Chapter 235

Mechanisms Underlying Context-Dependent VEGF Signaling For Distinct Biological Responses

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INTRODUCTION

VEGF signaling is essential for specification, morphogenesis, differentiation, and homeostasis of vessels during development and in the adult [1–5]. Furthermore, this signaling pathway is an integral component of pathological angiogenesis during tumor expansion [6]. In fact, decreased levels of VEGF result in suppression of vascular expansion, tumor growth, and metastasis [7, 8], making anti-angiogenesis an appealing cancer therapy. A decade ago, the promise of anti-angiogenic therapy rested on three main tenets: (1) tumor expansion is greatly dependent on angiogenesis, thereby providing a common target in the treatment of widely heterogeneous disease; (2) endothelial cells were considered to be less likely to develop a drug-resistant phenotype; (3) it was anticipated that tumor vessels were molecularly and functionally distinct from normal vessels by their inherent proliferative capacity, thus providing a differential target that would therefore avoid quiescent vessels present in normal tissues. Results from angiogenesis research during the last decade have questioned some of these earlier assumptions, and revealed that improvement of current therapies would require a more thorough understanding of the basic signaling pathways being targeted.

Several more detailed pre-clinical studies have now supported the findings in the clinic and revealed that indeed not all tumor vessels appear equally susceptible to a single modality of anti-angiogenic therapy. The reasons for this outcome are likely multiple. The molecular nature of tumor microvessels appears to be more variable than anticipated, and differences in the tumor microenvironment has been shown to influence therapeutic outcome. In fact, experimental evidence from several laboratories has shown that "context" poses a direct (and reciprocal) effect in the tumor cells and tumor vessels [9, 10]. In addition, the contribution of tumor cells to the vascular wall ("vascular mimicry") has been demonstrated in some cancers, including uveal carcinoma and melanoma [11]. Additional reasons for the "disconnect" between bench and clinical outcome has been attributed to the existence of alternative and interconnected signaling pathways [12, 13]. Regardless, the relative success of some anti-angiogenic therapeutic approaches, specifically those targeting the VEGF pathway (Avastin), has engendered hope for this modality of therapy in cancer. Further efforts in basic science are required to determine the basis for VEGF susceptibility to and understand the mechanisms of evasion regarding this therapy. This chapter will focus on the most recent developments in understanding VEGF signaling and how they can impact further therapeutic exploration.

DIVERSITY OF PROTEINS GENERATED BY THE VEGFA GENE

VEGFA (frequently referred simply as VEGF) is the more prevalent member of a family of five related genes: VEGFA, B, C, D, and E. VEGFA levels are under exquisite transcriptional and translational control, and slight alterations in expression levels can have devastating consequences during development. In fact, unlike most mammalian genes, inactivation of only one allele results in embryonic lethality at mid-gestation due to severe cardiovascular defects [14, 15]. Interestingly, organ-specific two-fold increase of VEGF can also lead to lethality [16]. Thus, both decreases and increases in VEGF levels produce significant pathological insults to the vasculature that affect the entire organism. In fact, regulation of VEGF synthesis, secretion, and availability modulates the angiogenic response.

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VEGFA exists in multiple isoforms, generated by alternative splicing, which are designated according to the number of encoded amino acids. To date, isoforms 121, 145, 148, 165, 183, 189, and 206 have been described in humans [17]. In mouse, the same isoforms exist but they are one amino acid shorter. Because work has been done using both species, we will refer to both in agreement with the use of either human or mouse in the context of the experiments described. All isoforms contain a receptor binding domain, but differ in their ability to bind heparan sulfate and extracellular matrix molecules due to differences in their C-terminal sequence (Figure 235.1; the most prevalent isoforms are depicted).

The gene encoding VEGFA comprises 14 kb and contains 8 exons, of which exons 3, 4, and 5 encode sequences involved in receptor binding. Exons 6a, 6b, and 7 encode for residues that bind to matrix proteins, and can undergo splicing to generate the different protein isoforms (Figure 235.1). Interestingly, the last exon (exon 8) is present in all isoforms, and this is the region required to bind neuropilin, a non-tyrosine kinase VEGF and semaphorin receptor. VEGF121 lacks exons 6a, 6b, and 7, and is the only highly soluble isoform. All other isoforms bind extracellular matrix proteins. As depicted in Figure 235.1, the affinity for matrix proteins is proportional to the length of the C-terminal matrix- binding domain.

