Integrin-linked kinase controls vascular wall formation by negatively regulating Rho/ROCK-mediated vascular smooth muscle cell contraction

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Vascular smooth muscle cells (VSMCs) form contractile layers around larger blood vessels in a process that is essential for the formation of a fully functional vasculature. Here, we show that integrin-linked kinase (ILK) is required for the formation of a unitary layer of aligned VSMCs around arterioles and the regulation of blood vessel constriction in mice. In the absence of ILK, activated Rho/ROCK signaling induces the elevated phosphorylation of myosin light chain leading to abnormally enhanced VSMC contraction in vitro and in vivo. Our findings identify ILK as a key component regulating vascular wall formation by negatively modulating VSMC contractility.

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The recruitment of pericytes and vascular smooth muscle cells (VSMCs), which form the outer layer of blood vessels, is a key process during angiogenesis and arteriovenous specification (Armulik et al. 2005). VSMCs, but not pericytes, envelope arteries and veins (Armulik et al. 2005). VSMCs also become highly elongated and aligned on small arteries (arterioles) to form a so-called unitary smooth muscle layer (Jäkkäräju et al. 2003). The unitary layer is interconnected electronically and ionically with a nexus of the adjoining VSMCs and acts as a functional syncytium to maintain vascular tone, contractility, and vascular wall rigidity (Jäkkäräju et al. 2003). Disorganiziation of the unitary layer and loss of VSMC contractility is a feature often associated with atherosclerosis and other vascular diseases (Hao et al. 2003). However, the importance of the unitary layer in vascular development remains to be established.

Individual VSMCs in the unitary layer also secrete extracellular matrix and develop attachments to the basement membrane through integrin-associated cell adhesions (Moi seeva 2001). Integrins are α/β heterodimeric transmembrane receptors that link the extracellular matrix to the actin cytoskeleton (Hynes 2002). This cell–matrix connection also provides additional stability to the vessel wall (Moi seeva 2001). Genetic studies have revealed that the adaptor molecule integrin-linked kinase (ILK) is essential for integrin function in vivo (Delon and Brown 2007). In developing striated muscle, ILK is recruited to sarcomeric adhesion plaques where integrins bind to the extracellular matrix (Zervas et al. 2001). This recruitment is necessary for the assembly of myocytes into myotubes and the function of striated muscle (Zervas et al. 2001).

Previously, we reported that the major β1 integrin subunit β1 contributes to vascular smooth muscle function (Abraham et al. 2008). Gene inactivation of β1 integrin in mice impairs VSMC spreading and differentiation, and increases VSMC proliferation leading postnatal lethality (Abraham et al. 2008). Here, we report that ILK plays a fundamental role in developing vascular wall assembly that is distinct from that of β1 integrin. Mice lacking ILK expression in vascular wall cells fail to assemble their VSMCs into a unitary layer, which results in defective vascular remodeling and embryonic lethality. Moreover, we show that loss of ILK causes increased VSMC contractility due to elevated myosin light-chain phosphorylation through activation of Rho/ROCK signaling.

Results and Discussion

Defective vessel wall formation and embryonic lethality in IlkPdgfrb-Cre mice

Since the early lethality of global Ilk knockout mice precludes studies in the vasculature, we bred mice carrying a loxP-flanked Ilk gene (Ilkfl/fl) and Pdgfrb-Cre mice to examine the consequences of Ilk gene deletion selectively in vascular wall cells. The offspring, herein referred to as IlkPdgfrb-Cre, resulted in a loss of ILK expression in dermal pericytes and developing VSMCs but not in endothelial cells (Supplemental Fig. 1A). IlkPdgfrb-Cre embryos developed local hemorrhage and edema, which were first evident around embryonic day 13.5 (E13.5) (Fig. 1A; Supplemental Fig. 1B). Growth and organ morphogenesis also appeared delayed in late-stage IlkPdgfrb-Cre embryos (E18.5), and no living mutants were obtained after birth (Fig. 1A; Supplemental Fig. 1B).

Characterization of the embryonic dermal vasculature by whole-mount immunostaining showed that arterioles of control littermates were continuously covered by VSMCs at E15.5 (Fig. 1B). In contrast, arteriolar VSMC coverage was incomplete in IlkPdgfrb-Cre mutants and further reduced at E18.5 (Fig. 1B,C). IlkPdgfrb-Cre pericytes also had an unusual round morphology by E18.5 (Fig. 1C). Our observations suggest that the phenotype associated with the loss of ILK first manifests itself...
in VSMCs and later in pericytes. Furthermore, a wild-type-like parallel arrangement of arterioles and veins was seen at E15.5, but the venous architecture was highly disorganized by E18.5, suggesting blood vessel stability was compromised in \textit{IlkPdgfrb-Cre} mice (Fig. 1B,C).

