Modulation of Receptor Tyrosine Kinase Activity through Alternative Splicing of Ligands and Receptors in the VEGF-A/VEGFR Axis

Megan Stevens * and Sebastian Oltean *

Institute of Biomedical & Clinical Sciences, Medical School, College of Medicine and Health, University of Exeter, Exeter EX1 2LU, UK

*** Correspondence: m.stevens2@exeter.ac.uk (M.S.); s.oltean@exeter.ac.uk (S.O.); Tel.: +44-1392-72-7417 (S.O.)

Abstract: Vascular endothelial growth factor A (VEGF-A) signaling is essential for physiological and pathological angiogenesis. Alternative splicing of the VEGF-A pre-mRNA gives rise to a pro-angiogenic family of isoforms with a differing number of amino acids (VEGF-A$_{xxx}$a), as well as a family of isoforms with anti-angiogenic properties (VEGF-A$_{xxx}$b). The biological functions of VEGF-A proteins are mediated by a family of cognate protein tyrosine kinase receptors, known as the VEGF receptors (VEGFRs). VEGF-A binds to both VEGFR-1, largely suggested to function as a decoy receptor, and VEGFR-2, the predominant signaling receptor. Both VEGFR-1 and VEGFR-2 can also be alternatively spliced to generate soluble isoforms (sVEGFR-1/sVEGFR-2). The disruption of the splicing of just one of these genes can result in changes to the entire VEGF-A/VEGFR signaling axis, such as the increase in VEGF-A$_{165}$a relative to VEGF-A$_{165}$b resulting in increased VEGFR-2 signaling and aberrant angiogenesis in cancer. Research into this signaling axis has recently focused on manipulating the splicing of these genes as a potential therapeutic avenue in disease. Therefore, further research into understanding the mechanisms by which the splicing of VEGF-A/VEGFR-1/VEGFR-2 is regulated will help in the development of drugs aimed at manipulating splicing or inhibiting specific splice isoforms in a therapeutic manner.

Keywords: VEGF; VEGFR; tyrosine kinase; alternative splicing

1. Introduction

Angiogenesis comprises the formation and maintenance of blood vessels. A variety of signaling molecules are involved in the regulation of angiogenesis, including vascular endothelial growth factor (VEGF), which is essential both for physiological and pathological angiogenesis [1]. The biological functions of VEGF proteins are mediated by a family of cognate protein tyrosine kinase receptors, known as the VEGF receptors (VEGFRs) [2]. Activation of the VEGF pathway has been implicated in a large number of disease processes ranging from cancer to autoimmunity.

There are several VEGF proteins; VEGF-A binds to and signals through VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1), VEGF-B signals solely through VEGFR-1, and VEGF-C and VEGF-D have a high affinity to VEGFR-3 (Flt-4) [1,2]. In addition, there are two neuropilin receptors, which are transmembrane glycoproteins, that function in the VEGF-VEGFR axis [2]; neuropilin-1 (NRP-1), a non-kinase co-receptor for VEGFR-2, functions to enhance the binding and signaling of certain isoforms of VEGF-A. NRP-2, on the other hand, is a non-kinase co-receptor for VEGFR-3. Since VEGFR-1 and VEGFR-2 are the receptor tyrosine kinases specific for VEGF-A, this review will focus on the splice variants of these two receptors only.

2. VEGFR Splice Variants and Functions

VEGF-A binds to two tyrosine kinase VEGFRs, VEGFR-1 and VEGFR-2. There are several isoforms of these VEGFRs that arise as a result of alternative splicing of the VEGFR pre-mRNA, which can alter the protein function, as detailed below (Figure 1). Both VEGFR-1 and VEGFR-2 have seven extracellular immunoglobulin (Ig)-like domains, which consist of a tetramer of two light chains and two heavy chains linked by disulphide bonds, a single transmembrane region, and an intracellular tyrosine kinase sequence interrupted by a kinase insert domain [3]. VEGF-A binds to the extracellular domain and the kinase insert domain acts as a binding site for intracellular proteins to carry out specific signaling cascades in response to ligand binding.
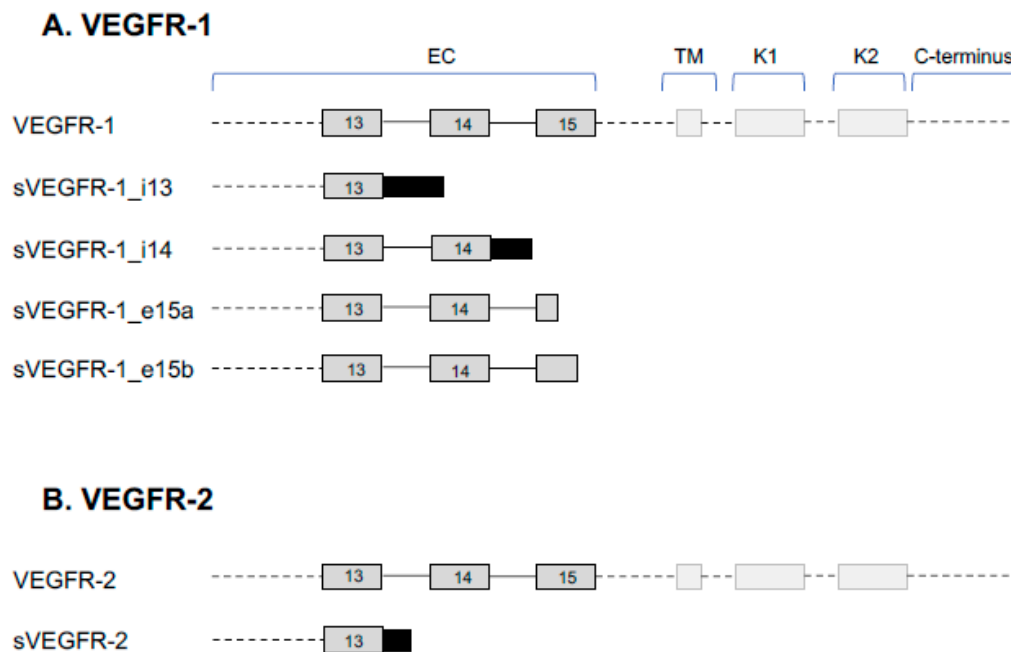

Figure 1. Alternative splice variants of VEGFR-1 and VEGFR-2. (**A**) Alternative splicing gives rise to five known splice variants of VEGFR-1: full length VEGFR-1, intron 13 retention (sVEGFR-1_i13), intron 14 retention (sVEGFR-1_i14), terminal exon 15a (sVEGFR-1_e15a), and terminal exon 15b (sVEGFR-1_e15b). The soluble isoforms only contain the extracellular (EC) domain and are missing the transmembrane (TM) and kinase (K1 and K2) domains. (**B**) Alternative splicing gives rise to two known splice variants of VEGFR-2: full length VEGFR-2 and sVEGFR-2, which result from intron 13 retention. The sVEGFR-2 only contains the EC domain.

2.1. VEGFR-1 Signaling

VEGFR-1 was the first receptor tyrosine kinase for VEGF-A to be identified in COS cells [4] and has since been reported to be widely expressed on many cell types; however, it has very poor tyrosine kinase activity and is not required for endothelial cell function [5]. VEGFR-1 binds VEGF-A with high affinity but there is conflicting evidence for the role of VEGFR-1 as it appears to signal differently depending on the cell type and stage of development [5]. VEGFR-1 gene expression is regulated by hypoxia in human umblical endothelial cells; the VEGFR-1 promoter contains a binding site for hypoxia inducible factor (HIF)-1α [6]. Relatively little is known about the function of VEGFR-1. Constitutive knock-out (KO) of VEGFR-1 results in embryonic lethality between embryonic days 8.5 and 9 [7]. This was later found to be the result of increased endothelial cell outgrowth and angioblast commitment, which prevented proper organization of the vascular network [8]. Previous reports have labelled VEGFR-1 as a decoy receptor, decreasing the amount of VEGF-A readily available to bind to and phosphorylate VEGFR-2 [9]. Further evidence for this is that deletion of just the intracellular

kinase domain for VEGFR-1 resulted in normal vascular development in mice [9]. Therefore, VEGFR-1 is hypothesized to sequester VEGF-A, preventing it from binding to its functional receptor, VEGFR-2.

2.2. Function of sVEGFR-1

The VEGFR-1 pre-mRNA can be alternatively spliced to produce the full-length membrane-spanning receptor described above, or the truncated soluble VEGFR-1 (sVEGFR-1), which includes the seven N-terminal immunoglobulin-like extracellular domains but not the transmembrane spanning or intracellular kinase domains, thus has a specific 31-amino acid c-terminus [1]. Full-length VEGFR-1 mRNA consists of 30 exons, whereas sVEGFR-1 only contains the first 13–14 exons due to intron retention and usage of an alternative polyadenylation signal and stop codon (isoforms detailed below). sVEGFR-1 is suggested to form non-signaling complexes with VEGFR-2, thus functioning as a modulator of VEGF-A signaling [10]. Like full length VEGFR-1, sVEGFR-1 has also been shown to act as a decoy receptor; VEGFR-1 KO mice die from vasuclar overgrowth due to increased signaling of VEGF-A through VEGFR-2; however, the administration of sVEGFR-1 to VEGFR-1 KO mice partially rescues this phenotype as it reduces the levels of VEGFR-2 phosphorylation [11].

There are currently five known VEGFR-1 protein coding isoforms (reviewed in [12]) (Figure 1A). Isoform 1 is denoted by the full-length VEGFR-1. Isoform 2 is termed sVEGFR-1, which comprises the 656 N-terminal residues followed by a specific 30-amino acid C-terminus and appears to have ubiquitous expression throughout most tissues [12]. Isoform 3 is a second soluble form generated by alternative splicing downstream of exon 14, termed sVEGFR-1_i14, which has been predominantly detected in the testes and brain [12]. Isoforms 4 and 5 result from the use of a new terminal exon, termed exon 15a and 15b, which is derived from an intronic sequence. These isoforms have been found to be highly expressed in the placenta [12]. Alternative splicing of VEGFR-1 involves *cis*-regulatory elements in the VEGFR-1 pre-mRNA within intron 13 [13]. Hypoxia is reported to increase the expression of transmembrane VEGFR-1 [6]; however, the effect of hypoxia on sVEGFR-1 expression is not so clear. In endothelial cells, hypoxia was shown to downregulate the expression of sVEGFR-1, which was not directly attributable to HIF-1α [14]. In contrast, exposure of macrophages/monocytes to granulocyte-macrophage colony-stimulating factor (GM-CSF) under hypoxic conditions results in HIF-2α-dependent changes in sVEGFR-1 expression [15]. In cytotrophoblasts, where the sVEGFR-1_i14 isoform is most commonly expressed, hypoxia increases both sVEGFR-1_i14 and sVEGFR-1 mRNA, which is proposed to be through HIF-1α [16]. Furthermore, sVEGFR-1_i14 secretion was shown to increase under hypoxic conditions through activation of the growth arrest and DNA damage-inducible 45a (Gadd45a) factor and p38 phosphorylation [17]. Several drugs and protein factors have been shown to modulate sVEGFR-1 expression, including Jumonji domain-containing protein 6, which interacts with the splice factor U2AF65 resulting in augmented levels of sVEGFR-1 in hypoxic conditions [18]. In addition, hnRNP D and arginine methylation have also been reported to play important roles in the regulation of sVEGFR-1 mRNA alternative polyadenylation [19]. Interestingly, VEGF-A can increase the expression of sVEGFR-1 through VEGFR-2-dependent activation of protein kinase C [20].

