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LKB1 in endothelial cells is required for angiogenesis and TGFβ-mediated vascular smooth muscle cell recruitment

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Inactivation of the tumor suppressor kinase *Lkb1* in mice leads to vascular defects and midgestational lethality at embryonic day 9-11 (E9-E11). Here, we have used conditional targeting to investigate the defects underlying the *Lkb1*$^{-/-}$ phenotype. Endothelium-restricted deletion of *Lkb1* led to embryonic death at E12.5 with a loss of vascular smooth muscle cells (vSMCs) and vascular disruption. Transforming growth factor beta (TGFβ) pathway activity was reduced in *Lkb1*-deficient endothelial cells (ECs), and TGFβ signaling from *Lkb1*$^{-/-}$ ECs to adjacent mesenchyme was defective, noted as reduced SMAD2 phosphorylation. The addition of TGFβ to mutant yolk sac explants rescued the loss of vSMCs, as evidenced by smooth muscle alpha actin (SMA) expression. These results reveal an essential function for endothelial *Lkb1* in TGFβ-mediated vSMC recruitment during angiogenesis.

KEY WORDS: LKB1, TGFβ, Vascular smooth muscle cell, Angiogenesis, Endothelium, Differentiation

INTRODUCTION

Inactivating germline mutations of the tumor suppressor kinase *LKB1* lead to Peutz-Jeghers Syndrome (PJS), which is characterized by gastrointestinal polyposis and an elevated risk for cancer (Hemminki et al., 1998). To investigate the physiological functions of *Lkb1* (*Stk11* – Mouse Genome Informatics), we, and others, have analyzed mice with homozygous null mutations of *Lkb1* (Bardeesy et al., 2002; Jishage et al., 2002; Ylikorkala et al., 2001). While heterozygous *Lkb1*$^{+/-}$ mice develop polyps similar to those seen in PJS patients (Bardeesy et al., 2002; Jishage et al., 2002; Miyoshi et al., 2002; Rossi et al., 2002), targeted disruption of both *Lkb1* alleles results in midgestational lethality between embryonic days 9 and 11 (E9-E11) (Bardeesy et al., 2002; Jishage et al., 2002; Ylikorkala et al., 2001). *Lkb1*$^{-/-}$ mouse embryos manifest multiple developmental abnormalities, such as deficient neural tube closure and somitogenesis, and vascular abnormalities (Ylikorkala et al., 2001). Formation of the primary blood vessel plexus (vasculogenesis) proceeds normally in *Lkb1*$^{-/-}$ embryos but further vascular development (angiogenesis), including remodeling, sprouting and maturation of blood vessels, is severely defective, and the expression of vascular endothelial growth factor (VEGF) is upregulated in mesenchymal cells (Ylikorkala et al., 2001).

Blood vessel growth and differentiation is regulated by interactions between the endothelium and the surrounding cells and extracellular matrix. Signaling molecules involved in this crosstalk include growth factors, integrins, chemokines, oxygen sensors and extracellular matrix molecules (Carmeliet, 2003). VEGF has an essential role in vessel formation (Carmeliet et al., 1996) and stabilization (Darland et al., 2003), whereas TGFβ, angiopoietin 1 (ANG1; ANGPT1 – Mouse Genome Informatics), and platelet-derived growth factor B (PDGFB) are key regulators of subsequent blood vessel maturation (Lindahl et al., 1997; Pepper, 1997; Suri et al., 1996).

Lkb1 encodes an intracellular serine-threonine kinase that phosphorylates and activates a number of kinases, including PAR-1/MARK (microtubule-affinity-regulating kinase) and AMPK (AMP-activated protein kinase), implicating it in multiple signaling pathways regulating, for example, cell metabolism and polarity (Katajisto et al., 2006). Of these, AMPK has been shown to regulate endothelial migration and tube formation in culture under hypoxic conditions (Nagata et al., 2003), whereas no specific role in vascular development has been reported for the other LKB1 substrate kinases. LKB1 has also been reported to associate with a number of other proteins, including LIP1 (Smith et al., 2001) and the PTEN tumor suppressor (Mehenni et al., 2005). The latter association may be linked to vascular function, as LKB1 was reported to phosphorylate and activate PTEN in human umbilical vein endothelial cells following peroxynitrite or high glucose treatment (Song et al., 2007).

Considering the ubiquitous expression pattern of *Lkb1* during embryogenesis (Luukko et al., 1999) and the broad spectrum of implicated signaling pathways, it has been unclear which cell types and signaling defects underlie the vascular abnormalities in *Lkb1*-deficient embryos. Here, we have used conditional mutagenesis to address this question and to investigate the cellular functions of LKB1 during mammalian development.

MATERIALS AND METHODS

Mice

The Lkb1 null (Ylikorkala et al., 2001) and conditional (Bardeesy et al., 2002) alleles, as well as the Mox2-Cre (Tallquist and Soriano, 2000) and Tie1-Cre (Gustafsson et al., 2001) mouse strains have been previously described. Mox2-Cre and Tie1-Cre mice were first bred onto a Lkb1$^{+/-}$ background and then mated with Lkb1$^{lox/lox}$ mice.

Histology and immunohistochemistry

For histological and immunohistochemical analyses, tissues were fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin, and sectioned and stained according to standard protocols. Whole-mount immunohistochemistry was done as previously described (Ylikorkala et al., 2001). Primary antibodies used were anti-phospho-SMAD2 ser465/467

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(Chemicon AB3849), anti-VE-cadherin (BD Pharmingen 555289), anti-smooth muscle actin (clone 1A4; Sigma A5691, A2547 and C6198), anti-PECAM1 (Pharmingen 553369) and anti-PDGFRβ (eBioscience 14-1402). Primary antibodies were detected by Alexa549-conjugated (Invitrogen), FITC-conjugated (Jackson ImmunoResearch) or HRP-conjugated (Dako) secondary antibodies. Alkaline phosphatase-conjugated anti-smooth muscle actin antibody (Sigma C6198) was visualized with the Vector Black Alkaline Phosphatase Substrate Kit (Vector SK-5200). For quantification of pSMAD2-positive cells, serial sections were examined through the yolk sac, and over 1000 endothelial cells and 2500 mesenchymal cells were scored.

