

# Non-redundant functions of the protein isoforms arising from alternative splicing of the VEGF-A pre-mRNA

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**T**he different protein isoforms generated from the vascular endothelial growth factor-A (VEGF-A) gene, an essential regulator of blood vessel formation, differ in biochemical property and functional activity. Despite the relevance of VEGF-A for both normal and pathologic angiogenesis, our understanding of the molecular mechanisms governing alternative splicing of its pre-mRNA is still in its infancy.

Members of the vascular endothelial growth factor (VEGF) family of secreted proteins play an essential role both during embryonic development and in the adult life. In mammals, the family consists of five members, each one encoded by a different gene (VEGF-A, -B, -C, -D and the placental growth factor PlGF). The proteins encoded by these genes act as homo- or hetero-dimers through their interaction with three structurally related tyrosine kinase receptors (VEGFR-1/Flt1, VEGFR-2/Flk-1/KDR and VEGFR-3/Flt-2) expressed on the surface of endothelial cells as well on that of several other cell types. In particular, VEGF-B and PlGF specifically interact with VEGFR-1, VEGF-C and VEGF-D with VEGFR-3, while VEGF-A interacts with both VEGFR-1 and VEGFR-2. In addition, the various VEGF family members also exert their varied functions by interacting with the co-receptors Neuropilin-1 and -2 (Nrp-1 and -2), and by the modulation of VEGFR activity through their interaction with other cell surface molecules such as integrins and heparan sulfate proteoglycans (HSPGs), (Fig. 1; reviewed in refs. 1 and 2).

A crucial angiogenic factor is VEGF-A, which is pivotal in driving vascular development in the embryo as well as regulating formation of new blood vessel and vessel homeostasis in the adult. In addition, this factor is the major angiogenic agent driving pathological blood vessel formation during tumorigenesis and vascular retinopathies, thus becoming an appealing target of various antiangiogenic therapies.

The need for timely and precisely regulated VEGF-A gene expression is highlighted by the remarkable observation that deletion of a single VEGF-A gene allele<sup>3,4</sup> or modest gene overexpression<sup>5</sup> both result in embryonic lethality. Not surprisingly, therefore, regulation of VEGF-A production occurs at multiple levels, including transcription, splicing, mRNA stability, translation and subcellular localization of the various factor isoforms.

## Protein Isoforms Generated by Alternative Splicing of the VEGF-A pre-mRNA

A schematic representation of the VEGF-A gene is shown in **Figure 2A**. The gene consists of eight exons and encompasses approximately 14 kilobases. Both the exons and introns are highly conserved between human, rodent, dog and chicken.

Canonical transcription of the VEGF-A gene is driven by a TATA-less promoter, which is exquisitely sensitive to hypoxia and to stimulation by various cytokines and growth factors; a second internal promoter instead directs basal levels of transcription in a more constitutive manner. The pre-mRNA that is generated then undergoes complex alternative processing,

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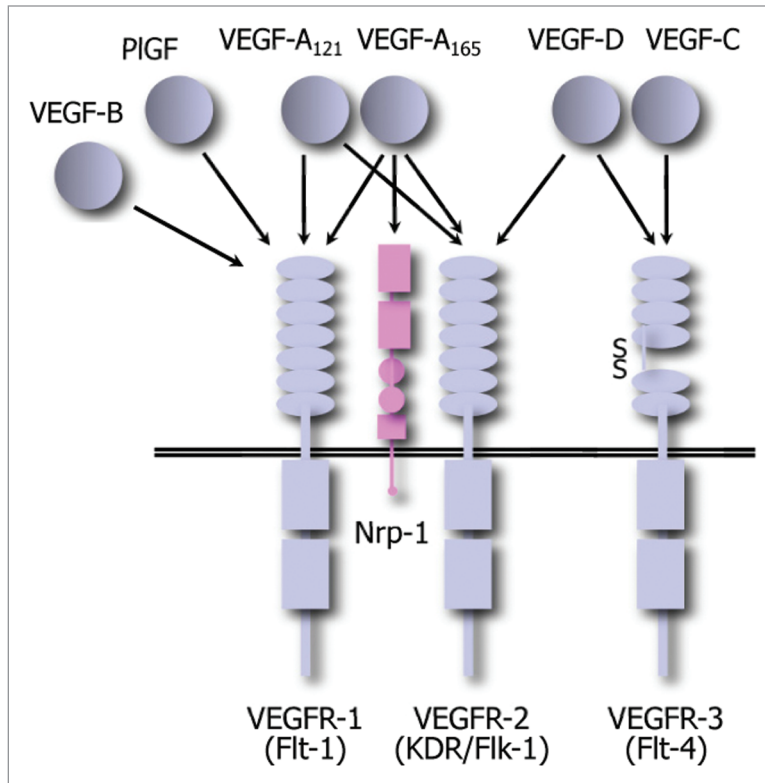
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**Figure 1.** Schematic representation of the interactions between the VEGF family of ligands and receptors.

