Fibulin-5 functions as an endogenous angiogenesis inhibitor

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Ablation of the fibulin-5 gene (fbln5) in mice results in loose skin, emphysematous lungs and tortuous vessels. Additionally, $fbln5^{-/-}$ animals display an apparent increase in vascular sprouting from systemic and cutaneous vessels. From these observations, we hypothesized that a de-regulation of vascular sprouting occurs in the absence of endogenous fibulin-5. To test this hypothesis, vascular sprouts from the long thoracic artery were quantified and polyvinyl alcohol sponges were implanted subcutaneously in wild-type and $fbln5^{-/-}$ mice to assess fibrovascular invasion. Results showed a significant increase in in situ sprouting from vessels in $fbln5^{-/-}$ mice and a significant increase in vascular invasion, with no increase in fibroblast migration, into sponges removed from $fbln5^{-/-}$ mice compared with wild-type mice. Localization of fibulin-5 in wild-type mice showed the protein to be present subjacent to endothelial cells (ECs) in established vessels at the periphery of the sponge, and as a component of the newly formed, loose connective tissue within the sponge. These results suggest that fibulin-5 could function as an inhibitor molecule in initial sprouting and/or migration of ECs. To elucidate the molecular mechanism that drives the increased angiogenesis in the absence of fibulin-5, expression of vascular endothelial growth factor (VEGF) and the angiopoietins (Angs) was determined in sponges implanted for 12 days in wild-type and $fbln5^{-/-}$ mice. Quantitative RT-PCR showed message levels for VEGF and all three Angs to be elevated by several fold in the area of invasion of sponges from $fbln5^{-/-}$ mice compared with wild-type mice. Expression of Ang-1 was also shown to be elevated (30-fold) in vitro in aortic smooth muscle cells isolated from $fbln5^{-/-}$ mice when compared with wild-type cells, with no change in the expression of the Ang-1 mediating transcription factor, ESE-1. Taken together, these results suggest that the normal angiogenic process is enhanced in the absence of fibulin-5.

Angiogenesis, the formation of new capillaries from pre-existing vessels, is a complex process that involves an orchestrated interplay between cells, extracellular matrix (ECM) and soluble pro- and anti-angiogenic factors.[1,2] In the healthy adult, there is little necessity for new vessels to form and, apart from the female reproductive cycle and wound repair, the vasculature is remarkably quiescent. In a variety of diseases, however, an angiogenic switch occurs tipping the balance in favor of de novo blood vessel formation.[3] The regulatory mechanisms that control this switch are not fully understood, despite a detailed knowledge of the sequence of events that occurs to allow vessels to sprout and the identification of numerous molecules that can stimulate or inhibit this process.

The ECM is an essential component of the vasculature not only as a physical scaffold to support the endothelial cells (ECs) and surrounding pericytes or smooth muscle cells (SMCs), but also in the transduction of signals via interactions with integrins on the surface of vascular cells.[4] During angiogenesis, one of the first steps that must occur is ECM degradation and the generation of a matrix that is permissive for EC proliferation, migration and morphogenesis. At later stages, the matrix must reassemble and re-establish cell-ECM contacts to stabilize the nascent vessel.[5,6] Although there is no doubt of the significant involvement of the ECM in neovessel formation, relatively little attention has been paid to the role of these insoluble molecules in controlling blood vessel growth when compared with the extensive emphasis that has been placed on angiogenic cytokines, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF).

Fibulin-5 was identified by two groups in an attempt to isolate novel regulators of vascular growth.[7,8] This 66 kDa

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ECM protein, has six calcium-binding (CB)-EGF motif repeats and a globular C-terminal domain typical of the other fibulins. The first and sixth CB-EGF motifs are divergent, with a proline-tyrosine-rich insert and eight cysteines, respectively. Fibulin-5 is strongly expressed in vascular SMCs in the fetal arterial system and is downregulated in most adult vascular beds.[8] Expression is reactivated during neointima formation following vascular injury and in vascular disease.[8,9] Taken together, these data suggest that fibulin-5 may be a key player in vascular development and injury.

To define further the role of fibulin-5, fibulin-5 gene (*fbln5*) knockout mice were generated by two independent groups.[10,11] Fibulin-5 null mice are viable but have tortuous and elongated blood vessels, emphysematous lungs and severe loose skin due to a remarkable absence of dermal elastic fibers. In addition to aberrant elastic fiber assembly, the skin of *fbln5*^{−/−} mice showed an apparent increase in cutaneous blood vessels. Similarly, excess vascular sprouting was also observed from larger systemic vessels. These observations led us to the hypothesis that fibulin-5 not only plays a role in ECM assembly, but that it also influences angiogenesis as an endogenous negative regulator. This hypothesis is supported by *in vitro* studies using fibulin-5-expressing murine brain microvascular ECs[12] and the observation that exogenous fibulin-5 can inhibit angiogenesis *in vivo*.[13] However, with a seemingly opposite effect, exogenously expressed fibulin-5 delivered by retrovirus has been reported to promote wound healing,[14] a process that requires new blood vessel growth.[15] In direct contrast, the lack of endogenous fibulin-5 has no effect on the rate of wound closure or the integrity of the wound after healing.[16] These results stress the fact that endogenous fibulin-5 may function by a different mechanism than the exogenously added protein.