Cell proliferation and TUNEL assay

Mitotic cells were visualized from paraffin sections with an anti-Ki-67 antibody (Dako M7249). Apoptotic cells were detected from paraffin sections by TUNEL assay according to the manufacturer's instructions (Apoptag S7101). For quantification of Ki-67-positive cells, serial sections were examined through the yolk sac and over 1000 endothelial cells were scored.

Treatment of yolk sacs with exogenous TGFβ1

E11.5 and E12.5 yolk sacs were divided in half, cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 0.3% fetal calf serum (FCS) with or without 1 ng/ml TGFβ1 for 1 or 8 hours at 37°C, and then fixed with 2% paraformaldehyde for 30 minutes and embedded in paraffin.

RNA interference

hTERT-immortalized human umbilical vein endothelial cells (HUVEC) (a kind gift from Sonja Koopal, University of Helsinki) were plated into 12-well plates, transfected with 100 nmol siRNA diluted in OptiMEM (Gibco) and incubated with oligofectamine (Invitrogen), according to manufacturers' instructions. Cells were collected for RNA extraction 72 hours after transfection. The sequences for LKB1 siRNA were 5′-UAG UUG AAU UUC CUU CUU CUU-3′ (LKB1-si1; Dharmicon) and 5′-UAC UCA AGC AUC CUU UCA UU-3′ (LKB1-si2; Dharmicon). A non-targeting siRNA (Dharmicon catalog number D-001210-04-20) was used as a control.

RNA extraction and real-time RT-PCR analysis

RNA from yolk sacs and immortalized HUVECs was isolated using an RNeasy isolation kit (Qiagen), according to the manufacturer's instructions. Total RNA was reverse transcribed using Taqman Reverse Transcription reagents (Applied Biosystems). Real-time PCR was performed on an ABI Prism 7500 using Power SYBR Green PCR Master Mix (Applied Biosystems). Relative mRNA amounts were assayed by using the 7500 Fast Real-Time PCR System Software. Primers used were:

mouse TGFβ1, 5′-CAC CAT CCA TGA CAT GAA CC-3′ (forward) and 5′-ACT TCC AAC CCA GGT CCT TC-3′ (reverse);

mouse GAPDH, 5′-TCA ACG ACC CCT TCA TTG AC-3′ (forward) and 5′-ATG CAG GGA TGA TGT TCT GG-3′ (reverse);

human TGFβ1, 5′-CCC TGG ACA CCA ACT ATT GC-3′ (forward) and 5′-GTC CAG GCT CCA AAT GTA GG-3′ (reverse);

human GAPDH, 5′-CGA CCA CTT TGT CAA GCT CA-3′ (forward) and 5′-AGG GGA GAT TCA GTG TGG TG-3′ (reverse); and

human PAI1, 5′-CAG GAA GCC CCT AGA GAA CC-3′ (forward) and 5′-ATG CGG GCT GAG ACT ATG AC-3′ (reverse).

RESULTS

Lkb1$^{-/-}$ embryos are not rescued by wild-type extraembryonic tissues

Abnormal development and lethality of embryos at E8.5-E11.5 is commonly due to defective development of extraembryonic vascularization, depriving the growing embryo of maternal oxygen and nutrient supplies (Copp, 1995) We have previously shown that *Lkb1*$^{-/-}$ yolk sac vascularization fails to develop beyond primary plexus stage and that allantoic blood vessels of *Lkb1*$^{-/-}$ embryos are unable to invade the placenta (Ylikorkala et al., 2001). To determine whether the defects seen in *Lkb1*$^{-/-}$ embryos are caused by extraembryonic dysfunction, we used an experimental approach to supply *Lkb1*-deficient embryos with wild-type placentas and yolk sac endoderm by using the *Mox2-Cre* (MORE) mouse strain in which Cre expression is restricted to epiblast-derived cells (Tallquist and Soriano, 2000). Crossing mice with floxed *Lkb1* alleles (Bardeesy et al., 2002) with *Mox2-Cre* mice deletes *Lkb1* in the embryo, allantois and yolk sac mesoderm, while *Lkb1* in trophoblast cells of the placenta and visceral endoderm of the yolk sac remains intact.

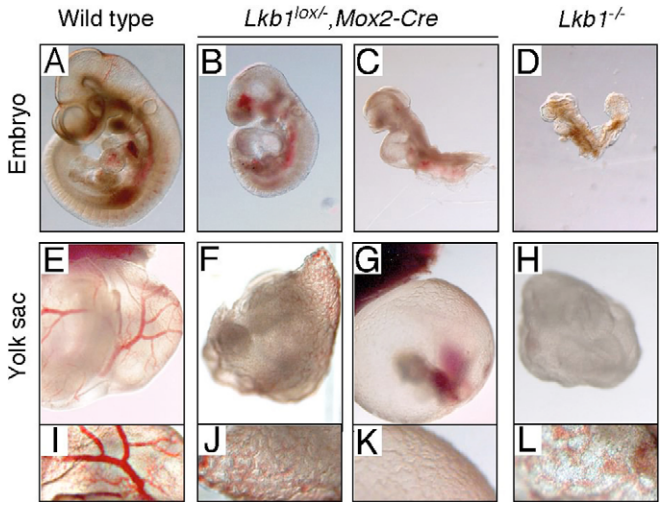

Fig. 1. Abnormal development of *Lkb1*$^{lox/-}$, *Mox2-Cre* conceptuses at E9.5. (**A-H**) Gross morphology of wild-type (A,E), *Lkb1*$^{lox/-}$, *Mox2-Cre* (B,C,F,G) and *Lkb1*$^{-/-}$ (D,H) embryos and yolk sacs (E-H). (**I-L**) Higher magnification of E-H, respectively. Defects observed in *Lkb1*$^{lox/-}$, *Mox2-Cre* embryos vary from mild abnormalities and reduced size (B) to severe developmental retardation (C) resembling the *Lkb1*$^{-/-}$ phenotype (D). Similar to *Lkb1*$^{-/-}$ yolk sacs (H,L), the yolk sacs of *Lkb1*$^{lox/-}$, *Mox2-Cre* embryos (F,G,J,K) fail to remodel the primary vascular plexus into large vitelline vessels, as seen in E9.5 wild-type yolk sacs (E,I).

