Endothelial cells drive organ fibrosis and dysfunction by inducing the transcription factor SOX9

Authors: Felix A. Trogisch,[1,2,3] Aya Abouissa,[1,2,3] Merve Keles,[1,2,3,‡] Anne Birke,[1] Manuela Fuhrmann,[1] Gesine M. Dittrich,[1,2,§] Nina Weinzierl,[1] Elvira Wink,[1] Julio Cordero,[2,4] Adel Elsherbiny,[4] Abel Martin-Garrido,[1] Steve Grein,[1,2] Shruthi Hemanna,[1,2] Ellen Hofmann,[1] Luka Nicin,[5,6,7] Sofia-Iris Bibli,[6,7,8] Rannar Airik,[9,¶] Andreas Kispert,[9] Ralf Kist,[10] Sun Quanchao,[11,#] Sina W. Kürschner,[12,13] Manuel Winkler,[12,13] Norbert Gretz,[11,†] Carolin Mogler,[14] Thomas Korff,[13,15] Philipp-Sebastian Koch,[12,13] Stefanie Dimmeler,[5,6,7] Gergana Dobreva,[2,4] and Joerg Heineke[1,2,3,*]

Affiliations:

[1]ECAS (European Center for Angioscience), Department of Cardiovascular Physiology, Mannheim Faculty of Medicine, Heidelberg University, 68467 Mannheim, Germany;

[2]DZHK (German Center for Cardiovascular Research), partner site Heidelberg/Mannheim, 68167 Mannheim, Germany;

[3]CFPM (Core Facility Platform Mannheim), Cardiac Imaging Center, Mannheim Faculty of Medicine, Heidelberg University, 68167 Mannheim, Germany;

[4]ECAS, Department of Cardiovascular Genomics and Epigenomics, Mannheim Faculty of Medicine, Heidelberg University, 68167 Mannheim, Germany;

[5]Institute for Cardiovascular Regeneration, Goethe University Frankfurt, 60590 Frankfurt am Main, Germany;

[6]Cardiopulmonary Institute, Goethe University Frankfurt, 60590 Frankfurt am Main, Germany;

[7]DZHK, partner site Frankfurt Rhine-Main, Frankfurt, 60590 Frankfurt am Main, Germany;

[8]Institute of Vascular Signaling, Centre for Molecular Medicine, Goethe University Frankfurt, 60590 Frankfurt am Main, Germany;

[9]Institute of Molecular Biology, Hannover Medical School, 30625 Hannover, Germany;

[10]School of Dental Sciences, Biosciences Institute, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne NE2 4BW, United Kingdom;

[11]Medical Research Center, Mannheim Faculty of Medicine, Heidelberg University, 68167 Mannheim, Germany;

[12]Department of Dermatology, Venereology and Allergology, University Medical Center and Medical Faculty Mannheim, Heidelberg University, and Center of Excellence in Dermatology, 68167 Mannheim, Germany;

[13]ECAS, Adjunct Faculty, Mannheim Faculty of Medicine, Heidelberg University, 68167 Mannheim, Germany;

[14]Institute of Pathology, School of Medicine, Technical University of Munich, 81675 Munich, Germany;

[15]Department of Cardiovascular Physiology, Heidelberg University, 69120, Heidelberg, Germany;

[‡]current address: National Heart and Lung Institute (NHLI), Faculty of Medicine, Imperial College London, London SW7 2AZ, UK

5 [§]current address: Department of Pharmacology and Toxicology, University Medical Center Göttingen, 37075 Göttingen, Germany;

[¶]current address: Division of Nephrology, Department of Pediatrics, University of Pittsburgh, Pittsburgh, PA 15224-1334, USA and Department of Developmental Biology, University of Pittsburgh, Pittsburgh, PA 15224-1334, USA;

10 [#]current address: Department of Thoracic Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 430030 Wuhan, China;

[†]deceased

*Corresponding author. Email: joerg.heineke@medma.uni-heidelberg.de

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One-Sentence Summary:

SOX9 in endothelial cells regulates organ fibrosis by inducing extracellular matrix,
5 inflammatory, and growth factor gene expression.