In the present study, the effect endogenous fibulin-5 on the process of angiogenesis was determined by measuring fibrovascular invasion into polyvinyl alcohol (PVA) sponges implanted subcutaneously in wild-type and *fbln5*^{−/−} mice. Implantation of PVA sponges in this manner elicits a robust invasion of cells from the dermis into the sponge and gives rise to a highly vascularized tissue.[17] As such, subcutaneous placement of PVA sponges has been used quite extensively to analyze a variety of pro- and anti-angiogenic molecules in a number of different species.[18–23] To determine whether fibulin-5 functioned as a constituent of the general connective tissue matrix or influenced ECs sprouting by being a component of the subendothelial matrix, immunofluorescence localization of fibulin-5 was carried out on sponges removed from wild-type mice. Additionally, expression levels of pro-angiogenic factors were determined in the sponges removed from wild-type and *fbln5*^{−/−} mice and in SMCs isolated from wild-type and *fbln5*^{−/−} aortas. Results from this study support the hypothesis that fibulin-5 is an endogenous inhibitor of angiogenesis, but unlike the exogenously added protein, has no effect on fibroblast migration. Evidence is also provided for a novel mechanism for fibulin-5 function in the control of aberrant vascular sprouting through the regulation of angiopoietins (Angs).

MATERIALS AND METHODS
Animals
Fibulin-5 gene knockout mice (*fbln5*^{−/−}) were generated as described previously.[11] For all experiments, age-matched wild-type, *fbln5*^{+/−} and *fbln5*^{−/−} mice on a C57Bl6/129SvEv background, at 2–3 months of age, were used. All studies were carried out in accordance with the McGill University Animal Care Committee regulations and protocols.

Analysis of the Long Thoracic Artery
Wild-type, *fbln5*^{+/−} and *fbln5*^{−/−} mice were used for the quantification of branches extending from the long thoracic artery. A central incision was made on the ventral surface of the animal and the skin reflected laterally to expose the posterior surface of the skin. Images were taken of the long thoracic artery using a Zeiss AxioCam digital camera mounted on a Stemi2000 dissecting microscope (Carl Zeiss, North York, ON, Canada). Images were compiled to show the complete length of the vessel using Adobe Photoshop software. The length of the artery was measured between two consistent branch points using National Institute of Health (NIH) ImageJ software (NIH, Bethesda, MD, USA) and all branches between these two branch points were counted. Data were evaluated with the Excel software program (Microsoft, Seattle, WA, USA) and examined for significance by a two-tailed unpaired Student's *t*-test. Significance was defined as $P < 0.05$.

Sponge Implantation
PVA foam discs (10 mm × 4 mm, M-PACT, Eudora, KS, USA) were sterilized in 95% ethanol overnight at 4°C. Sponges were rinsed and left in sterile phosphate-buffered saline (PBS) for 2–3 h before implantation. The general procedure for implantation followed that of Bradshaw *et al*.[20] Briefly, each mouse was anesthetized by an intraperitoneal injection of 50 mg/kg Somnotol (MTC Pharmaceuticals, Cambridge, ON, Canada) diluted in a 0.9% NaCl solution and a small area was shaved on the dorsal surface. An incision was made and two subcutaneous pockets were opened on either side of the incision with blunt-ended forceps. One sponge was inserted in each pocket before closing the incision with 6–0 sutures (Fine Science Tools, Foster City, CA, USA). The sponges remained in place for either 12 or 21 days. At the appropriate end point, the animal was killed, sponges dissected from the body, bisected and immersion fixed overnight in either 10% buffered formalin or zinc fixative (2.8 mM calcium acetate, 22.8 mM zinc acetate, 36.7 mM zinc chloride in 100 mM Tris, pH 7.4). Sponges were then dehydrated and paraffin-embedded.

Immunohistochemistry

Five micrometer paraffin sections, taken from the central 2 mm of the sponge and encompassing the entire sponge cross-section, were deparaffinized, rehydrated and blocked with TBS containing 0.1% bovine serum albumin and 1.5% normal rabbit serum (Vector Laboratories, Burlington, ON, Canada). Sections were then incubated with a rat monoclonal primary antibody specific for platelet endothelial cell adhesion molecule (PECAM-1; BD Pharmingen, Mississauga, ON, Canada) for 1 h at room temperature. After washing, the sections were incubated with a biotinylated rabbit anti-rat secondary antibody followed by immunoperoxidase staining using a Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions. Positive cells were visualized using NovaRED substrate (Vector Laboratories) and counterstained with hematoxylin.

Quantification of Fibroblast Invasion and Microvascular Density in Sponge Implants

To quantify fibroblast invasion, images encompassing the entire bisected area of hematoxylin and eosin-stained sections of sponges from wild-type ($n = 6$) and *fbln5$^{-/-}$* ($n = 5$) mice were captured using a Zeiss AxioCam digital camera mounted on a Zeiss Axioskop2 microscope (Carl Zeiss). Images were imported into NIH ImageJ software and percent fibroblast invasion for each sponge section was determined as the total area occupied by cellular invasion divided by the total area available for invasion. To quantify microvascular density, five $\times 20$ images of PECAM-1-stained sections were taken for each sponge removed from wild-type ($n = 6$) and *fbln5$^{-/-}$* ($n = 11$) mice. Using a grid overlay, intercepts overlying vessels (PECAM-1-positive areas) were quantify and calculated as a percentage of total intercepts overlying fibroblast-invaded areas. Statistical analysis of the data was conducted as described above.

Immunofluorescence Staining

Sponges removed from wild-type and *fbln5$^{-/-}$* animals were cryoprotected with increasing concentrations of sucrose in PBS. The sponges then were embedded in a fresh solution consisting of 2:1 ratio of 20% sucrose:tissue freezing medium (Triangle Biomedical Sciences, Durham, NC, USA). Frozen sections were fixed in cold acetone, quenched in 0.1 M ammonium chloride in PBS, and then blocked in 10% normal goat serum (Jackson Laboratories, West Grove, PA, USA) for 1 h. For double labeling, sections were incubated with an affinity purified rabbit polyclonal anti-fibulin-5 antibody raised against the pro-tyr rich region of the rat fibulin-5 N-terminus[11] and monoclonal rat anti-mouse PECAM-1. The primary antibodies were detected with FITC-conjugated anti-rabbit IgG (Cappel, Irvine, CA, USA) or Cy3-conjugated goat anti-mouse IgG (Jackson Laboratories). For Ang-1 staining, slides were incubated with a monoclonal mouse anti-human Ang-1 antibody (R&D, Minneapolis, MN, USA)

followed by a FITC-conjugated anti-mouse IgG secondary antibody (Cappel).

