Dysfunctional Microvasculature as a Consequence of Shb Gene Inactivation Causes Impaired Tumor Growth

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Abstract

Shb (Src homology 2 protein B) is an adapter protein downstream of the vascular endothelial growth factor receptor-2 (VEGFR-2). Previous experiments have suggested a role for Shb in endothelial cell function. Recently, the Shb gene was inactivated and Shb null mice were obtained on a mixed genetic background, but not on C57Bl6 mice. The present study was performed to address endothelial function in the Shb knockout mouse and its relevance for tumor angiogenesis. Tumor growth was retarded in Shb mutant mice, and this correlated with decreased angiogenesis both in tumors and in Matrigel plugs. Shb null mice display an abnormal endothelial ultrastructure in liver sinusoids and heart capillaries with cytoplasmic extensions projecting toward the lumen. Shb null heart VE-cadherin staining was less distinct than that of control heart, exhibiting in the former case a wavy and punctuate pattern. Experiments on isolated endothelial cells suggest that these changes could partly reflect cytoskeletal abnormalities. Vascular permeability was increased in Shb null mice in heart, kidney, and skin, whereas VEGF-stimulated vascular permeability was reduced in Shb null mice. It is concluded that Shb plays an important role in maintaining a functional vasculature in adult mice, and that interference with Shb signaling may provide novel means to regulate tumor angiogenesis. [Cancer Res 2009;69(5):2141–8]

Introduction

The microvasculature is an organ of importance for numerous pathophysiologic processes including tumor growth. Primarily, it supplies peripheral cells with nutrients and oxygen and disposes of waste. In addition, recent studies have implicated that endothelial cells are important in maintaining a stem cell niche or a differentiated cell phenotype (1–3).

The microvasculature must display plasticity to adapt to different functional demands. Expansion of the microvasculature can occur by angiogenesis or vasculogenesis. Angiogenesis is regulated by several growth factors of which vascular endothelial growth factor (VEGF) is the most important. The mammalian VEGF family consists of five members, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PLGF (4). These activate their corresponding receptors, VEGFR-1, VEGFR-2, and VEGFR-3. Among these, VEGF-A and VEGFR-2 are the most important for angiogenesis, and loss of these genes causes embryonic lethality (5–7). In addition to being a regulator of neoangiogenesis, VEGF-A increases vascular permeability and regulates the fenestration of the endothelium (8–10).

Shb is an Src homology 2 (SH2) domain adapter protein downstream of tyrosine kinase receptors, such as the platelet-derived growth factor receptors α and β, fibroblast growth factor receptor-1, and VEGFR-2 (11–14). The Shb SH2 domain interacts primarily with the activated receptors, and consequently, Shb generates signaling complexes through interactions with other components by binding to these via the proline rich motifs, the phosphotyrosine binding domain and phosphotyrosine residues present in Shb. Among the Shb interacting partners can be found Src family kinases, focal adhesion kinase (FAK), phospholipase C-γ, Crk, and c-Abl (11, 15–17).

Several studies have implicated a role of Shb in endothelial cell function. It was observed that Shb overexpression promotes apoptosis in response to the inhibitors of angiogenesis angiotatin and endostatin (18, 19). Furthermore, an Shb mutant with an inactive SH2 domain disrupted vascular tube formation in vitro in mouse endothelial cells or in differentiating embryoid bodies (EBs; refs. 20, 21). Shb overexpression, on the other hand, promoted tube formation in differentiating EBs in response to VEGF and platelet-derived growth factor. Shb was found to interact with VEGFR-2, and Shb knockdown reduced FAK activation and cell migration in response to VEGF (13, 15, 22). Shb knockdown in SVR endothelial angiosarcoma cells augmented their sensitivity in vivo to the angiogenesis inhibitor honokiol, causing diminished tumor growth (23). Taken as a whole, the data suggest an involvement of Shb in multiple aspects of endothelial cell function.

