VEGFA and tumour angiogenesis

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Abbreviations: AXL; AXL receptor tyrosine kinase, cdc42; cell division control protein 42 homolog, ECM; extracellular matrix, eNOS; endothelial nitric oxide synthase, Erk; extracellular protein kinase, FAK; focal adhesion kinase, FYN; FYN tyrosine kinase, GAB; Grb2-associated binder, IQGAP; IQ motifs and GTPase activating protein, MMP; matrix metalloproteinase, NCK; non-catalytic region of tyrosine kinase *adaptor* protein 1, NFAT; nuclear factor of activated T cells, NO; nitric oxide; p38MAPK; p38 mitogen-activated protein kinase, PAK; p21-activated kinase, PI3K; phosphoinositide 3' kinase, PKC; protein kinase C, PLCγ; phospholipase Cγ, pS/T; phosphoserine/phosphothreonine, pY; phosphotyrosine, RAC; small GTPase of the Rho family, RHO; small GTPase of the Rho family, ROS; reactive oxygen species, SHB; Src homology 2 protein B, SRC; sarcoma oncogene/tyrosine kinase, TSAd; T cell-specific adaptor, VE-cad; vascular endothelial cadherin, VEGF; vascular endothelial growth factor.

Abstract

In this review we summarize the current understanding of signal transduction downstream of vascular endothelial growth factor A (VEGFA) and its receptor VEGFR2, and the relationship between these signal transduction pathways and the hallmark responses of VEGFA, angiogenesis and vascular permeability. These physiological responses involve a number of effectors, including extracellular signal-regulated kinases (ERKs), Src, phosphoinositide 3 kinase (PI3K)/Akt, focal adhesion kinase (FAK), Rho family GTPases, endothelial NO and p38 mitogen-activated protein kinase (MAPK). Several of these factors are involved in the regulation of both angiogenesis and vascular permeability. Tumour angiogenesis primarily relies on VEGFA-driven responses, which to a large extent result in a dysfunctional vasculature. The reason for this remains unclear, although it appears that certain aspects of the VEGFA-stimulated angiogenic milieu (high level of microvascular density and permeability) promote tumour expansion. The high degree of redundancy and complexity of VEGFA-driven tumour angiogenesis may explain why tumours commonly develop resistance to anti-angiogenic therapy targeting VEGFA signal transduction.

Introduction

Growth of solid tumours is accompanied by stimulation of angiogenesis. Vascular endothelial growth factor-A (VEGFA; also referred to as VEGF) is one, but not the only, primary factor driving expansion of the tumour vascular bed. It is well established that the tumour microvasculature displays abnormal features, including high turnover of vessels, poor perfusion and increased leakage. In many instances vascular 'normalization' [1], i.e. reversal of these abnormalities in response to anti-angiogenic treatment, has been reported to lead to decreased tumour growth, despite the fact that the normalized tumour vessels appear more functional. Understanding this contradiction will be useful for designing effective anti-angiogenic therapy for cancer. Thus, it is important to determine the detailed mechanism of VEGF-dependent signalling.

'VEGF' refers to both the originally identified dimeric glycoprotein now termed VEGFA, and the family of VEGF-related polypeptides, i.e. VEGFA, VEGFB, VEGFC, VEGFD and placental growth factor (PLGF). VEGFA was originally shown to be an endothelial growth factor as well as a regulator of vascular permeability, and was also termed vascular permeability factor (VPF).

VEGFA is produced by most cells in the body but is upregulated in hypoxia [2]. In tumours, VEGF is produced by hypoxic tumour cells, endothelial cells (ECs) and infiltrating myeloid cells, termed tumour-associated macrophages (TAMs). Tumour angiogenesis is commonly associated with a pro-angiogenic, immunosuppressive 'M2' phenotype [3, 4]. Other infiltrating cells such as neutrophils may also have a role in tumour angiogenesis [5, 6].

The VEGF family members bind in an overlapping manner to three receptor tyrosine kinases, VEGFR1, VEGFR2 and VEGFR3. The *in vivo* angiogenic response to VEGFA is mainly mediated via activation of VEGFR2. This initiates signalling cascades that include numerous intermediates. Eventually, the characteristics of the EC will be affected by several distinct responses. These can be considered, simply, as five major responses: proliferation and survival, cell migration, vascular permeability, invasion into the surrounding tissue, and the endothelial inflammation. Here we describe several VEGFA-induced signalling pathways downstream of VEGFR2 and discuss how they are integrated to achieve an angiogenic response.

VEGFR2-dependent signalling pathways

The phospholipase Cγ–extracellular regulated kinase pathway

Phospholipase Cγ (PLCγ) is an important mediator of VEGFR2-dependent proliferation [7–9]. Tyrosine (Y) 1175, one of the major autophosphorylation sites in the human VEGFR2 (1173 in the mouse VEGFR2), has been identified as the main binding site for the Src homology 2 (SH2) domain of PLCγ. PLCγ is activated following VEGFR2-mediated tyrosine phosphorylation, resulting in hydrolysis of phosphatidyl inositol 4,5 bisphosphate (PIP2). Hydrolysis of PIP2 generates inositol 3,4,5 trisphosphate (IP3) and Ca^{2+} fluxes, as well as diacylglycerol, which in turn promotes activation of protein kinase (PK) C, followed by induction of extracellular regulated kinase (ERK) 1 and 2 activity, and proliferation [8, 9]. Alternative mechanisms of VEGF-regulated proliferation through the Erk pathway include binding of growth receptor bound 2 (Grb2) and the adaptor Shc directly to VEGFR2 [10], or indirectly via the scaffold proteins Gab1 and Gab2 (see below) [11], downstream of VEGFR2.

