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**Aberrant association between Vascular Endothelial Growth Factor
Receptor-2 and VE-cadherin in response to Vascular Endothelial
Growth Factor-A in *Shb*-deficient lung endothelial cells**

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Abstract

Vascular permeability is a hallmark response to the main angiogenic factor VEGF-A and we have previously described a reduction of this response in *Shb* knockout mice. To characterize the molecular mechanisms responsible for this effect, endothelial cells were isolated from lungs and analyzed *in vitro*. *Shb* deficient endothelial cells exhibited less migration in a scratch wound-healing assay both under basal conditions and after vascular endothelial growth factor-A (VEGF-A) stimulation, suggesting a functional impairment of these cells *in vitro*. Staining for VE-cadherin and vascular endothelial growth factor receptor-2 (VEGFR-2) showed co-localization in adherens junctions and in intracellular sites such as the perinuclear region in wild-type and *Shb* knockout cells. VEGF-A decreased the VE-cadherin/VEGFR-2 co-localization in membrane structures resembling adherens junctions in wild-type cells whereas no such response was noted in the *Shb* knockout cells. VE-cadherin/VEGFR-2 co-localization was also recorded using spinning-disc confocal microscopy and VEGF-A caused a reduced association in the wild-type cells whereas the opposite pattern was observed in the *Shb* knockout cells. The latter expressed slightly more of cell surface VEGFR-2. VEGF-A stimulated extracellular-signal regulated kinase, Akt and Rac1 activities in the wild-type cells whereas no such responses were noted in the knockout cells. We conclude that aberrant signaling characteristics with respect to ERK, Akt and Rac1 are likely explanations for the observed altered pattern of VE-cadherin/VEGFR-2 association. The latter is important for understanding the reduced *in vivo* vascular permeability response in *Shb* knockout mice, a phenomenon that has patho-physiological relevance.

Keywords

Vascular endothelial growth factor receptor-2, VE-cadherin, adherens junctions, *Shb*, signal-transduction, Rac1

1. Introduction

Increased vascular permeability is a hallmark response to the main angiogenic factor, VEGF-A¹ [1]. VEGF-induced vascular permeability is thought to result from either transport through vesiculo-vacuolar organelles [2] or a VEGFR-2 dependent dissociation of adherens junctions [3]. The cell-cell interacting molecule VE-cadherin is the primary organizer of adherens junctions [4, 5] and VEGF-A causes its dissociation from this site [6]. VE-cadherin and VEGFR-2 have been found to be in complex with each other [5] but how VEGF-A dissociates VE-cadherin from adherens junctions remains unresolved. VEGF-A induced vascular permeability has been reported to depend on signaling pathways involving c-Src [3, 7] and Rac [8].

The Src-homology 2 protein B (Shb) is an adapter protein operating downstream of VEGFR-2 [9]. An abnormal endothelial compartment was observed in *Shb* knockout mice that causes reduced tumor growth [10]. The endothelial ultrastructure and VE-cadherin and CD31 staining patterns were less distinct in knockout endothelial cells. These morphological aberrations correlated with reduced VEGF-A stimulated vascular permeability and decreased angiogenesis. The results were taken to reflect altered VEGFR-2 signaling. We subsequently observed in *Shb* knockout venules abnormal adherens junctions from which VE-cadherin did not dissociate upon VEGF-A addition. Consequently, reduced vascular permeability in response to ischemia after arterial ligation was observed [11].

The present study was conducted in order to understand the molecular mechanisms causing the aberrant characteristics of adherens junctions in *Shb* deficient endothelial cells. The latter fail to reduce membranous VE-cadherin/VEGFR-2 co-localization in response to VEGF-A as a consequence of signaling defects and this may have implications for *in vivo* regulation of adherens junctions and vascular permeability.

2. Materials and Methods

2.1 Animals

¹ VEGF= vascular endothelial growth factor
VEGFR-2= vascular endothelial growth factor receptor 2
Shb= Src-homology 2 protein B
ERK= extracellular-signal regulated kinase
FAK= focal adhesion kinase
PLC= phospholipase C
GAP=GTPase activating protein
NOS= nitric oxide synthase
PI3= phosphatidylinositol-3'

The *Shb* knockout mouse [12] was obtained on a FVBN/C57Bl6/129SvJ background. Breeding of wild-type and *Shb* knockout mice had been approved by the local animal ethics committee at the Uppsala county court. Mice of ages 2-3 months were sacrificed by cervical dislocation and used in the current study.

