Cardiovascular, Pulmonary and Renal Pathology

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

Lieve Umans,^{*,†} Luk Cox,^{*,†} Marc Tjwa,^{‡§}
Virginie Bito,[¶] Liesbeth Vermeire,^{*,†} Kjell Laperre,^{*,†}
Karin Sipido,[¶] Lieve Moons,^{‡§}
Danny Huylebroeck,^{*,†} and An Zwijsen^{*,†}

From the Department of Developmental Biology,^{*} VIB, Leuven, and Laboratory of Molecular Biology (Celgen),[†] Center for Human Genetics, K.U. Leuven, Leuven; Department of Transgene Technology and Gene Therapy,[‡] VIB, Leuven; The Center for Transgene and Gene Therapy (CTG),[§] K.U. Leuven, Leuven; and the Laboratory of Experimental Cardiology,[¶] K.U. Leuven, Leuven, Belgium

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; Smad5^{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 (*Flk1^{+/-Cre}; Alk3^{fl/fl}*) die between E10.5 and E11.5 and display multiple abnormalities in vascular development, including vessel remodeling and maturation that contribute to severe abdominal hemorrhage.⁷ Targeted gene disruption of *Smad1* or *Smad5* in mice results in embryonic lethality at

Supported by the Flanders Interuniversity Institute for Biotechnology (VIB 7), by the European Union (grant T-Angiovasc QLG1-CT-2001-01032), and by the Interuniversity Attraction Poles Programme IUAP-5/35.

L.U. and L.C. contributed equally to this work.

Accepted for publication January 25, 2007.

L.C. is a predoctoral fellow, and M.T. is a postdoctoral fellow of the Flanders Institute for the Promotion of Innovation by Science and Technology (IWT).

Address reprint requests to An Zwijsen, Department for Molecular and Developmental Genetics, VIB, Leuven, Belgium and Laboratory of Molecular Biology (Celgen), Center for Human Genetics, K.U. Leuven, Herestraat 49, O&N1, Box 812, 3000 Leuven, Belgium. E-mail: an.zwijsen@med.kuleuven.be.

~E9.5.^{8–11} *Smad5*-deficient¹² embryos 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*^{8,9} 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-Cre*¹⁹ or *Tie-2-Cre*²⁰ mice and *Sm22-Cre*²¹ 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*^{fl/fl}) 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-Cre*^{19,20}) or in smooth muscle cells (*Sm22-Cre*²¹). In addition, homozygous *Smad5*^{fl/fl}*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*^{fl/-} mice constitute the endothelium- or smooth muscle-restricted KO mice, whereas the *Smad5*^{fl/WT} 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 *Sm22-Cre*^{+/-}*Tie-1-Cre*^{+/-}*Smad5*^{fl/-} mice. For genotyping of the *Sm22-Cre* transgene, the primers *Sm22-Cre*FW, 5'-TCCAAAGCATGCAGAGAATGTC-3', and *Sm22-Cre*REV, 5'-GACCGGTAATGCAGGCAAT-3', were used to amplify a 400-bp fragment. For genotyping of the *Tie-1-Cre* transgene, the primers *Tie-1-Cre*FW, 5'-GGAGGACTACAGGCGACAAC-3', and *Tie-1-Cre*REV, 5'-CCGGCAAACGGACAGAAG-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*^{fl/-} 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 μ m, 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 = $(IV - I)/IV \times 100$, 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*^{+/-}; *Smad5*^{fl/-} mice, seven *Sm22-Cre*^{+/-}; *Smad5*^{fl/-} 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*^{fl/-} mice, nine control mice) and eight males (four *Sm22-Cre*^{+/-}; *Smad5*^{fl/-} 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 $\pm$ 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 tread 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 $\Delta\Delta C_t$ 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