Editor's summary:

Abstract:

Fibrosis is a hallmark of chronic disease. Although fibroblasts are known to be involved, it is unclear to what extent endothelial cells might contribute. We detected increased expression of the transcription factor *Sox9* in endothelial cells of fibrotic mouse organs in models of systolic heart failure by pressure overload, diastolic heart failure by high-fat diet and L-Name administration, pulmonary fibrosis after bleomycin treatment, and liver fibrosis as consequence of CDAA diet. We also observed endothelial *SOX9* upregulation in human heart tissue in patients with heart failure. To test whether this SOX9 induction was sufficient to cause disease, we generated mice with endothelial cell-specific overexpression of *Sox9*, which indeed triggered extensive fibrosis in multiple organs and promoted signs of heart failure. Endothelial *Sox9* deletion, in turn, prevented fibrosis and organ dysfunction in pre-clinical surgical and pharmacological mouse models of heart failure as well as of lung, and liver injury. Bulk and single cell RNA sequencing of endothelial cells across multiple vascular beds revealed that SOX9 induced extracellular matrix, growth factor, and inflammatory genes leading to matrix deposition by endothelial cells. Moreover, in part through triggering an increased expression of the secreted growth factor *Ccn2* as direct SOX9 target, endothelial cells activated neighboring fibroblasts to migrate and deposit matrix in response to SOX9. Endothelial *Sox9* deletion reversed these changes, suggesting a role for endothelial SOX9 as fibrosis-promoting transcription factor across organs in response to disease stimuli. Therefore, endothelial cells could be a promising target to counteract fibrotic heart, liver and lung disease.

Introduction

Fibrosis is a hallmark of chronic organ remodeling in response to many disease stimuli. Examples include persistent cardiac overload, chronic liver diseases, or pulmonary inflammation caused by pathogens, toxins or drugs. Acute extracellular matrix (ECM) deposition can be beneficial to a limited extent in non-regenerative tissues like the infarcted human myocardium, for instance to prevent organ rupture (*1*). Fibrosis is partially reversible in regenerative organs, such as the skin, lung and liver, but chronic fibrotic disease deteriorates organ function, eventually causing failure and death (*2*).

While fibroblasts were widely considered the main source of ECM during fibrotic remodeling (*2, 3*), the contribution of other cell types had been underestimated for a long time, but recently came more into focus (*4-7*). Even though vascular endothelial cells (ECs) are capable of ECM production (*8*), their transient induction of ECM genes in animal models of myocardial pressure overload and infarction in vivo was only recently described by us (*6, 9*). In particular, EC induction of a reversible, transient state, in which they hold endothelial and mesenchymal properties in dynamic proportions, without full differentiation to a mesenchymal cell type, gained recent interest (*10*) and has been observed in wound scarring (*11*), myocardial (*9, 12*) and vascular disease (*13*). This intermediate EC state was recently termed "transient mesenchymal activation" (*12*), in contrast to full endothelial-to-mesenchymal transition (EndoMT), which was reportedly absent in the diseased heart (*14, 15*). Capillary ECs constitute a critical interface between the systemic circulation and the organs' interstitial space, which enables these ECs to relay disease stimuli into tissue. Indeed, beside their crucial role in blood supply, ECs are known to release angiocrine factors to control organ function in physiology, disease, and aging (*16-18*). While other vascular cells like pericytes have recently been identified as source of myofibroblasts and fibrosis in distinct organs, such as kidney, brain, and heart (*19-23*), the functional relevance of ECs and their mesenchymal activation for the formation and maintenance of organ fibrosis remains unknown.

The transcription factor SOX9 [Sry (Sex-determining region Y protein)-related HMG-box 9] is essential during embryogenesis during which it regulates chondrogenesis and the development of the testes, bile ducts and heart valves [reviewed in (*24, 25*)]. SOX9 directly controls *Col2a1* (encoding for collagen type II, alpha 1 chain) transcription by binding to DNA motifs in the first intron of the gene (*26*). Moreover, SOX9 is involved in epithelial-to-mesenchymal transition (EMT) of multipotent neural crest cells and epicardial cells to generate cardiac fibroblasts during embryogenesis and in different forms of cancer (*27-29*). As an inducer of ECM deposition, SOX9 is implicated in the formation of fibrosis by mesenchymal cells (such as fibroblasts) in various organs. Recent work from our group revealed a maladaptive contribution of SOX9 following myocardial infarction that was ameliorated by fibroblast-restricted inactivation of *Sox9* (*30-33*).