We have recently generated an Shb knockout mouse that displays embryonic malformations (24). No Shb null pups were born on the C57Bl/6 background due to an early (before E8.5) embryonic defect, whereas on a mixed genetic background, Shb null mice were procured. Most malformed embryos carrying the Shb null allele showed no signs of vascular defects, although a few embryos exhibited petechiae. Despite the fact that viable mice are born on the Shb null background, we considered the possibility that Shb knockout mice display vascular abnormalities. The present study was conducted to investigate an Shb null vascular phenotype in relation to tumor angiogenesis.

Materials and Methods

Animals. The generation of mice carrying the Shb null allele has been previously described (24). These were either on a mixed genetic background...
was repeated twice, and the pooled supernatants were centrifuged at 6200 g.

Cells were broken apart by passing through a 14-gauge needle four to five times. Cells were then plated on 1.8 mg/mL collagen-coated plates in DMEM with high glucose, 20% FCS, 1 mmol/L nonessential amino acid, 1 mmol/L sodium pyruvate, 25 mmol/L HEPES (Invitrogen), 100 μg/mL ECGS (BTI), 100 μg/mL leupeptin (Sigma). The growth medium was replaced every 2 to 3 days. The procedure was an adaptation of the Brigham and Women’s Hospital cell biology core center for vascular biology (Harvard Medical School) endothelial cell isolation protocol.

**Immunostaining.** When paraffin-embedded sections were used, they were treated with trypsin (0.025%) for 30 min at 37°C. The sections (5–10 μm, treated or not with trypsin) were washed with PBS and incubated for 10 min with freshly prepared 3% H2O2 in methanol. These were blocked using the blocking solution (TSA Biotin System, Perkin-Elmer Life Sciences) for 1 h and reacted for 1 h with primary antibody (anti-mouse CD31, BD Bioscience). The sections were subsequently stained for 1 h with biotinylated anti-rat IgG (Vector Laboratories), followed by incubation for 1 h with Streptavidin–horseradish peroxidase (Perkin-Elmer Life Sciences). Peroxidase was revealed using the AEC peroxidase substrate kit (Vector Laboratories). Alternatively, a tyramide amplification step (TSA-Biotin System, Perkin-Elmer) was included.

**Permeability assay.** Vascular leakage was visualized using FITC-conjugated dextran (70 kDa, Sigma) injected i.v. through the tail vein at a dose of 2 mg/kg body weight. Organs were removed after 2 h. After injection with 4 mg/kg of the anti-histamine pyrilamine (Sigma) to avoid histamine-dependent release of the dye, wild-type, Shb−/−, and Shb knockout mice were injected with Evans blue (Sigma) at a dose of 25 mg/kg body weight. After 30 min, 400 ng VEGF (R&D Systems) dissolved in 50 μL HBSS or HBSS alone were injected intradermally (9). After an additional 30 min, the skin patches were dissected, weighed, and eluted at 70°C for immunohistochemistry.

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**Tumor growth.** Lewis lung carcinoma (LLC) cells (10⁶) or T241 fibrosarcoma cells (500,000) were injected s.c. in the back of 3-mo-old to 5-mo-old wild-type or Shb−/− mice. The mice were monitored for tumor growth beginning on day 7 and every other following day until 17 d or when the tumor size reached 1 cm², measuring the length and width of the tumor using calipers. Tumor volume was determined using the formula: volume = 0.44 × (width)² × (length).

Implanted tumors were excised and frozen. The tumor sections were immunostained with α-CD31 (BD Bioscience), α-CD68 (Serotec), Ng2 (Millipore), or α-A-selectin (BD Bioscience). To quantify tumor vasculature and macrophage infiltration, staining for CD31, CD68, Ng2, and E-selectin was numerically counted from representative photos and normalized per

(129Sv/C57Bl6/FVB) or on the C57Bl6/6 background. In the latter case, no Shb−/− mice were obtained. The average weight and blood pressure of Shb knockout mice on the mixed background was not significantly different from that of the corresponding controls (results not shown). All animal experiments had been approved by the local animal ethics committee at Uppsala University.