2.3. VEGFR-2 Signaling

VEGFR-2 is the main signaling receptor for VEGF-A. It is primarily located on endothelial cells and is essential for endothelial cell biology both during development and during physiological and pathological processes in adults. Like VEGFR-1, all VEGF-A isoforms contain residues that enable them to bind to VEGFR-2 and all bind with the same affinity. However, the affinity of VEGF-A for VEGFR-2 is 10-fold lower than that for VEGFR-1 [21,22]. A constitutive KO of VEGFR-2 results in embryonic lethality on day 8.5–9.5; mice lack mature endothelial and hematopoietic cells [23]. This is similar to the phenotype observed in VEGF-A KO mice [24]. Therefore, unlike VEGFR-1, VEGFR-2 signaling is crucial for vascular development.

Proteolytic hydrolysis of membrane-bound VEGFR-2 results in the generation of soluble VEGFR-2 (sVEGFR-2) [12]. sVEGFR-2 is proposed to function as an inhibitor of angiogenesis by binding to and

sequestering VEGF-A, blocking canonical VEGF-A-VEGFR-2 signaling [25,26]. A further sVEGFR-2 isoform generated by intron 13 retention has been described; as with VEGFR-1, retention of intron 13 yields a truncated transcript whose protein variant lacks the transmembrane and intracellular kinase domain of full length VEGFR-2 [27] (Figure 1B). This splice variant is reported to play a role in lymphangiogenesis by blocking VEGF-C [27]. Little is known regarding the mechanisms controlling this alternative splicing event.

3. VEGF-A Splice Variants

The human VEGF-A pre-mRNA consists of eight exons and seven introns. Alternative splicing of the VEGF-A pre-mRNA gives rise to a family of isoforms with a differing number of amino acids due to the exclusion/inclusion of various exons (e.g., VEGF-A$_{121}$, VEGF-A$_{165}$, VEGF-A$_{189}$, and VEGF-A$_{206}$, collectively known as VEGF-A$_{xxx}$a where xxx denotes the number of amino acids) (Figure 2). Such isoforms are widely known to be pro-angiogenic, pro-permeability factors. In addition, the selection of an alternative 3' splice site, known as the distal splice site, in exon 8 of the VEGF-A pre-mRNA results in a new family of VEGF-A isoforms, termed VEGF-A$_{xxx}$b [28]. The resulting VEGF-A$_{xxx}$b proteins differ in the C-terminal sequence by only six amino acids, resulting in radically different functional properties (Figure 2). In comparison to VEGF-A$_{xxx}$, VEGF-A$_{xxx}$b isoforms are collectively anti-angiogenic and reduce vessel permeability (anti-permeability). Sixteen isoforms of VEGF-A have been identified, including an additional isoform, VEGF-Ax, which arises from translational read-through of the VEGF-A transcript beyond the canonical stop codon (programmed translational read-through) [29].
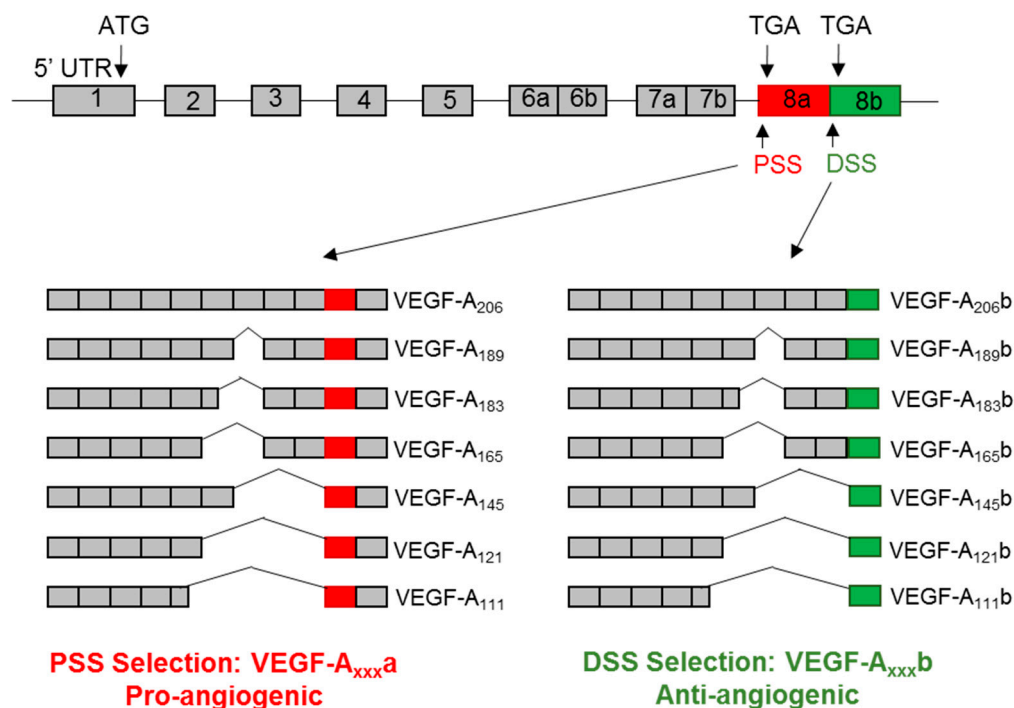

Figure 2. Alternative splicing of VEGF-A. The VEGF-A pre-mRNA is comprised of 8 exons. Inclusion/exclusion of exons 6a,b and 7a,b gives rise to VEGF-A isoforms with differing numbers of amino acids. The use of an alternative 3' splice site in exon 8 results in a differing c-terminal sequence of amino acids (VEGF-A$_{xxx}$b isoforms). The VEGF-A$_{xxx}$a family of isoforms have pro-angiogenic, pro-permeability properties whereas the VEGF-A$_{xxx}$b isoforms are anti-angiogenic and anti-permeability. Figure adapted from Stevens et al. 2018.

VEGF-A splicing is predominantly regulated by a group of RNA binding proteins known as serine/arginine (SR) proteins. SRSF1, SRSF2, SRSF5, and SRSF6 have all been reported to play

a role in VEGF-A alternative splicing [30]. Upon phosphorylation of multiple serine/arginine and proline/serine repeats, SR proteins are translocated from the cytoplasm to the nucleus where they bind to exonic sequence enhancers within the VEGF-A pre-mRNA, resulting in the splicing out of an exon [31]. The inclusion/exclusion of certain exons result in the different isoform properties of each VEGF-A protein. Exons 1–5 are constitutive exons; they encode a single sequence (exons 1/2), a glycosylation site (Asp74), a potential plasmin cleavage site (Arg110 and Ala111), as well as VEGFR binding residues [32,33]. Whereas exons 1–5 are present in all isoforms of VEGF-A, exons 6 and 7 are alternatively spliced. Heparin sulfate (HS) glycoproteins are present in the extracellular matrix (ECM) and can interact with both VEGF-A and VEGFRs, thus they are suggested to regulate the bioavailability of VEGF-A. Residues in exon 6a and 7 of VEGF-A are responsible for the interaction with HS [34]. VEGF-A$_{145}$, VEGF-A$_{189}$, and VEGF-A$_{206}$ all contain exon 6a and 7 resulting in a high affinity for HS; this results in these longer isoforms being tethered to the ECM. On the other hand, VEGF-A$_{111}$ and VEGF-A$_{121}$ lack exon 6 and 7, so they are unable to bind HS, making them freely diffusible in the ECM and more bioavailable [35]. The most dominant isoform is VEGF-A$_{165}$, which contains exon 7 but not 6. Therefore, VEGF-A$_{165}$ has an intermediate bioavailability as approximately 50% remains cell- or ECM-bound [36].

Regarding exon 8 of the VEGF-A gene, selection of either the proximal or distal splice site has been reported to be dependent on the type of external stimulus; proximal splice site selection is promoted by insulin like growth factor (IGF1) and tumor necrosis factor alpha (TNFα), whereas distal splice site selection is promoted by tumor growth factor beta 1 (TGF-B1) [37]. A widely reported example of exon 8 splicing regulation involves serine/threonine-protien kinase 1 (SRPK1) and CDC-like kinase 1 (Clk-1). SRPK1 activation has been shown to phosphorylate SRSF1, resulting in proximal splice site selection and the translation of VEGF-A$_{xxx}$a proteins [38]. On the other hand, Clk-1 signaling results in the phosphorylation of SRSF6, with the distal splice site being subsequently selected and VEGF-A$_{xxx}$b proteins translated [37]. Other reported regulators of VEGF-A exon 8 splicing are E2F1 and SRSF2, which were both shown to increase the VEGF-A$_{xxx}$b/VEGF-A$_{xxx}$a ratio [39].

4. VEGFR Signaling

4.1. Role of VEGFR-1 Signlaing and sVEGFR-1 Isoforms

As mentioned previously, the role of VEGFR-1 in vasculogenesis and angiogenesis has been ascribed to VEGF-A binding, thus regulating the amount of VEGF-A available for vascular development. VEGFR-1 is widely expressed but has poor kinase activity and is not required for endothelial cell function. Further evidence for this hypothesis arose from mice with a homozygous deletion of the VEGFR-1 tyrosine kinase domain developing healthy vasculature [9]. Therefore, the primary role of VEGFR-1 in embryonic angiogenesis is restricted to its extracellular region and is independent of its tyrosine kinase activity. As sVEGFR-1 contains the extracellular domain, it also acts as a decoy receptor [40]. sVEGFR-1 is also proposed to form non-signaling complexes with VEGFR-2 [10].

A study using VEGFR-1 KO embryonic stem cells showed that sVEGFR-1 is important for the modulation of endothelial cell migration and vascular sprouting during development [41]. During vessel morphogenesis, endothelial cells are suggested to form a VEGF-A gradient via the interaction of VEGF-A with sVEGFR-1, resulting in sequestration of VEGF-A and local inactivation of VEGFR-2 signaling [42]. Therefore, sVEGFR-1 is proposed to act as a guidance molecule during vessel sprouting, i.e., inactivating VEGF-A either side of the sprout to provide a VEGF-A-rich corridor for the emerging vessel [43]. sVEGFR-1 present in the ECM is also reported to play a role in α5β1 integrin signaling regarding the cell adhesion pathway [44]; however, these signaling pathways are not related to VEGF-A and are beyond the scope of this review.

Recent studies have highlighted that VEGF-B and PlGF are able to signal through VEGFR-1, eliciting a pro-angiogenic effect independent of VEGF-A [45,46]. In addition, increased levels of

sVEGFR-1 have been observed in vascular pathologies [45], indicating that VEGFR-1 may act as more than a decoy receptor/VEGFR-2 inhibitor.

The role of sVEGFR-1 in tumor development and progression has been widely reported. The expression of sVEGFR-1 has been found to be increased in many types of cancer, including glioblastoma, melanoma, breast, hepatocellular, lung, leukemia, colorectal, renal, and head and neck [47–55]. Increased circulating sVEGFR-1 is often correlated with poor prognosis; however, the balance between VEGF-A and sVEGFR-1 may be more important when considering the clinical outcome. For example, increased sVEGFR-1 and VEGF-A are correlated with poor prognosis in lung cancer patients [51]. On the other hand, increased VEGF-A combined with low levels of sVEGFR-1 are associated with a poor prognosis in breast cancer [56]. In addition to being a marker for tumor progression, sVEGFR-1 has also been shown to serve as a biomarker for tumor response to therapy. Using the example of bevacizumab, increased plasma levels of sVEGFR1 were reported to be inversely correlated with treatment response in breast cancer [57]. However, this appears to be dependent on the type of cancer as the sVEGFR-1 expression level was found to be decreased upon treatment of metastatic colorectal cancer [58].

