Chapter 2

VEGF and inhibitors of TGF-β type-I receptor kinase synergistically promote blood-vessel formation by inducing α5-integrin expression

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Abstract

Vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β) are potent regulators of angiogenesis. How VEGF and TGF-β signalling pathways crosstalk is not well understood. Therefore, we analyzed the effects of the TGF-β type-I-receptor inhibitors (SB-431542 and LY-2157299) and VEGF on endothelial cell (EC) function and angiogenesis. We show that SB-431542 dramatically enhances VEGF-induced formation of EC sheets from fetal mouse metatarsals. Sub-optimal doses of VEGF and SB-431542 synergistically induced EC migration and sprouting of EC spheroids, whereas overexpression of a constitutively active form of TGF-β type-I receptor had opposite effects. Using quantitative PCR, we demonstrated that VEGF and SB-431542 synergistically upregulated the mRNA expression of genes involved in angiogenesis, including the integrins α5 and β3. Specific downregulation of α5-integrin expression or functional blocking of α5 integrin with a specific neutralizing antibody inhibited the cooperative effect of VEGF and SB-431542 on EC sprouting. *In vivo*, LY-2157299 induced angiogenesis and enhanced VEGF- and basic-fibroblast-growth-factor-induced angiogenesis in a Matrigel-plug assay, whereas adding an α5-integrin neutralizing antibody to the Matrigel selectively inhibited this enhanced response. Thus, induction of α5-integrin expression is a key determinant by which inhibitors of TGF-β type-I receptor kinase and VEGF synergistically promote angiogenesis.

Keywords**:** Angiogenesis, Endothelial cell, Integrin, Signalling, SB- 431542, TGF-β, VEGF

Introduction

During embryogenesis, the formation of new blood vessels depends on vasculogenesis and angiogenesis. Angiogenesis refers to the formation of new blood vessels from pre-existing ones (1), and consists of an activation phase and a resolution phase (2). The activation phase is associated with vessel destabilization and increased permeability, degradation of the extracellular matrix (ECM), and endothelial cell (EC) proliferation and migration. During the resolution phase, ECs become quiescent and pericytes and vascular smooth muscle cells (VSMCs) are recruited to ensure stabilization and maturation of the newly formed vessels (1).

Angiogenesis is tightly regulated by pro- and anti-angiogenic signals, and plays an important role in pathophysiological and physiological processes such as wound healing, tissue remodeling, the female reproductive cycle, autoimmune diseases and cancer (3). Vascular endothelial growth factor (VEGF) is a key regulator of vasculogenesis and angiogenesis. Heterozygous mice lacking a single *VEGF* allele die at embryonic day (E)8.5 with severe vascular defects (4; 5). Overexpression of VEGF also results in embryonic lethality (6). VEGF signals through two distinct tyrosine- kinase receptors, VEGFR1 (also known as Flt-1) and VEGFR2 (also known as KDR and Flk-1) (7), and exerts multiple effects on ECs, including proliferation, survival (8), migration and the formation of capillary-like tubules (9).

Genetic studies in mice and humans have suggested that perturbation of TGF-β signalling results in vascular abnormalities (10). TGF-β exerts its biological effects by binding to and activating type-I and type-II transmembrane serine/threonine-kinase receptors. Binding of TGF-β to the TGF-β type II receptor (TβRII) leads to recruitment and phosphorylation of type I receptor [TβRI, or activin receptor like kinase 5 (ALK5)]. Activin and Nodal, which are structurally related to TGF-β, signal via ALK4 and ALK7, respectively. Activated ALK5, ALK4 and ALK7 propagate the signal into the cells by phosphorylating the downstream effector proteins Smad2 and Smad3 (10). In ECs, TGF-β can also activate ALK1, an alternate type-I receptor, which mediates phosphorylation of Smad1 and Smad5 (11, 12). The TGF-β-ALK5 pathway results in inhibition of EC proliferation and migration, whereas the TGF-β-ALK1 pathway results in the activation of proliferation and the migration of ECs. Balance between the TGF-β-ALK1 and TGF-β-ALK5 signalling pathways play an important role in angiogenesis (11, 12). In line with those results, the effects of TGF-β on angiogenesis are highly context dependent, e.g. at low concentrations, TGF-β promotes angiogenesis, whereas, at high concentrations, it inhibits it (13). In addition to its direct effects, TGF-β can exert effects on angiogenesis by regulating the expression of angiogenic factors such as VEGF and components of the ECM such as matrix metalloproteases (MMPs) and integrins (14; 15; 16).

Integrins are heterodimeric transmembrane proteins consisting of α- and β-subunits mediating cell-ECM interactions. Up to date, nine integrin heterodimers have been implicated in blood-vessel formation, namely α1β1, α2β1, α4β1, α5β1, αvβ1, αvβ3, αvβ5, αvβ8 and α6β4 (17). Despite the

fact that integrins lack intrinsic enzymatic activity, upon ligand-induced integrin clustering specific intracellular signals are initiated by the activation of intracellular associated kinases and adaptor proteins in focal adhesion complexes. Integrins regulate divergent biological events including cell adhesion, migration, proliferation, differentiation, survival and angiogenesis (17).

Several studies have provided evidence suggesting that the interplay of VEGF and TGF-β signalling pathways plays an important role in angiogenesis. TGF-β can induce the expression of VEGF by various cells in the tumor microenvironment, such as tumor cells, stromal fibroblasts and cells of the immune system (18; 19). SB-431542, an ALK5/4/7 kinase inhibitor (20) was shown to exert an inhibitory effect on VEGF secretion in human cancer cell lines (21; 22; 23). Moreover, it was shown that SB-431542 stimulated the formation of FLK1- positive embryonic stem cell (ESC)-derived EC sheets induced by VEGF (24).

However, the molecular mechanisms that regulate the cross-talk between the VEGF and TGF-β signalling pathways in angiogenesis have not been determined. To elucidate the interplay between VEGF and ALK5, we analyzed the effects of VEGF and SB-431542 alone or in combination using different angiogenesis assays. We show that VEGF and SB-431542 synergistically induce angiogenesis both *in vitro* and *in vivo*. Gene expression profiling and functional validation revealed that the upregulation of α5-integrin expression plays a crucial role by which VEGF and TGF-β-type-I-receptor- kinase inhibitor achieve their synergistic angiogenic response.

