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Non-Angiogenic Functions of VEGF in Breast Cancer

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Abstract This review advances the hypothesis that the function of vascular endothelial growth factor (VEGF) in breast cancer is not limited to angiogenesis, and that VEGF signaling in breast carcinoma cells is important for the ability of these cells to evade apoptosis and progress towards invasive and metastatic disease. In other terms, VEGF signaling provides a selective advantage for the survival and dissemination of breast carcinoma cells that may be independent of angiogenesis. The key component of this hypothesis is that breast carcinoma cells express specific VEGF receptors and that these receptors respond to autocrine VEGF, resulting in the activation of signaling pathways that impede apoptosis and promote cell migration. A related hypothesis, which is developed in this review, is that the $\alpha 6\beta 4$ integrin, which has been implicated in the survival and motility of breast cancer cells, can stimulate the translation of VEGF mRNA and, consequently, autocrine VEGF signaling. These findings imply that VEGF and VEGF receptor-based therapeutics, in addition to targeting angiogenesis, may also target tumor cells directly.

Keywords VEGF · Breast cancer · Integrin

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Introduction

Vascular endothelial growth factor (VEGF) exerts multiple effects on tumors that include stimulating the formation of new blood vessels and lymphatics, as well as increasing vascular permeability [1]. Indeed, the contribution of VEGF to vascular and lymph angiogenesis has dominated the study of this growth factor in breast and other cancers. Undoubtedly, this function of VEGF is essential for some aspects of tumor behavior. However, the fact that most tumors contain hypoxic pockets [2] implies that angiogenesis induced by VEGF in tumors is not sufficient to mitigate hypoxia. In this context, hypoxia provides a strong selective pressure for the survival of the most aggressive and metastatic cells. An understanding of the mechanisms that enable tumor cells to survive in hypoxia, therefore, is essential for deciphering the biology of cancer progression and for designing therapeutic interventions. One mechanism that has emerged from our work and that of other labs is that VEGF produced by either tumor or stromal cells engages VEGF receptors on tumor cells and initiates a signaling response that facilitates survival in hypoxia, as well as in response to other apoptotic stimuli [3–5]. Such a mechanism, which probably functions in concert with p53 inactivation, provides tumor cells with a degree of self-sufficiency that could facilitate their ability to form tumors and increase the probability that these tumors progress to a metastatic state. In other terms, we hypothesize that hypoxia selects for the survival of cells that are competent for VEGF signaling, and that the most aggressive tumor cells (metastatic cells) are characterized by their dependency on VEGF. A secondary consequence of VEGF signaling in breast tumor cells is that it can facilitate their ability to migrate and invade.

An important implication of the work to be discussed is that it challenges the notion that the function of VEGF in cancer is limited to angiogenesis and that therapeutic approaches based on the inhibition of VEGF function target

only angiogenesis. In fact, adjuvant therapy aimed at targeting either VEGF or VEGF receptors is showing progress in clinical trials [6, 7] and it is likely that such therapy is targeting VEGF signaling in tumor cells. For this reason, a rigorous understanding of the mechanisms used in this signaling and its relevance to breast cancer is essential.

VEGF Signaling in Breast Carcinoma Cells

Autocrine VEGF Survival Signaling

The complex microenvironment of solid tumors implies that tumor cells receive signals from multiple sources and, conversely, that they influence the function of other cells. Within this meshwork of paracrine signaling, however, it is becoming more apparent that tumor cells can acquire a certain degree of self-sufficiency by elaborating autocrine signaling pathways that facilitate key functions of growth, survival and invasion [8]. Autocrine pathways are more significant as tumors progress towards invasive and metastatic disease because the environment of such tumors is increasingly hostile. As such, autocrine signaling pathways represent a prime target for therapy aimed at impeding tumor dissemination. One of the first indications that VEGF may exhibit autocrine functions in carcinoma was provided by our study on invasive breast carcinoma cell lines [3]. Using an antisense oligonucleotide approach to reduce VEGF expression, we observed that an approximate 50% reduction in VEGF expression resulted in a significant increase in apoptosis, even in the presence of 10% serum. This finding supports the hypothesis that such cells had been selected *in vivo* for their dependency on VEGF as a survival factor. Subsequent studies by our own group and others have confirmed the importance of VEGF for the survival of carcinoma and other cancer cells [5, 9–11].