Excess circulating soluble isoforms of VEGFR-1 have been shown to contribute to the pathogenesis of pre-eclampsia in pregnant women [59,60]. The sVEGFR-1_i14 isoform is presumed to be a major contributor to this condition because it is selectively expressed by placental cytotrophoblasts; the increased sequestration of platelet-derived growth factor (PIGF) and VEGF-A by excess sVEGFR-1_i14 results in endothelial dysfunction and altered neutrophil activation and migration, ultimately causing hypertension, proteinuria, and glomerular endotheliosis in patients [60,61]. Indeed, increased levels of circulating sVEGFR-1_i14 is used as a biomarker for the development of pre-eclampsia [62].

As described above in pregnant women with pre-eclampsia, increased circulating levels of sVEGFR-1 is linked to endothelial dysfunction in the glomeruli of the kidney. VEGF-A is secreted by the glomerular epithelial cells (podocytes) to signal to VEGFR-2 on the glomerular endothelial cells, a process that is tightly regulated to maintain proper functioning of the glomerular filtration barrier. Plasma levels of sVEGFR-1 are higher in patients with chronic kidney disease (CKD), which are correlated with cardiovascular disease [63,64]. On the other hand, inducible over-expression of podocyte sVEGFR-1 has been shown to be therapeutic in a model of diabetic nephropathy where excess VEGF-A expression is observed [65]. In addition, sVEGFR-1 has been reported to bind to lipid microdomains in podocytes, which can alter cell morphology and the function of the glomerular filtration barrier [66].

sVEGFR-1 has also been shown to play a role in ocular pathologies through the inhibition of VEGF-A, including the preservation of cornea avascularity [67]. In addition, reduced levels of sVEGFR-1 where observed in patients with age-related macular degeneration [68]. Regarding inflammation, increased levels of sVEGFR-1 in the blood is indicated to act as a potential new biomarker of sepsis [69], and a predictor of endothelial dysfunction/activation of coagulation in acute pancreatitis [70].

On the other hand, in mouse xenograft models of melanoma, lung cancer, fibrosarcoma, and glioblastoma, exogenous administration of sVEGFR-1 (either transfection, recombinant protein, or adenovirus infection) inhibited tumor growth and neoangiogenesis, increasing the survival rate [71–74].

4.2. VEGF-A$_{xxx}$b Activation of VEGFR-1

Information on VEGFR-1 activation and signaling is sparse; however, a recent study has shown that VEGF-A$_{165}$b inhibits VEGFR-1 signaling in ischemic muscle in mice, and that VEGF-A$_{165}$b inhibition induces activation of VEGFR-1 [75]. Furthermore, in vitro studies showed that VEGF-A$_{165}$b failed to induce the activation of VEGFR-1-Y1333, reducing VEGFR-1-STAT3 signaling [75].

4.3. Mehcanisms of VEGFR-2 Signaling

As mentioned above, all VEGF-A isoforms can bind to VEGFR-2 with similar affinity; however, different isoforms result in different activation and signaling outcomes [32] (Figure 3). Upon binding of VEGF-A to its orthosteric ligand binding site, VEGFR-2 undergoes dimerization and a conformational twist in the extracellular region results in the rotation of transmembrane helices [76,77]. Both VEGF-A$_{165}$ and VEGF-A$_{165}$b have been shown to result in VEGFR-2 dimerization [77]. Conformational changes in the intracellular domain of VEGFR-2 follows; ATP binds to the flexible N-lobe cleft facilitating the intrinsic kinase activity of the receptor and phosphorylation of the tyrosine residues in the C-lobe [78]. Upon phosphorylation of these tyrosine residues, certain cytoplasmic proteins bind and distinct signaling pathways are initiated, included those involved in cell survival, migration, proliferation, vasodilatation, and permeability (reviewed in [79]). The tyrosine residues include Y1054 and Y1059 in the activation loop, which are required for maximal kinase activity of VEGFR-2 [80]; Y951 in the kinase insert domain, which serves as a binding site for T cell-specific adapter molecule (TSAd) [81], and is vital for HUVEC migration in response to VEGF-A [82]; and Y1175 and Y1214 in the COOH-terminal tail. Y1175 phosphorylation mediates cell proliferation through binding of phospholipase C (PLC)-γ [83]. VEGFR-2 is dephosphorylated by protein phosphatase 1b (PTP1b) in the endoplasmic reticulum, which highlights the importance of spatiotemporal trafficking on the activation of VEGFR-2 [84,85].
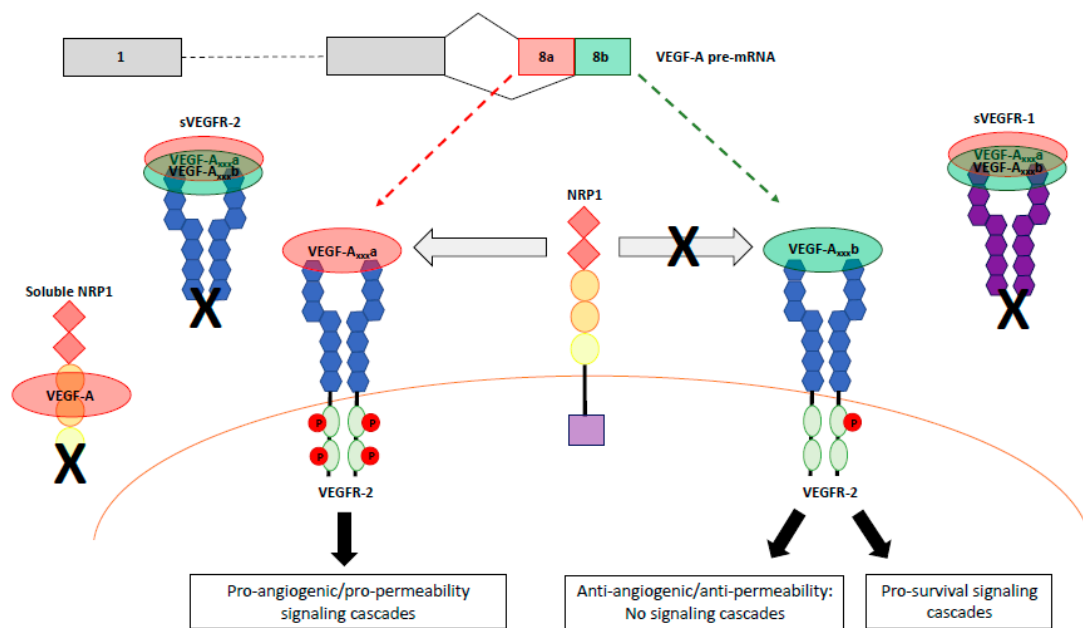

Figure 3. VEGF-A$_{xxx}$a and VEGF-A$_{xxx}$b signaling through VEGFR splice variants and NRP1. Both VEGF-A$_{xxx}$a and VEGF-A$_{xxx}$b can bind and dimerize VEGFR-2. VEGF-A$_{xxx}$a recruits NRP1, a co-receptor for VEGFR-2, which results in phosphorylation of the tyrosine kinase domains of VEGFR-2, producing pro-angiogenic and pro-permeability intracellular signaling cascades. In contrast, VEGF-A$_{xxx}$b is unable to recruit NRP1, resulting in weak, transient phosphorylation of VEGFR-2 and some pro-survival signaling cascades. Soluble isoforms of NRP1, as well as sVEGFR-2 and sVEGFR-1 lack transmembrane domains and act as decoy receptors, sequestering VEGF-A.

4.4. VEGFR-2 Signaling in Angiogenesis

During sprouting angiogenesis, endothelial cells within existing vessels form an angiogenic sprout towards a chemotactic stimulus, such as VEGF-A. The angiogenic sprout is orientated with a leading tip cell and trailing stalk cells. The extent of sprouting in neighboring endothelial cells is regulated by delta-like ligand 4 and Notch via lateral inhibition [86]. Lumen formation occurs once two sprouts anastomose, and the new vessel is stabilized by smooth muscle cell and basement membrane deposition [87].

Cell proliferation is required for angiogenesis. VEGF-A activates VEGFR-2 and stimulates proliferation through the activation of RAS, which then activates RAF kinase to phosphorylate mitogen-activated protein kinases (MAPK/ERK) [88]. VEGFR-2 stimulates ERK activation via Y1175-dependent phosphorylation of PLC-γ, resulting in the subsequent activation of protein kinase C (PKC) [82]. Mutation of Y1175 or administration of and antibody specific to Y1175 decreased VEGF-A-dependent cell proliferation in vitro [89]. Furthermore, mutation of Y1175 in mice results in embryonic lethality on day 5–9 due to a lack of blood vessel formation [90].

Endothelial cell migration is also essential for angiogenesis. One VEGFR-2 signaling pathway that has been implicated in endothelial cell migration is initiated via the phosphorylation of Y951, which allows for the binding of T cell specific adapter protein (TSAd) [81]. Both mutation of Y951 and knock-down of TSAd are reported to inhibit VEGF-A-mediated actin reorganization, and thus migration in cultured endothelial cells; however, proliferation remained unaffected [81]. Another example of a VEGFR-2 signaling pathway involves phosphorylation of Y1175 to induce focal adhesion kinase (FAK)-mediated endothelial cell migration [91].

4.5. VEGFR-2 Signaling in Cell Survival

VEGF-A activation of VEGFR-2 is associated with increased endothelial cell survival. VEGFR-2 activates phosphoinositide 3-kinase (PI3K), which enables membrane recruitment and phosphorylation of protein kinase B (PKB/AKT) [92]. Activation of the cell survival factor AKT results in the phosphorylation of Bcl-2 associated death promoter (BAD), inhibiting the activity of pro-apoptotic factors such as Bcl-2 and caspase 9 [93].

4.6. VEGFR-2 Signaling in Permeability

VEGF-A activation of VEGFR-2 induces extravasation of proteins and leukocytes in vivo [94]. This is suggested to occur through two mechanisms: the formation of transcellular endothelial pores and the transient opening of paracellular junctions [95]. However, the exact signaling mechanisms regulating these events are not yet clear. One suggested mechanism involves VEGF-A-dependent endothelial nitric oxide synthase (eNOS) activation through PLC-γ and AKT, resulting in the activation of the pro-permeability factor nitric oxide (NO) [96,97].

4.7. Role of sVEGFR-2

The alternatively spliced sVEGFR-2 isoform has been reported to act as an endogenous VEGF-C antagonist, preventing it from binding to VEGFR-3 and consequently inhibiting lymphatic endothelial cell proliferation [27]. In addition, like sVEGFR-1, sVEGFR-2 is a natural circulating decoy receptor for VEGF, thus acting as a ligand trap [98].