Table 1. Oligonucleotide Primers Used for Quantitative Gene Expression Analysis

Gene	Forward primer	Reverse primer
<i>Smad5</i>	5'-CCAGCCGTGAAGCGATTG-3'	5'-GCCTTTTCTGCCCCATTCTCT-3'
<i>Smad1</i>	5'-GTGGAACAGGGCGATGAAG-3'	5'-GCGTCCACAGCTTTCTCTGC-3'
<i>Id1</i>	5'-CCCTGAACGGCGAGATCA-3'	5'-TTTTTCCTCTTGCCTCCTGAA-3'
<i>Id2</i>	5'-GAAAGCCTTCAGTCCGGTGA-3'	5'-TGGTCCGACAGCTGTTTT-3'
<i>Id3</i>	5'-CTGAGGGCATGGATGAGCTT-3'	5'-AGGGTAAGTAAGAGGGCTGG-3'
<i>Tgf-β1</i>	5'-CCCGAACCCCATTCG-3'	5'-GCCAGGAATTGTTGCTATTTCTG-3'
<i>Tgf-β2</i>	5'-GCCGAGCAGCGGGATTG-3'	5'-GCGCTGGGTGGGAGATG-3'
<i>Pai1</i>	5'-CACCGTCTCTGTGCCCATG-3'	5'-TCAGTGATAGTTGAACCTTGCTCTG-3'
<i>Opn</i>	5'-CCGAGGTGATAGCTTGGCTTATGG-3'	5'-GGACTCCTTAGACTCACCGCTCTT-3'
<i>Anf</i>	5'-TCTGAGAGACGGCAGTGCTCTAG-3'	5'-TGCTTTTCAAGAGGGCAGATCT-3'
<i>BNP</i>	5'-GCTGCTGGAGCTGATAAGAGAAA-3'	5'-GCTGCTGGAGCTGATAAGAGAAA-3'
<i>α-Mhc</i>	5'-GGCAGAGCAGGAGCTGATTGAGAC-3'	5'-GCCTTCTCCTCTGCGTTCCTACAC-3'
<i>β-Mhc</i>	5'-GGAGTTCAACCAGATCAAGGCAGAG-3'	5'-TCATTGCGGCTGCGTGTCTC-3'
<i>Bmp9</i>	5'-TCCAACATCGTGCGGAGCTT-3'	5'-TGCCTCGGGATGGAGATGTTGA-3'
<i>Bmp10</i>	5'-CCACAGACCGGACCTCCAT-3'	5'-CTGGTTGAGAAAACAGATCTTCGTT-3'
<i>Gdf15</i>	5'-TGCAGGCAACTCTTGAAGACTTGG-3'	5'-CTTTGATCTGCGCATGCGGTGTT-3'
<i>Pdgf-C</i>	5'-GCTGGAAGATCCAGAAGACGAT-3'	5'-GCGTCCTAAAACACTTCCATCAC-3'
<i>Serca2</i>	5'-GGGTGGCTCTTTTCCGTTACCTG-3'	5'-TCCATCGAAGTCTGGGTTGTCCTC-3'

expressed as the fractional shortening, ie, normalized to resting cell length ($\Delta L/L_0$). Data are shown as mean $\pm$ SEM.

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*

Homozygous floxed *Smad5* mice (*Smad5^{fl/fl}*) were crossed with *Tie-1-Cre*, *Tie-2-Cre*, or *Sm22-Cre* mice that were heterozygous for *Smad5* (*Cre^{+/0};Smad5^{+/+}*). Both the smooth muscle-specific *Smad5* KO mice and the endothelium-specific *Smad5* KO mice were born at Mendelian ratios (*Sm22-Cre^{+/0};Smad5^{fl/-}*, 22%, $n = 303$; *Tie-1-Cre^{+/0};Smad5^{fl/-}*, 22%, $n = 212$; *Tie-2-Cre^{+/0};Smad5^{fl/-}*, 22%, $n = 95$), reached adulthood, were fertile, and showed no clear morphological defects. The Cre-mediated recombination of the *Smad5^{fl}* allele and the presence of the conventional KO allele (*Smad5^{fl/-}*) were confirmed by PCR analysis on DNA isolated from tail and organs of adult mice, or yolk sac of embryos as previously described.^{8,22} Genomic analysis of DNA isolated from kidney, liver, heart, and lung of adult *Sm22-Cre^{+/0};Smad5^{fl/-}* mice and *Tie-1-Cre^{+/0};Smad5^{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^{+/0};Smad5^{fl/-}* mice, ECs were derived from hearts and lungs of adult endothelium-specific KO mice. PCR on DNA isolated from such cell cultures demonstrated cor-

rect 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 *Smad5^{fl/fl}* mice onto a R26R background²³ 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^{+/0};Smad5^{fl/-};R26R* and *Tie-2-Cre^{+/0};Smad5^{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.^{19, 20} This demonstrates that endothelium-specific deletion of *Smad5* did not impair EC differentiation

