Aortic Aneurysm Generation in Mice With Targeted Deletion of Integrin-Linked Kinase in Vascular Smooth Muscle Cells


Rationale: Integrin-linked kinase (ILK) is located at focal adhesions and links the extracellular matrix (ECM) to the actin cytoskeleton via β1- and β3-integrins. ILK plays a role in the activation of kinases including protein kinase B/Akt and glycogen synthase kinase 3β and regulates cell proliferation, motility, and survival.

Objective: To determine the function of ILK in vascular smooth muscle cells (SMCs) in vivo.

Methods and Results: SM22Cre⁺Ilk⁺/⁺F/F conditional mutant mice were generated in which the Ilk gene was selectively ablated in SMCs. SM22Cre⁺Ilk⁺/⁺F/F conditional mutant mice survive to birth but die in the perinatal period exhibiting multiple vascular pathologies including aneurysmal dilatation of the aorta and patent ductus arteriosus (PDA). Defects in morphogenetic development of the aorta were observed as early as E12.5 in SM22Cre⁺Ilk⁺/⁺F/F mutant embryos. By late gestation (E16.5 to 18.5), striking expansion of the thoracic aorta was observed in ILK mutant embryos. Histological analyses revealed that the structural organization of the arterial tunica media is severely disrupted with profound derangements in SMC morphology, cell-cell, and cell-matrix relationships, including disruption of the elastic lamellae. ILK deletion in primary aortic SMCs results in alterations of RhoA/cytoskeletal signaling transduced through aberrant localization of myocardin-related transcription factor (MRTF)-A repressing the transcription and expression of SMC genes, which are required for the maintenance of the contractile SMC phenotype.

Conclusions: These data identify a molecular pathway linking ILK signaling to the contractile SMC gene program. Activation of this pathway is required for morphogenetic development of the aorta and ductus arteriosus during embryonic and postnatal survival. (Circ Res. 2011;109:616-628.)

Key Words: integrin-linked kinase ■ smooth muscle cells ■ aortic aneurysm ■ actin cytoskeleton ■ myocardin-related transcription factor-A

Vascular smooth muscle cells (SMCs) transduce a variety of signals from a highly structured extracellular matrix (ECM) that modulate cellular differentiation, migration, and proliferation.1 Adhesion of vascular SMCs to ECM components determines how cells respond to humoral factors, mechanical forces, and developmental cues.2,3 Vascular SMC-ECM interactions are mediated by integrin cell surface receptors.3 In vascular SMCs, integrins localize to membrane-associated dense plaques, which are structurally similar to the focal adhesions observed in cultured cells.4,5 Dense plaques transmit forces between the contractile apparatus and the ECM.6 Because integrin cytoplasmic domains generally lack actin-binding sites and enzymatic activity, signaling is implemented through a series of linker proteins including talin, α-actinin, vinculin, and kinases such as focal adhesion kinase and integrin-linked kinase (ILK).7–9 ILK is a 51-kDa protein originally identified in a search of proteins capable of physically associating with the cytoplasmic domain of the integrin β1 subunit.10 ILK was named on the basis of in vitro studies demonstrating enzymatic activity...
of its C-terminal kinase domain,11,12 which was shown to phosphorylate target proteins including protein kinase B/Akt, glycogen synthase kinase 3β, and myosin light chain.13 ILK plays a critical role in the organization of the actin cytoskeleton through its association with α and β-parvin, paclixin, and the focal adhesion protein vinculin.14 ILK also physically associates with particularly interesting new cysteine-histidine–rich protein (PINCH) 1 and 2,15 which in turn interact with Nck2, a SH2/SH3-adapter protein that associates with cytoskeletal-related signaling molecules including Wiskott-Aldrich syndrome protein and Pak (p21 activated serine/threonine kinase). ILK deletion in leukocytes modulates the activation of small GTPases, which are also important for membrane ruffling and lamellipodia formation, as well as chemokine-triggered cell movement.16 These findings suggest strongly that ILK plays a role as a molecular scaffold necessary for maintaining the integrity of the integrin-based cell adhesion and signaling complex.

To examine the function of ILK in vivo, investigators have used genetic models in multiple species. Deletion of Ilk gene in Caenorhabditis elegans leads to embryonic demise, recapitulating the phenotype observed in β-integrin knockouts.17 Similarly, mice harboring a null mutation in the Ilk gene exhibit peri-implantation lethality; as ILK is critical for the polarization of the epiblast.18 Endothelial-specific deletion of the murine Ilk gene inhibits vasculogenesis and also confers embryonic lethality.19 Surprisingly, mice harboring cardiomyocyte-specific deletion of the Ilk gene appear phenotypically normal at birth, but, during postnatal development, develop dilated cardiomyopathy and heart failure.20 Despite the critical role that focal adhesions and ECM play in modulating the response to vessel wall injury, the role of ILK in vascular SMCs has not been defined. Several studies have shown that ILK promotes calcium/calcmodulin-independent contraction through its capacity to phosphorylate myosin light chain in vitro.21,22 Although some in vitro studies have concluded that forced expression of ILK promotes SMC proliferation and migration,23,24 others have concluded that ILK helps to maintain SMCs in a stationary phenotype in the arterial wall.25 A recent study used platelet-derived growth factor receptor (PDGFR)-B–driven deletion of ILK in multiple vascular wall cells including dermal pericytes and vascular smooth muscle cells, resulting in embryonic demise associated with local hemorrhage and edema first evident around embryonic day (E) 13.5.26

In the studies described in this report, we have examined the function of ILK in vascular SMCs in the developing vasculature in vivo using ILK conditional mutant mice that were intercrossed with mice expressing Cre recombinase under the transcriptional control of the SMC-restricted SM22α promoter, SM22Cre transgenic mice expressing Cre recombinase under the transcriptional control of the SMC-restricted SM22α promoter (SM22Cre).27 Inheritance of the SM22-Cre transgene was determined by PCR, using the following oligonucleotide primers: forward, 5'-CTCTTCTCACGTCCACAAACGACAC-3'; reverse, 5'-GGCGATCCCTGAACATGTCC-3'. Screening of tail DNA for inheritance of the floxed Ilk gene was performed by PCR, as previously reported.28 Using 2 primers, 5'-CAGGTGGCAGAGGTAAGTA-3' and 5'-CAAGGAAATAGGTGAGCTCAGA-3', for simultaneous detection of wild-type, floxed, and excised Ilk genomic sequences. All animal experimentation was performed under protocols approved by the Massachusetts General Hospital and University of Pennsylvania IACUC and in accordance with National Institutes of Health guidelines.

### Transgenic and Genetically Targeted Mice

ILK conditional mutant mice containing a loxP-flanked (floxed) Ilk gene (IlkFlox) have been previously described in detail.19,27 To delete Ilk in vivo in SMCs, IlkFlox mice were intercrossed with SM22Cre-transgenic mice expressing Cre recombinase under the transcriptional control of the SMC-restricted SM22α promoter (SM22Cre).28 Inheritance of the SM22-Cre transgene was determined by PCR, using the following oligonucleotide primers: forward, 5'-CTCTTCTCACGTCCACAAACGACAC-3'; reverse, 5'-GGCGATCCCTGAACATGTCC-3'. Screening of tail DNA for inheritance of the floxed Ilk gene was performed by PCR, as previously reported.29 Using 2 primers, 5'-CAGGTGGCAGAGGTAAGTA-3' and 5'-CAAGGAAATAGGTGAGCTCAGA-3', for simultaneous detection of wild-type, floxed, and excised Ilk genomic sequences. All animal experimentation was performed under protocols approved by the Massachusetts General Hospital and University of Pennsylvania IACUC and in accordance with National Institutes of Health guidelines.

