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Review

Modulation of receptor tyrosine kinase activity through alternative splicing of ligands and receptors in the VEGF-A/VEGFR axis

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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

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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.

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.

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.

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 singlaing 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 predmoniantly detecte din 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].

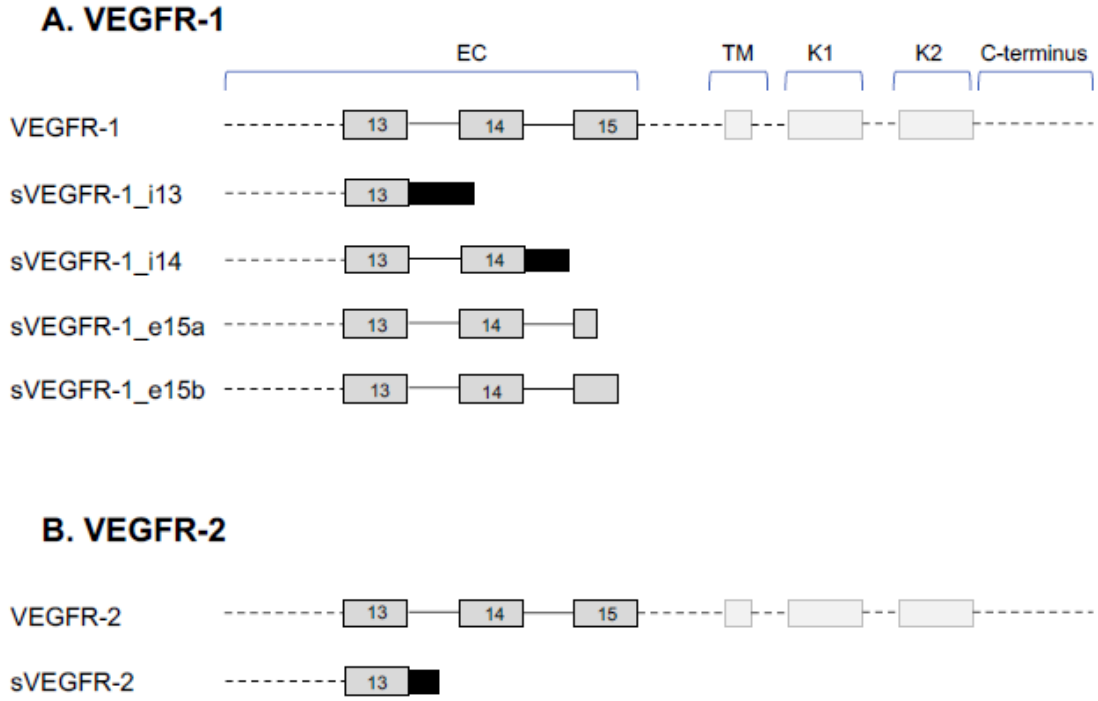


Figure 1. Alternative splice variants of VEGFR-1 and VEGFR-2. A) Alternative splicing gives rise to five known splice variants of VEGFR-1; full length VEGFR-1, intron 13 retention (sVEGFR-1_i13), intron 14 retention (sVEGFR-1_i14), terminal exon 15a (sVEGFR-1_e15a), and terminal exon 15b (sVEGFR-1_e15b). 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 results from intron 13 retention. The sVEGFR-2 only contains the EC domain.