A pertinent observation is that the exposure of invasive breast carcinoma cell lines to hypoxia actually decreased apoptosis induced by serum starvation conditions because it increased VEGF expression [12]. The mechanism by which autocrine VEGF sustains survival of breast carcinoma cells appears to involve constitutive activation of the PI3-kinase pathway based on the findings that reducing VEGF expression results in a significant decrease in the basal activity of PI3-kinase, hypoxia stimulates Akt activity, and inhibition of PI3-kinase induces apoptosis [12]. Other work has demonstrated that VEGF can mitigate the apoptosis of breast carcinoma cells by inducing expression of the anti-apoptotic protein Bcl-2 [13].

Expression of VEGF Receptors on Breast Carcinoma Cells

VEGF signaling in breast carcinoma cells implies that such cells express receptors that mediate VEGF interactions. The classic, tyrosine kinase VEGF receptors, which have been characterized on endothelial cells, are VEGFR-1 (*flt*-1) and VEGFR-2 (KDR, *flk*-1) [14]. Although it had been assumed initially that these receptors are expressed only on endothelial cells, it has become apparent in recent years that their expression is more widespread. Surprisingly, however, the relative expression of these tyrosine kinase receptors on breast carcinoma cells and their putative functions have not been resolved adequately. There is some consensus that VEGFR-2 (KDR) is not expressed at significant levels on most human breast carcinoma cell lines and breast carcinomas [3, 4, 15, 16], although other reports challenge this view [17]. There is evidence, however, that murine VEGFR-2 expression is linked to the progression of MMTV-MT mammary tumors and that its down-regulation in tumor cells isolated from these mice results in decreased proliferation [18]. With regard to VEGFR-1, studies from our lab indicate that its mRNA and protein are expressed in several well-studied breast carcinoma cell lines, as well as in a subset of breast tumors (Weigand and Mercurio, unpublished). This finding meshes with data from gene profiling of breast tumors, which revealed that VEGFR-1 expression is associated with a ‘poor prognosis’ signature [19]. These findings highlight the need for more mechanistic studies on the contribution of tyrosine kinase VEGF receptors to breast carcinoma.

One key receptor that has been implicated in VEGF signaling in breast carcinoma is neuropilin-1 (NP-1) [3, 20, 21]. NP-1 was identified initially as a neuronal receptor for semaphorins, which are axon guidance factors that function primarily in the developing nervous system [22, 23]. Subsequent studies revealed that NP-1 is also expressed on other cell types including endothelial, hematopoietic, and carcinoma cells, and that on these cells it can function as a receptor for the $VEGF_{165}$ isoform [16]. Our studies using metastatic breast cancer cell lines implicated NP-1 in survival signaling by functioning as a receptor for autocrine $VEGF_{165}$ [3]. Ablation of NP-1 expression in breast carcinoma cell lines results in a significant increase in their apoptosis and expression of NP-1 in NP-1-deficient cells enables these cells to evade apoptosis in hypoxia [3]. A more recent study using a peptide that corresponds to the NP-1 binding site on $VEGF_{165}$ demonstrated that this peptide induced apoptosis in NP-1 expressing breast carcinoma cells [21]. In contrast, a peptide directed against KDR had no effect. Although these data implicate NP-1 as a mediator of VEGF signaling in breast carcinoma cells, this receptor lacks intrinsic signaling functions and, presumably, functions together with another receptor that remains to be identified (see below).