VEGFR2-dependent PLCγ activation occurs after internalization of the receptor, in the endosomal compartment [12, 13]. The importance of Y1175/Y1173 for VEGFR2 signalling is demonstrated by embryonic lethality in mice carrying a tyrosine to phenylalanine exchange mutant at Y1173 in VEGFR2 [14]. However, Y1173 also binds to other signalling proteins, including the adaptors Shb (see below) and Sck [15], and thus the severe phenotype of a receptor lacking this binding site may be due to loss of multiple signalling pathways.

Src kinases

Src family kinases are cytoplasmic tyrosine kinases organized into one SH3 and one SH2 domain in the amino terminal of the molecule, followed by a tyrosine kinase domain. c-Src and the related c-Yes and c-Fyn are expressed in most if not all cell types, including ECs. Phosphorylation of Y416 in the kinase domain induces c-Src kinase activity whereas phosphorylation at the C-terminal Y527 leads to attenuation of kinase activity, by binding of phosphorylated (p)Y527 to the SH2 domain within the same molecule. VEGFR2 activates c-Src via the SH2 domain-containing T cell-specific adaptor (TSAd; see below) [16, 17], which binds to Y951 (Y949 in the mouse) in VEGFR2 [18]. Alternative pathways for c-Src activation involve binding to Y1059 in the VEGFR2 kinase domain [19] or to the scaffold proteins Gab1 and Gab2

[20]. Src family kinases may phosphorylate and thereby activate receptor tyrosine kinases to initiate downstream signalling in a ligand-independent manner. Src family kinases phosphorylate vascular endothelial cadherin (VE-cadherin) in adherens junctions, leading to elevated vascular permeability [21–23]. Substrates for Src kinases also include components that regulate the cytoskeleton such as paxillin, talin1, p120 catenin, cofilin and cortactin [24], suggesting that Src family members have a broad range of effects on EC biology.

Focal adhesion kinase

The non-receptor tyrosine kinase focal adhesion kinase (FAK) is an important regulator of EC shape, migration, adherens junction integrity, proliferation and survival [25]. FAK is localized in focal adhesions, sites of tight adhesion between cells in culture and their underlying matrix, which are established as a consequence of integrin ligation to the extracellular matrix [26, 27]. The sequential activation of FAK upon integrin ligation involves autophosphorylation of FAK at Y397 followed by Src family kinase-mediated phosphorylation of Y576, Y577, Y861 and Y925. This sequence of events is promoted by VEGFR2 [28] via c-Src [29], Shb [30] or RhoA [29] or by direct association between VEGFR2 and clustered integrins (focal adhesions) [26]. Direct interaction between integrins and VEGFR2 may exert reciprocal activation of these two components in a Src- and Rap1b-dependent manner [31, 32]. Downstream effectors of FAK in ECs include phosphoinositide 3-kinase (PI3K) [33] and ERK [27]. EC-specific inactivation of FAK results in embryonic lethality [between embryonic day (E)10.5 and (E)13.5], due to vascular defects, increased EC apoptosis and defective lamellipodia formation [34, 35]. EC-specific deletion in the adult mouse results in reduced vascular permeability [36].

The PI3K–PKB)Akt pathway

PI3K is an important regulator of angiogenesis [37, 38]. VEGFA activates PI3K via several pathways, including FAK [33], Shb [30], Gab1 [11], IQGAP1 [19] and TSAd/Src/Axl [17], and via direct binding of PI3K to pY1175 in VEGFR2 [39]. PI3K has been implicated in formation of EC tubules *in vitro* [40], and in proliferation, survival and vascular permeability [41, 42]. Rho family G proteins and Akt are considered to be the main PI3K downstream effectors [42].

Akt (also known as PKB) is a signalling intermediate downstream of PI3K of considerable importance in VEGF-regulated EC biology [42]. Akt exists in three

isoforms in ECs, Akt1–3, which are activated by PI3K via phosphoinositide-dependent kinase 1 (PDK1), mammalian target of rapamycin C2 (mTORC2) and inhibition of the phosphatase PTEN [43]. Active Akt has been linked to angiogenesis via EC survival, proliferation, vascular permeability, synthesis and release of matrix metalloproteinases (MMPs) and for conferring an inflammatory EC characteristic [44, 45]. Expression of constitutively active Akt in ECs causes enlarged and hyperpermeable tumour vessels [46]. *Akt1* gene targeting shows that this isoform is particularly important for normal vascular development [45] and lymphangiogenesis [47]. Downstream targets of Akt include: (i) the Bcl2 (B cell CLL/lymphoma 2)-associated death promoter BAD (Bcl2-associated agonist of cell death): (ii) endothelial nitric oxide synthase (eNOS), which regulates vessel diameter; (iii) glycogen synthase kinase (GSK)-3β, which regulates the expression of cell cycle proteins and MMPs; (iv) the NF kappa B inhibitor kinase (IKK) complex, which regulates nuclear factor (NF)-κB and thus promotes an inflammatory signature; (v) mTORC1 (mechanistic target of rapamycin complex), which increases protein synthesis; and (vi) the transcription factor FOXO (forkhead box O), which promotes an anti-apoptotic response [42].