In the extracellular matrix, heparin sulfate proteoglycans and several matrix proteins can bind to VEGF. Heparinase I or II induce the release of VEGF, providing further support for the notion that heparin-containing proteoglycans contain binding sites for this growth factor [18]. In addition, VEGF has been shown to bind to fibrinogen, fibrin, and fibronectin, and *in vivo* studies indicate that these bound forms of VEGF are functional – i.e., able to phosphorylate VEGF receptors [19–21]. Together these results contradict the dogmatic view that VEGF must be released to induce angiogenic responses. However, as will be discussed later, signaling initiated through matrix-bound VEGF leads to distinct cellular behaviors compared to that initiated by soluble VEGF.

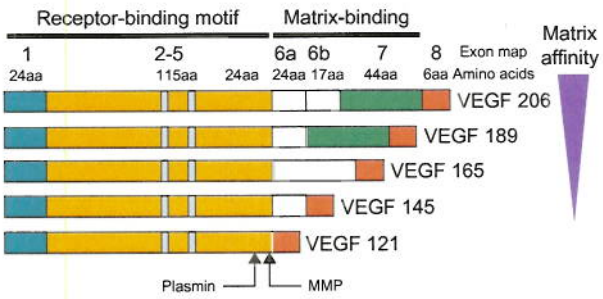

FIGURE 235.1 VEGF structure, splice forms and affinity for matrix. The VEGF gene is composed of 8 Exons. Exons 2–5 code for the receptor-binding domain; while exons 6 and 7 code for amino acids involved in binding to matrix proteins. Exon 8 is common to all isoforms.

VEGF Signaling

VEGF signaling can be induced by two receptor tyrosine kinases: VEGFR1 (flt-1 or FLT-1) and VEGFR2 (flk-1 or KDR) [22]. A non-tyrosine kinase receptor, neuropilin-1, also binds to VEGF specifically, and modulates the responses to the other two tyrosine kinases [23]. The two tyrosine kinase receptors share 44 percent homology, and consist of seven extracellular immunoglobulin-like domains, a single transmembrane region, a split tyrosine-kinase domain that is interrupted by a 70-amino-acid kinase insert, and a C-terminal tail.

VEGFR1 exists in both in membrane-bound and soluble forms and, although it displays high affinity for VEGF, it negatively regulates vasculogenesis and angiogenesis during early development [24]. Genetic inactivation of VEGFR1 results in increased hemangioblast commitment towards the endothelial fate, an outcome that leads to profuse vascular disorganization due to endothelial cell overgrowth [24]. These data, as well as additional information from several laboratories, indicate that VEGFR1 functions as a decoy receptor for regulation of VEGFR2 function through control of VEGF levels at the cell surface [25]. In adult settings, however, VEGFR1 has been shown to display pro-angiogenic properties, particularly during inflammatory responses. Its contribution appears to be relevant in rheumatoid arthritis and tumor neovascularization, although it is unclear whether this activation is mediated by VEGFA, VEGFB, PlGF, or a combination thereof [26, 27].

Our current understanding indicates that the sum of pro-angiogenic responses initiated by VEGF originates primarily through activation of VEGFR2 that regulates endothelial proliferation, migration, survival, and permeability [22]. How VEGFR2 alone coordinates downstream signals to specify each of these possible responses is not completely understood.

Exposure of VEGF to endothelial cells induces VEGFR2 phosphorylation at six sites: 951, 996, 1054, and 1059 are located in the kinase domain, while 1175 and 1214 are within the C-terminal tail [22]. Using a combination of genetic deletion and cell biological approaches, tyrosine 1175 has been identified as a VEGFA-dependent auto-phosphorylation site essential to developmental angiogenesis [27]. Other individual (and some combinations of) tyrosine sites have been thought to play different roles in signal transduction pathways that affect neovascularization of tumors and permeability events.

In terms of specific intracellular signal transduction pathways, it is well accepted that VEGFR2 activates PLCgamma and PI3Kinase through binding to phosphorylated VEGFR2Tyr1175 [28]. Activation of the PI3K pathway through Tyr1175 leads to signaling via AKT/PKB to regulate endothelial cell survival [29, 30], as well as binding to the adaptor protein Sck/ShcB [31]. Phosphorylated Tyr1175 also recruits Shb, although the biological significance of this binding is yet to be determined [32].

Tyrosine 951 also has important downstream signaling events. Once phosphorylated, this residue binds to TSAd (T cell-specific adaptor; also known as VEGF-receptor associated protein (VRAP)) [33]. The phosphorylated Tyr-951–TSAd complex regulates cell migration, and has been implicated in neovascularization of tumors [33]. VEGFA induces the formation of a complex between TSAd and Src, which indicates that TSAd might regulate Src activation and vascular permeability downstream of VEGFR2 [34].

