

Autocrine functions of VEGF in breast tumor cells

Adhesion, survival, migration and invasion

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Vascular endothelial growth factor A (VEGF-A) is well known for its key roles in blood vessel growth. Although most studies on VEGF and VEGF receptors have been focused on their functions in angiogenesis and in endothelial cells, the role of VEGF in cancer biology appears as an emerging area of importance. In this context, the presence of VEGF receptors in tumor cells strongly suggests that VEGF-A also promotes a wide range of functions, both in vitro and in vivo, all autocrine functions on tumor cells, including adhesion, survival, migration and invasion. Ultimately, refining our knowledge of VEGF signaling pathways in tumor cells should help us to understand why the current used treatments targeting the VEGF pathway in cancer are not universally effective in inhibiting metastasis tumors, and it should also provide new avenues for future therapies.

Vascular endothelial growth factor (VEGF-A) is a key mediator of angiogenesis, the effects of which on cancer growth and development have been well characterized.^{1,2} Alternative splicing of VEGF-A leads to several different isoforms, which are differentially expressed in various tumor types and have distinct functions in tumor blood vessel formation. Angiogenic isoforms are known as VEGF120, VEGF144, VEGF164 and VEGF188 in mice, and VEGF121, VEGF145, VEGF165 and VEGF189 in humans.³ Depending on the presence of genomic exons 6 and 7, these isoforms are either secreted as soluble forms (VEGF121 and VEGF165) or remain cell- or matrix-associated (VEGF189, VEGF206 and partially VEGF165).^{4,5} VEGF165 and VEGF121 are the most abundant isoforms and have been extensively studied. VEGF189 shows the strongest association to the cell membrane of all human VEGF isoforms. VEGF189 can also be cleaved in vitro into shorter bioactive forms by proteases, such as plasmin, urokinase plasminogen activator and MMPs,^{6–8} suggesting that native or cleaved proteins may have distinct roles.

Cellular responses to VEGF165 are mediated by two high-affinity type III tyrosine kinase receptors, VEGF-R2 (KDR/Flk1) and VEGF-R1 (Flt-1), and two receptors of the semaphorin receptor family, neuropilin-1 and neuropilin-2.^{9,10} Initially, VEGF-A (VEGF165) was believed to act exclusively on

endothelial cells through VEGF-R1 and VEGF-R2. VEGF-R2 is the main signaling VEGF receptor in endothelial cells, but it has been shown that its activity is balanced by VEGF-R1, which sequesters excess VEGF through its extracellular domain.^{11,12} VEGF165 and VEGF189 isoforms have different binding affinities for the VEGF receptors.¹³ Native VEGF189 binds preferentially to VEGF-R1 and NRP1,¹³ raising the possibility that specific functions may be driven by different isoforms in physiology or in pathology.^{13–16}

VEGF receptors are not restricted to vascular endothelial cells, and are also present in neurons, retinal epithelium, smooth muscle cells and tumor cells. The identification of VEGF receptor expression in a variety of solid tumors and in tumor cell lines has generated interest and autocrine functions of VEGF may be hypothesized in cancer.^{17–21} MCF-7 cells express VEGF-R1 and VEGF-R2. However, VEGFR1/2 expression is not always detectable in breast tumor cells. Moderate levels of VEGF-R1/Flt-1 and low levels of VEGF-R2/Flk-1/KDR mRNAs are present in a variety of breast cancer cell lines. In addition, neuropilins (NRP1 or NRP2) are expressed at high level in breast tumor cells (MDA-MB-231 and MDA-MB-435, respectively).^{13,17,20,22}

In the present review, we report that VEGF-A expressed by different types of cancer cells acts through an autocrine signaling pathway mediated by VEGF receptors and/or NRP1 to promote tumorigenesis. VEGF can promote proliferation, survival, adhesion, migration and chemotaxis of breast cancer cells, independently from angiogenesis.