**Morphology.** Mice of either 3 mo or 8 to 10 mo of age were sacrificed, and their organs were immediately excised and frozen or fixed. The number of mice used for morphologic analyses was four to five, and in most instances, more than one section from each mouse was analyzed. The duration between death and the tissue being in fixative was never >3 min. Frozen tissue was cryosectioned. Organs fixed in 4% paraformaldehyde were paraffin embedded. For electron microscopy, the organs were placed in 2.5% glutaraldehyde and immediately minced to 1 mm³–sized pieces. These were embedded in Epon812. Ultrathin sections were contrasted with uranylacetate and lead citrate. Electron microscopy was carried out using a Hitachi H-7100 transmission electron microscope at an accelerating voltage of 75 kV.

**Isolation of endothelial cells.** Livers were collected in a tube containing cold HBSS with antibiotics. These were finely minced and placed in a 50-mL tube with 30 to 40 mL of sterile 2.5 mg/mL Worthington type I collagenase in Dulbecco’s PBS mixed with Ca/Mg (DPBS) that had been predigested at 37°C for 1 h and mixed with gentle agitation for 20 min. Clumps were further broken apart by passing through a 14-gauge needle four to five times. Cells were centrifuged at 70 × g for 1 min, and the supernatants were saved. This was repeated twice, and the pooled supernatants were centrifuged at 620 × g at 4°C for 4 min before suspension in 2 to 3 mL DPBS with 0.1% bovine serum albumin (BSA). The cells were incubated for 20 min with rotation at 4°C with 25 to 50 μL beads (DYNAL, Invitrogen) that had been conjugated with CD31 antibody (BD Bioscience). Cells with beads were collected using a magnet and washed six to eight times with PBS/0.1% BSA. The cells were then plated on 1.8 mg/mL collagen-coated plates in DMEM with high glucose, 20% FCS, 1 mmol/L nonessential amino acid, 1 mmol/L sodium pyruvate, 25 mmol/L HEPES (Invitrogen), 100 μg/mL ECGS (BTI), 100 μg/mL leupeptin (Sigma). The growth medium was replaced every 2 to 3 days. The procedure was an adaptation of the Brigham and Women’s Hospital cell biology core center for vascular biology (Harvard Medical School) endothelial cell isolation protocol.

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area unit. To measure cell proliferation, the sections were stained for Ki-67 (Dako) and visualized with a fluorescent Alexa Fluor secondary antibody (Molecular Probes).

For tumor perfusion, 0.05 mg FITC-labeled Tomato Lectin (Vector Laboratories) was injected in the tail vein 20 min before sacrifice. Excised tumors were frozen and cryosectioned before CD31 staining. FITC-positive CD31-stained tumor vessels were scored by double immunofluorescence.

Western blot. Isolated endothelial cells were stimulated or not with 20 ng/mL VEGF-A for 2 min. The cells were lysed in SDS sample buffer. The samples were electrophoresed on SDS-polyacrylamide gels, protein was transferred on to Hybond-P filters (GE Healthcare), and these were then probed for pY-1175 VEGFR-2, total VEGFR-2, pY-397 FAK, total FAK, phosphorylated extracellular signal-regulated kinase (ERK), total ERK, and phosphorylated Akt, total Akt, phosphorylated myosin light chain (pMLC), pY-658 VE-cadherin, phosphorylated p38, and total p38 (all antibodies were from Cell Signaling, except for total VEGFR-2 from R&D Systems, pY-397 FAK from Biosource, pY-VE-cadherin from ProSci, and pMLC from Santa Cruz Biotechnology) before incubation with secondary antibodies and enhanced chemiluminescence.

Real-time reverse transcription–PCR. RNA was isolated from 3-month-old kidney, liver, and heart using the Qiagen RNeasy mRNA preparation kit (Qiagen). Alternatively, mRNA from isolated glomeruli was used. Real-time reverse transcription–PCR (RT-PCR) was performed on these RNA templates using the Qiagen QuantiTact SYBR-green RT-PCR kit on a Roche light cycle (Roche Diagnostics). Crossing points (Ct values) were calculated using the accompanying software. PCR primers and amplification conditions are given in Supplementary Table S1.