VEGF in Breast Carcinoma Migration and Invasion

Carcinoma cells acquire the ability to migrate and invade tissues as a function of malignant transformation and pro-

gression. Although environmental cues, such as gradients of chemoattractants, can promote carcinoma migration and invasion, it has become evident that the ability of cells to elaborate autocrine signaling pathways can enhance their response to external stimuli [24]. In this context, we were able to uncover a function for autocrine VEGF in the migration and invasion of breast carcinoma cells towards chemokines by depleting VEGF expression in the presence of caspase inhibitors, which prevent the apoptosis that results from loss of VEGF expression [12]. In such conditions, the ability of breast carcinoma cells to migrate and invade toward chemotactic stimuli is impaired significantly. One mechanism that accounts for the contribution of VEGF to these phenomena is that it can regulate the expression of the chemokine receptor CXCR4 [12]. This finding is relevant to breast cancer progression because stromal-derived factor-1, the ligand for this receptor, is present in tumor stroma and in tissues such as lymph and lung [25], which are the primary targets of invasive breast carcinoma cells, and CXCR4 inhibitors impair metastasis. Thus, in addition to its survival functions, VEGF autocrine signaling may contribute to tumor progression by inducing chemokine receptor expression, enabling tumor cells to migrate towards chemokine gradients.

Although chemoattractants such as stromal-derived factor-1 promote the directed migration of cells, it is becoming apparent that cell migration is also subject to negative regulation. A number of soluble members of the semaphorin family of axon guidance molecules promote growth cone collapse and axon repulsion [26]. Interestingly, this inhibitory pathway involves the semaphorin receptor, NP-1 [22, 23], which also supports VEGF autocrine signaling (see above). Given that breast carcinoma cells express NP-1, we hypothesized that an autocrine semaphorin 3A/NP-1 pathway may impede their migration. In fact, we established that breast carcinoma cell lines and primary tumors express semaphorin 3A and plexin A1, molecules necessary to support semaphorin autocrine signaling, and that the inhibition of this pathway enhances carcinoma migration [27]. Autocrine VEGF is important for breast carcinoma migration because it competes with semaphorin 3A for neuropilin-1 binding and overrides its migration suppressing activity. Interestingly, the concentration of VEGF relative to Sema3A in breast carcinoma cell lines increases proportionately with their chemotactic potential. Thus, in addition to its survival functions, autocrine VEGF could contribute to carcinoma progression by inhibiting the activity of an endogenous migration suppressor [27].

The ability of VEGF to antagonize semaphorin signaling may also impact the apoptotic potential of breast carcinoma cells. For example, semaphorin 3B is known to induce apoptosis of breast carcinoma cells and this effect can be antagonized by $VEGF_{165}$, which also competes with semaphorin 3B for binding to NP-1 [20]. One implication of these data is that the soluble semaphorins have tumor suppressive functions in their ability to stimulate apoptosis and impede migration.

The EMT and VEGF Signaling

The epithelial to mesenchymal transition (EMT) has become a useful paradigm for studying the genesis of invasive and metastatic carcinoma [28, 29]. The defining characteristic of an EMT is loss of expression or function of E-cadherin, and the consequent 'transdifferentiation' of epithelial cells into mesenchymal cells. Whether or not such a bona fide transdifferentiation occurs in cancer is a matter of current debate [30, 31], but it is evident that the loss of E-cadherin in breast carcinomas and the expression of 'mesenchymal' proteins such as N-cadherin are correlated with more aggressive disease [32]. Within this conceptual framework, an intriguing observation that emerges from an analysis of the existing data is that those cells that exhibit autocrine VEGF signaling and that depend upon VEGF for their survival tend to be cells that lack expression of E-cadherin, and exhibit properties associated with increased tumor aggressiveness [3, 27]. In contrast, E-cadherin positive cells do not appear to be as dependent on VEGF for their survival, and they exhibit a higher ratio of [semaphorin 3A] to [VEGF] than do E-cadherin negative cells [27]. Although the focus of the EMT and cancer has been on the acquisition of cell migration and invasion, the EMT may also contribute to the autonomous survival of carcinoma cells. Epithelial and highly differentiated carcinoma cells require cell–cell adhesion for their survival [5, 33]. The dissociation of these cells by calcium chelation, for example, can result in massive apoptosis. A *priori*, cells undergoing an EMT must either acquire mechanisms to facilitate their survival as single cells, or apoptose. We postulate that the acquisition of $VEGF_{165}$/NP-1 signaling is one mechanism for breast carcinoma cells to survive in response to an EMT. A key issue that derives from this hypothesis is how microenvironmental factors that promote an EMT regulate the expression of VEGF. To this end, we have shown that expression of the Ets-1 transcription factor is increased during the EMT of colon carcinoma and that it can regulate Flt-1 transcription [5].

