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VEGF₁₆₅b, a splice variant of VEGF-A, promotes lung tumor progression and escape from anti-angiogenic therapies through a β 1 integrin/VEGFR autocrine loop

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Running title: VEGF₁₆₅b and tumor progression

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Abstract

Vascular Endothelial Growth Factor-A (VEGF-A) is highly subjected to alternative pre-mRNA splicing that generates several splice variants. The VEGF_{xxx} and VEGF_{xxx}b families encode splice variants of VEGF-A that differ only at the level of six amino acids in their C-terminal part. The expression level of VEGF_{xxx} splice variants and their function as pro-angiogenic factors during tumor neo-angiogenesis have been well-described. The role of VEGF_{xxx}b isoforms is less well known, but they have been shown to inhibit VEGF_{xxx} mediated angiogenesis, while being partial or weak activators of VEGFR receptors in endothelial cells. On the opposite, their role on tumor cells expressing VEGFRs at their surface remains largely unknown. In this study, we find elevated levels of VEGF₁₆₅b, the main VEGF_{xxx}b isoform, in 36% of Non Small Cell Lung Carcinoma (NSCLC), mainly lung adenocarcinoma (46%), and show that a high VEGF₁₆₅b/VEGF₁₆₅ ratio correlates with the presence of lymph node metastases. At the molecular level, we demonstrate that VEGF₁₆₅b stimulates proliferation and invasiveness of two lung tumor cell lines through a VEGFR/ β 1 integrin loop. We further provide evidence that the isoform specific knock-down of VEGF₁₆₅b reduces tumor growth, demonstrating a tumor promoting autocrine role for VEGF₁₆₅b in lung cancer cells. Importantly, we show that bevacizumab, an anti-angiogenic compound used for the treatment of lung adenocarcinoma patients, increases the expression of VEGF₁₆₅b and activates the invasive VEGFR/ β 1 integrin loop. Overall, these data highlight an unexpected role of the VEGF₁₆₅b splice variant in the progression of lung tumors and their response to anti-angiogenic therapies.

Keywords: anti-angiogenic therapies; lung tumor; pre-mRNA alternative splicing; VEGF₁₆₅b.

Introduction

Angiogenesis is a complex process that contributes to various pathologies including cancer (1, 2). Vascular Endothelial Growth Factor-A (VEGF-A) is secreted by tumor cells and plays a crucial role in different aspects of tumor-induced angiogenesis. Binding of VEGF-A to its cognate tyrosine kinase receptors VEGFR-1 (Flt-1) or VEGFR-2 (Flk-1/KDR) and their co-receptors neuropilin-1 and neuropilin-2, promotes the activation of downstream signaling pathways that control proliferation, survival and migration of endothelial cells during neo-angiogenesis (3, 4). It has become apparent that the function of VEGF-A is not restricted to angiogenesis and vascular permeability (5). Besides acting on endothelial cells through paracrine signaling, VEGF-A also impacts tumor cells themselves, especially those that express VEGF receptors and neuropilins, including lung tumors (6). Such VEGF-A autocrine signaling is essential for tumor initiation, survival, proliferation or invasion as it promotes de-differentiation, and epithelial to mesenchymal transition, but also because it regulates the self-renewal of cancer stem cells (7-10). Recent studies have revealed VEGF-A biology to be even more complex. As an example, VEGF-A prevents tumor cell invasion and mesenchymal transition in glioblastoma through activation of a MET/VEGFR2 signaling pathway (11). In early squamous lung carcinoma patients, combination of high levels of VEGF-A, VEGFR1 and VEGFR2 is associated with a better rather than a worse outcome (12).

VEGF-A is expressed as multiple isoforms that result from pre-mRNA alternative splicing (13). Two families of isoforms, VEGF_{xxx} and VEGF_{xxx}b are generated through the use of alternative 3' splice acceptor sites in exon 8 (14, 15). This leads to the synthesis of proteins with the same length as the VEGF_{xxx} isoforms, but with a distinct C-terminal domain. Owing to the absence of basic carboxy-terminal amino acids essential for neuropilin-1 binding,

VEGF₁₆₅b, the main VEGF_{xxx}b isoform, is able itself to stimulate some VEGFR signaling (16-18). However, it cannot trigger a strong and sustained activation of VEGFR2, and it is also able to prevent VEGF₁₆₅ signaling that results in angiogenesis in endothelial cells (14, 19-22). Therefore, VEGF_{xxx}b isoforms inhibit the phosphorylation and angiogenic capacity of VEGF_{xxx} isoforms. In various tumors such as melanoma (20), a decrease of VEGF₁₆₅b correlates with a poor prognosis. On the opposite, high levels of VEGF_{xxx}b proteins have been observed in some tumors, such as infiltrating ductal breast carcinoma (17). Up to now, it is not known whether VEGF₁₆₅b impacts tumor cells themselves that express VEGFR receptors.

Owing to the crucial role of neo-angiogenesis in tumor development, several anti-angiogenic therapies have been developed that inhibit either VEGF-A binding or VEGFR tyrosine kinase activity. However, these therapies modestly improve overall survival. Recent studies have highlighted *VEGF-A* pre-mRNA splicing as a potential reservoir for predictive biomarkers to anti-angiogenic therapies. As an example, high plasma levels of the shortest and most diffusible VEGF-A isoform (VEGF₁₂₁) were correlated with improved Progression Free Survival (PFS) and/or Overall Survival (OS) in breast, gastric or pancreatic cancer patients treated with the humanized monoclonal anti-VEGF-A antibody, bevacizumab (23, 24). In addition, metastatic colorectal cancer patients with a low VEGF₁₆₅b:VEGF_{total} tumor ratio had a better outcome when treated with the combination of the FOLFOX4 chemotherapeutic protocol with bevacizumab compared to FOLFOX4 alone (25). Therefore, deciphering the expression pattern and specific functions of VEGF_{xxx} and VEGF_{xxx}b splice isoforms in tumors might be critical when considering the response to these therapies.