Results

VEGF and ALK5-kinase inhibitor synergistically enhance angiogenesis in fetal mouse metatarsal assay

To investigate the interplay between VEGF and TGF-β-ALK5 signalling in angiogenesis, we made use of an *ex vivo* fetal mouse metatarsal assay. This model provides a quantitative *ex vivo* assay with the complexity of an *in vivo* assay to study the formation of capillary-like structures (25; 26). Metatarsals of 17-day-old mouse embryos were isolated and cultured in 24-well plates for 72 hours to allow adherence, followed by stimulation with VEGF, TGF-β3, SB-431542 or combinations thereof. Capillary-like structures were visualized by staining the cultures using an EC-specific anti-CD31 antibody (Fig. 1A). Similar to what was previously reported for VEGF and TGF-β2 (25), quantitative image analysis showed that VEGF strongly stimulated the formation of vessel-like structures, which was inhibited by TGF-β3 (Fig. 1). Treatment of bone explants with the ALK4/5/7 inhibitor SB-431542 had no significant effects on basal outgrowth of tube-like structures. However, the combination of VEGF with SB-431542 (VEGF+SB-431542) significantly stimulated EC network formation in a synergistic manner. Interestingly, treatment of metatarsals with LY-2157299, another ALK5-

kinase inhibitor that is structurally divergent from SB-431542 (27), slightly inhibited endothelial sheet formation. However, similar to SB-431542, LY-2157299 strongly promoted VEGF-induced angiogenesis (Fig. 2A,C). Our results indicate that activation of VEGF and inhibition of TGF-β (and/or Activin and/or Nodal) signalling by the SB-431542 or LY-2157299 inhibitors synergistically stimulates angiogenesis *in vitro*.

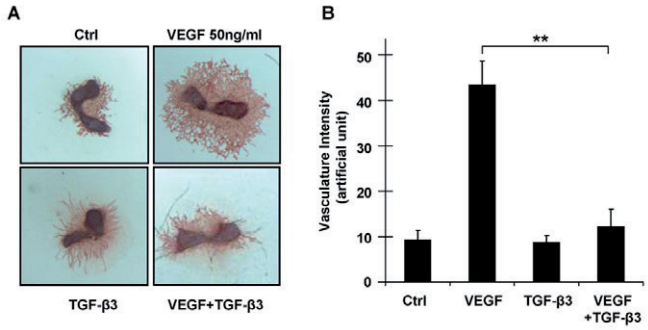

Fig. 1. *TGF-β inhibits VEGF-induced formation of the endothelial network in mouse metatarsal assays. Metatarsals of 17-day-old mouse fetuses were prepared, transferred to cell-culture plates and allowed to adhere, and were then stimulated with VEGF (50 ng/ml), TGF-β3 (5 ng/ml) or both. (A) Cultures were fixed and vessel-like structures were visualized by anti-CD31 staining. Six bones were stimulated per experimental group and one representative picture of each group is shown. Ctrl,control. (B) TGF-β3 did not significantly affect baseline formation of the endothelial network. Incubation with VEGF strongly stimulated the formation of vessel-like structures, which was dramatically decreased by addition of TGF-β3. **P≤0.01.*

ALK5 inhibitor and VEGF synergize in inducing EC sprouts in 3D spheroid culture

To study the effect of combined VEGF and ALK5 inhibitor on EC function, we performed a three-dimensional (3D)-culture collagen EC spheroid assay, which is a suitable model for the analysis of the early regulation of angiogenesis (28). ECs originating from the embedded spheroids invade the gel to form capillary-like structures. Spheroids, generated from human umbilical vein ECs (HUVECs), were embedded into type I collagen gels and stimulated with VEGF, SB-431542 or their combination for 24 hours (Fig. 3). In the absence of stimulation, hardly any EC sprouting was observed. Stimulation with different amounts of VEGF (10-50 ng/ml) dramatically increased the length and the number of sprouts (Fig. 3A). The addition of SB-431542 (1-10 μM) induced the formation of sprouting in a dose-dependent manner, but to a lesser extent compared with VEGF (Fig. 3B).

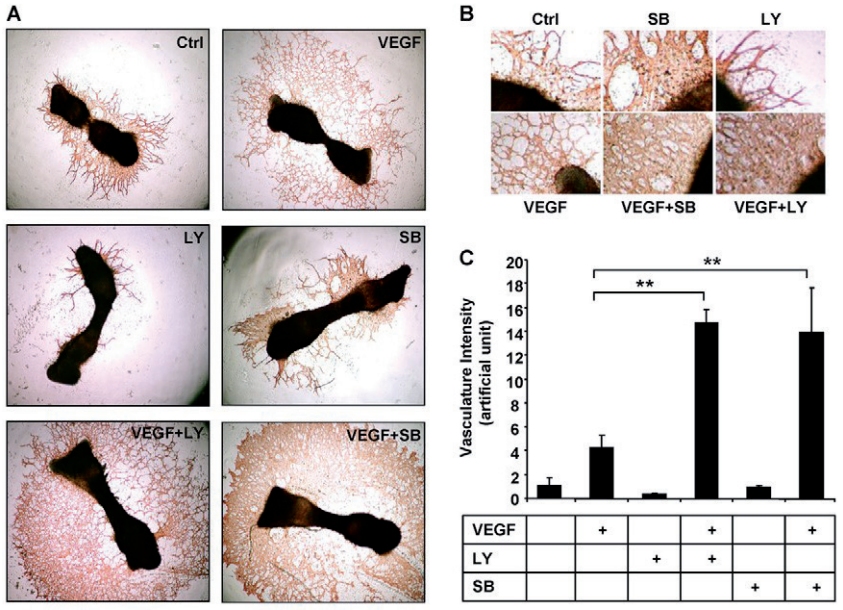

Fig. 2. *Effects of VEGF and SB-431542 on endothelial network formation in mouse metatarsal assays. (A) Metatarsals of 17-day-old mouse fetuses were prepared, transferred to cell-culture plates and allowed to adhere for 4 days. Medium was refreshed and bones were stimulated for 7 days with VEGF (50 ng/ml), SB-431542 [SB; or LY-2157299 (LY)] (10 μM) or both. Cultures were fixed and vessel-like structures were visualized by anti-CD31 staining. Six bones were stimulated per experimental group and one representative picture of each group is shown. Ctrl control. (B) Enlargements of the EC-sheet formation of the images in A. (C) SB-431542 did not significantly affect baseline formation of the endothelial network. LY-2157299 slightly inhibited formation of the endothelial network. Incubation with VEGF strongly stimulated the formation of vessel-like structures, which was dramatically promoted by co-stimulation with SB- 431542 or LY-2157299. **P≤0.01.*

Thus, both VEGF and SB-431542 promote EC sprouting. Interestingly, the addition of a VEGFR2-kinase inhibitor (PTK787) (29) blocked both VEGF- and SB-431542-induced sprouting (Fig. 3D). Because PTK787 can inhibit the stimulatory effect of SB-431542 on EC sprouting, we wondered whether SB-431542 enhances VEGFR2 phosphorylation. We observed no effect of SB-431542 on basal and VEGF-induced VEGFR2 phosphorylation levels or on the VEGF-induced activation of downstream pathways such as ERK and p38 MAP kinases (data not shown). Treatment of HUVECs with SB-431542 decreased basal phosphorylated Smad2, indicating the presence of TGF-β-like factors in media supplements or active TGFβ secreted by HUVECs. VEGF had no effect on the levels of Smad2 phosphorylation (data not shown). Taken together, our results indicate that SB-43152 and VEGF do not influence immediate VEGFR2- or ALK5-induced responses, respectively.