### Histological Analyses, Immunohistochemistry, and Immunofluorescence Studies

Timed matings were set up between male SM22CreIlkFlox and female IlkFlox mice in a mixed SV129 and C57BL/6 background. The presence of a vaginal plug was considered day 0 of pregnancy. Embryos harvested between embryonic day E11.5 and E18.5 were fixed in 4% paraformaldehyde for 24 to 48 hours. After gradient dehydration with ethanol, embryos were embedded in paraffin, sectioned, and stained with hematoxylin and eosin, or immunostained with the following antibodies: anti-ILK-1 (No. 3862, Cell Signaling Technology), anti-fibronectin (No. NB100-92247, Novus Biologicals), anti-fibrillin-1 (No. sc-20084, Santa Cruz), anti–smooth muscle(SM) α-actin (No. A5228, Sigma), anti-SM22α, and anti–SM-myosin heavy chain (No. BT-562, BioMedical Tech). Elastin was detected by van Gieson staining. To assess cell proliferation, transverse serial sections cut at the level of the aortic arch were prepared from E16.5 control and conditional mutant mice and immunostained with the anti-phospho-histone H3 antibody (No. 9706L, Cell Signaling Technology). The number of phospho-histone H3-positive cells per section was determined (n=9 sections). Data are reported as mean number of

---

**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-Cre</td>
<td>recombinant adenovirus-Cre recombinase</td>
</tr>
<tr>
<td>Ad-GFP</td>
<td>recombinant adenovirus–green fluorescent protein</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ILK</td>
<td>integrin-linked kinase</td>
</tr>
<tr>
<td>MRTF-A</td>
<td>myocardin-related transcription factor-A</td>
</tr>
<tr>
<td>PDA</td>
<td>patent ductus arteriosus</td>
</tr>
<tr>
<td>PDGFR</td>
<td>platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>SMCs</td>
<td>smooth muscle cells</td>
</tr>
<tr>
<td>SRF</td>
<td>serum response factor</td>
</tr>
</tbody>
</table>
phospho-histone H3-positive cells per section ± SEM. Detailed histology and immunohistochemistry protocols are available at www.uphs.upenn.edu/mrc/histology/histologyhome.html.

For immunocytofluorescence studies, SMCs plated on Lab-Tek chamber slides were washed twice with prewarmed PBS without Ca²⁺ and Mg²⁺, then fixed in 3.7% formaldehyde solution in PBS for 10 minutes, followed by permeabilization for 10 minutes with 0.2% Triton X-100 at room temperature. After permeabilization, cells were immunostained with the following antibodies: rabbit anti-ILK (No. I1907, Sigma), mouse anti–SM22α (No. Ab10135, Abcam), mouse–anti–FITC (No. H7411, Sigma), and rabbit anti–MRTF-A.30 For labeling F-actin, rhodamine-phalloidin (No. R415, Invitrogen) was used. Nuclei were counterstained with DAPI (Molecular Probes). Immunostained SMCs were visualized on a Nikon ECLIPSE ME600 fluorescence microscope.

**RNA Isolation and Real-Time qPCR**

Total RNA was isolated from whole length aortas from embryos at E18.5, using mechanical homogenization with a rotor-stator and RNeasy columns (Qiagen). After DNaseI digestion, equivalent amounts of RNA from each sample were reverse transcribed, using QuantiTect reverse transcription reagents (Qiagen). Real-time qPCR reactions were conducted with the Applied Biosystems 7500/7500 Fast Real-Time PCR System. Amplification plots were analyzed with the 7500 software. Gene expression was normalized to GAPDH as an internal control.

**Vascular SMC Isolation and Culture**

To isolate primary mouse aortic SMCs, aortas were isolated (from the aortic root to the iliac bifurcation) from 6-week-old Ilk⁺/⁺ mice. The adventitia was removed and the aorta was excised, and the intimal endothelium was removed. The remaining tissue was cut into pieces of approximately 2 mm² and then placed as explants in T25 flasks containing 1 mL RPMI 1640 with 20% fetal bovine serum (FBS) (3 aortas per flask).31 Once the SMC outgrowth had become 50% confluent, cells were grown and passaged in RPMI 1640 supplemented with 0.2% Triton X-100 at room temperature. After permeabilization, cells were immunostained with the following antibodies: rabbit anti-ILK (No. I1907, Sigma), mouse anti–SM22α (No. Ab10135, Abcam), mouse–anti–FITC (No. H7411, Sigma), and rabbit anti–MRTF-A.30 For labeling F-actin, rhodamine-phalloidin (No. R415, Invitrogen) was used. Nuclei were counterstained with DAPI (Molecular Probes). Immunostained SMCs were visualized on a Nikon ECLIPSE ME600 fluorescence microscope.

**Transduction of Primary Aortic SMCs With Recombinant Adenoviruses**

Replication defective adenoviruses (RDAV) encoding Cre recombinase (Ad-Cre),33 as well as the adenovirus control (Ad-GFP), have been described previously.33 Sixty percent to 70% confluent SMCs were transduced with the indicated RDAV at a multiplicity of infection (MOI) of ~100 in RPMI 1640 with 2% FBS for 3 hours at 37°C, and the media with adenovirus was replaced with 3 mL of a lysis buffer containing 1 mmol/L ATP to stabilize F-actin. The cell lysates were centrifuged at 100,000 g for 1 hour at 37°C, using a Beckman ultracentrifuge equipped with an L-80, SW50.1 rotor. The G-actin—containing supernatants were then separated from the F-actin—containing pellets and were immediately placed on ice. The pellets were resuspended to the same volume as the supernatants, using ice-cold water containing 10 μmol/L cytochalasin D, were incubated on ice for 1 hour, and sheared every 15 minutes. Equivalent volumes of the corresponding F- and G-actin fractions were loaded into lanes of an SDS-PAGE gel and analyzed by Western blot analysis. The ratio of F-actin versus G-actin was quantified with the use of image analysis software.

**Statistical Analysis**

Data are expressed as mean±SEM. Statistical comparison of means was performed by using the 2-tailed unpaired Student t test. Probability values <0.05 are considered statistically significant.

**Results**

**Targeted Ablation of the Ilk Gene in Vascular SMCs**

Mice harboring a null mutation in the Ilk gene die at the peri-implantation stage,18 precluding assessment of the function of ILK in vascular SMCs. To examine the function of ILK in SMCs in vivo, conditional mutant mice were generated in which the Ilk gene was selectively ablated in SMCs by intercrossing mice in which the Ilk gene has been conditionally targeted (Ilk⁺/⁻)35 with SM22Cre transgenic mice that express the Cre-recombinase under the transcriptional control of the SMC-restricted SM22α promoter (SM22Cre⁺).28 Of note, the gene-targeting strategy used resulted in excision of Ilk exons 5 through 12 after Cre-mediated recombination generating a true null allele.35 Cre-mediated excision of the loxp-flanked Ilk alleles in SM22Cre⁺ mice was confirmed using PCR-based protocol designed to amplify and distinguish wild-type, floxed, and excised Ilk simultaneously (Online Figure I).

To confirm that excision of the conditional Ilk allele results in a corresponding loss of ILK protein, immunohistochemical analyses were performed on tissue sections harvested from SM22Cre⁺Ilk⁺/⁺ mutant and Ilk⁺/⁻ control mice at E11.5 (Figure 1A through 1D) and E18.5 (Figure 1E and 1F), respectively. Of note, we have shown that SM22Cre transgenic mice promote highly efficient Cre-mediated recombination as early as E9.5 in vascular and visceral SMCs and in cardiac myocytes.36 At E11.5 in the control mice, abundant ILK protein (brown stain) was observed in the carotid artery (car) and 3rd (3), 4th (4), and 6th (6) branchial arch arteries (Figure 1A). High-power magnification revealed that ILK is
Conditional Deletion of ILK in SMCs Results in Perinatal Lethality

To examine the function of ILK in vascular SMCs in vivo, SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup> males and females were intercrossed. However, their offspring failed to generate the expected mendelian frequency of inheritance of all genotypes (Online Table I). Of 146 offspring analyzed at 2 weeks of age, no viable offspring were observed that carried both the SM22Cre allele and 2 copies of the targeted Ilk allele (SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup>). Subsequent analysis of 238 embryos harvested between E11.5 and late gestation (E18.5) revealed the expected mendelian ratio of SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup> mutant embryos (Online Table II). However, all SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup> mutant pups died between E18.5 and postnatal day (P1) in the immediate postnatal period (ie, mutant embryos were liveborn). These data demonstrate that expression of ILK in SMCs is required for postnatal survival beyond P1.

SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup> Mutants Exhibit Aneurysmal Dilation of the Aorta

To determine the cause of perinatal lethality in SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup> mutant pups, SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup> embryos were harvested from E18.5 through parturition (P1). SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup> pups were born alive, but shortly after birth, all SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup> mutant pups became markedly cyanotic and died within 2 to 3 hours (Figure 2A and 2B). Comparison of histological sections of E18.5 SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup> mutant embryos (Figure 2F through 2H) and control littermates (Figure 2C through 2E) revealed the extent of derangements in the vascular anatomy of the conditional mutant mice. These included marked enlargement of the aortic arch (Ao) extending through the descending thoracic aorta beyond the insertion site of the ductus arteriosus (compare Figure 2C and 2F and Online Figure II). In all mutant embryos, dilatation of the aorta caused displacement of the trachea and esophagus to the right of the midline (Figure 2G and 2H and Online Figure II I and II). At this plane of section, the thoracic aorta filled approximately 50% of the chest cavity in some embryos (Figure 2G and 2H). Moreover, the ductus arteriosus (DA) is grossly enlarged at the level of its insertion into the dilated thoracic aorta (Online Figure II J). Of note, only mild dilation of the ascending aorta (AAA) was observed extending to the origin of the right carotid artery (Online Figure II J). However, the medial wall of the AAA was consistently thicker in mutant than control embryos.

Vascular casting studies of 7 E18.5 SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup> mutant embryos revealed striking enlargement of the proximal aorta and thoracic aorta (DAO) as well as enlargement of the ductus arteriosus (DA) (compare Online Figure II A and II C, and Online Table III). Mechanistically, it is noteworthy that cardiac outflow tract and great arch artery patterning defects attributable to the cardiac neural crest including

the expression of ILK in the heart. Consistent with previous reports,<sup>20</sup> ILK protein (brown stain) was observed in cardiomyocytes of control embryos (Figure 1G), but it was not detectable in cardiac myocytes of E14.5 SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup> mutant embryos (Figure 1H).

Figure 1. ILK protein expression in Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup> control and SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup> mutant embryos. A through D, Tissue sections showing ILK expression (brown stain) in the carotid (car), 3rd (3), 4th (4), and 6th (6) branchial arch arteries of E11.5 Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup> control (A and C) and SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup> mutant (B and D) embryos. E and F, Tissue sections cut at the level of the aortic arch of E18.5 Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup> control (E) and SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup> mutant (F) embryos, demonstrating prominent staining ILK expression in the control sections and deletion of Ilk in SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup> mutant embryos. Ao indicates aorta; br, bronchiolae; and eso, esophagus; G and H, Tissue sections cut at the level of the heart of E14.5 Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup> control (G) and SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup> mutant (H) embryos demonstrating prominent staining within the embryonic myocardium of the control embryos. a indicates atrium; v, ventricle. Images acquired at magnification ×100 (A, B, E, and F) or ×400 (C, D, G, and H).

expressed in both medial SMCs and intimal endothelial cells at this developmental stage before remodeling of the branchial arch arteries (Figure 1C, arrows). By contrast, brown staining indicative of ILK expression was not observed in the tunica media of arteries of SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup> conditional mutant mice at E11.5 (Figure 1B and 1D). As anticipated in control mice at E18.5, ILK protein (brown stain) was readily observed in aortic intima and tunica media (Ao) (Figure 1E, arrows). Consistent with the SMC-restricted activity of the SM22α promoter<sup>26</sup>, ILK was not observed in the tunica media of the aorta in SM22Cre<sup>Ilk<sup>F<sub>F</sub>P<sub>P</sub></sup></sup> mutant embryos but was observed in intimal endothelial cells (Figure 1F, arrows). ILK was also expressed abundantly in the muscular layer of the esophagus (eso) (Figure 1E). Because SM22α promoter-driven expression of Cre recombinase also results in gene deletion in embryonic cardiac myocytes,<sup>28</sup> we also examined
persistent truncus arteriosus, double-outlet right ventricle, and right-sided aortic arch were not observed in the SM22Cre IlkF/F mutant embryos. Consistent with these findings, postmortem analysis of 13 SM22Cre IlkF/F mutant newborn pups confirmed vascular defects recapitulating those observed in E18.5 SM22Cre IlkF/F mutant embryos (Online Table III). In all control pups, anatomic constriction and luminal obliteration of the DA was observed within 2 to 3 hours of birth (Online Figure II, B). By contrast, the DA of SM22Cre IlkF/F mutant pups was widely patent and dilated at the time of death (Online Figure II, D). Given the timing of their death, it is most likely that the mice died from the PDA, which was a consistent finding. However, in some mice, aortic dissection was also observed. Together, these data demonstrate that ILK is required for morphogenetic development of the aorta and great vessels required for neonatal survival. The magnitude of the PDA in mutant mice explains, at least in part, the observed cyanosis and lethality of SM22Cre IlkF/F mutant pups.

Because expression of the SM22Cre transgene leads to efficient deletion of conditional alleles in SMCs as well as the embryonic heart, the hearts of SM22Cre IlkF/F mutant pups were also examined. Consistent with the phenotype of MCKCre IlkF/F mice in which the Ilk gene was specifically ablated in cardiac myocytes, hearts developed normally in the SM22Cre IlkF/F mutant embryos and were generally indistinguishable from those observed in IlkF/F control littermates, at least through E18.5 (data not shown). These data support the conclusion that the observed vascular phenotype is attributable to ILK deficiency in vascular SMCs as opposed to a primary cardiac defect. In addition, we examined bronchial SMCs and found no abnormalities that might contribute to the observed phenotype (data not shown).

Defective Morphogenetic Development of the Aorta in SM22Cre IlkF/F Mutants

To begin to examine the mechanism underlying aneurysmal dilation of the aorta observed in SM22Cre IlkF/F mutant mice, we compared the developmental time course of aortic morphogenesis in control and mutant embryos (Figure 3). The aorta arises from the left 4th branchial arch artery and the left dorsal aorta through a complex series of morphogenetic steps beginning at midgestation (for review, see Reference 37). At E11.0, the right and left 3rd (3), 4th (4), and 6th (6) branchial arch arteries arise from the aortic sac and insert into the paired right and left dorsal aortae, respectively. At the level of the bifurcation of the trachea, the paired dorsal aortae merge establishing a single midline dorsal aorta. Beginning at E11.5, the branchial arch arteries begin to asymmetrically remodel, which is functionally coupled to remodeling of the left and right dorsal aortae (dAo). As schematically depicted in Figure 3, by E12.0, the right dorsal aorta and right 6th branchial arch artery have regressed, whereas the left dorsal aorta enlarges, merging with the left 4th branchial arch artery, resulting in formation of the distal aspect of the arch of the aorta and the descending thoracic aorta.