Results

Human lung adenocarcinomas express high levels of VEGF_{165b}

In order to analyze the status of VEGF_{165b} and of its pro-angiogenic counterpart VEGF₁₆₅ in lung tumors, we first performed immunohistochemistry in a series of normal lung parenchyma tissues and 76 NSCLCs, including 41 adenocarcinomas (ADC) and 35 squamous cell lung carcinomas (SCC) using specific anti-VEGF₁₆₅ and anti-VEGF_{165b} antibodies we generated in the laboratory (see Materials and Methods and Supplementary Figure 1a). In normal lung, a moderate diffuse cytoplasmic staining of both VEGF₁₆₅ and VEGF_{165b} isoforms was observed in bronchial basal cells and hyperplastic type II pneumonocytes (Supplementary Figures 1b and 2). As compared to normal lung tissues, NSCLCs had a more heterogeneous pattern of VEGF_{165b} staining with 36% (27/76) of patients expressing high intra-tumoral VEGF_{165b} protein levels (Supplementary Table 1, Figures 1a, Figure 1b and Supplementary Figure 2). Higher intra-tumoral (Figure 1c) or circulating (Figure 1d) VEGF_{165b} levels were specifically observed in lung adenocarcinoma patients, as compared to squamous lung carcinoma or healthy control subjects respectively. In addition, elevated levels of VEGF_{165b} were found in various cellular models derived from lung ADC or SCC compared to immortalized human bronchial epithelial HBEK-3KT cells (Supplementary Figure 1c). Compared to SCC cell lines, most ADC cell lines expressed higher levels of intra-cellular (Supplementary Figure 1c) or circulating (Supplementary Figure 1d) VEGF_{165b} protein level. Moreover, we observed a differential pattern of expression between VEGF_{165b} and VEGF₁₆₅ in NSCLCs (Figures 1a and 1b, $p < 0.001$; Supplementary Table 1), indicating that both isoforms are not expressed in the same way. Importantly, we noticed that a high VEGF_{165b}:VEGF₁₆₅

ratio correlates with lymph node metastases in NSCLC (Figure 1e). Collectively, these results suggested that high levels of VEGF_{165b} are associated with NSCLC aggressivity.

VEGF_{165b} promotes cell proliferation and lung adenocarcinoma growth

To deepen the functions of VEGF_{165b} in Non Small Cell Lung Carcinoma, we generated stable VEGF_{165b}-overexpressing clones in two cellular models derived from lung adenocarcinoma, namely H358 and H1299. These clones secreted high level of VEGF_{165b} (Supplementary Figure 3a) and proliferated faster than their control counterparts (Figure 2a). Increased cell proliferation was also observed when conditioned medium derived from these clones was applied native cells (Figure 2b), or when cells were treated with recombinant human VEGF_{165b} ligand (rhVEGF_{165b}) (Figure 2c). On the other hand, transfecting cells with a siRNA specifically targeting the *VEGF_{165b}* isoform diminished cell proliferation compared to control *mismatch* transfected cells (Figure 2d). Taken together, these data provided the first evidence that VEGF_{165b} stimulates the proliferation of lung tumor cells.

To validate the results *in vivo*, we designed sub-cutaneous xenograft experiments in nude mice using H1299 cells stably overexpressing VEGF_{165b} or H358 cells stably expressing the splicing factor SRSF6. Hence, SRSF6 was previously reported to physiologically induce VEGF_{165b} expression in primary epithelial cells (26). We confirmed increased expression of VEGF_{165b} in H358-derived SRSF6 overexpressing cells compared to H358 control cells despite a reduction in overall VEGF-A level (Supplementary Figure 4a). In addition, HUVEC cells cultured with conditioned medium of the SRSF6 clones were unable to form tubes in matrigel, which was consistent with the anti-angiogenic properties of secreted VEGF_{165b}

(Supplementary Figure 4b). Importantly, we observed that both H1299-VEGF₁₆₅b and H358-SRSF6 xenografts grow faster than control tumors (Supplementary Figures 3b and 4c), reach higher tumor weights (Supplementary Figure 4d, left panel) and enhanced tumor growth was correlated with increased KI67 staining (Supplementary Figures 3c and 4d right panel) compared to control xenografts. Of note, microvascular density (as revealed by CD31 or α -SMA stainings of neo-vessels) did not significantly vary in H358-SRSF6 xenografts compared to control H358 tumors which rules out the possibility that VEGF₁₆₅b promotes growth by modulating tumor neo-angiogenesis in that case (data not shown).

To eliminate unspecific effects related to VEGF₁₆₅b overexpression, we designed a reverse in vivo experiment in which H358 cells were sub-cutaneously injected into nude mice, allowed to form tumors. Mice were then sub-divided in 2 groups that received either PBS + *mismatch* siRNA [mismatch] or PBS + VEGF₁₆₅b siRNA [VEGF₁₆₅b]. Inhibition of VEGF₁₆₅b expression was detected by immunohistochemistry, while VEGF₁₆₅ expression was not affected by VEGF₁₆₅b siRNA (Figure 2e). In agreement with a role of VEGF₁₆₅b in lung tumor growth, tumors deprived of VEGF₁₆₅b were smaller than control *mismatch* ones (Figure 2f). Level of hypoxia analyzed by CAIX or HIF1-alpha immunostaining (Supplementary Figure 5), or tumor neo-angiogenesis assessed by CD31 or α -SMA endothelial staining (data not shown) was not different between control tumors and tumors deprived of VEGF₁₆₅b. These results strongly suggested that tumor growth inhibition is not associated with decreased neo-angiogenesis. Consistently, the knock-down of VEGF₁₆₅b in H358-derived xenografts significantly decreased KI67 staining compared to control xenografts (Figure 2g). As a whole, these data demonstrated that VEGF₁₆₅b stimulates lung tumor proliferation and strongly suggested that autocrine rather than paracrine mechanisms are involved.

VEGF₁₆₅b activates a VEGFR-dependent autocrine loop to promote tumor cell proliferation

We previously showed that lung adenocarcinoma express VEGFR1 and VEGFR2, neuropilin-1 and neuropilin-2 receptors (6). Therefore, we tested the impact of VEGF₁₆₅b on VEGFR signalling in our lung tumor cells. We detected a persistent accumulation of activated P-VEGFR1(Tyr1213), P-VEGFR2(Tyr1054/1059) and P-VEGFR2(Tyr1214) proteins in H358 and H1299 cells treated for 72 hours with rhVEGF₁₆₅b (Figures 3a and 3b). Same results were found in H358 and H1299 clones overexpressing VEGF₁₆₅b (Figures 3c and 3d). Of note, the activation of VEGFR1/VEGFR2 receptors was detectable at 24 hours of rhVEGF₁₆₅b treatment, but was not clearly observed at earlier time points (Supplementary Figure 6). The activation of VEGFR1 and VEGFR2 was associated with the downstream accumulation of P-ERK1/2 (Figures 3a-d), and the inhibition of ERK signaling by the use of U0126, a MEK1 pharmacological inhibitor, slowed-down cell proliferation of H358-VEGF₁₆₅b clones (Figure 3e). Same results were obtained in H1299-VEGF₁₆₅b clones (data not shown). Taken together, these results demonstrated that VEGF₁₆₅b triggers a sustained activation of VEGFR receptors that correlates with ERK1/2 activation and cell proliferation. Importantly, the knock-down of VEGF₁₆₅b strongly decreased P-VEGFR1(Tyr1213) immunostaining in H358-derived xenografts (Figure 3f), whereas overexpression of VEGF₁₆₅b in H1299-derived xenografts (Supplementary Figures 3c and 3d) or in SRSF6-expressing H358 xenografts (Supplementary Figure 4e) increased P-VEGFR1(Tyr1213) and P-VEGFR2(Tyr1214) protein levels. These data indicated that VEGF₁₆₅b may stimulate tumor growth in vivo through activation of VEGFR1 and VEGFR2. Of note, we were not able to assess the phosphorylation status of VEGFR2 in xenografts as the antibody did not give a specific immunostaining pattern. Furthermore, we observed that *VEGFR1* and/or *VEGFR2* mRNA levels increase in cells treated with rhVEGF₁₆₅b (Figure 3g) and in clones overexpressing VEGF₁₆₅b (Figure 3h).