\textit{IlkPdgfrb-Cre} mice also exhibited altered basement membrane assembly and minor VSMC apoptosis at E18.5 (Supplemental Fig. 1C,D). However, no overt change was detected in caspase activation or PKB/Akt phosphorylation in \textit{IlkPdgfrb-Cre} tissues (data not shown).

Lack of unitary smooth muscle layers in \textit{IlkPdgfrb-Cre} mice

To investigate the primary cause of reduced VSMC coverage in \textit{IlkPdgfrb-Cre} mice, we examined the association of VSMCs with skin arterioles during early vascular wall formation at E13.5. Control VSMCs formed a unitary smooth muscle layer consisting of elongated and regularly orientated VSMCs [Fig. 2A]. In contrast, ILK-deficient VSMCs failed to align, and were associated with arterioles in a disorganized fashion [Fig. 2A]. Nevertheless, \(\beta_1\) integrin was still concentrated between adjacent VSMCs of mutant mice, as seen in control littermates [Fig. 2A]. Our in vivo observations indicate that ILK is required to organize VSMCs into the unitary smooth muscle layer.

It is generally thought that the increased transmural pressure within arterioles during maturation of the fetal circulation is responsible for promoting the alignment of VSMCs (Jakkaraju et al. 2003; Flavahan et al. 2005). To examine whether ILK is indeed necessary for the mechanical response to induce VSMC alignment, we subjected immortalized mouse control and ILK-deficient VSMCs to cyclic stretching in vitro. Control \textit{Ilkfl/fl} VSMCs aligned perpendicular to the stretching axis and developed ventral actin stress fibers [Fig. 2B]. In contrast, ILK-deficient [\textit{Ilkko}] cells were less aligned and contained prominent cortical actin bundles [Fig. 2B]. These data indicate that ILK is necessary to organize VSMC alignment in response to mechanical stretching.

Loss of ILK induces matrix contraction and vessel constriction

It is thought that ILK is involved in regulating extracellular matrix assembly by mediating an inside-out signaling cascade (Delon and Brown 2007). To investigate whether ILK deficiency affects the surrounding matrix,
we performed three-dimensional (3D) collagen matrix contraction assays using primary human VSMCs (hVSMCs). Examination of the 3D culture reveals that control hVSMCs were elongated and largely orientated in the same direction (Fig. 3A). In contrast, hVSMCs with reduced ILK expression following siRNA-mediated knockdown had a more chaotic arrangement that was reminiscent of that seen on blood vessels in Ilk$^{Dgfbh-Cre}$ mice [Figs. 2A, 3A; Supplemental Fig. 2]. Interestingly, the loss of ILK resulted in a significant increase in the ability of hVSMCs to promote matrix contraction [Fig. 3B,C]. Consistent with the increased collagen matrix contraction in vitro, we found that both arterioles and venules in E15.5 knockout skin were significantly more constricted than those of control mice [Fig. 3D]. Thus, the presence of ILK limits vascular constriction, thereby facilitating normal vessel wall development.

**Loss of ILK modulates VSMC contractility and focal adhesion assembly**

In culture, Ilk$^{ko}$ VSMCs were more contracted than control cells [Fig. 4A,B]. Moreover, phalloidin-positive actin stress fibers in knockout VSMCs were mainly locally concentrated rather than being evenly distributed throughout the cell [Fig. 4B]. These dramatic differences in cell morphology are due to an absence of ILK, as Ilk$^{ko}$ VSMCs expressing ILK-EGFP were spread and had actin stress fibers distributed throughout the cell [Supplemental Fig. 3]. Consistent with the differences in cell spreading and contractility, the organization of focal adhesions was also affected by the loss of ILK. The absence of ILK, however, did not affect cell adhesion on collagen-I or fibronectin (Supplemental Fig. 4). Live-cell imaging revealed that, in migrating control cells, Ilk$^{ko}$ cells showed little or no migration and displayed constitutive ruffling without forming paxillin-positive focal complexes (red arrows). The time in minutes and seconds is indicated in each panel. Bar, 5 μm.

