

VEGF-A Splice Variants: Do They Play a Role in Tumor Responses to Anti-Angiogenic Therapies?

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Abstract It has been known for two decades that *VEGF-A* encodes several VEGF-A splice variants, which are termed VEGF_{xxx}, according to the total number of amino acids in the mature protein. To date, nine VEGF_{xxx} isoforms have been described, displaying different biodistribution and pro-angiogenic activity. Adding another level of complexity to VEGF-A biology, a new family of VEGF-A isoforms, termed VEGF_{xxx}b, which exert anti-angiogenic functions was discovered in 2002 and only differ from VEGF_{xxx} polypeptides with regard to their C-terminal six amino acids. Therefore, reminiscent of what is observed, for instance, during apoptosis, the alternative splicing of *VEGF-A* pre-mRNA generates two types of isoforms with antagonistic biological functions. As anti-angiogenic therapies target both the VEGF_{xxx} and VEGF_{xxx}b families, *VEGF-A* pre-mRNA splicing may therefore impact tumor responses to these therapies. Consistently, recent clinical studies have highlighted VEGF-A splice variants as predictive biomarkers in response to bevacizumab. Hence, identification of the upstream signaling pathways that control *VEGF-A* pre-mRNA splicing, better characterization of the specific biological functions played by each VEGF-A splice variant, and/or analysis of the impact of anti-angiogenic therapies on *VEGF-A* pre-mRNA splicing are critical goals. The purpose of this chapter is to summarize the current knowledge in this field.

6.1 Proteome Diversity Through Pre-mRNA Alternative Splicing: *VEGF-A* Does Not Escape the Rule

Deep-sequencing of the human transcriptome has provided evidence that up to 90% of human multi-exon genes undergo pre-mRNA alternative splicing (AS) (Pan et al. 2008), and the role of AS in both physiological and pathological processes, such as cancer, is now well-documented. For instance, many splice variants have been found to be misregulated in cancers (David and Manley 2010), among them *VEGF-A*-encoded splice variants.

6.1.1 The *VEGF*_{xxx} Family

6.1.1.1 General Presentation

The human *VEGF-A* gene is located on chromosome 6 at 6p21.3 and spans 14 kb, with eight exons separated by seven introns (Vincenti et al. 1996) (Fig. 6.1). Two years after its discovery, *VEGF-A* was found to encode multiple protein isoforms through exonic alternative splicing (Houck et al. 1991). To date, nine human *VEGF-A* splice variants, termed *VEGF*_{xxx} (where xxx represents the total number of amino acids in the mature protein after signal sequence cleavage), have been described: *VEGF*₁₁₁, *VEGF*₁₂₁, *VEGF*₁₄₅, *VEGF*₁₄₈, *VEGF*₁₆₂, *VEGF*₁₆₅, *VEGF*₁₈₃, *VEGF*₁₈₉, and *VEGF*₂₀₆ (Ferrara 2004). At the structural level (Fig. 6.1), with the exception of *VEGF*₁₁₁ (containing exons 1-4), all *VEGF*_{xxx} isoforms contain exons 1-5 and differ only by the inclusion/exclusion of exon 6a, 6b, or 7. Hence, *VEGF*₁₈₉ lacks the residues encoded by exon 6b, *VEGF*₁₆₅ those encoded by exon 6, and *VEGF*₁₂₁ those encoded by exons 6 and 7. Exons 6a and 7 encode highly conserved sequences that are rich in basic residues that confer an affinity for heparin-containing proteoglycans (Fig. 6.2). Therefore, *VEGF*₁₂₁ is an acidic protein that does not bind heparin and is highly diffusible, whereas the *VEGF*₂₀₆, *189*, *183*, *162*, and *145* isoforms tightly bind to the heparan sulfate that is present at the cell surface and extracellular matrix [ECM; (Houck et al. 1992)]. The *VEGF*₁₆₅ isoform displays an intermediate pattern, as it contains only 15 basic amino acids within the 44 residues encoded by exon 7, and it is estimated that approximately 50-70% of the *VEGF*₁₆₅ pool is anchored at the cell surface or ECM (Houck et al. 1992). The *VEGF-A* isoforms in the ECM constitute a reservoir of growth factor that can be slowly released after exposure to heparin, heparan sulfate, and heparinases or more rapidly mobilized by specific proteolytic enzymes, such as plasmin and urokinase-type plasminogen activator (uPA) (Plouet et al. 1997). Importantly, the loss of the heparin-binding domain after proteolytic cleavage reduces the mitogenic activity of *VEGF-A* (Keyt et al. 1996). Therefore, *VEGF-A* pre-mRNA splicing controls both the biodistribution and bioactivity of *VEGF-A*, allowing the establishment of a gradient of *VEGF-A* during tumor angiogenesis, with the most diffusible isoform acting at distance to promote vascular recruitment, whereas the matrix-bound isoforms promote the local expansion of capillary beds (Grunstein et al. 2000).

All *VEGF-A* isoforms are secreted as antiparallel homodimers covalently linked by two disulfide bridges between Cys-51 and Cys-60 (Muller et al. 1997). At the structural level, all isoforms possess a dimerisation domain and a *VEGFR1* or *VEGFR2* binding domain (Fig. 6.2). However, the isoforms display different binding affinities for the *VEGF* receptors, *VEGFR1* (Flt-1) and *VEGFR2* (KDR/Flk-1), and for their co-receptors, neuropilin-1 (NRP1) and neuropilin-2 (NRP2) (Roskoski 2007). Hence, *VEGF*₁₆₅ binds to *VEGFR1* and *VEGFR2*, whereas *VEGF*₁₈₉ preferentially binds to *VEGFR1* unless cleaved by proteases. *VEGF*₁₂₁ exhibits a modestly reduced affinity for *VEGFR1* and *VEGFR2* in the presence of heparin