These data indicated that VEGF₁₆₅b also positively regulates total VEGFR expression. Consistently, VEGFR1 immunostaining decreased in H358-derived xenografts deprived of VEGF₁₆₅b compared to control xenografts (Figure 3f) and conversely slightly increased in VEGF₁₆₅b-overexpressing xenografts (Supplementary Figure 3c). Furthermore, a significant association between VEGF₁₆₅b and VEGFR1 levels (Figure 3i, p=0.009) was observed by immunohistochemistry in NSCLC patients, which was consistent with our in vitro results.

VEGF₁₆₅b promotes an invasive phenotype

When performing all the above studies, we noticed that cells treated with rhVEGF₁₆₅b or overexpressing VEGF₁₆₅b exhibited morphological changes that suggested phenotypic transformation (Figure 4a). Indeed, accumulation of EMT markers such as N-cadherin, fibronectin, vimentin, SNAIL or ZEB1 was observed upon treatment with rhVEGF₁₆₅b (Figure 4b), in H358-VEGF₁₆₅b and H1299-VEGF₁₆₅b clones (Figure 4c), in H1299-VEGF₁₆₅b xenografts (Supplementary Figure 3d), as well as in H358-SRSF6 xenografts (Supplementary Figure 4e). Furthermore, we found that H1299-VEGF₁₆₅b clones close wounds faster than control cells (Figure 4d). Of note, H358 cells were not tested in wound healing assay as they never reach confluency when grown in Petri dishes. However, in transwell assays, H358-VEGF₁₆₅b clones transmigrated more efficiently than control cells (Figure 4e). H1299-VEGF₁₆₅b clones also produced more pro-MMP9 and active MMP9 metalloproteinases (Figure 4f). Moreover, a reorganization of the actin cytoskeleton in stress fibers was observed in cells overexpressing VEGF₁₆₅b (Figure 4g) or cultured with rhVEGF₁₆₅b (Figure 4h). Cells exposed to rhVEGF₁₆₅b also had more focal adhesions (Figure 4i). As a whole, these results demonstrate that VEGF₁₆₅b promotes an invasive phenotype in H358 and H1299 lung adenocarcinoma cells.

VEGF₁₆₅b stimulates an invasive β 1 integrin/VEGFR2 signaling pathway which triggers the formation of actin stress fibers and focal adhesions

Next, we undertook a series of experiments to decipher the molecular mechanism(s) by which VEGF₁₆₅b exhibits invasive properties. In endothelial cells, Chen and colleagues previously reported that matrix-bound VEGF₁₆₅ is able to maintain the phosphorylation of VEGFR2 on its tyrosine 1214 residue leading to sustained activation of phospho-p38 MAPK. Such effects were dependent on the association of VEGFR2 with β 1 integrin (27). In our cellular models, we reproducibly observed that rhVEGF₁₆₅b triggers a sustained activation of P-VEGFR2(Tyr1214) (Figures 3a-d). We also noticed the activation of p38 MAPK in H358-VEGF₁₆₅b clones together with expression of phospho-FAK and phospho-cofilin proteins, two targets of β 1 integrin signaling involved in actin polymerization (Figure 5a). Therefore, we postulated that the effects of VEGF₁₆₅b could be mediated through modulation of β 1 integrin expression and/or activation. To test this hypothesis, we first immunoprecipitated activated β 1 integrin in H358-VEGF₁₆₅b clones or in H358 cells that had been exposed to rhVEGF₁₆₅b ligand. A slight but reproducible increased expression of activated β 1 integrin was detected in the immunoprecipitates of cells overexpressing VEGF₁₆₅b (Figure 5b) or treated with rhVEGF₁₆₅b (Figure 5c), whereas the total amount of β 1 integrin was not changed by any of the treatments. In agreement with a role of VEGF₁₆₅b on β 1 integrin activation, cell-surface activated β 1 integrin slightly increased in H358 or H1299 cells treated with rhVEGF₁₆₅b compared to untreated cells (Figure 5d). Altogether, these observations were consistent with the idea that VEGF₁₆₅b activates β 1 integrin to mediate its biological effects. To confirm the role of β 1 integrin, we used specific siRNA. We showed that β 1 integrin neutralization prevents the formation of actin stress fibers (Figure 5e) and focal adhesions (Figure 5f), cell migration (Figure 5g) and accumulation of phospho-VEGFR2(Tyr1214) and phospho-

p38MAPK proteins (Figure 5h) in response to VEGF₁₆₅b. Moreover, using co-immunoprecipitation experiments, we demonstrated that rhVEGF₁₆₅b treatment stimulates the formation of a complex between β 1 integrin and VEGFR2 (Figures 5i and 5j). Taken together, these results demonstrate that a cross-talk between VEGF₁₆₅b, VEGFR2 and β 1 integrin proteins promotes an invasive phenotype in lung adenocarcinoma cells.

Bevacizumab increases VEGF₁₆₅b expression and promotes an invasive phenotype in lung cancer cells

Although the humanized monoclonal antibody against VEGF-A, bevacizumab, is currently used in clinic for treatment of lung adenocarcinoma patients, the results are disappointing and the molecular mechanisms involved in tumor escape remain largely unknown. Interestingly, we showed that bevacizumab induces a dose-dependent intra-cellular accumulation of the VEGF₁₆₅b protein in H358 cells (Figure 6a). In contrast, the level of pan-VEGF-A decreased in the same conditions (Figure 6a). Same results were obtained using semaxanib (SU5416), a VEGFR tyrosine kinase inhibitor (Figure 6b) and in H1299 cells (data not shown). Furthermore, treatment of human H358-xenografts in mice with bevacizumab up-regulated intra-tumoral VEGF₁₆₅b protein level as detected by immunohistochemistry (Figure 6c). Bevacizumab is currently used in combination with platinum salts for the treatment of NSCLC patients. We showed that cisplatin also increases the expression level of VEGF₁₆₅b in lung adenocarcinoma cell lines (Figure 6d). Interestingly, a stronger accumulation of VEGF₁₆₅b occurred upon co-treatment with cisplatin and bevacizumab (Figure 6d), and an elevated VEGF₁₆₅b:VEGF-A_{total} ratio was observed in the sera of 4/10 lung adenocarcinoma patients following bevacizumab/cisplatin treatment (Supplementary Figure 7a, patients 6, 7, 9, 10). VEGF₁₆₅b was found to represent 20% to 60% of all VEGF-A_{total} in the sera of treated

patients (data not shown). As a whole, these results are the first evidence that anti-angiogenic therapies used alone or in combination with cisplatin increase the expression of VEGF₁₆₅b. Of note, the specific neutralization of VEGF₁₆₅b in lung adenocarcinoma cell lines increased caspase-3 cleavage in response to cisplatin (Supplementary Figure 7b), suggesting that VEGF₁₆₅b could also act as an inhibitor of apoptosis.