SMC Isolation and Culture

Primary cultures of vascular SMCs were obtained from age-matched, wild-type and fibulin-5 null mouse aortas by enzymatic digestion as described previously.[24] Two animals were used for each genotype and the cultures were maintained separately. Cultures were expanded to passage 5 (P5) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin, non-essential amino acids and 10% fetal bovine serum (FBS) (Wisent, St-Bruno, QC, Canada). At P5, 5×10^5 cells were plated in 100 mm plates and left for 24 h. The cells were then washed with PBS and incubated in serum-free DMEM or DMEM containing 10% FBS for an additional 24 h before isolation of RNA.

RNA Isolation and Quantitative RT-PCR Analysis

RNA was isolated from sponges removed from wild-type and *fbln5$^{-/-}$* mice at the 12 day end point. Sponges were frozen at -80°C and then powdered in liquid nitrogen and sonicated. Samples from similar genotypes were pooled in order to obtain sufficient RNA. RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A total of 2 µg total RNA was reverse transcribed with Superscript II RNase-H Reverse Transcriptase (Invitrogen) using a Touchgene Gradient PCR machine (Techne, Princeton, NJ, USA) with conditions of 5 min at 70°C, 50 min at 42°C and 5 min at 90°C. For the SMC cultures, RNA was isolated using an RNeasy kit (Qiagen, Mississauga, ON, Canada) and QIAshredder columns (Qiagen). RNA from the SMC cultures was kept separate to provide two independent samples from each genotype. Dnase I (Invitrogen) treatment and reverse transcription was performed using 5 µg total RNA with M-MLV Reverse Transcriptase (Invitrogen) at 37°C for 60 min.

Gene expression levels were quantified using Assays-on-Demand TaqMan primers and probes. For sponge experiments, mRNA expression of murine VEGF-A, Ang-1, Ang-2, Ang-3 and 18S (control gene) (Applied Biosystems, Foster City, CA) was determined. For aortic SMCs, mRNA expression of murine VEGF-A, Ang-1, PDGF-A, FGF-1, ESE-1 and 18S was determined. Expression levels were analyzed using an ABI PRISM Sequence Detection System and carried out with a thermal cycler profile of 15 s at 95°C and 1 min at 60°C for 50 repetitions. Dissociation curve analyses were performed to show the specificity of amplification. Results were analyzed using the comparative threshold cycle (C_T) method, where $\Delta C_T = C_T$ of gene of interest minus C_T of 18S and, $\Delta\Delta C_T = \Delta C_T$ of *fbln5$^{-/-}$* mice minus ΔC_T of wild-type mice. Relative expression was calculated as $2^{-\Delta\Delta CT}$. Gene expression for the sponge samples compared a pooled wild-type sample (five sponges) with a pooled fibulin-5 null sample (four sponges). qRT-PCR for each sample was run in

triplicate and results expressed as the mean ± s.d. For the SMC cultures, consistent results were obtained from cells isolated from two independent animals for each genotype. Data were reported as the mean ± s.d. of the average of triplicate runs for each animal for each genotypes.

RESULTS

In Vivo Examination of Cutaneous Vessels in Wild-Type and Fibulin-5 Null Mice

During our studies on the phenotype of the fibulin-5 null mouse, a tortuous and excessive vasculature was noted. To examine this further, images were taken of the posterior surface of the skin of wild-type, $fbln5^{+/-}$ and $fbln5^{-/-}$ mice where the long thoracic artery was clearly visible. In $fbln5^{-/-}$ mice, the vessel appeared to have an increased number of small, tortuous branches compared with $fbln5^{+/-}$ and wild-type mice (Figure 1a–c). To quantify this observation, branches off the long thoracic artery were counted between two consistent major branches that served as start and end points. A significant increase in branching was found from the vessels in $fbln5^{-/-}$ mice relative to wild-type mice ($P < 0.01$) (Figure 1d). The overall length of the vessel measured between the start and end points, however, was unchanged (Figure 1e), suggesting that the increased number of vessels branching from the long thoracic artery of $fbln5^{-/-}$ mice was not a consequence of a longer vessel.

Fibroblast Migration and Vascular Invasion of PVA Sponges

To determine the effect of endogenous fibulin-5 on the angiogenic process *in vivo*, PVA sponges were implanted subcutaneously in wild-type and $fbln5^{-/-}$ mice to trigger invasion of fibroblasts and ECs into the implant (Figure 2a).[17,21,23] Upon removal of the sponge, vessels could be clearly seen entering the matrix encapsulating the sponge (Figure 2b). To determine the degree of fibroblast migration into the sponge, sponges were removed after 12 days from wild-type and $fbln5^{-/-}$ mice, and sections were generated to cover the entire cross-sectional area of the center of the sponge (Figure 2c). After hematoxylin and eosin staining, the degree of infiltration appeared similar for both genotypes (Figure 2d and e). The extent of fibroblast migration was quantified by determining the area invaded divided by the total area available for invasion in the entire section. Quantification revealed that there was no significant difference in the degree of fibroblast migration into sponges removed from $fbln5^{-/-}$ and wild-type mice (30 *vs* 32%) (Figure 2f). Although sponges from both genotypes showed equivalent fibroblast migration, sponges removed from $fbln5^{-/-}$ mice displayed a considerable increase in vascular invasion compared with those from wild-type mice based on PECAM-1 staining (Figure 2g and h). Vascular invasion quantified by grid overlay showed a significant increase in the amount of vessels in PVA sponges removed from $fbln5^{-/-}$ mice compared with wild-type mice (20 *vs* 14%) (Figure 2i).

