Vascular endothelial growth factor-A regulation of blood vessel sprouting in health and disease

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Abstract

Introduction

Vascular endothelial growth factors (VEGFs) comprise of a multi-gene-encoded family of cytokines that display remarkable effects in different tissues including the vascular, epithelial and neuronal tissues. Such factors bind to both membrane-bound and soluble receptor isoforms that regulate the ability of these cytokines to trigger profound biochemical changes within cells, resulting in changes to metabolism, gene expression, cell proliferation, apoptosis and homeostasis. The cornerstone of this biochemical control is based on the interaction between soluble factors such as VEGFs and membrane-bound receptors such as VEGF receptor tyrosine kinases (VEGFRs). The endothelial monolayer that lines all blood vessels responds to VEGFs to regulate many aspects of vascular physiology. Dysfunction in the VEGF-A signalling pathways is implicated in a wide variety of diseases ranging from solid tumour metastasis to atherosclerosis. VEGF binding to membrane VEGFRs triggers intracellular signalling, post-translational protein modifications, trafficking and proteolysis. Temporal and spatial co-ordination of such events is critical for programming downstream responses by the endothelium. In this review, we have discussed the biochemical mechanisms that underlie new blood vessel sprouting mediated by VEGF-like cytokines.

Conclusion

The development of primary cell systems, multicellular models, organ culture and animal models, will dramatically increase our ability to understand the biochemical basis for how this important class of cytokines regulate animal physiology. Targeting such pathways directly or indirectly using humanised antibodies or small molecule inhibitors, is important for treating a wide variety of pathological states including solid tumour growth and metastasis, age-related macular degeneration and conditions involving blood vessel repair and regeneration.

Introduction

The discovery of vascular endothelial growth factor A (VEGF-A) more than three decades ago triggered an intense interest in the biochemical mechanisms underlying the sprouting of new blood vessels i.e ‘angiogenesis’. This is a complex process whereby new blood vessels sprout from a pre-existing vascular network¹,², which occurs when extrinsic levels of pro-angiogenic factors exceed those of the anti-angiogenic factors. This is a biochemical switch that regulates the quiescent vasculature to proliferate and sprout new blood vessels.¹ Physiological regulation of angiogenesis is crucial as dysfunction in angiogenic responses can cause serious pathological conditions such as diabetic retinopathy, atherosclerotic plaque rupture, solid tumour growth and metastasis². This review discusses VEGF-A regulation of blood vessels sprouting in health and disease.

Discussion

The authors have referenced some of their own studies in this review. The protocols of these studies have been approved by the relevant ethics committees related to the institutions in which they were performed.

VEGFs

The human VEGF family consists of five members: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PIGF), which regulate angiogenesis, vasculogenesis and/or lymphangiogenesis¹. The VEGF-A gene is located on the human chromosome 6p21.3³; the encoded pre-mRNA is approximately 14 kB in length, containing eight exons and seven introns⁴. Alternative splicing of this pre-mRNA transcript produces multiple VEGF isoforms (Figure 1). Each isoform contains exons 1–5 which encode the signal sequence (exon 1), N-terminus (exon 2), dimerisation domain, VEGF receptor tyrosine kinase 1 (VEGFR1) binding and N-glycosylation site (exon 3), and VEGFR2-binding site (exon 4) and a plasmin cleavage site (exon 5). The identity of each VEGF-A isoform is largely determined by variable inclusion of exons 6a, 6b, 7a and 7b, which encode the heparin-binding domain (Figure 1). In addition to generating VEGF-A isoforms that differ in length and domain composition, the pre-mRNA splicing machinery also gives

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 rise to anti-angiogenic isoforms by splice site selection events termed as proximal splice site selection and distal splice site selection. Splice site selection determines the six amino acids at the carboxy-terminal (exon 8), namely the pro-angiogenic sequence CDKPRR (exon 8a) or the anti-angiogenic sequence SLTRKD (exon 8b)\(^4\). There are at least seven pro-angiogenic isoforms of the human VEGF-A which encode polypeptides of 121, 145, 148, 165, 183, 189 or 206 residues (a isoforms) and five anti-angiogenic isoforms which encode polypeptides of 121, 145, 165, 183 and 189 residues (b isoforms e.g. VEGF-A\(_{121}\))\(^5\). However, there is debate about the existence of these b isoforms in the light of a recent study, suggesting that these were derived by real-time polymerase chain reaction artefacts\(^5\).

