dependent to the late phosphorylation-dependent activation of the endothelial nitric-oxide synthase in vascular endothelial growth factor-exposed endothelial cells. J Biol Chem. 2001;276(35):32663–9.
- [49] Mancini M, Toker A. NFAT proteins: emerging roles in cancer progression. Nat Rev Cancer. 2009;9(11):810–20.
- [50] Suehiro J, Kanki Y, Makihara C, Schadler K, Miura M, Manabe Y, et al. Genome-wide approaches reveal functional vascular endothelial growth factor (VEGF)-inducible nuclear factor of activated T cells (NFAT) c1 binding to angiogenesis-related genes in the endothelium. J Biol Chem. 2014;289(42):29044–59.
- [51] Hernández GL, Volpert OV, Iñiguez MA, Lorenzo E, Martínez-Martínez S, Grau R, et al. Selective inhibition of vascular endothelial growth factor-mediated angiogenesis by cyclosporin A: roles of the nuclear factor of activated T cells and cyclooxygenase 2. J Exp Med. 2001;193(5):607–20.
- [52] Schweighofer B, Testori J, Sturtzel C, Sattler S, Mayer H, Wagner O, et al. The VEGFinduced transcriptional response comprises gene clusters at the crossroad of angiogenesis and inflammation. Thromb Haemost. 2009;102(3):544–54.
- [53] Noren DP, Chou WH, Lee SH, Qutub AA, Warmflash A, Wagner DS, et al. Endothelial cells decode VEGF-mediated Ca<sup>2+</sup> signaling patterns to produce distinct functional responses. Sci Signal. 2016;9(416):ra20.

- [54] Cheng HW, James AF, Foster RR, Hancox JC, Bates DO. VEGF activates receptor-operated cation channels in human microvascular endothelial cells. Arterioscler Thromb Vasc Biol. 2006;26(8):1768–76.
- [55] Ge R, Tai Y, Sun Y, Zhou K, Yang S, Cheng T, et al. Critical role of TRPC6 channels in VEGF-mediated angiogenesis. Cancer Lett. 2009;283(1):43–51.
- [56] Hamdollah Zadeh MA, Glass CA, Magnussen A, Hancox JC, Bates DO. VEGF-mediated elevated intracellular calcium and angiogenesis in human microvascular endothelial cells in vitro are inhibited by dominant negative TRPC6. Microcirculation. 2008;15(7):605–14.
- [57] Singh AJ, Meyer RD, Navruzbekov G, Shelke R, Duan L, Band H, et al. A critical role for the E3-ligase activity of c-Cbl in VEGFR-2-mediated PLCgamma1 activation and angiogenesis. Proc Natl Acad Sci U S A. 2007;104(13):5413–8.
- [58] Cantley LC. The phosphoinositide 3-kinase pathway. Science. 2002;296(5573):1655-7.
- [59] Jiang BH, Liu LZ. PI3K/PTEN signaling in angiogenesis and tumorigenesis. Adv Cancer Res. 2009;102:19–65.
- [60] Missy K, Van Poucke V, Raynal P, Viala C, Mauco G, Plantavid M, et al. Lipid products of phosphoinositide 3-kinase interact with Rac1 GTPase and stimulate GDP dissociation. J Biol Chem. 1998;273(46):30279–86.
- [61] Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat Rev Cancer. 2002;2(7):489–501.
- [62] Pugazhenthi S, Nesterova A, Sable C, Heidenreich KA, Boxer LM, Heasley LE, et al. Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response elementbinding protein. J Biol Chem. 2000;275(15):10761–6.
- [63] Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, et al. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem. 1998;273(46):30336–43.
- [64] Gerber HP, Dixit V, Ferrara N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. J Biol Chem. 1998;273(21):13313–6.
- [65] Tran J, Rak J, Sheehan C, Saibil SD, LaCasse E, Korneluk RG, et al. Marked induction of the IAP family antiapoptotic proteins survivin and XIAP by VEGF in vascular endothelial cells. Biochem Biophys Res Commun. 1999;264(3):781–8.