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 (cHe) and lung (cLu) from *Sm22-Cre^{+/0};Smad5^{fl/-}* and *Tie-1-Cre^{+/0};Smad5^{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.

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: $\times 60$ (A); $\times 20$ (B, F); $\times 14$ (C); $\times 10$ (D, H); $\times 40$ (E, J, L); $\times 16$ (G); and $\times 100$ (I, K).

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.²¹ 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 (Figure 2, G, H, and L), X-gal staining was also observed in visceral smooth muscle cells (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/-}* embryos and *Tie-2-Cre^{+/0};Smad5^{fl/-}* embryos, all further experiments in adult mice were performed with the *Tie-1-Cre^{+/0};Smad5^{fl/-}* mice.

Figure 3. Immunohistochemical staining for vWF (A, B), CD31 (C, D), and α -SMA (E–H) on paraffin sections of liver (A, E), lung (B, F), kidney (C, G), and heart (D, H), demonstrating normal blood vessel morphology in organs of adult *Sm22-Cre^{+/0};Smad5^{fl/-}* mice. All veins of *Sm22-Cre^{+/0};Smad5^{fl/-}* mice are lined with ECs, and all arteries contain α -SMA-positive cells, comparable with control mice (not shown). Original magnifications, $\times 200$.

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^{+/0};Tie-2-Cre^{+/0};Smad5^{+/-}* mice and *Smad5^{fl/ml}* resulted in 13% of *Sm22-Cre^{+/0};Tie-2-Cre^{+/0};Smad5^{fl/-}* 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^{+/0};Smad5^{fl/-}* 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 branches of the hepatic artery were clearly positive for α -SMA (Figure 3E). In lung, all bronchioles were surrounded by smooth muscle cells, and the pulmonary arteries contained vWF- (Figure 3B) or CD31- (data not shown) and α -SMA-positive cells (Figure 3F). In kidney, the large blood vessels were positive for both vWF (data not shown) and CD31, whereas ECs in the glomerular capsule were only positive for CD31, but this

was comparable in mutant and control mice. The morphology of interlobular arteries and glomerular arterioles was normal (Figure 3, C and G). Also in the heart, no difference in blood vessel morphology was observed between *Sm22-Cre^{+/0};Smad5^{fl/-}* mice and control mice (Figure 3, D and H). Similar results were found in mice with loss of *Smad5* in endothelium (data not shown). This analysis of adult blood vessel morphology indicates that the formation and homeostasis of blood vessels is normal in adult tissues with an endothelium- or smooth muscle-restricted *Smad5* deletion.

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^{+/0};Smad5^{fl/-}* and *Sm22-Cre^{+/0};Smad5^{fl/-}* 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^{+/0};Smad5^{fl/-}* and seven *Sm22-Cre^{+/0};Smad5^{fl/-}* mice (data not shown) as well as in seven control littermates to induce blood vessels in the avascular cornea.²⁶ 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).

Figure 4. Flat mounted corneas of control mice (Ctrl; **A**) and *Tie-1-Cre^{+/0};Smad5^{fl/-}* mice (Tie-1 KO; **B**), immunofluorescently stained for CD31. A pellet containing basic fibroblast growth factor (circle) was implanted in the cornea and blood vessel growth from the limbal vasculature toward the pellet was recorded after 5 days. No difference was observed between control and mutant mice. Original magnifications, $\times 50$.