Results

Tumor growth. Tumor growth was tested in relation to the Shb gene and its role in angiogenesis and/or microvascular function. Tumor studies using LLC cells or T241 fibrosarcoma cells (25, 26) require the C57Bl/6 background. Shb–/– mice could not be obtained on that background, and we, thus, forced to use Shb–/+ mice instead. When LLC tumors reached a size of ~0.2 cm³, growth in Shb–/– mice became retarded, and on day 13.5, these displayed a significantly reduced tumor size (Fig. 1). The growth of T241 tumors was reduced at early time points and became significantly retarded on day 14 (Fig. 1). Subsequent morphologic analysis revealed the LLC and T241 tumors to have a lower vascular density, as assessed by CD31 staining (Figs. 1 and 2 and Supplementary Fig. S1). In addition to the reduced density of CD31-positive structures, the staining for E-selectin, which is a marker for activated endothelium (27), and CD68, which is a macrophage marker (28), were also suppressed in the LLC tumors grown on Shb–/– mice, indicating a generalized reduction in

![Figure 2.](image-url) Characteristics of LLC and T241 fibrosarcomas grown in Shb–/– and Shb–/+ mice. DNA synthesis (percentage of Ki-67–positive cells), vascular density (number of CD31-positive structures per area), E-selectin relative vascular density (percentage of E-selectin–positive structures over CD31-positive structures) indicating activated endothelium, macrophage infiltration (CD68-positive cells per area), pericyte density (Ng2-positive structures per area unit), tumor perfusion (CD31-positive structures that had bound FITC-Tomato Lectin), and vascular permeability (Evans blue leakage per tumor weight) were also determined (n = 4). Vascular densities and tumor proliferation were determined for LLC and T241; CD68 and E-selectin were determined in LLC and Ng2; Tomato Lectin binding and vascular permeability were determined in T241. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Students’ t test).
vascular and inflammatory activity (Fig. 2 and Supplementary Fig. S1). LLC and T241 proliferation, assessed by Ki67 staining, was dramatically diminished in the Shb−/− mice (Fig. 2).

Staining T241 tumors grown on Shb+/+ mice for the pericyte marker Ng2 (Supplementary Fig. S2) showed a reduced density of pericytes (Fig. 2). A reduced vascular density was further confirmed by staining for VE-cadherin (Supplementary Fig. S2). In addition, the VE-cadherin staining pattern of Shb+/− tumor endothelium was altered with less regular and distinct staining. Tumor perfusion, assessed as relative Tomato Lectin binding was significantly increased in the +/− tumors (Fig. 2). This indicates that the reduced vascular bed in the +/− tumors is more efficiently used. Vascular permeability was not different in the +/− tumors (Fig. 2). Endothelial ultrastructure was abnormal in the +/− tumors (Supplementary Fig. S2) bearing a distinct resemblance with the irregular appearance observed in the knockout heart (see below and Fig. 3). The endothelium of wild-type tumors showed increased and irregular cytoplasm, although in a manner distinct from that of the +/− situation. The data provide no indication of vascular normalization in the +/− tumors but rather suggest vascular dysfunction as a cause of reduced tumor growth.

The results suggest that loss of one Shb allele reduces tumor vascularization and that this hampers tumor growth.

**Angiogenesis in Matrigel plugs.** To further study Shb in relation to in vivo angiogenesis, Matrigel plugs containing VEGF-A and FGF-2 were used. For these experiments, Shb−/− mice on a mixed genetic background were used. In vivo angiogenesis was reduced in Shb null mice, both with respect to vascular density and distance of vascular growth into the plugs (Table 1 and Supplementary Fig. S3).

It is concluded that the absence of Shb reduces in vivo angiogenesis.

**Structure of microvasculature in Shb null liver and heart.** Staining wild-type liver for CD31 revealed the anticipated staining pattern of periportal vessels, a central vein and a capillary network. Certain areas in the liver vasculature are shown that certain sinusoids connecting the major vessels (results not shown). Certain areas in the Shb knockout liver exhibit vascular irregularities (Supplementary Fig. S4). Ultrastructural examination reveals that certain Shb−/− liver endothelial cells, unlike the wild-type cells, display irregular cytoplasmic extensions protruding toward the lumen (Supplementary Fig. S4). No signs of endothelial cell death were observed by staining for active (cleaved) caspase-3 or TUNEL (results not shown).