At E11.0, before branchial arch artery remodeling, patterning of the 3rd, 4th, and 6th branchial arch arteries in SM22Cre IlkF/F mutant embryos and IlkF/F control littermates was indistinguishable. By E12.5, in control embryos, the right 4th branchial arch artery has regressed significantly, whereas the left 4th branchial arch artery (L4BA) has increased in caliber, giving rise to the arch of the aorta (Figure 3A). By contrast, in E12.5 SM22Cre IlkF/F mutant embryos, the right 4th branchial arch artery (R4BA) is approximately equal in caliber to the left 4th branchial arch artery (L4BA) (Figure 3B). In addition, in E12.5 control embryos, the right 6th branchial arch artery (R6BA) has
regressed, whereas the left 6th branchial arch artery (L6BA) becomes the ductus arteriosus (DA) linking the pulmonary trunk to the descending thoracic aorta (Figure 3C). However, in SM22Cre+IlkF/F mutant embryos, the right 6th branchial arch artery (R6BA) is approximately equal in caliber to the left 6th branchial arch arteries and the right and left dorsal aorta (dAo) are shown. The planes of sections for A through F are also indicated. A through F, Hematoxylin and eosin–stained transverse sections were prepared from E12.5 IlkF/F control and SM22Cre+IlkF/F mutant embryos. A and B, Failure of the right 4th branchial artery (R4BA) to regress in the mutant embryo. C and D, Failure of the right 6th branchial arch artery (R6BA) to regress in the mutant embryo. E and F, Enlargement of the distal thoracic segment of the dorsal aorta in mutant embryos caused by merging of the right dorsal aorta (RdAo) and left dorsal aorta (LdAo) in the mutant mouse. These abnormalities include a right dorsal aorta of almost equal caliber to the left dorsal aorta, whereas control embryos show clear regression of this vessel. All images acquired at magnification ×40.
enlargement of the aortic arch and descending thoracic aorta are already observed in SM22Cre\(^+\)Ilk\(^{F/F}\)mutant embryos. These data reveal an unanticipated role of ILK in morphogenetic development of the aorta. In this regard, it is noteworthy that the observed defects involve anatomic structures containing both neural crest-derived and mesodermally-derived vascular SMCs populating the aortic arch and descending thoracic aorta beyond the insertion of the DA (or L6BA).

**Loss of ILK Disrupts Organization of the Arterial Tunica Media**

Comparison of hematoxylin and eosin-stained sections of thoracic aorta harvested from E13.5 to E18.5 SM22Cre\(^+\)Ilk\(^{F/F}\)mutant embryos and Ilk\(^{F/F}\)control embryos revealed marked derangements in the structural organization of the arterial tunica media (Figure 4A and 4B). In control embryos at E16.5, SMCs display a spindle-like morphology and are densely packed and circumferentially oriented around the arterial lumen (Figure 4A). Each layer of SMCs in the tunica media of the aorta contains an underlying layer of ECM containing elastin (Figure 4C). By contrast, in SM22Cre\(^+\)Ilk\(^{F/F}\)mutants, aortic SMCs appear very heterogeneous with obvious alterations in cell size, morphology, and orientation as well as marked alterations of cell-cell contact including obvious gaps between medial SMCs (Figure 4B). Consistent with these findings, the elastic fibers, which normally underlie each circumferentially oriented layer of vascular SMCs in the aorta, are not present (Figure 4D). High-power confocal imaging of fibronectin expression revealed striking disruption of cell-cell and cell-ECM structural organization that typifies a large elastic artery such as the aorta (compare Figure 4E and 4F). Interestingly, fibrillin-1 (Fbn-1), which has been implicated in aortic aneurysms observed in patients with Marfan disease,\(^ {38,39} \) was dramatically upregulated in the aorta of SM22Cre\(^+\)Ilk\(^{F/F}\)mutant embryos compared with controls (Figure 4G and 4H). Taken together, these data demonstrate that ILK expression in vascular SMCs plays a critical role in regulating SMC phenotype and cell-cell relationships in the tunica media of large elastic arteries, including the aorta, which is required for structural integrity of these arteries.

**ILK Is Required for Maintenance of a Contractile SMC Phenotype**

To determine whether ILK influences the vascular SMC phenotype, immunohistochemical analyses were performed with a panel of antibodies that distinguish contractile and synthetic SMC markers. Immunofluorescence staining of arteries from SM22Cre\(^+\)Ilk\(^{F/F}\)mutant mice demonstrated marked attenuation of SM-α-actin and SM22α, which are both associated with a “contractile SMC phenotype” (Figure 5A through 5D). Moreover, expression of SM–myosin heavy chain (SM-MHC), a specific and relatively late marker of the SMC lineage, was dramatically downregulated in SM22Cre\(^+\)Ilk\(^{F/F}\)mutant aorta. Downregulation of genes encoding SMC-restricted contractile proteins is accompanied by an increase in SMC proliferation, transverse sections of the aortic arch prepared from E16.5 control Ilk\(^{F/F}\)and SM22Cre\(^+\)Ilk\(^{F/F}\)mutant embryos were immunostained for phospho-histone H3 (pH3) and the number of pH3-positive aortic SMCs/section was quantified. As shown in Figure 5H, the mean number of pH3-positive cells per aortic section in SM22Cre\(^+\)Ilk\(^{F/F}\)mutant embryos demonstrated a statistically significant increase compared with aortic sections prepared from Ilk\(^{F/F}\)control embryos (P<0.05).
Cell Autonomous Alteration in SMC Phenotype in ILK-Deficient SMCs

To determine whether ILK signaling altered the vascular SMC phenotype in a cell-autonomous manner, primary aortic SMCs were isolated from IlkFl/Fl mice and transduced with either the Ad-Cre32 or control Ad-GFP replication-defective adenovirus (RdAV). As anticipated, Ad-Cre transduction conferred a dose-dependent increase in Cre protein expression, with concomitant diminution of ILK protein expression after 72 hours (Online Figure III, A and III, B). Decreased ILK expression was accompanied by decreases in immunofluorescent staining for SM22α/H9251 and SM–/H9251-actin protein (Figure 6A), similar to the diminished expression noted in vivo. Moreover, the rich array of cytoskeletal actin fibers that typify contractile SMCs was severely attenuated in Ad-Cre transduced IlkFl/Fl aortic SMCs (Figure 6A).

To determine whether deletion of ILK signaling directly regulated transcription of the SM22α and SM–α-actin genes, Ad-Cre-transduced IlkFl/Fl SMCs were transiently transfected with luciferase reporter plasmids under the transcriptional control of the SM22α promoter (SM22α.luc) or the SM–α-actin promoter/enhancer (Smα-actin.luc), respectively. Expression of the 441-bp SM22a promoter was reduced by 69%, whereas expression of the SM–α-actin promoter/enhancer was decreased by 71% compared with luciferase activity observed in untransduced and Ad-GFP transduced IlkFl/Fl SMCs (Figure 6B). Taken together, both in vivo and ex vivo experiments demonstrate that SMC-specific deletion of Ilk inhibits transcription and expression of genes encoding SMC contractile proteins. These data also suggest that ILK signaling influences cytoskeletal organization of vascular SMCs in a cell-autonomous fashion. Of note, SMC proliferation was not significantly altered in ILK-deficient SMC in vitro, as assessed by thymidine incorporation (data not shown).

Aberrant Subcellular Localization of Myocardin-Related Transcription Factor-A in ILK-Deficient Vascular SMCs

Both the SM22α and SM–α-actin promoter/enhancers are regulated by a serum response factor (SRF)/myocardin-dependent transcriptional regulatory program.34 In vascular SMCs, SRF-dependent transcription and SMC phenotype is
Influenced by multiple intracellular signals including myocardin-related transcription factors (MRTFs) [for review see1]. Myocardin related transcription factors (MRTF)-A, is a potent transcriptional coactivator that has been shown to transduce integrin signals from the cytoskeleton to the nucleus influencing expression of genes encoding SMC-contractile proteins.1,40 In contrast to nonmuscle cell lineages, MRTF-A, Ad-Cre–transduced cells were transiently transfected with 200 ng SM22α, or SMα-actin expression (green) and nuclear staining with DAPI (blue) are demonstrated. Images acquired at magnification \( \times 400 \). B, Twenty-four hours after RdAV transduction, cells were transiently transfected with 200 ng Sm22α.Luc, or Smα-actin.Luc, or control plasmid pGL3-Basic. Forty-eight hours after transfection, cells were harvested and luciferase activity was measured. Data are displayed as the mean fold induction in luciferase activity observed in cells transfected with Sm22α.Luc or Smα-actin.Luc versus control plasmid pGL3-Basic ± SEM *P<0.05, relative to untransduced cells and Ad-GFP transduced cells. Each experiment was repeated at least 3 times, with each reaction performed in duplicate.