Lkb1$^{lox/-}$, *Mox2-Cre* conceptuses were analyzed at E9.5, which is the stage when *Lkb1*$^{-/-}$ embryos show severe developmental defects (Ylikorkala et al., 2001). Out of 133 conceptuses, 27 were genotyped as *Lkb1*$^{lox/-}$, *Mox2-Cre*. Morphologically, six of these were indistinguishable from wild-type littermates; the remaining 21 embryos had variable phenotypes resembling those of *Lkb1*$^{-/-}$ embryos (Fig. 1D), with 15 being less severely affected (Fig. 1B) and six being similar although bigger (Fig. 1C). Yolk sac vasculature of all 21 *Lkb1*$^{lox/-}$, *Mox2-Cre* embryos remained at the primary vascular plexus stage (Fig. 1F,G,J,K). The phenotypic variation seen in *Lkb1*$^{lox/-}$, *Mox2-Cre* embryos could be due to incomplete recombination, as *Mox2-Cre* has been reported to be mosaic in activity (Hayashi et al., 2002). Consistent with this, efficiency of Cre-mediated deletion of the floxed *Lkb1* allele correlated with phenotype severity in individual *Lkb1*$^{lox/-}$, *Mox2-Cre* embryos, as assessed by semi-quantitative PCR (data not shown).

The analysis of placentas of severely affected *Lkb1*$^{lox/-}$, *Mox2-Cre* embryos revealed that the wild-type trophoblasts had formed a thick labyrinthine layer with prominent maternal blood lacunas (see Fig. S1B in the supplementary material), which was distinct from that of *Lkb1*$^{-/-}$ trophoblasts (Ylikorkala et al., 2001). Invasion of *Lkb1*$^{-/-}$ allantoic blood vessels into the trophoblast layer was, however, poor compared with wild type.

DEVELOPMENT

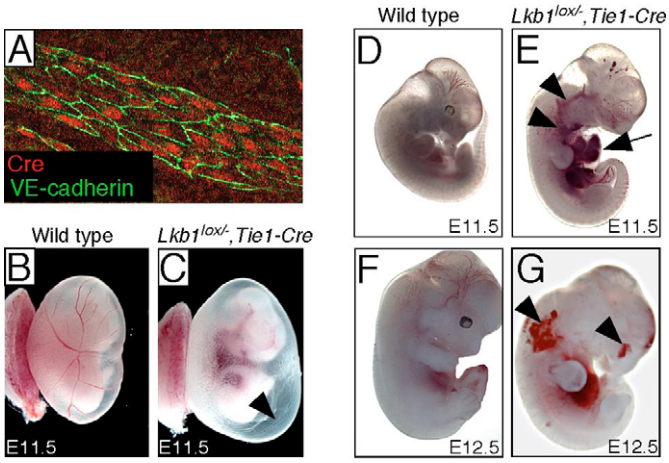

Fig. 2. Vascular disruption in embryos after *Tie1-Cre* induced inactivation of endothelial *Lkb1*. (**A**) Whole-mount immunofluorescence staining of Cre (red) and VE-cadherin (green) in E11.5 *Tie1-Cre*-positive yolk sac. (**B**,**C**) E11.5 wild-type (B) and $Lkb1^{lox/-}$, *Tie1-Cre* (C) yolk sacs. The $Lkb1^{lox/-}$, *Tie1-Cre* yolk sac is pale owing to a lack of blood in the vitelline vessels (arrow). (**D**,**E**) E11.5 wild-type (D) and $Lkb1^{lox/-}$, *Tie1-Cre* (E) embryos. Pericardial swelling (arrow) and dilated embryonic vessels (arrowheads) are apparent in the $Lkb1^{lox/-}$, *Tie1-Cre* embryo. (**F**,**G**) E12.5 wild-type (F) and $Lkb1^{lox/-}$, *Tie1-Cre* (G) embryos, demonstrating paleness and severe hemorrhaging in the mutant (arrowheads).

Taken together, these data demonstrate that wild-type trophoblast and visceral endoderm cells are not sufficient to restore extraembryonic vascularization and development of the embryo proper, and indicate that the defective development of $Lkb1^{-/-}$ mutant embryos is primarily due to defective function of embryonic cell lineages rather than to impaired placental or yolk sac function.

Endothelium-specific inactivation of *Lkb1* leads to vascular defects and embryonic death at E12.5

Embryonic cell lineages involved in vascular development include endothelial cells (ECs) and surrounding mesenchymal cells, which differentiate into vascular smooth muscle cells (vSMCs) and pericytes (Carmeliet, 2003). Both undifferentiated mesenchymal cells and differentiated vSMCs and pericytes produce VEGF, which stimulates the migration, proliferation and survival of ECs (Darland et al., 2003). Although VEGF expression is upregulated in mesenchymal cells in $Lkb1^{-/-}$ embryos (Ylikorkala et al., 2001), this is unlikely to underlie the severe vascular phenotype, as mice overexpressing VEGF show normal development of the vasculature up to E14 (Miquerol et al., 2000).

In order to determine whether the disruption of *Lkb1* leads to cell-autonomous defects in ECs, we generated mice with an endothelium-restricted deletion of *Lkb1* by using *Tie1-Cre* transgenic mice (Gustafsson et al., 2001). The *Tie1-Cre* mouse strain has been widely used and efficiently inactivates target genes in ECs starting at E8.5 (Carvalho et al., 2004; Gustafsson et al., 2001). In accordance with this, immunohistochemical staining of yolk sacs from $Lkb1^{+/-}$, *Tie1-Cre* and $Lkb1^{lox/lox}$ intercrosses using antibodies against Cre and the endothelial marker VE-cadherin showed efficient and EC-restricted expression of Cre (Fig. 2A).