**Figure 3.** Extracellular matrix contraction and vessel constriction facilitated by ILK-deficient VSMCs. (A) Images of hVSMC visualized with phalloidin reveal that mock-treated hVSMCs are regularly arranged, while ILK knockdown hVSMCs have random orientation. Bar, 50 μm. (B) Representative image of increased gel contraction [1.79 ± 0.03 times more than control] induced by hVSMCs treated with ILK siRNA oligo1. The outer and inner dotted lines indicate the area of the initial and contracted gel, respectively. (C) Quantification of gel contraction [n = 7] induced by hVSMCs treated with the indicated ILK siRNA oligos together with the corresponding Western blot showing the knockdown of ILK. (D) Quantification of blood vessel diameter at E15.5. The diameter is calibrated manually using Velocity software on the widest [red] and narrowest [green] points of a single vessel [n = 88 from seven mice].

**Figure 4.** Loss of ILK increases cell contractility and changes focal adhesion assembly. (A) Phase images showing that loss of ILK results in defects in VSMC spreading. Bar, 50 μm. (B) Confocal plane (xy) and vertical view (z) of α-SMA and actin cytoskeleton reveal that actin fibers in Ilk$^{ko}$ cells are concentrated within the cell rather than being distributed throughout the cell. Bar, 10 μm. (C) Paxillin immunostaining reveals that focal adhesions are enlarged and principally observed at cell periphery in Ilk$^{ko}$ VSMCs. Bar, 25 μm. The fluorescent and phase panels from time-lapse movies show the formation and dissociation of nascent focal adhesions at the expanding lamellipodia [red arrows]. In contrast to migrating control cells, Ilk$^{ko}$ VSMCs, which remain stationary, undergo constant ruffling and fail to establish focal complexes [red arrows]. The time in minutes and seconds is indicated in each panel. Bar, 5 μm.


ROCK1 or ROCK2 was also capable of reducing MLC phosphorylation and promoting spreading of Ilk$^{−/−}$ VSMCs (Supplemental Fig. 5). In addition, Ilk$^{−/−}$ VSMCs treated with Y-27632 also recovered the ability to orientate during mechanical stretching (Fig. 5D).

Consistent with the increased contractility, the level of active GTP-bound RhoA was also significantly elevated in Ilk$^{−/−}$ VSMCs, although the expression level of RhoA did not change (Fig. 5E; data not shown). Moreover, inhibition of RhoA signaling using the membrane-permeable TAT-C3 inhibitor was also able to reduce MLC phosphorylation and rescued the spreading of Ilk$^{−/−}$ VSMCs (Fig. 5B,C). The negative regulation of MLC phosphorylation by ILK is not restricted to VSMCs in culture. Immunostaining on tissue sections reveals that the absence of ILK also led to increased MLC phosphorylation in VSMCs associated with dural arterioles of IlkPdgfrb-Cre mice (Fig. 5F).

We demonstrated that ILK-controlled vascular wall coverage is essential during artery/vein specialization and normal embryonic development. This observation strongly supports previous findings in PDGF-B/PDGFR-β knockout mice that vascular wall immaturity destabilizes the vascular network and impairs angiogenic remodeling (Hellstrom et al. 1999). The genetic inactivation of PDGF-B/PDGFR-β or ILK results in embryonic lethality around E18.5, although they contribute to vascular wall formation in distinct ways. Unlike PDGF-B/PDGFR-β signaling, ILK does not appear to control the recruitment of pericytes to nascent vessels, but modulates subsequent steps of vessel wall maturation.

As mentioned above, ILK, but not β1 integrin, is important for the formation of a functional unitary layer. Consistent with this difference, we found that VSMCs lacking β1 integrin do not have altered levels of MLC phosphorylation or increased matrix contraction in vitro.