Autocrine Functions of VEGF in Breast Tumor Cells

Proliferation. Several studies demonstrate that VEGF-A induces tumor cell proliferation in mice models of breast cancer; increased tumor proliferation is observed in transgenic mice with VEGF165 targeted to mammary epithelial cells under the control of mouse mammary tumor virus (MMTV) promoter²³ or in xenograft mice model generated by the injection of MDA-MB-231 tumor cells transfected with a VEGF165 or VEGF189 plasmid under a bicistronic eukaryotic vector.¹³ In vitro, increased proliferation is observed in VEGF165- or VEGF189-overexpressing MDA-MB-231 cells, as compared with control cells.¹³ VEGF also induces cell proliferation in vitro in T47D cells.²³ MCF-7 breast cancer cells are estrogen (E2)-sensitive cells and E2-dependent for their proliferation. Estrogen signaling increases VEGF expression in MCF-7 cells, or MDA-MB231 transfected with ER α ,

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through an estrogen-responsive element within the VEGF promoter.²⁴ This effect is inhibited by 4-OH-tamoxifen (4-OH-T). Interestingly, higher VEGF secretion and increased VEGF-R2 signaling are observed in Tamoxifen-resistant cells compared with sensitive MCF-7 one.²⁵ Pharmacological inhibition of p38 kinase in combination with 4-OH-T gave an additive effect on inhibition of proliferation in tamoxifen-resistant cells, demonstrating an increased autocrine VEGF/VEGFR2 signaling through p38 mitogen-activated kinase in tamoxifen-resistant cells.²⁵

Survival. VEGF is an autocrine survival factor for breast cancer cells.^{20,26-30} Several mechanisms have been proposed to support VEGF-induced survival in breast cancer cells. In normoxia and hypoxia, VEGF blockade using VEGF neutralizing antibodies or siRNA VEGF resulted in direct tumor cell apoptosis, implicating the PI3K pathway, downregulation of Bcl-2 expression and increase of the pro-apoptotic protein Bad.^{20,26,27} In some studies, VEGF signaling has been shown to induce the survival of tumor cells through VEGF-R1 or VEGF-R2.^{23,29} A specifically targeted knockdown of VEGF-R1 expression by siRNA (siVEGF-R1) in MCF-7 or MDA-MB-231 cells significantly decreases the survival of breast cancer cells through downregulation of protein kinase B (AKT) phosphorylation, while a targeted knockdown of VEGF-R2 or NRP1 expression has no effect on the survival of these cancer cells.²⁹ Specifically, VEGFR1 was predominantly expressed internally, mainly in the nuclear envelope of breast cancer cell lines,²⁹ suggesting that VEGF mediate intracrine survival in breast carcinoma cells through internally expressed VEGF-R1/FLT1. The role of neuropilin 1 in the survival of breast cancer cells (MDA-MB-231) was also described by several groups,^{20,30} and VEGF was shown to promote breast carcinoma survival by stimulating the PI3-kinase pathway.²⁰ The role of NRP1 will be discussed in the next chapter due to the multifaceted role of neuropilins (see below). In our study,³⁰ we also compared the role of different VEGF isoforms on the survival of MDA-MB-231. Contrasting roles of VEGF165 and VEGF189 isoforms were found to affect tumor survival. Unexpectedly, we found that VEGF189 reduced survival in stress conditions as compared with VEGF165,³⁰ and the mechanisms remain to be established. Recently, the role of integrins was analyzed in several studies.^{31,32} Chung and Mercurio³¹ have reported that hypoxia-induced protection from apoptosis via VEGF was dependent on $\alpha 6\beta 1$ integrins in MDA-MB-435 breast cancer cells. In three dimensional cultures, a $\beta 1$ integrin inhibitory antibody induces apoptosis of breast cancer cells and inhibits tumor growth.³² Thus, the mechanisms of tumor survival, which is a fundamental process in metastasis, are probably heterogeneous depending on the nature of the tumor cell and/or VEGF receptors present in these cells.

Cell adhesion. The existence of multiple VEGF isoforms exhibiting different binding affinities toward heparin sulfate raises the possibility that individual isoforms may differently affect cell adhesion. Hutchings and Plouët³³ showed that endothelial cells could adhere and spread on VEGF189 and VEGF165, but not on VEGF121. Adhesion was mediated by the $\alpha 3\beta 1$ and $\alpha v\beta 3$ integrins and other αv integrins but not by the cognate VEGF receptors.³³ Using stable human breast carcinoma cells (MDA-MB-231) which overexpress VEGF165