leading to the production of at least seven different protein isoforms of 206, 189, 183, 165, 148, 145 and 121 amino acids after removal of the signal peptide (all isoforms are one amino acid shorter in mice). The three most abundant isoforms detected in vivo are VEGF-A<sub>121</sub>, VEGF-A<sub>165</sub> and VEGF-A<sub>189</sub>.

All the mRNAs coding for the different protein isoforms contain exons 1 to 5 in their 5' portion and are characterized by various combinations of exons 6, 7 and 8, or part of these, in their 3' regions (Fig. 2B–F). In the mRNA coding for the shorter isoform (VEGF-A<sub>121</sub>), exon 5 is directly spliced to the 6 aa-encoding exon 8 (Fig. 2B). Two mRNAs also contain exon 7 (coding for 44 aa) or its 5' portion (coding for 32 aa) only. In particular, in the mRNA coding for VEGF-A<sub>165</sub>, the whole exon 7 is included between exon 5 and exon 8, while in that coding for VEGF-A<sub>148</sub> the 5' part of exon 7 (7a) is spliced to exon 8, generating a different open reading frame that terminates protein translation in exon 8 by the inclusion of an additional single amino acid

(Fig. 2C). Three mRNA isoforms contain complete exon 6 (coding for 41 aa) or parts of exon 6 shorter at their 3' ends, in addition to complete exon 7. These are the mRNAs coding for the longest VEGF-A isoform (VEGF-A<sub>206</sub>, containing whole exon 6) and the mRNAs coding for VEGF-A<sub>189</sub> and VEGF-A<sub>183</sub>, which are generated using alternative 5' donor sites that shorten the coding region to 24 aa (exon 6a) and 18 aa (exon 6a'), respectively (Fig. 2D). Finally, only one isoform (VEGF-A<sub>145</sub>) selectively contains exon 6a inserted between exons 5 and 7 (Fig. 2E).

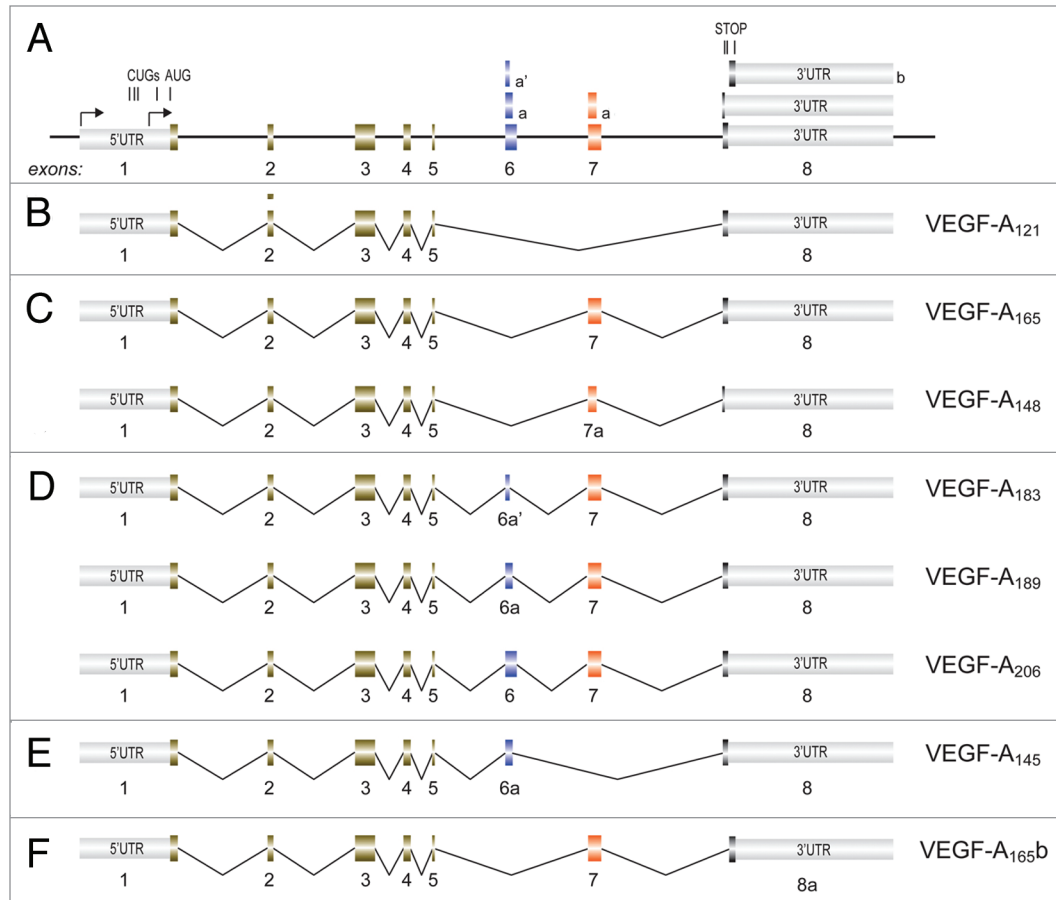
Additional variability is generated when a 3' splice site distal to that of exon 8 is used. This defines a shorter exon (exon 8b), the 5' of which codes for 6 different amino acids from those encoded by classic exon 8. This distal splice site choice can occur in conjunction with exon 6 or 7 inclusion or exclusion, thus leading to the production of a family of polypeptides differing from those described above by the last six amino acids. The most prominent of these polypeptides is VEGF-A<sub>165</sub>b (Fig. 2F). Interest for the protein isoforms

