Inactivation of Smad5 in Endothelial Cells and Smooth Muscle Cells Demonstrates that Smad5 Is Required for Cardiac Homeostasis

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Smads are intracellular signaling proteins that transduce signals elicited by members of the transforming growth factor (TGF)-β superfamily. Smad5 and Smad1 are highly homologous, and they mediate primarily bone morphogenetic protein (Bmp) signals. We used the Cre-loxP system and Sm22-Cre and Tie-1-Cre mice to study the function of Smad5 in the developing blood vessel wall. Analysis of embryos demonstrated that deletion of Smad5 in endothelial or smooth muscle cells resulted in a normal organization of embryonic and extra-embryonic vasculature. Angiogenic assays performed in adult mice revealed that mutant mice display a comparable angiogenic and vascular remodeling response to control mice. In Sm22-Cre; Smad5fl/fl mice, Smad5 is also deleted in cardiomyocytes. Echocardiographic analysis on those 9-month-old female mice demonstrated larger left ventricle internal diameters and decreased fractional shortening compared with control littermates without signs of cardiac hypertrophy. The decreased cardiac contractility was associated with a decreased performance in a treadmill experiment. In isolated cardiomyocytes, fractional shortening was significantly reduced compared with control cells. These data demonstrate that restricted deletion of Smad5 in the blood vessel wall results in viable mice. However, loss of Smad5 in cardiomyocytes leads to a mild heart defect. (Am J Pathol 2007, 170:1460–1472; DOI: 10.2353/ajpath.2007.060839)

Transforming growth factor (TGF)-β family ligands regulate a wide range of cellular functions and have essential roles in embryonic development. These growth factors signal by binding to a heteromeric complex of type I and type II serine/threonine kinase receptors. The type I receptors, also known as activin receptor-like kinases (Alks), act downstream of the type II receptors and activate intracellular signal mediators among Smads. Smad proteins can be divided into three subgroups: receptor-activated Smads (R-Smads, Smad1, -2, -3, -5, and -8), common Smad (Smad4), and inhibitory Smads (Smad6 and -7). R-Smads become phosphorylated, form heteromeric complexes with Smad4, and accumulate in the nucleus where they participate in transcriptional regulation of target genes.1–3 Smad1, -5, and -8 are closely related Smads and mediate primarily bone morphogenetic protein (Bmp) signaling, although in endothelial cells (ECs) they can also transduce signals from TGF-β via Alk1.4

Gene targeting studies in mice demonstrate that the TGF-β/Bmp signaling pathway is involved in the development of the vascular system and affects the function of endothelial and periendothelial cells.5,6 Mice with a conditional deletion of the Bmp receptor IA (Flk11+/Cre;Alk3fl/fl) die between E10.5 and E11.5 and display multiple abnormalities in vascular development, including vessel remodelling and maturation that contribute to severe abdominal hemorrhage.7 Targeted gene disruption of Smad1 or Smad5 in mice results in embryonic lethality at...
--E9.5.8–11 Smad5-deficient investigators have enlarged blood vessels surrounded by decreased numbers of vascular smooth muscle cells and an aberrant yolk sac, which contains red blood cells but lacks a well-organized vasculature.8,9 Smad1-deficient embryos die because of failure of chorioallantoic fusion.10,11 Smad8 homozygous mutant mice are viable and fertile.13,14 A profound genetic interaction was discovered between Smad1 and Smad5. Smad1+/−/Smad5+/− double heterozygotes die at E10.5 and display defects resembling those seen in Smad1 and Smad5 homozygous mutants.14 In humans, mutations in endoglin (ENG, HHT1) and ALK1 (HHT2), two components of the TGF-β signaling pathway, also lead to vascular dysfunction. Hereditary hemorrhagic telangiectasia (HHT, or Osler-Weber-Rendu syndrome) is an autosomal dominant vascular disease exhibiting multifocal vascular telangiectasias and arteriovenous malformations.15

In vitro, Tgf-β exerts bifunctional effects on ECs: activation of Alk1 will result in Smad1/5 phosphorylation and expression of proangiogenic genes that promote proliferation and migration, whereas activation of Alk5 will result in Smad2/3 phosphorylation and expression of maturation genes that inhibit both processes.16 Endoglin is required for efficient TGF-β/Alk1 signaling and indirectly inhibits TGF-β/Alk5 signaling. The balance between these two TGF-β signaling pathways plays a pivotal role in determining the properties of endothelium during angiogenesis, although contradictory results on these pathways have been published.17,18 These data show that the context and the EC type may influence the outcome of the in vitro experiments. This highlights the requirement of in vivo models to gather additional information on the role of Smad5 in blood vessel development.

The early embryonic lethality of Smad5+−/+ conventional knockout (KO) mice prevents the investigation of the role of Smad5 at later stages of embryonic development and in adult mice. The objective of our study was to determine whether Smad5 is essential in the endothelium and/or in the smooth muscle component of the blood vessel wall during development or pathological conditions in the adult. We generated tissue-specific Smad5 KO mice by crossing Tie-1-Cre19 or Tie-2-Cre20 mice and Sm22-Cre21 mice with floxed Smad5 mice.22 We demonstrated that Smad5 deficiency in either one or both components of the blood vessel wall is compatible with normal viability and reproduction. Loss of Smad5 in smooth muscle cells and cardiomyocytes resulted in decreased cardiac function in female mice, without signs of cardiomyocyte hypertrophy or impaired vascularization.