Rho family of monomeric G proteins (GTPases)

The Rho family includes Rho, Rac and Cdc42, which are primary regulators of cell shape and motility [48]. Cdc42 is involved in the generation of filopodia, while RhoA is involved in focal adhesion assembly and stress fibre formation. Rac has a prominent role in lamellipodia production, although Cdc42 and Rho are also involved in this process [48]. Guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) regulate the cycling of monomeric G proteins between the active GTP-bound and the inactive GDP-bound states and thus control the activity of the G protein. VEGFA stimulates the activities of Cdc42 [49], Rho [29, 50] and Rac [49, 51, 52]. Rho activity stimulates FAK activation by focal adhesion assembly [29]. Rac has been linked to various aspects of EC function in numerous studies. Endothelial specific Rac1 knockout mice embryos die *in utero* (at E9.5) due to impaired angiogenesis, suggesting an essential role of Rac in EC function [53]. In the adult mouse, Rac is involved in recovery from ischaemia [54]; however, it is only essential for tumour angiogenesis in the absence of αVβ3 integrin [55]. The lipid sphingosine 1-phosphate stimulates angiogenesis in a manner dependent on Rac activation [56] and 'normalizes' (causes mature, non-leaky capillaries) VEGFA-stimulated angiogenesis [57]. Angiopoietin-1-dependent normalization of VEGFA-

stimulated EC permeability may operate via a pathway that depends on sequestration by RhoA of Src kinases to mDia (diaphanous homologue) reducing the VEGFA-stimulated activation of Rac [58]. Furthermore, VEGFA-stimulated EC permeability requires Rac activation [51]. Accordingly, the downstream Rac kinase p21-activated kinase (PAK) becomes activated and phosphorylates VE-cadherin, thus causing disassembly of endothelial adherens junctions. Thus Rac can lead to closure and opening of endothelial adherens junctions, possibly depending on the precise angiogenic milieu in which it is operating. Rac has also been reported to increase the generation of reactive oxygen species (ROS) [59].

Rac can be activated by numerous GEFs. RhoG is a Rac-GEF that functions in ECs [60] but it is currently unknown whether this GEF also plays a role in VEGFA-activated Rac biology. On the other hand, the Rac1-GEF Vav2 has been shown to transduce VEGF-activated Rac1 in ECs [51, 52]. In non-endothelial cells, FAK increases Rac activity or localization, possibly via the Rac-GEF Tiam1 [61–63].

Multifunctional docking proteins and adaptors in VEGFR2 signalling

Shb

Shb is a multidomain adapter protein [64] that operates downstream of several tyrosine kinase receptors [65] including VEGFR2 [30]. Shb is associated with numerous signalling proteins [65], including Src kinases, PLCγ, PI3K, FAK, Crk, Grb2 and c-Abl [66] and has a role in generating signalling complexes in a context-dependent manner [65]. Shb binds to pY1175 in VEGFR2 and is required for activation of FAK and PI3K, stress fibre formation and EC migration in response to VEGFA [30]. Shb is also required for VEGFA-dependent angiogenesis in differentiating embryonic stem cells [67, 68]. It is not possible to generate *Shb* -/- mice on a C57Bl/6 background because of early developmental defects [69, 70]; however, *Shb* -/- mice were born on a mixed genetic background. In adulthood, these mice had vascular abnormalities detected as diffuse staining of CD31 and VE-cadherin and cytoplasmic extensions projecting towards the capillary lumen [71, 72]. Stress fibres were increased in ECs of *Shb*-deficient mice, which gave the cells an irregular shape and probably explained the irregular staining pattern *in vivo* [71]. These morphological aberrations correlate with reduced VEGF-stimulated vascular permeability and *in vivo* angiogenesis, which occur with deletion of only one *Shb* allele. RIP-Tag2 insulinoma [73] incidence and tumour expansion were also decreased in the absence of *Shb* [74]. The effects were attributed to reduced angiogenesis and an abnormal morphological appearance of ECs, thus excluding EC

'normalization' as a cause for the anti-angiogenic effect. VEGFA-dependent angiogenesis was reduced by *Shb* deficiency although the tumours were heterogeneous in their dependence on VEGFA and Shb with a subgroup of tumours having the ability to escape from the angiogenic restriction imposed by their absence. *Shb* deficiency did not increase metastasis, therefore the angiogenic restriction imposed by *Shb* deficiency inhibits tumour growth without increasing tumour metastasis.

TSAd

TSAd is an SH2 domain-containing adaptor protein which binds to phosphorylated Y951 (Y949 in the mouse) on VEGFR2 [18] via its SH2 domain and to the SH3 domain of c-Src via its proline-rich domain [16]. Consequently, TSAd presents an important docking mechanism for Src to VEGFR2. Exchange of Y951/Y949 for phenylalanine, as well as *Tsad* knockdown, prevents VEGFA-dependent activation of c-Src, actin reorganization and EC migration, but not proliferation [18]. Of note, *tsad* -/- mice fail to respond to VEGFA with increased vascular permeability due to loss of active c-Src from adherens junctions [16].

IQGAP1

The actin-binding protein IQGAP1 (IQ motif-containing GTPase-activating protein 1) becomes phosphorylated by c-Src in response to VEGFA and has been implicated in the regulation of cell–cell contacts at adherens junctions, proliferation and migration by participating in the activation of ERK and PI3K [19, 75]. A complex consisting of Rac1, TIAM1, Src, cortactin, $p47^{phox}$ and phospholipase D2 has been described in isolated pulmonary artery ECs [76].