Out of 64 offspring from $Lkb1^{+/-}$, *Tie1-Cre* and $Lkb1^{lox/lox}$ intercrosses, no $Lkb1^{lox/-}$, *Tie1-Cre* mice were recovered, demonstrating the embryonic lethality of this genotype. Analysis of the embryos revealed that, up to E10.5, $Lkb1^{lox/-}$, *Tie1-Cre* embryos were indistinguishable from wild-type littermates. However, at E11.5, almost half (21/46) of the $Lkb1^{lox/-}$, *Tie1-Cre* embryos had pale, bloodless yolk sacs (Fig. 2C) and pericardial swelling (Fig. 2E, arrow), indicative of embryonic circulatory defects. Blood vessels within these embryos appeared dilated and showed congested blood (Fig. 2E, arrowheads). By E12.5, all analyzed $Lkb1^{lox/-}$, *Tie1-Cre* embryos (n=25) were anemic and exhibited large areas of hemorrhages (Fig. 2G), demonstrating disruption of the vasculature. No live $Lkb1^{lox/-}$, *Tie1-Cre* embryos were recovered after E12.5.

To investigate blood vessel structure, $Lkb1^{lox/-}$, *Tie1-Cre* conceptuses were stained with antibodies against VE-cadherin and PECAM1. Whole-mount staining of the adherens junction protein VE-cadherin did not show abnormalities in endothelial junctions (Fig. 3B), and PECAM1 staining revealed normal capillary density and formation of arteries and veins, demonstrating that vascular remodeling and maturation had commenced in yolk sacs (Fig. 3D) and embryos (data not shown). Moreover, vascular sprouting and branching proceeded normally in $Lkb1^{lox/-}$, *Tie1-Cre* embryos, contrary to *Lkb1*-null embryos, where intersomitic branches terminated prematurely in the mesenchyme (Ylikorkala et al., 2001). $Lkb1^{lox/-}$, *Tie1-Cre* yolk sac vessels were, however, distorted and fragile, with small local dilations and irregular vessel wall structure (Fig. 3B,D). In the E11.5 $Lkb1^{lox/-}$, *Tie1-Cre* embryo proper, defects in superficial microvessels were less pronounced, but histological analysis revealed dramatic dilation of the large trunk vessels (Fig.

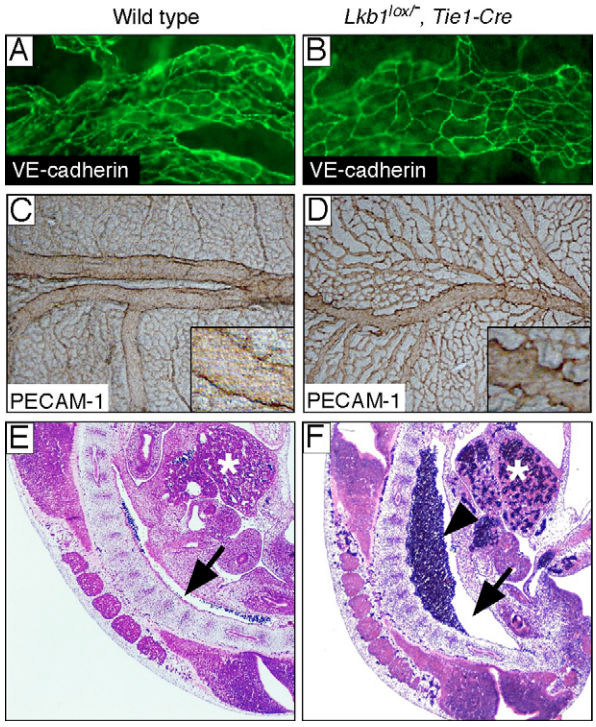

Fig. 3. Blood vessel structure of $Lkb1^{lox/-}$, *Tie1-Cre* conceptuses at E11.5. (**A**,**B**) Whole-mount VE-cadherin staining of wild-type (A) and $Lkb1^{lox/-}$, *Tie1-Cre* (B) yolk sacs. (**C**,**D**) Whole-mount PECAM1 staining of wild-type (C) and $Lkb1^{lox/-}$, *Tie1-Cre* (D) yolk sacs. Insets show higher magnification of single vessels. (**E**,**F**) Hematoxylin and Eosin-stained sagittal sections from wild-type (E) and $Lkb1^{lox/-}$, *Tie1-Cre* (F) embryos. Arrows indicate the aortas; asterisks, the livers. Arrowhead in F indicates congested blood in the $Lkb1^{lox/-}$, *Tie1-Cre* aorta.