Materials and Methods

Mouse Strains and Genotyping

Mice homozygous for the floxed Smad5 allele (Smad5+/−) were bred with transgenic mice (Cre+/−/Smad5+/−). These mice are heterozygous for the Smad5 KO allele and express the Cre-recombinase specific in ECs (Tie-1-Cre, Tie-2-Cre19,20) or in smooth muscle cells (Sm22-Cre21). In addition, homozygous Smad5+/−/ROSA26 reporter mice (R26R23) were generated and crossed with Cre+/−/Smad5+/− mice to examine the efficiency of Cre-mediated recombination and to visualize the endothelial or smooth muscle cells in mutant mice. In litters of such crosses, the Cre+/−/Smad5+/− mice constitute the endothelial- or smooth muscle-restricted KO mice, whereas the Smad5+/− mice represent the control mice (Ctrl). All mice used in the experiments have a variable mixed background (CD1, 129/ola, and C57BL6). All animal procedures were performed according to the guidelines of the Animal Care Committee of Katholieke Universiteit Leuven, Leuven, Belgium.

Mouse genotyping of the floxed Smad5 allele, detection of the Cre-mediated recombination, and genotyping of the conventional KO allele (Smad5−/−) was done as described.8,22 Specific primer sets to discriminate the Tie-1-Cre and Sm22-Cre transgene were used to genotype the Smad5−/− embryos. For genotyping of the Sm22-Cre transgene, the primers Sm22-CreFW, 5’-TCCAAAAGCATGCAGAATGTC-3’, and Sm22-CreREV, 5’-GACGGGTATGCGAGGCAAAT-3’, were used to amplify a 400-bp fragment. For genotyping of the Tie-1-Cre transgene, the primers Tie-1-CreFW, 5’-GGAGACTACAGGCCGAACTC-3’, and Tie-1-CreREV, 5’-CGGCAACGGAGCAGAAG-3’, were used to amplify also a 400-bp fragment.

Isolation of Endothelial Cells

Neonatal heart and lung ECs were derived from hearts and lungs of adult Tie-1-Cre+/−/Smad5−/− mice and control littermates using a two-step Percoll gradient protocol.24

Histology, Immunohistochemistry, and Morphometry

Mice were perfused with saline and with 4% paraformaldehyde for 7 minutes before the isolation of organs. Embryos and adult tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline at 4°C overnight, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (H&E) by standard procedures. Heart sections were stained with Masson trichrome to examine interstitial fibrosis. Immunostaining with α-smooth muscle actin (α-SMA) antibody (no. U7033; DAKO, Glostrup, Denmark) was performed to visualize smooth muscle cells, the von Willebrand factor (vWF) antibody (no. A0082; DAKO) and CD31 antibody (no. 557355; BD Pharmingen, San Diego, CA) were used to visualize ECs, and the laminin antibody (no. L9393; Sigma, St. Louis, MO) was used to visualize the basement membrane. Standard immunohistochemical protocols were used, according to the manufacturers’ instructions.

The capillary-to-myocyte ratio was morphometrically quantitated on CD31-stained sections in four randomly chosen optical fields in the left ventricle (LV) wall and the...
interventricular septum. The myocyte size was measured on laminin-stained sections. A minimum of 100 myocytes per heart were measured in at least five randomly chosen optical fields. LV diameter was analyzed on four entire sections with KS 300 image analysis software (Carl Zeiss Inc., Thornwood, NY). Whole mount 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) staining was performed according to standard procedures on E8.0–E12.5 embryos and E15.5 and E18.5 isolated hearts. Stained embryos and hearts were postfixed in 4% paraformaldehyde at 4°C overnight and paraffin-embedded for histological analyses.

**Carotid Artery Ligation Model**

Carotid artery ligation was performed as described.\(^{25}\) In brief, mice were anesthetized by intraperitoneal injection of ketamine and xylazine. The left carotid artery was dissected and ligated just proximal to its bifurcation with silk ligature. Twenty-eight days after surgery, mice were euthanized, the left carotid artery was dissected close to the ligature, and 1 cm of the artery was embedded transversely in paraffin, sectioned, and stained with H&E. Immunohistochemical staining with anti-α-SMA and anti-vWF was performed. Perimeters of the lumen, the internal elastic lamina, and the external elastic lamina were obtained by tracing the contours on digitized images. The intimal and medial areas were calculated by subtracting the area of the lumen from the area defined by the internal elastic lamina and the area defined by the internal elastic lamina from the area defined by the external elastic lamina, respectively. The percentage of stenosis was determined by the formula % stenosis = (IV − I)/IV × 100, where I = luminal surface and IV = internal elastic lamina area.

**Corneal Micropocket Assay**

The corneal micropocket assay was performed as described.\(^{26}\) Pellets containing vascular endothelial growth factor (300 ng) or basic fibroblast growth factor (12.5 ng) were implanted in the corneas of seven Tie-1-Cre\(^{+/-}\), Smads\(^{5/−}\) mice, seven Sm22-Cre\(^{+/-}\),Smad5\(^{5/−}\) mice, and seven control littermates. Five days after pellet implantation, mice were euthanized, the corneas were isolated and fixed in Dent’s fix overnight, and whole mount fluorescent immunostaining with CD31 (catalog no. 01954D; Pharmingen) was performed to visualize the blood vessels. The maximal vessel length extending from the limbal vasculature toward the pellet and the zone of neovascularization was compared between KO and control mice.