At the molecular level, we observed that bevacizumab induces the accumulation of phosphorylated VEGFR1/VEGFR2 and p38 MAPK in both H358 and H1299 cells (Figure 6e and Supplementary Figure 8a). This was prevented by the specific knock-down of VEGF₁₆₅b. Increased P-VEGFR1(Tyr1213) immunostaining was also observed in bevacizumab-treated H358 xenografted mice, that was prevented by the knock-down of VEGF₁₆₅b (Supplementary Figure 8b). These results clearly indicated that bevacizumab activates the VEGF₁₆₅b/VEGFR autocrine loop in H358 and H1299 lung adenocarcinoma cell lines. We did not detect any significant effect of bevacizumab in vitro on ERK1/2 activation (Figure 6e and Supplementary Figure 8a) or cell proliferation (data not shown), nor on tumor growth in vivo (data not shown). However, the knock-down of VEGF₁₆₅b by siRNA significantly prevented the growth of bevacizumab-treated tumors, indicating that VEGF₁₆₅b is required for tumor growth both in untreated (Figure 2f) and bevacizumab-treated (data not shown) H358 xenografts. In addition, treatment of H1299 cells with bevacizumab for a long period of time (7 days) induced the expression of EMT markers such as N-cadherin, vimentin, fibronectin and α -SMA proteins (Supplementary Figure 8c). Although less pronounced, increased expression of vimentin was observed in H358 cells after 3 days of treatment (Figure 6e), as well as in bevacizumab-treated H358 xenografts (Supplementary Figure 8b). Furthermore, H358 cells exposed to bevacizumab also had more focal adhesions (Figure 6f) and more actin

stress fibers (Figure 6g). All these events were prevented by the neutralisation of VEGF₁₆₅b using siRNA (Figure 6e, Figure 6g and Supplementary Figures 8a and 8b). These data strongly suggested that VEGF₁₆₅b also triggers invasive functions upon bevacizumab treatment.

Finally, we investigated the contribution of the cross-talk between VEGF₁₆₅b, VEGFR2 and β 1 integrin to the response to bevacizumab. We found that bevacizumab treatment slightly but reproducibly induces β 1 integrin activation (Figure 7a) and more importantly stimulates the formation of a complex between activated β 1 integrin and VEGFR2 which was prevented by the knock-down of VEGF₁₆₅b (Figure 7b). In addition, the knock-down of β 1 integrin was found to prevent the formation of actin stress fibers (Supplementary Figure 9a) and focal adhesions (Supplementary Figure 9b) in H358 cells, and to inhibit cell migration in H1299 cells (Supplementary Figure 9c) upon treatment. Taken together, these results highly suggested that the cross-talk between VEGF₁₆₅b, β 1 integrin and VEGFR2 proteins leads to an invasive phenotype in lung adenocarcinoma cell lines in response to bevacizumab treatment.

Discussion

The VEGF₁₆₅b splice variant has been shown to inhibit angiogenic signaling of VEGF₁₆₅ and its downregulation in primary melanoma was associated with subsequent metastasis (14, 19, 20, 28). It has not previously been shown to be upregulated in cancer, rather it is generally considered to be reduced as a proportion of total VEGF-A. In contrast, we find high levels of endogenous VEGF₁₆₅b protein in a significant proportion of lung adenocarcinoma. Furthermore, we observe an association between a high VEGF₁₆₅b/VEGF₁₆₅ ratio and lymph node metastases. Therefore, in parallel with VEGF₁₆₅ triggering both invasive and anti-invasive functions (11), we highlight a complex role of VEGF₁₆₅b in tumors. In this new scheme, VEGF₁₆₅b cannot be viewed solely as a tumor suppressive anti-angiogenic factor, but has now also to be considered to be able to be an endogenous tumor promoting factor when cancer cells express VEGFRs. Importantly, we demonstrate that autocrine rather than paracrine functions of VEGF₁₆₅b are involved in this setting.

We provide the first evidence that VEGF₁₆₅b stimulates the proliferation and invasion of lung tumor cells. Importantly, we confirm the results in tumor xenografts and show that this is associated with a modulation of the KI67 proliferative index while CD31 or α -SMA endothelial staining, two markers of tumor neo-angiogenesis, do not significantly vary. Paradoxically, we previously demonstrated that overexpression of VEGF₁₆₅b correlates with reduced angiogenesis in lung adenocarcinoma xenografts (29). In this former report, we only investigated the early paracrine effects of VEGF₁₆₅b on neo-angiogenesis as mice were sacrificed 7 days after induction of VEGF₁₆₅b. In the current study, mice were sacrificed 41-45 days after tumor cells injection, thereby supporting the idea that autocrine tumor

promoting functions of VEGF₁₆₅b may surpass its paracrine anti-angiogenic functions to promote lung tumor growth (Figure 8). Our results are in agreement with Catena and co-workers who noticed that stable overexpression of VEGF₁₆₅b promotes the development of lung ADC xenografts without affecting vascular density (17). In addition, our data are consistent with previous observations in various tumors such as renal cell carcinoma (30) or infiltrating breast carcinoma (17), where VEGF_{xxx}b isoforms are not always down-regulated. On the other hand, VEGF₁₆₅b was also reported to slow-down the growth of colon cancer xenografts through inhibition of tumor neovascularization, without any effects on tumor proliferative rate (31-33). Therefore, whether VEGF₁₆₅b exerts anti- versus pro-tumoral functions may depend on the tumor type. One plausible but not exclusive explanation of these dual functions could come from the expression level of VEGFR receptors in tumors. In lung cancer that express VEGFR1/2 (6), we show that VEGF₁₆₅b increases VEGFR1 and VEGFR2 mRNA levels, and triggers a sustained phosphorylation of both receptors which correlates with ERK1/2 and p38MAPK activation. In addition, we demonstrate that VEGF₁₆₅b induces a β 1 integrin/VEGFR2/FAK/cofilin signaling pathway that promotes actin stress fiber formation, and show that β 1 integrin is required for the invasive functions of VEGF₁₆₅b. Taken together, these data provide a molecular rationale for the previous observation that upregulation of β 1 integrin correlates with advanced stages, lymph node metastases, increased relapse rate and decreased overall survival in lung adenocarcinoma patients (34).

The identification of predictive biomarkers of response to anti-angiogenic therapies is a crucial issue in the clinic. Eventhough these therapies were initially developed to target endothelial cells only, it is of prime importance to evaluate their impact on tumor cells themselves. In this study, we show that anti-angiogenic therapies combined (or not) with

platinum salts regulate *VEGF-A* alternative pre-mRNA splicing and specifically increase VEGF_{165b} levels in lung adenocarcinoma cell lines. In addition, we demonstrate that bevacizumab activates the VEGF_{165b}/β1 integrin/VEGFR signaling network leading to an invasive phenotype. Therefore, besides its ability to promote lung tumor growth, VEGF_{165b} appears to be also a determinant of the response to anti-angiogenic therapies. In this setting, our results support the very recent observation that integrin alpha 9 (ITGA9), known to form a heterodimeric receptor with activated β1 integrin, belongs to a 10 gene-signature that predicts outcome in advanced non squamous NSCLC patients treated with a combination of bevacizumab and erlotinib (35, 36). They also fit well with the previous demonstration that β1 integrin and its downstream effector kinase FAK are activated in bevacizumab-resistant glioblastoma patients and contribute to bevacizumab resistance (37, 38).