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. Quan-

titative 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, $71\% \pm 23$; *Tie-1-Cre^{+/0};Smad5^{fl/-}*, $60\% \pm 34$, $P = 0.85$ versus control; *Sm22-Cre^{+/0};Smad5^{fl/-}*, $70\% \pm 21$, $P = 0.55$ 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

Figure 5. Neointimal lesion development in the arteries of control mice (Ctrl; **A, B, E, F, I, J**) and *Sm22-Cre^{+/0};Smad5^{fl/-}* mice (Sm22KO; **C, D, G, H, K, L**) 28 days after ligation of the left carotid artery. **A–D**: Representative pictures of transverse sections stained with H&E demonstrating no difference in neointimal lesion development between control mice and mutant mice. In control mice as well as in mutant mice, the cells in the intimal lesion are mainly positive for α -SMA (**E–H**), but also vWF-positive cells can be observed (**I–L**). Original magnifications, $\times 200$.

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^{+/0};Smad5^{fl/-}* 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^{+/0};Smad5^{fl/-}* mice were *Smad5*-deficient in smooth muscle cells and cardiomyocytes. Hence, we assessed cardiac function. Echocardiographic analysis of *Sm22-Cre^{+/0};Smad5^{fl/-}* mice and control littermates 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 littermates ($n = 4$). 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^{+/0};Smad5^{fl/-}* mice did not show overt cardiomyopathy, and heart weights of *Sm22-Cre^{+/0};Smad5^{fl/-}* 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^{+/0};Smad5^{fl/-}* 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^{+/0};Smad5^{fl/-}* hearts (KO, $147 \mu\text{m} \pm 20$; Ctrl, $154 \mu\text{m} \pm 22$; $P = 0.5$) 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^{+/0};Smad5^{fl/-}* mice and control mice (KO, $145\% \pm 13$; Ctrl, $146\% \pm 14$; $P = 0.83$) (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^{+/0};Smad5^{fl/-}* mice was analyzed. Statistical analysis demonstrated that *Sm22-Cre^{+/0};Smad5^{fl/-}* 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^{+/0};Smad5^{fl/-}* 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^{+/0};Smad5^{fl/-}* females and Ctrl females. *Sm22-Cre^{+/0};Smad5^{fl/-}* mice had no change in heart to body weight ratio compared with Ctrl mice ($11.54 \pm 1.06 \text{ mg/g}$ in KO, $n = 4$ versus $9.79 \pm 0.75 \text{ mg/g}$ 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 ($123.5 \pm 4.1 \mu\text{m}$ in KO versus $133.2 \pm 5.1 \mu\text{m}$ in Ctrl) or in cell width between the two groups ($25.7 \pm 1.0 \mu\text{m}$ in KO versus $24.9 \pm 0.7 \mu\text{m}$ in Ctrl) (Figure 7B), confirming the data obtained on sections. The fractional shortening ($\Delta L/L_0$) 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^{+/0};Smad5^{fl/-}* 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^{+/0};Smad5^{fl/-}* 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 , ie, *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

Figure 6. **A:** Echocardiographic analysis on 9-month-old *Sm22-Cre^{+/0};Smad5^{-/-}* (KO) and control female littermates (Ctrl). Significantly larger systolic left ventricle internal diameters (LVIDs) and diastolic left ventricle internal diameters (LVIDd) and significantly decreased fractional shortening (FS) are observed in *Sm22-Cre^{+/0};Smad5^{-/-}* females ($n = 10$) compared with control mice ($n = 9$). No difference was observed between *Sm22-Cre^{+/0};Smad5^{-/-}* males ($n = 4$) and control mice ($n = 4$). * $P < 0.05$. **B:** Laminin staining was performed on heart sections to measure the myocyte size. No difference was observed between control (Ctrl, **A**) and *Sm22-Cre^{+/0};Smad5^{-/-}* mice (KO, **E**). Masson's trichrome staining on sections of hearts isolated from a control (**B**) and a *Sm22-Cre^{+/0};Smad5^{-/-}* (Sm22KO; **F**) female littermate revealing no interstitial fibrosis in mutant or control hearts. **C** and **G:** CD31 staining of ECs demonstrating comparable capillary density in control (**C**) and *Sm22-Cre^{+/0};Smad5^{-/-}* mice (**G**). **D** and **H:** Immunostaining for α -SMA showing positive staining in coronary arteries in hearts of control (**D**) and *Sm22-Cre^{+/0};Smad5^{-/-}* (**H**) littermates. **C:** A treadmill experiment was performed both on males and females. After 1 day of training on the treadmill, the running endurance of all mice was analyzed. The time (in seconds) of running until exhaustion was recorded for each mouse. Statistical analysis demonstrated that *Sm22-Cre^{+/0};Smad5^{-/-}* (KO) females performed significantly ($P = 0.005$) worse than control females (Ctrl) but no differences could be observed between both male genotypes ($P = 0.4$). Original magnifications: $\times 400$ (**A**, **C**, **E**, **G**); $\times 200$ (**B**, **D**, **F**, **H**).