**Echocardiography**

Echocardiograms were obtained after urethane anesthesia (1.4 g/kg) with a 15-MHz linear probe on an HP5500 Sonos machine. Nineteen females (10 Sm22-Cre\(^{+/-}\), Smad5\(^{5−}\) mice, nine control mice) and eight males (four Sm22-Cre\(^{+/-}\),Smad5\(^{5−}\) mice, four control mice) between 7 and 9 months of age were used for this experiment. M-mode analysis of LV end-diastolic and end-systolic diameters and interventricular septum and posterior wall end-diastolic thicknesses was performed, and fractional shortening was calculated by an investigator blinded for the genotype, as described by Janssens and coworkers.\(^{27}\) Data are shown as mean ± SEM.

**Treadmill Experiment**

A four-lane treadmill with a speed and inclination adaptable belt was used (Columbus Instruments, Columbus, OH). The first day, a training test was performed in which the mice had to run three times for 15 minutes with a treadmill speed of 5 m/minute (10° inclination) and a rest period of 5 minutes after each run. The experiment was performed the day after the training test. Every 5 minutes, the speed was increased with 2 m/minute starting from 6 m/minute until mice stopped running from exhaustion. A mild electric shock was presented to promote running and mice were removed from the experiment after being on the shock grid for 15 consecutive seconds. The time (in seconds) of running until exhaustion was recorded for each mouse. All mice were tested on the same day.

**Quantitative Polymerase Chain Reaction (PCR)**

Quantitative PCR analysis was done on RNA isolated from hearts of 3-month-old (eight KO, eight Ctrl) and 9- to 11-month-old (four KO, four Ctrl) mice. cDNA was synthesized using random hexamers, PCR analysis was performed with SYBR Green Mastermix (Invitrogen, Carlsbad, CA) and gene-specific primers (designed with Primer Express; Applied Biosystems, Foster City, CA). Relative quantification was achieved using the ΔΔCt method with glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as an internal control. Expression of the following mRNAs was analyzed: Smad1, Smad5, Smad8, inhibitor of DNA binding-1 (Id1), Id2, Id3, plasminogen activator inhibitor (Pai1), Bmp9, Bmp10, atrial natriuretic factor (Anf), brain natriuretic peptide (Bnp), growth differentiation factor 15 (Gdf15), sarcoplasmic reticulum Ca\(^{2+}\) ATPase (Serca 2), platelet-derived growth factor-C (Pdgf-C), osteopontin (Opn), α-myosin heavy chain (α-Mhc), and β-Mhc. Primer sequences are shown in Table 1.

**Myocyte Isolation and Measurement of Contraction**

Ventricular myocytes were enzymatically isolated from the hearts of 11-month-old female mice, as described previously.\(^{28}\) Cells were kept at room temperature, and experiments performed at 30°C within 6 hours of isolation. Cells were placed in a perfusion chamber on the stage of an inverted microscope (Nikon Diaphot; Tokyo, Japan) and stimulated by platinum field electrodes at 1, 2, and 4 Hz, at values 20% greater than threshold. Unloaded cell shortening was measured with a video edge-detector (Ionoptix, Milton, MA). The cell shortening is
mediated recombination of the Smad5fl and showed no clear morphological defects. The Cre-SEM.

tissues such as yolk sac and chorioallantoic placenta, as protected in all ECs within the embryo and extra-embryonic adults. M, marker, 1-kb DNA ladder. Two primer sets were used: a CreFW and CreREV primer (top) were used for amplification by PCR analysis demonstrating Cre-mediated recombination in DNA isolated from tail and lungs of adult endothelium-specific KO mice. PCR on DNA isolated from such cell cultures demonstrated correct recombination of the floxed Smad5 allele (Figure 1), but we could not determine whether all ECs were Smad5-deficient because the primary cultures also contained fibroblasts (data not shown).

Normal Blood Vessel Development in Endothelium-Specific or Smooth Muscle-Specific Smad5 KO Embryos

We bred the Smad5fl/ mice onto a R26R background to analyze the onset and the pattern of the Cre-mediated recombination and to visualize the endothelial and smooth muscle cells in the mutant mice. Whole mount staining with X-gal of Tie-1-Cre;Smad5fl/;R26R and Tie-2-Cre;Smad5fl/;R26R embryos from E8.5 until E12.5 and subsequent sectioning showed that already at initial stages of vasculogenesis, ie, at E8.0, X-gal staining was detected in almost all ECs of the developing vasculature. At later stages, β-galactosidase activity was detected in all ECs within the embryo and extra-embryonic tissues such as yolk sac and chorioallantoic placenta, as described. This demonstrates that endothelium-specific deletion of Smad5 did not impair EC differentiation.

Statistical Analysis

The statistical analysis was performed by a two-tailed Student’s t-test or a two-way analysis of variance followed by the posthoc Fisher’s test for contraction measurements. Probability values of \( P \leq 0.05 \) were considered as significant.

Results

Generation of Mice with Endothelium- or Smooth Muscle-Restricted Deletion of Smad5

Homzygous floxed Smad5 mice (Smad5fl/fl) were crossed with Tie-1-Cre, Tie-2-Cre, or Sm22-Cre mice that were heterozygous for Smad5 (Cre+/−; Smad5+/−). Both the smooth muscle-specific Smad5 KO mice and the endothelium-specific Smad5 KO mice were born at Mendelian ratios (Sm22-Cre+/−; Smad5fl/fl, 22%, \( n = 303 \); Tie-1-Cre+/−; Smad5fl/fl, 22%, \( n = 212 \); Tie-2-Cre+/−; Smad5fl/fl, 22%, \( n = 95 \)), reached adulthood, were fertile, and showed no clear morphological defects. The Cre-mediated recombination of the Smad5 allele and the presence of the conventional KO allele (Smad5 WT) were confirmed by PCR analysis on DNA isolated from tail and organs of adult mice, or yolk sac of embryos as previously described. Genomic analysis of DNA isolated from kidney, liver, heart, and lung of adult Sm22-Cre+/−; Smad5fl/fl mice and Tie-1-Cre+/−; Smad5fl/fl mice clearly demonstrated Cre-mediated recombination in all organs analyzed (Figure 1).