when compared with VEGF₁₆₅. Although all VEGF₁₈₉, VEGF₁₆₅, and VEGF₁₂₁ isoforms are able to bind NRP1 (Delcombel et al. 2013), only VEGF₁₆₅ bridges the formation of VEGFR2/NRP1 ternary complexes (Soker et al. 2002), whereas VEGFR2 enhances the interaction between NRP1 and VEGF₁₆₅ only (Pan et al. 2007a). Therefore, as compared with the other VEGF_{xxx} isoforms, VEGF₁₆₅ displays optimal features in terms of biological potency. Indeed, VEGF₁₆₅ is the only isoform that can fully rescue the tumorigenic phenotype in mouse VEGF^{-/-} cells (Grunstein et al. 2000). Interestingly, matrix-bound VEGF₁₆₅, but not soluble VEGF₁₆₅, is able to trigger a sustained phosphorylation of VEGFR2 in endothelial cells, followed by a specific activation of the p38 MAPK pathway (Chen et al. 2010). These data suggest that VEGF_{xxx} isoforms transactivate specific downstream VEGFR-dependent signaling pathways depending on their ECM-binding ability.

6.1.1.2 VEGF_{xxx} Isoforms: Distinct Functions and Pattern of Expression in Tumors

As determined by the cDNA analysis of a variety of cell types and tissues and tumor specimens, most VEGF-producing cells appear to preferentially to express *VEGF₁₂₁*, *VEGF₁₆₅* and *VEGF₁₈₉* mRNAs, with *VEGF₁₆₅* being the predominant isoform. *VEGF₁₂₁* mRNA is abundant in lung and kidney, and *VEGF₁₈₉* mRNA is highly expressed in cardiac and vascular tissues, albeit to a lesser extent than *VEGF₁₆₅* and *VEGF₁₂₁* (Zygalaki et al. 2008). *VEGF₁₈₉* mRNA is preferentially expressed under conditions of increased stretch-induced cell tension and is thought to play a role in vascularization during bone repair (Faure et al. 2008). *VEGF₁₈₃* also has a wide tissue distribution and may have avoided earlier detection through confusion with VEGF₁₈₉ (Jingjing et al. 1999). By contrast, *VEGF₁₄₅* and *VEGF₂₀₆* mRNAs are rarely expressed and are seemingly restricted to cells of placental origin (Cheung et al. 1995).

In tumors, RT-PCR or RT-QPCR analyses have further revealed differential patterns of VEGF_{xxx} expression and have correlated this pattern with clinico-pathological features. In lung and colon carcinomas, *VEGF₁₆₅* and *VEGF₁₂₁* mRNAs appear to be the most predominant transcripts (Zygalaki et al. 2007). *VEGF₁₆₅* mRNA expression in many tumor types has been associated with progression, invasion and metastasis, microvessel density, decreased survival rate, and a poor prognosis. In colon, renal cell, and lung carcinomas, the expression of *VEGF₁₈₉* mRNA was associated with a worse prognosis (Ohta et al. 1996), and only *VEGF₁₈₉* mRNA expression was found to be an independent prognostic indicator in a series of 130 Non Small Cell Lung Carcinomas (NSCLCs) (Matsuyama et al. 2009). *VEGF₁₈₉* was also found to be necessary for the growth of NSCLC xenografts in vivo, with VEGF₁₈₉-overexpressing tumors being larger and displaying higher vessel perfusion and permeability than those overexpressing either VEGF₁₂₁ or VEGF₁₆₅ (Yuan et al. 2001). However, in lung cancer, high levels of *VEGF₁₂₁* mRNA have also been associated with a poor prognosis and lymph node metastasis (Oshika et al. 1998), and VEGF₁₂₁ was found to promote lymphangiogenesis via the secretion of VEGF-C (Kawai et al. 2008). In addition, expression of *VEGF₁₂₁* mRNA was associated with the tumor progression of bladder and renal cell carcinoma (Li et al. 2000; Ljungberg et al. 2003) and with a worse prognosis in acute myeloid leukemia (Mourah et al. 2009), and the VEGF₁₂₁ isoform was found to be the most tumorigenic in prostate and breast cancers (Catena et al. 2007). In 2007, it was reported that VEGF₁₁₁ mRNA was expressed in various tumor cell lines following treatment with such genotoxic agents as UV-B, camptothecin, and mitomycin C (Mineur et al. 2007).

Altogether, these data demonstrate that *VEGF-A* pre-mRNA splicing likely contributes to tumorigenesis by providing an another way to finely regulate VEGF-A bioactivity in addition

to the activation of *VEGF-A* transcription. Such a notion is fully consistent with the data of Tozer and colleagues, which provided evidence that mouse fibrosarcoma cells that express only a single isoform of VEGF_{xxx} produce tumors with different vascularization patterns, vessel wall structure, and barrier function (Tozer et al. 2008).