2.2 Isolation of primary endothelial cells

Lungs were collected in a tube containing cold Hanks' Balanced Salt Solution (HBSS) with antibiotics. These were finely minced and placed in a 50 ml tube with 30-40 ml of sterile 2.5mg/ml Worthington (Lakewood, NJ, USA) type I collagenase in Dulbecco's PBS mixed with Ca/Mg (DPBS) that had been predigested at 37 °C for one hour and mixed with gentle agitation for 20 minutes. Cells were centrifuged at 70g for 1 minute and the supernatants saved. This was repeated twice and the pooled supernatants were centrifuged at 620g at 4 °C for 4 minutes before suspended in 2-3 ml DPBS with 0.1% BSA. The cells were incubated for 20 minutes with rotation at 4 °C with 25-50 ul beads (DYNAL, Invitrogen, Carlsbad, CA, USA) that had been conjugated with CD31 antibody (BD Bioscience, San Jose, CA, USA). Cells with beads were collected using a magnet and washed 6-8 times with PBS/0.1% BSA. The cells were then plated on 1% gelatin coated plates in DMEM with high glucose, 20% FCS, 1 mM nonessential amino acids, 1mM sodium pyruvate, 25 mM HEPES (Invitrogen), 100 ug/ml BTI endothelial mitogen (ECGS, Biomedical Technologies, Stoughton, MA, USA), 100 ug/ml heparin (Sigma, St. Louis, MO, USA). The growth medium was replaced every 2-3 days. Alternatively, aortic endothelial cells were isolated as described [13]. The isolated lung endothelial cells showed a high percentage (>80%) of CD31 positive cells and a negligible content (<5%) of LYVE-1 positive lymphendothelial cells (results not shown).

2.3 Scratch wound-healing assay

Gelatin pre-coated plastic dishes with cultures of confluent lung or aortic endothelial cells were scraped in a straight line with a sterile plastic pipette tip, after which wound healing in the absence or presence of 20 ng/ml VEGF-A (R&D Systems, Minneapolis, MN, USA) was monitored. Photographs were taken at the time points indicated by phase contrast light microscopy at 100× original magnification.

2.4 VE-cadherin/VEGFR-2 co-localization

For VE-cadherin/VEGFR-2 double stains, cells were cultured for 4 days on cover slips and maintained in medium without serum for the last 3 hours of that period before incubated (or not) for 10 minutes with 100 ng/ml VEGF-A. The cells were

washed and fixated in 4% paraformaldehyde, blocked in 2% BSA before staining for VE-cadherin (R&D systems) and VEGFR-2 (BD Bioscience) at room temperature for 2 hours. After washing, the cells were incubated with the corresponding secondary antibodies (donkey anti-goat Alexa 488, donkey anti-rat Alexa 594, from Invitrogen). After final washing and mounting with Fluoromount G (Southern Biotech, Birmingham, AL, USA) the slides were subjected to confocal microscopy (TE2000 C-1 with Plan Fluor ELWD 60x/1.40 objective, Nikon, Japan).

2.5 Time-lapse imaging of VE-cadherin/VEGFR-2 co-localization

Isolated lung endothelial cells were attached on 1% gelatin coated coverslips 24 hours prior to the experiment. Firstly, the cells were stained in the medium on ice with VE-cadherin (1/100) for 30 minutes. After washing, the cells were stained for 30 minutes on ice with Alexa 488 anti-goat secondary antibody (1/500) together with PE-conjugated VEGFR-2 antibody (BD Biosciences, #555308, 1/50), washed twice and incubated in buffer (125 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgCl₂, 3 mM glucose and 25 mM HEPES, pH 7.40). The association between VE-cadherin and VEGFR-2 was recorded with a Yokogawa CSU-10 spinning disk confocal unit (Andor Technology, Belfast, Northern Ireland) attached to a Nikon TE2000 microscope with a 60x 1.40-NA objective (Nikon, Japan) as described previously [14]. The 488 nm line of an argon laser (ALC 60, Creative Laser Production, Munich, Germany) and the 561 nm line of a diode-pumped solid-state laser (Jive, Cobolt AB, Stockholm, Sweden) were used for excitation of Alexa 488 and phycoerythrin, respectively. The laser beam was homogenized by a rotating light shaping diffuser (Physical Optics Corp, Torrance, CA, USA) before being refocused into the confocal scanhead. Fluorescence emission was selected by interference filters (527 nm with 27 nm half bandwidth for Alexa 488 and 586/20 nm for phycoerythrin, Semrock Inc, Rochester, NY, USA) and detected with a back-illuminated EM-CCD camera (DU-888, Andor Technology) under MetaFluor software control. Image pairs were acquired every 10 s. VE-cadherin and VEGFR-2 co-localization was quantified as follows: Puncta in the red channel representing VEGFR-2 stained structures were localized through the movie using the findspot function in MetaMorph (Universal Imaging, Sunnyvale, CA, USA). At each detected location, the fluorescence in the green channel (VE-cadherin) was measured in a circle of 0.6 μm (c). The corresponding average fluorescence of the surrounding annulus (a) with an outer