To verify whether all ECs were recombined in the Tie-1-Cre+/−; Smad5fl/fl mice, ECs were derived from hearts and lungs of adult endothelium-specific KO mice. PCR on DNA isolated from such cell cultures demonstrated correct recombination of the floxed Smad5 allele (Figure 1), but we could not determine whether all ECs were Smad5-deficient because the primary cultures also contained fibroblasts (data not shown).

### Table 1. Oligonucleotide Primers Used for Quantitative Gene Expression Analysis

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<th>Gene</th>
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<td>5′-TCCATCGAAGTCTGAGGGTCTC-3′</td>
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Figure 1. PCR analysis demonstrating Cre-mediated recombination in DNA isolated from kidney (Ki), liver (Li), heart (He), lung (Lu), and cell cultures derived from heart (He) and lung (Lu) from Sm22-Cre+/−; Smad5fl/fl and Tie-1-Cre+/−; Smad5fl/fl adult mice. M, marker, 1-kb DNA ladder. Two primer sets were used: a CreFW and CreREV primer (top) were used for amplification of the 400-bp Cre fragment, primers 1 and 3 for the recombined 387-bp Smad5 fragment only.

expressed as the fractional shortening, ie, normalized to resting cell length (ΔL/L0). Data are shown as mean ± SEM.

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Homzygous floxed Smad5 mice (Smad5fl/fl) were crossed with Tie-1-Cre, Tie-2-Cre, or Sm22-Cre mice that were heterozygous for Smad5 (Cre+/−; Smad5+/−). Both the smooth muscle-specific Smad5 KO mice and the endothelium-specific Smad5 KO mice were born at Mendelian ratios (Sm22-Cre+/−; Smad5fl/fl, 22%, \( n = 303 \); Tie-1-Cre+/−; Smad5fl/fl, 22%, \( n = 212 \); Tie-2-Cre+/−; Smad5fl/fl, 22%, \( n = 95 \)), reached adulthood, were fertile, and showed no clear morphological defects. The Cre-mediated recombination of the Smad5 allele and the presence of the conventional KO allele (Smad5 WT) were confirmed by PCR analysis on DNA isolated from tail and organs of adult mice, or yolk sac of embryos as previously described. Genomic analysis of DNA isolated from kidney, liver, heart, and lung of adult Sm22-Cre+/−; Smad5fl/fl mice and Tie-1-Cre+/−; Smad5fl/fl mice clearly demonstrated Cre-mediated recombination in all organs analyzed (Figure 1).

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We bred the Smad5fl/fl mice onto a R26R background to analyze the onset and the pattern of the Cre-mediated recombination and to visualize the endothelial and smooth muscle cells in the mutant mice. Whole mount staining with X-gal of Tie-1-Cre+/−; Smad5fl/fl;R26R and Tie-2-Cre+/−; Smad5fl/fl;R26R embryos from E8.5 until E12.5 and subsequent sectioning showed that already at initial stages of vasculogenesis, ie, at E8.0, X-gal staining was detected in almost all ECs of the developing vasculature. At later stages, β-galactosidase activity was detected in all ECs within the embryo and extra-embryonic tissues such as yolk sac and chorioallantoic placenta, as described. This demonstrates that endothelium-specific deletion of Smad5 did not impair EC differentiation.
and resulted in the formation of normal blood vessels (Figure 2, A–D, I, and J).

The expression in adult organs of the Sm22-Cre transgene has been reported. Here, using Sm22-Cre\(^{+/0}\);Smad5\(^{fl/+}\);R26R embryos, we demonstrated an efficient recombination by Cre at several embryonic stages. Already at E8.0 to E8.5, blue staining was visible in the vessels that form in the yolk sac, in the dorsal aorta, but also in the developing heart tube (Figure 2E). An unexpected clear blue staining in the amnion was also observed from E8.0 onwards (Figure 2K). At E9.5, positive cells were observed in the heart and dorsal aorta, demonstrating a normal progression of blood vessel development in the mutant embryos (Figure 2F). Sections through the head showed that both cephalic mesenchyme and surface ectoderm, regions known to express Sm22,\(^{29}\) were positive. In E10.5 and E12.5 embryos, X-gal staining was observed in nearly all vascular smooth muscle cells and cardiomyocytes (data not shown). Staining of hearts isolated from E15.5 and E18.5 embryos and subsequent sectioning revealed normal, blue-stained pericardial and myocardial cells (data not shown). These data demonstrate that, although conventional Smad5 mutant embryos die and show severe cardiovascular defects at midgestation, deletion of Smad5 in endothelial or smooth muscle cells/cardiomyocytes did not result in a lethal blood vessel or cardiac phenotype. Because we did not observe any morphological difference between Tie-1-Cre\(^{+/0}\);Smad5\(^{fl/+}\);R26R embryos and Tie-2-Cre\(^{+/0}\);Smad5\(^{fl/+}\);R26R embryos, all further experiments in adult mice were performed with the Tie-1-Cre\(^{+/0}\);Smad5\(^{fl/+}\) mice.