Replacement of the carboxy-terminal six amino acids from VEGF-A\(_{165a}\) to that of VEGF-A\(_{165b}\) has a substantial effect on its biochemical activity. VEGF-A\(_{165a}\) binding to VEGFR2 and neuropilin 1 (NRP1), leads to a conformational change resulting in internal rotation and autophosphorylation of the VEGFR2 intracellular domain, thus triggering multiple signalling outputs. However, although VEGF-A\(_{165b}\) binds to VEGFR2 with similar affinity as exhibited by VEGF-A\(_{165a}\), it is proposed to elicit insufficient torsional rotation of the tyrosine kinase domain, resulting in rapid closing of the VEGFR2 adenosine triphosphate binding site, rapid inactivation and weak transient activation of downstream p42/44 (extracellular signal-regulated kinases 1/2 [ERK 1/2]) mitogen-activated protein kinase (MAPK)\(^4\). In addition, VEGF-A\(_{165b}\) binds to VEGFR2 but not NRP1 due to its altered carboxy-terminus. Thus, a combination of weak VEGFR2 activation, lack of co-receptor binding and competition for binding sites with pro-angiogenic VEGF-A\(_{165a}\) could explain the anti-angiogenic properties of VEGF-A\(_{165b}\)\(^4,6\). Furthermore, as VEGF-A usually functions as a disulphide-linked homodimer, there is the theoretical possibility that heterodimers of either non-paired, non-equivalent length isoforms (e.g. VEGF-A\(_{206}\) and VEGF-A\(_{121}\)) or paired, equivalent length but non-identical isoforms (VEGF-A\(_{165a}\) and VEGF-A\(_{165b}\)) exist, causing increased complexity in regulating the angiogenic response\(^4\).

Membrane receptors that bind VEGFs VEGF-A binds with picomolar affinity to two different receptor tyrosine kinases (VEGFR1 and VEGFR2), with additional binding specificity for NRP co-receptors, NRP1 and NRP2.

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Review

Figure 2: VEGFR1 and VEGFR2 and their associated ligands. Black boxes on the receptors indicate important tyrosine residues. Yellow boxes show tyrosine residues 1054 and 1059 which are important for kinase activity.

c-Akt, protein kinase B; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; ERK1/2, extracellular signal-regulated kinase 1/2; FAK, focal adhesion kinase; HPC, hematopoietic progenitor cells; HSP27, heat shock protein-27; MAPK, mitogen-activated protein kinase; MEK, MAPK and ERK kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLCγ1, phospholipase Cγ1; PIGF, placental growth factor; Shb, SH2 domain-containing adaptor protein b; TSAd, T-cell–specific adaptor, VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor tyrosine kinase.

NRPs are approximately 140 kDa transmembrane, non-tyrosine kinase, glycoprotein co-receptors for both the semaphorin and the VEGF family1. They comprise a large extracellular domain of approximately 850 residues, a transmembrane region and a relatively short cytoplasmic domain (approximately 40 residues). This cytoplasmic domain is likely to function as a docking site for various downstream signalling molecules1. NRPI binds VEGF-A165a, PIGF-152 (the heparin sulphate binding isoform) and both VEGF-B isoforms, whereas VEGFR1 (Flt-1) has an estimated molecular mass of 151 kDa, but the mature protein undergoes processing and glycosylation to produce a species of approximately 200–230 kDa, as detected by SDS-PAGE, which lack tyrosine kinase activity7. VEGFR1 and VEGFR2 belong to the Class III receptor tyrosine kinase family and comprise of seven immunoglobulin-like repeats within their extracellular domain, a single transmembrane region and a split cytoplasmic tyrosine kinase domain. Both VEGFR1 and VEGFR2 play important roles in vasculogenesis and angiogenesis as gene knockout mice die in utero at E8.5 and E9.5, respectively8,9.

VEGFR1 (Flt-1) has an estimated molecular mass of 151 kDa, but the mature protein undergoes processing and glycosylation to produce a species of approximately 180 kDa, as detected by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). VEGFR1 binds to VEGF-A, VEGF-B and PIGF1. VEGFR1 has highest affinity for VEGF-A (Kd≈10–30 pM)10 but displays relatively weak tyrosine kinase activity following VEGF-A binding2,11. These biochemical properties have led to the suggestion that the primary physiological role of VEGFR1 is to act as a negative regulator of angiogenesis, by ‘trapping’ VEGF-A to reduce its bioavailability for VEGFR212. The VEGFR1 gene is located on chromosome 13q12 and contains 30 exons; alternative splicing of this pre-mRNA gives rise to both soluble and membrane-bound protein isoforms13. VEGFR1 is expressed by both quiescent and actively proliferating endothelial cells suggesting that VEGFR1 has an important role in endothelial homeostasis2.