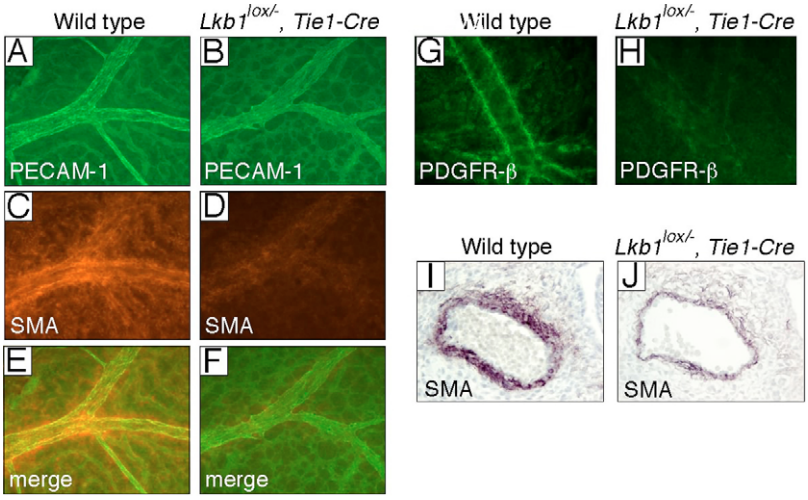

Fig. 4. SMA and PDGFRβ staining demonstrates a reduction of vSMCs in *Lkb1*$^{lox/-}$, *Tie1-Cre* conceptuses at E11.5 (**A-F**) Whole-mount immunofluorescence staining of PECAM1 (green) and SMA (red) in wild-type (A,C,E) and *Lkb1*$^{lox/-}$, *Tie1-Cre* (B,D,F) yolk sacs, demonstrating loss of SMA staining in the mutant. (**G**,**H**) Whole-mount immunofluorescence staining of PDGFRβ in wild-type (G) and *Lkb1*$^{lox/-}$, *Tie1-Cre* (H) yolk sacs, showing loss of PDGFRβ expression in *Lkb1*$^{lox/-}$, *Tie1-Cre* yolk sacs. (**I**,**J**) SMA staining of cross sections of the aorta at the thoracic level of wild-type (I) and *Lkb1*$^{lox/-}$, *Tie1-Cre* (J) embryos demonstrates reduced SMA staining in the mutant.

3F) and blood was congested in these vessels and in the liver, indicating insufficient circulation (Fig. 3F). This is likely to be caused by defects in the blood vessels, as no obvious alterations in heart morphology were noted (data not shown).

The observed dilation of blood vessels following loss of the *Lkb1* tumor suppressor in ECs could be due to increased EC proliferation, as has been previously noted in knockout mouse models with similar vascular phenotypes (Hamada et al., 2005; Lai et al., 2003). These include the EC-specific knockout of the *PTEN* tumor suppressor, which results in enhanced proliferation of ECs, cardiac failure and fused capillaries, leading to embryonic death at E11.5 (Hamada et al., 2005).

To evaluate the proliferation of ECs in *Lkb1*$^{lox/-}$, *Tie1-Cre* conceptuses, yolk sacs were stained for the proliferation marker Ki-67. No significant differences were observed between wild-type and *Lkb1*$^{lox/-}$, *Tie1-Cre* yolk sacs in the percentage of Ki-67-positive ECs (27±7% and 26±12%, respectively) at E11.5 (see Fig. S2A-D in the supplementary material). Similarly, TUNEL assays revealed very low numbers of apoptotic ECs, with no difference between wild-type and *Lkb1*$^{lox/-}$, *Tie1-Cre* yolk sacs (see Fig. S2E,F in the supplementary material). These observations suggest that alterations in EC proliferation or death rates do not contribute to the vascular defects following loss of endothelial *Lkb1*, and further imply that the mechanisms underlying the *Lkb1*$^{lox/-}$, *Tie1-Cre* vascular phenotype are distinct from those in PTEN mutants, which involve the PTEN/PI3K pathway.

Loss of vascular smooth muscle cells in *Lkb1*$^{lox/-}$, *Tie1-Cre* conceptuses

The stabilization and maturation of blood vessels requires the formation of vascular smooth muscle cells (vSMCs) around the nascent endothelial tube, a process regulated by signaling from the endothelium to the surrounding mesenchymal cells and differentiating vSMCs (Armulik et al., 2005). Staining wild-type E11.5 yolk sacs with antibodies against smooth muscle cell-specific α-smooth muscle actin (SMA) identified vSMCs around the blood vessels as expected (Fig. 4C). Interestingly, SMA staining was strongly reduced or absent in *Lkb1*$^{lox/-}$, *Tie1-Cre* yolk sacs (Fig. 4D), although PECAM1 staining revealed an intact endothelium (Fig. 4B), as noted before. Similarly, staining with another vSMC marker, platelet-derived growth factor receptor β (PDGFRβ), identified vSMCs in wild-type yolk sacs (Fig. 4G) but not in *Lkb1*$^{lox/-}$, *Tie1-Cre* yolk sacs (Fig. 4H). Staining of vSMCs in the embryo proper also revealed a significant reduction of vSMCs at E11.5 in *Lkb1*$^{lox/-}$, *Tie1-Cre* embryos compared with wild-type littermates, as is shown in the aorta wall (Fig. 4I,J).

These results show that EC-specific deletion of *Lkb1* results in a marked reduction of vSMCs, suggesting that *Lkb1*$^{-/-}$ ECs are defective in signaling to adjacent vSMCs. The observed loss of vSMCs is likely to cause vessel fragility and vascular disruption of *Lkb1*$^{lox/-}$, *Tie1-Cre* mutants, as has been shown in previous studies describing similar phenotypes (Carvalho et al., 2004; Jadrich et al., 2006; Li et al., 1999).

Deletion of *Lkb1* from ECs leads to defective TGFβ signaling

The PDGFB/PDGFRβ, sphingosine-1-phosphate (S1P)/S1P$_1$ and TGFβ pathways are well-known mediators of signaling from ECs to adjacent mesenchymal cells and differentiating vSMCs (Armulik et al., 2005). Based on mouse knockout studies, the de novo formation of vSMCs from mesenchymal cells around blood vessels is dependent upon endothelial TGFβ signaling (Carvalho et al., 2007; Carvalho et al., 2004; Larsson et al., 2001; Li et al., 1999; Oh et al., 2000; Urness et al., 2000). PDGFB and S1P are required at later embryonic stages (E12-E18), promoting vSMC migration along new blood vessel sprouts (Hellstrom et al., 1999; Liu et al., 2000), and are therefore unlikely to underlie the phenotype in the *Lkb1*$^{lox/-}$, *Tie1-Cre* mice noted at an earlier developmental stage. Loss-of-function mutants of several central TGFβ pathway components (ALK1, ALK5, endoglin, SMAD5) (Larsson et al., 2001; Li et al., 1999; Oh et al., 2000; Yang et al., 1999), as well as of the TGFβ signaling modulator TAK1 (Jadrich et al., 2006), are embryonic lethal at E10-E11, with vascular abnormalities and defective vSMC formation. The necessity of intact endothelial TGFβ signaling for vascular development has been demonstrated by EC-specific knockouts of ALK5 and TGFβRII, and by the knockout of endoglin, an endothelial TGFβ co-receptor, all of which lead to vascular disruption and midgestational lethality (Carvalho et al., 2007; Carvalho et al., 2004).