4.8. VEGF-A Isoform Specific Activation of VEGFR-2

The canonical VEGF-A$_{xxx}$a isoforms are widely described as pro-angiogenic, pro-permeability factors as they activate the aforementioned signaling pathways via VEGFR-2 binding and dimerization. On the other hand, VEGF-A$_{xxx}$b isoforms are anti-angiogenic and anti-permeability, which is due to their effect on VEGFR-2 activation. Like VEGF-A$_{xxx}$a, VEGF-A$_{xxx}$b is still able to bind and dimerize VEGFR-2, but whether they result in phosphorylation of the tyrosine residues in the intracellular domain is not clear. The six-amino acid frame shift that occurs when the distal splice site is selected in the VEGF-A pre-mRNA results in the replacement of a positively charged arginine residue with neutral aspartic acid and lysine, which are predicted to decrease VEGFR-2 activation [99]. In pulmonary arterial endothelial (PAE) cells, VEGF-A$_{165}$b was shown to induce VEGFR-2 activation (Y1052, Y1057) compared to untreated controls, but not to the same extent as that induced by VEGF-A$_{165}$ [99]. Another report suggested that recombinant VEGF-A$_{165}$b can induce Y1175 activation to almost the same extent as VEGF-A$_{165}$ in HEK293-VR2 cells [100]. In addition, VEGF-A$_{165}$b can induce VEGFR-2 Y1175 phosphorylation to the same extent as VEGF-A$_{165}$ in endothelial cells [75]. However,

anti-VEGF-A$_{165}$b treatment of HUVECs and cultured visceral adipose tissue resulted in increased Y951 phosphorylation [101,102], indicating that VEGF-A$_{165}$b antagonized Y951 phosphorylation. Furthermore, treatment of glomerular endothelial cells with VEGF-A$_{165}$b did not result in any increases in the overall phosphorylated state of VEGFR-2 (immunoprecipitation of VEGFR-2 followed by immunoblotting with a phospho-tyrosine antibody) [103]. Taken together, these findings indicate that VEGF-A$_{165}$b acts as a VEGFR-2 partial agonist/antagonist via the differential modulation of site-specific phosphorylation on VEGFR-2.

In some pathologies, VEGF-A$_{165}$b expression has been shown to be down-regulated relative to VEGF-A$_{165}$a. For example, in the late stages of human diabetic nephropathy when the kidney is not filtering properly, kidney VEGF-A$_{165}$b levels are down-regulated relative to VEGF-A$_{165}$a; however, during the early stages of diabetic nephropathy when the kidney is functioning well, the VEGF-A$_{165}$b isoform is increased [104]. Therefore, VEGF-A$_{165}$b may play a protective role in early nephropathy but when the expression is decreased, increased angiogenesis and permeability occur, resulting in a worse phenotype. Indeed, several studies in mouse models have shown the VEGF-A$_{165}$b isoform to have reno-protective effects regarding glomerular permeability [103–106]. These protective effects are indicated to be due to VEGF-A$_{165}$b decreasing the phosphorylation of VEGFR-2, which has been shown in glomerular endothelial cells [103]. Decreased levels of VEGF-A$_{165}$b have also been observed in certain cancers, including colon cancer and renal cell carcinoma [28,107]. This reduction in VEGF-A$_{165}$b is often accompanied by an increase in the pro-angiogenic VEGF-A$_{165}$a, which contributes to angiogenesis within the tumor. Administration of VEGF-A$_{165}$b, or manipulation of VEGF-A splicing to promote VEGF-A$_{165}$b expression (such as with SRPK1 inhibitors), has been shown to be therapeutic in many tumor models through inhibition of VEGF-A$_{xxx}$a mediated angiogenesis [108,109]. On the other hand, VEGF-A$_{165}$b has also been shown to promote lung tumor progression and specific knock-down of just the VEGF-A$_{165}$b isoform reduced tumor growth in lung cancer cells [110]. Thus, the role of VEGF-A$_{165}$b signaling may depend on the tissue it is expressed in.

VEGF-A$_{121}$a is a shorter freely diffusible VEGF-A isoform. In contrast to VEGF-A$_{165}$a, VEGF-A$_{121}$a has been shown to exhibit both partial and full agonist effects. On one hand, VEGF-A$_{121}$a acts as a partial agonist of VEGFR-2 in both in vivo and in vitro measurements of angiogenesis and signaling, respectively [5,99], as well as slowing HUVEC proliferation and reducing sprouting in comparison to VEGF-A$_{165}$a [111,112]. In contrast, VEGF-A$_{121}$a-induced angiogenic sprouting ex vivo has been reported to be both comparable [33] and reduced [113] in comparison to VEGF-A$_{165}$a. Similar trends are seen regarding vascular permeability [114–116].

VEGF-A$_{145}$a and VEGF-A$_{189}$a are ECM-bound isoforms that also show reduced agonistic effects on VEGFR-2 signaling in comparison to VEGF-A$_{165}$a. In HUVECs, VEGF-A$_{145}$a had a reduced effect on proliferation and permeability relative to VEGF-A$_{165}$a, but comparable effects on migration [114]. This was indicated to be due to reduced phosphorylation of VEGFR-2 in addition to reduced activation of AKT and ERK [114]. Similarly, VEGF-A$_{189}$a resulted in decreased cell survival and proliferation in BAECs, but comparable effects to VEGF-A$_{165}$a on migration [117,118].

5. VEGFR Signaling Complexes

5.1. VEGFR Heterodimerization

Computational modeling has predicted VEGFR-1/2 heterodimers to comprise 10–50% of signaling VEGFR complexes, which are favored over VEGFR-1 homodimers when the VEGFR-2 abundance is higher [119]. There is evidence that suggests that VEGF-A stimulation of VEGFR-2 homodimers, VEGFR-1 homodimers, and VEGFR-1/2 heterodimers results in different efficacies of signal transduction; the pattern of Ca2+ flux was found to be unique for each type of receptor dimer in porcine aortic endothelial cells [120]. VEGF-A, VEGF-C, and VEGF-D have also been shown to induce the heterodimerization of VEGFR-2/3, which is required for certain ligand-dependent cellular responses mediated by VEGF-C and VEGF-D [121].

5.2. Roles of Neuropilins NRP1 and NRP2

Neuropilins can function as coreceptors with VEGFR-1 and VEGFR-2. There are two homologs of NRP, NRP1 and NRP2, which consist of a single transmembrane spanning domain with a small cytoplasmic domain lacking intrinsic catalytic function [122]. NRP1 was firstly suggested to bind in exon 7 of VEGF-A, which is present in isoforms such as VEGF-A$_{165}$, forming a ternary complex with VEGFR-2 [112], thus primarily acting as a co-receptor for VEGFR-2. More recent studies have implicated the exon 8a-encoded arginine residue in the binding of VEGF-A to the b1 domain of NRP1 [123]. Binding of VEGF-A to NRP1 enhances VEGF-A signaling in endothelial cells with respect to migration and survival [124–126]. Furthermore, NRP1 is reported to be essential for VEGF-A-induced vessel sprouting and branching in angiogenesis [127]. NRP1 has also been shown to be associated with the adapter Synectin (GIPC), which is associated with the intracellular trafficking of VEGFR-2 [125]. In contrast, NRP2 acts as a co-receptor for VEGFR-3 and is therefore not involved with VEGF-A signal transduction [128]. In mice, both overexpression and disruption of NRP1 results in embryonic lethality on E12.5-13.5 due to vascular abnormalities [129]. Furthermore, siRNA [113] or antibody [112] blocking of NRP1 led to a decrease in VEGF-A$_{165}$a-induced phosphorylation of VEGFR-2 in vitro.

In contrast to VEGF-A$_{165}$, VEGF-A$_{189}$, and VEGF-A$_{145}$, fluorescent real-time ligand binding assays revealed that VEGF-A$_{165}$b and VEGF-Ax are unable to bind to NRP1 as they lack the exon 7-8a-encded residues [130]. This provides further evidence for the lack of VEGFR-2 singling induced by the weak agonist VEGF-A$_{xxx}$b isoforms. There is conflicting data regarding the binding of VEGF-A$_{121}$a to NRP1 as it lacks exon 7, with most studies suggesting that although VEGF-A$_{121}$a can bind NRP1, albeit at a lower affinity, it is unable to bridge the NRP1/VEGFR-2 complex (reviewed in [131]).

5.3. NRP1 and NRP2 Splice Variants

NRP1 exists as a full-length membrane-bound form in addition four soluble isoforms. Full-length NRP1 is comprised of 17 exons. On the other hand, two soluble splice variants, s$_{12}$NRP1 and s$_{11}$NRP1, are generated during pre-mRNA processing via intron read-through in the NRP1 gene, resulting in proteins that lack transmembrane and cytoplasmic domains of full-length NRP1 [132,133]. Functionally, these soluble isoforms of NRP1 were reported to bind VEGF-A$_{165}$, although not VEGF-A$_{121}$, thus inhibiting VEGF-A$_{165}$-induced phosphorylation of VEGFR-2 in endothelial cells resulting in reduced tumor growth (anti-tumor properties) [133]. Therefore, s$_{12}$NRP1 and s$_{11}$NRP1 appear to act as VEGF-A$_{165}$ antagonists. Two further soluble isoforms of NRP1 have also been described, s$_{III}$NRP and s$_{IV}$NRP, which are proposed to have similar biological and biomechanical properties as s$_{12}$NRP1 and s$_{11}$NRP1 [134]. The s$_{III}$NRP1 isoform results from the deletion of exons 10 and 11, while exon 12 is still present, followed by retention of the beginning of intron 12 (28 bp). The s$_{IV}$NRP1 isoform is missing exon 11, also resulting in intron 12 retention [134]. Both s$_{III}$NRP and s$_{IV}$NRP have been shown to be expressed in normal and cancerous tissues and are capable of binding VEGF-A$_{165}$, indicating that these two isoforms are antagonists for NRP1-mediated cellular activities [134]. The final isoform of NRP1 is NRPΔE16, which results from the skipping of exon 16 and replacement with an "AAG" Arg triple; however, this isoform does not have a functional difference to full length NRP1 [135].

NRP2 can also exist as a membrane bound or soluble form. The membrane bound form of NRP2 has two splice variants, NRP2a and NRP2b, which differ in the last 100 amino acids of the c-terminus. Therefore, these two splice variants are proposed to bind different proteins and govern different molecular pathways [132]. NRP2b has been reported to have a prometastatic role in non-small cell lung cancer, whereas NRP2a in promoting metastasis and therapy resistance [136]. However, further studies are needed to clarify the roles of each of these splice variants with respect to VEGF-A binding and signaling.

6. Regulation of Splicing as a Therapeutic Intervention

Research into the VEGF-A-VEGFR signaling axis in disease has recently taken a new direction focused on manipulating the splicing of these genes as a potential therapeutic avenue. One example of this is the regulation of the VEGF-A$_{xxx}$a/VEGF-A$_{xxx}$b ratio. Small molecule inhibitors of SRPK1, known as SRPIN340 and SPHINX31, have been shown to upregulate the VEGF-A$_{xxx}$b isoforms relative to VEGF-A$_{xxx}$a, which had a therapeutic effect in animal models of retinopathy [137,138]. Furthermore, a natural blueberry extract as also been shown to increase VEGF-A$_{165}$b/VEGF-A$_{164}$a in the kidneys of diabetic mice, exerting a therapeutic effect through a decrease in kidney fibrosis and permeability [139]. Regarding the VEGFRs, exogenous administration of sVEGFR-1 (either transfection, recombinant protein, or adenovirus infection) was reported to inhibit tumor growth and neoangiogenesis, increasing the survival rate in mouse xenograft models of melanoma, lung cancer, fibrosarcoma, and glioblastoma [71–74]. Therefore, further research into the regulation of VEGFR splicing is warranted to explore the potential therapeutic benefits of switching VEGFR splicing.

7. Conclusions

The VEGF-A-VEGFR axis is critical in both physiological and pathological angiogenesis and vessel permeability. The disruption of the splicing of just one of the genes involved in the VEGF-A-VEGFR axis (VEGF-A, VEGFR-1, VEGFR-2) can result in changes to the entire signaling axis, such as the increase in VEGF-A$_{165}$a relative to VEGF-A$_{165}$b resulting in increased VEGFR-2 signaling and aberrant angiogenesis in cancer. Further research into understanding the mechanisms by which the splicing of VEGF-A/VEGFR-1/VEGFR-2 is regulated will help in the development of drugs aimed at manipulating splicing or inhibiting specific splice isoforms in a therapeutic manner.