Localization of Fibulin-5 within the PVA Sponges

The lack of fibulin-5 resulted in a significant increase in vascularization of the implanted PVA sponges. To provide insight as to the mechanism by which fibulin-5 may affect EC invasion, the location of fibulin-5 relative to invading ECs was determined in PVA sponges removed from wild-type
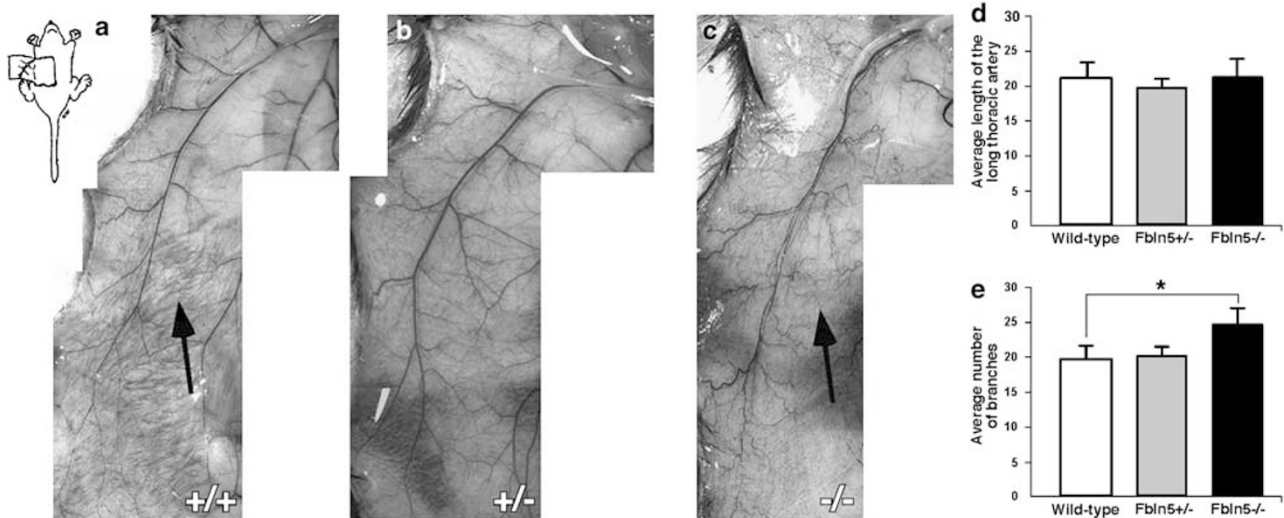

Figure 1 Analysis dermal vasculature and the length and branches from the long thoracic artery. On the posterior surface of the skin, a qualitative difference in the degree of vascularization can be seen among wild-type (**a**), $fbln5^{+/-}$ (**b**) and $fbln5^{-/-}$ (**c**) mice. Note the tortuous nature of the small vessels branching from the long thoracic artery in the $fbln5^{-/-}$ mouse and the general increase in number of these vessels in the skin compared with wild-type mice (arrows). Quantification showed no significant difference in length of the long thoracic artery among wild-type, $fbln5^{+/-}$ and $fbln5^{-/-}$ mice (**d**) and confirmed a significant increase in the number of vessels branching from the long thoracic artery of $fbln5^{-/-}$ mice relative to wild-type mice (**e**). Results are expressed as mean ± s.d. Asterisk, $P < 0.05$. Wild-type ($n = 11$), $fbln5^{+/-}$ ($n = 7$), $fbln5^{-/-}$ ($n = 11$). Color version of this figure is available in the HTML version online.
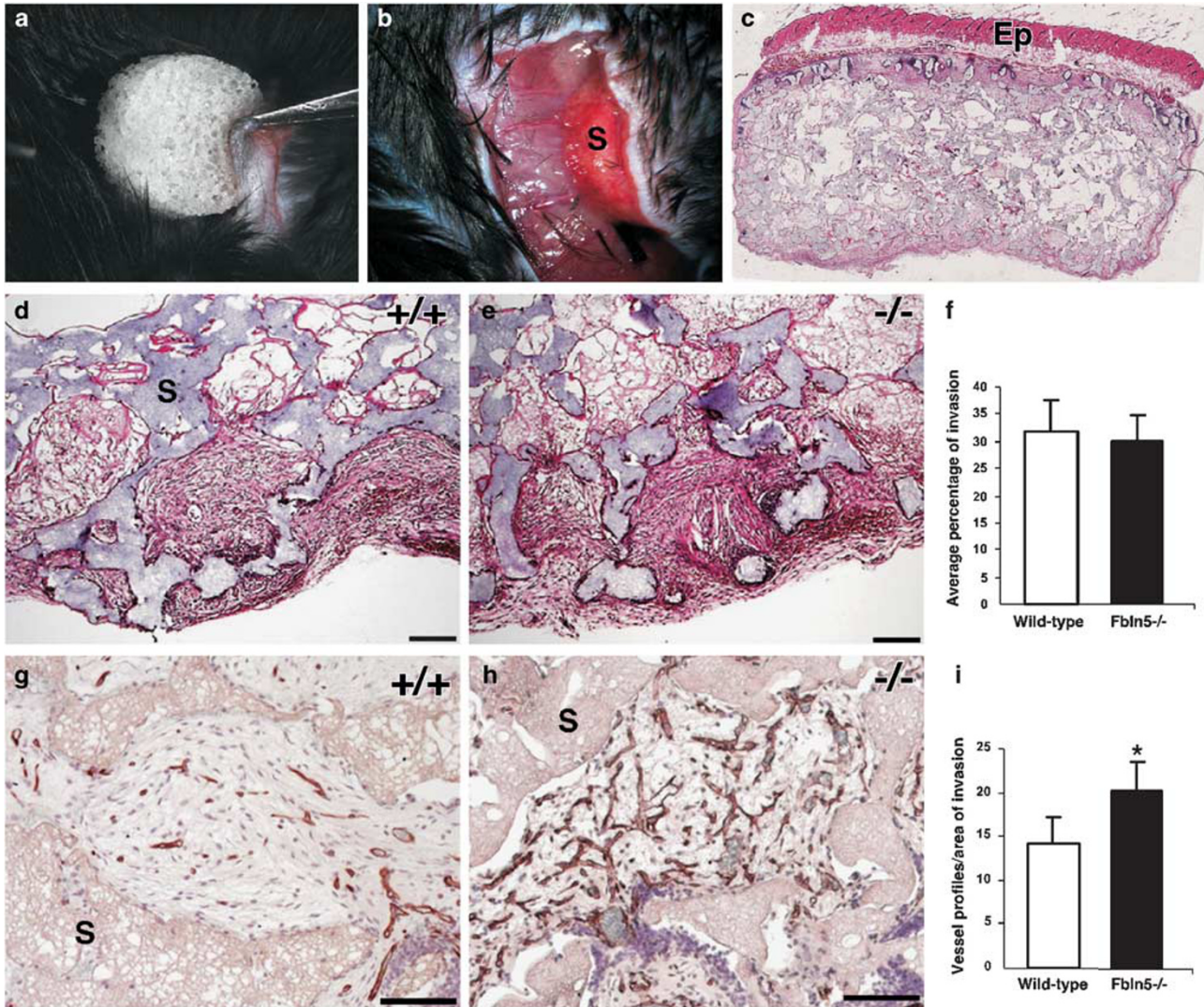
Figure 2 Fibrovascular invasion of PVA sponges. PVA sponge discs (10 mm × 4 mm) were subcutaneously implanted on the dorsum of mice (**a**). After 12 days, sponges were encased in a highly vascularized capsule (**b**). Sponges were removed with the overlying epidermis (Ep) left intact, bisected, embedded, and sections prepared that encompassed the entire central portion of the sponge (**c**). The extent of cellular invasion into sponges implanted in either wild-type (**d**) or *fbln5*^{−/−} (**e**) mice appeared similar following hematoxylin and eosin staining. No significant difference was determined in the degree of fibroblast migration, calculated as total area occupied by fibroblasts divided by the total area in the sponge available for invasion (**f**). In contrast, a clear difference in the extent of vascular invasion could be seen from PECAM-1-stained sections (**g** and **h**). Quantification of the PECAM-1-positive vessels confirmed a significant difference between wild-type and *fbln5*^{−/−} mice (**i**). S, sponge. Bars = 100 μm. Asterisk, $P < 0.05$.