To conclude, we propose that a VEGF_{165b}/VEGFR/β1 integrin autocrine loop contributes to the resistance of NSCLCs to anti-angiogenic therapies by promoting an invasive phenotype. This model is distinct but not contradictory to the results of Bates and colleagues who proposed that colon carcinoma patients exhibiting low levels of VEGF_{165b} are more sensitive to bevacizumab + FOLFOX treatment compared to FOLFOX alone (25). In that case, paracrine rather than autocrine functions of VEGF_{165b} are presumed to be at play. As a whole, these and our data highlight the need for addressing the respective status of VEGF-A splice variants and their paracrine/autocrine functions when investigating the global response to anti-angiogenic treatments.

Materials and Methods

Patients, Tissue samples and Serum

Seventy six human Non Small Cell Lung Carcinoma (NSCLC) and 17 matched normal lung parenchymas were included in this study (Supplementary Table 1 and Supplementary Figures 1 and 2). Tissue banking and research conduct followed national ethical guidelines and were approved by the Ministry of Research (approval AC-2010-1129) and by the regional IRB (CPP 5 Sud Est). Histopathology diagnosis was made on formalin-fixed, paraffin-embedded samples using the current WHO classification (39, 40). For quantification of VEGF₁₆₅b in sera, blood samples from nine healthy subjects and ten patients with chemotherapy-naïve ADC enrolled in the Pharmacogenoscan study (NCT00222404; Grenoble University Hospital) were used (41). Informed consent was obtained from all subjects.

Immunohistochemistry

Antibodies recognizing specifically VEGF₁₆₅ or VEGF₁₆₅b splice isoform were produced by immunizing rabbits with synthetic peptides specific of the C-terminal part of either VEGF₁₆₅ (LNERTCRCDKPRR) or VEGF₁₆₅b (LNERTCRSLTRKD) protein (Covalab, Villeurbanne, France). The other antibodies used for immunohistochemistry are listed in Supplementary Table 2. Immunostaining was performed on 7mm tissue sections as described previously (42) (43), and assessed by a pathologist (EB) who was blinded to the group allocation when scoring. Scoring (0-300) was established by multiplying the percentage of labelled cells (0 to 100%) by the staining intensity (0, null; 1, low; 2, moderate; 3, strong). Scores obtained for alveolar type II pneumocytes and bronchial cells in normal lung tissues taken at distance from the tumor were considered as normal scores for adenocarcinoma and squamous cell

carcinoma, respectively. Tumors were sub-divided in three classes for VEGF₁₆₅b (class 0: low ≤ 50 ; class 1: moderate 51-199; class 2: high ≥ 200) and VEGF₁₆₅ expression (class 0: low ≤ 50 ; class 1: moderate 51-149; class 2: high ≥ 150). For VEGFR1, tumors were sub-divided into two classes with low (< 125 , class 0) or high (≥ 125 , class 1) expression.

Cells, Cell Culture and Reagents

Human lung adenocarcinoma cell lines (H358, H1299) were recently authenticated by DNA STR profiling (ATCC cell line Authentication Service, LGC standards, Molsheim, France) and tested for mycoplasma contamination. Cells were cultured in RPMI-1640 medium + 2mM L-glutamine and 10% (v/v) Fetal Calf Serum (FCS) as described previously (29). Bevacizumab (Avastin®) was provided by Roche/Genetech (Indianapolis, USA). Recombinant rhVEGF₁₆₅b and rhVEGF₁₆₅ proteins were purchased from R&D Systems, SU5416 from Sigma-Aldrich and cisplatin and U0126 from Calbiochem. Stable VEGF₁₆₅b clones were obtained after transfection of H358 or H1299 cells using Fugene® 6 reagent (Roche Diagnostic, France) with either pcDNA3 empty vector or pcDNA3-VEGF₁₆₅b encoding plasmids. Geneticin (G418, 800 $\mu\text{g/ml}$) was added post-transfection for at least 4 weeks in order to select stable transfected clones. Two representative VEGF₁₆₅b-overexpressing clones were chosen for H358 (clones 13 and 36) and H1299 (clones 2 and 5) cells.

Apoptosis, wound healing, invasion, zymography and immunofluorescence studies

Apoptosis was evaluated by scoring the percentage of apoptotic cells on 500 cells after Hoescht 33342 staining (42, 44). Wound-healing assays were performed using 4-well lab-tek

chamber Permanox® slides, previously coated with fibronectin (10µg/mL) and visualized using an inverted microscope equipped with a camera MicroMAX N/B (Princeton Instruments). Invasion assays in transwells using 50ng/mL β-FGF as a chemoattractant and analysis of the activity of MMP9 using gelatin zymography were performed as previously described (45, 46). Immunofluorescence studies for activated β1 integrin (using 9EG7 antibody), phospho-paxillin(Tyr118) and F-actin were performed on cells coated onto 2µg/ml fibronectin, fixed in 4% PFA and permeabilized or not with PBS 1X/Triton 0.2%. Cells were then mounted in a solution containing 4,6-diamino-2-phenylindole (DAPI) (Roti ®-Mount FluorCare DAPI, ROTH), visualized by fluorescent microscopy using Axioimager microscope (Carl Zeiss, Jena, Germany) with the AxioVision® software at a 60x magnification and processed with Image J software.

Analysis of activated β1 integrin by flow cytometry

Cells re-suspended in PBS/BSA 1% (either with or without Mn²⁺ 0.5 mM) to induce β1 integrin activation were incubated with the primary antibody HUTS4 (MAB2079Z, Sigma-Aldrich) for 30 min. After two washes with PBS, cells were incubated with Alexa⁴⁸⁸ anti-rat antibody, fixed with 4% PFA and resuspended in PBS before flow cytometry analysis. The percentage of β1 integrin activation was calculated using the formula: % activation= (Mean FL1-A [Ac] / Mean FL1-A [Ac+Mn²⁺]) x 100.

ELISA assays

VEGF₁₆₅b was quantified on cellular extracts, cellular supernatants or serum using the R&D system Duoset ELISA kit (cat#DY3045) and pan-VEGF using either the Human VEGF

Standard ELISA Development Kit from PeproTech (cat# 900-K10) or the ELISA assay from R&D System (DVE00).