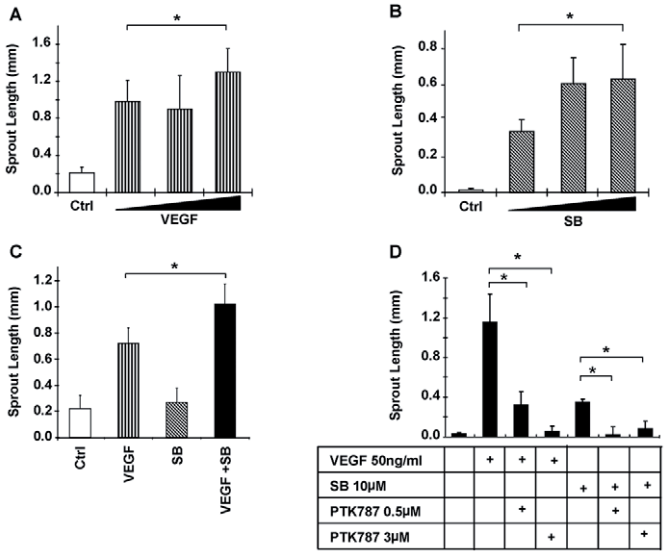

Fig. 3. *Synergistic effect of SB-421542 or TGF-β neutralizing antibody with VEGF on EC sprouting. (A) HUVEC spheroids embedded in collagen were stimulated with increasing amounts of VEGF (10, 25 or 50 ng/ml). (A)HUVEC spheroids embedded in collagen were stimulated with increasing amounts of SB-431542 (SB; 1, 5 or 10 μM). (C) HUVEC spheroids embedded in collagen were stimulated with VEGF (50 ng/ml), SB-431542 (10 μM) or both for 24 hours. (D) EC spheroids were stimulated with VEGF or SB-431542 in the presence or absence of the VEGF-receptor-kinase inhibitor PTK787. (A-D) Quantitative analysis of the mean total sprout length was performed on at least ten spheroids per experimental group. *P≤0.05.*

Although stimulation of the spheroids with VEGF (50 ng/ml) and SB-431542 (10 μM) further increased sprouting (Fig. 3C), this effect was additive and not synergistic. Therefore, we hypothesized that the concentrations of VEGF and SB-431542 are near-to-plateau levels, and we analyzed the effect of sub-optimal concentrations of both VEGF and the ALK5 kinase inhibitor. Stimulation of EC spheroids with 1 ng/ml VEGF or 0.2 μM SB-431542 alone resulted in a small induction of sprouting compared with control (Fig. 4A,B). Interestingly, the combination of low levels of VEGF and SB-431542 synergistically enhanced EC sprouting (Fig. 4A,B). Similar results were obtained when the TGF-β type I receptor kinase inhibitor LY-2157299 was used. The combination of sub-optimal concentrations of LY-2157299 and VEGF resulted in enhanced sprouting compared with VEGF or the inhibitor alone (Fig. 4C,D).

Because SB-431542 interferes with signalling not only of the TGFβ type I receptor ALK5, but also with the activity of the Actvin and Nodal type I receptors ALK4 and ALK7, we investigated whether the effects seen are due to inhibition of TGF-β or antagonism of Actvin-Nodal signalling. We therefore used the 1D11 TGF-β neutralizing antibody (29 30., 1989). EC spheroids were stimulated with a low concentration of VEGF in the presence or absence of the TGF-β neutralizing antibody or an isotype-

matched control antibody (Fig. 4E). The isotype-matched control antibody had no effect on basal or VEGF-induced sprouting. Addition of the TGF-β neutralizing antibody did not induce EC sprouting. However, the combination of TGF-β neutralizing antibody with VEGF significantly induced EC sprouting compared with VEGF or TGF-β blocking antibody alone (Fig. 4E). As expected, ectopic expression of a constitutively active form of ALK5 receptor (caALK5) exerted the opposite response of SB-431542 treatment and inhibited VEGF-induced HUVEC sprouting in the 3D spheroid system (supplementary material Fig. S1A). Moreover, caALK5 overexpression inhibited basal cord formation of mouse embryonic endothelial cells (MEECs) (supplementary material Fig. S1B). Taken together, our results suggest that inhibition of the TGF-β signalling pathway significantly enhances VEGF-induced EC sprouting *in vitro*.

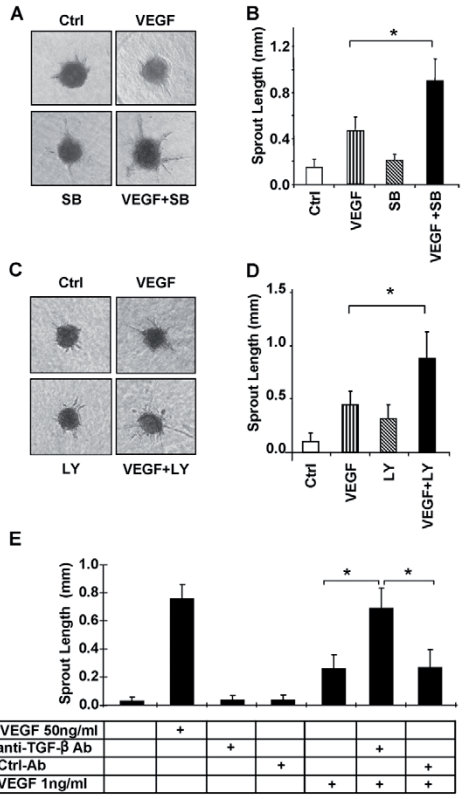

Fig. 4. *Synergistic effect of SB-421542 or TGF-β neutralizing antibody with VEGF on EC sprouting. (A,B) HUVEC spheroids embedded in collagen were stimulated with VEGF (1 ng/ml), SB-431542 (SB; 0.2 μM) or both for 24 hours. One representative picture of each group is shown. Quantitative analysis of the mean total sprout length was performed on at least ten spheroids per experimental group. (C,D) Spheroids were stimulated with VEGF (1 ng/ml), LY-2157299 (LY; 0.2 μM) or both for 24 hours. One representative picture of each group is shown. Quantitative analysis of the mean total sprout length per experimental group is shown. (E) EC spheroids were stimulated with VEGF (1 ng/ml), TGFβ-neutralizing antibody (30 μg/ml), isotype control antibody (10 μg/ml) or a combination for 24 hours. Quantitative analysis of the mean total sprout length per experimental group is shown. *P≤0.05.*

VEGF and ALK5 inhibitor promote EC migration

To further investigate the mechanisms by which VEGF and SB-431542 exert their synergistic effect on angiogenesis, we analyzed their effects on EC migration. To study the effect of VEGF and SB-431542 on migration, serum-starved monolayers of HUVECs were wounded by scratching and were stimulated for 6 hours. There was no effect on migration when cells were stimulated by VEGF or the ALK5-kinase inhibitor alone. However, the combination of VEGF and SB-431542 significantly induced EC migration (Fig. 5A,B). Consistent with these results, over-expression of caALK5 in MEECs resulted in reduced migration, using the transwell migration assay, and invasion, using a Transwell invasion assay with Matrigel coating (11) (supplementary material Fig. S2A,B).