The analysis of Shb knockout vasculature was further examined by CD31 staining of heart (Fig. 3A and B). As observed in the liver, the Shb knockout heart exhibited areas with an abnormal endothelial cell appearance. These seemed to contain a fuzzy lining toward both the lumen and the basal membrane. Counting the number of CD31-positive cells showing these features gave a value of 11.4 ± 5.2% in the Shb knockout compared with 2.7 ± 1.3% in the wild-type control (P < 0.01 with paired Student’s t test). Ultrastructural examination revealed control heart endothelium with dense and even endothelial cytoplasm surrounding the lumen, whereas the Shb null heart endothelial cytoplasm was in some cells diffuse and irregular without distinct boundaries (Fig. 3C and D). These also contained processes extending toward the lumen (Fig. 3E).

In addition, the heart was stained for the endothelial adherens marker (29, 30) VE-cadherin marking cellular junctions (Fig. 3F and G). Longitudinal sections of heart vasculature were compared by confocal microscopy between Shb null and wild-type mice, revealing in the former an irregular and discontinuous staining pattern that appeared punctuate and wavy. In contrast, the wild-type heart featured continuous staining along the capillary that was localized to distinct, thin, and almost straight lines. This difference could be measured as an increase by 46% (P < 0.001) in the thickness of VE-cadherin staining in the knockout heart capillaries relative to control.
Staining for the smooth muscle cell marker α-smooth muscle actin revealed no distinct difference between knockout and wild-type heart (Fig. 3H and I).

In summary, Shb knockout liver and heart endothelium displays certain morphologic aberrations with cytoplasmic extensions toward the lumen.

Gene expression of VEGF-A and VEGFR-2. A possible explanation for the observed endothelial ultrastructure in Shb null mice would be alterations in the expression of VEGF-A and/or VEGFR-2. For this purpose, these genes were analyzed together with CD31 as a control for total vascular cell RNA. Whereas expression of VEGFR-2 was increased in the consequence of altered VEGF-A and VEGFR-2 expression.

Vascular permeability. The number of structures with extracapillary fluorescence was increased in the Shb null heart compared with control (Supplementary Fig. S5) after injection with FITC-dextran.

To quantify the increased vascular leakage, mice were injected with Evans blue. Shb+/− skin, heart, and kidney all contained significantly increased amounts of dye compared with wild-type control (Fig. 4). In the lung, however, no difference was noted. The increased vascular leakage could relate to the altered VE-cadherin staining pattern (Fig. 3).

The VEGF-induced vascular permeability of Shb−/− and Shb+/− mice was significantly reduced compared with the wild-type controls, which showed a robust response (Fig. 4 and Supplementary Fig. S6). The VEGF-induced vascular permeability assay includes pretreatment with pyrilamine to reduce histamine-dependent vascular permeability, and this regimen reduces the vascular leakage in the skin, but not in the kidney of Shb−/− mice (results not shown).

A potential consequence of increased vascular permeability could be edema. No signs of edema in organs from Shb null mice were noted, as visualized in the s.c. vasculature (results not shown). Staining the cells for VE-cadherin and CD31 as a control for total vascular cell RNA. Whereas expression of VEGFR-2 was increased in Shb−/− cells, the staining of knockout cultures was patchy and discontinuous whereas the wild-type endothelial cultures exhibited continuous VE-cadherin staining along the cellular boundaries suggesting altered cell-cell communications in the Shb-deficient cells (Supplementary Fig. S7A).

Visualizing the cytoskeleton revealed stress fibers in both knockout and wild-type cells, although the number of these was higher in untreated knockout cells (Fig. 5A and Supplementary Fig. S7B). The Shb null cells consistently showed a less regular cell shape with numerous extensions. Addition of VEGF-A to the control cells produced changes that made the cells resemble the Shb null cells in the absence of VEGF-A. No clear difference in the cytoskeleton between knockout and wild-type cells was noted after stimulation with VEGF-A.