encoded by these exon 8b-containing mRNAs has recently risen, due to their potentially inhibitory, rather than stimulatory, effect on the VEGF receptors (reviewed in ref. 6).

### Non-Redundant Functions of VEGF-A Isoforms

What is the biological significance of such a complex pattern of alternative splicing regulation? The N-terminal portion of VEGF-A, encoded by exons 1–5, is sufficient to bind and activate the canonical VEGF receptors. As a consequence, all the VEGF-A splicing isoforms can interact with VEGFR-2 and, with an affinity that is 10 times higher, with VEGFR-1. Exons 6 and 7 at the C-terminus of the protein, however, are essential in determining its bioavailability and biodistribution. In particular, the 24 aa and 44 aa stretches encoded by exons 6a and 7, respectively, are highly enriched in clusters of basic amino acids, defined by their capacity to bind negatively charged heparin (heparin binding domains, HBDs). These HBDs confer upon the protein isoforms containing them the capacity to bind cell surface and extracellular matrix heparan sulfate proteoglycans (HSPGs) and thus essentially modulate their localization in the extracellular space. VEGF-A<sub>121</sub>, the shortest isoform, lacks any HBD and is weakly acidic; thus, it is freely diffusible upon secretion. In contrast, the longer splice forms VEGF-A<sub>189</sub> and VEGF-A<sub>206</sub> have two HBDs, and are almost completely sequestered in the extracellular matrix and on the cell surface. VEGF-A<sub>165</sub>, the prototypic and most abundant VEGF-A isoform, differs from VEGF-A<sub>121</sub> only by the inclusion of exon 7; its moderate affinity for heparin enables this isoform to act as both a soluble and a cell-bound factor (reviewed in ref. 7 and 8).

Based on these observations, it was originally proposed that binding to the insoluble extracellular matrix allowed the HBD-containing VEGF-A isoforms to form a concentration gradient that radiates from the ischemic areas, and that this gradient was essential for proper angiogenic stimulation. Consistent with this notion, various studies have shown that soluble VEGF-A<sub>121</sub> is less effective than



**Figure 2.** Major mRNAs generated by alternative splicing of the VEGF-A pre-mRNA. (A) Schematic representation of the VEGF-A gene intron and exon organization. Arrows indicate transcription start site. The positions of the translation initiation and termination codons are shown. (B) mRNA with exon 5/8 junction. (C) mRNAs including exon 7. (D) mRNAs including exons 6 and 7. (E) mRNA including exon 6. (F) mRNA containing alternative exon 8b.

the heparin-binding isoforms in supporting the neovascularization required for tumor growth.<sup>9,10</sup>