The relevance of endothelial TGFβ signaling for vascular smooth muscle formation and the similarities in phenotypes of the mouse knockouts of TGFβ signaling components with that of *Lkb1*$^{lox/-}$, *Tie1-Cre* suggested that loss of endothelial *Lkb1* might lead to defective TGFβ signaling in ECs. This hypothesis was supported by the recent observation that loss of *Lkb1* in mouse embryonic fibroblasts (MEFs) decreases the amount of active TGFβ in culture supernatants (Katajisto et al., 2008).

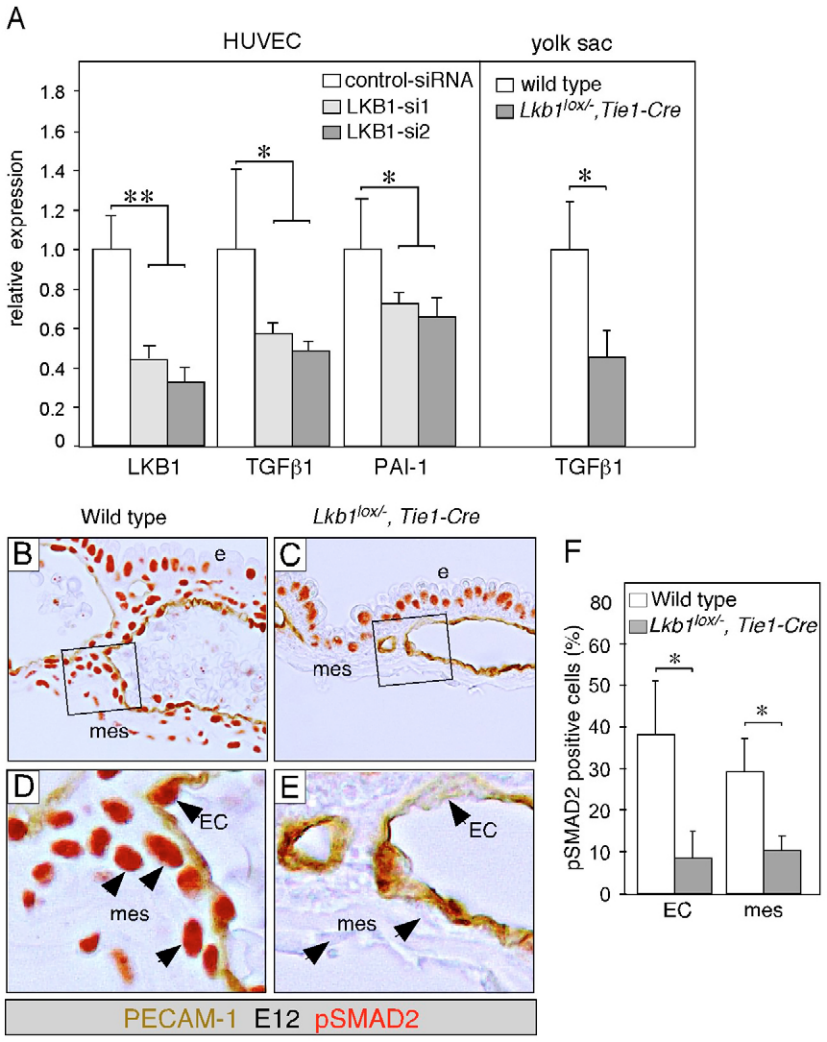

Fig. 5. Suppressed TGFβ signaling in *Lkb1*-deficient ECs. (**A**) Quantitative RT-PCR analysis of LKB1, TGFβ1 and PAI1 mRNA levels in immortalized HUVECs, or wild-type and *Lkb1*$^{lox/-}$, *Tie1-Cre* yolk sacs, as indicated. HUVECs were transfected with a control-siRNA or two independent siRNAs targeting *LKB1* (LKB1-si1 and LKB1-si2). Measurements were normalized according to GAPDH mRNA levels and are presented relative to control samples (control-siRNA transfected HUVECs or wild-type yolk sac). Bars represent the mean (±s.d.) of two samples (siRNA treated), four samples (control-siRNA) or three samples (yolk sacs; **P*<0.05, ***P*<0.01). (**B**,**C**) pSMAD2 (red) and PECAM1 (brown) staining of E12 wild-type (B) and *Lkb1*$^{lox/-}$, *Tie1-Cre* (C) yolk sac sections. (**D**,**E**) Higher magnifications of boxed areas in B and C. (**F**) Percentage of pSMAD2-positive ECs and mesenchymal cells in wild-type and *Lkb1*$^{lox/-}$, *Tie1-Cre* yolk sacs, with means (±s.d.) of three embryos indicated (**P*<0.05). e, endoderm; EC, endothelial cell; mes, mesenchymal cell.

The effect of LKB1 deficiency on endothelial TGFβ signaling was investigated in hTERT-immortalized HUVEC cells, by using siRNA-mediated knockdown of *LKB1* with two siRNAs, which led to a 56% (LKB1-si1) and a 67% (LKB1-si2) decrease in *LKB1* mRNA levels (Fig. 5A). Subsequently, mRNA expression of *TGFβ1* and plasminogen activator inhibitor 1 (*PAI1*; *SERPINE1* – Human Gene Nomenclature Database), a known downstream target of the TGFβ (Goumans et al., 2002; Hackett and Campochiaro, 1993), was analyzed. Interestingly, both *TGFβ1* and *PAI1* mRNAs were decreased following *LKB1* knockdown, and levels correlated with the efficiency of the knockdown (Fig. 5A). As both *PAI1* and *TGFβ1* (Letarte et al., 2005; Van Obberghen-Schilling et al., 1988) are regulated by TGFβ pathway activity, this result suggests that LKB1 regulates TGFβ signaling in endothelial cells. Importantly, a decrease in *Tgfβ1* mRNA levels was also noted in *Lkb1*$^{lox/-}$, *Tie1-Cre* yolk sacs compared with the yolk sacs of wild-type littermates (Fig. 5A).