VEGFR2 (KDR) has a molecular mass of 152 kDa, but the mature protein undergoes processing and glycosylation to produce a species of approximately 200–230 kDa, as detected by SDS-PAGE. Only this mature, glycosylated form of VEGFR2 can be efficiently autophosphorylated14. The binding affinity of VEGFR2 for VEGF-A is 5–10 fold lower than that of VEGFR1 (Kd≈75–125 pM)15; however, VEGFR2 has a higher tyrosine kinase activity1,15, VEGFR2 can also bind to VEGF-C, VEGF-D and the virally encoded VEGF-E variant16,17. In contrast to VEGFR1, VEGFR2 expression is downregulated in quiescent adult vasculature16, this is likely due to the fact that most of the pro-angiogenic gene expression is regulated by VEGFR22. VEGFR2 activation is primarily responsible for the majority of the biological effects linked to VEGF-A bioactivity, including vascular permeability, cell migration, cell survival, cell proliferation and smooth muscle relaxation (Figure 2). Thus, VEGFR2-blocking therapies which are tailored to attenuate specific intracellular signalling events or pathways, could reduce clinical side effects of current VEGF therapies19, including excessive vascular permeability, inflammation, tumour growth and metastasis.
NRP2 binds VEGF-A\textsubscript{145} VEGFA\textsubscript{165d}, PIGF-152 and VEGF-C. It was originally thought that neither NRP1 nor NRP2 could bind VEGF-A\textsubscript{121}, however, Pan et al. (2007)\textsuperscript{21} reported that the VEGF-A\textsubscript{121} splice variant can bind to NRP1 in vitro but cannot promote the formation of a VEGFR2/NRP1 complex\textsuperscript{21,22}. Co-expression of VEGFR2 and NRP1 in stably transfected porcine aortic endothelial cells leads to a stronger VEGF-A-stimulated response compared to cells expressing VEGFR2 alone\textsuperscript{6}.

VEGFR tyrosine phosphorylation and intracellular signalling

A key aspect of receptor tyrosine kinase function is the capacity to become activated upon binding ligands such as growth factors. In the case of VEGFRs, binding to VEGF dimers triggers receptor dimerisation followed by trans-autophosphorylation on specific cytoplasmic tyrosine residues. VEGFRs can also undergo heterodimerisation, but the functional significance remains unclear\textsuperscript{23-25}. Ligand-stimulation of VEGFR1 elevates levels of urokinase plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1), which in turn regulate extracellular matrix degradation and cell migration. Both uPA and PAI-1 modulate actin dynamics via p38 MAPK, thus regulating endothelial cell migration\textsuperscript{2,7,26,27}. VEGFR1 is also capable of recruiting phospholipase Cγ1 (PLCγ1) after phosphorylation of residue Y1169, creating a binding site for the Src homology (SH2) domains in PLCγ1\textsuperscript{11} (Figure 2). However, activated VEGFR1 does not seem to directly impact cellular responses such as cell proliferation.

Activated VEGFR2 is phosphorylated on 6–7 tyrosine residues, notably residues Y1175, Y951 and Y1214\textsuperscript{1,19}. Such post-translational modifications generate binding sites for a variety of regulatory proteins containing SH2 or phosphotyrosine binding domains\textsuperscript{1} (Figure 2). PLCγ1 binds to the pY1175 site and to the phosphotyrosine-based epitope within VEGFR2\textsuperscript{1,19}, and its recruitment to the plasma membrane triggers phosphatidylinositol-4,5-bisphosphate hydrolysis to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP\textsubscript{3}). Generation of IP\textsubscript{3} triggers activation of the IP\textsubscript{3} receptor, which acts as a calcium channel on the endoplasmic reticulum, by mediating IP\textsubscript{3}-activated translocation of calcium ions from the endoplasmic reticulum to the cytosol. In contrast, DAG activates the protein kinase C (PKC) enzyme, which triggers MAPK pathway activation and signalling. Additionally, VEGFR2 phosphorylation stimulates the activation of the phosphatiidylinositol 3-kinase (PI3K) pathway, leading to increased c-Akt (protein kinase B) activity, subsequent endothelial nitric oxide synthase (eNOS) phosphorylation and activation, resulting in nitric oxide (NO) production\textsuperscript{1,19}. Increased levels of NO in response to VEGF stimulation, promote a positive feedback loop in angiogenesis: NO stimulates expression and transcriptional activity of the hypoxia inducible factor complex which stimulates VEGF-A mRNA transcription\textsuperscript{28}. Activated PKC enzymes are postulated to phosphorylate and activate MEK (MAPK and ERK kinases), which subsequently phosphorylates and activates p42/44 MAPK, leading to changes in gene expression and cell proliferation. However, studies implicate the PKC\textalpha as a key regulator of VEGF-A-stimulated phosphorylation and activation of c-Akt and eNOS\textsuperscript{29}.