diameter of 1.1 μm was subtracted from the average circle fluorescence (c-a) to correct for local background [15]. The resulting values were averaged for each frame. In addition, total average fluorescence for each cell monitored was determined.

2.6 Detection of cell surface VEGFR-2

Isolated lung endothelial cells (wild-type and *Shb* knockout were always analyzed in parallel) cultured in gelatin pre-coated plastic dishes (2.5 cm diameter) were stimulated or not for 10 minutes with 100 ng/ml VEGF-A, after which they were kept on ice for the remainder of the procedure. After three washes with PBS, they were incubated with 0.15 mg/ml sulfo-NHS-SS-biotin (Thermo Scientific, Rockford, IL, USA) for 10 minutes after which they were washed once with TBA (25 mM Tris, pH=8.0, 137 mM NaCl, 5 mM KCl, 2.3 mM CaCl_2 , 0.5 mM MgCl_2 , 1 mM Na_2HPO_4) and three times with PBS. The cells were solubilized in 20 mM Tris (pH=7.5), 125 mM NaCl, 10% glycerol, 1% NP40, 1 $\mu\text{g/ml}$ PMSF, centrifuged for 10 minutes at 14000 $\times g$ and an aliquot was taken for total VEGFR-2 determination. Streptavidin-agarose beads (Upstate Biotechnology, Lake Placid, NY, USA) were added to the bulk of the samples (20 μl beads per 100 μl lysate) and incubated under rotation for 2 hours. The beads were pelleted by centrifugation for 10 minutes at 14000g, supernatants collected, washed three times in lysis buffer and dissolved in SDS-sample buffer for Western blot analysis for VEGFR-2 together with supernatants and lysate aliquots.

2.7 Western blot analysis

Isolated wild-type or *Shb* knockout lung endothelial cells in gelatin pre-coated 96-well tissue culture plates were stimulated (or not) with 100 ng/ml VEGF-A for 5 and 15 min. The cells were dissolved in SDS-sample buffer and subjected to Western blot analysis. The blots were incubated with antibodies recognizing activated (phosphorylated) ERK and Akt (Cell Signaling, Beverly, MA), as well as the corresponding total ERK and Akt antibodies. Antibody reactivity was detected with ECL plus (GE Healthcare, Uppsala, Sweden). Rac1 activity was determined using a kit provided by Thermo Scientific (Rockford, IL, USA) after VEGF-A stimulation (100 ng/ml) for 5 minutes or not.

2.8 Statistical analysis

Means $\pm$ SEM are given for the number of observations described in each legend. Students' *t*-tests were used to provide an assessment for chance differences between the groups compared.

3. Results

3.1 Scratch-wound healing assay

A scratch-wound healing assay was employed to determine if isolated lung endothelial cells from *Shb* knockout mice exhibit functional abnormalities. *Shb* knockout lung and aortic endothelial cells displayed reduced gap wound healing in response to VEGF-A (Fig. 1). In addition, the basal gap closure rate was lower in the *Shb* knockout lung endothelial cells.

3.2 VE-cadherin/VEGFR-2 co-localization

We have previously reported an altered *in vivo* VE-cadherin staining pattern and ultrastructural abnormalities indicating aberrant properties of adherens junctions as a consequence of *Shb* deficiency [10, 11]. An explanation for these observations is abnormal characteristics of VEGFR-2/VE-cadherin co-localization due to signaling defects since VE-cadherin and VEGFR-2 have been reported to interact and it is likely that such an interaction has implications for junction integrity. To address this possibility, isolated lung endothelial cells were stimulated or not with VEGF-A and stained for VE-cadherin (green) and VEGFR-2 (red). Staining for VEGFR-2 was detected diffusely throughout the cell being more intense in some membranous structures and the perinuclear area. Stimulation of wild-type cells with VEGF-A appeared to cause a more distinct staining pattern with further concentration of staining to the perinuclear region in larger and more distinct structures. Wild-type cells under basal conditions displayed a partial co-localization of VE-cadherin and VEGFR-2 to membranous structures representing the *in vitro* counterpart of adherens junctions (Fig. 2). In addition there was co-localization of these in the perinuclear region corresponding to the perinuclear recycling compartment [16]. VEGF-A reduced the co-localization of VE-cadherin/VEGFR-2 in the junctions of the wild-type cells (Fig. 2) and instead caused increased co-localization in the perinuclear region. VEGF-A exerted no major effect on the staining pattern of the *Shb* knockout endothelial cells compared with the unstimulated situation. These staining patterns demonstrate that VEGF-A caused a rapid alteration of VEGFR-2/VE-cadherin association only in the wild-type cell-cell junctions, whereas in the *Shb* knockout cells no major effects were noted.