In order to clarify the role and the overall contribution of endothelial cells to organ fibrosis, we generated endothelial-cell specific gain and loss-of function models of the fibrogenic transcription factor SOX9 in mice. We demonstrated here that overexpression of *Sox9* in endothelial cells triggers fibrosis and dysfunction in the heart and multiple other organs, whereas its ablation in endothelial cells reduces cardiac, pulmonary and liver fibrosis in pre-clinical disease models by inhibiting endothelial mesenchymal activation, leading to improved organ function. Therefore, our data revealed a fundamental contribution of endothelial cells to organ fibrosis.

Results

Endothelial *Sox9* is upregulated in fibrotic organs in pre-clinical models and human disease

5 To start investigating a potential role of endogenous endothelial SOX9 in fibrosis, we analyzed *Sox9* mRNA expression in isolated endothelial cells (ECs) from early stage fibrotic organs in mouse disease models. qPCR analyses revealed a profound induction of *Sox9* mRNA in cardiac ECs during pressure overload by transverse aortic constriction (TAC, a model of systolic heart failure) versus control mice, as well as in a model of diastolic heart failure (induced by combined
10 application of a high fat diet (HFD) and L-NAME) (*34*) (Fig. 1A). Furthermore, endothelial *Sox9* mRNA was induced in mouse pulmonary fibrosis (due to bleomycin injection) and in liver fibrosis (due to choline-deficient, l-amino acid-defined (CDAA) diet) (Fig. 1A). These data show endothelial *Sox9* mRNA induction in heart, lung, and liver during early fibrotic disease in established pre-clinical models. Recently, single nuclei RNA sequencing was performed to map
15 ACE & ACE2 expression in healthy and diseased human heart tissue from patients with heart failure (*35*). In data from this study, a subcluster of ECs was observed which expressed mesenchymal as well as endothelial cell markers, and therefore could reflect endothelial mesenchymal activation ("ECFB" cluster). Remarkably, while *SOX9* was hardly detectable in healthy cardiac ECs, both EC and ECFB clusters showed increased *SOX9* expression during
20 human systolic heart failure (heart failure with reduced ejection fraction, or HFrEF) (Fig. 1B). Accordingly, staining of human cardiac biopsy sections showed that endothelial SOX9 protein abundance correlated with the extent of cardiac tissue fibrosis in diseased hearts (Fig. 1C, Data file S1A), suggesting that our findings from murine models of cardiac disease might also apply to human heart failure.

25 **Inducible *Sox9* overexpression in ECs triggers fibrosis and organ dysfunction**

In order to assess the consequences of increased *Sox9* expression in ECs, we induced endothelial *Sox9* overexpression by administering tamoxifen to adult mice expressing a tamoxifen-inducible *Cdh5*-driven Cre recombinase (*36*) and an inactive, floxed *Sox9* insert in the endogenous *Hprt* locus, which becomes activated in response to Cre mediated recombination (*36, 37*) (Fig. 2A,
30 fig. S1A-C). EC-specific *Sox9* overexpression (*Sox9*$^{\text{EC-OE}}$) resulted in a mild augmentation in body weight as well as small increases in heart, lung, liver and spleen weights, but not in skeletal muscle, kidney and brain weights, where the degree of endothelial *Sox9* overexpression was very mild or even absent (fig. S2A-B). Heart, lung, liver and spleen showed augmented fibrosis after 4 weeks of EC-restricted *Sox9* overexpression, as shown by Sirius red staining and specific
35 collagen I and III immunostaining (Fig. 2B-D, fig. S2C-D, Movie S1-2, Data file S1B-F). We noticed an overlay of collagen and EC marker staining (IB4 and CD31, respectively) in immunofluorescence microscopy, implying ECs as source of collagen production (Fig. 2D, fig. S2C-D, Movie S1-2, Data file S1F). In the heart, endothelial *Sox9* overexpression triggered diastolic dysfunction as well as cardiac hypertrophy (shown by echocardiography and
40 immunofluorescence assessment of cardiomyocyte size) after 2-4 weeks (Fig. 2E, fig. S3A-C; decreased global longitudinal strain (GLS) and augmented left-ventricular posterior wall thickness (LVPW) suggested diastolic impairment with cardiac hypertrophy; a detailed explanation of the physiologic parameters is displayed in Table S1). Endothelial *Sox9* overexpression also triggered systolic heart failure after 2 months as demonstrated by
45 echocardiography and cardiac catheterization (Fig. 2F, fig. S3D-F; decreased left-ventricular