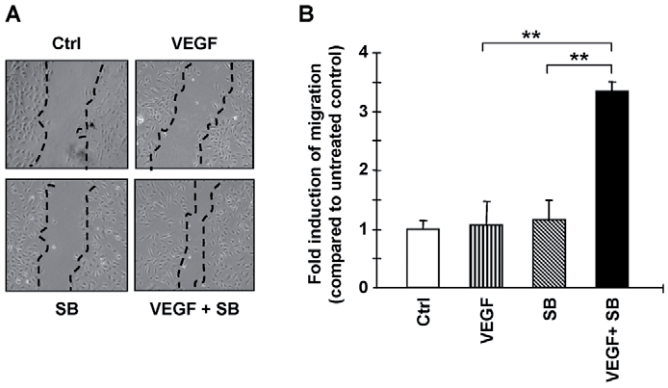

Fig. *5. Effects of SB-431542 and VEGF on EC migration. VEGF and SB- 431542 (SB) synergistically stimulate HUVEC migration. HUVECs were allowed to grow to confluence and serum-starved for 5 hours. Monolayers were wounded and stimulated with VEGF (1 ng/ml), SB-431542 (0.2 μM) or both for 6 hours. (A) One representative picture of each group is shown. Dashed lines indicate the wound edge. (B) Wound closure was measured after 6 hours using ImageJ software. **P≤0.01.*

Transcriptional profiling of the VEGF+SB-431542-induced EC response

Trying to unravel the mechanistic basis of the VEGF and SB-431542 synergistic effects on angiogenesis (EC sprouting), we analyzed expression of a number of genes essential to EC function by quantitative real-time PCR. We used a highly sensitive commercial PCR-based array system containing up to 84 genes related to EC function. From the genes analyzed, the expression of 20 of them was significantly upregulated when cells were stimulated by the combination of VEGF and SB-431542, but not by VEGF or SB- 431542 alone (Table 1). In addition, VEGF+SB-431542 stimulation resulted in the downregulation of 30 genes. Interestingly pro- and anti-apoptotic genes were differentially regulated following VEGF+SB-431542 stimulation. There was an upregulation in expression of the anti-apoptotic gene *Bcl2* and a significant downregulation of pro-apoptotic genes, such as annexin A5, *Bax* and caspase 6 (Table 1), suggesting that VEGF and SB-431542 induce

pathways that promote EC survival. VEGF and SB-431542 synergistically induced the expression of several other genes implicated in angiogenesis, such as angiotensin receptor I, *ACE*, *CCL5*, *IL3* and *IL7* (Table 1). Integrins have been shown to play an important role in EC migration and survival, as well as in capillary sprouting during angiogenesis. In line with this notion, VEGF+SB-431542 stimulation synergistically induced α5 integrin and β3 integrin mRNA expression. To verify these results, we performed quantitative PCR analysis using PCR primer sets different from those used in the array (supplementary material Fig. S3A). Similar results were obtained when mRNA was isolated from EC spheroids embedded in collagen after VEGF and/or SB-431542 stimulation (supplementary material Fig. S3B).

***Table** 1. Summary of genes identified as significantly altered by VEGF/SB-431542 stimulation of ECs, using an EC function PCR-based array system.*

Fold difference compared to control				
Gene	**VEGF**	**SB**	**VEGF/SB**	**p value***
Apoptosis/survival				
Bcl2	2.8	4.0	1×10^{3}	<0.01
Bax	1.1	1.8	0.2×10^{-3}	<0.01
Annexin A5	1.1	2.2	8×10^{-3}	<0.01
CASP6	1.0	1.6	0.1×10^{-3}	<0.01
Angiotensin system				
AGT	0.5	0.8	0.5	0.2
AGTR1	0.5	0.8	0.5×10^{3}	<0.01
ACE	1.6	2.7	0.6×10^{3}	<0.01
Chemokines				
CCL5 (RANTES)	0.93	2.1	5×10^{3}	<0.01
Interleukins				
IL1b	1.0	2.6	21	0.5
IL3	1.0	1.0	2×10^{2}	<0.01
IL6	0.5	0.87	7	<0.05
IL7	0.7	0.99	3×10^{3}	<0.01
IL11	0.9	1.8	45	0.08
Integrins				
ITGA5	1.0	1.31	1×10^{3}	<0.01
ITGB3	7.6	14.7	51	<0.01

**p value of VEGF/SB stimulated versus untreated control cells.*

Crucial role of α5 integrin in VEGF+SB-431542-induced angiogenesis

Stimulation of VEGF and inhibition of the TGFβ type I receptor kinase significantly induces integrins α5 and β3 mRNA expression. Interestingly, pretreatment of HUVECs with cyclohexamide (inhibitor of protein synthesis) diminished the synergistic effect of VEGF+SB-431542 on α5-integrin expression (supplementary material Fig. S4). Those results indicate that the synergistic effect of VEGF and SB-431542 on integrin expression is not direct, but it requires de novo protein synthesis.

To investigate whether integrins α5 and β3 have a functional role in the synergistic effects of VEGF and SB-431542 on capillary sprouting, we studied the loss-of-function effects using an RNA-interference approach. HUVECs were transfected with siRNA oligonucleotides for α5 integrin and β3 integrin or with control siRNA and subsequently seeded to form spheroids, which were then embedded in collagen. In α5 integrin- and β3 integrin- siRNA-transfected cells, the levels of α5-integrin and β3 integrin RNA expression decreased on average 50% (data not shown). Quantification of sprouting demonstrated a significant decrease in VEGF+SB-431542-induced sprout length in the α5 and β3 integrin siRNA transfected spheroids compared with control siRNA (Fig. 6A). It is well recognized that integrin-mediated signalling plays a crucial role in angiogenesis. To exclude the possibility that downregulation of α5 integrin or β3 integrin results in a general block in angiogenesis, we tested the effect of α5 and β3 integrin siRNA on basic fibroblast growth factor (bFGF)- and VEGF (high concentration)-induced sprouting in the spheroid assay. Whereas α5-integrin downregulation diminished the VEGF+SB-431542-induced sprouting, it had no effect on bFGF- or high dose VEGF-induced capillary formation (Fig. 6A). Interestingly, downregulation of β3 integrin by siRNA affected VEGF+SB-431542 as well as bFGF- or high dose VEGF-induced EC sprouting (Fig. 5A). Moreover, α5-integrin downregulation had no effect on VEGF- (1 ng/ml) or SB-431542- (0.2 μM) induced EC sprouting (Fig. 6B).