Phosphorylation of Tyr1214 has been implicated in VEGF-induced actin remodeling through the sequential activation of cdc42 and p38MAPK [35]. However, targeted mutation of Tyr1212Phe (corresponding to human Tyr1214) results in mice that are viable and fertile [27]. Inhibition of the p38MAPK augments VEGF-induced angiogenesis in the CAM [33, 36] without an accompanying increased in vascular permeability [36]. Upon VEGF activation of VEGFR2, p38MAPK induces the phosphorylation of the heat-shock protein-27, a molecular chaperone that positively regulates VEGF-induced actin reorganization and migration [37, 38]. In light of these findings, it is unclear how to reconcile the outcome of the mutant mouse Tyr1212Phe [27]. Either additional sites are involved, or compensatory mechanisms might overcome the mutation.

Additional downstream effectors associate with activated VEGFR2, but their direct or indirect links to specific tyrosine residues have not been resolved. In particular, focal adhesion kinase (FAK), its substrate paxilin [39, 40], integrins [41], and VE-cadherin [42] interact and affect VEGFR2 signaling, leading to adhesion, motility, and permeability. Endothelial cell motility is also to be regulated through IQGAPI, a binding partner of phosphorylated VEGFR2, which also binds to and activates Rac1 by inhibiting its intrinsic GTPase activity [42, 43]. IQGAPI co-localizes with phosphorylated VEGFR2 at the leading edge of migrating endothelial cells, and knockdown by siRNA prevents VEGF-induced migration [44].

It is extremely likely that the context of VEGF (matrix-bound or soluble) alters the association of VEGFR2 with some of these molecules to modulate the cellular responses that result in alternative modes of vascular expansion: hyperplasia or vascular sprouting (Figure 235.2).

INTERACTION OF VEGF WITH EXTRACELLULAR MATRIX MOLECULES

The relevance of matrix–VEGF interactions and the biological significance of multiple isoforms have intrigued investigators for a long time. Binding and activation of all three VEGF receptors – VEGFR1 (or Flt-1), VEGFR2 (or KDR, flk1), and neuropilin – are equivalent amongst all of the isoforms, and the intracellular signaling pathways that result from their activation also appear to be similar. It should be emphasized that although initial studies

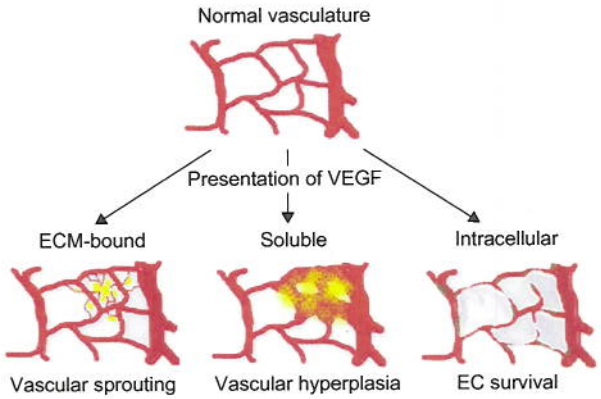

FIGURE 235.2 Presentation of VEGF impacts the cellular outcome of vascular responses.

evaluating binding of VEGF to neuropilin stated its inability to bind to VEGF121, it was later determined that this was not the case. It has now been established by several laboratories that indeed the six amino acids encoded by Exon 8 are responsible for interaction with neuropilin, and are shared by all of the isoforms described so far [45] (Figure 235.1).

Biological insight regarding the significance of the different VEGF isoforms has come from genetic studies in which the coding region for VEGF120, 164, or 188 was substituted for the VEGF gene [46]. This strategy eliminated the option for splicing, and thereby generated mice expressing only one isoform. Analysis of these VEGF-variant mice revealed the contribution of matrix-association in the formation of VEGF gradients and vascular patterning. Briefly, VEGF188-expressing mice showed increased vascular sprouting and concurrent increased capillary density with thinner vessels, as well as higher filopodial extension from endothelial tip cells. In contrast, mice expressing only VEGF120 displayed lower vascular density, with wider-caliber capillaries and relative loss of filopodial extensions when compared to wild-type controls [46]. As anticipated, VEGF164 mice phenocopied wild-type controls, as this is the most predominant form expressed in most tissue and during development. A large body of literature has also emerged from the initial studies and reveals the importance of VEGF isoforms in several developmental settings, including growth of long bones, and cardiac morphogenesis. For example, endochondral bone formation is impaired in VEGF120 mice, indicating a requirement for matrix-bound VEGF in the process of bone morphogenesis [47, 48]. Cardiac outflow tract development also requires matrix-bound VEGF (isoforms 164 and 188). Mice that express only VEGF120 are highly susceptible to developing Tetralogy of Fallot, a condition characterized by pulmonary stenosis and interventricular septal defects [49].