Reduced F-Actin in ILK-Deficient SMC

Previous studies have shown that cytoskeletal actin dynamics control SRF-dependent pathways, at least in part by regulating the subcellular localization of its coactivator MRTF-A.41,42 Therefore, we examined whether ILK signaling regulates F-actin polymerization and/or F-actin disassembly in vascular SMCs. We first quantified the ratio of filamentous actin (F-actin) to monomeric actin (G-actin) by differential centrifugation and Western blotting. These studies demonstrated that deletion of ILK significantly reduced the amount of F-actin in ILK-deficient cells (Figure 8A and 8B). ILK\(^{+/−}\) SMCs were also transduced with Ad-Cre or left untreated and then stained with rhodamine-phalloidin to visualize cytoskeletal actin filaments. As anticipated, in untransduced or Ad-GFP transduced SMCs, a rich array of stress fibers (Figure 8C and 8D) was observed. By contrast, F-actin staining of Ad-Cre transduced SMCs demonstrated an absence of organized actin stress fibers, with clumps of staining concentrated at the periphery of cells, which were sometimes retracted (Figure 8E and 8F). Next, we examined whether overexpression of wild-type ILK (AdWT-ILK) could rescue the cellular pathology observed in the cells rendered ILK-deficient by Ad-Cre. As seen in Figure 8G, forced expression of ILK rescued the striking F-actin and stress fiber abnormalities as assessed by rhodamine-phalloidin staining.

Impaired RhoA Activation in ILK-Deficient SMC

Prior studies have demonstrated that genes encoding SMC contractile proteins, such as SM22α and SMα-actin, are regulated by RhoA-mediated actin polymerization.43 We therefore tested whether deletion of ILK in vascular SMCs modulated the activation of RhoA by measuring the active GTP-bound form of the molecule. Indeed, we observed a significant decrease in basal levels of GTP-bound RhoA in SMC transduced with Ad-Cre as compared with untransduced or Ad-GFP transduced control cells (Online Figure IV). Taken together, these data are consistent with a molecular model wherein ILK signaling activates RhoA, which in turn influences cytoskeletal dynamics, promoting the nuclear localization of MRTF-A and activation of SRF-dependent SMC contractile genes.

Discussion

The capacity of vascular SMCs to modulate their phenotype in response to environmental stimuli plays a critical role in the homeostatic response of the cardiovascular system. ILK is ideally positioned in the vascular SMC to transduce extracellular signals received from the extracellular membrane through β-integrin cell surface receptors.44 It plays a role in the phosphorylation of vital substrates in the SMC, which regulate cell morphology, migration, contraction, prolifera-
tion, and survival. Therefore, it was of interest to examine the vascular phenotype of mice in which the Ilk gene was selectively ablated in vascular SMCs. Surprisingly, Ilk conditional mutant mice survived to birth but die in the immediate postnatal period, coincident with redirection of the fetal circulation. Ilk conditional mutant mice exhibit profound abnormalities in structural organization of great arteries including the aorta and ductus arteriosus. Defects in morphogenetic development of the aorta were observed as early as E12.5. Examination of large elastic arteries in Ilk mutant embryos and pups revealed profound disruption of the tunica media. SMC-specific deletion of ILK alters RhoA activation and cytoskeletal dynamics, which is transduced, at least in part, to the nucleus through MRTF-A attenuating the transcription and expression of SMC genes associated with the contractile SMC phenotype. These data demonstrate that ILK plays a critical role in morphogenetic development of large elastic arteries and that ablation of the Ilk gene in vascular SMCs severely disrupts the structural integrity of arterial structures required for postnatal survival.

Figure 7. Deletion of Ilk in primary aortic SMCs disrupts the nucleocytoplasmic localization of MRTF-A. Aortic SMCs isolated from Ilk<sup>Fl/Fl</sup> mice were transduced with Ad-Cre RdAV or left untransduced (UT). A, B, E, and F, Expression of HA-tagged recombinant MRTF-A protein. Twenty-four hours after RdAV transduction, cells were transfected with an expression plasmid encoding HA epitope-tagged MRTF-A. Forty-eight hours after transfection, the intracellular localization of MRTF-A was examined by anti–HA-FITC antibody staining (MRTF-A in green, DAPI in blue). C, D, G, and H, Expression of native MRTF-A protein. Seventy-two hours after RdAV transduction, cells were fixed, permeabilized, and immunostained with anti–MRTF-A antibody. MRTF-A protein translocated from the nucleus to the cytoplasm in cells transduced with Ad-Cre. Representative images from 1 of 3 experiments are shown. Images acquired at magnification ×400.

Figure 8. Loss of ILK reduces actin polymerization in SMCs. Aortic SMCs isolated from Ilk<sup>Fl/Fl</sup> mice were transduced with Ad-Cre, or Ad-GFP RdAV, or left untransduced (UT). Seventy-two hours after transduction, A, the ratio of F-actin and G-actin was assessed by differential centrifugation and Western blot with an anti-actin antibody, and B, the ratio of F-actin versus G-actin was quantified using image analysis software. The mean F:G actin ratio ± SEM is shown. *P<0.05. C through G, Cells were also stained with phalloidin (red), which binds to actin filaments. To rescue F-actin stress fiber formation (G), SMCs were transduced with adenovirus encoding wild-type ILK (AdWT-ILK) for 3 hours before cotransduction with Ad-Cre. Representative images from 1 of 3 experiments are shown. Images acquired at magnification ×400 (C, E, and G). Higher magnification of the framed area in C and E are shown in D and F, respectively.
Prior studies in genetically targeted Ilk mice revealed a critical role for ILK in cardiovascular biology. Endothelium-specific deletion of the murine Ilk gene inhibits vascularization and results in embryonic lethality.\textsuperscript{19} Targeted ablation of ILK in the murine heart ultimately results in a dilated cardiomyopathy and congestive heart failure.\textsuperscript{20} With regard to SMC biology, one prior study has demonstrated that angiotensin II increases ILK protein expression and kinase activity in vitro.\textsuperscript{24} Adenoviral gene transfer experiments using a dominant-negative construct suggested that ILK is necessary for angiotensin II-mediated SMC migration and proliferation in vitro. Other investigators have demonstrated that ILK may modulate calcium-independent, myosin-mediated contraction of triton-skinned rat carotid artery preparations. More recently, it was also demonstrated that ILK protein levels were decreased after balloon injury of the rat carotid artery.\textsuperscript{25} ILK knockdown by RNA silencing led to augmented cell movement and enhanced wound closure in an in vitro model, which contrasts with the prior published study that used adenoviral overexpression constructs and found diminished SMC migration.\textsuperscript{24} The present study thus extends the prior work by specifically defining a role for ILK in the morphogenetic development of the aorta and in the structural integrity of large elastic arteries in vivo. In this regard, it is noteworthy that alterations in development of the aorta may result from vascular SMC autonomous defects directly attributable to ILK signaling and/or indirectly via alterations in blood flow resulting from alterations in vascular SMC phenotype/contractility or vascular SMC-ECM interactions.

A recent study evaluated PDGFR-B-driven deletion of ILK in multiple vascular wall cells, including dermal pericytes and vascular smooth muscle cells, resulting in embryonic demise associated with local hemorrhage and edema first evident around embryonic day E13.5.\textsuperscript{26} Although extensive pathology in the embryonic dermal vasculature was noted, no pathology of the great vessels was described using the PDGFR-B-driven deletion of ILK in mice. Of note, the investigators also described a mechanism by which ILK knockdown in immortalized, murine SMCs activated Rho/ROCK signaling and induced the phosphorylation of myosin light chain and abnormally enhanced vascular SMC contraction in vitro and in vivo. However, the observation of downregulation of contractile SMC genes in the mutant ducus arteriosus (and aorta) of Sm22Cre\textsuperscript{−/IlkF/F} mice raises questions about the relevance of this finding in vivo. Undoubtedly, the use of different cell types and promoters contribute to these different findings, but together the data underscore a clear role for ILK in SMC homeostasis.