or VEGF189 isoforms, we reported an increase of the adhesion of VEGF165-overexpressing MDA-MB-231, as compared with control cells; however, the adhesion of VEGF189-overexpressing MDA-MB-231 cells on fibronectin and vitronectin was increased as compared with VEGF165-overexpressing MDA-MB-231.¹³ Neutralizing antibodies against $\alpha 5\beta 1$, but not $\alpha v\beta 3$ and $\alpha v\beta 5$, significantly inhibited the adhesion of V189 clones to fibronectin.¹³ Furthermore, the adhesion of V189 to vitronectin was significantly reduced by neutralizing antibodies against anti- $\alpha v\beta 5$, but not by anti- $\alpha 5\beta 1$ antibodies. The role of NRP1 in the VEGF-induced adhesion of MDA-MB-231 cells was suggested in our studies.¹³ Recently, a new VEGF-NRP2 signaling pathway was described in MDA-MB-435 cells;³⁴ VEGF165-NRP2 activates the $\alpha 6\beta 1$ integrin, making it able to form focal adhesions on laminin.³⁴

Migration and invasion. An autocrine loop exists for VEGF to induce breast cancer cell migration/invasion. MCF-7 cells that are not a tumorigenic breast cancer cell line without estrogen supplementation and do not induce metastasis in mice have a low capacity of migration in vitro. MCF-7 cells express lower levels of VEGF than MDA-MB-231 cells which have high invasive and migration capacities. The first experiments demonstrating the autocrine effect of VEGF165 in the migration or invasion were described for MDA-MB-231 and T47D breast cancer cell lines.^{18,35} The expression of VEGF and its receptor NRP1 in MDA-MB-231 was correlated with the aggressiveness of the cells.³⁵ This invasive property involves NRP1 but not VEGF-R2, which is poorly expressed in these cells.³⁵ A possible mechanism is a competition between VEGF and Semaphorin 3A (SEMA 3A) for NRP1 in this cell line, as shown in experiments in which the protein levels of SEMA3A and VEGF in breast cancer cell lines were correlated with the chemotactic activity of the cells.³⁵ Cells with the highest SEMA3A/VEGF protein ratio exhibited the lowest migration rate. In contrast, those that had the lowest ratio of SEMA3A/VEGF protein exhibited the highest migration rates. Other experiments have shown that the expression of VEGF induced the expression of CXCR4 which is responsible for the invasiveness.

In our laboratory we isolated two subpopulations from the MDA-MB-231 parental cells through matrigel in vitro. In this way, we could isolate one population with poor invasive capacity (REF cells) from another with high capacity (INV cells). The characteristics of the invasive cells were a loss of extracellular matrix and endothelial attachment, an increase in survival and in metastasis colonization in nude mice model.³⁷ The analysis of the genes differently regulated by these two cell lines revealed that the more invasive cells (INV) expressed VEGF more than 2-fold, as compared with REF cells. Also we could demonstrate by western blot analysis that the expression of the NRP1 receptors in the invasive cells was increased. However, we could not demonstrate an activation of CXCR4 in these cells. Also, we could not see differences in AKT activation between INV and REF cells.³⁷ Cell migration and invasion result from a balance between cell adhesion and detachment, both of which are required for motility. If the adhesion force is too strong, the cells attach tightly to the substrate and are unable to disperse.

Indirectly, VEGF-induced migration has also been reported to be mediated by Snail, a transcription repressor of E-cadherin.³⁸ VEGF and NRP1 increase snail expression by suppressing the activity of glycogen synthase kinase 3 (GSK3), a negative regulator of Snail. The subsequent attenuation of E-cadherin expression favors the emergence of a more invasive EMT phenotype.³⁸ The autocrine pathway of VEGF in migration was also described for others cancer cell types. For example VEGF via VEGF-R2 followed by the activation of ras/ERK1 pathway increased the migration of PC3 prostate cell line.³⁹ VEGF has been shown to increase the migration of hepatocarcinoma cells,⁴⁰ and skin cancer cells.⁴¹ In conclusion, it seems clear that an autocrine loop exists for VEGF to induce cancer cell migration/invasion, but further experiments are required to understand the pathway(s) involved in the autocrine stimulation of migration/ invasion by VEGF.