Summary and Perspective

The data discussed support the hypothesis that VEGF signaling in breast carcinoma cells facilitates their ability to survive in the face of apoptotic stimuli and to migrate towards gradients of chemoattractants. It can also be inferred that those cells capable of VEGF signaling have a selective

advantage for survival in the tumor microenvironment and for progression to metastatic disease. An interesting twist to this hypothesis is that VEGF signaling may be manifested as a consequence of the EMT, but more data are needed on the EMT of breast carcinoma in this regard. Although these conclusions are attractive and compelling, they are based largely on *in vitro* data and they need to be validated using mouse models. In this direction, Schoeffner et al. reported that MMTV-MT mammary tumors that were derived from mice engineered to express human $VEGF_{165}$ in the mammary gland were more aggressive and metastatic than were control MMTV-MT tumors [18]. Analysis of these tumors revealed that expression of human $VEGF_{165}$ resulted in increased angiogenesis and increased tumor proliferation and survival. These data are provocative but they do not distinguish rigorously between the effects of $VEGF_{165}$ on tumor cells and endothelial cells. Targeted deletion of specific VEGF receptors on tumor cells should provide more definitive information.

The finding that breast carcinoma cells express VEGF receptors is significant but much more work needs to be done to assess the expression of these receptors as a function of transformation and progression, including the EMT, and to decipher the mechanisms by which these receptors influence tumor cell behavior. Arguably, NP-1 is the most interesting VEGF receptor on breast tumor cells, but little is known about how this receptor, which lacks intrinsic signaling functions, mediates $VEGF_{165}$ signaling. The literature on endothelial cells suggests that it may function in concert with either VEGFR1 or VEGFR2 [34, 35], but this possibility remains to be established for breast carcinoma cells. The alternative possibility is that NP-1 cooperates with non-VEGF receptors in carcinoma cells such as the plexins, which transmit NP-1 signals in neurons [36]. Indeed, our studies indicate that plexin A1 is expressed in breast carcinoma cells, and can influence cell migration [27]. Pursuit of the involvement of plexins in NP-1 signaling will require a much better understanding of plexin expression and function in breast, as well as in other cancers. Moreover, more rigorous data are needed on the localization and relative expression of NP-1 within the mammary gland, as well as in human breast tumors.

Studies on VEGF signaling in breast carcinoma cells, to date, have focused on VEGF-A and its isoforms, but other members of the VEGF family, notably VEGF-C and VEGF-D, have been implicated in angiogenesis and lymphangiogenesis in breast tumors [37, 38]. Clearly, the ability of breast carcinoma cells to respond to these VEGFs in either a paracrine or autocrine fashion should be studied. In this direction, there is some evidence that breast carcinoma cells can respond to VEGF-D in an autocrine fashion [39], but more data are needed to substantiate this possibility.

Regulation of VEGF Expression in Breast Carcinoma Cells by the α6β4 Integrin

An important issue that arises from the foregoing discussion is an understanding of the mechanisms that regulate VEGF expression in breast carcinoma cells. Such mechanisms are important not only for VEGF signaling in tumor cells, but also for the contribution of tumor-derived VEGF to angiogenesis and vascular permeability. Hypoxia is a potent inducer of VEGF transcription and mRNA stability [40, 41], but other factors can contribute to this process. This possibility is supported, for example, by the fact that many breast carcinoma cell lines produce copious amounts of VEGF in normoxic culture [3]. Moreover, as discussed above, the EMT can stimulate VEGF expression in the absence of hypoxia [5].