To further substantiate the role of α5 integrin in the synergistic effect of VEGF and SB-431542 on angiogenesis, we made use of an α5 integrin neutralizing antibody. Addition of α5 integrin function blocking antibody inhibited the synergistic effect of VEGF+SB-431542 on EC sprouting (Fig. 6C). However, α5 integrin neutralizing antibody had no effect on high dose VEGF-induced capillary formation (Fig. 6D). Our results suggest that α5 integrin is necessary for VEGF+SB-431542-mediated synergistic induction of EC sprouting in the 3D EC spheroid assay.

VEGF and ALK5-kinase inhibitor synergistically enhance in vivo angiogenesis in a Matrigel-plug assay in mice

In order to characterize the effect of inhibition of the ALK5 receptor in VEGF-induced angiogenesis, we analyzed the *in vivo* angiogenesis response by placing Matrigel plugs without and with supplements (LY-2157299 and/or VEGF and bFGF) under the skin of mice. Because VEGF

alone did not induce angiogenesis in the *in vivo* Matrigel-plug assay (data not shown), we used a combination of VEGF and bFGF. Vascularization was assayed by CD31 staining of sections from the recovered plugs (Fig. 7B,C). Matrigel plugs without any stimulus showed no vascularization. Addition of LY- 2157299 or VEGF+bFGF combination significantly induced angiogenesis (Fig. 7 A,B).

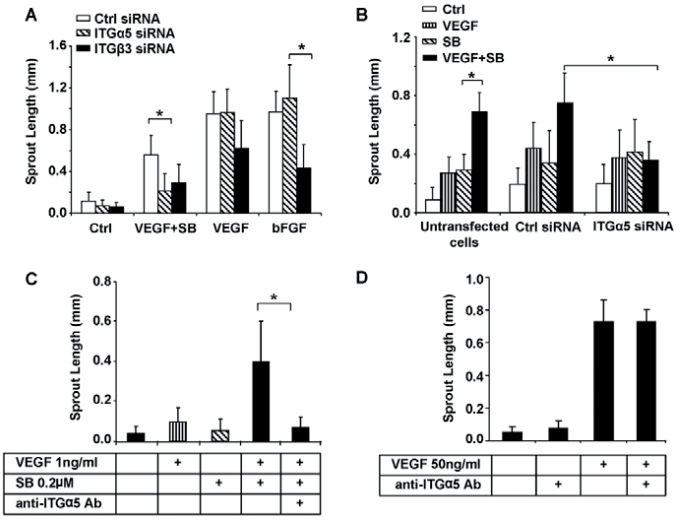

Fig. 6. *Integrin α5 and β3 mediate the synergistic effects of SB and VEGF induced angiogenesis. (A) Downregulation of integrin α5 and β3 inhibits VEGF/SB-431542- induced EC sprouting. HUVECs transiently transfected with control, integrin α5 (ITGα5) or β3 (ITGβ3) siRNA were allowed to form spheroids. Spheroids were embedded in collagen and stimulated with VEGF (1 ng/ml) and SB-431542 (0.2 μM), VEGF (50 ng/ml) or bFGF (100 ng/ml) for 24hr. Quantitative analysis of the mean total sprout length per experimental group is shown. (B) HUVECs were transiently transfected with control or ITGα5 siRNA were plated to form spheroids. Spheroids were embedded in collagen and stimulated with VEGF (1 ng/ml), SB-431542 (0.2 μM) or their combination for 24hr. Quantitative analysis of the mean total sprout length per experimental group is shown. (C) Functional blocking of integrin α5 suppresses VEGF/SB-431542-induced angiogenesis. Spheroids were embedded in collagen and stimulated with VEGF (1 ng/ml), SB-431542 (0.2 μM), VEGF (1 ng/ml) and SB-431542 (0.2 μM), VEGF/SB-431542 or VEGF/SB-431542 and integrin α5 neutralizing antibody (10 μg/ml). Quantitative analysis of the mean total sprout length per experimental group is shown. (D) Functional blocking of α5 integrin does not affect VEGF (high dose)-inducedangiogenesis. Spheroids were embedded in collagen and stimulated with VEGF (50 ng/ml) with or without integrin α5 neutralizing antibody (10 μg/ml). Quantitative analysis of the mean total sprout length was performed on at least 10 spheroids per experimental group using the Olympus Analysis software.*

Addition of LY-2157299 with VEGF+bFGF (VEGF+bFGF+LY) further significantly enhanced the blood-vessel formation (Fig. 7A). To corroborate the role of α5 integrin in VEGF+bFGF+LY synergistic effect on *in vivo* angiogenesis we examined the effect of the α5 integrin neutralizing antibody in the Matrigel plug assay. Whereas addition of α5 integrin neutralizing antibody but not a control antibody inhibited VEGF+bFGF+LY-induced angiogenesis, it had no significant effect on VEGF+bFGF-induced angiogenesis. These results illustrate that inhibition of TGFβ-ALK5 signalling *in vivo* can potentiate the pro-angiogenic effect of VEGF and bFGF, which is critically dependent on the induction of α5 integrin signalling.

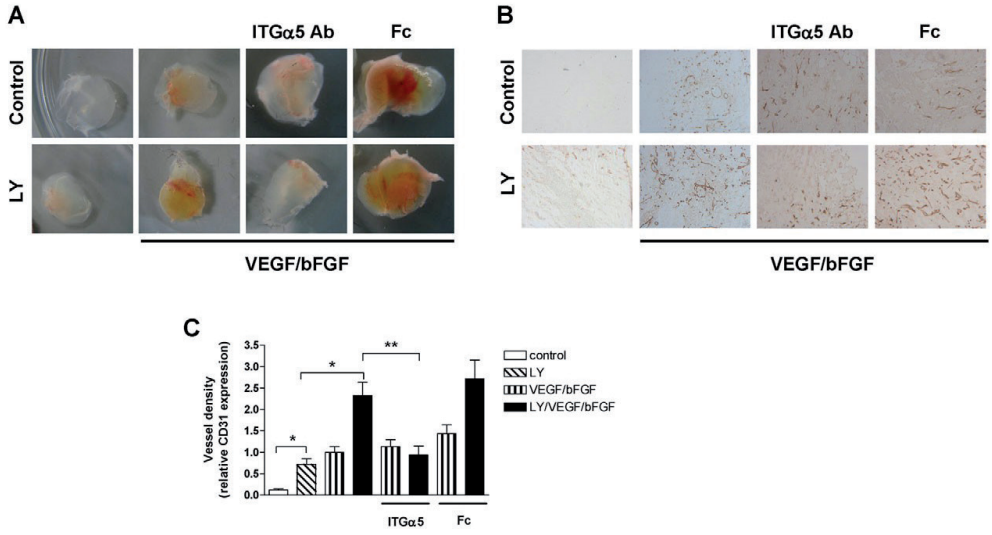

Fig. 7. *LY-2157299 induces angiogenesis in vivo in a Matrigel-plug assay and promotes VEGF+bFGF-induced angiogenesis. Matrigel plugs combined with either PBS (control), VEGF (300 ng/ml) and bFGF (700 ng/ml), and/or LY-2157299 (LY; 0.1 μM), in the presence or absence of α5-integrin-neutralizing antibody (20 μg/ml) or Fc control protein (20 μg/ml), were subcutaneously injected into mice. LY-2157299 induces angiogenesis and enhances VEGF+bFGF-induced angiogenesis. The synergistic effect of LY+VEGF+bFGF on angiogenesis is inhibited by an anti-α5-integrin antibody but not by Fc. (B) Histological analysis of CD31-stained sections recovered from Matrigel plugs with different stimuli. (C) Quantification of the vascular density by CD31 immunostaining as a percentage of the lesional area. *P≤0.05; **P≤0.01.*