RNA interference, RNA extraction and quantitative RT-PCR

β 1 integrin silencing was performed using SMARTpool siGENOME ITGB1 siRNA (Dharmacon) and Oligofectamine RNAi max (Invitrogen). The siRNA specifically targeting VEGF₁₆₅b was: forward, 5'-GAACGUACUUGCAGAUCUCUC-3'; reverse, 5'-GAGAGAUCUGCAAGUACGUUC-3'. The scrambled siRNA oligonucleotides used as a control for all RNA interference experiments were as follows: forward 5'-UCGGCUCUUACGCAUUC-3' and reverse 5'-CAAGAAAGGCCAGUCCAAG-3' or ON-TARGET plus non targeting control pool (Dharmacon). Transfection was performed using Oligofectamine™ reagent (Invitrogen) and cells were analysed 72 hours post-transfection. The sequence for control siRNA used in *in vivo* experiments was designed as forward, 5'-CUUACGCUCACUACUGCGA-3'; and reverse, 5'-UCGCAGUAGUGAGCGUAA-G-3'. Total RNA was extracted using Trizol® reagent (Invitrogen) and subjected to reverse transcription using iScript™ Reverse Transcription Supermix (BIO-RAD). Quantitative PCR was performed with GoTaq® qPCR Master Mix (Promega). VEGFR1/FLT1 (Hs.654360) and VEGFR2/KDR (Hs.479756) primer assays were purchased from TEBU-Bio (Le Perray-en-Yvelines, France). Relative gene expression was calculated, for each sample, as the ratio of specific target gene to GAPDH gene (reference gene), thus normalizing the expression of target gene for sample to sample differences in RNA input.

Immunoblotting and immunoprecipitation

Immunoblotting and immunoprecipitation experiments were performed as previously described (47). The antibodies used are listed in the Supplementary Table 2. Sera for immunoprecipitation or detection of total (serum 227) or activated (TS2/16) β 1 integrin were provided by Dr Corinne Albiges-Rizo (Institute For Advanced Biosciences, La Tronche, France). For immunoprecipitation, 5 mM sodium molybdate was added to the lysis buffer to enhance the cross-linking of the complexes. Irrelevant rabbit or mouse IgG was used as control for immunoprecipitation. Immunoblots were quantified using Image J software and the mean \pm standard deviation of at least three independent experiments was calculated. Owing to space constraints in some main figures, quantification of western blotting experiments is also provided in Supplementary Figure 10 and Supplementary Figure 11.

***In vivo* studies**

All animal experiments were conducted in agreement with the “Principles of Laboratory Animal Care“ (NIH publication n°86-23, revised 1985) and were approved by the ComEth Grenoble ethic committee (n° of approval 165_IAB-U823-MK-11). In all experiments, H358, H358-Tet-On, H358-SRSF6, H1299-Luc or H1299-Luc-VEGF₁₆₅b cells were implanted subcutaneously (10-20x10⁶ cells) into 5 to 6 weeks-old female Nmri nude mice (Janvier, Le Genest-Isle, France). Tumor volumes were measured once a week as previously described (48). H358 cells were allowed to form tumors for 3-4 weeks. When tumor volumes reached 30-40mm³, mice were weighed, randomly divided into 4 homogeneous groups according to their weight (10 mice each) and were alternatively SC- or IP-injected four times a week, with either *mismatch* siRNA as a control or VEGF₁₆₅b siRNA. Once a week, siRNAs were combined with either bevacizumab (7.5mg/kg) or PBS as a control. Transfection of siRNA

was performed using in vivo-jetPEI™-Gal (Polyplus-transfection). Tumor volumes were monitored throughout the experiment. In all experiments, the investigators (AB, SG, MK, MG) were blinded to the group allocation during the experiment and when assessing the outcome. Mice were sacrificed after 6 weeks of treatment, tumors were removed, photographed, weighed, washed, fixed in Buffered Formaldehyde and processed for immunohistochemical analysis as previously detailed. Immunostainings were quantified using Image J software as previously described (49). In some experiments, tumoral samples were lysed in RIPA buffer supplemented just before use with proteases and phosphatases inhibitors and western blot experiments were performed on these samples. Statistical analysis of tumor volumes between groups was performed using a two-tailed non-parametric Mann-Whitney t test (at least 9 animals per group).

Statistical Analyses

In all in vitro experiments on cell lines, at least three independent experiments were performed. MTS, apoptosis, proliferative and ELISA assays were always done in duplicate or triplicate for each experiment. Non-parametric, two-tailed Mann-Whitney or ANOVA tests were performed using Statview (Abacus Concepts) or Prism software. Descriptive analyses comparing continuous and two-level categorical variables were carried out using the Mann-Whitney test. P values < 0.05 were considered significant.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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Figure legends

Figure 1. VEGF₁₆₅b is highly expressed in a significant proportion of Non Small Cell Lung Carcinomas (NSCLCs) and a high VEGF₁₆₅b:VEGF₁₆₅ ratio is associated with lymph node metastases. (a, b) Upper panels: representative VEGF₁₆₅b or VEGF₁₆₅ immunostaining from paraffin-embedded sections of acinous lung adenocarcinoma (a) or well-differentiated squamous cell lung carcinoma (b). Negative controls (no Ab, IgG) for immunostainings are illustrated in left panels for each histological sub-type. Lower panels: distribution of VEGF₁₆₅ and VEGF₁₆₅b scores for each tumor case. Statistical analyses were performed using a Student paired t test. Scale bar = 50 μ m. (c) Mean levels \pm standard deviation of VEGF₁₆₅b immunohistochemical scores according to the histological sub-type. (d) Quantification (Elisa) of VEGF₁₆₅b in blood samples of healthy controls and chemotherapy-naïve adenocarcinoma patients (ADC). Median levels are indicated. Of note, circulating VEGF₁₆₅b levels never correlated with platelet count, nor the time at which these sera were collected (from 4 to 12 weeks after the start of treatment) (data not shown). (e) Distribution of the VEGF₁₆₅b:VEGF₁₆₅ ratio (IHC) according to the nodal status in NSCLC. (c, d, e) Statistical analyses were performed using a non-parametric Mann-Whitney test.

Figure 2. VEGF₁₆₅b promotes cellular proliferation and lung tumor growth. (a) Cell proliferation was assessed by counting trypan blue negative cells in two stable VEGF₁₆₅b clones derived from either H358 (named 13 and 36) or H1299 (named 2 and 5) cells respectively, or (b) in H358 and H1299 native cells cultured with supernatants from either control H358 or H1299 cells or VEGF₁₆₅b-clones as indicated, or (c) in H358 and H1299 cells treated with rhVEGF₁₆₅b (0.1 ng/ml) for 72 hours or (d) in H358 and H1299 cells transfected with either *mismatch* or *VEGF₁₆₅b* siRNA for 72 hours. The means \pm SD of three independent