To assess the dynamics of the VE-cadherin and VEGFR-2 co-localization, spinning disc confocal microscopy imaging was employed to visualize this process in live isolated lung endothelial cells with images acquired every 10 seconds. Quantitation of total average VE-cadherin (green) and VEGFR-2 intensity (red) in the monitored cells is shown in Fig. 3A. The staining intensity in both channels decreased with time in a manner that was indistinguishable between the genotypes and unaffected by VEGF-A addition, probably reflecting photobleaching and/or internalization and degradation of the fluorophore. VE-cadherin and VEGFR-2 co-localization was also assessed (Fig. 3B). Initially, the intensity of VE-cadherin staining co-localizing with VEGFR-2 appeared higher in the wild-type cells, but this trend failed to reach statistical significance. There was no immediate response to VEGF-A addition (arrow) but 3 minutes later a trend of reduced co-localization was initiated that continued for the rest of the experiment. The *Shb* knockout cells exhibited the reverse pattern with an increased trend of co-localization starting 3 minutes after VEGF-A addition. The values were normalized for total association between VE-cadherin and VEGFR-2 within each trace to better visualize these changes (Fig. 3B). It then became apparent that wild-type and *Shb* knockout cells showed opposite responses to VEGF-A beginning 3 minutes after addition with reduced association in the wild-type and increased association in the *Shb* knockout. These differences were significant in five of the eight last time points of the recordings. The reduced association in response to VEGF-A in the wild-type cells is likely to reflect the altered staining pattern observed in Fig. 2 with less co-localization in the junction-like structures after VEGF-A addition. In contrast, the *Shb* knockout cells were unable to respond to VEGF-A with dissociation between VEGFR-2 and VE-cadherin.

3.3 VEGFR-2 cell surface expression and signaling

One process that may have an impact on the dynamics of VEGFR-2 and VE-cadherin association is the recycling of VEGFR-2. We determined the proportion of cell surface expressed VEGFR-2 with or without addition of VEGF-A in wild-type and *Shb* knockout cells (Fig. 4). Relatively less cell surface VEGFR-2 expression was noted in the wild-type cells. VEGFR-2 dependent signaling was also investigated under the different conditions in isolated lung endothelial cells (Fig. 5). *Shb* knockout lung endothelial cells displayed reduced ERK and Akt activities in response to VEGF-A. These differences were more pronounced than those previously noted in liver endothelial cells [10]. In addition, Rac1 activity (GTP-Rac1) was determined

after 5 minutes of stimulation with VEGF-A (Fig. 5). The wild-type cells responded to VEGF-A with increased GTP-Rac1 relative total lysate Rac1, whereas the *Shb* knockout cells displayed elevated basal activity that did not increase further upon VEGF-A stimulation. Phosphorylation of Y397 at FAK was also investigated and was statistically significantly increased by 15 ± 4 % ($p=0.017$, $n=7$) in wild-type endothelial cells whereas no stimulatory response was recorded in the *Shb* knockout cells (3 ± 5 %, $p=0.57$, $n=7$). The altered signaling characteristics of *Shb* deficient lung endothelial cells provide a biochemical explanation for the defects in angiogenesis and vascular permeability previously [10, 11] and presently observed.