A small adaptor molecule called Shb (SH2 domain-containing adaptor protein b) also binds to the VEGFR2-pY1175 phospho-epitope and activates PI3K, which sequentially stimulates activation of c-Akt and eNOS, thus promoting cell survival and NO production, respectively\textsuperscript{1,19}. Binding of a T-cell–specific adaptor protein (TSAd) to the same phospho-epitope is also implicated in c-Src–regulated endothelial cell migration and vascular permeability. Generation of the VEGFR2-pY1214 phospho-epitope is linked to increased actin remodelling and cell migration through a pathway involving CDC42, p38 MAPK and heat shock protein 27 kDa. Furthermore, the VEGFR2-pY1214 site is linked to focal adhesion turnover and cell migration through focal adhesion kinase and its substrate paxillin.

VEGFR ubiquitination and proteolysis

A key feature of receptor-ligand complex formation is temporal and spatial regulation linked to subsequent cellular responses. For example, conventional models of receptor tyrosine kinase activation postulate that binding to ligands at the plasma membrane is followed by trafficking through the endosome-lysosome system, which is linked to receptor-ligand proteolysis. Interestingly, VEGFR1 and VEGFR2 display markedly different profiles in this respect. VEGFR1 levels appear to be relatively insensitive to VEGF-A stimulation, unlike VEGFR2\textsuperscript{30,31}. Work from our group suggests that only 20% of VEGFR1 is available at the plasma membrane, whereas the remaining pool is located within an intracellular Golgi-like compartment\textsuperscript{31}. Other studies suggest that VEGFR1 displays both cytoplasmic and nuclear pools in both endothelial and non-endothelial cells\textsuperscript{22,33}. Nonetheless, calcium-regulated VEGFR1 trafficking from an intracellular membrane-bound compartment to the plasma membrane may be one way of generating a negative-feedback loop to modulate VEGF-A–stimulated VEGFR2 response in endothelial cells\textsuperscript{31,34}.

VEGFR2 displays a complex pattern of distribution to the secretory pathway, plasma membrane and endosome-lysosome system, with marked proteolytic sensitivity\textsuperscript{30,31,35}. VEGF-A binding stimulates VEGFR2 trafficking through endosome-lyso-
some compartments\textsuperscript{30,36-38} and the nucleus\textsuperscript{33}. A key aspect of VEGFR2 activation by VEGF-A binding is ubiquitination and proteolysis\textsuperscript{30,38} (Figure 3). VEGFR2 proteolysis is also tightly regulated with at least two different proteolytic activities postulated for its cleavage within the endosome-lysosome system: a 26S proteasome-regulated step associated with early endosomes for processing of the cytoplasmic domain and lysosomal activity for processing of the extracellular/luminal domain\textsuperscript{30,38} (Figure 3).

The process of ubiquitination involves reversible post-translational modification of protein substrates\textsuperscript{99} which programmes proteolysis and/or intracellular re-distribution and thus regulates altered protein function. Intriguingly, inactive VEGFR2 may also be ubiquitinated as it undergoes slow proteolysis in the absence of VEGF-A\textsuperscript{13,40}. One view is that stimulation of VEGF-A accentuates endogenous VEGFR2 ubiquitination, thus promoting intracellular re-distribution and clearance. Sequential complex assembly, based on multiple ubiquitin–ubiquitin-binding domain interactions, enables the propagation of signalling events, which control the dynamics of receptor trafficking through the endosome-lysosome system\textsuperscript{41,42}. Ubiquitin conjugation to target proteins involves E1, E2 and E3 enzymes or ligases. Thus a specific E1/E2/E3 cascade must regulate VEGFR2 ubiquitination in response to VEGF-A stimulation. Notably, de-ubiquitinating enzymes (DUBs) have been implicated in regulating receptor tyrosine kinase function\textsuperscript{43-45}.