experiments are illustrated (a, one-way ANOVA; c,d non parametric Mann-Whitney test). * $p < 0.05$; ** $p < 0.01$. (d) Lower panel: Western blot analysis using anti-VEGF₁₆₅b or anti-VEGF₁₆₅ antibody was performed to assess the specific knock-down of VEGF₁₆₅b. Tubulin was used as a loading control. (e, f, g) H358 cells were sub-cutaneously injected into nude mice to form tumors. Animals were then sub-divided in two groups (n=9 for each group), receiving *mismatch* or *VEGF₁₆₅b* siRNA. (e) Upper panels: representative VEGF₁₆₅b and VEGF₁₆₅ immunostainings in H358 xenografts having received either control (*mismatch*) or VEGF₁₆₅b (*siVEGF₁₆₅b*) siRNA. Scale bar = 50 μ m. Lower histograms: quantification of immunostainings as described in the material and methods section. Statistical analyses were performed using non-parametric Mann-Whitney test. * $p < 0.05$; NS: not significant. (f) Upper panels: representative tumors. Lower panel: mean tumor volume \pm SD in each condition. Statistical analyses were performed using non-parametric Mann-Whitney test. * $p < 0.05$. (g) Left panels represent immunostainings of KI67. Scale bar = 50 μ m. Right panel: histogram of the percentage of KI67-stained tumor cells for each condition. Five fields containing at least 100 cells were scored for each tumor xenograft. ** $p < 0.01$ using non parametric Mann-Whitney test.

Figure 3. VEGF₁₆₅b activates a VEGFR autocrine loop to promote tumor cells proliferation. (a, c) Immunoblots for indicated proteins in H358 or H1299 cells treated with rhVEGF₁₆₅b (0.1 ng/ml) for 72 hours (a) or in H358 or H1299 VEGF₁₆₅b-overexpressing clones (c). Actin was used as a loading control. (b, d) Quantification of immunoblots in (a) and (c) respectively, using Image J software. An arbitrary value of 1 was given to untreated or control (Co) cells (black histogram). Values are mean \pm SD of 3 independent experiments. Non-parametric Mann-Whitney statistical test. (** $p < 0.01$, * $p < 0.05$, NS: not significant). (e) H358 control or H358 VEGF₁₆₅b-clones were treated or not for 48 hours with 10 μ M U0126 as

indicated. Cell number was estimated following trypan blue staining. Values are mean \pm SD of 3 independent experiments. (Non-parametric Mann-Whitney test, *** $p < 0.001$). (f) Representative immunostaining of P-VEGFR1(Tyr1213) or VEGFR1 proteins in H358 xenografts having received either *mismatch* or *VEGF₁₆₅b* siRNA as indicated. Scale bar = 50 μ m. (g) H358 or H1299 cells were treated (rhVEGF₁₆₅b, hatched bars) or not (NT, white bars), for 72 hours with rhVEGF₁₆₅b (0.1 ng/ml) as indicated. *VEGFR1* or *VEGFR2* mRNA levels were quantified by RT-QPCR in each condition. *GAPDH* was used as an internal control. (h) *VEGFR1* (white bars) or *VEGFR2* (hatched bars) mRNA levels were quantified by RT-QPCR in control cells (pcDNA3.1 transfected) or H358 or H1299 clones overexpressing VEGF₁₆₅b. *GAPDH* was used as an internal control. (i) Mean levels \pm standard deviation of VEGF₁₆₅b immunohistochemical scores according to the expression of VEGFR1 (high and low) in NSCLC patients. (g, h, i) Statistical analyses were performed using non-parametric Mann-Whitney test (** $p < 0.01$; *** $p < 0.001$).

Figure 4. VEGF₁₆₅b promotes invasion. (a) Microscopic visualization of morphological differences between H358/H1299 control and VEGF₁₆₅b-overexpressing cells. (b, c) Immunoblots for Epithelial to Mesenchymal Transition (EMT) markers in H358 cells treated or not with 0.1 ng/ml rhVEGF₁₆₅b for 72 hours (b) or in control (Co), H358 and H1299-VEGF₁₆₅b clones (c). Actin or tubulin was used as a loading control. (d) Wound healing assay in control and H1299-VEGF₁₆₅b clones (5 and 2). Scratch recovering was estimated after 13 hours. Right histogram represents the scratch coverage percentage. $n = 3$ independent experiments. Values are mean \pm SD (non parametric Mann-Whitney test). ** $p < 0.01$. (e) Boyden chamber assay in control and H358-VEGF₁₆₅b clones (13 and 36) using serum gradient and β -FGF as attractants. Trans-migrating cells were stained with Hoechst as illustrated in upper panels. $n = 3$ independent experiments. Values are mean \pm SD (non

parametric Mann-Whitney test). * $p < 0.05$ ** $p < 0.01$. **(f)** Representative immunoblots of MMP-9 gelatin zymography in H1299-VEGF₁₆₅b clones. The supernatant from HT1080 cell line was used as a control for MMPs activation. **(g)** F-actin staining using phalloidin-TRITC in H1299 control or -VEGF₁₆₅b clones. **(h)** Representative F-actin staining of H1299 cells treated, or not (Co), with rhVEGF₁₆₅b (1 ng/ml) for 60 min (upper panel). Percentages of cells with actin stress fibers are presented for each condition (lower panel). $n = 4$ independent experiments. 500 cells were counted per experiment. Values are mean \pm SD (Mann-Whitney test). **(i)** H1299 were plated onto fibronectin glass coverslips for 24 hours and treated or not for 1 hour with rhVEGF₁₆₅b (1 ng/ml). Co-immunofluorescence analyses of activated $\beta 1$ integrin (clone 9EG7, green) and phospho-paxillin(Tyr118) (red) were performed. DAPI was used to counterstain the nucleus. Histograms representing quantification of cells exhibiting focal adhesion was performed on at least 300 cells (Mann-Whitney test) * $p < 0.05$. Scale bar = 20 μm .

Figure 5. VEGF₁₆₅b activates a $\beta 1$ integrin signaling pathway to trigger actin cytoskeleton reorganization. **(a)** Immunoblots of the indicated proteins in control and H358-VEGF₁₆₅b clones. Tubulin was used as a loading control. Same results were obtained in H1299-VEGF₁₆₅b clones (data not shown). **(b, c)** Immunoprecipitation (IP) of activated $\beta 1$ integrin in cellular extracts from **(b)** H358 control or -VEGF₁₆₅b clones or **(c)** H358 treated with rhVEGF₁₆₅ or rhVEGF₁₆₅b (0.1 ng/ml) for 60 min. IgG was used as an irrelevant antibody. Total amount of $\beta 1$ integrin in protein extracts was assessed by western blotting (lower panels). Numbers represent the results of densitometric quantification of the signal using image J software. The ratio activated $\beta 1$ integrin:total $\beta 1$ integrin in untreated cells was arbitrarily assigned the value 1. **(d)** $\beta 1$ integrin activation measured by flow cytometry in