Discussion

Here we show that inhibition of TGFβ type-I receptor (ALK5) and VEGF synergistically promotes angiogenesis. Treatment with the ALK5 inhibitors SB-43152 or LY-2157299 strongly enhanced VEGF-induced EC capillary formation and EC sprouting. Similar results were obtained using a TGFβ-neutralizing antibody (30), indicating that inhibition of TGFβ, and not of Activin- Nodal signalling, is likely to cooperate with VEGF in inducing angiogenesis. Addition of the VEGFR2-kinase inhibitor PTK787 (29) blocked not only the VEGF- but also the SB- 431542-induced EC sprouting, suggesting that inhibition of TGFβ signalling potentiates autocrine VEGF signalling in ECs. Importantly, the TGFβ receptor kinase inhibitor promoted basal and VEGF+bFGF-induced angiogenesis *in vivo* in a Matrigel-plug assay. Conversely, ectopic expression of caALK5 potently inhibited all VEGF-induced pro-angiogenic responses of ECs.

Recently it was reported that inhibition of TGFβ antagonized the pro-angiogenic response on human normal dermal microvascular ECs (MVECs) *in vitro* (31). There are several explanations for the discrepancy with our results. First, the pro-angiogenic effects of TGFβ were inhibited using TGFβ antagonistic peptides derived from the TGFβ type III receptor sequence. By contrast, we used pharmacological inhibitors of the TGFβ and/or Activin receptors and a pan TGFβ-neutralizing antibody in combination with VEGF. TGFβ type III receptor facilitates TGFβ signalling by enhancing the binding of ligand to TβRII (32). It was recently shown that the TGFβ type III receptor can bind multiple members of the BMP subfamily, including BMP2, BMP4 and BMP7, and thereby potentiate BMP signalling. BMP2, BMP4 and BMP7 have been shown to induce angiogenesis (33; 25; 34; 35; 36). Consequently, it is possible that the peptide antagonists used by Serrati and co-workers inhibit endogenous BMP signalling (30). Second, ECs of different origin were used. Seratti used MVECs, whereas we used HUVECs. These ECs might respond differently to TGFβ signalling owing to, e.g. expression of different receptors or different levels of TGFβ production. We show that treatment of HUVEC spheroids with the TGFβ-receptor-kinase inhibitor (or TGFβ neutralizing antibody) resulted in decreased basal Smad2 phosphorylation and increased sprouting, suggesting that endogenous TGFβ signalling or TGFβ present in media supplements or produced by the cells inhibits VEGF signalling in HUVECs.

Transcriptional profiling revealed that the VEGF+SB-431542 stimulation of HUVECs synergistically regulates a number of genes involved in angiogenesis. Our results showed that VEGF+SB- 431542 stimulation of ECs resulted in a dramatic decrease in the expression of pro-apoptotic genes and an increase in that of anti-apoptotic genes. TGFβ was shown to induce apoptosis of ECs via autocrine and/or paracrine stimulation of VEGF expression and signalling by VEGFR2 via downstream activation of p38 (37). We observed no effects of SB-431542 on VEGF-induced ERK and p38 phosphorylation (data not shown). Thus, on the basis of our results, we suggest that stimulation of ECs with VEGF and SB-431542 protects ECs from apoptosis and enhances their survival and proliferation. This might explain the dramatic induction of EC-sheet formation we observed upon VEGF+SB- 431542 challenge in the ex vivo metatarsal assay.

α5 integrin, a cell-surface receptor for fibronectin, is one of the genes that is synergistically upregulated by VEGF+SB-431542 co-stimulation. We confirm the importance of this upregulation in VEGF+SB-431542-induced EC sprouting because genetic ablation and function neutralizing antibodies of α5 integrin inhibit VEGF+SB-431542-induced EC sprouting *in vitro* and *in vivo*. Our results underline the important role of α5 integrin demonstrated before in blood vessel development; α5 integrin null embryos exhibit abnormal blood vessel formation and a lower complexity vascular network (38). Furthermore, α5β1 integrin function blocking antibody was shown to inhibit HUVEC tube formation *in vitro* (39), and to inhibit tumor neovascularization and growth in animal models (40).

VEGF and TGFβ signalling play a central role in tumor angiogenesis and thus in tumor development and metastasis. Several therapeutic strategies targeting TGFβ signalling have been shown to prevent the growth and metastasis of certain cancers (41). Interestingly, a recent study using low doses of the TβRI kinase inhibitor (LY364947) in experimental tumors decreased VSMC coverage of the tumor endothelium and promoted the accumulation of anticancer drugs in the tumor tissue (42). Our results suggest that inhibition of TGFβ signalling renders the ECs more sensitive to VEGF-induced sprouting *in vitro* and *in vivo*. Therefore, anti-TGFβ-based therapeutic strategies must be carefully considered before administration of TGFβ antagonists because there might be adverse effects, including the induction of tumor angiogenesis. A better treatment modality, as suggested by this study, is combining VEGF and TGFβ pathway inhibitors to inhibit tumor cell metastasis and angiogenesis.

In conclusion, we investigated the effect of TGFβ type I receptor inhibitor(s) or TGF-β neutralizing antibody on VEGF-induced EC sprouting. Our results suggest that combining VEGF and inhibition of TGFβ-ALK5 signalling synergistically promotes angiogenesis *in vitro* and *in vivo* by inducing a cascade of expression of genes that play important roles in EC survival and in angiogenesis. Functional studies revealed that the induction and function of α5 integrin is a key determinant in VEGF+SB-431542-induced angiogenesis.

Materials and Methods

Recombinant proteins, inhibitors and antibodies

The VEGF165 isoform was purchased from R&D Systems and bFGF from Peprotech. SB-431542 was purchased from Tocris Biotrend, LY-2157299 from Calbiochem and PTK787 from Novartis. α5-integrin neutralizing antibody was purchased from BD Biosciences, the TGFβ-neutralizing antibody and isotype control obtained from Genzyme, and the Fc domain of IgG1 (MOPC-21) from Bio Express, West Lebanon, NH.