We have focused our efforts on the contribution of the α6β4 integrin to VEGF expression in breast carcinoma cells for several reasons. This integrin, which was characterized initially as laminin receptor expressed on basal epithelial cells and invasive carcinoma cells, plays a pivotal role in functions associated with carcinoma progression (reviewed in [42]). A key finding made by our group is that α6β4 can promote the survival of breast carcinoma cells in stress conditions [9, 43], a phenomenon that is linked to the ability of this integrin to activate the PI3-K/Akt pathway. An insightful extension of this work was the observation that α6β4-mediated survival is dependent on VEGF [9]. In cells in which VEGF expression had been reduced, α6β4 lost its ability to prevent apoptosis in stress conditions. More recently, studies employing three-dimensional cultures have substantiated the importance of α6β4 for the survival of breast carcinoma cells. Initially, it was reported that activation of α6β4 signaling in three-dimensional mammary epithelial cells conferred resistance to apoptosis by maintaining polarized tissue architecture [44]. Additional studies have suggested that α6β4 can mediate the anchorage-independent survival of malignant mammary epithelial cells by a mechanism that involves secretion of laminin-5 followed by ligation of α6β4 and subsequent activation of a Rac GTPase/NF-κB signaling pathway [45]. Although the involvement of VEGF was not examined in these studies, it is worth noting that VEGF can activate NF-κB [46], suggesting that the mechanisms that have been proposed for α6β4-mediated survival signaling are not mutually exclusive.

Our pursuit of the mechanism by which α6β4 may influence VEGF expression led to the finding that the expression and signaling properties of this integrin can stimulate the translation of VEGF mRNA. The mechanism involves the ability of this integrin to enhance the phosphorylation of 4E-Binding Protein (4E-BP1), a protein that binds and sequesters translation initiation factor eIF-4E [47]. Phosphorylation of 4E-BP1 by mTOR disrupts its binding