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- β 1 and Tgf- β 2 mRNA levels were found unaffected (Figure 8). Bmp10 is a heart-

specific Bmp that is dependent on intact Alk3 signaling in myocardium.³⁰ Alk3 is an upstream receptor of Smad5. Bmp9, but also Bmp10, has recently been reported as a high-affinity ligand for Alk1.^{31,32} Both Bmp10 and Bmp9 expression were unaltered at 3 months of age and were hardly detectable in hearts of

Figure 7. **A:** Pooled data of heart weight/body weight ratios from five Ctrl versus four *Sm22-Cre^{+/+};Smad5^{fl/-}* mice (KO). **B:** Pooled data of primary cell dimensions (cell length and cell width) from 40 cells per animal ($n = 5$ for Ctrl females versus $n = 4$ for *Sm22-Cre^{+/+};Smad5^{fl/-}* females). **C:** Averaged data on the contractile properties of Ctrl (29 cells from five mice) and *Sm22-Cre^{+/+};Smad5^{fl/-}* (22 cells from four mice) cells. FS, fractional shortening; TTP, time to peak shortening; T to RT₅₀, time to half-relaxation. * $P < 0.05$. BW, body weight; HW, heart weight.

9-month-old mice (Figure 8). Cardiac-specific overexpression of *Pdgf-C* results in dilated cardiomyopathy in female mice³³ but was found unchanged in our mice (Figure 8).

Discussion

In the mouse, gene targeting of *Smad5* as well as *Tgf-β1*, *Tbrl1*, *Alk5*, *Alk1*, and *endoglin* have shown that the indi-

Figure 8. Real-time RT-PCR gene expression analysis in *Smad5^{fl/WT}* (Ctrl) and *Sm22-Cre^{+/+};Smad5^{fl/-}* (KO) hearts. Histograms represent relative mRNA expression of each gene after normalization to GAPDH mRNA. Quantitative PCR analysis revealed a significant decrease in *Smad5* mRNA in the mutant hearts, but no difference in expression levels of the *Bmp* target genes *Id1* and *Id2* was observed. Expression of *Id3* and *Pai1*, *Bmp*, and *Tgf-β* target genes were moderately increased at 9 months of age. *Anf*, α -MHC, and β -MHC expression was also moderately increased in older mice. No change in expression was observed for all of the other genes analyzed.

vidual 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.^{5,34} 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* (*Tβr1*) or *Alk1* induce the phosphorylation of *Smad2/Smad3* or *Smad1/Smad5*, respectively, in ECs.^{17,35} However, contradictory results on these pathways have been published,^{18,36} 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^{fl/-};R26R* embryos. The recombination in cardiomyocytes is consistent with the recently reported recombination profile of the *Sm22-Cre* transgene in embryos³⁷ and the expression pattern of the *Sm22α* promoter, with cardiac expression as early as day E7.0.^{37,38} Consequently, *Sm22-Cre^{+/+};Smad5^{fl/-}* mice can be considered as smooth muscle- and cardiomyocyte-specific *Smad5* KOs. Echocardiographic analysis of the heart of adult *Sm22-Cre^{+/+};Smad5^{fl/-}* and control littermates demonstrated that deletion of *Smad5* 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^{fl/-}* female mice also performed significantly worse in a graded treadmill experiment than control littermates. In 9-month-old *Sm22-Cre^{+/+};Smad5^{fl/-}* 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^{fl/-}* 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};Smad5^{fl/-};R26R* and *Tie-1-Cre^{+/0};Smad5^{fl/-};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-B^{lox/-}* mice.⁴¹

The fact that the *Tie-1-Cre^{+/0};Smad5^{fl/-}* mice and *Sm22-Cre^{+/0};Smad5^{fl/-}* mice survive without obvious morphological defects may result from functional compensation by other Bmp-Smads or by *Smad5*-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};Smad5^{fl/-}* 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 *Smad1*⁴³ (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,⁵ 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, *Smad1Smad5* 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};Smad1^{fl/fl};Smad5^{fl/fl}* and *Cre^{+/0};Smad1^{fl/fl};Smad5^{fl/fl};Smad8^{fl/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.