Our in vivo and in vitro results demonstrate that SMC-specific deletion of ILK represses the transcription and expression of SMC genes, which are required for the maintenance of the contractile SMC phenotype. A particularly novel finding in ILK deletion in SMC in vitro relates to the observed effects on MRTF-A, which is believed to transduce Rho GTPase-dependent signaling from the cell membrane to the nucleus and is critical to maintenance of the contractile SMC phenotype.\textsuperscript{1} Our present studies are consistent with prior work demonstrating that ILK deletion in leukocytes modulates the activation of small GTPases, which are also important for membrane ruffling and lamellipodia formation, as well as chemokine-triggered cell movement. It has also been shown that pharmacological agents that inhibit actin polymerization and/or forced expression of non-polymerizing actin mutant proteins stimulate the export of MRTF-A.\textsuperscript{1} However, disruption of MRTF-A signaling alone cannot explain the vascular derangements observed in ILK conditional mutant mice, because mice harboring a null mutation in MRTF-A appear phenotypically normal. This is not surprising, because activity of SRF is modulated by multiple overlapping and redundant signaling pathways including signals transduced through the related transcriptional coactivators myocardin and MRTF-B.\textsuperscript{1}

Additional abnormalities observed in the ILK-deficient mice may also be contributing to the vascular pathology. Confocal imaging of the vessel wall demonstrated striking disruption of the extracellular matrix, consistent with prior studies demonstrating a role for ILK in fibronectin matrix assembly.\textsuperscript{47} Matrix abnormalities could, in turn, be influencing cell migration and proliferation in the ILK-deficient aortas. The observed changes in elastin staining are similarly notable, because elastin is known to affect actin polymerization through an integrin-independent but a RhoA-dependent pathway.\textsuperscript{48} It is tempting to speculate that these disruptions of vascular SMC-ECM signaling in Sm22Cre\textsuperscript{−/IlkF/F} mutant embryos may have led to compensatory expression of fibrillin-1 highlighting the complex but critical role that ECM signaling plays in regulating development of the cardiovascular system.

Although our studies provide unequivocal evidence that ILK plays a critical role in the morphogenetic development and structural organization of the aorta required for survival beyond the perinatal period, the role of ILK in maintenance of cardiovascular homeostasis remains to be determined. Given these data and the critical role that ILK plays in transduction of external signals and the phenotypic abnormalities observed in ILK-deficient aortic SMCs, it is tempting to speculate that ILK may be involved directly or indirectly in the pathogenesis of vascular proliferative syndromes including atherosclerosis and/or the pathogenesis of congenital or acquired vascular aneurysms. In support of this hypothesis, we have recently observed that ILK expression is markedly diminished in a mouse model of abdominal aortic aneurysm disease (Online Figure V). In any case, further studies examining the function of ILK in the postnatal vasculature are warranted.

Acknowledgments

We gratefully acknowledge support from the National Institutes of Health to R.E.G. (R01HL-65584), J.D.R. (R01HL-96779), and R01HL-094520 and R01HL-102968 to M.S.P. We also acknowledge the excellent technical assistance of Melinda J. Palma.

Sources of Funding

We gratefully acknowledge support from the National Institutes of Health to R.E.G. (R01HL-65584) and R01HL-094520 and R01HL-102968 to M.S.P.

Disclosures

None.
References


---

### Novelty and Significance

**What Is Known?**

- Integrin-linked kinase (ILK) is a protein that associates with the cytoplasmic domain of β1 integrins and is thus well poised to transmit signals from within the cell to the extracellular milieu, and vice versa.
- The in vivo role of ILK has been difficult to study because complete knockout confers early embryonic lethality, and thus investigators have turned to tissue specific deletion of this protein.

**What New Information Does This Article Contribute?**

- Deletion of ILK in vascular smooth muscle cells (SMC) in mice confers striking aneurysmal dilation of the aorta.
- ILK deletion in primary aortic SMC triggers alterations in intracellular signaling, which ultimately manifest in the repression of the transcription and expression of genes that are required for the maintenance of the contractile SMC phenotype.

The molecular pathways that orchestrate the formation of the major arteries remain incompletely defined. To examine the function of ILK in SMCs in vivo, conditional mutant mice were generated in which the *Ilk* gene was selectively ablated in SMCs by intercrossing mice in which the *Ilk* gene has been conditionally targeted with SM22Cre transgenic mice that express the Cre-recombinase under the transcriptional control of the SMC-restricted SM22α promoter. The phenotyping of these mice revealed multiple vascular pathologies including aneurysmal dilatation of the aorta and patent ductus arteriosus. These studies define a previously unanticipated molecular pathway linking ILK signaling to the contractile SMC gene program. Activation of this pathway is required for morphogenetic development of the aorta and ductus arteriosus during embryonic and postnatal survival. Further, our data suggest that ILK may be involved directly or indirectly in the pathogenesis of vascular proliferative syndromes, including atherosclerosis and/or the pathogenesis of congenital or acquired vascular aneurysms.
Aortic Aneurysm Generation in Mice With Targeted Deletion of Integrin-Linked Kinase in Vascular Smooth Muscle Cells

_Circ Res._ 2011;109:616-628; originally published online July 21, 2011;
doi: 10.1161/CIRCRESAHA.110.239343
_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/109/6/616

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2011/07/21/CIRCRESAHA.110.239343.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the _Permissions and Rights Question and Answer_ document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/
Online Methods

Transgenic and genetically targeted mice.
ILK conditional mutant mice containing a loxP-flanked (floxed) Ilk gene (Ilk^{F/F}) have been previously described in detail. To delete Ilk in vivo in SMCs, Ilk^{F/F} mice were intercrossed with SM22-Cre transgenic mice expressing Cre recombinase under the transcriptional control of the SMC-restricted SM22α promoter (SM22Cre^{+}). Inheritance of the SM22-Cre transgene was determined by PCR using the following oligonucleotide primers: forward, 5'-CTCCTTCCAGTCCAAACGACC-3'; reverse, 5'-GGCGATCCCTGACATGTC-3'. Screening of tail DNA for inheritance of the floxed Ilk gene was performed by PCR as previously reported, using two primers, 5'-CCAGGTGGCAGAGTGAAGTA-3' and 5'-CAAGGAATAAGGTGAGCTCAGAGA-3', for simultaneous detection of wild-type (WT), floxed, and excised Ilk genomic sequences. All animal experimentation was performed under protocols approved by the Massachusetts General Hospital and University of Pennsylvania IACUC and in accordance with NIH guidelines.

Histological analyses, immunohistochemistry, and immunofluorescence studies.
Timed matings were set up between male SM22Cre^{+}Ilk^{F/+} and female Ilk^{F/F} mice in a mixed SV129 and C57BL/6 background. The presence of a vaginal plug was considered day 0 of pregnancy. Embryos harvested between embryonic day E11.5 and E18.5 were fixed in 4% paraformaldehyde for 24-48 hours. After gradient dehydration with ethanol, embryos were embedded in paraffin, sectioned, and stained with hematoxylin and eosin, or immunostained with the following antibodies: anti-ILK-1 (# 3862, Cell Signaling Technology), anti-fibronectin (# sc-20084, Santa Cruz), anti-fibrillin-1(# sc-20084, Santa Cruz), anti-smooth muscle(SM)-α-actin (# A5228, Sigma), anti-SM22α, and anti-SM-myosin heavy chain (# BT-562, Biomedical Tech) . Elastin was detected by van Gieson staining. To assess cell proliferation, transverse serial sections cut at the level of the aortic arch were prepared from E16.5 control and conditional mutant mice and immunostained with the anti-phospho-histone H3 antibody (#9706L, Cell Signaling Technology). The number of phospho-histone H3-positive cells per section was determined (n > 9 sections). Data are reported as mean number of phospho-histone H3-positive cells per section+ S.E.M. Detailed histology and immunohistochemistry protocols are available at www.uphs.upenn.edu/mcrc/histology/histologyhome.html. For immunocytofluorescence studies, SMCs plated on Lab-Tek chamber slides were washed twice with pre-warmed PBS without Ca^{2+} and Mg^{2+}, then fixed in 3.7% formaldehyde solution in PBS for 10 minutes, and followed by permeabilization for 10 minutes with 0.2% Triton X-100 at room temperature. After permeabilization, cells were immunostained with the following antibodies: rabbit anti-ILK (#I1907, Sigma), rabbit anti-Cre (#69050-3, Novagen), mouse mAb anti-SM-α-actin-FITC (#F3777, Sigma), rabbit anti-SM22α (#Ab10135, Abcam), mouse mAb anti-HA-FITC (#H7411, Sigma), and rabbit polyclonal anti-MRTF-A. For labeling F-actin, rhodamine-phalloidin (#R415, Invitrogen) was used. Nuclei were counterstained with DAPI (Molecular Probes). Immunostained SMCs were visualized on a Nikon ECLIPSE ME600 fluorescence microscope.