**VEGFR2 regulation by ESCRTs**

Following VEGF-A binding and activation at the plasma membrane, the VEGFR2-VEGF-A complex is endocytosed and transported to early endosomes where an ubiquitin-linked receptor complex, endosomal sorting complex required for transport (ESCRT-0), mediates sorting and delivery to late endosomes\textsuperscript{41,46} (Figure 3). An array of endocytic regulators working with the endosomal ubiquitin receptors, namely Hrs, STAM and Eps15, initially engage activated receptor tyrosine kinase–ligand complexes in early endosomes for subsequent delivery to multivesicular body endosomes\textsuperscript{87}; this is followed by trafficking towards the lysosome for terminal degradation\textsuperscript{48}. The ESCRT-0 heterodimer can bind several ubiquitin moieties simultaneously via the ubiquitin-interacting motif of the signal transducing adaptor molecule (STAM) subunit and the di-ubiquitin motif of the Hrs subunit\textsuperscript{41}. The Hrs/STAM complex shows increased co-distribution with VEGFR2 after VEGF-A stimulation\textsuperscript{30}, suggesting active sorting of the receptor–ligand complex further into the endosome-lysosome system. After sorting, ubiquitin attached to cargo proteins can be removed by endosomal DUBs, such as the associated molecule with the SH3 domain of STAM (AMSH) and ubiquitin-specific protease Y (UBPY)\textsuperscript{49}. The process of de-ubiquitination enables the ESCRT machinery to dissociate from its bound cargo and recycle for subsequent use in the trafficking of other ubiquitinated molecules. Due to their functional role in epidermal growth factor receptor (ErbB1) trafficking, UBPY and AMSH\textsuperscript{49} can thus mediate VEGFR2 recycling from early endosomes back to the plasma membrane.

**Figure 3:** VEGFR2 trafficking and degradation. Non-stimulated VEGFR2 undergoes constitutive internalisation and recycling back to the plasma membrane. Following internalisation, VEGF-A–stimulated VEGFR2 is either recycled or degraded. VEGFR2 can recycle independently (Rab4a pathway) or via a NRP1 mediated pathway (Rab11 pathway). Interaction with the ESCRT machinery in early endosomes regulates transport to late endosomes where the VEGFR2 C-terminal domain can be proteolytically cleaved prior to final degradation in the lysosome.

**Legend:**
- EE, early endosome; ESCRT, endosomal sorting complex required for transport;
- LE, late endosome; Lys, lysosome; MVB, multivesicular body; NRP, neuropilin; RE, recycling endosome; VEGFR, vascular endothelial growth factor receptor tyrosine kinase.

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Trafficfing of the VEGFR2-NRP1 complex

Interaction between the NRP1 binding adaptor synectin and myosin VI, mediates the internalisation of VEGFR2 into Rab5a-positive early endosomes. Internalised, activated VEGFR2–VEGF-A complexes can be recycled to the plasma membrane primarily from Rab4a-positive recycle-ling endosomes. NRP1 itself recycles via Rab11a-positive vesicles, following transport from Rab4a-positive vesicles. The NRP1 C-terminal domain contains a carboxy-terminal tripeptide motif (SEA*) that binds a PDZ-binding motif within synnectin/ GIPC. VEGF-stimulated interaction between VEGFR2 and NRP1, is linked to actin-dependent recycling through a Rab5a-Rab4a-Rab11a endosomal pathway.

VEGFR2 regulation by VE-cadherin and c-Src

VEGFR2 can be recycled through a non-conventional pathway in response to ligand stimulation. Phosphorylated VEGFR2 is delivered into endosomes via a vascular endothelial (VE)-cadherin–linked, clathrin-regulated endocytic pathway; here, receptor stability is retained, leading to prolonged signalling outputs which ultimately result in increased cell migration and proliferation. Activated VEGFR2 recruits TAOs, which subsequently enables recruitment of the tyrosine kinase c-Src which is a potent effector of cell proliferation. Phosphorylation of c-Src by VEGFR2 promotes activation and downstream phosphorylation of p21-activated kinase 2 (PAK2). Activated PAK2 phosphorylates key serine residues on VE-cadherin within the binding region for p120catenin. Dissociation of p120catenin exposes a short endocytic motif (DEE) within VE-cadherin, promoting endocytosis, disruption of endothelial adherens junctions and increased vascular permeability. However, VEGFR2 and VE-cadherin appear to follow different endocytic routes from the plasma membrane. The c-Src enzyme shows increased co-distribution with intracellular stores of VEGFR2 upon VEGF-A stimulation; however, there is uncertainty as to whether this is a late secretory (e.g. trans-Golgi network) or endosomal pool of VEGFR2. Nonetheless, both VEGFR2 and c-Src are recycled through the same endocytic recycling pathway. In quiescent endothelial cells, VEGFR2 is stored in intracellular Rab4/Rab11-negative vesicles and delivered to the plasma membrane in a c-Src-activation-dependent manner in response to VEGF-A, highlighting unique differences in comparison to other receptor tyrosine kinases.