mice after 21 days of implantation. Colocalization of fibulin-5 and PECAM-1 showed an extensive fibulin-5 matrix and distinct vascular structures within the sponge (Figure 3a and b). These results demonstrate that fibulin-5 forms a network of fibers within the ECM independent of, but concurrent with, the invasion and formation of vessels. In addition to forming a network of fibers in the invaded areas of the sponge, colocalization of fibulin-5 and PECAM-1 also showed consistent localization of fibulin-5 to the blood vessels at the sponge periphery. These larger vessels displayed an intact endothelium surrounded by a layer of fibulin-5-positive staining (Figure 3c).

Expression of VEGF and the Angs *In Vivo*

To investigate possible downstream mediators of the activity of fibulin-5, localization of Ang-1 within and surrounding the sponge was carried out. Previous studies have shown that Ang-1 promotes vessel sprouting[25] and that overexpression of Ang-1 in murine skin can result in more numerous and highly branched vessels.[26] Consistent with these studies, frozen sections of sponges removed from *fbln5*^{−/−} mice after 12 days showed increased Ang-1 immunoreactivity compared with wild-type mice in both the invaded areas of the sponge and in the overlying dermis (Figure 4a–d). To confirm and quantify this observation, RNA was extracted from sponges
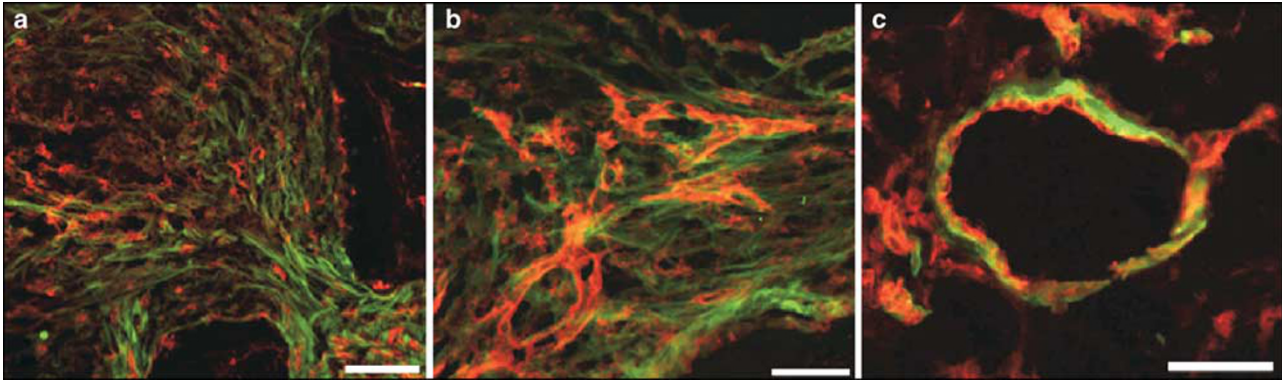
Figure 3 Localization of fibulin-5 and PECAM-1 within the sponge and adjacent vessels in wild-type mice after 21 days of implantation. Within the sponge, a network of fibulin-5 fibers (green) can be seen among, and relatively independent of, PECAM-1-positive (red) cells (**a**) and microvessels (**b**). In contrast, at the periphery of the sponge, the larger vessels that supply the sponge with new vasculature show a close association of fibulin-5 (green) with the endothelium of the vessel (red). Bars = 100 μm (**a**), 50 μm (**b** and **c**).