Mechanisms of Autocrine Function in Cancer Cells: Role of Neuropilins

Neuropilin (NRP)-1 is a transmembrane protein expressed by endothelial cells and several other cell types, such as dendritic cells and malignant tumor cells. It has been shown that NRP1 regulates both endothelial cells and tumor cell functions. NRP1 is an essential receptor for angiogenesis. In cultured endothelial cells, NRP1 enhances VEGF-R2 signaling by binding to VEGF165 and promoting a complex formation between the three molecules.^{42,43} In vivo blockade of neuropilin-1 function induced an effect additive to that of anti-VEGF to inhibit tumor growth.⁴⁴ NRP2 shares many properties with NRP-1. The binding of NRP2 with VEGFR-2 and VEGFR-3 has also been reported to promote endothelial cell survival and migration.⁴⁵ Blockade of NRP2 function inhibits tumor cell metastasis.⁴⁶

NRP1 and NRP2, which are multifunctional proteins frequently expressed by cancer cells, have been linked to tumor progression^{47,48} or metastasis.⁴⁶ Evidence indicates that NRPs may contribute to the function of cancer cells by several specific mechanisms. NRP1 or NRP2 are strongly expressed in aggressive breast cancer cells, MDA-MB-231 or MDA-MB-435 respectively. The increase of NRP in tumor cells was found to correlate with survival, migration and chemoresistance.⁴⁹ However, the complexity of NRP-VEGF interactions is underlined by the fact that NRP1 binds members of two different ligand families: the VEGF family and the class 3 semaphorin family (Sema 3A and Sema3B) of axonal guidance regulators.⁵⁰ The multifaceted role of neuropilins in cancer has been reviewed recently by Ellis⁴⁷ and Tamagnone.⁴⁸

Neuropilins as VEGF receptors. Using the BIACore system, we recently demonstrated that both VEGF165 and 189 binds directly to neuropilin-1.¹³ VEGF189 binds more strongly to NRP1 than VEGF165.¹³ In contrast, VEGF121 does not bind to NRP1. Thus, NRP1 appears as a receptor that recognized the heparin-binding VEGF splice forms. The binding of VEGF189 to NRP2 has not been reported until now. NRP1 was initially reported to bind to the exon 7-encoded region of VEGF-A (164/165). However, analysis of the crystal structure of

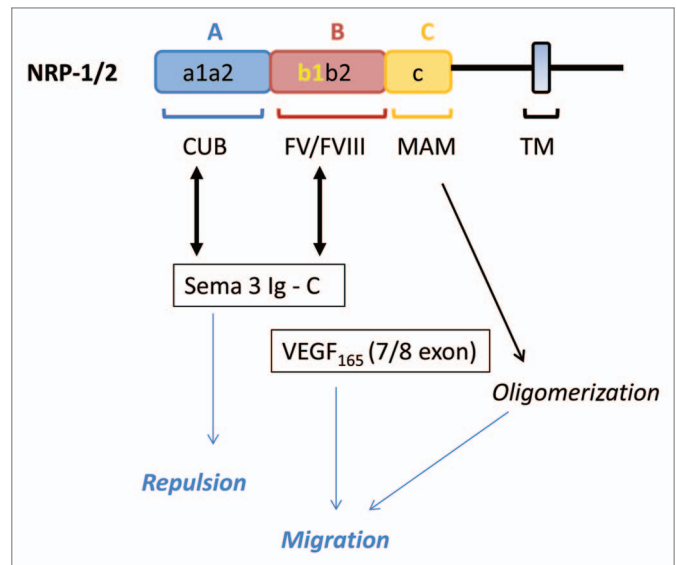


Figure 1. Potential complexes of VEGF and SEMA class 3 with NRPs. Electronegative charges in the B domain interact with both basic region in C and exon 7/8 domains of Sema 3 and VEGF, respectively. Semaphorins class 3 also interact with the a1/a2 domain via the Sema 3 and Ig sequences. The MAM domain induces oligomerization that is important to induce biological activities of both Sema and VEGF.