fractional shortening (LV-FS) correlates with LV systolic dysfunction, and decreased LV pressure-time gradient during systole (dp/dt max) and diastole (dp/dt min) demonstrated systolic and diastolic heart failure). Especially early deterioration of GLS, but also maintenance of global circumferential strain (GCS) and radial strain (RS) are each hallmarks of fibrotic cardiac impairment (Fig. 2E, fig. S3F). Neither systolic nor diastolic blood pressure were changed upon endothelial *Sox9* overexpression for up to 6 months (fig. S3G). Induction of pulmonary fibrosis led to a progressive reduction of blood oxygen saturation (measured with peripheral pulse oximetry) and caused an increased pulmonary artery resistance, which is shown by a reduction in the ratio of pulmonary artery acceleration over ejection time (PAT/ET) (Fig. 2G, fig. S4A). This eventually induced moderate right-ventricular cardiac hypertrophy without RV failure (fig. S4B; indicated by augmented right ventricular free wall (RVFW) thickness with maintained RV fractional shortening (RV-FS) and unchanged ratio of early to late diastolic RV filling, tricuspid valve E/A ratio). Liver remodeling was accompanied by switching from discontinuous to continuous endothelium 6 months after start of the endothelium-restricted *Sox9* overexpression (indicated by reduction in LYVE1 expression, fig. S4C), which, however, was not accompanied by strong tissue damage or reduction in liver function as suggested by unchanged plasma concentrations of free liver enzymes, protein, and non-pathologic sonographic liver parenchymal appearance (fig. S4D-F). In contrast, portal vein hypertension (indicated by increasing portal vein diameter) as marker of pathologic liver remodeling consistently worsened over time (fig. S4G). Taken together, our results revealed the development of organ fibrosis, mild hypertrophy and progressive organ dysfunction due to induced endothelial cell restricted overexpression of *Sox9* in adult mice.

Endothelial SOX9 leads to activation of mesenchymal and inflammatory genes

To investigate how endothelial SOX9 triggers fibrosis and deteriorates organ function, we performed bulk RNA sequencing of isolated ECs from heart, lung, and liver 4 weeks after *Sox9* induction by *Cdh5/Hprt*-mediated overexpression in ECs. Both individual analysis of regulated genes as well as overlay of regulated gene-ontology (GO) terms of these three organs indicated a strong up-regulation of ECM organization-, immune response-, growth-, and cell cycle-related processes and downregulation of nerve development-related GO terms (fig. S5A-D). These were verified by immunostaining for selected markers in heart, lung, and liver tissue (fig. S6A-C). For example, we detected more leukocyte abundance (CD45, protein tyrosine phosphatase receptor type C, staining) and less nerve endings (TBB3, tubulin beta-3 chain, staining) in these organs of *Sox9*$^{EC-OE}$ mice, and an increase in ECM deposition (Fn1, fibronectin 1, staining or collagen) (fig. S6A-C). A high proportion of upregulated genes were allocated to the extracellular space, suggesting increased inter-cellular communication.

To determine the subset of ECs in which SOX9-dependent gene-expression occurs, we analyzed cardiac ECs by single cell RNA sequencing (Fig. 3A-C, fig. S7A-C). We identified 12 different clusters in cardiac ECs from control and *Sox9*$^{EC-OE}$ mice, of which all except clusters # 6, 8, 10 and 11 showed high expression of the EC markers *Cdh5* (cadherin 5) and *Pecam1* (platelet and endothelial cell adhesion molecule 1), wherein clusters #0, 1, 3 and 7 were enriched for *Sox9* and *Ccn2* (cellular communication network factor 2) (Fig. 3A-C, fig. S7A-C). Among these endothelial clusters, cluster #0 (expressing genes related to the immune system and to extracellular matrix organization) and cluster #1 (expressing genes related to apoptosis, cell cycle, cell growth and the response to viruses) were markedly expanded in *Sox9*$^{EC-OE}$ mice compared to control mice. Clusters #0 and #1 also displayed the highest abundance of *Sox9* expression. In contrast, fewer cells were found in cluster #2 (expressing genes related to