6.1.2 The VEGF_{xxx}b Family: Six Amino Acids that Make the Difference

6.1.2.1 General Presentation

In 2002, a new family of VEGF-A splice variants, termed VEGF_{xxx}b, was discovered (Bates et al. 2002). VEGF_{xxx}b splice variants share 94-98% homology with VEGF_{xxx} and result from the use of an alternative 3' splice acceptor site in the final exon 8 of *VEGF-A* (Fig. 6.1). Hence, the use of a proximal splice site (PSS) in exon 8 leads to the inclusion of exon 8a and generates the VEGF_{xxx} family, whereas the use of a distal splice site (DSS) located 66 nucleotides further downstream from PSS allows the inclusion of exon 8b and the generation of VEGF_{xxx}b transcripts. At the protein level, VEGF_{xxx} and VEGF_{xxx}b isoforms display the same lengths and amino acids sequences and differ only at the level of their C-terminal six amino acids. Indeed, exon 8a in VEGF_{xxx} mRNAs encodes a CDKPRR sequence, whereas exon 8b in VEGF_{xxx}b transcripts encodes an SLTRKD sequence (Ladomery et al. 2007). This distinct C-terminal extremity strongly affects the VEGF-A tertiary structure (Cui et al. 2004), as the Cys-160 that forms a disulfide bond with Cys-146 in exon 7 is replaced by an aspartic acid residue. In addition, the two highly charged arginine residues of the VEGF_{xxx} proteins are replaced by a lysine and aspartic acid in the VEGF_{xxx}b proteins, with a substitution of a proline by an arginine. As a consequence, the VEGF_{xxx}b isoforms retain both the VEGF tyrosine kinase receptor binding and dimerization domains but do not bind neuropilins and are not able to fully activate VEGFR-dependent signaling pathways (Cebe Suarez et al. 2006; Kawamura et al. 2008; Delcombel et al. 2013). Indeed, VEGF₁₆₅b does not exert its effect at full rotation on VEGFR2, which results in the rapid blockage of the ATP binding site, leading to transient and non-efficient VEGFR2 auto-phosphorylation (Harper and Bates 2008). Thus, although VEGF₁₆₅b is able to stimulate endothelial cell proliferation and subsequent ERK activation under certain conditions, this occurs with less potency than with VEGF₁₆₅ (Catena et al. 2010). In addition, unlike VEGF₁₆₅, which induces VEGFR2 internalization and recycling to the plasma membrane through Rab11 vesicles by a mechanism involving NRP1, VEGF₁₆₅b promotes the accumulation of phospho-VEGFR2 in Rab7 vesicles, which is indicative of receptor degradation (Ballmer-Hofer et al. 2011). At the biological level, in in vitro endothelial proliferation and migration assays, ex vivo isolated resistance vessel myographs, and in vivo neo-vascular and tumor growth models, VEGF₁₆₅b acts as a dominant negative isoform that specifically competes with and inhibits VEGF₁₆₅ effects on endothelial cell proliferation, migration, and vasodilatation, thereby inhibiting angiogenesis and tumor growth (Bates et al. 2002; Woolard et al. 2004; Rennel et al. 2008). VEGF₁₂₁b also inhibits the migration of endothelial cells and reduces xenografted tumor growth through a direct effect on endothelial cells (Rennel et al. 2009). Interestingly, VEGF₁₆₅b failed to bind heparin despite possessing exon 7 (Delcombel et al. 2013), implicating exon 8a or an inhibitory function of exon 8b in this interaction. Therefore, as compared with VEGF_{xxx}, VEGF_{xxx}b isoforms could also possess distinct bioavailability.

6.1.2.2 VEGF_{xxx}b Isoforms: Do They Exhibit a Differential Pattern of Expression in Tumors?

VEGF_{165b} mRNA was the first *VEGF_{xxx}b* mRNA identified by RT-PCR in kidney cortex tissue (Bates et al. 2002). Subsequently, *VEGF_{121b}* (Rennel et al. 2009), *VEGF_{145b}* (Perrin et al. 2005), and *VEGF_{189b}* mRNAs (Miller-Kasprzak and Jagodzinski 2008) were identified in various human tissues. Indeed, VEGF_{xxx}b isoforms are thought to form over 50% of the total VEGF-A protein in normal, non-angiogenic tissues, such as dorsal root ganglia (71%), lung (82%), colon (>95%), skin (>95%) (Qiu et al. 2009), and vitreous (66%) (Perrin et al. 2005). By contrast, VEGF_{xxx}b represents a small proportion of total VEGF-A in placenta (1.4%) (Bates et al. 2006). Importantly, although VEGF_{xxx} isoforms are overexpressed in many tumor types, the VEGF_{xxx}b isoforms appear to be mainly downregulated in several cancers, including melanoma (Pritchard-Jones et al. 2007), prostate (Woolard et al. 2004), colon (Varey et al. 2008), kidney (Bates et al. 2002), and Wilm's (Amin et al. 2011) carcinomas. In colorectal tumors, the downregulation of VEGF_{165b} was associated with advanced stages, vascular invasion, and lymph node metastases (Diaz et al. 2008), and the absence of VEGF_{xxx}b expression was found to predict metastatic spread in melanoma patients (Pritchard-Jones et al. 2007). Moreover, VEGF_{165b} expression was downregulated as compared with VEGF₁₆₅ in neuroblastoma, and rhVEGF_{165b} was found to reduce neuroblastoma tumor growth rate in established xenografts (Peiris-Pages et al. 2010). Therefore, a switch between the VEGF_{xxx} and VEGF_{xxx}b isoforms in favor of VEGF_{xxx} exists in human tumors. Regardless, the persistence of VEGF_{xxx}b expression, together with VEGF_{xxx}, has also been reported in tumors. This is indeed the case in 70% of clear renal cell carcinoma (Grepin et al. 2012) and in breast cancers in which the VEGF_{xxx}b isoforms are upregulated compared to nonmalignant breast tissues (Catena et al. 2010). VEGF_{165b} expression was also detected in stromal cells surrounding colorectal cancers and correlated with lower microvessel density (Tayama et al. 2011). Moreover, Bates and colleagues recently showed that a differential pattern of VEGF_{165b} protein expression exists in colon carcinoma, with tumors displaying low or high levels as compared with normal tissues (Bates et al. 2012). Accordingly, we observed that approximately 35% and 15% of NSCLCs display a low or high level of VEGF_{165b} protein expression, respectively, as compared with normal lung tissues (Boudria et al. in preparation). More interestingly, we found a significant association between high levels of VEGF_{165b} and lymph node metastases in NSCLC. Thus, the role of VEGF_{xxx}b isoforms during tumorigenesis might be more complex than previously imagined.