the exon 7/8-encoded VEGF-A heparin binding domain in complex revealed that exon 8-encoded residues within the NRP1-b1 domain are implicated.⁵¹ The two neuropilins have a similar domain structure, containing an A domain (containing a1/a2), a B domain (containing b1/b2), a C domain that is thought to be possibly important for the interaction of NRP to other receptors and a transmembrane domain that has recently been shown to be important for homodimerization of neuropilins. The b1 domain of NRP1 is an essential binding site for the VEGF165 ligand (Fig. 1), the b2 domain being required for optimal binding. In addition, VEGF-164 binds 50-fold more strongly to NRP1 than NRP2.⁵² Differences in the amino acid composition of NRP L1 loop are responsible for the different affinities of VEGF for NRP1/NRP2; direct repulsion between the electronegative exon 7-encoded residues of the heparin binding domain and the electronegative L1 loop found only in NRP2 significantly contributes to the observed selectivity.⁵² Following the characterization of NRP as a VEGF receptor (Fig. 2B), it was found that NRPs are also able to function as receptors for the VEGF family members placental growth factor 2 (PlGF2), VEGF-B or VEGF-E.

NRP1 as a major regulator of VEGF and class 3 semaphorin (SEMA3) signaling. As previously mentioned, VEGF165 exerts an autocrine effect, including survival and chemotaxis, on MDA-MB-231 breast cancer cells through neuropilin-1. It has been shown that competing autocrine pathway involving alternative neuropilin-1 ligands regulates chemotaxis or survival of carcinoma cells. Secreted semaphorins can exert either inhibitory or stimulatory functions in multiple tumor cell types.^{53,54} For example, increasing VEGF165 could modulate SEMA 3A signaling pathway, leading to increased chemotaxis.⁵⁵ Reducing SEMA 3A and NRP1 expression by RNA interference signaling enhanced