VEGFR-2 activation of isolated liver endothelial cells, as visualized by pY1173 VEGFR-2 phosphorylation, was not affected by the absence of Shb (Fig. 5B). FAK activity was comparable in all groups studied, although VEGF exerted a minimal increase in activity in wild-type endothelial cells. Immunostaining cells for pY397-FAK revealed an effect of VEGF-A in the wild-type cells (Supplementary Fig. S7C). VEGF caused an accumulation or targeting of pY-FAK to the processes extending from the stimulated cells (examples indicated by arrows), whereas no detectable changes were noted in the Shb−/− cells. The cytoskeleton may also be under the control of Rho family GTPase (G-protein) activity (31) that can be measured by levels of MLC phosphorylation (32–34). We noted increased MLC phosphorylation under basal conditions and a blunted response compared with control cells to VEGF-A in the Shb−/− cells (Fig. 5B), indicating deregulated Rho family G-protein activity as a contributing factor of relevance to the altered cytoskeleton. Shb null liver endothelial cells exhibited an elevated

### Table 1. Matrigel vascularization in Shb−/− and Shb+/+ mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>Shb−/−</th>
<th>Shb+/+</th>
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<tbody>
<tr>
<td>Vascular density (no. CD31-positive structures/mm² at the rim of the plug)</td>
<td>28.3 ± 5.8⁴ (5)</td>
<td>47.2 ± 6.7 (5)</td>
</tr>
<tr>
<td>Vascular ingrowth (distance from edge to central regions with CD31-positive structures in mm)</td>
<td>0.52 ± 0.09⁴ (5)</td>
<td>0.90 ± 0.16 (5)</td>
</tr>
<tr>
<td>Percentage of the Matrigel plug area vascularized</td>
<td>13.0 ± 2.9¹ (5)</td>
<td>33.4 ± 6.9 (5)</td>
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NOTE: Vascular density and length of vascular ingrowth into Matrigel plugs in Shb knockout and wild-type mice. Matrigel plugs were placed s.c., and 7 d later, the mice were sacrificed. The Matrigel plugs were retrieved, frozen, and sectioned for analysis by CD31 and MLC staining of vascular density in the plug and length of vascular ingrowth. For ingrowth or percentage area vascularized, overview pictures were taken and the average distance from the edge to the area is devoid of CD31 staining was measured. These values were also used to calculate the percentage area vascularized (by relating to the corresponding average radius of the plug) by using the formula [1 – (plug radius – plug distance ingrowth)² / (plug radius)²]. For density, six random pictures at the rim of the Matrigel plugs were taken and vascular density was counted as an average number of CD31 staining structures for each plug.

Five animals were analyzed in each group.

⁴P < 0.05 with a paired Student’s t test.
¹P < 0.001 with a paired Student’s t test.
²P < 0.05 with a Student’s t test.
basal ERK activity measured in the absence of VEGF. Akt activity was unaffected, whereas p38 mitogen-activated protein kinase (MAPK) activity was elevated in Shb-deficient endothelial cells.

In summary, Shb deficiency causes altered endothelial cell signaling with elevated basal ERK, Rho family G-protein and p38 activities, and a blunted response to VEGF with respect to Rho family G-protein activation. Changes in the activities of p38 and Rho family G proteins may all contribute to the altered cytoskeleton and consequently lead to a dysfunctional microvasculature that exhibits a reduced capacity to support tumor growth.

Discussion

In vitro studies have previously suggested that Shb is pleitropic, and the recently generated Shb knockout is currently under investigation with the aim of more precisely defining role(s) of Shb in animal pathology and physiology. One distinctive response to Shb overexpression or knockdown in vitro was an endothelial phenotype. This included altered apoptosis, cytoskeleton, differentiation, migration, and vascular tube formation (13, 17). For these reasons, we decided to investigate the microvasculature in mice deficient in Shb. The vascular physiology of Shb-deficient mice showed no major abnormalities under resting conditions. The mice are fertile (24), demonstrating that the vasculature has the capability to support placental and embryonic growth. Although a few Shb-deficient embryos exhibit petechiae (24), most embryos seem normal. However, because the mice were never provoked or stressed, it is uncertain how they would perform under heavy burden.