6.1.2.3 Analysis of VEGF-A Splice Isoforms in Tumors: What We Should Keep in Mind

As shown above, the analysis of distinct VEGF_{xxx} isoforms in human tumors has been mainly performed by RT-PCR or RT-QPCR using primers specifically designed to amplify each *VEGF_{xxx}* splice variant. However, in the vast majority of these studies, these primers were later shown to also co-amplify *VEGF_{xxx}b* mRNAs. The same is true regarding the analysis of the VEGF_{xxx} protein isoforms. Indeed, the antibodies used in the vast majority of in situ studies are pan-VEGF antibodies that recognize all VEGF-A isoforms, including VEGF_{xxx}b isoforms. Hence, the commercial antibodies previously thought to specifically target VEGF_{xxx} isoforms were later found to cross-react with rhVEGF_{xxx}b proteins. To date, there is no tool for the specific detection of VEGF_{xxx} protein isoforms by western blotting, as the use of a pan-VEGF antibody will allow the discrimination among VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉ but not between the VEGF_{xxx} and VEGF_{xxx}b isoforms. Nonetheless, mouse monoclonal antibodies directed against the C-terminal nine amino acids of VEGF_{165b} (CTRLTRKD) have been developed; one (MVRL56/1) now commercially available, exhibits specificity for

the VEGF_{165b} isoform versus VEGF₁₆₅ and does not cross-react with VEGF₁₆₅ or VEGF₁₂₁ recombinant proteins (Woolard et al. 2004). Therefore, until tools allowing the specific detection of VEGF_{xxx} isoforms become available, a concomitant analysis of both total VEGF-A (using a pan-VEGF-A antibody) and VEGF_{xxxb} (using the specific MVRL56/1 anti-VEGF_{xxxb} antibody) proteins should be performed before drawing conclusions regarding the status of VEGF-A in tumors. In addition, because *VEGF-A* is regulated at multiple post-transcriptional levels in addition to pre-mRNA splicing and it is not known whether the mRNA and protein levels are strictly correlated, concomitant analyses of both VEGF_{xxx}/VEGF_{xxxb} mRNA and protein levels should also be performed.

6.1.3 Molecular Mechanisms Regulating VEGF-A Pre-mRNA Splicing

Several transcription factors, such as hypoxia-inducible factor 1 (HIF-1), inflammatory cytokines, growth factors, or oncogenes, have been identified as upstream transcriptional activators of *VEGF-A* (Ferrara 2004). By contrast, although VEGF-A splice isoforms were described for the first time in 1989, very little is known about the molecular mechanisms that control *VEGF-A* pre-mRNA splicing (Fig. 6.3).

6.1.3.1 Molecular Determinants of VEGF_{xxx} Pre-mRNA Splicing

It was previously shown that the splicing factor SAM68 (Cohen et al. 2005), the U2AF65-related protein CAPER (Dowhan et al. 2005), and a putative chloride channel, pICn (Li et al. 2004), control *VEGF_{xxx}* pre mRNA splicing. Progesterone selectively was reported to increase the expression of the VEGF₁₈₉ isoform in the human uterus (Ancelin et al. 2002). Moreover, an exonic splicing silencing (ESS) sequence was recently described in exon 6a, though the regulatory proteins interacting with it remain unknown (Wang et al. 2009). In endometrial cancer cells, low pH (acidosis) significantly upregulated the *VEGF₁₂₁* mRNA level when compared with the 145, 165, or 189 isoforms through a mechanism involving the p38 MAPK pathway and splicing factors of the SR proteins family, namely SRSF1 (formerly SF2/ASF), SRp20, and SRp40 (Elias and Dias 2008). In addition, incubation with phorbol myristate acetate (PMA), an activator of PKC activity, induced the expression of VEGF₂₀₆ in human skin mast cells, which normally express the 121, 165, and 189 isoforms (Grutzkau et al. 1998). As previously discussed, *VEGF₁₁₁* mRNA was specifically induced by genotoxic stress in several tumor cell lines and human xenografts in nude mice upon treatment with camptothecin (Mineur et al. 2007); however, the molecular mechanisms involved remain unknown.

6.1.3.2 Molecular Pathways that Control the Balance Between VEGF_{xxx} and VEGF_{xxxb} Splice Variants

Bates's group has mainly contributed to the identification of upstream regulators of the VEGF_{xxxb} isoforms. In 2008, Nowak and colleagues first demonstrated in primary epithelial cells that insulin growth factor (IGF1) and tumor necrosis factor alpha (TNF- α) treatment favor the selection of the PSS, thereby increasing VEGF_{xxx} production, whereas tumor growth factor beta 1 (TGF- β 1) favors DSS selection, increasing VEGF_{xxxb} (Nowak et al. 2008) (Fig. 6.3). Using epithelial cells overexpressing various SR proteins, these authors further demonstrated that SRSF1 favors PSS selection by binding a 35-nucleotide sequence of exon