With the marked exception of one study, the regulation of isoform-specific synthesis during development has not

been extensively characterized [50]. However, modulation of soluble and bound isoforms can also be mediated extracellularly.

Irrespective of the isoform secreted, the interaction of VEGF with the extracellular matrix can also be altered post-translationally via proteolytic processing. Thus, in tissues, the relative abundance of C-terminal region displayed by the distinct isoforms can be further altered in the extracellular matrix, depending upon the availability of active proteases. In particular, plasmin and MMPs have been shown to cleave VEGF in a manner that severs the receptor-binding domain from the matrix-binding motif (Figure 235.1). The resulting cleaved VEGF can bind and activate both VEGFR1 and VEGFR2, although it cannot interact with neuropilin.

VEGF can be cleaved by plasmin at aa110–111 and by MMPs at aa113 (Figure 235.1). A large cohort of MMPs can process VEGF, including: MMP3, MMP7, MMP9 (only when in the presence of heparin), and MMP19. These endoproteolytic processing events can modulate the levels of soluble and matrix-bound VEGF in tissues, and elicit the formation of either enlarged vessels or sprouting angiogenesis (Figure 235.2).

Although much emphasis has been placed on understanding transcriptional and post-transcriptional regulation of VEGF transcripts, little attention has been given to extracellular regulation of VEGF activity. In fact, release of VEGF from matrix stores has been implicated in the angiogenic switch facilitating the transition from hyperplastic to malignant tumors [51]. Thus, the ability of proteases to further modulate the release of VEGF from the extracellular matrix bears significance with respect to regulation of angiogenesis by VEGF in the context of specific tissues and/or pathological settings.

Cleavage by plasmin (aa110) or by MMPs (aa113) can target any of the VEGF isoforms (including VEGF121). Since both cleaved and bound VEGF can phosphorylate the two main tyrosine receptors, the significance of these proteolytic processing events to activation of signaling has not been clear. Nonetheless, the presence of matrix-bound or soluble VEGF provides important contextual differences in signaling. In fact, these differences result in alternative modes of vascular expansion, the first leading to sprouting angiogenic events and increased vascular density and the second resulting in vascular hyperplasia (Figure 235.2).

The findings that support these conclusions came from studies with engineered forms of VEGF designed to mimic a cleaved soluble form (VEGF113) and a mutant VEGF form that is resistant to cleavage by either plasmin or MMPs [21]. Using these distinct VEGF forms, we found that soluble VEGF resulted in hyperplasia of existent vessels and vascular beds with lower vascular density and poor branching. In contrast, matrix-bound uncleavable VEGF induced highly branched and thin vessels that greatly facilitated perfusion of tissues and tumor growth [21]. This outcome resembles the findings with isoform-specific mice and data from tumor studies with overexpression of isoform-specific VEGF [46, 52] – that is, that VEGF120 (more soluble) results in the formation of larger vessels and low sprouting, similarly to "cleaved-VEGF." In contrast, VEGF188 resulted in high capillary density and greater number of sprouts.

To gain further insight into the cellular effects mediated by soluble and bound VEGF, we evaluated their effects *in vitro*. Purified growth factors at identical concentrations were included in fibrinogen/fibronectin gels before polymerization and the behavior of endothelial cells bound to sepharose beads was examined. Our data showed that uncleavable VEGF induced capillary morphogenesis, while cleaved VEGF (VEGF113) induced the proliferation of endothelial cells in sheets [21]. These results indicate that local and discrete VEGF164 cleavage is likely to occur as endothelial cells migrate and grow as sheets, whereas in the absence of such digestion, VEGF mediates the organization of cord-like structures. It is likely that signaling via VEGF, bound or soluble, leads to the phosphorylation of a specific cohort of tyrosine residues in VEGFR2, resulting in the activation of different downstream signaling pathways. Alternatively, receptor clustering and/or internalization kinetics could account for the distinct responses. Clearly, a deeper understanding of how soluble and bound VEGF functions in the context of VEGF signaling is required.