Gab1 and Gab2

Gab1 and Gab2 are scaffold proteins that operate downstream of VEGFR2 [11, 20, 77]. Upon tyrosine phosphorylation, Gab1 binds to Grb2, SHP2, the p85 subunit of PI3K and PLCγ, and Gab2 interacts with Grb2, SHP2, p85 PI3K and Shc. In ECs *in vitro*, Gab1 has been found to mediate activation of ERK and PI3K in response to VEGFA [11]. Decreased Gab1 expression reduced EC migration [77], a essential part of active angiogenesis. Gab2 also contributes to VEGFA-signalling in ECs [20]; however, Gab2 knockdown caused a paradoxical upregulation of Src, ERK and Akt activities, possibly via increased Gab1 levels. *In vivo*, EC-specific Gab1 inactivation caused reduced angiogenesis in response to ischaemia; in addition, PKA and eNOS

activities were decreased [78], suggesting that these are the primary components of relevance to Gab1-dependent angiogenesis.

Nck and Crk

Nck and Crk are SH2 and SH3 domain adapter proteins that are associated with activated VEGFR2 in ECs [79]. These proteins affect the cytoskeleton; little is known about Crk biology, but the action of Nck involves N-WASP and LIM-kinase [80]. The binding site for Nck on VEGFR2 was identified as Y1214 [79], which also binds the Src-family kinase Fyn. Nck and Fyn operate in a concerted manner downstream of VEGFR2 to activate p38MAPK and PAK2 to cause cytoskeletal alterations. In addition, the Nck–PAK signalling cascade has a role in the assembly of focal adhesions [81]. VEGF-dependent angiogenesis requires p38MAPK activation and this response is dependent on the VEGFR2 co-receptor neuropilin-1, along with the VEGFR2 co-receptor neuropilin-1, for VEGF-dependent angiogenesis [82, 83]. Furthermore, it has been reported that the VEGFR2-dependent phosphorylation of Nck requires the c-Abl tyrosine kinase [84].

Other signalling responses downstream of VEGFR2

Calcineurin/nuclear factor of activated T cells

Ca^{2+} fluxes as a consequence of PLCγ activation cause Ca^{2+}-/calcineurin-dependent activation of nuclear factor of activated T cells (NFAT), thus leading to changes in gene transcription that resemble the ’inflammatory response‘, for example that of interleukin-1 (IL-1) [7]. NFAT signalling also seems to be required for VEGFA-induced eNOS expression and NO production [85]. DSCR1 is an inhibitor of calcineurin and thus confers a negative feedback signal to the VEGFA–NFAT pathway. In numerous studies, VEGFA has been shown to increase DSCR1 expression [86–89] through increased cytoplasmic Ca^{2+} and activation of PKCδ [90]. Both DSCR1 overexpression [91] and targeted deletion or knockdown of DSCR1 suppress tumour growth by inhibition of angiogenesis (EC migration, tube formation and *in vivo* angiogenesis) [86, 87, 90, 92]. The uncertainty as to whether DSCR1 is anti- or pro-angiogenic may result from it being expressed as two isoforms, one (Ex4) that blocks EC proliferation and the other (Ex1) that stimulates angiogenesis [92].

ROS

ROS are induced downstream of VEGFR2 activation [93] as a consequence of NADPH oxidase (Nox) activity. ECs express Nox1, Nox2, Nox4 and Nox5, which in an NADPH-dependent manner generate a superoxide anion that is converted to hydrogen peroxide by superoxide dismutase [94]. In many systems, this process is stimulated by Rac [59] and by phosphorylation of $p47^{phox}$ by PKC, Akt, Src kinases, MAPK and PAK [94], all of which have a role in EC biology. The Rac-dependent effect has been suggested to involve p66Shc [95] and is at least partly attributed to mitochondrial generation of ROS [96]. Nox1 ablation in mice reduces EC migration and *in vivo* angiogenesis [97], accompanied by increased PPAR (peroxisome proliferator-activated receptor)-α expression and reduced VEGF-dependent NF-κB activation. These changes in transcriptional activity decrease expression of VEGFA and MMP-9. VEGFA causes $p47^{phox}$-dependent direct oxidation of VEGFR2 and Src, and this reaction leads to complex formation with effects on Akt activation [98]. Inhibition of VEGFR2 and Src oxidation by $p47^{phox}$ knockdown reduces Akt activation [98], providing a mechanistic explanation for the Nox1-dependent effects on NF-κB activation. Moreover, the results suggest a positive feedback loop in which early VEGFA-stimulated Rac-dependent activation of ROS generation will further enhance VEGFR2 signalling.

VEGFR2-dependent EC responses

Angiogenesis

VEGFA-induced pathways are tightly regulated spatially and kinetically to coordinate EC proliferation/survival, migration and invasion into the surrounding tissue, leading to formation of lumen-containing structures. EC proliferation/survival is stimulated primarily via the ERK [9] and PI3K/Akt pathways [42], which in turn are activated by numerous signalling intermediates as described above. ERK activation may occur in response to PLCγ, IQGAP1, FAK and Gab1 signalling and PI3K-activation via signalling through Shb, IQGAP1, AXL, Gab1 and FAK. Sustained activation of proliferation/survival without migration will produce vascular structures with stacked ECs projecting towards the lumen. EC migration is regulated through multiple pathways, often converging on PI3K stimulation that stimulates activation of Rho family G proteins. EC invasion occurs by release of MMPs, which degrade the basal membrane and extracellular matrix in order to allow migration of nascent ECs, leading to the formation of capillary sprouts. VEGFA has been shown to induce MT (membrane type)-MMP, MMP-2, MMP-9 and urokinase plasminogen activator [42,

99]. The primary mechanism by which VEGFA upregulates expression of these MMTs appears to be via Akt-dependent activation of β-catenin and NF-κB [42]. Activation of MMTs via ROS has also been reported [97].