Author Contributions: M.S. wrote the first draft of the manuscript and reviewed intermediate versions; S.O. reviewed intermediate and the final versions.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Shibuya, M. Structure and function of VEGF/VEGF-receptor system involved in angiogenesis. *Cell Struct. Funct.* **2001**, *26*, 25–35. [CrossRef] [PubMed]
2. Ferrara, N.; Gerber, H.P.; LeCouter, J. The biology of VEGF and its receptors. *Nat. Med.* **2003**, *9*, 669–676. [CrossRef]
3. Terman, B.I.; Carrion, M.E.; Kovacs, E.; Rasmussen, B.A.; Eddy, R.L.; Shows, T.B. Identification of a new endothelial cell growth factor receptor tyrosine kinase. *Oncogene* **1991**, *6*, 1677–1683.
4. De Vries, C.; Escobedo, J.A.; Ueno, H.; Houck, K.; Ferrara, N.; Williams, L.T. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* **1992**, *21*, 989–991. [CrossRef]
5. Cebe-Suarez, S.; Zehnder-Fjallman, A.; Ballmer-Hofer, K. The role of VEGF receptors in angiogenesis; complex partnerships. *Cell. Mol. Life Sci.* **2006**, *63*, 601–615. [CrossRef] [PubMed]
6. Gerber, H.P.; Condorelli, F.; Park, J.; Ferrara, N. Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is upregulated by hypoxia. *J. Biol. Chem.* **1997**, *272*, 23659–23667. [CrossRef]
7. Fong, G.H.; Rossant, J.; Gertsenstein, M.; Breitman, M.L. Role of Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* **1995**, *376*, 66–70. [CrossRef]
8. Fong, G.H.; Zhang, L.; Bryce, D.M.; Peng, J. Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. *Development* **1999**, *126*, 3015–3025.
9. Hiratsuka, S.; Minowa, O.; Kuno, J.; Noda, T.; Shibuya, M. Flt-1 lacking the tyrosine domain is sufficient for normal development and angiogenesis in mice. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 9349–9354. [CrossRef] [PubMed]

10. Kendall, R.L.; Wang, G.; Thomas, K.A. Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. *Biochem. Biophys. Res. Commun.* **1996**, *226*, 324–328. [CrossRef] [PubMed]

11. Roberts, D.M.; Kearney, J.B.; Johnson, J.H.; Rosenberg, M.P.; Kumar, R.; Bautch, V.L. The vascular endothelial growth factor (VEGF) receptor Flt-1 (VEGFR-1) modulates Flk-1 (VEGFR-2) signaling during blood vessel formation. *Am. J. Pathol.* **2004**, *164*, 1531–1535. [CrossRef]

12. Abou-Faycal, C.; Hatat, A.S.; Gazzeri, S.; Eymin, B. Splice variants of the RTK family: Their role in tumor progression and response to targeted therapy. *Int. J. Mol. Sci.* **2017**, *18*, 383. [CrossRef] [PubMed]

13. Thomas, C.P.; Raikwar, N.S.; Kelley, E.A.; Liu, K.Z. Alternate processing of Flt1 transcripts is directed by conserved cis-elements within an intronic region of FLT1 that reciprocally regulates splicing and polyadenylation. *Nucleic Acids Res.* **2010**, *38*, 5130–5140. [CrossRef] [PubMed]

14. Ikeda, T.; Sun, L.; Tsuruoka, N.; Ishigaki, Y.; Yoshitomi, Y.; Yoshitake, Y.; Yonekura, H. Hypoxia down-regulates sFlt-1 (sVEGFR-1) expression in human microvascular endothelial cells by a mechanism involving mRNA alternative processing. *Boichem. J.* **2011**, *436*, 399–407. [CrossRef]

15. Eubank, T.D.; Roda, J.M.; Liu, H.; O'Neil, T.; Marsh, C.B. Opposing roles for HIF-1α and HIF-2α in the regulation of angiogenesis by mononuclear phagocytes. *Blood* **2011**, *117*, 323–332. [CrossRef]

16. Thomas, R.; Kim, M.H. A HIF-1alpha-dependent autocrine feedback loop promotes survival of serum-deprived prostate cancer cells. *Prostate* **2009**, *69*, 263–275. [CrossRef] [PubMed]

17. Xiong, Y.; Liebermann, D.A.; Tront, J.S.; Holtzman, E.J.; Huang, Y.; Hoffman, B.; Geifman-Holtzman, O. Gadd45a stress signaling regulates sFlt-1 expression in preeclampsia. *J. Cell. Physiol.* **2009**, *220*, 632–639. [CrossRef]

18. Boeckel, J.N.; Guarani, V.; Koyanangi, M.; Roexe, T.; Lengeling, A.; Schermuly, R.T.; Gellert, P.; Braun, T.; Zeiher, A.; Dimmeler, S. Jumonji domain-containing protein 6 (Jmjd6) is required for angiogenic sprouting and regulates splicing of VEGF-receptor 1. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 3276–3281. [CrossRef]

19. Ikeda, T.; Yoshitomi, Y.; Shimasaki, T.; Yamaya, H.; Kobata, T.; Ishigaki, Y.; Tomosugi, N.; Yoshitake, Y.; Yonekura, H. Regulation of soluble Flt-1 (VEGFR-1) production by hnRNP D and protein arginine methylation. *Mol. Cell. Biochem.* **2016**, *413*, 155–164. [CrossRef]

20. Raikwar, N.S.; Liu, K.Z.; Thomas, C.P. Protein kinase C regulates FLT1 abundance and stimulates its cleavage in vascular endothelial cells with the release of a soluble PIGF/VEGF antagonist. *Exp. Cell Res.* **2013**, *319*, 2578–2587. [CrossRef] [PubMed]

21. Fuh, G.; Li, B.; Crowley, C.; Cunningham, B.; Wells, J.A. Requirements for binding and signaling of the kinase domain receptor for vascular endothelial growth factor. *J. Biol. Chem.* **1998**, *273*, 11197–11204. [CrossRef]

22. Shinkai, A.; Ito, M.; Anazawa, H.; Yamaguchi, S.; Shitara, K.; Shibuya, M. Mapping of the sites involved in ligand association and dissociation at the extracellular domain of the kinase insert domain-containing receptor for vascular endothelial growth factor. *J. Biol. Chem.* **1998**, *273*, 31283–31288. [CrossRef]

23. Shalaby, F.; Rossant, J.; Yamaguchi, T.P.; Gertsenstein, M.; Wu, X.F.; Breitman, M.L.; Schuh, A.C. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* **1995**, *376*, 62–66. [CrossRef] [PubMed]

24. Ferrara, N.; Carver-Moore, K.; Chen, H.; Dowd, M.; Lu, L.; O'Shea, K.S.; Powell-Braxton, L.; Hillan, K.J.; Moore, M.W. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **1996**, *380*, 439–442. [CrossRef] [PubMed]

25. Kou, B.; Li, Y.; Zhang, L.; Zhu, G.; Wang, X.; Li, Y.; Xia, J.; Shi, Y. In vivo inhibition of tumor angiogenesis by a soluble VEGFR-2 fragment. *Exp. Mol. Pathol.* **2004**, *76*, 129–137. [CrossRef] [PubMed]

26. Collet, G.; Lamerant-Fayel, N.; Tertil, M.; El Hafny-Rahbi, B.; Stepniewski, J.; Guichard, A.; Foucault-Collet, A.; Klimkiewicz, K.; Petoud, S.; Matejuk, A.; et al. Hypoxia-regulated overexpression of soluble VEGFR2 controls angiogenesis and inhibits tumor growth. *Mol. Cancer Ther.* **2014**, *13*, 165–178. [CrossRef] [PubMed]

27. Albuquerque, R.J.; Hayashi, T.; Cho, W.G.; Kleinman, M.E.; Dridi, S.; Takeda, A.; Baffi, J.Z.; Yamada, K.; Kaneko, H.; Green, M.G.; et al. Alternatively spliced vascular endothelial growth factor receptor-2 is an essential endogenous inhibitor of lymphatic vessel growth. *Nat. Med.* **2009**, *15*, 1023–1030. [CrossRef] [PubMed]

28. Bates, D.O.; Cui, T.G.; Doughty, J.M.; Winkler, M.; Sugiono, M.; Shields, J.D.; Peat, D.; Gillatt, D.; Harper, S.J. VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. *Cancer Res.* **2002**, *62*, 4123–4131. [PubMed]

29. Eswarappa, S.M.; Potdar, A.A.; Koch, W.J.; Fan, Y.; Vasu, K.; Linder, D.; Willard, B.; Graham, L.M.; DiCorleto, P.E.; Fox, P.L. Programmed translational readthrough generates antiangiogenic VEGF-Ax. *Cell* **2014**, *157*, 1605–1618. [CrossRef] [PubMed]

30. Guyot, M.; Pages, G. VEGF splicing and the role of VEGF splice variants: From physiological-pathological conditions to specific pre-mRNA splicing. *Methods Mol. Biol.* **2015**, *1332*, 3–23. [PubMed]

31. Stevens, M.; Oltean, S. Modulation of VEGF-A alternative splicing as a novel treatment in chronic kidney disease. *Genes* **2018**, *9*, 98. [CrossRef] [PubMed]

32. Woolard, J.; Bevan, H.S.; Harper, S.J.; Bates, D.O. Molecular diversity of VEGF-A as a regulator of its biological activity. *Microcirculation* **2009**, *16*, 572–592. [CrossRef] [PubMed]

33. Holmes, D.I.; Zachary, I.C. Vascular endothelial growth factor regulates stanniocalcin-1 expression via neuropilin-1-dependent regulation of KDR and synergism with fibroblast growth factor-2. *Cell Signal.* **2008**, *20*, 569–579. [CrossRef] [PubMed]

34. Krilleke, D.; DeErkenez, A.; Schubert, W.; Giri, I.; Robinson, G.S.; Ng, Y.S.; Shima, D.T. Molecualr mapping and functional characterization of the VEGF164 heparin-binding domain. *J. Biol. Chem.* **2007**, *282*, 28045–28056. [CrossRef]

35. Lee, T.Y.; Folkman, J.; Javaherian, K. HSPG-binding peptide corresponding to the exon 6a-encoded domain of VEGF inhibits tumor growth by blocking angiogenesis in a murine model. *PLoS ONE* **2010**, *5*, e9945. [CrossRef]

36. Houck, K.; Leung, D.W.; Rowland, A.M.; Winer, J.; Ferrara, N. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *J. Biol. Chem.* **1992**, *267*, 26031–26037.