ANTI-ANGIOGENIC THERAPY TARGETING VEGF

Since there is a great deal of experimental support for the notion that inhibition of VEGF suppresses tumor angiogenesis, several different strategies have been designed to target the VEGF–VEGFR signaling axis. Clinically, the most successful has been the VEGF-neutralizing antibody Bevacizumab/Avastin, which was approved by the United States Food and Drug Administration for clinical use early in 2004 [53]. Other strategies to target the VEGF pathway include soluble VEGFRs (Traps), receptor tyrosine-kinase inhibitors (RTKI) that target VEGFR2, as well as neutralizing aptomers. RTK inhibitors generally target several kinases in addition to VEGFR2, and inhibit tumor cell proliferation and survival. The kinase inhibitors Sorafenib (BAY43-9006) and Sunitinib (SU11248) have been tested in large phase III clinical trials for treatment of metastatic kidney cancer and gastrointestinal stromal tumors, respectively, and represent promising monotherapies [54, 55].

It has been suggested that anti-VEGF treatment transiently "normalizes" the tumor vasculature, which in turn provides a more efficient delivery of the chemotherapeutic drugs [56]. Thus, anti-angiogenic therapy would not only suppress neo-angiogenesis, but while pruning the vasculature it would also improve perfusion, facilitating drug exposure. In fact, when given in combination with chemotherapy,

Bevacizumab prolongs the survival of patients with lung and colon cancer [54]. It is unclear, however, whether Avastin inactivates both soluble and matrix-bound VEGF, or one form more effectively than the other.

In some cases resistance to anti-angiogenic therapy (with Avastin specifically) has been documented. The molecular basis for the development of this resistance and for the lack of efficacy are not well understood. Current speculation, in the absence of strong experimental evidence, points to compensatory mechanisms by other signaling pathways, such as PDGF, to explain for VEGF resistance [57]. While additional pathways could also be functioning, data from our laboratory have supported a direct correlation between autocrine/intracrine signaling and resistance to VEGF therapy.

INTRACRINE VEGF SIGNALING

Studies on the distribution of VEGF in normal adult mice are scant, and, considering the rapid advance of clinical trials focused on this pathway, it has become critical to known the distribution and penetration of constitutive VEGF expression in normal tissues. Several laboratories have used two LacZ reporters to answer this question [16]. Detailed analysis of these mice revealed several unexpected findings, the most significant was identification of VEGF promoter activity in a large population of endothelial cells [58, 59]. Endothelial VEGF was detected in large and small vessels, with a bias towards the arterial arm of the vasculature. Tissues such as lung, kidney, intestine, and uterus showed higher levels than skeletal muscle and brain. Although expression of VEGF by endothelial cells has been described previously, the observations have been restricted to *in vitro* analysis [60]. The biological relevance of this expression was further explored by generating a knockout model in the endothelial cell compartment using Cre-lox [59, 61]. Removal of VEGF from the endothelium (VEGF-ECKO) results in 55 percent lethality by 6 months of age. VEGF-ECKO mice do not display any apparent abnormality, and do not show any signs of pathology prior to the onset of lethality. Death results form cardiac microinfarcts due to obstruction of coronary arteries. In fact, macroscopic examination of hearts from homozygous mice identified multiple ischemic regions, and histological analysis revealed intravascular thromboembolitic events with accumulation of fibrinogen/fibrin and von Willebrand's factor. These findings indicate that absence of VEGF from the endothelium results in intravascular thrombosis. Ultrastructural evaluation of cardiac specimens from these mice revealed endothelial cell death in both capillaries and large vessels. Thus, the intraembolitic events are attributed to the progressive loss of endothelium leading to the accumulation of platelets and resultant clots, which, if dislodged by the circulation, could potentially result in fatal outcomes [59].

Analysis of the marrow-hematopoetic lineage in VEGF-ECKO mice revealed no anomalies, excluding a hematopoietic contribution to this phenotype. Bone-marrow transplantation experiments with normal and mutant marrow transplanted into mutant and wild-type hosts respectively were also performed. The phenotype was reiterated in the mutant host transplanted with wild-type marrow, indicating that the cause was endothelial [59]. In addition, a complete analysis of liver coagulation proteins revealed no variations from those in the wild-type range.

Together, these findings provided evidence for the existence of an autocrine VEGF signaling loop of high biological significance to both embryonic vascular development and adult homeostasis. These results also provide an explanation for the side-effects reported for Avastin [55], and indicate a call for caution when considering long-term treatment with anti-VEGF therapy.