removed from $fbln5^{-/-}$ and wild-type mice and qRT-PCR for VEGF, Ang-1, Ang-2 and Ang-3 was performed. Results showed that expression levels for VEGF and all three Angs were increased in sponges removed from $fbln5^{-/-}$ mice in comparison to those from wild-type mice (Figure 4e). VEGF expression was increased eightfold and message levels for all members of the Ang family, especially Ang-2, were significantly elevated in the sponges removed from $fbln5^{-/-}$ mice.

Expression of Pro-Angiogenic Factors *In Vitro*

To provide a more direct link between fibulin-5 and the regulation of angiogenic factors, vascular SMCs were isolated from wild-type and fibulin-5 null mouse aortas and analyzed for expression of Ang-1, VEGF, FGF-1 and PDGF-A after 24 h in serum-free media or 10% serum (Figure 5a). Relative to wild-type SMCs, $fbln5^{-/-}$ SMCs showed an approximate 30-fold increase in Ang-1 expression in the absence of serum, which was slightly reduced if the cells were incubated with serum. In constrast, VEGF showed reduced expression in $fbln5^{-/-}$ SMCs in the absence of serum and only a small increase in the presence of serum. A similar expression pattern was seen for PDGF-A. FGF-1 expression was slightly reduced in both the absence and presence of serum.

ESE-1 has been shown to be a transcriptional mediator of Ang-1 expresssion in inflammation.[27] To determine if a similar mechanism was mediating the increased expression of Ang-1 in the $fbln5^{-/-}$ SMCs, expression levels of ESE-1 were compared between wild-type and $fbln5^{-/-}$ SMCs *in vitro*. qRT-PCR showed reduced levels of ESE-1 expression in the $fbln5^{-/-}$ SMCs when compared with wild-type cells, both with and without serum, indicating that the elevated expression of Ang-1 in $fbln5^{-/-}$ SMCs was independent of the transcriptional activity of ESE-1 (Figure 5b).

DISCUSSION

In the present study, we have quantitatively documented a significant increase in vascular sprouting from the long thoracic artery of fibulin-5 null mice compared with wild-type mice confirming previous qualitative observations of excess microvessels in the $fbln5^{-/-}$ mouse. As this insinuated that angiogenesis may be de-regulated in the absence of fibulin-5, the degree of fibrovascular invasion was determined in subcutaneously implanted PVA sponges in mice of the two genotypes. A significant increase in vascular invasion into the sponges of $fbln5^{-/-}$ mice was found indicating that an ECM in the absence of fibulin-5 provides a more permissive angiogenic environment.

The observation of increased angiogenesis in the absence of fibulin-5 corroborates *in vitro* data showing that murine brain ECs, retrovirally infected to express fibulin-5, have reduced tubulogenesis, proliferation and migration when compared with GFP-infected control cells,[12] and confirms the finding that exogenous fibulin-5 added to matrigel plugs can reduce the degree of bFGF-stimulated vascular invasion when implanted into wild-type mice.[13] The increased angiogenic response seen in the sponges removed from $fbln5^{-/-}$ mice in our study, however, occurred in the absence of any increased fibroblast migration into the sponge. This result suggests that fibulin-5 has a differential effect on the migration and/or proliferation of ECs and fibroblasts. This notion is supported by our recent work showing; (1) a normal rate of wound healing in mice deficient for fibulin-5 despite increased angiogenesis in the wound bed, (2) no difference in migration or proliferation between of dermal fibroblasts isolated from wild-type and $fbln5^{-/-}$ mice, and (3) no difference in the rate of outgrowth of keratinocytes from skin explants removed from mice of the two genotypes.[16] In direct contrast to our results, it has been reported that fibulin-5 promotes wound healing[14] and induces fibroblast migration into matrigel plugs implanted into wild-type mice.[13] In the wound healing study, dermal fibroblasts in the wound were infected with fibulin-5 retrovirus shown *in vitro* to result in high levels of fibulin-5 secretion.[14] It is possible, therefore, that this excess exogenous fibulin-5, which would not be assembled into the matrix in its normal architecture, could have a stimulatory
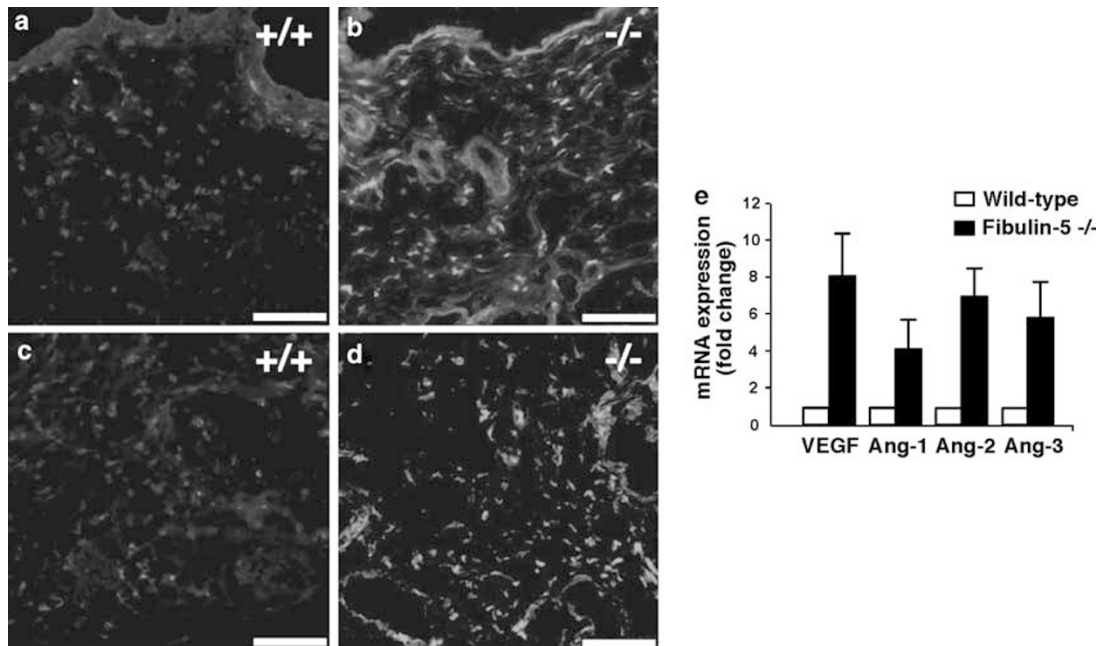
Figure 4 Localization of Ang-1 within sponges and overlying dermis and expression of VEGF and Angs in sponges after 12 days of implantation. In contrast to the overlying dermis (**a**) and within the sponge (**c**) from wild-type mice, the dermis (**b**) and fibroblast invaded areas of sponges (**d**) from *fbln5*^{−/−} mice showed increased staining for Ang-1. Using RNA isolated from the sponges, a significant increase was observed in expression for VEGF, Ang-1, Ang-2 and Ang-3 in *fbln5*^{−/−} mice compared with wild-type mice (**e**). Columns represent mean values normalized to wild-type levels. Bars = 100 μm. Color version of this figure is available in the HTML version online.