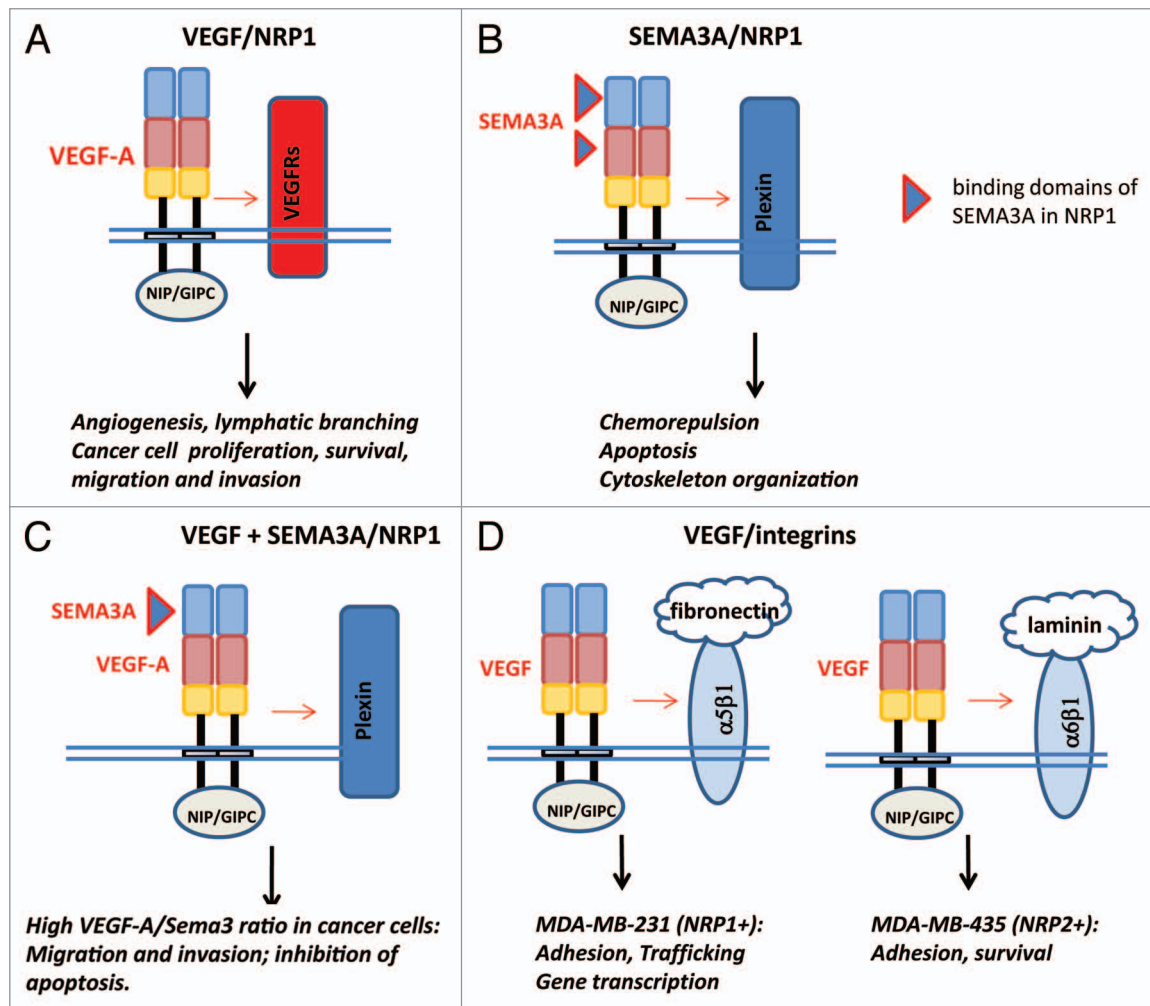


Figure 2. Schematic representation of molecular complexes involving neuropilins of the plasma membrane of cancer and/or endothelial cells. (A) Interaction between VEGF and neuropilin/VEGF receptor complexes; (B) interaction between class3 semaphorin and neuropilin/plexin complexes; (D) interaction between VEGF and neuropilin/integrin complexes. Oligomerization of NRPs molecule is induced by VEGF or SEMA3A as mentioned in Figure 1 via MAM domain (yellow box). NRPs forms complex with VEGFR (A) or plexin (B) to induce angiogenesis or repulsion and apoptosis. (C) Competition between VEGF and class3 semaphorin for neuropilin binding and activation of signaling pathway. Competition of VEGF and SEMA3A occurs at the level of b1 domain in NRP1. This mechanism is particularly relevant in tumor cell migration. (D) NRPs also participate in cell adhesion via $\alpha 5 \beta 1$, $\alpha \nu \beta 5$ and $\alpha 6 \beta 1$ integrins. NRPs can also induce independent activities via interaction of the SEA domain with NIP/GIPs.

migration.⁵⁵ In another study, SEMA 3B has been reported to induce apoptosis in lung and breast cancer, whereas VEGF165 antagonizes this effect.⁵⁵

Class 3 semaphorins are the only secreted semaphorins, while the others are membrane bound. The main receptors for semaphorins are the plexins. However, a subset of the secreted semaphorins requires the presence of neuropilins to bind to Plexin receptors (Fig. 2A). Semaphorins class 3 molecules interact with the A domain (containing $\alpha 1/\alpha 2$) of NRP-1 and NRP-2 (see review in ref. 48). In addition, the b1 domain mediates the high affinity of NRPs to the basic domain of Sema3 proteins.⁵⁶⁻⁵⁹ SEMA 3A and VEGF (165) have a common binding region in the b1 domain of NRP-1 via their C-terminal basic sequences (Ig domain for SEMA, c.f. Fig. 1). These two ligands can compete for their binding to the b1 domain of NRPs^{60,61} (Fig. 2C). While VEGF-A preferentially binds NRP1 rather than NRP2,⁵²

semaphorins do not display the NRP-specific binding to the b1 domain. Furthermore, the proteolytic protein furin processes SEMA class 3 molecules to liberate the basic region recognized by NRP-1/2 binding domains. The furin-dependent proteolytic processing of Sema3 is important to allow the exposure of the NRP1-binding C-terminal sequence of Sema3 and to regulate the chemorepulsive activity of secreted semaphorins.^{62,63} Cell and tissue-specific proteolytic processing of semaphorin family members may thus represent an important mechanism controlling the production of repellent migration and anti-angiogenic activity of semaphorins.

NRP-integrin signaling. NRP was originally identified as a surface protein mediating cell adhesion.⁶⁴ The possibility that NRP1/2 influences the activation and function of specific integrins [$\alpha 5 \beta 1$ (fibronectin), $\alpha \nu \beta 5$ (vitronectin) and $\alpha 6 \beta 1$ (laminin)] to contribute to tumor adhesion has been mentioned above.

Autocrine VEGF is also necessary for the survival of serum-deprived cells through $\alpha 6\beta 1^{31}$ (Fig. 2D).