4. Discussion

Adherens junctions are of importance for regulation of vascular permeability and conditions that cause increased vascular permeability often alter their structural features [4]. A key player in regulation of these events is the cell adhesion protein VE-cadherin [5] and VEGF-A is thought to via its receptor VEGFR-2 affect the localization and functional characteristics of VE-cadherin [3]. The underlying mechanisms are poorly understood but VEGFR-2 and VE-cadherin are known to associate with each other [5]. Both VE-cadherin and VEGFR-2 have been shown to dissociate from adherens junctions and internalize upon VEGF-A stimulation [8, 17]. Whereas VEGFR-2 recycles to a large extent to the cell surface [16] the fate of VE-cadherin is less well described. Our previously reported *in vivo* observations in cremaster venules suggest that VE-cadherin dissociates from junctions to other cellular compartments in response to VEGF-A [11]. As visualized by scanning electron microscopy, this had consequences for the structural features of junctions that could only be observed in wild-type and not in *Shb* knockout venules. In the present study using isolated endothelial cells corresponding changes in the staining patterns of VEGFR-2 and VE-cadherin were observed. These results suggest that VEGFR-2 and VE-cadherin normally dissociate from junctions in response to VEGF-A and re-associate at intracellular sites such as early endosomes and the perinuclear region. This is in agreement with a previous study demonstrating VE-cadherin/VEGFR-2 dissociation upon VEGF addition [3]. The present data show that *Shb* knockout endothelial cells failed to reproduce this pattern of events: VEGF-A did not cause a detectable dissociation of VEGFR-2 and VE-cadherin at the junction-like structures as determined by both standard confocal microscopy and spinning disc confocal microscopy. Our data do not directly address the functional significance of

the observed VEGF-induced dissociation between VE-cadherin and VEGFR-2 but suggest that this is a crucial step in the recycling of these proteins that ultimately is important for the regulation of adherens junctions. They also suggest the involvement of *Shb* in this process. Indeed, it is clear from our previous publications [10, 11, 18] that *Shb* deficiency has consequences for the structure of adherens junctions *in vivo* and that this has functional implications. The latter is most clearly demonstrated by the altered physiological response to arterial ligation, in which the vascular permeability was reduced 24 hours after the procedure [11].

One possible explanation for the disturbed pattern of VE-cadherin/VEGFR-2 co-localization at the membrane as a consequence of *Shb* deficiency is the increased cell surface expression of VEGFR-2, which makes dissociation between the two proteins less likely. The cues responsible for VEGFR-2 internalization are poorly understood but endocytic trafficking of VEGFR-2 has been shown to involve synectin and the phosphatase PTP1b followed by the intracellular activation of certain signaling events [13, 17]. Presumably, there are signals responsible for triggering VEGFR-2 internalization and it is conceivable that *Shb* deficiency will interfere with these. On the other hand, the increased cell surface expression could be the result of accelerated trafficking of intracellular VEGFR-2 to the plasma membrane, which in turn could be effected by deficient signaling.

Alternatively, the failure of *Shb* deficient endothelial cells to dissociate VE-cadherin from VEGFR-2 in junctions in response to VEGF-A can be explained by the aberrant signaling signature observed in the current study. Activation of VEGFR-2 causes Src-dependent dissociation between VEGFR-2 and VE-cadherin and Src-dependent VE-cadherin phosphorylation, which are likely to contribute to the loosening of junctions [3, 7, 19]. A parallel pathway involves Rac-activation and serine-phosphorylation of VE-cadherin and presumably its dissociation from VEGFR-2 in junctions [8]. Consequently, the aberrant Rac1 activation pattern presently observed in the *Shb* deficient endothelial cells is a plausible underlying cause for the altered VEGFR-2/VE-cadherin co-localization pattern observed. Filamin B deficiency exerts effects that resemble those of *Shb* knockout endothelial cells and in that situation elevated basal Rac1 activity was also observed [20]. In addition, Akt has previously [21] been shown to regulate vascular permeability and thus the junctional rearrangements presently observed in *Shb* deficient endothelium may additionally be explained by differences in the signaling characteristics of isolated lung endothelial cells with

respect to Akt activation. However, a significant component of Akt-dependent VEGF-A stimulated vascular permeability appears to reside in Akt-dependent phosphorylation of eNOS [21]. The presently recorded effects of *Shb* deficiency on endothelial cell signaling characteristics are more pronounced than those previously reported in isolated liver endothelial cells [10], in which reduced responsiveness to VEGF-A was reported for ERK but not for Akt. It may appear as if *Shb* knockout endothelial cells exhibited elevated basal ERK activity but this effect was variable and noted only in three of five experiments, thus failing to achieve statistical significance. Increased basal signaling activity in *Shb* deficient cells has also been reported in oocytes [22] and T lymphocytes [23]. Regardless, ERK and Akt are crucial signaling intermediates for various aspects of endothelial function [24] and consequently, major perturbations in the activities of these will have significant consequences for endothelial cell function. The mechanistic explanations to the reduced signaling responses to VEGF in *Shb* knockout endothelial cells remain elusive. *Shb* is a multi-domain adapter protein that may interact with Src kinases, PLC γ , PI3kinase, FAK, Crk and ras-GAP [25], and consequently, perturbations in one or several of these signaling pathways may yield the presently observed results.