effect on the cells within the wound bed. Similarly, exogenous fibulin-5 within a matrigel plug would not be in the context of its normal binding partners and thus may have stimulatory domains exposed. Taken together, our present findings and those of the above-mentioned studies strongly suggest that the natural role of fibulin-5 in tissues may be different than that added exogenously or overexpressed locally in adult tissues. Nevertheless, the function of fibulin-5 in both environments is equally important to study, as the endogenous role of fibulin-5 must be determined to fully understand the consequences of human mutations in *fbln5*, and the response to exogenously added fibulin-5 may clearly have therapeutic value.

A number of ECM molecules have been shown to influence EC survival, growth, migration, and/or tube formation (reviewed by Sottile[6]). Some matrix proteins, such as collagens, laminin, vitronectin and fibronectin, promote these functions and are thus pro-angiogenic, whereas others, such as thrombospondin (TSP)-1 and -2, are inhibitory. A remarkably large number of fragments derived from ECM components have also been identified to inhibit EC functions important for angiogenesis. However, the precise mechanism by which many of these fragments act, and whether they exist *in vivo* to function as endogenous regulators of angiogenesis, is still not clear. Interestingly, immunoreactivity of fibulin-5 in human reticular dermis decreases with age and sun-exposure,[28] suggesting that fibulin-5 may be susceptible to proteolytic cleavage. This notion was recently supported by

work demonstrating the existence of a truncated form of fibulin-5 that increases with age in mouse skin.[29] The cleavage event removes 10 kDa from the *N*-terminus and can be inhibited *in vitro* by a serine protease inhibitor. These results are intriguing in light of the anti-angiogenic properties of many ECM fragments and the necessity for ECM degradation to occur to allow for vascular sprouting.

Apart from bone and cartilage, the ECM exists in two predominant structural forms; (1) a fibrillar 3-dimensional connective tissue network that surrounds stromal cells and (2) a highly organized sheet-like matrix, the basement membrane, that acts as a barrier between epithelial cells and mesenchymal tissues, and which underlies ECs.[30] To begin to define the mechanism of a matrix protein on cell function, the location and assembly of that protein within the ECM must be determined. Localization of fibulin-5 in and around the sponge suggests that fibulin-5 may function at two levels to influence angiogenesis. First, fibulin-5 was found in the subendothelial region of small vessels surrounding the sponge. Although it remains to be determined if the protein is specifically localized to the basement membrane, and/or binds to basement membrane proteins such as type IV collagen, entactin or perlecan, the presence of fibulin-5 in this region supports a role for fibulin-5 in regulating initial EC sprouting. Fibulin-5 in this capacity may also stabilize the vessel and contribute to a nonpermissive ECM by binding EC and/or SMC surface integrins.[7,10] Indeed, fibulin-5 has been shown to suppress SMC migration and proliferation,[9] adding
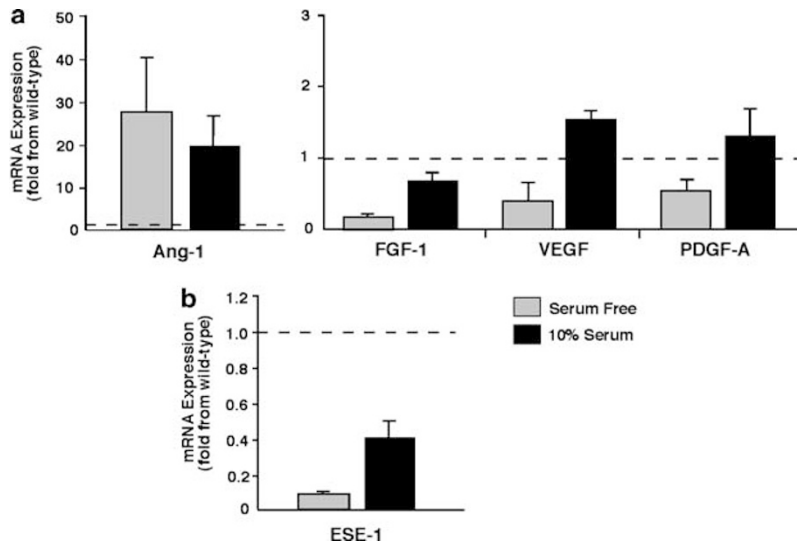
Figure 5 Expression of pro-angiogenic factors in wild-type and fibulin-5 null aortic SMCs. SMCs isolated from the aortas of wild-type and $fbln5^{-/-}$ mice were grown for 24 h in the absence or presence of 10% serum. RNA was isolated and expression of Ang-1, FGF-1, VEGF and PDGF-A was determined by qRT-PCR (**a**). Values were plotted as fold change for $fbln5^{-/-}$ cells over wild-type cells. Only Ang-1 showed a dramatic increase in expression in the fibulin-5 null SMCs when compared with wild-type SMCs. Expression of the Ang-1 regulating transcription factor, ESE-1, was slightly downregulated in $fbln5^{-/-}$ SMCs (**b**).