To analyze TGFβ pathway activity directly in ECs of *Lkb1*$^{lox/-}$, *Tie1-Cre* yolk sacs, the levels of phosphorylated and activated SMAD2 (pSMAD2), an intracellular signal transducer of TGFβ, were investigated by immunohistochemistry. Interestingly, the number of pSMAD2-positive ECs was significantly lower in *Lkb1*$^{lox/-}$, *Tie1-Cre* yolk sacs (Fig. 5B-F) than in the yolk sacs of wild-type littermates, indicating impaired TGFβ signaling in *Lkb1*$^{lox/-}$, *Tie1-Cre* ECs. Furthermore, expression of pSMAD2 was dramatically reduced in mesenchymal cells of *Lkb1*$^{lox/-}$, *Tie1-Cre* yolk sacs (Fig. 5B-F), indicating suppressed TGFβ signaling from ECs and a concurrent downregulation in TGFβ signaling in mesenchymal cells. These results show that TGFβ1 production and SMAD-dependent TGFβ signaling in ECs requires *Lkb1*.

Defects caused by LKB1 deficiency are partially restored by exogenous TGFβ

To investigate whether the defects resulting from endothelial *LKB1* loss in immortalized HUVECs could be rescued by exogenous TGFβ1, mRNA levels of *TGFβ1* and *PAI1* were analyzed before and after TGFβ treatment of LKB1-siRNA or control-siRNA transfected HUVECs. A significant increase in the levels of both mRNAs was noted following TGFβ treatment of LKB1-siRNA treated HUVECs, resulting in 81% *TGFβ1* and 87% *PAI1* mRNA levels in LKB1-siRNA compared with controls (Fig. 6A), and demonstrating that exogenous TGFβ1 can partly rescue the TGFβ signaling defects of LKB1-deficient endothelial cells.

To address the significance of reduced endothelial TGFβ production in vivo, wild-type and *Lkb1*$^{lox/-}$, *Tie1-Cre* E11.5 or E12.5 yolk sacs were split following dissection and incubated in medium with or without added TGFβ1 (1 ng/ml). Interestingly, after only one hour of TGFβ1 treatment the number of pSMAD2-