Essential evidence of the non-redundant function of the different VEGF-A proteins was further provided by a series of genetic studies in isoform-specific knock-in mice. Whereas animals expressing only VEGF-A<sub>164</sub> develop and grow normally, animals expressing exclusively VEGF-A<sub>120</sub> die perinatally. These animals also show impaired coronary vascularization and ischemic cardiomyopathy, with the formation of irregular, tortuous and dilated capillaries, indicative of incomplete vessel remodeling.<sup>11</sup> Incomplete pericyte coverage of vessels was also detected in other organs such as kidney and retina.<sup>12,13</sup> Conversely, the insoluble isoform VEGF-A<sub>188</sub> alone was also incapable of driving normal vessel development, resulting in abnormalities in arterial formation and vessel

branching.<sup>12</sup> Thus, these animal studies seem to indicate that a balance between diffusibility and gradient formation through HSPG binding, such as that achieved by VEGF-A<sub>164</sub>, is required for proper vascular formation.

Might the profound developmental defects observed in mice lacking VEGF-A<sub>164</sub> and the effects on tumor vascularization only derive from the differential capacity of the various VEGF-A isoforms to bind HSPGs? An additional, crucial level of complexity to our understanding of the significance of the VEGF-A isoforms, possibly providing an answer to this question, was provided by the discovery that the 44 aa encoded by VEGF-A exon 7 are also essential to bind the co-receptor Nrp-1.<sup>14</sup> This non-tyrosine kinase receptor was originally discovered as the main receptor for class 3 semaphorins, which provide repulsive guiding cues to axonal growth cones in the developing

nervous system. On endothelial cells, Nrp-1 significantly increases the affinity of VEGF-A<sub>165</sub>, but not of VEGF-A<sub>121</sub> lacking exon 7, to VEGFR-2, enhancing receptor activity and signaling.<sup>15-17</sup>

We have recently observed that the prolonged expression of VEGF-A<sub>165</sub> in both normoperfused and ischemic skeletal muscle or hearts upon AAV-mediated gene transfer exerts a profound angiogenic effect, inducing the formation of a large network of capillary vessels and arterioles covered by NG2<sup>+</sup>,  $\alpha$ -SMA<sup>+</sup> pericytes.<sup>18-20</sup> In sharp contrast, however, we also observed that gene transfer and prolonged expression of VEGF-A<sub>121</sub> markedly increases capillary sprouting, but is ineffective in determining maturation of the newly formed vasculature.<sup>18</sup> These differential effects of the two VEGF-A isoforms were correlated with their differential capacity to recruit, to the sites of neoangiogenesis, a novel population of CD11b<sup>+</sup>,

Gr-1<sup>+</sup> circulating cells, deriving from the bone marrow and expressing Nrp-1. More recently, we have observed that NEMs act in a paracrine fashion by secreting PDGF-B, TGF $\beta$  and various chemokines that promote vessel maturation, thus determining a higher degree of  $\alpha$ -SMA<sup>+</sup>/NG2<sup>+</sup> mural cell coverage and decreased vascular leakiness (Carrer A et al., manuscript in preparation). Taken together, these observations strongly suggest that VEGF-A<sub>121</sub> is insufficient for normal vasculogenesis in the embryo and angiogenesis in the adult, due not only to its incapacity to determine a concentration gradient through HSPG binding, but also because it fails to induce peri-endothelial cell coverage and vessel maturation through NEM recruitment by exon 7-mediated Nrp1 binding.

Finally, VEGF-A<sub>165b</sub> differs from VEGF-A<sub>165</sub> only in the C-terminal six amino acids. The factor still binds VEGF receptors with equal affinity as the other VEGF-A ligands, but is a poor agonist of VEGFR-2, determining a distinct pattern of tyrosine phosphorylation and, most notably, fails to bind HSPGs and interact with Nrp-1, similar to VEGF-A<sub>121</sub>.<sup>21,22</sup> Thus, VEGF-A<sub>165b</sub> might act competitively with the other VEGF-A isoforms and quench their angiogenic activity.