Acknowledgments

We thank L. Desmet and M. Missoul for mouse care; L. Beek and F. Cornelis for genotyping; Y. Man for the assistance in the cornea micropocket assay; R. Moura for the helpful advice in the carotid artery experiment; S. Terclavers for his advice with the morphometric analysis; A. Carton, A. Manderveld, and B. Vanwetswinkel for help with the histological analysis; F. Zwerts and M. Van de Wouwer for their help with the isolation of endothelial cells from adult organs; P. Holemans for help with myocyte experiments; and R. Fässler, M. Yanagisawa, and M. Gotthardt/J. Herz for making available *Tie-1-Cre*, *Tie-2-Cre*, and *Sm22-Cre* mice, respectively.

References

- Attisano L, Wrana JL: Smads as transcriptional co-modulators. *Curr Opin Cell Biol* 2000, 12:235–243
- ten Dijke P, Goumans MJ, Itoh F, Itoh S: Regulation of cell proliferation by Smad proteins. *J Cell Physiol* 2002, 191:1–16
- Verschueren K, Huylebroeck D: Remarkable versatility of Smad proteins in the nucleus of transforming growth factor-beta activated cells. *Cytokine Growth Factor Rev* 1999, 10:187–199
- Bruno E, Horrigan SK, Van Den Berg D, Rozler E, Fitting PR, Moss ST, Westbrook C, Hoffman R: The Smad5 gene is involved in the intracellular signaling pathways that mediate the inhibitory effects of transforming growth factor-beta on human hematopoiesis. *Blood* 1998, 91:1917–1923
- Goumans MJ, Mummery C: Functional analysis of the TGFbeta receptor/Smad pathway through gene ablation in mice. *Int J Dev Biol* 2000, 44:253–265
- Bertolino P, Deckers M, Lebrin F, ten Dijke P: Transforming growth factor-beta signal transduction in angiogenesis and vascular disorders. *Chest* 2005, 128:585S–590S
- Park C, Lavine K, Mishina Y, Deng CX, Ornitz DM, Choi K: Bone morphogenetic protein receptor 1A signaling is dispensable for hematopoietic development but essential for vessel and atrioventricular endocardial cushion formation. *Development* 2006, 133:3473–3484
- Chang H, Huylebroeck D, Verschueren K, Guo Q, Matzuk MM, Zwijsen A: Smad5 knockout mice die at mid-gestation due to multiple embryonic and extraembryonic defects. *Development* 1999, 126:1631–1642
- Yang X, Castilla LH, Xu X, Li C, Gotay J, Weinstein M, Liu PP, Deng CX: Angiogenesis defects and mesenchymal apoptosis in mice lacking SMAD5. *Development* 1999, 126:1571–1580
- Lechleider RJ, Ryan JL, Garrett L, Eng C, Deng C, Wynshaw-Boris A, Roberts AB: Targeted mutagenesis of Smad1 reveals an essential role in chorioallantoic fusion. *Dev Biol* 2001, 240:157–167
- Tremblay KD, Dunn NR, Robertson EJ: Mouse embryos lacking Smad1 signals display defects in extra-embryonic tissues and germ cell formation. *Development* 2001, 128:3609–3621
- Chang H, Zwijsen A, Vogel H, Huylebroeck D, Matzuk MM: Smad5 is essential for left-right asymmetry in mice. *Dev Biol* 2000, 219:71–78
- Hester M, Thompson JC, Mills J, Liu Y, El-Hodiri HM, Weinstein M: Smad1 and Smad8 function similarly in mammalian central nervous system development. *Mol Cell Biol* 2005, 25:4683–4692
- Arnold SJ, Maretto S, Islam A, Bikoff EK, Robertson EJ: Dose-dependent Smad1, Smad5 and Smad8 signaling in the early mouse embryo. *Dev Biol* 2006, 296:104–118
- van den Driesche S, Mummery CL, Westermann CJ: Hereditary hemorrhagic telangiectasia: an update on transforming growth factor beta signaling in vasculogenesis and angiogenesis. *Cardiovasc Res* 2003, 58:20–31
- Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P: Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *EMBO J* 2002, 21:1743–1753
- Lamouille S, Mallet C, Feige JJ, Bailly S: Activin receptor-like kinase 1 is implicated in the maturation phase of angiogenesis. *Blood* 2002, 100:4495–4501
- Oh SP, Seki T, Goss KA, Imamura T, Yi Y, Donahoe PK, Li L, Miyazono K, ten Dijke P, Kim S, Li E: Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc Natl Acad Sci USA* 2000, 97:2626–2631