Figure 2. Detection of Cre activity in ECs and smooth muscle cells and gross morphology of Cre Smad5\(^{fl/+}\);R26R embryos. Top: Embryos were whole mount-stained for β-galactosidase activity. E8.0 (A), E9.5 (B), E10.5 (C), and E12.5 (D) Tie-2-Cre\(^{+/0}\);Smad5\(^{fl/+}\);R26R (A, B, D) and Tie-1-Cre\(^{+/0}\);Smad5\(^{fl/+}\);R26R (C) embryos demonstrating that already at initial stages of vasculogenesis staining was detected in almost all ECs of the developing vasculature (A). At later stages, β-galactosidase activity was detected in nearly all ECs within the embryo and extra-embryonic tissues such as the yolk sac (B–D). E–H: Whole mount β-galactosidase activity in Sm22-Cre\(^{+/0}\);Smad5\(^{fl/+}\);R26R embryos at E8.0 (E), E9.5 (F), E10.5 (G), and E12.5 (H), demonstrating that at E8.0 blue staining is already detected in the heart and yolk sac, and at later stages, β-galactosidase activity is observed also in the aorta, cephalic mesenchyme, and yolk sac. Bottom: Cross section through an E10.5 Tie-1-Cre\(^{+/0}\);Smad5\(^{fl/+}\);R26R embryo (I) and an E11.5 Tie-2-Cre\(^{+/0}\);Smad5\(^{fl/+}\);R26R embryo (J) demonstrating blue staining in ECs of the dorsal aorta (da) and endocardial cushions in the heart (he). Cross section through E8.5 (K) and E10.5 (L) Sm22-Cre\(^{+/0}\);Smad5\(^{fl/+}\);R26R embryos showing blue-stained cells in the heart (he), yolk sac (ys), amnion (am), and dorsal aorta. Original magnifications: ×60 (A), ×20 (B, F), ×14 (C), ×10 (D, H), ×40 (E, J, L), ×16 (G), and ×100 (I, K).
Deletion of Smad5 in the Blood Vessel Wall Results in Viable Offspring

Because of the close interaction between ECs and smooth muscle cells and their transdifferentiation capacities, EC-restricted deletion of Smad5 can potentially be rescued by the presence of Smad5 in smooth muscle cells and vice versa. Therefore, we also generated mice that were Smad5-deficient both in ECs and smooth muscle cells. Crossing of Sm22-Cre<sup>+/−</sup>;Tie-2-Cre<sup>+/−</sup>, Smad5<sup>−/−</sup> mice and Smad5<sup>fl/fl</sup> resulted in 13% of Sm22-Cre<sup>+/−</sup>;Tie-2-Cre<sup>+/−</sup>;Smad5<sup>−/−</sup> mice (n = 108), which is the expected Mendelian frequency. Mice were viable and healthy and showed no obvious morphological defects. Clearly, the absence of Smad5 both in ECs and smooth muscle cells did not result in a lethal phenotype.

Normal Blood Vessel Morphology in Organs of Adult Mutant Mice

Liver, heart, kidney, and lung of adult endothelium- or smooth muscle-specific Smad5 KO mice and control littermates were immunohistochemically stained for α-SMA, vWF, and CD31 on paraffin sections for assessment of blood vessel morphology. In all these organs, no differences could be observed between mutant and control mice. In all Sm22-Cre<sup>+/−</sup>;Smad5<sup>−/−</sup> organs analyzed, both capillaries and larger blood vessels were positive for CD31, whereas only larger blood vessels were positive for vWF. In liver, all central and portal veins were lined with ECs (Figure 3A), comparable with control liver (data not shown), and all arteries contain α-SMA-positive cells, comparable with control mice (not shown). Original magnifications, ×200.

Vascular Endothelial Growth Factor- and Basic Fibroblast Growth Factor-Induced Angiogenesis Is Not Affected in Endothelium- or Smooth Muscle-Specific Smad5 KO Mice

The normal development of Tie-1-Cre<sup>+/−</sup>;Smad5<sup>−/−</sup> and Sm22-Cre<sup>+/−</sup>;Smad5<sup>−/−</sup> mice suggested that developmental angiogenesis does not require Smad5. Furthermore, mutant female mice bred equally well as controls, indicating that Smad5 is also not essential in angiogenic processes during pregnancy (data not shown). However, these data do not exclude a role for Smad5 in pathological angiogenesis. To evaluate further the angiogenic response in adult mice, pellets containing vascular endothelial growth factor (data not shown) or basic fibroblast growth factor (Figure 4) were implanted in corneas of seven Tie-1-Cre<sup>+/−</sup>;Smad5<sup>−/−</sup> and seven Sm22-Cre<sup>+/−</sup>;Smad5<sup>−/−</sup> mice (data not shown) as well as in seven control littermates to induce blood vessels in the avascular cornea. The induction of blood vessel growth from the limbal vasculature toward the pellet was analyzed 5 days later. Immunofluorescent staining for CD31 on flat-mounted corneas showed no differences in induced angiogenic responses between mutant and control animals (Figure 4, A and B).
Vascular Remodeling Is Not Affected in Smad5 Mutant Mice

To determine whether Smad5 deficiency in endothelial or smooth muscle cells affects vascular remodeling in adult mice, we induced neointima formation by ligating the left carotid artery and compared the morphology of the carotid artery of seven Tie-1-Cre^+/0;Smad5^fl/+ mice (data not shown) and seven Sm22-Cre^+/0;Smad5^fl/+ mice (Figure 5) with control littermates 28 days after ligation. Quantitative measurements of the intimal area on H&E-stained sections of control and mutant carotid arteries showed no significant difference in percentage of stenosis between mutant mice and control mice (control mice, 71% ± 23; Tie-1-Cre^+/0;Smad5^fl/+ 60% ± 34, P = 0.85 versus control; Sm22-Cre^+/0;Smad5^fl/+ 70% ± 21, P = 0.55 versus control), demonstrating that mutant mice display the same vascular remodeling response as control mice (Figure 5, A–D). Immunohistochemical staining with a smooth muscle-specific or an endothelium-specific marker
showed that the intimal lesions contained mainly α-SMA-positive cells (Figure 5, E–H), but vWF-positive cells (Figure 5, I–L) were also observed. There was no difference in expression pattern between control and mutant mice.