8a upstream of PSS, whereas SRSF6 (formerly SRp55) favors DSS by binding to a 35-nucleotide region of the 3'UTR immediately downstream of the stop codon in exon 8b. TGF- β 1 action requires the activity of p38 MAPK and CLK/STY kinases, whereas IGF1 involves both PKC and SRPK1/2, the latter being the specific kinases of SR proteins (Nowak et al. 2010). Consistent with a crucial role of SR proteins in controlling the balance between VEGF_{xxx} and VEGF_{xxx}b isoforms both under normal and pathological conditions, elevated VEGF₁₆₅b expression was correlated with the increased expression of TGF- β 1 and SRSF6 in keratinocytes, fibroblasts, endothelial cells, and perivascular inflammatory cells of patients suffering from systemic sclerosis (Manetti et al. 2011). In addition, we provided the first evidence that the transcription factor E2F1 favors VEGF₁₆₅b expression via a mechanism involving SRSF2 (formerly SC35), another SR protein, in lung adenocarcinoma cell lines and xenografts in nude mice (Merdzhanova et al. 2010). Later, Bates and colleagues showed that mutations in WT1, the Wilms' tumor suppressor gene, suppress VEGF₁₆₅b expression and cause abnormal gonadogenesis, renal failure, and Wilms' tumors (Amin et al. 2011). In WT1 mutants, SRPK1 was upregulated through the derepression of its transcription, leading to the phosphorylation and nuclear accumulation of SRSF1, thereby favoring VEGF_{xxx} isoforms. Interestingly, sodium butyrate (NaBu), a histone deacetylase inhibitor, increased *VEGF_{121b}*, *VEGF_{165b}*, and *VEGF_{189b}* mRNA and protein levels in immortalized human lung microvascular endothelial cells (HLMECs) (Ciura and Jagodzinski 2010). As we demonstrated that the treatment of human lung adenocarcinoma cell lines with NaBu or cisplatin increased the expression level of SRSF2 (Edmond et al. 2011a; Edmond et al. 2011b), it is tempting to speculate that therapies could modify *VEGF-A* pre-mRNA splicing through the modulation of SR protein expression (see 6.2.2). In addition, as pre-mRNA splicing is known to occur co-transcriptionally, one might postulate that SR proteins could cooperate with the transcription factors that control *VEGF-A* transcription to modulate *VEGF-A* pre-mRNA splicing, as we found for E2F1 and SRSF2. Whether HIF1 plays a role in this scenario remains a very intriguing question.

6.2 Does *VEGF-A* Pre-mRNA Splicing Play a Role in the Response to Anti-Angiogenic Therapies?

6.2.1 Anti-Angiogenic Therapy Failure: Patient Selection Is Critical

One of the main problems with the use of anti-angiogenic therapies is the absence of effective predictive biomarkers. In a meta-analysis of published randomized controlled trials (RCTs), Ranpura and colleagues showed that the addition of bevacizumab is associated with an increased risk of fatal adverse events (FAEs) in patients receiving taxanes or platinum salts compared with chemotherapy alone (Ranpura et al. 2011) and was not restricted to an anti-VEGF antibody. Indeed, another meta-analysis derived from 10 randomized RCTs and including 4679 patients receiving either sorafenib, sunitinib, or pazopanib also found an increased risk of FAEs following the use of these VEGFR TKIs compared with control patients (Schutz et al. 2012). Therefore, biomarkers are urgently needed to select patients that will benefit from such therapies. In addition, a better understanding of the molecular mechanisms involved in primary or acquired tumor resistance to these therapies is crucial in the development of new therapeutic strategies.

6.2.2 Anti-Angiogenic Therapies Used Alone or in Combination Regulate VEGF-A Pre-mRNA Splicing

Several clinical studies have shown acute increases or decreases in circulating total VEGF-A levels after bevacizumab delivery (Lambrechts et al. 2013). By contrast, the impact of anti-angiogenic therapies on *VEGF-A* pre-mRNA splicing has not yet been investigated. We recently addressed this question and provided the first evidence that treating human lung adenocarcinoma cell lines with bevacizumab alters the VEGF_{165b}:pan-VEGF ratio in favor of VEGF_{165b} (Boudria et al. in preparation). Similar results were obtained when SU5416, a VEGFR TKI, was used. Consistent with our data, intravitreal bevacizumab injection in an oxygen-induced retinopathy mouse model also increased the relative protein expression of the VEGF_{165b} isoform while inhibiting the protein level of total VEGF-A isoforms (Shi et al. 2013). Although these results must be extended to other tumor types and/or other VEGFR TKIs, such as sorafenib or sunitinib, they demonstrate the impact of anti-angiogenic therapies on *VEGF-A* pre-mRNA splicing.

Bevacizumab is never used alone and is always combined with conventional chemotherapies for the treatment of breast, lung, colon, and kidney carcinomas. However, as it has been shown that chemotherapies increase *VEGF-A* mRNA expression in cancer cells (Fan, Gray et al. 2008), it is possible that chemotherapies either used alone or in combination with anti-angiogenic therapies also impact *VEGF-A* pre-mRNA splicing. In NSCLCs, bevacizumab is combined with platinum salts (cisplatin or carboplatin) and with paclitaxel or gemcitabin. Interestingly, we found that treating NSCLC cell lines with these drugs significantly increased the VEGF_{165b} protein level, though decreased the expression of total VEGF-A (Boudria et al. in preparation). More interestingly, when bevacizumab was added to these chemotherapies, a stronger accumulation of VEGF_{165b} protein was observed in these cells, and VEGF_{165b} prevented cisplatin-induced apoptosis. Therefore, the combination of bevacizumab and cisplatin could select tumor cells that are more resistant to apoptosis through the upregulation of VEGF_{165b}. Of note, cells overexpressing VEGF_{165b} were also less sensitive to hypoxia-induced apoptosis, thereby suggesting that VEGF_{165b} could be a general inhibitor of apoptosis. Our results are consistent with recent data demonstrating that VEGF_{165b} exerts a neuroprotective function in hippocampal, cortical, and retinal neurons by activating VEGFR2 and p42/44 MAPK and preventing apoptosis (Beazley-Long et al. 2013).

Therefore, such anti-angiogenic therapies as bevacizumab, alone or in combination with chemotherapies, appear able to control *VEGF-A* pre-mRNA splicing and to favor the expression of VEGF_{xxx}b isoforms, at least in NSCLC cell lines. Such an effect might play a role in tumor adaptative resistance (see 6.2.3 and 6.2.4). Whether SR proteins are involved in this alternative switch remains largely unknown, but we observed that cisplatin, bevacizumab, and SU5416 increased SRSF2 and SRSF6 protein levels in our cellular models, a result that was consistent with VEGF_{xxx}b accumulation. In addition, whether chemotherapies and/or anti-angiogenic therapies control the expression level of distinct VEGF_{xxx}/VEGF_{xxx}b splice variants from each VEGF-A sub-family remains to be determined.