- Gustafsson E, Brakebusch C, Hietanen K, Fässler R: Tie-1-directed expression of Cre recombinase in endothelial cells of embryoid bodies and transgenic mice. *J Cell Sci* 2001, 114:671–676
- Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, Yanagisawa M: Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev Biol* 2001, 230:230–242
- Holtwick R, Gotthardt M, Skryabin B, Steinmetz M, Potthast R, Zetsche B, Hammer RE, Herz J, Kuhn M: Smooth muscle-selective deletion of guanylyl cyclase-A prevents the acute but not chronic effects of ANP on blood pressure. *Proc Natl Acad Sci USA* 2002, 99:7142–7147
- Umans L, Vermeire L, Francis A, Chang H, Huylebroeck D, Zwijsen A: Generation of a floxed allele of Smad5 for cre-mediated conditional knockout in the mouse. *Genesis* 2003, 37:5–11
- Soriano P: Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 1999, 21:70–71
- Lodge PA, Haisch CE, Thomas FT: A simple method of vascular endothelial cell isolation. *Transplant Proc* 1992, 24:2816–2817
- Kumar A, Hoover JL, Simmons CA, Lindner V, Shebuski RJ: Remodeling and neointimal formation in the carotid artery of normal and P-selectin-deficient mice. *Circulation* 1997, 96:4333–4342
- Kenyon BM, Voest EE, Chen CC, Flynn E, Folkman J, D'Amato RJ: A model of angiogenesis in the mouse cornea. *Invest Ophthalmol Vis Sci* 1996, 37:1625–1632
- Janssens S, Pokreisz P, Schoonjans L, Pellens M, Vermeersch P, Tjwa M, Jans P, Scherrer-Crosbie M, Picard MH, Szelid Z, Gillijns H, Van de Werf F, Collen D, Bloch KD: Cardiomyocyte-specific overexpression of nitric oxide synthase 3 improves left ventricular performance and reduces compensatory hypertrophy after myocardial infarction. *Circ Res* 2004, 94:1256–1262
- Antoons G, Mubagwa K, Nevelsteen I, Sipido KR: Mechanisms underlying the frequency dependence of contraction and $[Ca^{2+}]_i$ transients in mouse ventricular myocytes. *J Physiol* 2002, 543:889–898
- Zhang JC, Kim S, Helmke BP, Yu WW, Du KL, Lu MM, Strobeck M, Yu Q, Parmacek MS: Analysis of SM2alpha-deficient mice reveals unanticipated insights into smooth muscle cell differentiation and function. *Mol Cell Biol* 2001, 21:1336–1344
- Gaussin V, Van de Putte T, Mishina Y, Hanks MC, Zwijsen A, Huylebroeck D, Behringer RR, Schneider MD: Endocardial cushion and myocardial defects after cardiac myocyte-specific conditional deletion of the bone morphogenetic protein receptor ALK3. *Proc Natl Acad Sci USA* 2002, 99:2878–2883
- Brown MA, Zhao Q, Baker KA, Naik C, Chen C, Pukac L, Singh M, Tsareva T, Parice Y, Mahoney A, Roschke V, Sanyal I, Choe S: Crystal structure of BMP-9 and functional interactions with pro-region and receptors. *J Biol Chem* 2005, 280:25111–25118
- David L, Mallet C, Mazerbourg S, Feige JJ, Bailly S: Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) endothelial cells. *Blood* 2007, 109:1953–1961
- Pontén A, Li X, Thoren P, Aase K, Sjöblom T, Ostman A, Eriksson U: Transgenic overexpression of platelet-derived growth factor-C in the mouse heart induces cardiac fibrosis, hypertrophy, and dilated cardiomyopathy. *Am J Pathol* 2003, 163:673–682
- Zwijsen A, van Grunsven LA, Bosman EA, Collart C, Nelles L, Umans L, Van de Putte T, Wuytens G, Huylebroeck D, Verschueren K: Transforming growth factor beta signalling in vitro and in vivo: activin ligand-receptor interaction, Smad5 in vasculogenesis, and repression of target genes by the deltaEF1/ZEB-related SIP1 in the vertebrate embryo. *Mol Cell Endocrinol* 2001, 180:13–24
- Lebrin F, Goumans MJ, Jonker L, Carvalho RL, Valdimarsdottir G, Thorikay M, Mummery C, Arthur HM, ten Dijke P: Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. *EMBO J* 2004, 23:4018–4028
- Pece-Barbara N, Vera S, Kathirkamathamby K, Liebnier S, Di Guglielmo GM, Dejana E, Wrana JL, Letarte M: Endoglin null endothelial cells proliferate faster and are more responsive to transforming growth factor beta1 with higher affinity receptors and an activated