**Decreased Cardiac Contractility in Sm22-Cre<sup>+/-</sup>;Smad5<sup>fl/fl</sup> Mice**

Analysis of Sm22-Cre-mediated recombination in R26R mice demonstrated that Sm22-Cre not only recombines floxed alleles in vascular smooth muscle cells but also in myocardium (Figure 2); consequently, our Sm22-Cre<sup>+/-</sup>; Smad5<sup>fl/fl</sup> mice were Smad5-deficient in smooth muscle cells and cardiomyocytes. Hence, we assessed cardiac function. Echocardiographic analysis of Sm22-Cre<sup>+/-</sup>; Smad5<sup>fl/fl</sup> mice and control littersmates at 9 months of age showed significantly larger systolic (P = 0.017) and diastolic (P = 0.044) LV internal diameters and significantly decreased fractional shortening (P = 0.032) in female mutants (n = 10) compared with female control mice (n = 9, Figure 6A). In contrast, analysis of these same mice at earlier age did not show these differences (data not shown), and no significant differences in cardiac function were observed between mutant (n = 4) and control male littersmates (n = 4). In both sexes, there were no obvious differences in heart rate or wall thicknesses (Figure 6A, data not shown).

At autopsy, hearts of Sm22-Cre<sup>+/-;Smad5<sup>fl/fl</sup></sup> mice did not show overt cardiomyopathy, and heart weights of Sm22-Cre<sup>+/-;Smad5<sup>fl/fl</sup></sup> mice were not increased compared with Ctrl (data not shown). A detailed histological analysis was performed on isolated hearts of the female mice (10 KO, nine Ctrl) after diastolic arrest. The LV diameter was measured on cross sections (H&E staining) demonstrating a mild dilation in Sm22-Cre<sup>+/-;Smad5<sup>fl/fl</sup></sup> hearts compared with control hearts (data not shown, P = 0.09). Analysis of laminin-stained sections revealed that myocyte size did not increase in Sm22-Cre<sup>+/-;Smad5<sup>fl/fl</sup></sup> hearts (KO, 147 μm ± 20; Ctrl, 154 μm ± 22; P = 0.5) demonstrating that impaired cardiac function was not attributable to cardiac hypertrophy (Figure 6B, A and E). Fibrosis often accompanies cardiac hypertrophy, but Masson’s trichrome staining of heart sections did not show interstitial fibrosis in mutant mice (Figure 6B, B and F). Immunohistochemical staining for CD31 demonstrated that the capillary to myocyte ratio was comparable in Sm22-Cre<sup>+/-;Smad5<sup>fl/fl</sup></sup> mice and control mice (KO, 145% ± 13; Ctrl, 146% ± 14; P = 0.83) (Figure 6B, C and G). No difference was observed in the number of capillaries and coronary vessels surrounded by α-SMA-stained cells (Figure 6B, D and H). Thus, loss of Smad5 in smooth muscle cells and cardiomyocytes resulted in decreased cardiac function but without signs of cardiomyocyte hypertrophy or impaired vascularization.

**Smooth Muscle-Specific Smad5 KO Mice Perform Worse in a Treadmill Experiment**

To evaluate the functional consequences of decreased cardiac contractility in the smooth muscle-specific Smad5-deficient females, a treadmill experiment was performed both on males and females. After 1 day of training on the treadmill, the endurance capacity of control and Sm22-Cre<sup>+/-;Smad5<sup>fl/fl</sup></sup> mice was analyzed. Statistical analysis demonstrated that Sm22-Cre<sup>+/-;Smad5<sup>fl/fl</sup></sup> females (n = 10) performed significantly (P = 0.005) worse than control females (n = 9), but as expected, no differences could be observed between Sm22-Cre<sup>+/-;Smad5<sup>fl/fl</sup></sup> males (n = 6) and control males (n = 7) (P = 0.4) (Figure 6C).

**The Fractional Shortening Is Reduced in Smad5-Deficient Myocytes Compared with Ctrl Myocytes**

To investigate the role of Smad5 in controlling systolic/diastolic diameter and fractional shortening, we isolated cardiomyocytes from hearts of Sm22-Cre<sup>+/-;Smad5<sup>fl/fl</sup></sup> females and Ctrl females. Sm22-Cre<sup>+/-;Smad5<sup>fl/fl</sup></sup> mice had no change in heart to body weight ratio compared with Ctrl mice (11.54 ± 1.06 mg/g in KO, n = 4 versus 9.79 ± 0.75 mg/g in Ctrl, n = 5) (Figure 7A). The yield of quiescent, regularly striated, viable myocytes was comparable for both groups (20 to 40%), and there were no evident morphological differences in transmitted light images. We found no difference in cell length (123.5 ± 4.1 μm in KO versus 133.2 ± 5.1 μm in Ctrl) or in cell width between the two groups (25.7 ± 1.0 μm in KO versus 24.9 ± 0.7 μm in Ctrl) (Figure 7B), confirming the data obtained on sections. The fractional shortening (ΔL/L<sub>o</sub>) was measured at different frequencies of stimulation in Ctrl and KO myocytes. Fractional shortening was significantly reduced in KO compared with Ctrl myocytes for 1 and 2 Hz, and posthoc testing at 4 Hz was not significant because there are fewer recordings at this frequency. Time to peak and half-time relaxation were not significantly different between the two groups (Figure 7C).