Vascular polymer casting.
E18.5 embryos and newborns (P1) were euthanized, the anterior chest wall was dissected away, and the thymus was removed. The left ventricle was then injected with polymer casting material (#07349; Polysciences Inc.), and the embryo was immediately photographed.

RNA isolation and Real-time qPCR.
Total RNA was isolated from whole length aortas from embryos at E18.5 using mechanical homogenization with a roto-stator and RNeasy columns (Qiagen). After DNaseI digestion, equivalent amounts of RNA from each sample were reverse transcribed using QuantiTect reverse transcription reagents (Qiagen). Real-time qPCR reactions were conducted with the Applied Biosystems 7500/7500 Fast Real-Time PCR System. Amplification plots were analyzed with the 7500 software. Gene expression was normalized to GAPDH as an internal control.

**Vascular SMC isolation and culture.**

To isolate primary mouse aortic SMCs, aortas were isolated (from the aortic root to the iliac bifurcation) from 6-week old IlkFl/Fl mice. The adventitia was removed and the aorta was excised and the intimal endothelium was removed. The remaining tissue was cut into pieces of approximately 2mm², and then placed as explants in T25 flasks containing 1mL RPMI 1640 with 20% fetal bovine serum (FBS) (3 aortas per flask). Once the SMC outgrowth had become >50% confluent, cells were grown and passaged in RPMI 1640 supplemented with 20% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine. Cells from passages three to six were used for experiments. Staining with a FITC conjugated anti-mouse-SM-α-actin (Sigma) mAb confirmed >99% SMC purity, with an isotype-matched IgG control (Sigma) verifying specificity.

**Transduction of primary aortic SMCs with recombinant adenoviruses.**

Replication defective adenoviruses (RdAV) encoding Cre recombinase (Ad-Cre), as well as the adenovirus control (Ad-GFP) have been described previously. 60%-70% confluent SMCs were transduced with the indicated RdAV at a multiplicity of infection (MOI) of ~100 in RPMI 1640 with 2% FBS for 3 hours at 37°C, and the media with adenovirus was replaced with 3 ml of fresh medium supplemented with 20% FBS. Cells were then cultured and analyzed as detailed in the figure legends. For rescue experiments, SMCs were transduced with an adenovirus encoding wild type ILK (AdWT-ILK) for 3 hours prior to co-transduction with Ad-Cre.

**Plasmids, transient transfection and luciferase reporter assay.**

Transient transfection analyses were performed as described previously. In brief, 24 hours post RdAV transduction, SMCs were transiently transfected with 200 ng of the indicated luciferase reporter plasmid using Fugene 6 (Roche Applied Science) with a Fugene6/DNA ratio of 3:1. The luciferase reporter plasmids Sm22α.luc, Smα-actin.luc (or, pPIAct-luc) and promoterless control pGL3-Basic plasmid have been described previously. 48 hours post-transfection, SMCs were harvested, and luciferase activities were measured by Steady-Glo Luciferase Assay System (Promega). Each experiment was repeated at least five times. Data are reported as the mean-fold induction in luciferase activity (relative units) ± S.E.M. To determine the nucleo-cytoplasmic localization of MRTF-A, primary mouse SMCs were transfected with 500 ng of pcR3.1-HA-MRTF-A expression plasmid encoding HA epitope-tagged-mouse MRTF-A fusion protein.

**F- Actin/G- Actin assay.**

The ratio of F-actin to G-actin in smooth muscle cells was analyzed using a commercially available kit according to the manufacturer’s protocol (Cytoskeleton Inc., # BK037). Briefly, 72 hours post RdAV transduction, SMCs in 100mm dishes were lysed with 2 ml of a lysis buffer containing 1 mM ATP to stabilize F-actin. The cell lysates were centrifuged at 100,000 g for 1 hour at 37°C using a Beckman ultracentrifuge equipped with an L-80, SW50.1 rotor. The G-actin containing supernatants were then separated from the F-actin containing pellets and were immediately placed on ice. The pellets were resuspended to the same volume as the supernatants using ice cold water containing 10 µM cytochalasin D and were incubated on ice for 1 hour, and sheared every 15 minutes. Equivalent volumes of the corresponding F- and G-actin fractions were loaded into lanes of an SDS-PAGE gel and analyzed by western blot analysis. The ratio of F-actin versus G-actin was quantified using image analysis software.
**RhoA activation Assay.**
Quantification of RhoA activation was performed using a commercially available kit according to the manufacturer’s instructions (Cytoskeleton Inc., # BK036). In brief, Ilk^{FL/F1} SMCs transduced with Ad-Cre or Ad-GFP or left untreated in 100 mm dishes. 72 hours post-transduction, SMCs were harvested at 4°C. 1 mg clarified whole-cell lysates were incubated with 15 µg rhotekin-RBD beads for 60 min at 4°C with rocking. The beads were washed once with Wash buffer, and the GTP-bound form of RhoA was released from beads by the addition of 20 µl of 2× Laemmli reducing sample buffer and heating to 98°C for 5 min. The samples were analyzed by SDS-PAGE and Western blot and probed with an anti-RhoA antibody provided in the kit.

**Western blot analysis.** Western blot analysis for Cre and ILK expression was performed as described previously. Anti-ILK-1(#3862, Cell Signaling) and anti-Cre (#69050-3, Novagen) antibodies were employed. β-actin antibody (Abcam) was used to confirm uniform protein loading.

**Murine abdominal aortic aneurysm model.** ApoE-deficient mice were treated with angiotensin (Ang) II (1000 ng · kg⁻¹ · min⁻¹; Sigma Chemical Co) or normal saline via ALZET model 2004 osmotic pumps (DURECT Corp, Cupertino, Calif) that were implanted subcutaneously as described previously. After 4 weeks of infusion, mice were euthanized for aorta harvest. Total RNA was isolated from suprarenal aortas from mice perfused with buffer RLT (Applied Biosystems, Foster City, Calif) using Trizol reagent (Invitrogen) and RNeasy columns (Qiagen). RT-QPCR was performed as described previously. Amplification plots were analyzed with MX4000 software, version 3.0. Gene expression was normalized to β2-microglobulin as an internal control.

**Statistical analysis.**
Data are expressed as means ± S.E.M. Statistical comparison of means was performed by using the two-tailed unpaired Student t test. P values less than 0.05 were considered statistically significant.
Online Figure I

- Excised ILK, 230bp
- Floxed ILK, 2.1kb
- WT ILK, 1.9kb
- SM22Cre
Online Figure II

Control Mutant

A  B  C  D

AAo  DA  DAo  DA

ICA  DA  DAo  DA

Arch  SVC  Eso  Tr

E  F  G  H

AAo  DA  DAo  DA

Eso  Tr  Arch  SVC

I  J  K  L

AAo  DA  DAo  DA

Eso  Tr  Arch*  SVC

Arch*  DA  DAo*  DAo*
<table>
<thead>
<tr>
<th></th>
<th>Ad-GFP</th>
<th>+</th>
<th>-</th>
<th>Active RhoA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-Cre</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Total RhoA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cre</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ILK</td>
</tr>
</tbody>
</table>
Online Figure V

**ILK mRNA expression** (arbitrary units)

- **no aortic aneurysm**
- **aortic aneurysm**

*** $P < 0.0001$
Online Figure Legends

Online Figure I. Genotyping of Ilk$^{Fv/Fv}$ and Ilk$^{Fv/+}$ conditionally targeted embryos (E18.5).