37. Nowak, D.G.; Woolard, J.; Amin, E.M.; Konopatskaya, O.; Saleem, M.A.; Churchill, A.J.; Ladomery, M.R.; Harper, S.J.; Bates, D.O. Expression of pro- and anti-angiogenic isoforms of VEGF is differentially regulated by splicing and growth factors. *J. Cell Sci.* **2008**, *121*, 3487–3495. [CrossRef]

38. Amin, E.M.; Oltean, S.; Hua, J.; Gammons, M.V.; Hamdollah-Zadeh, M.; Welsh, G.I.; Cheung, M.K.; Ni, L.; Kase, S.; Rennel, E.S.; et al. WT1 mutants reveal SRPK1 to be a downstream angiogenesis target by altering VEGF splicing. *Cancer Cell* **2011**, *20*, 768–780. [CrossRef]

39. Merdzhanova, G.; Gout, S.; Keramidas, M.; Edmond, V.; Coll, J.L.; Brambilla, C.; Brambilla, E.; Gazzeri, S.; Eymin, B. The transcription factor E2F1 and the SR protein SC35 control the ratio of pro-angiogenic verus antiangiogenic isoforms of vascular endothelial growth factor-A to inhibit noevascularization in vivo. *Oncogene* **2010**, *29*, 5392–5403. [CrossRef]

40. Inoue, T.; Kibata, K.; Suzuki, M.; Nakamura, S.; Motoda, R.; Orita, K. Identification of a vascular endothelial growth factor (VEGF) antagonist, sFlt-1, from a human hematopoietic cell line NALM-16. *FEBS Lett.* **2000**, *469*, 14–18. [CrossRef]

41. Kearney, J.B.; Kappas, N.C.; Ellerstrom, C.; DiPaola, F.W.; Bautch, V.L. The VEGF receptor flt-1 (VEGFR-1) is a positive modulator of vascular sprout formation and branching morphogenesis. *Blood* **2004**, *103*, 4527–4535. [CrossRef] [PubMed]

42. Kappas, N.C.; Zeng, G.; Chappell, J.C.; Kearney, J.B.; Hazarika, S.; Kallianos, K.G.; Patterson, C.; Annex, B.H.; Bautch, V.L. The VEGF receptor Flt-1 spatially modulates Flk-1 signaling and blood vessel branching. *J. Cell Biol.* **2008**, *181*, 847–858. [CrossRef]

43. Chappell, J.C.; Taylor, S.M.; Ferrara, N.; Bautch, V.L. Local guidance of emerging vessel sprouts requires soluble Flt-1. *Dev. Cell* **2009**, *17*, 377–386. [CrossRef] [PubMed]

44. Orecchia, A.; Lacal, P.M.; Schietroma, C.; Morea, V.; Zambruno, G.; Failla, C.M. Vascualr endothelial growth factor receptor-1 is deposited in the extracellular matrix by endothelial cells and is a ligand for the alpha 5 beta integrin. *J. Cell Sci.* **2003**, *116*, 3479–3489. [CrossRef] [PubMed]

45. Failla, C.M.; Carbo, M.; Morea, V. Positive and negative regulation of angiogenesis by soluble vascular endothelial growth factor receptor-1. *Int. J. Mol. Sci.* **2018**, *19*, 1306. [CrossRef] [PubMed]

46. Li, X.; Tjwa, M.; Van Hove, I.; Enholm, B.; Neven, E.; Paavonen, K.; Jeltsch, M.; Juan, T.D.; Sievers, R.E.; Chorianopoulos, E.; et al. Reevaluation of the role of VEGF-B suggests a restricted role in the revascularization of the ischemic myocardium. *Arterioscler. Thromb. Vasc. Biol.* **2008**, *28*, 1614–1620. [CrossRef] [PubMed]

47. Yamaguchi, T.; Bando, H.; Mori, T.; Takahashi, K.; Matsumoto, H.; Yasutome, M.; Weich, H.; Toi, M. Overexpression of soluble vascular endothelial growth factor receptor 1 in colorectal cancer: Association with progression and prognosis. *Cancer Sci* **2007**, *98*, 405–410. [CrossRef]

48. Lamszus, K.; Ulbricht, U.; Matschke, J.; Brockmann, M.A.; Fillbrandt, R.; Westphal, M. Levels of soluble vascular endothelial growth factor (VEGF) receptor 1 in astrocytic tumors and its relation to malignancy, vascularity, and VEGF-A. *Clin. Cancer Res.* **2003**, *9*, 1399–1405.

49. Toi, M.; Bando, H.; Ogawa, T.; Muta, M.; Hornig, C.; Weich, H.A. Significance of vascular endothelial growth factor (VEGF)/soluble VEGF receptor-1 relationship in breast cancer. *Int. J. Cancer* **2002**, *98*, 14–18. [CrossRef]

50. Nagaoka, S.; Yoshida, T.; Akiyoshi, J.; Akiba, J.; Hisamoto, T.; Yoshida, Y.; Abe, M.; Koga, H.; Toirimura, T.; Ueno, T.; et al. The ratio of placenta growth factor to soluble vascular endothelial growth factor receptor-1 predicts the prognosis of hepatocellular carcinoma. *Oncol. Rep.* **2010**, *23*, 1647–1654.

51. Ilhan, N.; Ilhan, N.; Deveci, F. Functional significance of vascular endothelial growth factor and its receptor (receptor-1) in various lung cancer types. *Clin. Biochem.* **2004**, *37*, 840–845. [CrossRef]

52. Ruffini, F.; Failla, C.M.; Orecchia, A.; Bani, M.R.; Dorio, A.S.; Fortes, C.; Zambruno, G.; Graziani, G.; Giavazzi, R.; D'Atri, S.; et al. Expression of soluble vascular endothelial growth factor receptor-1 in cutaneous melanoma: Role in tumour progression. *Br. J. Dermatol.* **2011**, *164*, 1061–1070. [CrossRef]

53. Wierzbowska, A.; Robak, T.; Wrzesien-Kus, A.; Krawczynska, A.; Lech-Maranda, E.; Urbanska-Rys, H. Circulating VEGF and its soluble receptors sVEGFR-1 and sVEGFR-2 in patients with acute leukemia. *Eur. Cytokine Netw.* **2003**, *14*, 149–153. [PubMed]

54. Harris, A.L.; Reusch, P.; Barleon, B.; Hang, C.; Dobbs, N.; Marme, D. Soluble Tie2 and Flt1 extracellular domains in serum of patients with renal cancer and response to antiangiogenic therapy. *Clin. Cancer Res.* **2001**, *7*, 1992–1997.

55. Kulapaditharom, B.; Boonkitticharoen, V.; Sritara, C. Plasma vascular endothelial growth factor dysregulation in defining aggressiveness of head and neck squamous cell carcinoma. *J. Oncol.* **2012**, *2012*, 687934. [CrossRef] [PubMed]

56. Bando, H.; Weich, H.A.; Brokelmann, M.; Horiguchi, S.; Funata, N.; Ogawa, T.; Toi, M. Association between intratumoral free and total VEGF, soluble VEGFR-1, VEGFR-2 and prognosis in breast cancer. *Br. J. Cancer* **2005**, *92*, 553–561. [CrossRef] [PubMed]

57. Tolany, S.M.; Boucher, Y.; Duda, D.G.; Martin, J.D.; Seano, G.; Ancukiewicz, M.; Barry, W.T.; Goel, S.; Lahdenrata, J.; Isakoff, S.J.; et al. Role of vascular density and normalization in response to neoadjuvant bevacizumab and chemotherapy in breast cancer patients. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 14325–14330. [CrossRef] [PubMed]

58. Willett, C.G.; Duda, D.G.; di Tomaso, E.; Boucher, Y.; Ancukiewicz, M.; Sahani, D.V.; Lahdenranta, J.; Chung, D.C.; Fischman, A.J.; Lauwers, G.Y.; et al. Efficacy, safety, and biomarkers of neoadjuvant bevacizumab, radiation therapy, and fluorouracil in rectal cancer: A multidisciplinary phase II study. *J. Clin. Oncol.* **2009**, *27*, 3020–3026. [CrossRef]

59. Maynard, S.E.; Min, J.Y.; Merchen, J.; Lim, K.H.; Li, J.; Mondal, S.; Libermann, T.A.; Morgan, J.P.; Selke, F.W.; Stillman, I.E.; et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J. Clin. Investig.* **2003**, *111*, 649–658. [CrossRef]

60. McKeeman, G.C.; Ardill, J.E.; Caldwell, C.M.; Hunter, A.J.; McClure, N. Soluble vascular endothelial growth factor receptor-1 (sFlt-1) is increased throughout gestation in patients who have preeclampsia. *Am. J. Obstet. Gynecol.* **2004**, *191*, 1240–1246. [CrossRef] [PubMed]

61. Krysiak, O.; Bretschneider, A.; Zhong, E.; Webb, J.; Hopp, H.; Verlohren, S.; Fuhr, N.; Lanowska, M.; Nonnenmacher, A.; Vetter, R.; et al. Soluble vascular endothelial growth factor receptor-1 (sFLT-1) mediates downregulation of FLT-1 and prevents activated neutrophils from women with preeclampsia from additional migration by VEGF. *Circ. Res.* **2005**, *97*, 1253–1261. [CrossRef]

62. Palmer, K.R.; Tong, S.; Kaitu'u-Lino, T.J. Plcental-specific sFLT-1: Role in pre-eclamptic pathophysiology and its translational possibilities for clinical prediction and diagnosis. *Mol. Hum. Reprod.* **2017**, *23*, 69–78. [PubMed]

63. Di Marco, G.S.; Kentrup, D.; Reuter, S.; Mayer, A.B.; Golle, L.; Tiemann, K.; Fobker, M.; Engelbertz, C.; Breithardt, G.; Brande, E.; et al. Soluble Flt-1 links microvascular disease with heart failure in CKD. *Basic Res. Cardiol.* **2015**, *110*, 30. [CrossRef] [PubMed]

64. Di Marco, G.S.; Reuter, S.; Hillebrand, U.; Amler, S.; Konig, M.; Larger, E.; Oberleithner, H.; Brand, E.; Pavenstadt, H.; Brand, M. The soluble VEGF receptor sFlt1 contributes to endothelial dysfunction in CKD. *J. Am. Soc. Npehrol.* **2009**, *20*, 2235–2245. [CrossRef]

65. Ku, C.H.; White, K.E.; Dei Cas, A.; Hayward, A.; Webster, Z.; Bilous, R.; Marshall, S.; Viberti, G.; Gnudi, L. Inducible overexpression of sFlt-1 in podocytes ameliorates glomerulopathy in diabetic mice. *Diabetes* **2008**, *57*, 2824–2833. [CrossRef] [PubMed]

66. Jin, J.; Sison, K.; Li, C.; Tian, R.; Wnuk, M.; Sung, H.K.; Jeansson, M.; Zhang, C.; Tucholska, M.; Jones, N.; et al. Soluble FLT1 binds lipid microdomains in podocytes to control cell morphology and glomerular barrier function. *Cell* **2012**, *151*, 384–399. [CrossRef] [PubMed]

67. Ambati, B.K.; Nozaki, M.; Singh, N.; Takeda, A.; Jani, P.D.; Suthar, T.; Albuquerque, R.J.; Richter, E.; Sakurai, E.; Newcomb, M.T.; et al. Corneal avascualrity is due to soluble VEGF receptor-1. *Nature* **2006**, *443*, 993–997. [CrossRef]

68. Uehara, H.; Mamalis, C.; McFadden, M.; Taggart, M.; Stagg, B.; Passi, S.; Earle, P.; Chakravarthy, U.; Hogg, R.E.; Ambati, B.K. The reduction of serum soluble Flt-1 in patients with neovascular age-related macular degeneration. *Am. J. Ophthalmol.* **2015**, *159*, 92–100. [CrossRef] [PubMed]

69. Shapiro, N.I.; Yano, K.; Okada, H.; Fischer, C.; Howell, M.; Spokes, K.C.; Ngo, L.; Angus, D.C.; Aird, W.C. A prospective, observational study of soluble FLT-1 and vascular endothelial growth factor in sepsis. *Shock* **2008**, *29*, 452–457. [CrossRef] [PubMed]