Vascular permeability

Vascular permeability is essential for normal tissue homoeostasis, and dysregulated permeability leading to chronic oedema is an important feature in cancer and circulatory diseases, such as peripheral ischaemia and heart failure [100]. Fenestrae, which can be induced by VEGFA, are especially numerous in specialized vascular beds in hormone-secreting tissues [101]. VEGFA-induced extravasation of proteins or white blood cells is mediated by VEGFR2 *in vivo* [102].

Two main mechanisms have been implicated in VEGFA-induced vascular permeability, formation of transcellular pores and transient opening of paracellular junctions. First, transcellular pores such as vesiculo-vacuolar organelles (VVOs) are believed to assemble from lipid microdomains termed caveolae that form vesicles/vacuoles spanning the venular endothelium [103, 104]. VEGFR2 is localized in caveolae and forms a complex with caveolin-1 through its C-terminal tail [105]. Caveolin-1-targeted mice, which lack caveolae, retain VVOs and demonstrate increased permeability to plasma albumin and increased tumour growth [106].

Secondly, VEGFA-induced permeability depends on transient opening of adherens junctions and tight junctions. Adherens junctions are cell-cell interactions that primarily arise by hemophilic binding of VE-cadherin [23]. VEGFA stimulation causes dissociation of the homophilic VE-cadherin bonds [107] in a pathway involving formation of a complex including VEGFR2, VE-cadherin and Src [21, 22]. Transient opening of endothelial cell–cell junctions in response to VEGFA *in vitro* is well documented and involves dissolution of tight junctions as well as adherens junctions [108]. VEGFA dissolves adherens junction complexes, composed of VE-cadherin, *β*-catenin, p120-catenin and *α*-catenin [109], through activation of Src and Yes [21]. Src and Yes phosphorylate VE-cadherin [22, 110] and *β*-catenin [111, 112], leading to increased permeability in cell culture models [21, 113] and in mice [22]. Src-dependent vascular permeability in response to VEGFA requires the adaptor protein TSAd [16]. Angiopoietin-1 antagonizes VEGFA-induced permeability; this may occur via reduced Src-dependent phosphorylation of VE-cadherin through sequestration of

Src to mDia [58]. Alternatively, it has been suggested that the Rho family G protein Rac, via PAK, causes serine phosphorylation of VE-cadherin and its dissociation from junctions [51]. This will lead to increased endocytosis of VE-cadherin. A third proposed mechanisms of VE-cadherin dissociation from junctions involves the phosphotyrosine phosphatase VE-PTP which has been shown to associate with both VEGFR2 and VE-cadherin [114, 115]. The precise mechanism of how VE-PTP could exert such an effect has not been clarified, but may involve angiopoietin-1 and -2, as VE-PTP also interacts with their receptor Tie2 [116]. Reduced tyrosine phosphatase activity may contribute to the increased tyrosine phosphorylation of VE-cadherin, thus accelerating the opening of junctions [114, 117]. *Shb* deficiency reduces VEGFA-induced vascular permeability [71] and VE-cadherin dissociation from adherens junctions [118]. This effect may be related to aberrant signalling via Rho family G proteins [71]. In parallel to VE-cadherin dissociation, cell retraction may be required for a maximally stimulated breakup of the endothelial barrier [23] and this process is probably accomplished by the cytoskeleton and its regulation by Rho family G proteins and FAK as evidenced by reduced vascular permeability after conditional FAK knockout in ECs [36]. Rac activation is a possible mechanism of regulation of both VE-cadherin dissociation from junctions and cell retraction.

Activation of eNOS contributes to vascular permeability by production of NO followed by vasodilatation. Constitutively active Akt promotes vascular permeability independent of VEGF [46] and this effect involves activation of mTOR and phosphorylation of eNOS. VEGFA-dependent activation of eNOS can occur through the direct association between eNOS and Ca^{2+}/calmodulin (CaM) or through its phosphorylation by Akt [119, 120], AMP-activated protein kinase [121], CaM-dependent kinase II [122] and PKA [123]. Whether VEGFA causes AMP kinase-dependent activation of eNOS has been disputed [124]. The PKA-activated eNOS response seems to be primarily related to Gab1 signalling [78]. CaM-dependent kinase regulation of eNOS is a consequence of PLCγ activation and is further regulated by endothelial differentiation factor-1 (EDF-1) [125]. On the other hand, sphingosine 1-phosphate may cause direct stimulation of VEGFR2 and consequently phosphorylation of Akt and eNOS [126]. Akt appears to be an important signal for VEGFA-dependent eNOS activation.

Vascular inflammation

VEGFA is not an inflammatory cytokine; however, the EC response to VEGFA resembles the gene expression profile of IL-1β [7]. VEGFA-induced activation of

PLCγ causes an increase in Ca^{2+} and this will, via calcineurin, activate the transcription factor NFAT (see above). This pathway is pro-angiogenic but it also induces an 'inflammatory' gene expression pattern similar to that of IL-1β. Activation of NF-κB downstream of Akt will also induce an inflammatory-type response [42], resulting in numerous alterations in the gene expression profile. The precise role of the inflammatory EC response is not well understood, but it could promote attraction of inflammatory cells that participate in the angiogenic response.