to its potential to maintain vessels in a quiescent state. Fibulin-5 was also found as part of the connective tissue network newly assembled within the sponge by the invading cells. In this location, fibulin-5 would be encountered by the ECs, as they migrated into the invaded area and could potentially directly inhibit EC migration and proliferation. Alternatively, but not mutually exclusive, fibulin-5 may indirectly influence the activity of the ECs by altering cell signaling or bioavailability of other molecules. For instance, fibulin-5 has been reported to antagonize the pro-angiogenic signaling of VEGF and upregulate TSP-1,[12] which is itself a potent angiogenesis inhibitor.[31–33] Whether such activities are significant for fibulin-5 function *in vivo* remains to be determined.

Among the soluble angiogenic factors described to date, VEGF and the Angs are recognized as some of the most critical regulators of angiogenic sprouting.[34–36] As these molecules promote neovessel formation and morphogenesis in a cooperative manner,[37,38] and the signal transduction activity of VEGF has already been shown to be antagonized by fibulin-5 *in vitro*,[12] we hypothesized that the expression of VEGF and the Angs may be altered in concert in the absence of fibulin-5. Consistent with this hypothesis, expression levels for VEGF and all three Angs were found to be significantly elevated in the areas of fibrovascular invasion of sponges removed from $fbln5^{-/-}$ mice. Additionally, an increase in Ang-1 protein was observed in and around the sponge in the null animals. It is well established that VEGF is a major factor in the induction of angiogenesis by promoting EC proliferation and migration.[3,39,40] Ang-1 subsequently functions to remodel and stabilize these newly formed vessels by promoting recruitment of pericytes and SMCs.[36,41] Although Ang-2 has a seemly opposite effect of vessel destabilization,[36] in the presence of high VEGF levels, Ang-2 actually facilitates the angiogenic response and cooperates with VEGF to induce angiogenesis by rendering the vasculature more amenable to

sprouting.[42] Indeed, the results obtain in the presence study are entirely consistent with the induction of angiogenesis that is associated with tumor formation where the key factor is not so much the absolute levels of Angs, but a shift in the ratio of Ang-1:Ang-2 to favor Ang-2.[43] Interestingly, fibulin-5 expression is downregulated in most human tumors,[44] suggesting that its absence could contribute to the angiogenic switch in cancer. Considerably less is known about Ang-3, however, Ang-3 has also been shown to strongly induce the survival of primary cultured mouse ECs and induce angiogenesis *in vivo*.[45]

Consistent with the increased expression of Ang-1 seen in sponges removed from fibulin-5 null mice, cultures of $fbln5^{-/-}$ aortic SMCs showed a notable 30-fold increase of Ang-1 expression over wild-type SMCs. Other pro-angiogenic factors, such as FGF-1 and PDGF-A, were generally unaffected. In contrast to the eightfold increase in VEGF expression seen in sponges removed from $fbln5^{-/-}$ mice, however, little change in expression of VEGF between $fbln5^{-/-}$ and wild-type aortic SMCs was observed *in vitro*. These results suggest that there may be a fundamental difference between macro- and micro-vascular SMCs with respect to VEGF expression. Indeed, VEGF localization in the adult mouse shows no expression in the medial layer of the aorta, but remarkably, very strong expression in the ECs.[46] Additionally, pericytes *in vivo* have been shown to express VEGF when in contact with ECs during the progression of vessel formation.[47,48] Thus, in the *in vivo* setting of vascular invasion into the sponges, it is likely that the mesenchymal cells (pericyte precursors) and pericytes are responsible for the expression of VEGF upon interacting with the newly sprouting ECs. Based on the subendothelial localization of fibulin-5 in the small dermal vessels, it is conceivable that this mesenchymal–EC interaction may be enhanced in the absence of fibulin-5, leading to augmentation of VEGF expression.

Mutations in *fbln5* have been identified in individuals afflicted with cutis laxa,[49–51] a heterogeneous group of inherited and acquired disorders characterized by loose, redundant skin, and in cases of age-related macular degeneration (ARMD).[51,52] Although aberrant angiogenesis has not been reported in cutis laxa, neovascularization within the retina at the macula can be a contributing factor to the devastating loss of central vision in ARMD.[53] Research from the present study strongly supports a role for endogenous fibulin-5 in regulating angiogenesis during normal development through the control of VEGF and Angs. Further studies designed to elucidate the precise mechanism by which fibulin-5 can directly or indirectly influence the expression of these pro-angiogenic molecules could thus have important implications in the management of tumor growth and progression of ARMD.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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