70. Dumnicka, P.; Kusnierz-Cabala, B.; Sporek, M.; Mazur-Laskowska, M.; Gil, K.; Kuzniewski, M.; Ceranowicz, Z.; Warzacha, Z.; Dembinski, A.; Bonior, J.; et al. Serum concentrations of angiopoietin-2 and soluble fms-like tyrosine kinase 1 (sFlt-1) are associated with coagulopathy among patients with acute pancreatitis. *Int. J. Mol. Sci.* **2017**, *18*, 753. [CrossRef]

71. Goldman, C.K.; Kendll, R.L.; Cabrera, G.; Soroceanu, L.; Heike, Y.; Gillespie, G.Y.; Siegal, G.P.; Mao, X.; Bett, A.J.; Huckle, W.R.; et al. Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 8795–8800. [CrossRef] [PubMed]

72. Verrax, J.; Defresne, F.; Lair, F.; Vandermeulen, G.; Rath, G.; Dessy, C.; Preat, V.; Feron, O. Delivery of soluble VEGF receptor 1 (sFlt1) by gene electrotransfer as a new antiangiogenic cancer therapy. *Mol. Pharm.* **2011**, *8*, 701–708. [CrossRef] [PubMed]

73. Takayama, K.; Ueno, H.; Nakanishi, Y.; Sakamoto, T.; Inoue, K.; Shimizu, K.; Oohashi, H.; Hara, N. Suppression of tumor angiogenesis and growth by gene transfer of a soluble form of vascular endothelial growth factor receptor into a remote organ. *Cancer Res.* **2000**, *60*, 2169–2177. [PubMed]

74. Shiose, S.; Sakamoto, T.; Yoshikawa, H.; Hata, Y.; Kawano, Y.; Ishibashi, T.; Inomata, H.; Takayama, K.; Ueno, H. Gene transfer of a soluble receptor of VEGF inhibits the growth of experimental eyelid malignant melanoma. *Investig. Ophthalmol. Vis. Sci* **2000**, *41*, 2395–2403.

75. Ganta, V.C.; Choi, M.; Kutateladze, A.; Annex, B.H. VEGF165b modulates endothelial VEGFR1-STAT3 signaling pathway and angiogenesis in human and experimental peripheral arterial disease. *Circ. Res.* **2017**, *120*, 282–295. [CrossRef] [PubMed]

76. Ruch, C.; Skiniotis, G.; Steinmetz, M.O.; Walz, T.; Ballmer-Hoffer, K. Structure of a VEGF-VEGF receptor complex determined by electron microscopy. *Nat. Struct. Mol. Biol.* **2007**, *14*, 249–250. [CrossRef] [PubMed]

77. Sarabipour, S.; Ballmer-Hofer, K.; Hristova, K. VEGFR-2 conformational switch in response to ligand binding. *Elife* **2016**, *5*, e13876. [CrossRef] [PubMed]

78. Manni, S.; Kisko, K.; Schleier, T.; Missimer, J.; Ballmer-Hofer, K. Functional and structural characterization of the kinase insert domain and the carboxy terminal domain in VEGF receptor 2 activation. *FASEB J.* **2014**, *28*, 4914–4923. [CrossRef] [PubMed]

79. Koch, S.; Tugues, S.; Li, X.; Gualandi, L.; Claesson-Welsh, L. Signal transduction by vascular endothelial growth factor receptors. *Biochem. J.* **2011**, *437*, 169–183. [CrossRef]

80. Dougher, M.; Terman, B.I. Autophosphorylation of KDR in the kinase domain is required for maximal VEGF-stimulated kinase activity and receptor internalization. *Oncogene* **1999**, *18*, 1619–1627. [CrossRef] [PubMed]

81. Matsumoto, T.; Bohman, S.; Dixelius, J.; Berge, T.; Dimberg, A.; Magnusson, P.; Wang, L.; Wikner, C.; Qi, J.H.; Wernstedt, C.; et al. VEGF receptor-2 Y951 signlaing and a role for the adapter molecule TSAd in tumor angiogenesis. *EMBO J.* **2005**, *24*, 2342–2353. [CrossRef] [PubMed]

82. Zeng, H.; Sanyal, S.; Mukhopadhyay, D. Tyrosine residues 951 and 1059 of vascular endothelial growth factor receptor-2 (KDR) are essential for vascular permeability factor/vascular endothelial growth factor-induced endothelium migration and proliferation, respectively. *J. Biol. Chem.* **2001**, *276*, 32714–32719. [CrossRef] [PubMed]

83. Takahashi, T.; Yamaguchi, S.; Chida, K.; Shibuya, M. A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells. *EMBO J.* **2001**, *20*, 2768–2778. [CrossRef]

84. Lanahan, A.A.; Lech, D.; Dubrac, A.; Zhang, Z.W.; Eichmann, A.; Simons, M. PTP1b is a physiologic regulator of vascular endothelial growth factor signaling in endothelial cells. *Circulation* **2014**, *130*, 902–909. [CrossRef] [PubMed]

85. Haj, F.G.; Markova, B.; Klaman, L.D.; Bohmer, F.D.; Neel, B.G. Regulation of receptor tyrosine kinase signaling by protein tyrosine phosphatase-1B. *J. Biol. Chem.* **2003**, *278*, 739–744. [CrossRef] [PubMed]

86. Jackobsson, L.; Bentley, K.; Gerhardt, H. VEGFRs and Notch: A dynamic collaboration in vascular patterning. *Biochem. Soc. Trans.* **2009**, *37*, 1233–1236. [CrossRef] [PubMed]

87. Fantin, A.; Vieira, J.M.; Gestri, G.; Denti, L.; Schwarz, Q.; Prykhozhij, S.; Peri, F.; Wilson, S.W.; Ruhrberg, C. Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip induction. *Blood* **2010**, *116*, 829–840. [CrossRef] [PubMed]

88. Meadows, K.N.; Bryant, P.; Pumiglia, K. Vascular endothelial growth factor induction of the angiogenic phenotype requires Ras activation. *J. Biol. Chem.* **2001**, *276*, 49289–49298. [CrossRef] [PubMed]

89. Takahashi, T.; Ueno, H.; Shibuya, M. VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. *Oncogene* **1999**, *18*, 2221–2230. [CrossRef] [PubMed]

90. 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*, 1076–1081. [CrossRef] [PubMed]

91. Abu-ghazaleh, R.; Kabir, J.; Jia, H.; Lobo, M.; Zachary, I. Src mediates stimulation by vascular endothelial growth factor of the phosphorylation of focal adhesion kinase at tyrosine 861, and migration and anti-apoptosis in endothelial cells. *Biochem. J.* **2001**, *360*, 255–264. [CrossRef] [PubMed]

92. Gerber, H.P.; McMurtrey, A.; Kowalski, J.; Yan, M.; Keyt, B.A.; Dixit, V.; Ferrara, N. Vascualr endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J. Biol. Chem.* **1998**, *273*, 30336–30343. [CrossRef] [PubMed]

93. Cardone, M.H.; Roy, N.; Stennicke, H.R.; Salvesen, G.S.; Frank, T.F.; Stanbridge, E.; Frisch, S.; Reed, J.C. Regulation of cell death protease caspase-9 by phosphorylation. *Science* **1998**, *282*, 1318–1321. [CrossRef] [PubMed]

94. Bates, D.O.; Harper, S.J. Regulation of vascular permeability by vascular endothelial growth factors. *Vasc. Pharmacol.* **2002**, *39*, 225–237. [CrossRef]

95. Garrido-urbani, S.; Bradfield, P.F.; Lee, B.P.; Imhof, B.A. Vascular and epithelial junctions: A barrier for leucocyte migration. *Biochem. Soc. Trans.* **2008**, *36*, 203–211. [CrossRef] [PubMed]

96. Fulton, D.; Gratton, J.P.; McCabe, T.J.; Fontana, J.; Fujio, Y.; Walsh, K.; Franke, T.F.; Papapetropoulos, A.; Sessa, W.C. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* **1999**, *399*, 597–601. [CrossRef] [PubMed]

97. Dimmeler, S.; Fleming, I.; Fisslthaler, B.; Hermann, C.; Busse, R.; Zeiher, A.M. Activation of nitric oxide synthase in endothelial cells in Akt-dependent phosphorylation. *Nature* **1999**, *339*, 601–605. [CrossRef] [PubMed]

98. Ebos, J.M.; Bocci, G.; Man, S.; Thorpe, P.E.; Hicklin, D.J.; Zhou, D.; Jia, X.; Kerbel, R.S. A naturally occurring soluble form of vascular endothelial growth factor receptor 2 detected in mouse and human plasma. *Mol. Cancer Res.* **2003**, *2*, 315–326.

99. Kawamura, H.; Li, X.; Harper, S.J.; Bates, D.O.; Claesson_Welsh, L. Vascualr endothelial growth factor (VEGF)-A165b is a weak in vitro agonist for VEGF receptor-2 due to lack of coreceptor binding and deficient regulation of kinase activity. *Cancer Res.* **2008**, *68*, 4683–4692. [CrossRef]

100. Catena, R.; Larzabal, L.; Larrayoz, M.; Molina, E.; Hermida, J.; Agorreta, J.; Montes, R.; Ruben, P.; Montuenga, L.M.; Calvo, A. VEGF$_{121}$b and VEGF$_{165}$b are weakly angiogenic isoforms of VEGF-A. *Mol. Cancer* **2010**, *9*, 320. [CrossRef]

101. Kikuchi, R.; Nakamura, K.; MacLauchlan, S.; Ngo, D.T.; Shimizu, I.; Fuster, J.J.; Katanasaka, Y.; Yoshida, S.; Qiu, Y.; Yamaguchi, T.P.; et al. An antiangiogenic isoform of VEGF-A contributes to impaired vascularization in peripheral artery disease. *Nat. Med.* **2014**, *20*, 1464–1471. [CrossRef]

102. Ngo, D.T.; Farb, M.G.; Kikuchi, R.; Karki, S.; Tiwari, S.; Bigornia, S.J.; Bates, D.O.; LaValley, M.P.; Hamburg, N.M.; Vita, J.A.; et al. Antiangiogenic actions of vascular endothelial growth factor-A165b, an inhibitory isoform of vascular endothelial growth factor-A, in human obesity. *Circulation* **2014**, *130*, 1072–1080. [CrossRef] [PubMed]

103. Stevens, M.; Neal, C.R.; Salmon, A.H.J.; Bayes, D.O.; Harper, S.J.; Oltean, S.O. VEGF-A$_{165}$b protects against proteinuria in a mouse model with progressive depletion of all endogenous VEGF-A splice variants from the kidney. *J. Physiol.* **2017**, *595*, 6281–6298. [CrossRef] [PubMed]

104. Oltean, O.; Qiu, Y.; Ferguson, J.K.; Stevens, M.; Neal, C.; Russell, A.; Kaura, A.; Arkill, K.P.; Harris, K.; Symonds, C.; et al. Vascular endothelial growth factor-A165b is protective and restores endothelial glycocalyx in diabetic nephropathy. *J. Am. Soc. Nephrol.* **2015**, *26*, 1889–1904. [CrossRef] [PubMed]

105. Stevens, M.; Neal, C.R.; Salmon, A.H.J.; Bates, D.O.; Harper, S.J.; Oltean, O. Vascular endothelial growth factor-A165b restores normal glomerular water permeability in a diphtheria-toxin mouse model of glomerular injury. *Nephron* **2018**, *139*, 51–62. [CrossRef] [PubMed]