angiogenesis, cell migration, hypoxia response and nerves, but exerting relatively little *Sox9* expression) in *Sox9*$^{EC\text{-}OE}$ derived ECs. Clusters #0-2 were identified as arterial ECs by their typical gene expression (fig. S8). Venule ECs (identified in Cluster #7) were also expanded by Sox9 and expressed angiogenesis, apoptosis and ECM related genes as well as genes related to negative regulation of nerve development (Fig. 3A-B, fig. S8).

***SOX9* regulates collagen expression in cultured human ECs**

Because we found increased fibrosis by endothelial *Sox9* overexpression in vivo, we wanted to assess whether this was an autonomous effect of ECs, or whether other cell types (such as fibroblasts) were required. For this purpose, we stimulated human ECs (HUVECs) with TGF-β1, which is a known stimulus of mesenchymal activation. TGF-β1 promoted increased SOX9 protein and *SOX9* mRNA expression as well as a uniform induction of endothelial and mesenchymal genes (fig. S9A-C). Overexpression of *Sox9* in HUVECs by adenoviral infection (by adenovirus *Sox9*, ad*Sox9*, fig. S9D-E) induced a specific subset of mesenchymal genes (Fig. 3D, fig. S9F). TGF-β1 stimulation as well as ad*Sox9* treatment in human cardiac microvascular ECs (HCMECs) showed, in part, an even stronger mesenchymal gene activation than observed in HUVECs (Fig. 3E, fig. S9G-H). In addition, *Sox9* overexpression resulted in impaired endothelial function, such as reduced tube formation on Matrigel (fig. S9I). Accordingly, *Sox9*$^{EC\text{-}OE}$ mice exerted reduced myocardial capillary density 4 weeks after starting *Sox9* overexpression (fig. S9J). These results show that SOX9 directly induced mesenchymal activation in ECs, which was accompanied by reduced angiogenic function.

We next investigated whether *SOX9* was required for mesenchymal activation in ECs, in addition to being sufficient. Following *SOX9*-downregulation by siRNA, the induction of mesenchymal genes by TGF-β1 was blunted and endothelial marker expression was unaffected in both HUVECs and HCMECs (fig. S9K).

Deletion of *Sox9* in ECs and inhibition of TGF-β counteracts pressure-induced fibrosis and systolic heart failure in vivo

Because downregulation of *SOX9* in cultured ECs inhibited mesenchymal activation, we aimed to test this effect in vivo. We generated mice with an inducible, EC-specific knock-out of *Sox9* (*Sox9*$^{EC\text{-}KO}$) by mating mice with homozygous floxed *Sox9* alleles (*38*) with *Cdh5-CreER*-mice (Fig. 4A). Upon tamoxifen administration, we observed reduced endothelial *Sox9* mRNA expression in heart, lung and liver of *Sox9*$^{EC\text{-}KO}$ mice (fig. S10A). Cardiac pressure overload by TAC surgery resulted in reduced cardiac hypertrophy and lung congestion 2 weeks after aortic constriction and markedly reduced myocardial fibrosis after both 2 and 10 weeks in *Sox9*$^{EC\text{-}KO}$ mice versus littermate *Sox9*$^{flox/flox}$ control mice despite a similar degree of pressure overload in both genotypes (Fig. 4B-E, fig. S10B, Data file S2A-B). Immunostaining for collagen I and III in cardiac cryosections showed that interstitial fibrosis, which overlapped with ECs, as well as perivascular fibrosis of bigger vessels, were each reduced by endothelial *Sox9* ablation (fig. S10C-D).