In the absence of exogenous ligand, base-line phosphorylation of VEGFR2 is increased under hypoxic stress (59). Receptor activation is ligand-dependent, since endothelial cells from ECKO mice are unable to phosphorylate VEGFR2 in the absence of exogenous ligand [59]. Further understanding of this signaling loop was revealed from experiments using human endothelial cells exposed to either Avastin or SU4312 (3-(4-dimethylaminobenzylidene)-1,3-dihydroindol-2-one). While Avastin inactivates extracellular VEGF, SU4312 is a small molecule inhibitor that can gain access to the cell and binds competitively with ATP and non-competitively with the intracellular domain of VEGFR2. The compound shows high specificity for VEGFR2, with a K_i of 0.04 μM for VEGFR2 and K_i 19.4 μM for PDGFR. Interestingly, in the absence of exogenous ligand only SU4312 can block VEGFR2 phosphorylation in a dose-dependent manner. These findings suggest that endothelial VEGF could be activating VEGFR2 in an intracrine fashion, and likely within the secretory pathway where both ligand and receptor could co-segregate and interact. The fact that VEGF is upregulated by endothelial cells under low oxygen (i.e., stress) suggests that VEGF might be needed to protect cells against hypoxia and possibly other insults (Figure 235.2).

CONCLUSIONS

It has become increasingly clear that the presentation of VEGF – i.e., soluble, bound, or intracellular – impacts the array of cellular responses conveyed by VEGFR2. For example, intracrine VEGF-mediated activation does not mediate angiogenic or permeability responses; these are normally transmitted by paracrine activation via cell surface receptors. Instead, intracrine VEGF is essential for survival pathways that are not entirely redundant, as exogenous VEGF cannot rescue defects in cell survival in the absence of intracellular VEGF. The next challenge will

be to define the distinct signaling responses initiated by soluble, bound, and intracrine VEGF. It seems likely that context-dependent differences in second messengers could ultimately direct alternative vascular outcomes triggered by this multifaceted ligand.