NRP co-receptors for other receptors. NRP forms complexes with additional cell surface receptors. As an example, NRP1 binds latent and active transforming growth factor (TGF)- $\beta 1$, and activates the latent form latency-associated peptide (LAP)-TGF- $\beta 1$ in some breast cancer cells.⁶⁵ Since TGF- β promotes metastasis, this finding is highly relevant to cancer biology.

NRP-synectin binding. There is evidence that the neuropilins may function independently of tyrosine kinase VEGF receptors. NRP have short intracellular domains that were considered to be too short to support independent signal transduction. Indeed, NRP1 possesses an active cytoplasmic motif (C-terminal three amino-acid), known as SEA, which promotes its association by bridging to an adaptor protein, the PDZ protein of synectin (also known as GIPC1/NIP).⁶⁶⁻⁶⁹ This interaction was first shown to mediate VEGF/NRP1-dependent endothelial migration in angiogenesis. Neuropilin is not only subjected to endocytosis upon ligand binding, but is also implicated to regulate the trafficking of associated molecules at the cell surface, such as the vesicular trafficking of integrin $\alpha 5\beta 1^{70,71}$ (Fig. 2D). The SEA motif also promotes fibronectin assembly *in vitro*. Altogether, these findings support the hypothesis that the activation state of the VEGF-NRP signaling is tightly regulated by autocrine and paracrine factors present in the tumor environment.

Conclusions and Perspectives

In conclusion, *in vitro* data and transgenic models have enabled an evaluation of the various effects of VEGF-A on mammary tumor development and metastasis. VEGF-A(164/189) involves both blood vessels and tumor cells to increase neovascularization and vasodilation/vessel maturation, and acts by selective autocrine effects to stimulate tumor cell proliferation, survival, adhesion and chemotaxis. The production of VEGF by breast tumor cells, and the activation of VEGF receptors at the surface of cancer cells indicate the existence of a distinct autocrine signaling loop that enables breast cancer cells to promote their own growth, survival and migration by phosphorylation and activation of VEGFR-1/2 or VEGF-induced NRP signaling.

Understanding the mechanisms that mediate autocrine and paracrine VEGF/VEGFR/NRP signaling is of primary

importance due to the growing therapeutic use of cancer inhibitors.⁷² The monoclonal anti-VEGF antibody bevacizumab prevents VEGF binding to the VEGFR-1/2 receptor with successful inhibition of tumor angiogenesis and shows improvement in some metastatic cancers, but not in others. The negative response to angiogenic therapies might be due to an acquired resistance of tumor cells themselves to anti-VEGF, or by recruitment of circulating endothelial progenitors. Cancer stem cells (CSCs) have been described in various cancers. Using a mouse model of skin tumors, Beck et al.⁴¹ have investigated the impact of the vascular niche and VEGF signaling on controlling the “stemness” (the ability to self-renew and differentiate) of cancer cells (CSCs) during the early stages of tumor progression. CSCs of skin papillomas are localized in a perivascular niche, in the immediate vicinity of endothelial cells. Furthermore, blocking VEGF-R2 induces tumor regression not only by decreasing the microvascular density, but also by reducing CSC pool size and impairing CSC renewal properties. Thus, a vascular niche and a VEGF-NRP1 loop regulate the initiation and stemness of skin tumors. A direct autocrine effect of VEGF on tumor cells may account for the efficiency of VEGF-blockade therapies. However, the effects of targeting specific VEGF isoforms *in vivo* have received little attention in the clinical setting. The recent identification of novel splice variants of VEGF with anti-angiogenic properties (VEGFb) provided some insight on the lack of current treatment efficacy.^{73,74} The selective effects of VEGFb isoforms on breast cancer cells, as compared with pro-angiogenic isoforms, are unknown. However, another type of resistance could be caused by increased VEGF/VEGFR2 signaling or VEGF/NRP signaling. An increased autocrine VEGF/VEGFR2 signaling through p38 mitogen-activated kinase has been reported in tamoxifen-resistant MCF7 cells.²⁵ High levels of NRP1 were significantly associated with chemoresistance and a poor outcome in patients with breast cancers,⁴⁹ and NRP2 expression correlates with poor prognosis and lymph node metastasis formation. In conclusion, the acknowledgment of the VEGF autocrine pathway provides a new insight in cancer therapy and has to be investigated in the future. This autocrine loop represents an attractive therapeutic target.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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