- [66] Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature. 1999;399(6736):601–5.
- [67] Parsons SJ, Parsons JT. Src family kinases, key regulators of signal transduction. Oncogene. 2004;23(48):7906–9.

- [68] Sun Z, Li X, Massena S, Kutschera S, Padhan N, Gualandi L, et al. VEGFR2 induces c-Src signaling and vascular permeability in vivo via the adaptor protein TSAd. J Exp Med. 2012;209(7):1363–77.
- [69] Caron C, Spring K, Laramée M, Chabot C, Cloutier M, Gu H, et al. Non-redundant roles of the Gab1 and Gab2 scaffolding adapters in VEGF-mediated signalling, migration, and survival of endothelial cells. Cell Signal. 2009;21(6):943–53.
- [70] Duval M, Le Boeuf F, Huot J, Gratton JP. Src-mediated phosphorylation of Hsp90 in response to vascular endothelial growth factor (VEGF) is required for VEGF receptor-2 signaling to endothelial NO synthase. Mol Biol Cell. 2007;18(11):4659–68.
- [71] Alavi A, Hood JD, Frausto R, Stupack DG, Cheresh DA. Role of Raf in vascular protection from distinct apoptotic stimuli. Science. 2003;301(5629):94–6.
- [72] Meyer RD, Sacks DB, Rahimi N. IQGAP1-dependent signaling pathway regulates endothelial cell proliferation and angiogenesis. PLoS One. 2008;3(12):e3848.
- [73] Eliceiri BP, Paul R, Schwartzberg PL, Hood JD, Leng J, Cheresh DA. Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. Mol Cell. 1999;4(6):915–24.
- [74] Weis S, Shintani S, Weber A, Kirchmair R, Wood M, Cravens A, et al. Src blockade stabilizes a Flk/cadherin complex, reducing edema and tissue injury following myocardial infarction. J Clin Invest. 2004;113(6):885–94.
- [75] Wallez Y, Cand F, Cruzalegui F, Wernstedt C, Souchelnytskyi S, Vilgrain I, et al. Src kinase phosphorylates vascular endothelial-cadherin in response to vascular endothelial growth factor: identification of tyrosine 685 as the unique target site. Oncogene. 2007;26(7):1067–77.
- [76] Dejana E, Tournier-Lasserve E, Weinstein BM. The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. Dev Cell. 2009;16(2):209–21.
- [77] Mahabeleshwar GH, Feng W, Reddy K, Plow EF, Byzova TV. Mechanisms of integrinvascular endothelial growth factor receptor cross-activation in angiogenesis. Circ Res. 2007;101(6):570–80.
- [78] Mitra SK, Hanson DA, Schlaepfer DD. Focal adhesion kinase: in command and control of cell motility. Nat Rev Mol Cell Biol. 2005;6(1):56–68.
- [79] Calalb MB, Polte TR, Hanks SK. Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. Mol Cell Biol. 1995;15(2):954–63.
- [80] Owen JD, Ruest PJ, Fry DW, Hanks SK. Induced focal adhesion kinase (FAK) expression in FAK-null cells enhances cell spreading and migration requiring both auto- and activation loop phosphorylation sites and inhibits adhesion-dependent tyrosine phosphorylation of Pyk2. Mol Cell Biol. 1999;19(7):4806–18.

- [81] Le Boeuf F, Houle F, Huot J. Regulation of vascular endothelial growth factor receptor 2-mediated phosphorylation of focal adhesion kinase by heat shock protein 90 and Src kinase activities. J Biol Chem. 2004;279(37):39175–85.
- [82] Le Boeuf F, Houle F, Sussman M, Huot J. Phosphorylation of focal adhesion kinase (FAK) on Ser732 is induced by rho-dependent kinase and is essential for proline-rich tyrosine kinase-2-mediated phosphorylation of FAK on Tyr407 in response to vascular endothelial growth factor. Mol Biol Cell. 2006;17(8):3508–20.