REFERENCES

1. Damert A, Miquerol L, Gertsenstein M, Risau W, Nagy A. Insufficient VEGFA activity in yolk sac endoderm compromises haematopoietic and endothelial differentiation. *Development* 2002;**129**:1881–92.
2. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nature Med* 2003;**9**:669–76.
3. Gerber HP, Hillan KJ, Ryan AM, Kowalski J, Keller GA, Rangell L, Wright BD, Radtke F, Aguet M, Ferrara N. VEGF is required for growth and survival in neonatal mice. *Development* 1999;**126**:1149–59.
4. Gerber HP, Malik AK, Solar GP, Sherman D, Liang XH, Meng G, Hong K, Marsters JC, Ferrara N. VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. *Nature* 2002;**417**:954–8.
5. Helmlinger G, Endo M, Ferrara N, Hlatky L, Jain RK. Formation of endothelial cell networks. *Nature* 2000;**405**:139–41.
6. Inoue M, Hager JH, Ferrara N, Gerber HP, Hanahan D. VEGF-A has a critical, nonredundant role in angiogenic switching and pancreatic beta cell carcinogenesis. *Cancer Cell* 2002;**1**:193–202.
7. Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nature Rev Drug Discov* 2004;**3**:391–400.
8. Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* 1993;**362**:841–4.
9. Bissell MJ, Radisky D. Putting tumours in context. *Nature Rev Cancer* 2001;**1**:46–54.
10. Radisky D, Hagios C, Bissell MJ. Tumors are unique organs defined by abnormal signaling and context. *Semin Cancer Biol* 2001;**11**:87–95.
11. Hendrix MJ, Seftor RE, Seftor EA, Gruman LM, Lee LM, Nickoloff BJ, Miele L, Sheriff DD, Schatteman GC. Transendothelial function of human metastatic melanoma cells: role of the microenvironment in cell-fate determination. *Cancer Res* 2002;**62**:665–8.
12. Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. *Nature Rev Cancer* 2002;**2**:727–39.
13. Ellis LM, Hicklin DJ. Pathways mediating resistance to vascular endothelial growth factor-targeted therapy. *Clin Cancer Res* 2008;**14**:6371–5.
14. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 1996;**380**:435–9.
15. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 1996;**380**:439–42.
16. Miquerol L, Langille BL, Nagy A. Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression. *Development* 2000;**127**:3941–6.
17. Harper SJ, Bates DO. VEGF-A splicing: a key to anti-angiogenic therapeutics? *Nature Rev Cancer* 2008;**8**:880–7.
18. Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *J Biol Chem* 1992;**267**:26,031–26,037.
19. Sahni A, Francis CW. Vascular endothelial growth factor binds to fibrinogen and fibrin and stimulates endothelial cell proliferation. *Blood* 2000;**96**:3772–8.
20. Wijelath ES, Murray J, Rahman S, Patel Y, Ishida A, Strand K, Aziz S, Cardona C, Hammond WP, Savidge GF, Rafii S, Sobel M. Novel vascular endothelial growth factor binding domains of fibronectin enhance vascular endothelial growth factor biological activity. *Circ Res* 2002;**91**:25–31.
21. Lee S, Jilani SM, Nikolova GV, Carpizo D, Iruela-Arispe ML. Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. *J Cell Biol* 2005;**169**:681–91.
22. Shibuya M, Claesson-Welsh L. Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. *Exp Cell Res* 2006;**312**:549–60.
23. Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* 1998;**92**:735–45.
24. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 1995;**376**:66–70.
25. Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D, Kroll J, Plaisance S, De Mol M, Bono F, Kliche S, Fellbrich G, Ballmer-Hofer K, Maglione D, Mayr-Beyrle U, Dewerchin M, Dombrowski S, Stanimirovic D, Van Hummelen P, Dehio C, Hicklin DJ, Persico G, Herbert JM, Communi D, Shibuya M, Collen D, Conway EM, Carmeliet P. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nature Med* 2003;**9**:936–43.
26. Luttun A, Autiero M, Tjwa M, Carmeliet P. Genetic dissection of tumor angiogenesis: are PlGF and VEGFR-1 novel anti-cancer targets? *Biochim Biophys Acta* 2004;**1654**:79–94.
27. Sakurai Y, Ohgimoto K, Kataoka Y, Yoshida N, Shibuya M. Essential role of Flk-1 (VEGF receptor 2) tyrosine residue 1173 in vasculogenesis in mice. *Proc Natl Acad Sci USA* 2005;**102**:1076–81.
28. 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- and DNA-synthesis in vascular endothelial cells. *EMBO J* 2001;**20**:2768–78.
29. 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**:17,686–17,692.
30. Fujio Y, Walsh K. Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. *J Biol Chem* 1999;**274**:16,349–16,354.
31. Sakai R, Henderson JT, O'Bryan JP, Elia AJ, Saxton TM, Pawson T. The mammalian ShcB and ShcC phosphotyrosine docking proteins function in the maturation of sensory and sympathetic neurons. *Neuron* 2000;**28**:819–33.
32. Holmqvist K, Cross MJ, Rolny C, Hagerkvist R, Rahimi N, Matsumoto T, Claesson-Welsh L, Welsh M. 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**:22,267–22,275.
33. Matsumoto T, Bohman S, Dixelius J, Berge T, Dimberg A, Magnusson P, Wang L, Wikner C, Qi JH, Wernstedt C, Wu J, Bruheim S,

Mugishima H, Mukhopadhyay D, Spurkland A, Claesson-Welsh L. VEGF receptor-2 Y951 signaling and a role for the adapter molecule TSAd in tumor angiogenesis. *EMBO J* 2005;**24**:2342–53.