**Gene Expression Analysis in Hearts from Control and Sm22-Cre<sup>+/-;Smad5<sup>fl/fl</sup></sup> Mice**

The expression levels of cardiovascular genes and genes related to the Bmp/TGF-β pathway were investigated in hearts from 3- and 9- to 11-month-old control and Sm22-Cre<sup>+/-;Smad5<sup>fl/fl</sup></sup> mice. Quantitative PCR analysis showed a significant decrease in Smad5 expression in the hearts of smooth muscle cell-deficient Smad5 KO mice of both ages, confirming the efficient Cre-mediated recombination (Figure 8). Smad1 expression levels were not increased (Figure 8), whereas Smad8 expression could hardly be detected in control and mutant hearts (not shown). We could not detect a clear difference in expression levels of target genes for Bmp or TGF-β, i.e., Id1, Id2, Id3, Pai1, between Smad5 mutant and control hearts, although the expression levels for Id3 and Pai1 were slightly increased at 9 months (Figure 8). Quantitative PCR analysis also demonstrated normal expression levels at the age of 3
months for Opn, Anf, Bnp, α-Mhc, β-Mhc, Gdf15, and Serca2, which are established markers for cardiac disease. A small increase in Anf expression, but not for the other cardiac markers, was observed in hearts from 9-month-old mice. Tgf-1 and Tgf-2 mRNA levels were found unaffected (Figure 8). Bmp10 is a heart-specific Bmp that is dependent on intact Alk3 signaling in myocardium. Alk3 is an upstream receptor of Smad5. Bmp9, but also Bmp10, has recently been reported as a high-affinity ligand for Alk1. Both Bmp10 and Bmp9 expression were unaltered at 3 months of age and were hardly detectable in hearts of...
9-month-old mice (Figure 8). Cardiac-specific overexpression of Pdgf-C results in dilated cardiomyopathy in female mice but was found unchanged in our mice (Figure 8).

**Discussion**

In the mouse, gene targeting of Smad5 as well as Tgf-β1, TβrI, Alk5, Alk1, and endoglin have shown that the individual loss of these Tgf-β signaling components leads to midgestational lethality. These mutants all develop a primitive vascular plexus in the yolk sac but show impaired remodeling and maturation of the vasculature leading to fragile vessels with decreased vessel wall integrity. Generally, Tgf-β is accepted to be a dual factor that can both stimulate and inhibit the proliferation of ECs. In vitro, TGF-β signals mediated via Alk5 (TβrI) or Alk1 induce the phosphorylation of Smad2/Smad3 or Smad1/Smad5, respectively, in ECs. However, contradictory results on these pathways have been published, revealing that the context and the EC type may influence the outcome of the in vitro experiments.

The Smad5-null phenotype demonstrates that Smad5-mediated signaling is essential in blood vessel development. To analyze the role of Smad5 in angiogenic processes and blood vessel remodeling, we generated endothelium- and smooth muscle-specific Smad5 KO mice. Unexpectedly, mice that lack Smad5 in ECs and/or smooth muscle cells survive. Both the analysis of embryos and the documentation of induced angiogenesis and vascular-remodeling assays in adult mice did not reveal clear morphological differences between control and KO mice.

Besides efficient rearrangement of floxed alleles in vascular smooth muscle cells, we also demonstrated efficient recombination in cardiomyocytes in Sm22-Cre^{+/−};Smad5^{−/−} mice. Inactivation of Smad5 in ECs resulted in a significantly dilated LV cavity and a decreased cardiac function in 9-month-old female mice. In isolated stimulated myocytes, the fractional shortening was also significantly reduced in Smad5-deficient cells compared with control cells. These data clearly indicate that the in vivo contractile dysfunction results primarily from a defect in the cardiac myocytes and is not the consequence of a vascular defect. These data suggest a role for Smad5 in regulating contractile function at the myocyte level, through changes in calcium-handling proteins or myofilament proteins. Associated with the decreased heart performance, Sm22-Cre^{+/−};Smad5^{−/−} female mice also performed significantly worse in a graded treadmill experiment than control littermates. In 9-month-old Sm22Cre^{+/−};Smad5^{−/−} female mice compared with control mice, heart weights were not increased, the ventricular walls were not thickened, hypertrophy markers were inconsistently increased, and there was no increased fibrosis or cardiomyocyte cell diameters; hence, there were no signs of cardiac hypertrophy. We assume that the cardiomyopathy might be related to a form of primary myopathy that might aggravate progressively with age. At the age of 9 months, no signs of cardiac failure were observed in Sm22-Cre^{+/−};Smad5^{−/−} mice (eg, liver or
lung congestion, intra-atrial thrombosis; not shown), and there was no increased mortality up to 12 months of age.