**Figure 5.** Increased MLC phosphorylation by activated ROCK signaling in the absence of ILK. [(A)] Immunostaining with the indicated antibodies demonstrates that MLC phosphorylation is dramatically increased in Ilk$^{−/−}$ cells. Addition of the ROCK inhibitor Y-27632 suppressed the increased MLC phosphorylation and promoted the redistribution of actin stress fibers and cell spreading. Bar, 25 μm. [(B)] Western blot analysis reveals that phosphorylation of MLC (p-MLC; Thr18/S19) and MYPT1 (p-MYPT1; Thr853) are significantly increased in Ilk$^{−/−}$ cells. Inhibition of ROCK with Y-27632 (Y27) and RhoA with TAT-C3 (C3) but not MLCK kinase (ML9) reduced phosphorylation of MLC in Ilk$^{−/−}$ VSMCs. [(C)] Inhibition of ROCK (Y27) and RhoA (C3) but not MLCK kinase (ML9) also promoted increased spreading of Ilk$^{−/−}$ VSMCs. (n.s.) No significance. [(D)] Immunofluorescence analysis reveals that mechanical stretching of Ilk$^{−/−}$ VSMCs in the presence of Y-27632 (Y27) results in increased alignment (n = 412). Bar, 20 μm. [(E)] Loss of ILK increases RhoA activation in VSMCs (n = 4). [(F)] Immunofluorescence analysis of E15.5 skin (nine sections from seven mice) with the indicated antibodies reveals that loss of ILK results in increased MLC phosphorylation in VSMCs associated with arterioles in IlkPdgfrb-Cre mice. Bar, 20 μm.
purchased from Invitrogen and GE Healthcare, respectively. (clone MB1.2), collagen type IV, and pT853-MYPT1 (clone SA19) were from Abcam; ILK (clone 65.1.9), 13.3) and paxillin (clone 165) were from BD Biosciences; desmin and Ilkfl/fl control

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individual clones were tested by immunoblotting to confirm gene deletion 0.5 mg/mL Geneticin for 2 wk. To obtain a homogeneous cell line, 21

immorto Ilkfl/fl is isolation, background (Grashoff et al. 2003; Foo et al. 2006). For mouse VSMC

transiently transfected into VSMCs, a cre cDNA-contained plasmid (pBS598; a gift from B. Sauer) was with a few modifications (Foo et al. 2006). To obtain immortalized Mouse VSMC isolation, culture, and immunofluorescence

whether ILK is actually a functional Ser/Thr kinase VSMC contraction (Muranyi et al. 2002; Wilson et al. 2005). Nevertheless, there is considerable debate regarding whether ILK is actually a functional Ser/Thr kinase (Boudeau et al. 2003; Sakai et al. 2003; Vouret-Craviari et al. 2004). Our characterization of tissue-specific mutant mice and cultured knockout VSMCs firmly place ILK upstream of Rho/ROCK signaling and show that it is a crucial regulator of VSMC contractility. Moreover, this essential role of ILK in vascular morphogenesis suggests that it may also play a role in disease conditions in which VSMC contractility is lost or deregulated. The task ahead is to determine the molecular mechanism by which ILK attenuates Rho/ROCK signaling.

Materials and methods

Antibodies

α-Smooth muscle actin (α-SMA, clone 1A4), MLC [clone MY-21], and α-tubulin (clone B-5-1-2) were from Sigma-Aldrich; PECAM-1 (clone MEC-13.3) and Paxillin [clone 165] were from BD Biosciences, desmin and MYPT1/2 (clone YE36) were from Abcam; ILK (clone 65.1.9), β1 integrin (clone MB1.2), collagen type IV, and pT853-MYPT1 (clone SA19) were from Millipore; and pT18/S19-MLC were from Cell Signaling Technologies. Secondary antibodies coupled with either Alexa dyes or HRP were purchased from Invitrogen and GE Healthcare, respectively.

Mice and immunohistochemistry

All animal experiments were approved by the Cancer Research UK Animal Ethics Committee. Pdgfrb-Cre mice and mouse carrying a loxP-flanked Ilk gene (IlkLox/Lox) were bred to obtain mutant IlkLox/Cre and control IlkLox/Lox embryos as controls in a mixed 129SV/C57BL6 genetic background (Grashoff et al. 2003; Foo et al. 2006). For mouse VSMC isolation, immmorto IlkLox/Lox mice harboring temperature-sensitive SV40 large T-antigen were obtained as described previously (Foo et al. 2006). All progeny were genotyped by PCR prior to further analyses.

Antibody stainings of whole-mount tissue and skin parafin sections were performed as described previously (Foo et al. 2006). All images were collected using an upright LSM510 confocal microscope (Carl Zeiss) and reconstituted using Volocity software (Improvis).