VEGFR-2 signaling

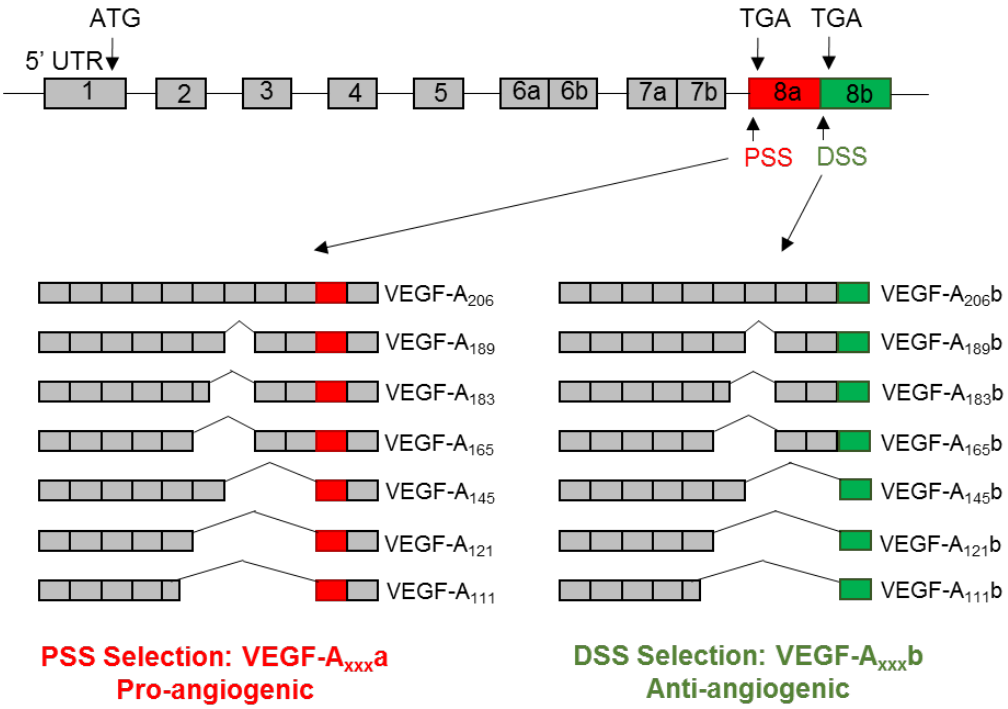
VEGFR-2 is the main signaling receptor for VEGF-A. It is primarily located on endothelial cells and is essential for endothelial cell biology both during development and during physiological and pathological processes in adults. Like VEGFR-1, all VEGF-A isoforms contain residues that enable them to bind 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.

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

142 isoforms of VEGF-A have been identified, including an additional isoform, VEGF-Ax, which arises
143 from trasnaltional readthrough of the VEGF-A transcript beyond the canonical stop codon
144 (programmed translational read-through) [29].



145

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

152 VEGF-A splicing is predominantly regulated by a group of RNA binding proteins known as
153 serine/arginine (SR) proteins. SRSF1, SRSF2, SRSF5, and SRSF6 have all been reported to play a
154 role in VEGF-A alternative splicing [30]. Upon phosphorylation of multiple serine/arginine and
155 proline/serine repeats, SR proteins are translocated from the cytoplasm to the nucleus where they bind
156 to exonic sequence enhancers within the VEGF-A pre-mRNA, resulting in the splicing out of an exon
157 [31]. The inclusion/exclusion of certain exons result in the different isoform properties of each VEGF-
158 A protein. Exons 1-5 are constitutive exons; they encode a single sequence (exons 1/2), a
159 glycosylation site (Asp74), a potential plasmin cleavage site (Arg110 and Ala111), as well as VEGFR
160 binding residues [32,33]. Whereas exons 1-5 are present in all isoforms of VEGF-A, exons 6 and 7
161 are alternatively spliced. Heparin sulfate (HS) glycoproteins are present in the extracellular matrix
162 (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].

VEGFR Signaling

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 form non-signaling complexes with VEGFR-2 [10].

192 A study using VEGFR-1 KO embryonic stem cells showed that sVEGFR-1 is important for the
193 modulation of endothelial cell migration and vascular sprouting during development [41]. During
194 vessel morphogenesis, endothelial cells are suggested to form a VEGF-A gradient via the interaction
195 of VEGF-A with sVEGFR-1, resulting in sequestration of VEGF-A and local inactivation of VEGFR-
196 2 signaling [42]. Therefore, sVEGFR-1 is proposed to act as a guidance molecule during vessel
197 sprouting, i.e. inactivating VEGF-A either side of the sprout to provide a VEGF-A-rich corridor for
198 the emerging vessel [43]. sVEGFR-1 present in the ECM is also reported to play a role in α5β1
199 integrin signaling regarding the cell adhesion pathway [44]; however, these signaling pathways are
200 not related to VEGF-A and are beyond the scope of this review.
201 Recent studies have highlighted that VEGF-B and PIGF are able to signal through VEGFR-1,
202 eliciting a pro-angiogenic effect independent of VEGF-A [45,46]. In addition, increased levels of
203 sVEGFR-1 have been observed in vascular pathologies [45], indicating that VEGFR-1 may act as
204 more than a decoy receptor/VEGFR-2 inhibitor.
205 The role of sVEGFR-1 in tumor development and progression has been widely reported. The
206 expression of sVEGFR-1 has been found to be increased in many types of cancer, including
207 glioblastoma, melanoma, breast, hepatocellular, lung, leukemia, colorectal, renal, and head and neck
208 [47-55]. Increased circulating sVEGFR-1 is often correlated with poor prognosis; however, the
209 balance between VEGF-A and sVEGFR-1 may be more important when considering the clinical
210 outcome. For example, increased sVEGFR-1 and VEGF-A are correlated with poor prognosis in lung
211 cancer patients [51]. On the other hand, increased VEGF-A combined with low levels of sVEGFR-1
212 are associated with a poor prognosis in breast cancer [56]. In addition to being a marker for tumor
213 progression, sVEGFR-1 has also been shown to serve as a biomarker for tumor response to therapy.
214 Using the example of bevacizumab, increased plasma levels of sVEGFR1 was reported to be inversely
215 correlated with treatment response in breast cancer [57]. However, this appears to be dependent on
216 the type of cancer as the sVEGFR-1 expression level was found to be decreased upon treatment of
217 metastatic colorectal cancer [58].
218 Excess circulating soluble isoforms of VEGFR-1 have been shown to contribute to the
219 pathogenesis of pre-eclampsia in pregnant women [59,60]. The sVEGFR-1_i14 isoform is presumed
220 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].