Endosome-associated VEGFR2 cytoplasmic domain proteolysis

VEG-A stimulation results in the degradation of the mature, glycosylated approximately 230 kDa VEGFR2 species and an increase in the levels of a novel 160 kDa VEGFR2 cytoplasmic domain-related proteolytic polypeptide. Production of this proteolytic 160 kDa fragment requires VEGF-A–stimulated VEGFR2 activity and subsequent endosomal trafficking. Mono-ubiquitination of VEGFR2 precedes the production of this proteolytic fragment; this modification could act to mediate the 26S proteasomal recognition of early endosomal VEGFR2, before subsequent terminal lysosomal degradation. Carboxy-terminal post-translational modifications and recruitment of signalling effectors bestow the VEGFR2 cytoplasmic domain with important roles in endothelial function. Inhibition of this limited cytoplasmic domain proteolysis alters endothelial cell migration and VEGF-A-regulated downstream signalling, prolonging the phosphorylation and activation of p42/44 MAPK, c-Akt and eNOS. Thus cleavage of the VEGFR2 cytoplasmic domain may be a key event in controlling the duration and intensity of endothelial signalling in response to VEGF-A.

PKC regulation of VEGFR2 function

VEGF-A may promote degradation of VEGFR2 by downstream activation of non-classical PKC isozymes. Direct or indirect PKC-mediated phosphorylation of VEGFR2 cytoplasmic domain residues S1188 and/or S1191, marks the receptor for PKC-regulated internalisation and proteasomal degradation. Thus, VEGF-A-stimulated PKC activation may promote the 26S proteasome-regulated removal of the VEGFR2 cytoplasmic domain. Recent findings by Nakayama et al. (2013) report that atypical protein kinase C (aPKC) activity negatively regulates the endocytosis and turnover of VEGFR2 within mature established blood vessels (where levels of aPKC are high) but not so much in sprouting vessel tips (were levels of aPKC are low).

Conclusion

Emerging concepts in different areas of classical biochemistry and cell biology highlight the importance of integration between signalling pathways with membrane trafficking, protein biosynthesis and proteolysis, to regulate cell and tissue function. This is exquisitely illustrated in the VEGF regulation of animal physiology as the interaction between such ligands and their cognate receptors fine tunes animal biology in both physiological and pathological states. A major challenge in this area is to understand the complexity of VEGF isoforms in programming differential receptor signalling, turnover and function. Many lines of evidence suggest that VEGFs have subtle effects on cell homoeostasis in the immune, epithelial and nervous systems, but these underlying mechanisms remain elusive. The development of primary cell systems, multicellular models, organ culture and animal models, will dramatically increase our ability to
understand the biochemical basis for how this important class of cytokines regulate animal physiology. Targeting such pathways directly or indirectly using humanised antibodies or small molecule inhibitors is important for treating a wide variety of pathological states including solid tumour growth and metastasis, age-related macular degeneration and conditions involving blood vessel repair and regeneration.

**Abbreviations list**
AMSH, associated molecule with the SH3 domain of STAM; aPKC, atypical protein kinase C; DAG, diacylglycerol; DUB, de-ubiquitinating enzyme; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; IP3, inositol-1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; NO, nitric oxide; NRP, neuropilin; PAI-1, plasminogen activator inhibitor-1; PAK2, p21-activated kinase 2; PlGF, placental growth factor; P13K, phosphatidylinositol 3 kinase; PKC, protein kinase C; PCLy1, phospholipase Cγ1; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SH, Src homology; STAM, associated molecule with the SH3 domain of STAM; uPA, urokinase plasminogen activator; VE-cadherin, vascular endothelial-cadherin; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor tyrosine kinase.

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