5. Conclusions

The present data demonstrate altered signaling characteristics of *Shb* deficient endothelial cells that are likely explanations to the aberrant interactions between VE-cadherin/VEGFR-2. These findings provide a mechanistic understanding of the previously observed aberrant *in vivo* vascular permeability response to VEGF-A in *Shb* knockout mice and thus have patho-physiological implications

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7. Author contribution

GZ, GC, GT, MHOR, EV and MW performed the experiments. GZ, GC, MP, SB, AT and MW designed, analyzed and interpreted the experiments. GZ, GC, MP, AT and MW wrote the paper. GZ and GC have contributed equally to the study.

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9. Figure legends

Figure 1: Scratch wound healing assay of wild-type (WT) and *Shb* knockout (KO) isolated aortic or lung endothelial cells. The gap closure in percent was estimated at the time points given in the absence or presence of VEGF-A in three to four separate experiments. Means $\pm$ SEM are given. *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, when compared with the corresponding *Shb* knockout value.

Figure 2: Staining of untreated or VEGF-A treated (100 ng/ml) wild-type (WT) or *Shb* knockout (KO) isolated lung endothelial cells. Panel A shows VE-cadherin (green), VEGFR-2 (red) and dapi (blue) stained endothelium. Original magnification 600x. Panel B shows VE-cadherin/VEGFR-2 co-localization quantified as the relative fraction of junction-like structures showing co-localization (yellow, exemplified by arrow in non-stimulated wild-type image or *Shb* knockout images), $p < 0.05$, $n = 4$ images each. Junction-like structures in VEGF-A stimulated wild-type cells showed less co-localization (see arrow for example in WT VEGF-A).

Figure 3: Time-lapse imaging of VE-cadherin/VEGFR-2 co-localization in isolated lung endothelial cells. Pictures were taken every 10 seconds and VE-cadherin co-localization with VEGFR-2 was determined using Metamorph software. In top two panels (A), total average VE-cadherin and VEGFR-2 fluorescence in the monitored cells are shown. Average VE-cadherin fluorescence co-localizing with VEGFR-2 puncta (c-a) and fraction of VE-cadherin puncta that were co-localized with VEGFR-2 are shown in lower two panels (B). For determining the fraction of VE-cadherin fluorescence co-localizing with VEGFR-2, co-localizing puncta were scored when their VE-cadherin (c-a) value fell within the highest 20-percentile of all (c-a) values from a cell. Arrows indicate addition of VEGF-A. The values were determined on 10 observations in each genotype. * indicates $p < 0.05$ when comparing wild-type with *Shb* knockout.

Figure 4: Cell surface expression of VEGFR-2 wild-type or *Shb* knockout isolated lung endothelial cells. In A, percent cell surface expression of VEGFR-2 given as means $\pm$ SEM for 6 experiments. * indicates $p < 0.05$ when compared with corresponding wild-type value with a paired Students' t-test.

Figure 5: Signaling characteristics of wild-type or *Shb* knockout isolated lung endothelial cells Isolated lung endothelial cells were stimulated for different time points with 100 ng/ml VEGF-A after a 3.5 hour serum deprivation period. Lysates were subjected to Western blotting for pY1173-VEGFR-2, VEGFR-2, pS473-Akt, Akt, pT/Y202/204-ERK and total ERK. Panels show densitometric quantification (in arbitrary units) of the corresponding Western blotting data in 3-5 experiments. Means $\pm$ SEM are given. * indicates $p < 0.05$ with a paired Students' t-test when compared with value without VEGF-A. Rac1-GTP assay with or without VEGF-A stimulation for 5 minutes was also performed. Densitometric quantitation relative corresponding lysate total Rac1 was determined and related to VEGF-A stimulated wild-type value in three separate experiments. Means $\pm$ SEM are given. * indicates $p < 0.05$ when compared with non-stimulated wild-type.

Figure 1

Figure 1

Lung EC scratch healing

Aortic EC scratch healing

Figure 2

Figure 3

Figure 4

Figure 4

Figure 5

VEGF-A (min): 0 5 15 0 5 15

WT

Shb KO

VEGF-A (5 min):

0

+

0

+

WT

Shb KO

Rac1-GTP/Rac1