Signs of vascular dysfunction are obtained through the tumor studies. Tumor growth is retarded in these mice, and the tumors...
Impaired Tumor Growth in Shb Knockout

3. Lammert E, Cleaver O, Melton D. Induction of angiogenesis in Shb-deficient mice, thus suggesting a dysfunctional microvasculature as the likely cause of the reduced tumor expansion. When analyzing the vasculature, morphologic changes in endothelial cells are observed in tumors, liver, and heart. These display certain common features, such as irregular and hypertrophic cytoplasm with extensions projecting toward the capillary lumen that show no immediate similarities to those observed in other mouse models exhibiting pathologic vascular phenotypes (9, 35–41). In Pdgf-h-deficient or Pdgfr-β-deficient mice, fetal brain endothelial cells display an altered endothelial cell morphology that partly resembles the changes presently observed (42). The underlying mechanisms generating this endothelial phenotype remain uncertain but could involve alterations in the endothelial cytoskeleton.

The presently observed morphology of the Shb knockout heart is distinct from that described in response to ischemia (43) or β3-integrin deficiency (39) but may involve VE-cadherin because staining for this protein is altered as a consequence of Shb deficiency. In heart endothelial cells, VEGFR-2 and VE-cadherin form a complex that is under the control of Src family kinases (43). Shb has also been shown to interact with Src (11), and this observation may provide a rationale for a linkage between altered VE-cadherin staining, Src activity, and Shb deficiency.

The vasculature of Shh null mice exhibits functional aberrations with increased basal vascular leakage in skin, kidney, and heart. The increased permeability occurs without edema and could be related to the irregular VE-cadherin staining pattern, because VE-cadherin is a component of cell-cell interactions controlling intercellular permeability (44). Besides the possible involvement of VE-cadherin in vascular permeability, Src kinases have also been shown to participate in this event (43, 45). In previous studies, heart ischemia has been shown to cause increased vascular leakage by a process involving an Src-dependent dissociation of a VEGFR-2/VE-cadherin complex (43).

The altered endothelial phenotype is maintained after isolation in culture, suggesting that the effects caused by the Shb deficiency are inherent to endothelial cells and not secondary to some in vivo phenomenon. Indeed, the Shh null endothelial phenotype presently observed in tumors, liver, and heart could reflect these cytoskeletal changes observed in vitro; cytoplasmic hypertrophy with processes extending toward the lumen, aberrant VE-cadherin staining, vascular leakage, and reduced angiogenesis. The signaling pathways responsible for the cytoskeletal alterations may in part involve FAK and Rho family G proteins. Previously, reduced activation of FAK in response to VEGF upon Shb depletion was noted (13), and this is currently also observed. In addition, increased basal p38 MAPK activity was noted. p38 MAPK has been related to in vitro vascular tube formation (46) and regulation of the endothelial cell cytoskeleton in an Nck-dependent and Fyn-dependent manner (47). No effect of Akt activity was presently noted, and this is of particular relevance because elevated Akt activity has been shown to cause increased vascular leakage (48, 49). However, that phenotype was associated with tissue edema, which presently was not noted in the nontumor tissues.

In summary, our data suggest that Shh is involved in maintaining a normal endothelial cytoskeleton and that Shh depletion causes microvascular dysfunction due to aberrant cytoskeleton. Apparently, this plays a minor role under resting conditions but causes reduced tumor growth; thus, interference with the Shh signaling pathway may provide means to expand our repertoire of anticancer therapy.

Disclosures of Potential Conflicts of Interest
We declare no financial interests related to this article.

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References
18. Claesson-Welsh L, Welsh M, Ito N, et al. Angiostatin induces endothelial cell apoptosis and activation of focal adhesion kinase independently of...
47. Lamalice L, Houle F, Huot J. Phosphorylation of Tyr1214 within VEGFR-2 triggers the recruitment of Nck and activation of Fyn leading to SAPK2/p38 activation and endothelial cell migration in response to VEGF. J Biol Chem 2006;281:34009–10.
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