H358 or H1299 cells treated or not (NT) for 1 hour with rhVEGF_{165b} (10 ng/ml). The mean \pm SDs of four independent experiments are presented. (non parametric Mann-Whitney test). (e) F-actin staining using phalloidin-TRITC in H1299-VEFG_{165b} clones transfected for 72 hours with *mismatch* (siMis) or *β 1 integrin* (si β 1) siRNA. Scale bar = 20 μ m. Same results were obtained in H1299 cells transfected for 72 hours with *mismatch* (siMis) or *β 1 integrin* (si β 1) siRNA and treated or not with rhVEGF_{165b} (0.1 ng/ml) for 30 min (data not shown). (f) H1299 cells were transfected with *mismatch* or *β 1 integrin* siRNA for 48 hours, plated onto fibronectin coated glass coverslips for 24 hours and treated, or not, for 1 hour with rhVEGF_{165b} (1 ng/ml). Co-immunofluorescence staining was performed to detect β 1 integrin (green) and phospho-paxillin(Tyr118) (red). Dapi was used to counterstain the nucleus. Of note, the green fluorescence strongly decreased in β 1 integrin knocked-down cells consistent with the efficiency of β 1 integrin knock-down. Scale bar = 20 μ m. (g) A wound healing assay was performed in H1299-VEFG_{165b} clones transfected with control (siMis) or siRNA against β 1 integrin (si β 1). Scratch recovery was estimated after 13 hours. The right panel represents the scratch coverage percentage. n = 3 independent experiments. Values are mean \pm SD (non parametric Mann-Whitney test) *p<0.05. (h) H1299 were transfected for 72 hours with *mismatch* (siMis) or *β 1 integrin* (si β 1) siRNA in the presence or absence of rhVEGF_{165b} (0.1 ng/ml) as indicated. Western-blot analysis of the indicated proteins was performed. Tubulin was used as a loading control. (i, j) Immunoprecipitation of (i) β 1 integrin or (j) VEGFR2 was performed in H358 cells treated or not with increasing amounts (0.1, 1 ng/ml) of rhVEGF_{165b} for 1 hour. IgG was used as an irrelevant antibody. Numbers represent the results of densitometric quantification of the signal using image J software. For VEGFR2 and β 1 integrin interaction, the VEGFR2: β 1 ratio was calculated in each condition. The value 1 was arbitrarily assessed in untreated conditions.

Figure 6. Anti-angiogenic therapies such as bevacizumab increase VEGF₁₆₅b protein level and promote the invasive phenotype. (a, b) Intra-cellular dosage (Elisa) of VEGF₁₆₅b and pan-VEGF-A in H358 cells, treated or not (NT), with (a) bevacizumab for 72 hours or (b) semaxanib (SU5416) for 48 hours. n = 3 independent experiments. Values are mean ± SD (non-parametric Mann-Whitney test; * p<0.05, ** p<0.01). Same results were obtained in H1299 cells (data not shown). (c) Representative immunostaining of VEGF₁₆₅b in H358 subcutaneous xenografts having received or not bevacizumab (7.5 mg/kg) once a week. Scale bar = 50 μm. (d) H358 or H1299 cells were treated, or not (NT), for 72 hours with either 5 μM cisplatin or a combination of 5 μM cisplatin and 1 μg/ml bevacizumab. ELISA assays were performed in intracellular extracts. n = 3 independent experiments. Values are mean ± SD (non-parametric Mann-Whitney; * p<0.05; ** p< 0.01). (e) Immunoblots for indicated proteins in H358 cells transfected with *mismatch* or *VEGF₁₆₅b* siRNA for 72 hours and incubated or not with increasing doses of bevacizumab as indicated. Tubulin was used as a loading control. (f) H1299 were plated onto fibronectin glass coverslips for 24 hours and treated or not for 72 hours with bevacizumab. Co-immunofluorescence analyses of β1 integrin (green) and phospho-paxillin(Tyr118) (red) were performed. DAPI was used to counterstain the nucleus. Scale bar = 10 μm. Histograms represent quantification of cells exhibiting focal adhesion on at least 300 cells (non-parametric Mann-Whitney; **p<0.01). (g) F-actin staining using phalloidin-TRITC in H1299 cells transfected with *mismatch* or *VEGF₁₆₅b* siRNA and treated or not with BVZ (10μg/ml) for 72 hours. Scale bar = 10 μm.

Figure 7. Bevacizumab promotes the formation of β1 integrin/VEGFR2 complex by a mechanism involving VEGF₁₆₅b. (a) Left panels: Immunoprecipitation (IP) of activated β1 integrin in cellular extracts from H358 cells treated or not with increasing concentrations of

bevacizumab for 72 hours as indicated. IgG was used as an irrelevant antibody. Total amount of $\beta 1$ integrin in protein extracts was assessed by western blotting (lower panels). Numbers represent the results of densitometric quantification of the signal using image J software. Activated $\beta 1$ integrin:total $\beta 1$ integrin ratio in untreated cells was arbitrarily assigned to 1. Right panel: Densitometric quantification (mean \pm SD) of the activated $\beta 1$ integrin:total $\beta 1$ integrin ratio in three independent experiments. The value 1 was arbitrarily assigned to the ratios obtained in untreated cells (non-parametric Mann-Whitney test; * $p < 0.05$). (b) Left panels: Immunoprecipitation of activated $\beta 1$ integrin in H358 cells transfected with *mismatch* or *VEGF₁₆₅b* siRNA and treated or not (-) with bevacizumab (10 μ g/ml) for 72 hours. Lower panel: efficiency of VEGF₁₆₅b knock-down. IgG was used as an irrelevant antibody. Numbers represent the results of densitometric quantification using image J software of the VEGFR2: $\beta 1$ integrin ratio in each condition. The value 1 was arbitrarily assigned to untreated conditions. Right panel: Densitometric quantification (mean \pm SD) of the VEGFR2: $\beta 1$ integrin ratio in three independent experiments. The value 1 was arbitrarily assigned to the ratio obtained in untreated cells (non-parametric Mann-Whitney test; * $p < 0.05$, ** $p < 0.01$).

Figure 8. A model for the dual functions of VEGF₁₆₅b in NSCLC. In lung tumors, VEGF₁₆₅b can exert both paracrine anti-angiogenic functions on endothelial cells (our former study, (29)), as well as pro-tumoral functions on tumor cells themselves through activation of a $\beta 1$ integrin/VEGFR autocrine loop (this study). We propose that in later stages of tumor development, the pro-tumoral proliferative functions of VEGF₁₆₅b may bypass the anti-tumoral angiogenic ones to promote tumor progression. VEGF₁₆₅b-expressing tumor cells also acquire more aggressive and invasive features as evidenced by the appearance of EMT markers that could contribute to the metastatic spreading of tumor cells. Anti-angiogenic

therapies impact on *VEGF-A* pre-mRNA splicing and favor the expression of VEGF₁₆₅b in tumor cells that could promote their escape from treatment. Based on preliminary results indicating that VEGF₁₆₅b-overexpressing cells are also more resistant to hypoxia, we propose that hypoxia induced by anti-angiogenic therapies may also contribute to the emergence of VEGF₁₆₅b-expressing tumor cells. As a whole, our data suggest that modulating *VEGF-A* pre-mRNA splicing together with β 1 integrin signaling might offer alternative therapeutic strategies to overcome resistance to anti-angiogenic therapies in lung adenocarcinoma.