- Alk1 pathway. *J Biol Chem* 2005, 280:27800–27808
37. Frutkin AD, Shi H, Otsuka G, Leveen P, Karlsson S, Dichek DA: A critical developmental role for *tgfb β 2* in myogenic cell lineages is revealed in mice expressing SM22-Cre, not SMMHC-Cre. *J Mol Cell Cardiol* 2006, 41:724–731
38. Li L, Miano JM, Cserjesi P, Olson EN: SM22 alpha, a marker of adult smooth muscle, is expressed in multiple myogenic lineages during embryogenesis. *Circ Res* 1996, 78:188–195
39. Babiker FA, De Windt LJ, van Eickels M, Grohe C, Meyer R, Doevendans PA: Estrogenic hormone action in the heart: regulatory network and function. *Cardiovasc Res* 2002, 53:709–719
40. Du XJ: Gender modulates cardiac phenotype development in genetically modified mice. *Cardiovasc Res* 2004, 63:510–519
41. Enge M, Bjarnegard M, Gerhardt H, Gustafsson E, Kalen M, Asker N, Hammes HP, Shani M, Fassler R, Betsholtz C: Endothelium-specific platelet-derived growth factor-B ablation mimics diabetic retinopathy. *EMBO J* 2002, 21:4307–4316
42. Carvalho RL, Jonker L, Goumans MJ, Larsson J, Bouwman P, Karlsson S, Dijke PT, Arthur HM, Mummery CL: Defective paracrine signalling by TGF β 2 in yolk sac vasculature of endoglin mutant mice: a paradigm for hereditary haemorrhagic telangiectasia. *Development* 2004, 131:6237–6247
43. Flanders KC, Kim ES, Roberts AB: Immunohistochemical expression of Smads 1–6 in the 15-day gestation mouse embryo: signaling by BMPs and TGF-betas. *Dev Dyn* 2001, 220:141–154
44. Chang H, Matzuk MM: Smad5 is required for mouse primordial germ cell development. *Mech Dev* 2001, 104:61–67
45. Bosman EA, Lawson KA, Debruyjn J, Beek L, Francis A, Schoonjans L, Huylebroeck D, Zwijsen A: Smad5 determines murine amnion fate through the control of bone morphogenetic protein expression and signalling levels. *Development* 2006, 133:3399–3409
46. Singbrant S, Moody JL, Blank U, Karlsson G, Umans L, Zwijsen A, Karlsson S: Smad5 is dispensable for adult murine hematopoiesis. *Blood* 2006, 108:3707–3712