6.2.3 VEGF-A Splice Variants: Can They Predict Responses to Anti-Angiogenic Treatments?

6.2.3.1 The VEGF_{xxx}b/pan-VEGF Ratio: A Predictive Biomarker of Responses to Bevacizumab

The discovery of the VEGF_{xxx}b sub-family has complicated the simple view that targeting VEGF-A could be beneficial to patients. Hence, VEGF-A was found to encode both “bad” (pro) and “good” (anti)-angiogenic isoforms, which are both targeted by bevacizumab with the same affinity, leading to the question of whether *VEGF-A* pre-mRNA splicing is the key to anti-angiogenic therapeutics (Harper and Bates 2008). The first evidence demonstrating that VEGF₁₆₅b expression has an impact on the efficacy of bevacizumab came from the study of Varey and colleagues (Varey et al. 2008), who showed that xenografts in mice derived from human VEGF₁₆₅b-overexpressing colon cancer cells grow more slowly than those bearing VEGF₁₆₅-overexpressing cancer cells. Consistent with an impairment of tumor angiogenesis, the VEGF₁₆₅b-overexpressing xenografts were less vascularized and displayed greater areas of necrosis. More importantly, the authors further demonstrated that the dose of bevacizumab required to prevent tumor growth in VEGF₁₆₅-overexpressing tumors had no effect on the VEGF₁₆₅b-expressing tumors. This in vitro model predicting that bevacizumab would be less efficient in colorectal cancer patients who exhibited an excess of VEGF_{xxx}b over VEGF_{xxx} compared with those with an excess of VEGF_{xxx} was recently validated in situ. In 149 metastatic colorectal cancer patients from the E3200 clinical trial of FOLFOX4±bevacizumab, the unadjusted analysis of the progression-free survival (PFS) showed a significantly better outcome for patients with intra-tumoral VEGF₁₆₅b:VEGF_{total} ratio scores below the median treated with FOLFOX4+BVZ compared with FOLFOX4 alone (Bates et al. 2012). Although these data need to be extended to a larger series of patients and to other tumor types, they suggest that a low VEGF₁₆₅b:VEGF_{total} ratio might be a predictive marker for response to bevacizumab, at least in metastatic colorectal cancer. In addition, studies investigating the predictive potential of VEGF_{xxx}b in response to other anti-angiogenic therapies used clinically, such as VEGFR TKIs, are lacking. As the VEGF_{xxx}b isoforms antagonize VEGFR1/VEGFR2 angiogenic signaling, one might predict that patients with significant levels of VEGF_{xxx}b would be less sensitive to VEGFR TKIs.

Importantly, a lack of correlation between intra-tumoral and plasma VEGF-A protein levels was recently demonstrated (Hegde et al. 2013); however, it is not known to date whether this is also true for VEGF_{xxx}b isoforms. Therefore, both the circulating and tumoral VEGF_{total} and VEGF_{xxx}b levels should be taken into account when seeking a correlation between VEGF-A status and tumor response to anti-angiogenic treatments in situ.

6.2.3.2 Plasma Level of Short VEGF-A Isoforms but Not of Total VEGF-A Predict Outcome after Anti-Angiogenic Therapies

In NSCLC and colorectal and renal cell carcinomas, an association between a high circulating VEGF-A level and a shortened progression-free or overall survival was recently described, regardless of bevacizumab treatment (Hegde et al. 2013). Therefore, the plasma levels of total VEGF-A can serve as a prognostic rather than a predictive marker. However, when an ELISA assay that preferentially detects short VEGF-A isoforms (VEGF₁₂₁ and VEGF₁₁₁) was used, an association between high basal plasma levels of VEGF₁₂₁ and improved PFS and/or overall survival (OS) after bevacizumab treatment was found in breast, pancreatic, and gastric

carcinomas but not in metastatic colorectal, renal, and lung cancers (Lambrechts et al. 2013). Therefore, VEGF₁₂₁ appears to be a promising candidate biomarker for bevacizumab response, at least in certain cancers. As previously discussed, the discrepancy between tumors might reflect a distinct role of the VEGF_{xxx} isoforms depending on the tumor type and/or chemotherapies combined with anti-angiogenic treatments. In addition, the used of citrated plasma, which has minimal effect on platelet activation, rather than EDTA-treated plasma could also affect the results.

6.2.4 VEGF-A Splice Variants as Predictive Biomarkers for Anti-Angiogenic Therapies: How Does It Work?

6.2.4.1 Paracrine Effects on Endothelial Cells

Several hypotheses have been proffered regarding VEGF_{165b} potential as a predictive biomarker for bevacizumab response. First, it has been proposed that the concentration of effective bevacizumab could be lowered in patients with higher VEGF_{165b} concentrations, as more of the antibody is bound to VEGF_{165b} and not to VEGF₁₆₅, thereby diminishing the anti-angiogenic activity of VEGF_{165b} while increasing the amount of free pro-angiogenic VEGF₁₆₅. Conversely, patients with lower VEGF_{165b} levels may have a more angiogenic-progressive tumor than the high VEGF_{165b} group, thereby rendering the tumor more susceptible to bevacizumab+FOLFOX (Bates et al. 2012). This more angiogenic profile could also explain why patients with higher levels of soluble VEGF₁₂₁ are more susceptible to bevacizumab, as this diffusible short isoform may more accurately reflect the levels of bioactive VEGF-A and the vascular dependence of the tumor. This hypothesis is also consistent with another study identifying a VEGF-dependent gene signature specifically expressed in the tumor vascular compartment in which high expression correlated with enhanced VEGF-A downstream bioactivity and with an increased response to bevacizumab in colorectal cancers (Brauer et al. 2013). Alternatively, as bevacizumab does not prevent interaction between VEGF-A and NRP1 or NRP2, blocking the VEGF-A/VEGFR signaling pathways could induce compensatory mechanisms leading to the activation of VEGF/NRP-dependent networks. Consistently, it has been shown that anti-NRP1 antibodies have an additive effect on anti-VEGF therapy in reducing tumor growth through vessel normalization (Pan et al. 2007b). As the VEGF_{xxx} and VEGF_{xxx}b isoforms do not display the same affinity for NRP, such a compensatory response to anti-angiogenic therapies could thereby be dependent on the balance between both VEGF-A sub-families.