Ex vivo metatarsal angiogenic assay

All animal experiments were approved by the local animal ethics committee. Metatarsals from 17-day-old mouse fetuses were dissected as described earlier (26). Six bones per experimental group were transferred to 24-well tissue culture plates containing α-MEM (Gibco), 10% FBS and penicillin/streptomycin (PS), and allowed to adhere for 4 days. Then, medium was replaced by fresh medium containing the stimuli. Cultures were fixed 7 days after stimulation and vessel formation was visualized by anti-CD31 staining (Deckers 25, 2001).

Cell culture

HUVECs cells were cultured in Medium 199 with Earle's salt and L-glutamine (Gibco), 10% FCS, heparin (LEO pharma), bovine pituitary extract (Gibco) and PS on plates coated with 1% gelatin, at 37°C, 5% CO2. HUVECs were used up to passage 4. Experiments were confirmed with HUVECs from different donors. MEECs were cultured in DMEM (Gibco) supplemented with 10% FCS and PS on 0.1% gelatin- coated plates, at 37°C, 5% CO2.

3D-culture spheroid assay

HUVECs (400 cells per spheroid) were suspended in Medium M199 containing Earle's salt and L-glutamine, 10% FBS, heparin, bovine pituitary extract, PS and seeded in non-adherent round-bottom 96-well plates. After 24 hours, spheroids were embedded into collagen and stimulated with corresponding stimuli in the presence or absence of inhibitors or neutralizing antibodies for another 24 hours. EC sprouts were measured by Olympus Analysis software.

Migration scratch assay

HUVECs were seeded in six-well plates coated with 1% gelatin and allowed to grow to confluence. After serum starvation, monolayers were wounded with three scratches and medium

was replaced by fresh medium containing stimuli. Cell migration was measured in five areas per well directly after wounding and 6 hours later by automated image analysis using ImageJ software.

Matrigel plugs

Male 7- to 8-week-old C57BL/6 mice (Charles River Laboratories, Sulzfeld, Germany) were injected subcutaneously near their abdominal midline with 0.4 ml of Matrigel basement membrane, high concentration (BD Biosciences, San Jose, CA) combined with either PBS, VEGF (300 ng/ml) and bFGF (700 ng/ml), and/or LY- 2157299 (0.1 μM), in the presence or absence of α5-integrin-neutralizing antibody (20 μg/ml) or Fc control protein (20 μg/ml). Groups of four plugs were injected for each treatment and the experiment was repeated twice. Seven days later Matrigel plugs were removed, fixed in formalin and embedded in paraffin. Sections were subjected to histological analysis with eosin. Sections were deparaffinized. Quenching of endogenous peroxidase activity was done using 0.3% H2O2 in methanol for 20 minutes at room temperature (RT), followed by antigen retrieval using citrate buffer and blocking with 1% BSA in PBS for 1 hour at RT. The primary antibody against CD31 (1:1000, Santa Cruz Biotechnologies, Santa Cruz, CA) was incubated in 1% BSA in PBS overnight at RT. Biotin-conjugated secondary antibodies were applied followed by amplification using the strep-AB-complex/HRP (DAKO, Hamburg, Germany). Finally, diaminobenzidine substrate (Sigma) was added to visualize peroxidase activity. The area covered by CD31-positive staining was quantified with image analysis.

Cell transfection and RNA interference

Cells were seeded in six-well plates and the following day were transiently transfected with control siRNA or siRNA against α5 integrin and β3 integrin, purchased from Dharmacon, according to the manufacturer's instructions. One day after transfection, cells were trypsinized and seeded for spheroid formation or used for RNA isolation.

RNA isolation and EC PCR array

Total DNA-free cellular RNA was extracted with RNeasy kit (Macherery-Nagel) according to the manufacturer's instruction. RNA from HUVECs grown in monolayers that were either non-treated or treated with VEGF, SB-431542 or VEGF+SB-431542 for 24 hours was isolated from three independent biological experiments and separately analyzed on the array. A commercial EC PCR-array (purchased from Superarray) was used to investigate gene expression profiling of selected angiogenesis- related genes involved in EC function (http://www.sabiosciences.com/rt_pcr) product/HTML/PAHS-015A.html). The experiment was performed in triplicate. The manufacturer's instructions were strictly followed (http://www.sabiosciences.com/). Gene expression levels were determined by using data analyzer template provided by Superarray (http://www.sabiosciences.com/pcrarraydataanalysis.php), using GAPDH, β-actin and ribosomal protein L13a as reference. The non-stimulated condition was set to 1.

Adenoviral infection

HUVECs or MEECs growing in 70-80% confluent monolayers were infected with an adenovirus expressing either caALK5 or lacZ with an MOI of 500. At 48 hours after infection, cells were trypsinized and re-seeded to be used in different assays.

EC cord formation

Matrigel Basement Membrane Matrix Growth Factor Reduced (Becton Dickinson) was added at 50 µl to each well of a 96-well plate and allowed to polymerize for 1 hour at 37°C. Cells were removed from culture by trypsinization and resuspended at 30,000 cells/ml. 100 µl cell suspension were plated in each well in triplicates and plates incubated for 48 hours. Pictures were acquired with a phase-contrast microscope in four different fields. The length of branches was quantified using automated image analysis using the Olympus Analysis software.

Transwell migration and invasion assays

Transwell migration was performed in 24-well plates with filter inserts of a pore size of 0.8 µm (Costar). For the Transwell invasion assay, filters were coated with Matrigel. 30,000 cells were seeded in the upper chamber. Experiments were done in triplicates. Cells were fixed after 20 hours with 4% paraformaldehyde and stained with 0.1% crystal violet. Pictures of the filters were acquired with phase-contrast microscopy at 10x magnification. Three fixed positions were imaged of each membrane and the number of cells was counted.

RNA isolation and quantitative PCR analysis

RNA from HUVECs growing in monolayers or EC spheroids embedded in collagen [non-treated, or VEGF- (1 ng/ml), SB-431542- (0.2 µM) or VEGF+SB-431542-treated for 24 hours) was isolated from three independent biological experiments with RNeasy kit (Macherery-Nagel) and subjected to cDNA synthesis with RevertAid H Minus first strand cDNA synthesis kit (Fermentas) according to the manufacturer's instruction. Expression of α5 integrin (ITGα5) and house-keeping gene acidic ribosomal phosphoprotein (ARP) were analyzed using the following primers:

ITGα5 forward: 5-ATACTCTGTGGCTGTTGGTGAATTC-3;
ITGα5 reverse: 5-ATTAAGGATGGTGACATAGCCGTAA-3;
ARP forward: 5-CACCATTGAAATCCTGAGTGATGT-3;
ARP reverse: 5-TGACCAGCCGAAAGGAGAAG-3.

Taqman PCR reactions were performed using the ABI prism HT7900 sequence-detection system (Applied Biosystem). All samples were plated in duplicates. Gene expression levels were determined with the comparative ΔCt method using ARP as reference and the non-stimulated condition was set to 1.