Summary of EC responses to VEGFA

Multiple signalling pathways converge in distinct patterns eventually resulting in different biological responses such as angiogenesis, vascular permeability and inflammation. For angiogenesis, combined stimulation of ERK, Akt, Src, FAK and family G proteins seems to be required (Fig. 1), whereas Src-kinases, Rac G protein, phosphatases and eNOS are essential for vascular permeability (Fig. 2). With regard to the inflammatory component, NFAT and NF-κB are the primary regulators. Such an apparent redundancy in the operating signalling pathways is likely to reflect a need for fine-tuned and differential control of the biological effects in response to VEGFA. Thus, parallel input signals may augment or diminish specific aspects of the response. It seems plausible that the tumour angiogenic phenotype is precisely a consequence of this: a skewed stimulation of the VEGFA response that primarily satisfies the particular needs of that specific tumour.

The tumour vasculature and future perspectives

Vascular normalization

The abnormal tumour vasculature forms a chaotic network often with blind ends and with vessels that are variable in size. The endothelial lining is irregular with stacked cells or cells that lack junctional connections. The pericyte coverage is reduced. Overall, the vasculature is hyperpermeable, creating oedema and leukocyte extravasation [127].

One simple explanation for these characteristics of the tumour vasculature is that hypoxia produces a chronic hypersecretion of VEGFA resulting in persistent stimulation of ECs. The different aspects of angiogenesis described above are thus promoted and the vasculature may consequently expand unrestrained, without becoming mature and fully functional. This simplistic approach to understanding the

tumour vasculature may have some validity but may also not provide the full picture. There are several means by which the tumour environment may correct for the excess VEGF stimulation. For example, the tumour vasculature is influenced by the angiopoietin-1/angiopoietin-2 ratio, so that a relatively greater abundance of angiopoietin-1 will promote vascular quiescence. Sphingosine-1 phosphate will also cause vascular normalization in conjunction with VEGFA. Platelet-derived growth factor influences pericyte coverage. Thus, the tumour is able to modify the vasculature for its own benefit by co-producing angiogenic factors other than VEGFA. However, why the abnormal, dysfunctional vasculature of tumours seems to be compatible with tumour growth remains an enigma.

The results of a number of studies have indicated that vessel normalization occurs whenever tumour growth is hampered by anti-angiogenic treatment [1]. Vascular normalization is thought to improve tumour oxygenation through an increase in blood perfusion rate. However, lower vascular density as a consequence of inhibition of angiogenesis may balance the improved blood perfusion due to a longer diffusion distance between the capillaries and the tumour cells located most distally from the capillary. Therefore, the supply of oxygen and nutrients may not be increased to all tumour cells as a consequence of vascular normalization, but may depend on the balance between increased tumour perfusion and decreased vascular density/increased diffusion distance. Differences in the vascular supply of oxygen and nutrients to the tumour cells as a consequence of reduced vascular density after anti-angiogenic treatment could accelerate the rate of tumour evolution and cell heterogeneity.

The role of VEGF-induced vascular permeability

Downstream effectors of VEGFR2 show a considerable overlap in their ability to stimulate angiogenesis and vascular permeability. It therefore seems that the two responses are essentially inseparable. How does vascular permeability affect angiogenesis? It is likely that vascular permeability is a prerequisite for the VEGFA-dependent angiogenic signature. Increased permeability could promote angiogenesis by allowing more rapid dissemination of extracellular proteases that are required for vessel sprouting and by allowing deposition of a provisional matrix on which the newly formed sprout grows [128]. In addition, extravasation of leukocytes may promote the early angiogenic response by enhancing the local production of cytokines/chemokines and by releasing proteases. Although leukocyte extravasation could simply be a consequence of loosened junctions, it could also reflect active,

VEGFA-dependent processes occurring on both ECs [129] and leukocytes [130]. Taken together, these effects may allow optimal revascularization of hypoxic areas.

Solid tumours are generally hypoxic; this leads to continuous production of VEGF, with persistent stimulation of the tumour vasculature, which as a consequence is highly permeable. For several reasons, this may promote increased metastatic spread. The reduced vessel integrity may facilitate exit of metastases from the primary tumours [131]. Moreover, poor vessel functionality promotes further increased hypoxia, which may drive the malignant process towards increased invasiveness [132].

VEGF and inflammation

Although leukocytes can kill tumour cells via innate and/or acquired immunity, the tumour also has the potential to utilize leukocytes to promote its survival and expansion. One example of this is macrophage M2 polarization as described above. A second example is upregulation of the chemokine receptor CXCR4 on rectal cancer cells upon VEGFA blockade [133, 134] thus leading to tumour dissemination. Consequently, tumours may utilize the inflammatory response to VEGFA-stimulated vascular permeability to promote their expansion and dissemination.

VEGF inhibition in current and future clinical use

Table 1 shows the clinical conditions in which exacerbated VEGFA signalling may have a pathogenetic role, and the current status with regard to clinical use or testing of VEGFA inhibition in these conditions. The importance of VEGFA in tumour biology has stimulated the development of inhibitors of VEGFA and its downstream effectors which are either in current use or being tested in clinical settings (Table 2). These (potential) therapies include inhibition of VEGFA using a blocking antibody, a 'VEGF trap' consisting of a soluble extracellular domain of VEGFR2, or chemical kinase inhibitors. In addition to the use of anti-angiogenic therapy for the treatment of cancer, VEGF inhibition is being used for treatment of macular degeneration. It is possible that as well as therapies to directly target VEGFA and its receptor, agents that influence key downstream effectors could be clinically beneficial. Of those described here, TSAd, Axl and Shb appear to be the most specific for pathological angiogenesis and thus constitute the most promising novel leads for developing potential therapies.