6.2.4.2 Autocrine Effects on Tumor Cells

Compared with the large number of publications related to VEGF-A endothelial functions, only a few studies have analyzed its autocrine effects on tumor cells that express VEGFR and/or NRP. Moreover, almost nothing is known regarding the functions of distinct VEGF-A splice variants in this setting. This situation is somewhat surprising because anti-angiogenic therapies, first designed to target endothelial cells, might directly impact the tumors themselves via the regulation of this autocrine loop. Alternatively, VEGF-A-regulated autocrine loops could contribute to primary or acquired resistance to these therapies.

VEGF-A: Not Only a Pro-Tumoral Factor in Tumor Cells?

It is currently acknowledged that VEGF-A exerts pro-proliferative/survival functions on tumor cells (Lichtenberger et al. 2010). As an example, the VEGF-A/VEGFR2/NRP1 autocrine loop was found to promote glioma stem-like cell viability and tumor growth and to limit the impact of bevacizumab treatment (Hamerlik et al. 2012). However, recent studies have highlighted unexpected VEGF-A anti-tumoral functions, increasing the complexity of the approach that blocking VEGF-A would always be beneficial. Indeed, VEGF₁₈₉ was found to induce NRP1-dependent apoptosis in breast cancer cell lines, whereas VEGF₁₆₅ acted as a survival factor (Vintonenko et al. 2011). In addition, the combination of high VEGF-A, VEGFR1, and VEGFR2 protein expression was associated with an improved progression-free survival in early squamous cell carcinoma of the lung in those who are not eligible for bevacizumab treatment owing to severe complications (Pajares et al. 2012). It is noteworthy that this effect was independent of endothelial cells, as no correlation was found between VEGF or its receptors and microvessel density. More strikingly, VEGF₁₆₅ was highlighted as a negative regulator of tumor cell invasion in glioblastoma through the inhibition of c-Met activity (Lu et al. 2012). In resistant glioblastoma patients, bevacizumab increased MET activation and contributed to the emergence of cells with a more invasive phenotype, which correlated with the expression of mesenchymal markers (Lu et al. 2012; Jahangiri et al. 2013). Taken together, these data highlight the distinct autocrine functions of VEGF_{xxx} splice variants in tumors and their potential as determinants of tumor responses to anti-angiogenic therapies.

VEGF_{165b}: A Pro-Invasive Factor in NSCLCs

In human lung adenocarcinoma cell lines and mouse xenografts, we found that bevacizumab treatment triggered a more invasive phenotype that correlated with the activation of both the VEGFR1 and VEGFR2 signaling pathways and with the acquisition of some mesenchymal markers and the secretion of metalloproteases (Boudria et al in preparation). Importantly, because the specific neutralization of VEGF_{165b} by siRNA was found to reverse the effects mediated by bevacizumab and to inhibit tumor growth, all these effects were dependent on VEGF_{165b}, but not VEGF₁₆₅, expression. Conversely and consistent with VEGF_{165b} controlling a pro-tumoral autocrine loop in NSCLCs, xenografts derived from VEGF_{165b}-overexpressing cells, despite being less angiogenic, grew more rapidly than control tumors and displayed markers of EMT. Altogether, our data support a model in which bevacizumab induces a more invasive phenotype through the activation of a VEGF_{165b}-dependent autocrine loop (Boudria et al in preparation). Our results are somewhat consistent with the results of Catena and colleagues who showed that the stable overexpression of either VEGF_{121b} or VEGF_{165b} protein in A549 human lung adenocarcinoma cells resulted in a significant increase in tumor development over the controls, even though no significant differences in vascular density were found. Additionally, in prostate PC-3 xenografted tumors, a reduction, as opposed to an increase, in the number of apoptotic cells was observed when VEGF_{xxx}b-overexpressing cells were injected, as compared with controls (Catena et al. 2010). Although we were not able to examine the correlation between VEGF_{165b} status and response to bevacizumab in situ in NSCLC patients, our findings correlate well with our observations associating high VEGF_{165b} protein levels with lymph node metastases in NSCLC. The data also suggested that NSCLC patients with high VEGF_{165b} levels could be less sensitive to bevacizumab, whereas bevacizumab could contribute to the emergence of more invasive tumor cells in patients displaying lower VEGF_{165b} levels. Therefore, the balance between VEGF_{xxx} and VEGF_{xxx}b isoforms also controls the autocrine loops potentially involved in tumor responses to anti-angiogenic therapies.

Taken together, all these studies provide evidence that both VEGF-A-dependent paracrine and autocrine loops must be taken into account when investigating the overall response to anti-angiogenic therapies. In addition, until more details are known regarding the role and expression of distinct VEGF-A splice variants, it should be considered that the impact of anti-angiogenic therapies could be drastically different, even deleterious, depending on the tumor type and/or which VEGF-A splice variant is expressed.