106. Oltean, O.; Neal, C.R.; Mavrou, A.; Patel, P.; Ahad, T.; Alsop, C.; Lee, T.; Sison, K.; Qiu, Y.; Harper, S.J.; et al. VEGF165b overexpression restores normal glomerular water permeability in VEGF164-overexpressing adult mice. *Am. J. Physiol. Renal. Physiol.* **2012**, *303*, F1026–F1036. [CrossRef] [PubMed]

107. Varey, A.H.; Rennel, E.S.; Qiu, Y.; Bevan, H.S.; Perrin, R.M.; Raffy, S.; Dixon, A.R.; Paraskeva, C.; Zaccheo, O.; Hassan, A.B.; et al. VEGF 165 b, an antiangiogenic VEGF-A isoform, binds and inhibits bevacizumab treatment in experimental colorectal carcinoma: Balance of pro- and antiangiogenic VEGF-A isoforms has implications for therapy. *Br. J. Cancer* **2008**, *98*, 1366–1379. [CrossRef] [PubMed]

108. Mavrou, A.; Brakspear, K.; Hamdollah-Zadeh, M.; Damodaran, G.; Babaei-Jadidi, R.; Oxley, J.; Gillatt, D.A.; Ladomery, M.R.; Harper, S.J.; Bates, D.O.; et al. Serine-arginine protein kinase 1 (SRPK1) inhibition as a potential novel targeted therapeutic strategy in prostate cancer. *Oncogene* **2015**, *34*, 4311–4319. [CrossRef]

109. Rennel, E.S.; Hamdollah-Zadeh, M.A.; Wheatley, E.R.; Magnussen, A.; Schuler, Y.; Kelly, S.P.; Finucane, C.; Ellison, D.; Cebe-Suarez, S.; Ballmer-Hofer, K.; et al. Recombinant human VEGF165b protein is an effective anti-cancer agent in mice. *Eur. J. Cancer* **2008**, *44*, 1883–1894. [CrossRef] [PubMed]

110. Boudria, A.; Abou Faycal, C.; Jia, T.; Gout, S.; Keramidas, M.; Didier, C.; Lemaitre, N.; Manet, S.; Coll, J.L.; Toffart, A.C.; et al. VEGF$_{165}$b. a splice variant of VEGF-A, promotes lung tumor progression and escape from anti-angiogenic therapies through a B1 integrin/VEGFR autocrine loop. *Oncogene* **2018**. [CrossRef]

111. Keyt, B.A.; Berleau, L.T.; Nguyen, H.V.; Chen, H.; Heinsohn, H.; Vandlen, R.; Ferrara, N. The carboxyl-terminal domain (111-165) of vascular endothelial growth factor is critical for its mitogenic potency. *J. Biol. Chem.* **1996**, *271*, 7788–7795. [CrossRef] [PubMed]

112. Pan, Q.; Chathery, Y.; Wu, Y.; Rathore, N.; Tong, R.K.; Peale, F.; Bagri, A.; Tessier-Lavigne, M.; Koch, A.W.; Watts, R.J. Neuropilin-1 binds to VEGF121 and regulates endothelial cell migration and sprouting. *J. Biol. Chem.* **2007**, *282*, 24049–24056. [CrossRef] [PubMed]

113. Fearnley, G.W.; Bruns, A.F.; Wheatcroft, S.B.; Ponnambalam, S. VEGF-A isoform-specific regulation of calcium ion flux, transcriptional activation and endothelial cell migration. *Biol. Open* **2015**, *4*, 731–742. [CrossRef] [PubMed]

114. Fearnley, G.W.; Smith, G.A.; Abdul-Zani, I.; Yuldasheva, N.; Mughal, N.A.; Homer-Vanniasinkam, S.; Kearney, M.T.; Zachary, I.C.; Tomlinson, D.C.; Harrison, M.A.; et al. VEGF-A isoforms program differential VEGFR2 signal transduction, trafficking and proteolysis. *Biol. Open* **2016**, *5*, 571–583. [CrossRef] [PubMed]

115. Xu, D.; Fuster, M.M.; Lawrence, R.; Esko, J.D. Heparin sulfate regulates VEGF165- and VEGF121-mediated vascular hyperpermeability. *J. Biol. Chem.* **2011**, *286*, 737–745. [CrossRef] [PubMed]

116. Becker, P.M.; Waltenberger, J.; Yachechko, R.; Mirzapoiazova, T.; Sham, J.S.; Lee, C.G.; Elias, J.A.; Verin, A.D. Neuropilin-1 regulates vascular endothelial growth factor-mediated endothelial permeability. *Circ. Res.* **2005**, *96*, 1257–1265. [CrossRef] [PubMed]

117. Herve, M.A.; Buteau-Lozano, H.; Mourah, S.; Calvo, F.; Perrot-Applanat, M. VEGF189 stimulates endothelial cells proliferation and migration in vu=itro and up-regulates the expression of Flk-1/KDR mRNA. *Exp. Cell Res.* **2005**, *309*, 24–31. [CrossRef] [PubMed]

118. Yamamoto, H.; Rundqvist, H.; Branco, C.; Johnson, R.S. Autocrine VEGF isoforms differentially regulate endothelial cell behavior. *Front. Cell Dev. Biol.* **2016**, *4*, 99. [CrossRef]

119. Mac Gabhann, F.; Popel, A.S. Dimerization of VEGF receptors and implications for signal transduction: A computational study. *Biophys. Chem.* **2007**, *128*, 125–139. [CrossRef] [PubMed]

120. Huang, K.; Andersson, C.; Roomans, G.M.; Ito, N.; Claesson-Welsh, L. Signaling properties of VEGF receptor-1 and -2 homo- and heterodimers. *Int. J. Biochem. Cell Biol.* **2001**, *33*, 315–324. [CrossRef]

121. Alam, A.; Herault, J.P.; Barron, P.; Favier, B.; Fons, P.; Delesque-Touchard, N.; Senegas, I.; Laboudie, P.; Bonnin, J.; Cassan, C.; et al. Heterodimerization with vascular endothelial growth factor receptor-2 (VEGFR-2) is necessary for VEGFR-3 activity. *Biochem. Biophys. Res. Commun.* **2004**, *324*, 909–915. [CrossRef] [PubMed]

122. Fujisawa, H.; Kitsukawa, T.; Kawakami, A.; Takagi, S.; Shimizu, M.; Hirata, T. Roles of a neuronal cell-surface molecule, neuropilin, in nerve fiber fasciculation and guidance. *Cell Tissue Res.* **1997**, *290*, 465–470. [CrossRef] [PubMed]

123. Vander Kooi, C.W.; Jusino, M.A.; Perman, B.; Neau, D.B.; Bellamy, H.D.; Leahy, D.J. Structural basis for ligand and heparin binding to neuropilin B domains. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 6152–6157. [CrossRef]

124. Soker, S.; Takashima, S.; Miao, H.Q.; 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*, 735–745. [CrossRef]

125. Wang, L.; Zeng, H.; Wang, P.; Soker, S.; Mukhopadhyay, D. Neuropilin-1-mediated vascular permeability factor/vascular endothelial growth factor-dependent endothelial cell migration. *J. Biol. Chem.* **2003**, *278*, 48848–48860. [CrossRef] [PubMed]

126. Favier, B.; Alam, A.; Barron, P.; Bonnin, J.; Laboudie, P.; Fons, P.; Mandron, M.; Herault, J.P.; Neufeld, G.; Savi, P.; et al. Neuropilin-2 interacts with VEGFR-2 and VEGFR-3 and promotes human endothelial cell survival and migration. *Blood* **2006**, *108*, 1243–1250. [CrossRef] [PubMed]

127. Kawamura, H.; Li, X.; Goishi, K.; van Meeteren, L.A.; Jakobsson, L.; Cebe-Suarez, S.; Shimizu, A.; Edholm, D.; Ballmer-Hofer, K.; Kjellen, L.; et al. Neuropilin-1 in regulation of VEGF-induced activation of p38MAPK and endothelial cell organization. *Blood* **2008**, *112*, 3638–3649. [CrossRef] [PubMed]

128. Gluzman-Poltorak, Z.; Cohen, T.; Shibuya, M.; Neufeld, G. Vascualr endothelial growth factor receptor-1 and neuropilin-2 form complexes. *J. Biol. Chem.* **2001**, *276*, 18688–18694. [CrossRef]

129. Fujisawa, H.; Kitsukawa, T. Receptors for collapsing/semaphorins. *Curr. Opin. Neurobiol.* **1998**, *8*, 587–592. [CrossRef]

130. Peach, C.J.; Mignone, V.W.; Arruda, M.A.; Alcobia, D.C.; Hill, S.J.; Kilpatrick, L.E.; Wollard, J. Molecular pharmacology of VEGF-A isoforms: Binding and signaling at VEGFR2. *Int. J. Mol. Sci.* **2018**, *19*, 1264. [CrossRef]

131. Sarabipour, S.; Mac Gabhann, F. VEGF-A121a binding to neuropilins- A concept revisited. *Cell Adhes. Migr.* **2018**, *12*, 204–214. [CrossRef] [PubMed]

132. Rossignol, M.; Gagnon, M.L.; Klagsburn, M. Genomic organization of human nueopilin-1 and neuropilin-2 genes: Identification and distribution of splice variants and soluble forms. *Genomics* **2000**, *70*, 211–222. [CrossRef] [PubMed]

133. Gagnon, M.L.; Bienlenberg, D.R.; Gechtman, Z.; Miao, H.Q.; Takashima, S.; Soker, S.; Klagsbrun, M. Identification of a natural soluble neuopilin-1 that binds vascular endothelial growth factor: In vivo expression and antitumor activity. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 2573–2578. [CrossRef] [PubMed]

134. Cackowski, F.C.; Xu, L.; Hu, B.; Cheng, S.Y. Identification of two novel alternatively spliced Neuropilin-1 isoforms. *Genomics* **2010**, *84*, 82–94. [CrossRef]

135. Tao, Q.; Spring, S.C.; Terman, B.I. Characterization of a new alternatively spliced neuropilin-1 isoform. *Angiogenesis* **2003**, *6*, 39–45. [CrossRef] [PubMed]

136. Germmill, R.M.; Nasarra, P.; Nair-Menon, J.; Cappuzzo, F.; Landi, L.; D'Incecco, A.; Uramoto, H.; Yoshida, T.; Haura, E.B.; Armeson, K.; et al. The neuropilin 2 isoform NRP2b uniquely supports TGFbeta-mediated progression in lung cancer. *Sci. Signal.* **2017**, *10*, eaag0528. [CrossRef] [PubMed]

137. Gammons, M.V.; Dick, A.D.; Harper, S.J.; Bates, D.O. SRPK1 inhibition modulates VEGF splicing to reduce pathological neovascularization in a rat model of retinipathy of prematurity. *Investig. Ophthalmol. Vis. Sci.* **2013**, *54*, 5797–5806. [CrossRef] [PubMed]

138. Batson, J.; Toop, H.D.; Redondo, C.; Babaei-Jadidi, R.; Chaikuad, A.; Wearmouth, S.F.; Gibbons, B.; Allen, C.; Tallant, C.; Zhang, J.; et al. Development of potent, selective SRPK1 inhibitors as potential topical therapeutics for neovascular eye disease. *ACS Chem. Biol.* **2017**, *12*, 825–832. [CrossRef]

139. Stevens, M.; Neal, C.R.; Craciun, E.C.; Dronca, M.; Harper, S.J.; Oltean, S. The natural drug DIAVIT is protective in a type II mouse model of diabetic nephropathy. *PLoS ONE* **2019**, *14*, e0212910. [CrossRef] [PubMed]