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

250 inhibition induces activation of VEGFR-1 [75]. Furthermore, *in vitro* studies showed that VEGF-
251 A_{165}b failed to induce the activation of VEGFR-1-Y1333, reducing VEGFR-1-STAT3 signaling [75].
252
253 *Mehcanisms of VEGFR-2 signaling*
254 As mentioned above, all VEGF-A isoforms can bind to VEGFR-2 with similar affinity; however,
255 different isoforms result in different activation and signaling outcomes [32] (Figure 3). Upon binding
256 of VEGF-A to its orthosteric ligand binding site, VEGFR-2 undergoes dimerization and a
257 conformational twist in the extracellular region results in the rotation of transmembrane helices
258 [76,77]. Both VEGF-A_{165} and VEGF-A_{165}b have been shown to result in VEGFR-2 dimerization [77].
259 Conformational changes in the intracellular domain of VEGFR-2 follows; ATP binds to the flexible
260 N-lobe cleft facilitating the intrinsic kinase activity of the receptor and phosphorylation of the tyrosine
261 residues in the C-lobe [78]. Upon phosphorylation of these tyrosine residues, certain cytoplasmic
262 proteins bind and distinct signaling pathways are initiated, included those involved in cell survival,
263 migration, proliferation, vasodilatation, and permeability (reviewed in [79]). The tyrosine residues
264 include Y1054 and Y1059 in the activation loop, which are required for maximal kinase activity of
265 VEGFR-2 [80]; Y951 in the kinase insert domain, which serves as a binding site for T cell-specific
266 adapter molecule (TSAd) [81], and is vital for HUVEC migration in response to VEGF-A [82]; and
267 Y1175 and Y1214 in the COOH-terminal tail. Y1175 phosphorylation mediates cell proliferation
268 through binding of phospholipase C (PLC)-γ [83]. VEGFR-2 is dephosphorylated by protein
269 phosphatase 1b (PTP1b) in the endoplasmic reticulum, which highlights the importance of
270 spatiotemporal trafficking on the activation of VEGFR-2 [84,85].