34. Weis SM, Cheresh DA. Pathophysiological consequences of VEGF-induced vascular permeability. *Nature* 2005;**437**:497–504.
35. 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**:434–45.
36. Issbrucker K, Marti HH, Hippenstiel S, Springmann G, Voswinckel R, Gaumann A, Breier G, Drexler HC, Suttorp N, Clauss M. p38 MAP kinase – a molecular switch between VEGF-induced angiogenesis and vascular hyperpermeability. *FASEB J* 2003;**17**:262–4.
37. McMullen ME, Bryant PW, Glembotski CC, Vincent PA, Pumiglia KM. Activation of p38 has opposing effects on the proliferation and migration of endothelial cells. *J Biol Chem* 2005;**280**:20,995–21,003.
38. Rousseau S, Houle F, Landry J, Huot J. p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. *Oncogene* 1997;**15**:2169–77.
39. Abedi H, Zachary I. Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. *J Biol Chem* 1997;**272**:15,442–15,451.
40. 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**:39,175–39,185.
41. Byzova TV, Goldman CK, Pampori N, Thomas KA, Bett A, Shattil SJ, Plow EF. A mechanism for modulation of cellular responses to VEGF: activation of the integrins. *Mol Cell* 2000;**6**:851–60.
42. Weis S, Cui J, Barnes L, Cheresh D. Endothelial barrier disruption by VEGF-mediated Src activity potentiates tumor cell extravasation and metastasis. *J Cell Biol* 2004;**167**:223–9.
43. Hart MJ, Callow MG, Souza B, Polakis P. IQGAP1, a calmodulin-binding protein with a rasGAP-related domain, is a potential effector for cdc42Hs. *EMBO J* 1996;**15**:2997–3005.
44. Yamaoka-Tojo M, Ushio-Fukai M, Hilenski L, Dikalov SI, Chen YE, Tojo T, Fukai T, Fujimoto M, Patrushev NA, Wang N, Kontos CD, Bloom GS, Alexander RW. 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**:276–83.
45. Pan Q, Chathery Y, Wu Y, Rathore N, Tong RK, Peale F, Bagri A, Tessier-Lavigne M, Koch AW, Watts RJ. Neuropilin-1 binds to VEGF121 and regulates endothelial cell migration and sprouting. *J Biol Chem* 2007;**282**:24,049–24,056.
46. Ruhrberg C, Gerhardt H, Golding M, Watson R, Ioannidou S, Fujisiwa H, Betsholtz C, Shima DT. Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel morphongenesis. *Genes Dev* 2002;**16**:2684–98.
47. Zelzer E, McLean W, Ng YS, Fukai N, Reginato AM, Lovejoy S, D'Amore PA, Olsen BR. Skeletal defects in VEGF (120/120) mice reveal multiple roles for VEGF in skeletogenesis. *Development* 2002;**129**:1893–904.
48. Maes C, Carmeliet P, Moermans K, Stockmans I, Smets N, Collen D, Bouillon R, Carmeliet G. Impaired angiogenesis and endochondral bone formation in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Mech Dev* 2002;**111**:61–73.
49. van den Akker NMS, Molin DGM, Peters PWM, Maas S, Wisse LJ, Brempt R, Munsteren CJ, Barteligs MM, Poelmann RE, Carmeliet P, Gittenberger-de-Groot AC. Tetralogy of Fallot and alterations in vascular endothelial growth factor-A signaling and Notch signaling in mouse embryos solely expressing the VEGF120 isoform. *Circ Res* 2007;**100**:842–56.
50. Ng. YS, Rohan R, Sunday ME, Demello DE, D'Amore PA. Differential expression of VEGF isoforms in mouse during development and in the adult. *Dev Dyn* 2001;**220**:112–21.
51. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, Hanahan D. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nature Cell Biol* 2000;**2**:737–44.
52. Grunstein J, Masbad JJ, Hickey R, Giordano F, Johnson RS. Isoforms of vascular endothelial growth factor act in a coordinate fashion to recruit and expand tumor vasculature. *Mol Cell Biol* 2000;**20**:7281–2.
53. Ferrara N, Hillan KJ, Novotny W. Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. *Biochem Biophys Res Commun* 2005;**333**:328–35.
54. Jain RK, Duda DG, Clark JW, Loeffler JS. Lessons from phase III clinical trials on anti-VEGF therapy for cancer. *Nature Clin Pract Oncol* 2006;**3**:24–40.
55. Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumor activity. *Nature Rev Cancer* 2008;**8**:579–91.
56. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 2005;**307**:58–62.
57. Tejada ML, Yu L, Dong J, Jung K, Meng G, Peale FV, Frantz GD, Hall L, Liang X, Gerber HP, Ferrara N. Tumor-driven paracrine platelet-derived growth factor receptor alpha signaling is a key determinant of stromal cell recruitment in a model of human lung carcinoma. *Clin Cancer Res* 2006;**12**:2676–88.
58. Maharaj AS, Saint-Geniez M, Maldonado AE, D'Amore PA. Vascular endothelial growth factor localization in the adult. *Am J Pathol* 2006;**168**:639–48.
59. Lee S, Chen TT, Barber CL, Jordan MC, Murdock J, Desai S, Ferrara N, Nagy A, Roos KP, Iruela-Arispe ML. Autocrine VEGF signaling is required for vascular homeostasis. *Cell* 2007;**130**:691–703.
60. Namiki A, Brogi E, Kearney M, Kim EA, Wu T, Couffinhal T, Varticovski L, Isner JM. Hypoxia induces vascular endothelial growth factor in cultured human endothelial cells. *J Biol Chem* 1995;**270**:31,189–31,195.
61. Alva J, Zovein AC, Monvoisin A, Murphy T, Salazar A, Harvey NL, Carmeliet P, Iruela-Arispe ML. VE-Cadherin-Cre-recombinase transgenic mouse: a tool for lineage analysis and gene deletion in endothelial cells. *Dev Dyn* 2006;**235**:759–67.