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Table 1. Clinical conditions with enhanced VEGFA-dependent signalling

Condition	Current clinical status: therapeutic use or testing of VEGF inhibition
Cancer, metastatic spread	Yes
Retinopathy, macula degeneration	Yes
Psoriasis	No
Chronic inflammation	No
Coronary ischaemia	No

For discussion of the use of VEGF neutralization in psoriasis, chronic inflammation and coronary ischaemia (i.e. diseases characterized by increased vascular permeability and vascular dysfunction), see [1, 22].

Table 2. VEGFA-inhibiting compounds in clinical use or development

Compound	Mode of action	Applications
Bevacizumab; ranibizumab	Neutralization of VEGFA antibody, either full-length (bevacizumab) or F(ab)2 fragment (ranibizumab)	Approved and being investigated for numerous cancers, approved for macular degeneration
Aflibercept	VEGF trap	Approved for macular degeneration, currently being investigated for various cancers
Ramucirurab	Neutralization of VEGFR2 antibody	Currently being investigated for various cancers and macular degeneration
Sunitinib	VEGFR2 inhibitor; broad effect on numerous tyrosine kinases	Approved for Renal cell carcinoma, gastrointestinal stromal tumour (GIST), pancreatic cancer
Sorafenib	VEGFR2 inhibitor; broad effect on numerous tyrosine kinases	Approved for Renal cell carcinoma, hepatocellular cancer
Pazopanib	VEGFR2 inhibitor; broad effect on numerous tyrosine kinases	Approved for Renal cell carcinoma, soft tissue sarcoma

For further details, see http://www.cancer.gov/cancertopics/druginfo/.

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Figure legends

Fig. 1 Summary of the key VEGFA/VEGFR2 signal transduction pathways. Schematic diagram showing the pathways arising from different phosphorylation sites pY951, pY1175 and pY1212 in VEGFR2 (left) that are thought to be important for VEGFA-induced migration, proliferation/survival and tubular morphogenesis and which are integral to angiogenesis. Arrows may indicate direct or indirect effects. For abbreviations and further details, see text.

Fig. 2 Summary of signalling events downstream of VEGFR2 in regulation of VEGFA-induced vascular permeability. Arrows indicate pathways resulting in activation of (i) the VEGFR2–TSAd–Src–VE-cadherin pathway, (ii) the FAK–Rac pathway leading to cell retraction and (iii) the PI3K–Akt–eNOS–NO pathway. For abbreviations and details, see text.