Mouse VSMC isolation, culture, and immunofluorescence

The isolation and culture of VSMCs was performed as described previously with a few modifications (Foo et al. 2006). To obtain immortalized IlkLox VSMCs, a cre CDNA-contained plasmid (pBSS98, a gift from B. Sauer) was transiently transfected into IlkLox cells, and IlkLox cells were selected with 0.5 mg/mL Geneticin for 2 wk. To obtain a homogeneous cell line, 21 individual clones were tested by immunoblotting to confirm gene deletion of Ilk as well as by the changes in cell morphology. As all 21 clones displayed a similar contracted cell shape, a single clone was expanded and used for all subsequent experiments. Smooth muscle identity was confirmed by the immunoreactivity for α-SMA and SM22α. Immunofluorescence was performed on cells plated onto glass coverslips coated with rat tail type I collagen-coated (BD Biosciences). Images were obtained on an inverted Zeiss Axiovert 200M microscope (Carl Zeiss) using a Hamamatsu ORCA-ER camera (Hamamatsu Photonics) controlled by Volocity software.

Application of cyclic strain

Mouse VSMCs were plated onto rubber-bottom BioFlex Culture Plates coated with type I collagen (Flexcell International Corp.) at a density of 3 × 10⁵ cells per well. After 48 h, subconfluent cells were subjected to equibiaxial cyclic strain under computer-controlled vacuum device FX-4000T with the following parameters: 10%–16% amplitude, 1 Hz constant frequency, and 18-h exposure. The stretched cells were fixed with 4% paraformaldehyde in PBS for 10 min and subsequently labeled with phallolidin (Invitrogen). Using Integrated Morphometry Analysis program on MetaMorph software (Molecular Devices), the angle of cell orientation was automatically measured to determine absolute scores up to 90°. The frequency of cell alignment was determined from (the number of aligned cells)/(total cell number).

siRNA and gel contraction assay

Human VSMCs [six to nine passages] at 70% confluence were transfected with siRNA duplexes using Lipofectamine 2000 (Invitrogen). A mock experiment was performed on cells transfected with 10 nM control BLOCK-IT Fluorescent Oligo (Invitrogen). ILK knockdown was performed on cells transfected with 10 nM BLOCK-IT and 20 nM ON-TARGET plus human ILK siRNA oligos1–6. Oligo1 (5′-GGGCCAGCAUCUAAUGUAU-3′), oligo2 (5′-CAUAUGCCGUAGUGUAUAUG-3′), oligo3 (5′-CGACCCAAAUUUGACAUAG-3′), and oligo4 (5′-CCACAAAUUUUGCCUGGUA-3′) were obtained from Dharmacon. Oligo5 (5′-GGAAGAGGCGGACUCUAATT-3′) and oligo6 (5′-CGACCCCGAGTCCCGGAGATAA-3′) were obtained from Quagen.

Knockdown of ILK was validated by Western blotting from days 2 to 6 after the transfection. After 3 d, cells were suspended at 0.5 × 10⁵ cells per milliliter in 1.0 mg/mL type I collagen, 8% FBS, and 0.8 × DEEMI adjusted to the appropriate pH with 0.1 M NaOH. The cell–matrix mixture was dropped onto BSA-coated 24-well plates with culture medium on the top to induce free contraction. After 30 h, the pixel area of the gels was measured using [image] for quantitative evaluation. Contraction index was calculated from (well area – gel area)/[well area] and then normalized to the control. The contracted gels were fixed with 4% paraformaldehyde in PBS for 30 min and subsequently labeled with phallolidin and DAPI.

Time-lapse imaging

To trace the dynamics of integrin-based adhesions, DsRed-Paxillin was transfected into VSMCs on type I collagen-coated 35-mm glass-bottom dish (MatTek Co.) using an Amaxa Nucleofector device (Amaxa). Images were obtained every 30 sec on an inverted Nikon TE2000-E (Nikon Instruments) using an Andor Ikon single-photon CCD camera controlled by MetaMorph.

Inhibitor treatment and RhoA activation assay

Mouse VSMCs were plated at 50% confluence and, the next day, cells were treated with DMSO (for mock), 10 μM ML9, or 5 μM Y-27632 (Calbiochem) for 6 h, or TAT-C3 for 18 h, and subjected to immunostaining or automatic cell area measuring as described previously (Foo et al. 2006). Quantification of RhoA activation was performed using G-LISA RhoA Activation Assay Biochem Kit following the manufacturer’s instruction (Cytokeleton, Inc.).

Statistical analysis

The data in the graphs are presented as mean and standard deviation (SD) of the mean. Box and whisker plots are generated using median, quartiles,
and highest and lowest values. The data were analyzed by two-tailed ANOVA or Student’s t-test using Excel software. A P-value of <0.05 is considered statistically significant. Three asterisks (*** ) indicate P < 0.001.

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