### Regulation of VEGF-A Gene Splicing

Despite the important differences in the biological activity of the different VEGF-A isoforms, information on the molecular mechanisms involved in alternative splicing of the VEGF-A gene in physiologic and pathologic conditions is still very scant. It is very likely that some of the molecular mechanisms leading to increased transcription from the inducible VEGF-A promoter might also determine activation of various SR proteins involved in splicing regulation. Consistent with this possibility, in endometrial carcinoma cells, increased production of VEGF-A in response to hypoxia and acidosis was paralleled by a shift of isoform ratio towards the production of VEGF-A<sub>121</sub> concomitant with p38 MAPK activation and consequent increase in different SR proteins (SF2/ASF, SRp20 and SRp40) activity.<sup>23</sup> In breast cancer cells, siRNA-mediated

silencing of one member (CAPER $\alpha$ , also known as hCC1.3) of the U2AF<sup>65</sup> family of splicing regulators, which couple splicing to transcriptional activation in the hormone receptor-responsive promoters, was also found to selectively increase VEGF-A<sub>121</sub> production compared to the longer isoforms, suggesting that this protein might conversely favor proximal splice site selection.<sup>24</sup>

Very little information is as yet available on the specific molecular mechanisms leading to exon 7 inclusion or exclusion. Cohen and coworkers<sup>25</sup> reported that this exon might contain a purine-rich exonic splicing enhancer potentially binding the alternative splicing regulatory protein SLM-2. Consistent with this conclusion, expression of this protein in podocytes was found to correlate with relatively higher production of VEGF-A<sub>165</sub> compared to other isoforms.

The difference between VEGF-A<sub>189</sub>, having high affinity for HSPGs, and the more soluble VEGF-A<sub>165</sub> is the presence of the alternatively spliced exon 6A, which is present in the longer isoform and missing in the shorter. By use of a reporter minigene, Wang and coworkers identified the presence of an exonic splicing silencer (ESS) within exon 6a; the identity of the proteins interacting with this ESS are still uncharacterized.<sup>26</sup>

As far as exon 8b is concerned, this is likely to be included in the mRNA in place of canonical exon 8 as a consequence of distal splice site selection. Bioinformatics analysis indicates that there is a cluster of ASF2/ASF (which favor proximal splice site selection) adjacent to the proximal splice site and a cluster of SRp55-binding sites just distal to the distal splice sites.<sup>27</sup> In a consistent manner, treatment of retinal pigment epithelial cells and podocytes with TGF $\beta$ 1 was found to determine accumulation of poorly angiogenic exon 8b-containing VEGF-As by increasing the levels of SRp55, possibly indirectly through the activation of the MAPK p38.<sup>27</sup> In contrast, cell treatment with IGF-1 also increased the amount of total VEGF-A; however, it decreased the levels of the isoforms containing 8b through a cascade of signals involving first activation of protein kinase C (PKC) and later that of the splicing factor kinase SRPK1,

the end results being activation of nuclear ASF/SF2 and consequent proximal splice site selection.<sup>28</sup>

### The Importance of Understanding VEGF-A Gene Alternative Splicing

Despite the vast body of information already available on the different functions of the various VEGF-A isoforms, it can be safely concluded that our understanding of the molecular mechanisms regulating alternative splicing of the VEGF-A pre-mRNA and, most notably, their connection with physiological and pathologic conditions is still in its infancy. However, this appears to be an important topic for investigation for a number of reasons. First, the non-equivalent function of the different VEGF-A isoforms indicates that their precise regulation is critical for blood vessel formation. In the adult, the modulation of VEGF-A pre-mRNA splicing might thus lead to unanticipated effects on tumor vascularization or impact on tumor vessel stabilization, with potentially important consequences on tumor growth, invasion and propensity to metastasize. Second, the induction of therapeutic angiogenesis by gene therapy holds great promise for the revascularization of otherwise ischemic tissues. A correct choice of the VEGF-A isoform cDNA to be delivered thus appears essential. In this respect, the apparent lack of satisfactory results so far obtained might be, at least in part, also attributed to improper choice of the delivered VEGF-A cDNA. Third, over the last several years, it has become evident that expression of the VEGF receptors is not restricted to endothelial cells and that their ligands exert a variety of fundamental functions in other cell types, including cardiomyocytes, muscle satellite cells, neurons, hepatocytes, osteoblasts, monocytes and hematopoietic stem cells (reviewed in ref. 18, 29 and 30). Very little is so far known, however, on the differential role of the various VEGF-A isoforms on all these non-angiogenic activities of the factor.

Given all of the above mentioned unknown elements and the central importance of VEGF-A and its receptors in so many different biological and clinical settings, further investigation on the

molecular mechanisms regulating splicing of its pre-mRNA are definitely warranted.

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