Cyclohexamide experiments

HUVECs growing in monolayers were either untreated or pretreated with 5 µg/ml cyclohexamide for 1 hour, and subsequently unstimulated or treated with 1 ng/ml VEGF, 0.2 µM SB-431542 or a combination of both VEGF and SB-431542 for 24 hours. RNA was isolated and subjected to cDNA synthesis followed by quantitative real-time PCR analysis.

Statistical analysis

All results are expressed as the mean ± s.d. Statistical differences were examined by two-tailed Student's t-test and $P \leq 0.05$ was considered to be statistically significant (in the figures, *$P \leq 0.05$ and **$P \leq 0.01$).

Acknowledgments

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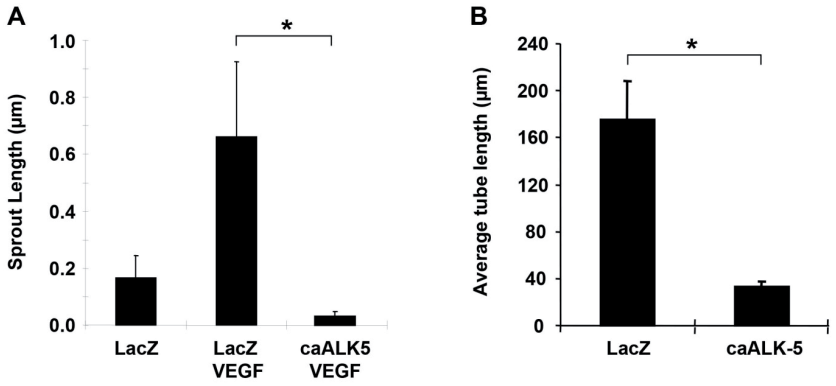
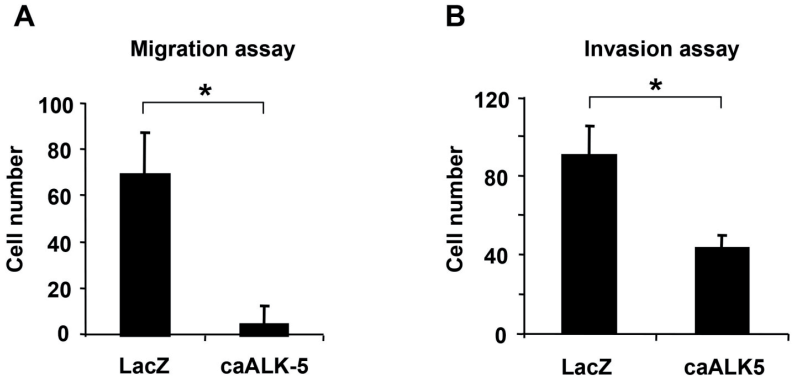
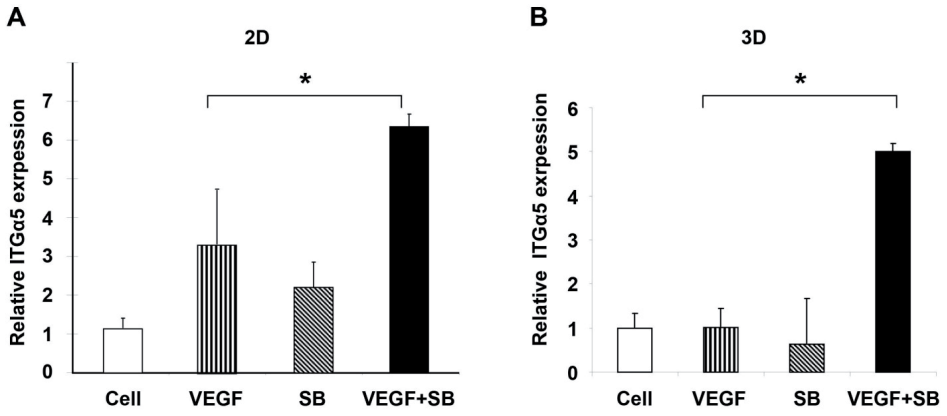
Supplementary Information



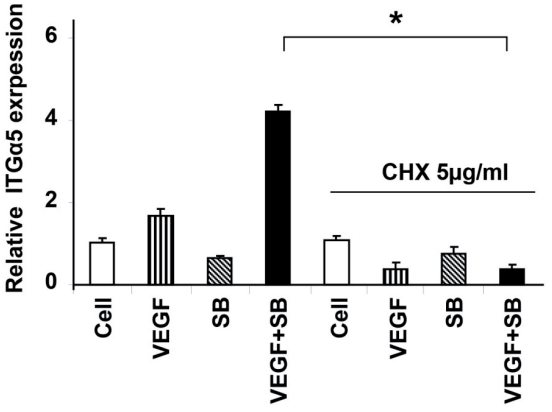
Supplemental Fig. 1 *Overexpression of constitutively active (ca)ALK5 inhibits EC sprouting and cord formation. (A) HUVECs infected with adenoviruses expressing caALK5 or lacZ were plated to form spheroids and embedded in collagen were stimulated with VEGF (50 ng/ml) for 24 hours. One representative picture of each group is shown. Quantitative analysis of the mean total sprout length was performed on at least ten spheroids per experimental group. (B) MEECs infected with adenoviruses expressing caALK5 or lacZ were plated on Matrigel-coated plates for cord formation for 48 hours. One representative picture of each group is shown. Quantitative analysis of the mean total cord length per experimental group is shown.*



Supplemental Fig. 2 *Overexpression of caALK5 inhibits EC Transwell migration and invasion. MEECs infected with adenoviruses expressing caALK5 or lacZwere plated on the upper chamber of 24-well plates with filter inserts. (A) Cells were fixed after 20 hours with 4% paraformaldehyde, stained with 0.1% crystal violet and the number of migrating cells was counted in three different fields. (B) For the Transwell invasion assay filters were coated with Matrigel. After 20 hours, cells were fixed, stained and the number of invaded cells was counted in three different fields*



Supplemental Fig 3 *SB-431542 and VEGF synergistically induce expression of integrin α5 in 2D and 3D EC cultures. HUVECs growing in monolayers (A) or in EC spheroids treated (B) with VEGF (1 ng/ml), SB-431542 (0.2 μM) or their combination for 24 hours. Extracted mRNA was reverse transcribed and used in a quantitative PCR array to determine relative integrin α5 mRNA expression patterns. The mRNA levels of integrin α5 in each sample was first normalized to the expression of ARP in that sample and then normalized to the expression of that gene in untreated HUVECs.*



Supplemental Fig 4 *VEGF+SB-431542-induced α5-integrin expression depends on new protein synthesis. HUVECs growing in monolayers were pretreated with 5 μg/ml cyclohexamide (CHX), followed by VEGF (1 ng/ml), SB-431542 (0.2 μM) stimulation or their combination for 24 hours. Extracted mRNA was reverse transcribed and used in a quantitative PCR array to determine relative α5-integrin mRNA expression patterns.*