The embryos were genotyped by PCR using tail DNA. Representative PCR analyses are shown here. Lane 1, DNA marker; lane 2, SM22Cre Ilk$^{Fv/Fv}$; lane 3, SM22Cre Ilk$^{Fv/+}$; lane 4, SM22Cre Ilk$^{Fv/+}$.

Online Figure II. SM22Cre Ilk$^{Fv/+}$ mutants exhibit aneurismal dilation of the aorta and patent ductus arteriosis (PDA). (A-D) Representative images of the great arteries of E18.5 (A, C) and P1 (B, D) Ilk$^{Fv/Fv}$ control (A, B) and SM22Cre Ilk$^{Fv/+}$ mutant (C, D) embryos (A, C) and pups (B, D). In these experiments, the aortae were injected with a vascular casting dye (red). Marked enlargement of the aortic arch extending beyond the insertion site of the DA was observed in all SM22Cre Ilk$^{Fv/Fv}$ mutant embryos (compare panels A and C). Arrows denote dye excluded from closed DA (B) and open DA with dye in lumen (D). (E-L) To define the extend of the aneurysmal dilation, a series of H&E-stained transverse sections (shown in order from rostral to caudal) were prepared from E18.5 Ilk$^{Fv/Fv}$ control embryos (E-H) and SM22Cre Ilk$^{Fv/+}$ mutant (I-L) embryos. Marked aneurysmal dilation was observed involving the arch of the aorta (Arch*) and descending thoracic aorta (DAo*) of SM22Cre Ilk$^{Fv/+}$ mutant embryos extending beyond the insertion site of the ductus arteriosus (DA) in all mutant embryos (n = 7). Note, the aortic aneurysm displaced the esophagus (Eso) and trachea (Tr). Prominent, but much less marked enlargement was observed at the level of the aortic root extending through the ascending aorta (AAo) to the level of the aortic arch. AAo – ascending thoracic aorta; Ao – aorta; Arch – aortic arch; DA – ductus arteriosus; DAo – descending thoracic aorta; Eso – esophagus; ICA – internal carotid artery; PA – pulmonary artery; SVC – superior vena cava; Tr – trachea. All images acquired at 20X.

Online Figure III. Reduction in the expression of ILK in primary cultures of Ilk$^{Fv/Fv}$ SMCs transduced with Ad-Cre RdAV. Primary aortic SMCs isolated from Ilk$^{Fv/Fv}$ mice were transduced with Ad-Cre, Ad-GFP or left untransduced (UT). (A) 72 hours post-transduction, cells were harvested for Western blot to determine Cre expression and ILK deletion. β-actin was used as a loading control, and genomic PCR was performed to confirm DNA excision. (B) Cells grown on Lab-Tek chamber slides were fixed, permeabilized, and immunostained with anti-Cre and anti-ILK antibody (green) to examine Cre and ILK, and DAPI (blue) staining was performed to visualize nuclei. Very little non-specific staining for Cre was observed in the untransduced cells (UT), while Cre expression was expressed in >90% of Ad-Cre-transduced SMCs. ILK was observed in the cytoplasm of untransduced cells, and transduction with Ad-Cre led to a dramatic reduction in ILK protein. Images acquired at 200X.
**Online Figure IV. Effect of ILK deletion on the RhoA activation.** Aortic SMCs isolated from $IIk^{F/F}$ mice were transduced with Ad-Cre or Ad-GFP or left untreated. 72 hours post-transduction, cell lysates were prepared for the analysis of active RhoA (GTP-bound form), total RhoA, Cre, and ILK by immunoprecipitation and Western blotting.

**Online Figure V. Decreased expression of ILK mRNA in a mouse model of abdominal aortic aneurysm (AAA).** ILK mRNA expression was quantified by qPCR in suprarenal aortic segments harvested from ApoE mice infused with angiotensin (Ang) II for 4 weeks. Data show the relative levels of ILK expression in aneursymally-dilated aortic segments versus uninvolved aortic segments (n=10 mice/group; *** P<0.0001).
Online Tables

Online Table I.

Offspring (2-week old) genotype distribution of mating pairs (\(SM22\text{Cre}^{+/+}/Ilk^{FL/+}\) mice × \(SM22\text{Cre}^{+/+}/Ilk^{FL/+}\) mice) following \(SM22\text{Cre}\)-mediated target deletion of ILK in VSMC

<table>
<thead>
<tr>
<th>Genotype</th>
<th>(SM22\text{Cre}^{+}) (Actual/expected)</th>
<th>(SM22\text{Cre}^{-}) (Actual/expected)</th>
<th>P value (X(^2) analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ilk(^{+/+})</td>
<td>34/34</td>
<td>11/11</td>
<td></td>
</tr>
<tr>
<td>Ilk(^{FL/+})</td>
<td>62/68</td>
<td>27/22</td>
<td></td>
</tr>
<tr>
<td>Ilk(^{FL/FL})</td>
<td>0/34</td>
<td>12/11</td>
<td>1.72002E-08</td>
</tr>
</tbody>
</table>
**Online Table II.**

Genotype distribution of embryos (from E11.5 through E18.5) and newborns (P1) following \textit{SM22Cre}-mediated target deletion of ILK in VSMC (Mating pair: \textit{SM22Cre}^{+/+} \textit{Ilk}^{FL/} \textit{mice} × \textit{Ilk}^{FL/} \textit{mice})

<table>
<thead>
<tr>
<th>Genotype</th>
<th>E11.5</th>
<th>E12.5</th>
<th>E13.5</th>
<th>E14.5</th>
<th>E16.5</th>
<th>E18.5</th>
<th>P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{SM22Cre} \textit{Ilk}^{FL/+}</td>
<td>15</td>
<td>4</td>
<td>8</td>
<td>5</td>
<td>10</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>\textit{SM22Cre}' \textit{Ilk}^{FL/Fl}</td>
<td>13</td>
<td>9</td>
<td>3</td>
<td>8</td>
<td>8</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>\textit{SM22Cre}^{+} \textit{Ilk}^{FL/+}</td>
<td>10</td>
<td>5</td>
<td>14</td>
<td>5</td>
<td>8</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td>\textit{SM22Cre}' \textit{Ilk}^{FL/Fl}</td>
<td>13(26%)</td>
<td>6(25%)</td>
<td>9(27%)</td>
<td>5(22%)</td>
<td>8(24%)</td>
<td>16(24%)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>51</td>
<td>24</td>
<td>34</td>
<td>23</td>
<td>34</td>
<td>72</td>
<td>37</td>
</tr>
</tbody>
</table>
Online Table III.

Summary of phenotypes of embryos (E18.5) and/or newborns (P1) following SM22Cre-mediated target deletion of ILK in VSMC

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>E18.5</th>
<th>P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilated aorta</td>
<td>7/7</td>
<td>13/13</td>
</tr>
<tr>
<td>Dilated ductus arteriosus</td>
<td>7/7</td>
<td>13/13</td>
</tr>
<tr>
<td>Patent ductus arteriosus</td>
<td>N/A*</td>
<td>13/13</td>
</tr>
<tr>
<td>Interrupted aortic arch branches</td>
<td>7/7</td>
<td>13/13</td>
</tr>
<tr>
<td>Ventricular septal defect</td>
<td>7/7</td>
<td>NC*</td>
</tr>
</tbody>
</table>

* N/A: not available; NC: not checked
Online References