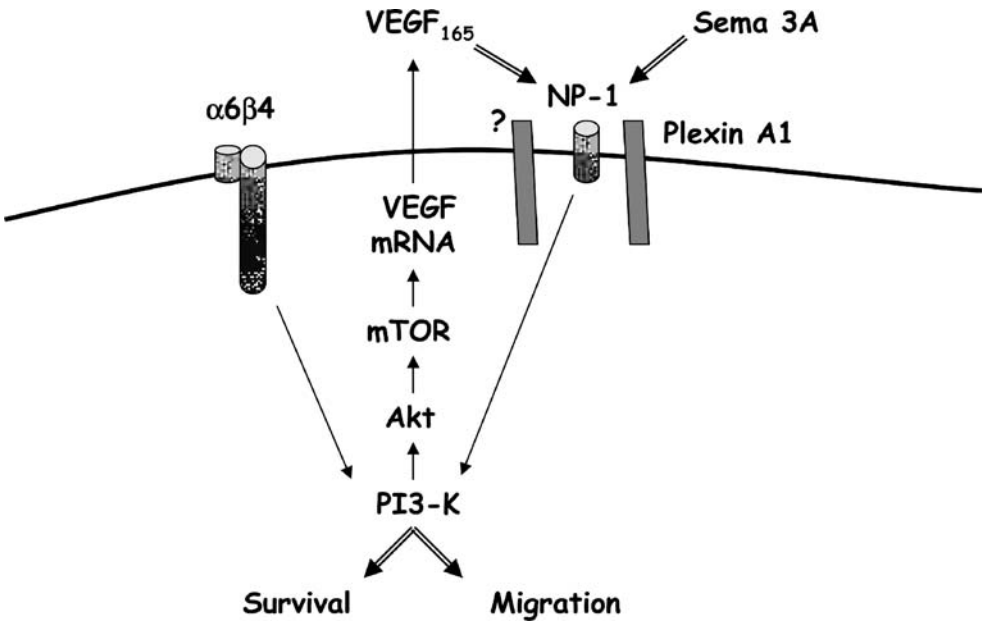

Figure 1 Autocrine VEGF signaling in breast carcinoma cells and its regulation by the α6β4 Integrin. This schematic synthesizes existing data on VEGF autocrine signaling in breast carcinoma cells and one potential mode of regulation of this pathway by the α6β4 integrin. The central component of this proposed model is the expression of $VEGF_{165}$ by breast carcinoma cells and its interaction with NP-1. This interaction can result in the stimulation of signaling pathways (PI3-K/Akt) that provide resistance to apoptotic stimuli. In this context, NP-1 probably functions in concert with a co-receptor (?) that remains to be identified. $VEGF_{165}$ also competes for binding to NP-1 with semaphorin 3A, which is also expressed by breast carcinoma cells. Semaphorin 3A, and possibly other semaphorins, can impede cell migration by engaging NP-1 and plexin A1. Binding of $VEGF_{165}$ to NP-1 would 'turn-off' these inhibitory signals and enhance the ability of breast carcinoma cells to respond to chemotactic stimuli. Soluble semaphorins may also induce apoptosis in breast carcinoma cells, a process that can be inhibited by $VEGF_{165}$. Signaling through the α6β4 integrin is one potential mechanism for stimulating VEGF expression in breast carcinoma cells that may function independently of hypoxia. Activation of the PI3-K/Akt pathway by α6β4 has been established, and it can result in mTOR activation and a consequent increase in the translation of VEGF mRNA. An important implication of the proposed model is that activation of the PI3-K/Akt pathway and VEGF expression is sustained by a positive feedback loop involving both NP-1 and α6β4.

to eIF-4E, which results in the activation of this translation initiation factor [48, 49]. The regulation of 4E-BP1 phosphorylation by α6β4 derives from the ability of this integrin to activate the PI-3K/Akt pathway and, consequently, mTOR (Fig. 1). Indeed, a considerable literature supports the hypothesis that activation of PI3-K/Akt by α6β4 is central to the numerous functions that have been ascribed to this integrin in cancer biology [50–53]. The mechanism by which α6β4 activates PI3-K has been reported to involve its regulation of insulin receptor substrate proteins [3] and its cooperation with growth factor receptors such as erbB2 [51]. The important point that emerges from these data is that the α6β4-mediated activation of PI3-K and the consequent increase in VEGF expression coupled with the ability of VEGF to activate PI3-K results in an amplification of PI3-K activation. In fact, this pathway could provide one mechanism for the high basal activity of PI3-kinase that has been noted in invasive carcinomas [3] and that may be essential for the behavior of these cells.

Recently, we assessed the importance of α6β4 to breast tumor progression *in vivo* and its putative relationship to VEGF [54]. For this purpose, an RNA interference strategy was used to deplete expression of α6β4 in SUM-159 cells. Loss of α6β4 expression inhibited colony formation in soft agar assays, suggesting a vital role for α6β4 in survival signaling and anchorage-independent growth. In three-dimensional Matrigel cultures, decreased expression of the α6β4 integrin led to enhanced apoptosis. Consistent with our hypothesis, recombinant $VEGF_{165}$ significantly inhibited the cell death observed in the β4-deficient cells in Matrigel, substantiating the importance of VEGF expression in this survival pathway. Orthotopic injection of the β4-deficient cell line into the mammary fat pad of immunocompromised mice yielded significantly fewer and smaller tumors than the control cell line, revealing a role for the α6β4 integrin in tumor formation. Furthermore, loss of α6β4 expression resulted in enhanced apoptosis and reduced expression of VEGF in breast carcinoma cells *in vivo*. The specificity of α6β4 in both the *in vitro* and *in vivo* assays was demonstrated by showing that re-expression of the β4 subunit into the β4-deficient cell line could rescue the functional phenotype. Taken together, these data implicate the α6β4 integrin in tumor formation by regulating tumor cell survival in a VEGF-dependent

manner. This conclusion is substantiated by the earlier report that VEGF is necessary for the ability of human breast carcinoma cells to form tumors in nude mice [55].