6.2.5 Pre-mRNA Splicing: A Reservoir of Biomarkers for Anti-Angiogenic Treatments

In addition to VEGF-A, many proteins involved in the angiogenic cascade are alternatively spliced isoforms. As an example, the pre-mRNA alternative splicing of VEGFR1, VEGFR2, or NRP1 generates soluble splice receptors that can, or are predicted to, act as natural inhibitors. Consistent with a role of these splice variants in responses to anti-angiogenic treatments, posttreatment changes in plasma sVEGFR2 were significantly correlated with tumor shrinkage in NSCLC patients treated with pazopanib (Nikolinakos et al. 2010). In addition, sVEGFR1 plasma levels were inversely correlated with the outcome of patients treated with either bevacizumab or anti-VEGFR TKIs in rectal, metastatic colorectal, and breast cancers and hepatocellular carcinoma (Lambrechts et al. 2013). Conversely, low intratumoral VEGFR1 expression was found to correlate with improved progression-free or overall survival after bevacizumab delivery in colorectal and gastric carcinomas, respectively. Therefore, it appears that a switch in *VEGFR1* pre-mRNA splicing occurs in tumors and could play a role in the response to anti-angiogenic therapies. Such an effect could be related to a recent study demonstrating that VEGF-A itself induces the expression of sVEGFR1 while leaving expression of the full-length VEGFR1 unchanged in human vascular endothelial cells via a mechanism involving the VEGFR2 protein kinase cMEK signaling pathway (Saito et al. 2013). Although further analyses dissecting the role played by these splice variants during the response to anti-angiogenic therapies are needed, it is likely that pre-mRNA splicing will highlight new potential biomarkers in the future (Fig. 6.4).

6.2.6 Combining Anti-Angiogenic Therapies with Drugs Targeting the Pre-mRNA Splicing Machinery: New Therapeutic Strategies in the Future

Based on the above data, one might propose that novel therapeutic strategies combining both anti-angiogenic therapies with drugs targeting the pre-mRNA splicing machinery should be developed (Fig 6.4). SRPK inhibitors are among these new drug candidates, as their use is thought to reduce the synthesis of pro-angiogenic isoforms of VEGF-A, consequently decreasing angiogenesis. Consistent with such a hypothesis, selective SRPK inhibitors reduce choroidal neovascularization in vivo when administered topically (eye drop) in rats and mice with exudative age-related macular degeneration (wet AMD) (Gammons et al. 2013). In addition, the injection of SRPIN340, an SRPK1/2 inhibitor, reduced angiogenesis in a mouse model of retinal neovascularization (Nowak et al. 2010). SRPKs could also be a good target in cancer as they are overexpressed in many tumor types compared with normal tissues, including NSCLC (Gout et al. 2012). Indeed, SRPK1 inhibition prevented angiogenesis and associated tumor growth in Wilm's tumors (Amin et al. 2011). Although decreasing the expression of pro-angiogenic VEGF_{xxx} isoforms while increasing that of anti-angiogenic

VEGF_{xxx}b might not always be beneficial (see 6.2.4), SRPK inhibitors could cooperate with anti-angiogenic therapies to prevent neovascularization and tumor growth in certain tumor types. More generally, pharmacological inhibitors of other components of the spliceosome are currently tested in cancer, one of which, spliceostatin A, was found to block angiogenesis by reducing the total amount of VEGF transcript (Furumai et al. 2010). Therefore, additional experiments are needed to select the most appropriate drug and combination. In addition, further experiments are needed to improve our fundamental knowledge regarding the contribution of pre-mRNA splicing to angiogenesis and response to anti-angiogenic therapies. To this end, the development of new tools/assays that recognize specific VEGF-A splice variants is crucial to clarify what, if any, role distinct VEGF-A splice isoforms have during the tumorigenic process and the response to anti-angiogenic therapies.

Figure captions

Fig. 6.1 **a** The human *VEGF-A* gene structure, consisting of eight exons and seven introns. **b** Focus on the C-terminal exon 8 of *VEGF-A*, the splicing of which generates either the VEGF_{xxx} or VEGF_{xxx}b sub-family. The proximal splice site (PSS) and distal splice site (DSS) are indicated. The nucleotide and amino acid sequences for exons 8a and 8b are depicted. **c** Illustration of all VEGF-A splice variants in each sub-family. The extracellular matrix affinity of the corresponding VEGF-A isoforms is indicated.

Fig. 6.2 Scheme of the functional domains of the VEGF-A protein encoded by each exon. The basic residues that confer affinity to heparin-containing proteoglycans are indicated. Because VEGF₁₆₅b does not bind heparin, it has been proposed that exon 8b could exert a negative effect on heparin binding. Alternatively, exon 8a is required for heparin binding. Exons 3 and 4 encode VEGFR1 and VEGFR2 binding domains. Exons 5, 7, and 8a encode sequences that are required for VEGF-A binding to neuropilins. As a consequence, VEGF_{xxx}b isoforms do not bind neuropilins.

Fig. 6.3 **a** The upstream stimuli that have been shown to modify the balance between VEGF_{xxx} and VEGF_{xxx}b isoforms. **b** The cellular signaling pathways that affect the choice between the proximal splice site (PSS) and distal splice site (DSS) in exon 8, thereby allowing the expression of either the VEGF_{xxx} or VEGF_{xxx}b splice variant. The SRSF1 protein favors PSS selection, whereas the SRSF6 or SRSF2 protein favors DSS. The potential link between RNA and DNA machineries are illustrated. In this setting, transcription factors, such as E2F1, could cooperate with SR proteins to control pre-mRNA splicing. In addition, the elongation rate of DNA polymerase II could also affect pre-mRNA splicing. TF: transcription factor.

Fig. 6.4 Relationships between pre-mRNA splicing and the response to anti-angiogenic therapies. Pre-mRNA splicing is proposed as a reservoir for predictive biomarkers. New therapeutic strategies combining both anti-angiogenic therapies and drugs targeting the pre-mRNA splicing machineries are illustrated as such a combination that could modulate the expression level of these pre-mRNA splice variants and thereby improve patient outcomes.

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