Echocardiographic analyses revealed that remodeling in *Sox9*$^{EC\text{-}KO}$ mice after TAC was mainly driven by lengthening of the ventricle and less by increased wall thickness (fig. S11A-B). This conclusion was suggested by increased LV length in *Sox9*$^{EC\text{-}KO}$ mice and a reduced increase in wall thickness after TAC. Whereas cardiac function in *Sox9*$^{fl/fl}$ control mice was continuously deteriorating, leading to systolic and diastolic heart failure 10 weeks after TAC, *Sox9*$^{EC\text{-}KO}$ mice maintained systolic heart function for up to 6 weeks and were protected from diastolic failure at least until 10 weeks after TAC surgery (Fig. 4F, fig. S11C-E). Fluorescence staining of

cardiomyocytes revealed reduced concentric hypertrophic remodeling (suggested by reduced cross-sectional area) in CMs of *Sox9*$^{EC\text{-}KO}$ mice 2 and 10 weeks after TAC, and mildly enhanced elongation of cardiomyocytes 10 weeks after TAC (fig. S12A). Myocardial capillary density was reduced in *Sox9*$^{EC\text{-}KO}$ mice early after TAC, but was increased at the 10-week time point compared to control mice (fig. S12B). Therefore, endothelial *Sox9* deletion delayed the development of systolic dysfunction, prevented diastolic dysfunction during cardiac pressure overload and reduced myocardial fibrosis. TGF-β signaling induces fibrotic and hypertrophic responses during pressure-overload, so we wanted to test the potential of TGF-β inhibition on TAC-induced heart failure (fig. S12C-D). Pan-TGF-β inhibition was recently used in anti-cancer and lung fibrosis research (*39-41*) and resulted in Sox9-downregulation in ECs, fibroblasts, and other cells following 2 weeks of TAC (fig. S12E**).** TGF-β inhibition also led to prevention of myocardial fibrosis during 2 weeks TAC and rescued maladaptive remodeling and loss of systolic function to a lesser extent than endothelial *Sox9* deletion (fig. S12F-L, Data file S2C).

Endothelial loss of *Sox9* protects multiple organs from fibrotic disease

To address whether endothelial *Sox9* deletion also ameliorated tissue fibrosis in other diseases, we challenged *Sox9*$^{fl/fl}$ control and *Sox9*$^{EC\text{-}KO}$ mice with either a combined administration of high-fat diet (HFD) and the NOS inhibitor L-NAME (a model of diastolic heart failure), administration of bleomycin (BLM, a model of lung fibrosis), or choline-deficient, l-amino acid-defined (CDAA) diet (a model of non-alcoholic steatohepatitis-associated liver fibrosis). During 10 weeks of HFD and L-NAME administration (Fig. 4G), *Sox9*$^{fl/fl}$ control and *Sox9*$^{EC\text{-}KO}$ mice similarly developed obesity, glucose intolerance, hypertension and impaired endothelial function (as indicated by reduced coronary flow reserve, CFR) (fig. S13A-D). In contrast, both cardiac hypertrophy and lung congestion were abolished in *Sox9*$^{EC\text{-}KO}$ mice (Fig. 4H, fig. S13E-F). Echocardiographic and invasive hemodynamic analyses indicated preserved systolic heart function in both groups (similar %LV-FS and end-systolic LV pressure), whereas indicators of diastolic heart dysfunction (increased LV end-diastolic pressure, LVEDP; decreased %GLS; increased early mitral valve blood flow over tissue velocity ratio, E/E´; increased left atrial size) were only detected in *Sox9*$^{fl/fl}$ control, but not in *Sox9*$^{EC\text{-}KO}$ mice (Fig. 4I-K, fig. S13G-S14A). Stress echocardiography showed a normalized ventricular strain and contraction response during dobutamine stress treatment in *Sox9*$^{EC\text{-}KO}$ mice (fig. S14B). Both Sirius red and collagen immunostaining indicated reduced myocardial fibrosis upon endothelial *Sox9* deletion in the HFD and L-NAME treated mice (Fig. 4L-M, fig. S14C, Data file S2D).