Summary and Perspective

A potentially interesting link between the α6β4 integrin and VEGF in breast cancer is beginning to emerge that may enhance the ability of tumor cells to evade apoptosis. An important consequence of this mechanism is that VEGF expression can be sustained in the absence of hypoxia, providing tumor cells with a selective advantage for survival and progression to metastatic disease. Although the xenograft experiments described above support the ability of α6β4 to sustain VEGF expression *in vivo*, additional data derived from transgenic models of mammary cancer in which expression of the β4 gene has been deleted are warranted. Such tumors would be expected to have a significant defect in their ability to produce VEGF. Analysis of tumors obtained from these mice, as well as from tumors that contain a targeted deletion of specific VEGF receptors in the tumor cells themselves (see above), should provide a composite assessment of the contribution of the α6β4/VEGF axis to mammary tumorigenesis. A critical issue that needs to be resolved in this regard is the relative contribution of hypoxia and α6β4 to regulate VEGF expression in breast tumor cells.

An unexpected aspect of α6β4-mediated, autocrine VEGF signaling in breast cancer that arises from the xenograft study using β4-deficient cells is that such signaling may be necessary for tumor formation. This possibility is actually supported from recent work in both the α6β4 and VEGF fields. Enhanced tumor growth in polyoma virus middle T-antigen mice was observed in a transgenic model targeting overexpression of VEGF to mammary epithelial cells [18]. This increased tumor growth was attributed, in part, to inhibition of apoptosis by autocrine VEGF signaling. A role for autocrine VEGF in tumor formation and growth has also been observed in xenografted human leukemias and lymphomas [11, 56]. In addition, a study using a mouse model of human squamous carcinoma concluded that α6β4 is necessary for tumor formation [57]. Although the potential involvement of VEGF was not assessed in these studies, it does merit investigation in light of the other data.

An issue that arises from the foregoing discussion is why α6β4-induced VEGF expression is necessary for tumor formation. The most obvious hypothesis is that the environment in which such tumors form is pro-apoptotic and VEGF signaling protects tumor cells from this apoptosis. This hypothesis is supported by our observation that tumors that arise from β4-deficient cells are fewer in number and those that do form are more apoptotic and express less VEGF than do control tumors. Another possibility, which is not mutually exclusive, is that α6β4-induced VEGF stimulates tumor angiogenesis. Indeed, VEGF produced by both tumor and stromal cells can impact angiogenesis [58]. Although we did not observe gross differences in the vasculature between control and β4-deficient tumors in our xenograft study, we cannot exclude some effect of α6β4 expression on angiogenesis in these experiments. In this direction, recent studies have examined the expression of α6β4 on endothelial cells and its possible role in angiogenesis. Based on the analysis of α6β4 expression in vascular endothelial cells during the development of the mouse whisker pad, it was inferred that this integrin actually inhibits the angiogenic switch [59]. In contrast, another study argued that α6β4 promotes the migration and invasion of endothelial cells during the invasive phase of tumor angiogenesis [60]. Interestingly, however, loss of α6β4 signaling in the latter study did not affect tumor angiogenesis in an orthoptopic model of mammary carcinogenesis, suggesting that the role of α6β4 in tumor angiogenesis may not relate to breast cancer [60].

In summary, the existing data substantiate the hypothesis that α6β4 plays an important role in regulating the expression of VEGF in carcinoma cells and that VEGF functions as a survival factor in nascent tumors. We emphasize, however, that a role for α6β4 and VEGF in the survival of nascent tumors does not preclude their involvement in later stages of invasion and metastasis. In this direction, the use of a conditional system to express β4-shRNA, as well as shRNAs targeted to VEGF receptors, at specific stages of tumor formation and progression will be valuable.

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