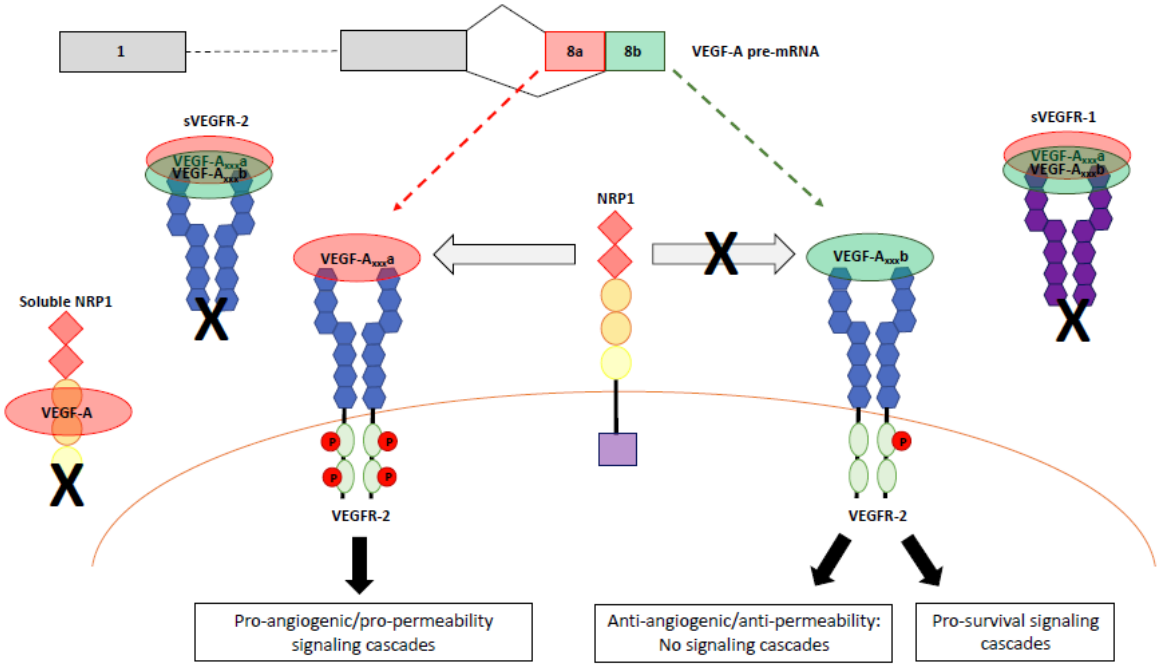


Figure 3. VEGF-A$_{xxx}$a and VEGF-A$_{xxx}$b signaling through VEGFR splice variants and NRP1. Both VEGF-A$_{xxx}$a and VEGF-A$_{xxx}$b can bind and dimerize VEGFR-2. VEGF-A$_{xxx}$a recruits NRP1, a co-receptor for VEGFR-2, which results in phosphorylation of the tyrosine kinase domains of VEGFR-2, 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.

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].

293 Endothelial cell migration is also essential for angiogenesis. One VEGFR-2 signaling pathway
294 that has been implicated in endothelial cell migration is initiated via the phosphorylation of Y951,
295 which allows for the binding of T cell specific adapter protein (TSAd) [81]. Both mutation of Y951
296 and knock-down of TSAd are reported to inhibit VEGF-A-mediated actin reorganization, thus
297 migration in cultured endothelial cells; however, proliferation remained unaffected [81]. Another
298 example of a VEGFR-2 signaling pathway involves phosphorylation of Y1175 to induce focal
299 adhesion kinase (FAK)-mediated endothelial cell migration [91].
300
301 *VEGFR-2 signaling in cell survival*
302 VEGF-A activation of VEGFR-2 is associated with increased endothelial cell survival. VEGFR-2
303 activates phosphoinositide 3-kinase (PI3K), which enables membrane recruitment and
304 phosphorylation of protein kinase B (PKB/AKT) [92]. Activation of the cell survival factor AKT
305 results in the phosphorylation of Bcl-2 associated death promoter (BAD), inhibiting the activity of
306 pro-apoptotic factors such as Bcl-2 and caspase 9 [93].
307
308 *VEGFR-2 signaling in permeability*
309 VEGF-A activation of VEGFR-2 induces extravasation of proteins and leukocytes *in vivo* [94]. This
310 is suggested to occur through two mechanisms: the formation of transcellular endothelial pores and
311 the transient opening of paracellular junctions [95]. However, the exact signaling mechanisms
312 regulating these events are not yet clear. One suggested mechanism involves VEGF-A-dependent
313 endothelial nitric oxide synthase (eNOS) activation through PLC-γ and AKT, resulting in the
314 activation of the pro-permeability factor nitric oxide (NO) [96,97].
315
316 *Role of sVEGFR-2*
317 The alternatively spliced sVEGFR-2 isoform has been reported to act as an endogenous VEGF-C
318 antagonist, preventing it from binding to VEGFR-3 and consequently inhibiting lymphatic endothelial
319 cell proliferation [27]. In addition, like sVEGFR-1, sVEGFR-2 is a natural circulating decoy receptor
320 for VEGF, thus acting as a ligand trap [98].
321

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 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].

VEGFR Signaling Complexes

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 suggest 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].

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]).

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 [136]. NRP2b has been reported to have a prometastatic role in non-small cell lung cancer, whereas NRP2a in promoting metastasis and therapy resistance [137]. However, further studies are needed to clarify the roles of each of these splice variants with respect to VEGF-A binding and signaling.

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 [138,139]. Furthermore, a natural blueberry extract as also been shown to increase VEGF-A_{165}b/VEGF-A_{164}a in the kidney of diabetic mice, exerting a therapeutic effect through a decrease in kidney fibrosis and permeability [140]. 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.

Conclusion

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.

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