In mice, as well as in humans, sex-dependent differences in cardiovascular mortality are believed to be attributable to the protective action of female hormones, evidenced by increased risk in women after menopause and by cardiovascular benefits of estrogen.39 In the majority of mouse strains, pathological phenotypes are detected only in male mice, and females display a less severe phenotype as compared with their male counterparts.40 An association of the female gender with increased susceptibility of myocardial dysfunction has been described only in a few models. In mice with cardiac-restricted overexpression of platelet-derived growth factor-C, only female mice showed dilated cardiomyopathy, heart failure, and sudden death.33 Sm22-Cre-mediated deletion of Smad5 also results in LV dilation only in female mice. The reason for this difference is currently not clear but Smad5 does not seem involved in the expression regulation of Pdgf-C.

The unexpected survival of the blood vessel-specific Smad5 KO mice could be attributable to an inefficient deletion of the floxed Smad5 allele. The loxP sites of the floxed Smad5 allele are functional.22 Based on PCR on DNA isolated from yolk sac and adult organs and X-gal staining of Sm22-Cre+/0;Smad5lox/lox;R26R and Tie-1-Crelox/lox;Smad5lox/lox;R26R embryos, we also concluded that the Sm22-Cre and Tie-1-Cre mouse strains efficiently recombine the floxed Smad5 allele in smooth muscle cells or ECs. However, we cannot exclude that the Cre-mediated recombination of the floxed Smad5 allele is not 100% and varies from mouse to mouse, as was reported for the Tie-1-Cre;Pdgf-Blox/– mice.41

The fact that the Tie-1-Cre+/0;Smad5lox/– mice and Sm22-Cre+/0;Smad5lox/– mice survive without obvious morphological defects may result from functional compensation by other Bmp-Smads or by Smad6-mediated signaling in adjacent tissues. The close interaction between the endothelium and the smooth muscle layer was shown in yolk sacs of endoglin KO embryos. In this mouse, alteration of the Tgf-β pathway in ECs resulted in reduced phosphorylation of Smad2 in the adjacent mesothelial layer. This decreased phosphorylation of Smad2 resulted from decreased Tgf-β secretion by the endothelium.42 Therefore we generated Tie-1-Cre+/0; Sm22-Cre+/0;Smad5lox/– mice to investigate the effect of Smad5 deficiency both in ECs and smooth muscle cells. However, these mice are also normal. This excludes the possibility that Smad5 deficiency in ECs can be rescued by Smad5 presence in smooth muscle cells or vice versa. The most likely explanation at this stage is that the tissue-specific deletion of Smad5 can be compensated by Smad1 or Smad8, which are highly homologous to Smad5. During development, Smad1 and Smad5 are coexpressed in many tissues, but differences in protein localization and expression levels are observed. Relevant for our study is that an intense immunostaining for Smad5 was observed in the hearts of E15.5 embryos in both the atrial and ventricular myocardium whereas the Smad1 protein level is much lower. Both Smad1 and Smad5 protein levels are low in the endocardial cushion tissue that contributes to the formation of the valves. In endothelium of blood vessels, relatively high Smad5 protein levels are detected, whereas Smad1 protein levels are much lower. The underlying smooth muscle layer is only positive for Smad5 and not for Smad143 (L.U., L.C., and A.Z, unpublished). The abundant presence of Smad5 in heart and blood vessels suggested that Smad5 might be the crucial Bmp-Smad in cardiovascular development.

Ubiquitous deletion of Smad1 and Smad5 results in distinct lethal phenotypes at ~E10.5. Smad1–/– embryos die because of defects in the formation of the allantois and placenta, whereas lethality of the Smad5–/– embryos is probably caused by defects in the circulatory system.8–11 By contrast, Smad8-null homozygotes survive and are phenotypically normal.13,14 Smad1–/– and Smad5–/– embryos also have some defect in common. The deletion of Smad1 and Smad5 results in both impaired primordial germ cell development and an allantois phenotype, but loss of Smad1 has a more pronounced impact on allantois formation.11,44,45 Vestigial heart development, a delay in ventral closure, and incomplete turning are often observed in Smad5–/– embryos but are also detected in some Smad1–/– embryos. Neither the Smad1-null homozygotes nor the Smad5-null homozygotes completely resemble the phenotype of one of the Bmp KO embryos,46 which conveys that a single Bmp can activate multiple Bmp-Smads, that the phenotype of the Smad1 and Smad5 KO embryos can be masked by compensation of related Bmp-Smads or, alternatively, that non-Bmp-Smad signaling may be more prevalent in several processes. Although Smad1+/– and Smad5+/– mice survive and are primarily normal, Smad1/Smad5 double-heterozygous embryos die at ~E10.5 having either Smad1–/– features, either Smad5–/– features, or a combination of both.14 This demonstrates that dosage of Smad1/Smad5 is critical and makes it conceivable that functional compensation occurs to a large extent in our models. Deletion of the floxed Smad5 gene in many different tissues investigated so far does not result in apparent morphological defects either (personal communications), ie, in adult hematopoiesis.46 Functional compensation by Smad1 is therefore likely to occur in many of these mouse models. Hence, it becomes necessary to generate Cre+/0;Smad1fl/fl;Smad5fl/fl and Cre+/0; Smad1fl/fl;Smad5fl/fl;Smad8fl/fl mice to investigate the requirement of Smad-dependent Bmp/TGF-β signaling cascades in, among others, blood vessel and heart development.

In this article, we demonstrated that restricted inactivation of Smad5 in the blood vessel wall resulted in normal, viable offspring with no morphological defects. Echocardiographic analysis showed decreased cardiac contractility in smooth muscle cell-specific Smad5 KO female mice. In addition, we showed that the fractional shortening is also decreased in Smad5 KO myocytes. Altogether, this demonstrates that Smad5 is dispensable in the vasculature but is required in the adult mouse heart.
References