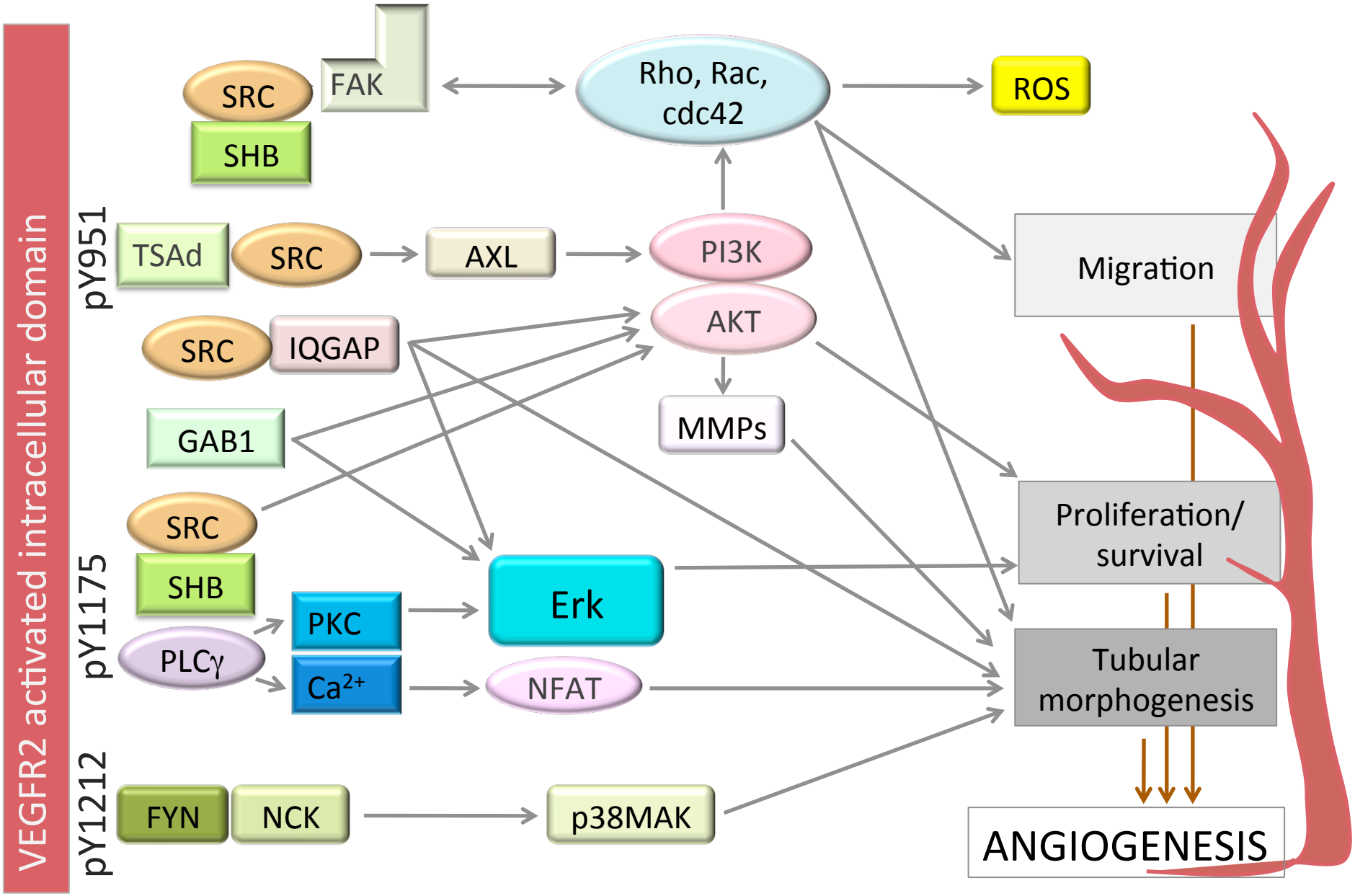
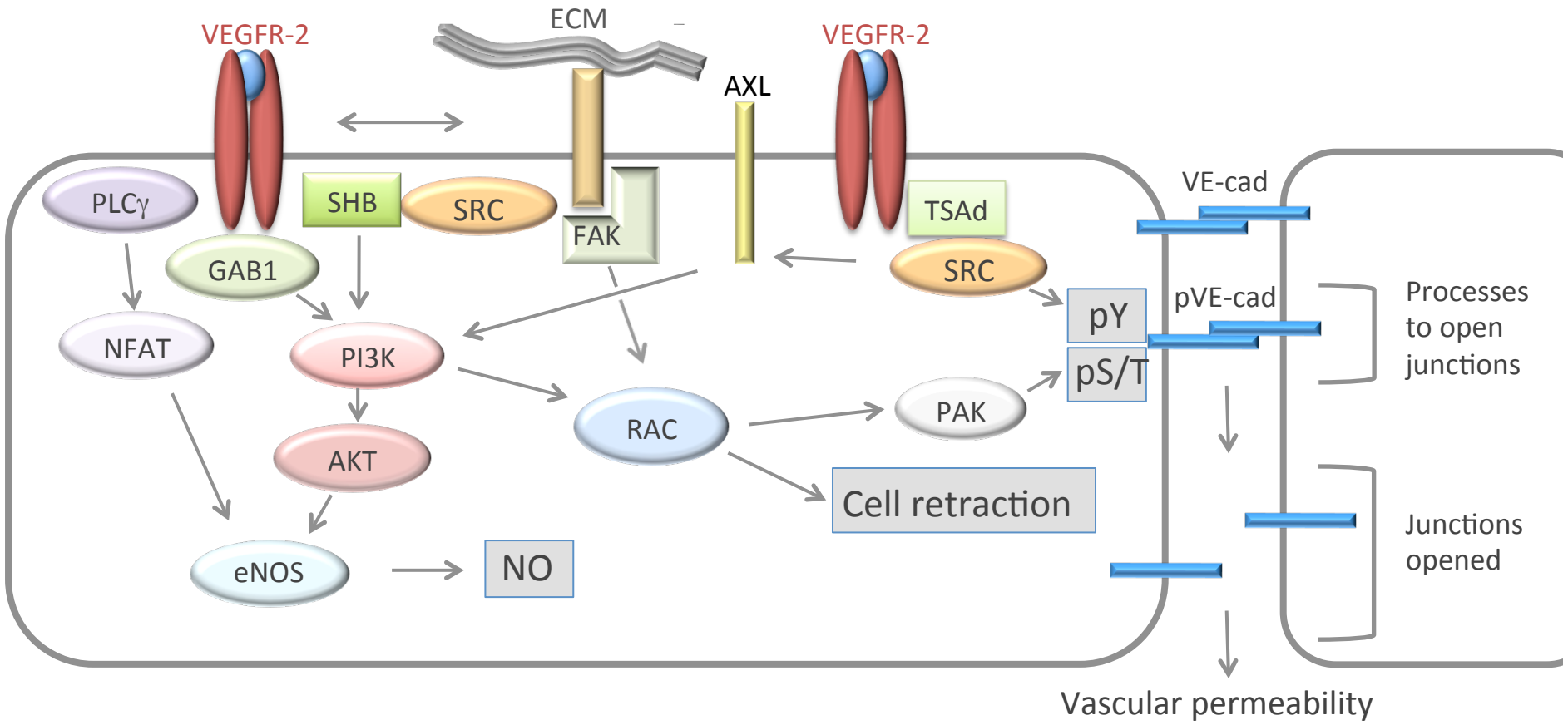

VEGFR2 activated intracellular domain
pY951
pY1175
pY1212
SRC
SHB
FAK
Rho, Rac, cdc42
ROS
TSAd
SRC
AXL
PI3K
Migration
AKT
SRC
IQGAP
GAB1
MMPs
SRC
SHB
Proliferation/ survival
PKC
PLCγ
Ca2+
Erk
NFAT
Tubular morphogenesis
FYN
NCK
p38MAK
ANGIOGENESIS

Fig. 1


VEGFR-2
ECM
VEGFR-2
AXL
PLCγ
SHB
SRC
FAK
TSAd
GAB1
SRC
VE-cad
pY
pVE-cad
NFAT
PI3K
pS/T
Processes
to open
junctions
RAC
PAK
AKT
Cell retraction
Junctions
opened
eNOS
NO
Vascular permeability