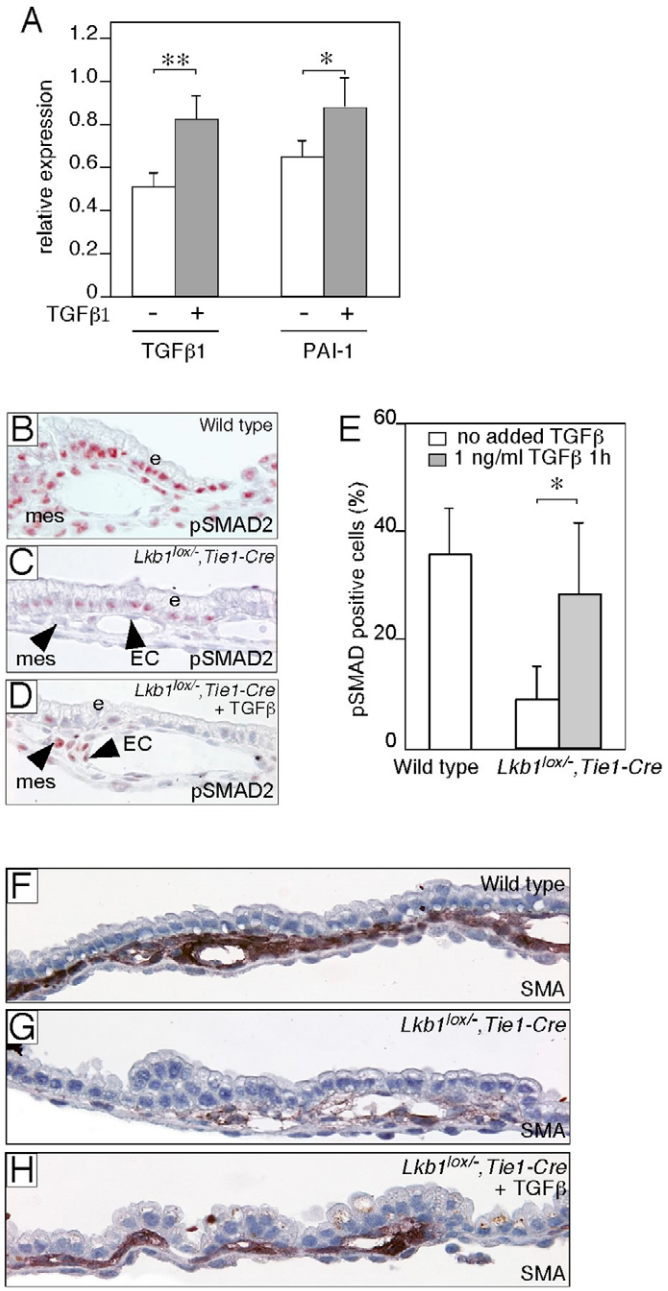

Fig. 6. Partial rescue of LKB1 deficiency by exogenous TGFβ. (**A**) Relative TGFβ1 and PAI1 mRNA levels in LKB1-siRNA transfected HUVECS with (+) or without (–) TGFβ1 treatment (1 ng/ml, 4 hours). Measurements were normalized according to GAPDH mRNA levels and the ratio (±s.d.) of LKB1-siRNA HUVECs to control HUVECs, which were normalized to one, is presented. Bars represent means of LKB1-si1 and LKB1-si2 duplicate samples compared with four controls (*P<0.05, **P<0.01). The data in non-treated samples (–TGFβ1) is summarized from Fig. 5A and is shown for reference. (**B-D**) pSMAD2 staining of E12.5 wild-type (B), *Lkb1*$^{lox/–}$, *Tie1-Cre* (C) and TGFβ-treated *Lkb1*$^{lox/–}$, *Tie1-Cre* (D) yolk sac sections. (**E**) Percentage of pSMAD2-positive cells in E12.5 wild-type and *Lkb1*$^{lox/–}$, *Tie1-Cre* yolk sacs with or without exogenous TGFβ. Means±s.d. of three embryos are indicated (*P<0.05). (**F-H**) SMA staining of E12.5 wild-type (F), *Lkb1*$^{lox/–}$, *Tie1-Cre* (G) and TGFβ-treated *Lkb1*$^{lox/–}$, *Tie1-Cre* (H) yolk sac sections, demonstrating loss of SMA staining in *Lkb1*$^{lox/–}$, *Tie1-Cre* embryos and partial recovery following TGFβ1 treatment. e, endoderm; EC, endothelial cell; mes, mesenchymal cell.

positive cells was already increased in the mutant yolk sacs (Fig. 6B-E). Moreover, the added TGFβ1 was able to induce SMA expression in mesenchymal cells of *Lkb1*$^{lox/–}$, *Tie1-Cre* yolk sacs, noted at 8 hours (Fig. 6F-H).

Together, these data imply that the loss of *Lkb1* in ECs reduces the availability of TGFβ protein to adjacent mesenchymal cells and differentiating vSMCs, which leads to an impaired formation of smooth muscle cell coverage around the maturing vessels.

DISCUSSION

In this study, we show that endothelial-specific deletion of the tumor suppressor kinase *Lkb1* leads to fragile and distorted blood vessels, resulting in vascular disruption and embryonic death at E12.5. The primary cause of aberrant vessel structure was identified as being a loss of supporting vSMCs, demonstrating defective signaling from ECs to adjacent differentiating vSMCs. Moreover, TGFβ signaling was downregulated in *Lkb1*-deficient ECs, indicating that normal TGFβ signaling in ECs requires *Lkb1*.

The mechanism by which downregulation of LKB1 attenuates TGFβ signaling and subsequent TGFβ production in ECs warrants further studies. Based on results from LKB1-deficient MEFs, where attenuated TGFβ signaling is associated with a comparable drop in both active and total TGFβ in culture supernatants (Katajisto et al., 2008) (K.V., Eeva Ventelä, M.T. and T.P.M., unpublished), it does not appear likely that TGFβ signaling defects reflect an altered extracellular activation of latent TGFβ. Another possible mechanism is suggested by the reported ternary complexes between LKB1, LIP1 and SMAD4 (Smith et al., 2001), although we have not been able to detect these complexes (data not shown). In this case, loss of LKB1 would regulate pSMAD2 indirectly through the autocrine regulation of TGFβ production. The mechanism involved may be shared with other cell types in which LKB1 appears to regulate TGFβ signaling, including fibroblasts (Katajisto et al., 2008) (K.V., Eeva Ventelä, M.T. and T.P.M., unpublished), and possibly epithelial cells, based on the observation that *Lkb1* overexpression in HeLa cells increases expression of the TGFβ target genes PAI1 and SM22 (Lin-Marq et al., 2005).

Comparison of vascular phenotypes in *Lkb1*$^{lox/–}$, *Tie1-Cre* mice and *Lkb1*$^{–/–}$ mice (Ylikorkala et al., 2001) demonstrates significant similarities, suggesting that lack of endothelial *Lkb1* function contributes to the phenotype noted in *Lkb1*-null embryos. The later onset of defects (E11.5) and lethality (E12.5) in *Lkb1*$^{lox/–}$, *Tie1-Cre* embryos compared with the lethality of *Lkb1*$^{–/–}$ embryos (E9.5-E10.5) could be due to the contribution of defects in other *Lkb1*-deficient cell types to the null phenotype, or to the LKB1 protein half-life following Tie1-Cre-mediated *Lkb1* excision starting at E8.5 (Gustafsson et al., 2001).

Defective blood vessel maturation is a feature shared not only between these genotypes, but also with mice lacking core TGFβ pathway components TGFβ1, TGFβRII, ALK1, ALK5 and SMAD5 (Dickson et al., 1995; Larsson et al., 2001; Oh et al., 2000; Oshima et al., 1996; Yang et al., 1999), which also display variation in the severity of vascular phenotypes, with lethality between E10.5-E11.5. The most severe phenotypes include the inability of the yolk sac vascular plexus to mature and the defective vascular smooth muscle cell recruitment noted in *Lkb1*, *Alk5* and *Smad5* knockout embryos (Larsson et al., 2001; Yang et al., 1999; Ylikorkala et al., 2001) *Lkb1*$^{lox/–}$, *Tie1-Cre* embryos were able to initiate angiogenesis and defects were observed at a later developmental stage than in endothelial knockouts of TGFβ receptors (Carvalho et al., 2007; Carvalho et al., 2004). Interestingly, defects caused by deficiencies in genes with a regulatory role in TGFβ signaling, such as endoglin

(Carvalho et al., 2004) and *Tak1* (Jadrich et al., 2006) are similar but arise later, and share phenotypes with the *Lkb1*$^{lox/-}$*, Tie1-Cre* mice. Knockout of TGFβRII specifically in smooth muscle cells leads to a very similar phenotype to that of *Lkb1*$^{lox/-}$*, Tie1-Cre* mice (Carvalho et al., 2007), supporting the notion that the major defect in *Lkb1*-deficient ECs is decreased TGFβ production. This would also be consistent with the noted progression of the initial stages of angiogenesis before vSMC recruitment, in the absence of endothelial *Lkb1*. Taken together, these observations suggest that regulation of TGFβ signaling in ECs represents a vital function of the LKB1 kinase during mammalian development.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/13/2331/DC1

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