- [83] Takai Y, Sasaki T, Matozaki T. Small GTP-binding proteins. Physiol Rev. 2001;81(1): 153–208.
- [84] van der Meel R, Symons MH, Kudernatsch R, Kok RJ, Schiffelers RM, Storm G, et al. The VEGF/Rho GTPase signalling pathway: a promising target for anti-angiogenic/antiinvasion therapy. Drug Discov Today. 2011;16(5–6):219–28.
- [85] Sulpice E, Ding S, Muscatelli-Groux B, Bergé M, Han ZC, Plouet J, et al. Cross-talk between the VEGF-A and HGF signalling pathways in endothelial cells. Biol Cell. 2009;101(9):525–39.
- [86] Gavard J, Gutkind JS. VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. Nat Cell Biol. 2006;8(11): 1223–34.
- [87] Garrett TA, Van Buul JD, Burridge K. VEGF-induced Rac1 activation in endothelial cells is regulated by the guanine nucleotide exchange factor Vav2. Exp Cell Res. 2007;313(15):3285–97.
- [88] Lamalice L, Houle F, Jourdan G, Huot J. Phosphorylation of tyrosine 1214 on VEGFR2 is required for VEGF-induced activation of Cdc42 upstream of SAPK2/p38. Oncogene. 2004;23(2):434–45.
- [89] Annerén C, Lindholm CK, Kriz V, Welsh M. The FRK/RAK-SHB signaling cascade: a versatile signal-transduction pathway that regulates cell survival, differentiation and proliferation. Curr Mol Med. 2003;3(4):313–24.
- [90] Holmqvist K, Cross M, Riley D, Welsh M. The Shb adaptor protein causes Src-dependent cell spreading and activation of focal adhesion kinase in murine brain endothelial cells. Cell Signal. 2003;15(2):171–9.
- [91] Granum S, Andersen TC, Sørlie M, Jørgensen M, Koll L, Berge T, et al. Modulation of Lck function through multisite docking to T cell-specific adapter protein. J Biol Chem. 2008;283(32):21909–19.
- [92] Berge T, Sundvold-Gjerstad V, Granum S, Andersen TC, Holthe GB, Claesson-Welsh L, et al. T cell specific adapter protein (TSAd) interacts with Tec kinase ITK to promote CXCL12 induced migration of human and murine T cells. PLoS One. 2010;5(3):e9761.

- [93] Lettau M, Pieper J, Gerneth A, Lengl-Janssen B, Voss M, Linkermann A, et al. The adapter protein Nck: role of individual SH3 and SH2 binding modules for protein interactions in T lymphocytes. Protein Sci. 2010;19(4):658–69.
- [94] Stoletov KV, Ratcliffe KE, Spring SC, Terman BI. NCK and PAK participate in the signaling pathway by which vascular endothelial growth factor stimulates the assembly of focal adhesions. J Biol Chem. 2001;276(25):22748–55.
- [95] Hedman AC, Smith JM, Sacks DB. The biology of IQGAP proteins: beyond the cytoskeleton. EMBO Rep. 2015;16(4):427–46.
- [96] Yamaoka-Tojo M, Ushio-Fukai M, Hilenski L, Dikalov SI, Chen YE, Tojo T, et al. IQGAP1, a novel vascular endothelial growth factor receptor binding protein, is involved in reactive oxygen species-dependent endothelial migration and proliferation. Circ Res. 2004;95(3):276–83.
- [97] Yamaoka-Tojo M, Tojo T, Kim HW, Hilenski L, Patrushev NA, Zhang L, et al. IQGAP1 mediates VE-cadherin-based cell-cell contacts and VEGF signaling at adherence junctions linked to angiogenesis. Arterioscler Thromb Vasc Biol. 2006;26(9):1991–7.
- [98] Dance M, Montagner A, Yart A, Masri B, Audigier Y, Perret B, et al. The adaptor protein Gab1 couples the stimulation of vascular endothelial growth factor receptor-2 to the activation of phosphoinositide 3-kinase. J Biol Chem. 2006;281(32):23285–95.
- [99] Laramée M, Chabot C, Cloutier M, Stenne R, Holgado-Madruga M, Wong AJ, et al. The scaffolding adapter Gab1 mediates vascular endothelial growth factor signaling and is required for endothelial cell migration and capillary formation. J Biol Chem. 2007;282(11):7758–69.
- [100] Cébe-Suarez S, Zehnder-Fjällman A, Ballmer-Hofer K. The role of VEGF receptors in angiogenesis; complex partnerships. Cell Mol Life Sci. 2006;63(5):601–15.
- [101] Raimondi C, Ruhrberg C. Neuropilin signalling in vessels, neurons and tumours. Semin Cell Dev Biol. 2013;24(3):172–8.
- [102] Fantin A, Herzog B, Mahmoud M, Yamaji M, Plein A, Denti L, et al. Neuropilin 1 (NRP1) hypomorphism combined with defective VEGF-A binding reveals novel roles for NRP1 in developmental and pathological angiogenesis. Development. 2014;141(3):556–62.
- [103] Plein A, Fantin A, Ruhrberg C. Neuropilin regulation of angiogenesis, arteriogenesis, and vascular permeability. Microcirculation. 2014;21(4):315–23.
- [104] Ballmer-Hofer K, Andersson AE, Ratcliffe LE, Berger P. Neuropilin-1 promotes VEGFR-2 trafficking through Rab11 vesicles thereby specifying signal output. Blood. 2011;118(3):816–26.
- [105] Chen TT, Luque A, Lee S, Anderson SM, Segura T, Iruela-Arispe ML. Anchorage of VEGF to the extracellular matrix conveys differential signaling responses to endothelial cells. J Cell Biol. 2010;188(4):595–609.

- [106] Grazia Lampugnani M, Zanetti A, Corada M, Takahashi T, Balconi G, Breviario F, et al. Contact inhibition of VEGF-induced proliferation requires vascular endothelial cadherin, beta-catenin, and the phosphatase DEP-1/CD148. J Cell Biol. 2003;161(4):793–804.
- [107] Gampel A, Moss L, Jones MC, Brunton V, Norman JC, Mellor H. VEGF regulates the mobilization of VEGFR2/KDR from an intracellular endothelial storage compartment. Blood. 2006;108(8):2624–31.
- [108] Scott A, Mellor H. VEGF receptor trafficking in angiogenesis. Biochem Soc Trans. 2009;37(Pt 6):1184–8.
- [109] Simons M, Gordon E, Claesson-Welsh L. Mechanisms and regulation of endothelial VEGF receptor signalling. Nat Rev Mol Cell Biol. 2016;17(10):611–25.
- [110] Lampugnani MG, Orsenigo F, Gagliani MC, Tacchetti C, Dejana E. Vascular endothelial cadherin controls VEGFR-2 internalization and signaling from intracellular compartments. J Cell Biol. 2006;174(4):593–604.
- [111] Nagy JA, Dvorak AM, Dvorak HF. Vascular hyperpermeability, angiogenesis, and stroma generation. Cold Spring Harb Perspect Med. 2012;2(2):a006544.
- [112] Oda N, Abe M, Sato Y. ETS-1 converts endothelial cells to the angiogenic phenotype by inducing the expression of matrix metalloproteinases and integrin beta3. J Cell Physiol. 1999;178(2):121–32.
- [113] Heo SH, Choi YJ, Ryoo HM, Cho JY. Expression profiling of ETS and MMP factors in VEGF-activated endothelial cells: role of MMP-10 in VEGF-induced angiogenesis. J Cell Physiol. 2010;224(3):734–42.