A single intra-tracheal injection of BLM (Fig. 5A) in mice caused an increased lung weight, right ventricular (RV) hypertrophy (elevated Fulton´s index and RV free wall hypertrophy), a progressive reduction in capillary blood oxygen saturation (Fig. 5B, fig. S15A-C) and pulmonary fibrosis until 12 days after the injection. *Sox9*$^{EC\text{-}KO}$ mice exerted less right ventricular hypertrophy and improved oxygen saturation compared to *Sox9*$^{fl/fl}$ control mice. Better oxygenation also led to reduced pulmonary artery (PA) resistance (increased PAT/ET), and maintained RV function (%RV-FS) in *Sox9*$^{EC\text{-}KO}$ mice, whereas *Sox9*$^{fl/fl}$ had reduced RV function (Fig. 5C-D, fig. S15D-F). Lung echography (expressed as MoLUS score), Sirius red, and αSMA staining as well as qPCR analysis of mesenchymal genes from lung tissue revealed ameliorated fibrosis following BLM injection in the absence of *Sox9* in ECs (Fig. 5E-F, fig. S15G-J, Data file S2E-F).

Due to 15 weeks of CDAA diet (Fig. 5G), all mice developed increased body and liver weight, elevated plasma cholesterol concentrations, lipid droplet accumulation, and an echo dense liver appearance (Fig. 5H, fig. S16A-F, Data file S2G). Moreover, we found CDAA-mediated

alterations in sinusoidal endothelial marker expression, as shown by decreased staining for the discontinuous EC marker LYVE1, while metabolic liver zonation was unaltered (fig. S16G). Ameliorated fibrotic remodeling in *Sox9*$^{EC-KO}$ mice was indicated by reduced acoustic signal attenuation (Fig. 5I). Augmented portal vein dilation and spleen congestion as well as marked liver fibrosis in Sirius red stained liver sections of *Sox9*$^{fl/fl}$ mice suggested advanced fibrotic liver disease in the CDAA model that was reduced by EC-restricted ablation of *Sox9* (Fig. 5J-L, fig. S16F, Data file S2H-I). Taken together, endothelial deletion of *Sox9* attenuated fibrotic remodeling of heart, lung, and liver in four different pre-clinical models.

SOX9 deletion downregulates pro-fibrotic gene-expression in ECs

Next, we isolated ECs from heart, lung, or liver during the early stage of the disease models in *Sox9*$^{EC-KO}$ versus *Sox9*$^{fl/fl}$ control mice, before organ failure became apparent (fig. S17A-E), and conducted bulk RNA sequencing. These analyses showed differential regulation of different gene sets between the organs and disease models upon endothelial *Sox9* deletion (fig. S17F-M). Upon TAC, cardiac ECs of *Sox9*$^{EC-KO}$ mice upregulated genes of the GO terms (biological process) heat response, negative regulation of cell proliferation, but downregulated (among others) cell cycle, inflammatory and ossification genes versus *Sox9*$^{fl/fl}$ control mice. During HFD and L-NAME, cardiac ECs of *Sox9*$^{EC-KO}$ mice upregulated cell cycle, cell division, and translation genes. Extracellular matrix, cell migration, and inflammatory genes were downregulated in this model compared to *Sox9*$^{fl/fl}$ mice. During lung injury, ECs of *Sox9*$^{EC-KO}$ mice upregulated inflammatory and chemotaxis genes, while downregulating angiogenesis, cell migration, and extracellular matrix development genes. During CDAA, upregulated genes in ECs of *Sox9*$^{EC-KO}$ mice comprised signal transduction, such as VEGF signaling, oxygen transportation, and cellular detoxification genes, while inflammatory, extracellular matrix, and cell cycle and cell division genes were decreased in comparison to liver ECs from *Sox9*$^{fl/fl}$ mice.

We next compared the endothelial transcriptomic analyses of *Sox9*$^{EC-OE}$ and *Sox9*$^{EC-KO}$ mice in an organ specific manner to identify genes that were upregulated by SOX9 overexpression and downregulated by *Sox9* ablation or vice versa (fig. S5 & S17). This comparison revealed which genes were more likely to be directly regulated by SOX9 in ECs (fig. S18-19). Among these potential targets, genes related to extracellular matrix were upregulated by SOX9 in ECs of the heart after TAC, in HFD with L-NAME, as well as in the lung due to BLM, and in the liver during CDAA diet. Inflammation-related genes were upregulated by SOX9 in cardiac ECs during TAC and in HFD with L-NAME, as well as in the liver after CDAA diet, but not in the lung treated with BLM, where these genes were downregulated by SOX9. Furthermore, endothelial SOX9 downregulated neuronal genes in the heart during TAC and HFD with L-NAME (fig. S18-19).

With single cell RNA sequencing, we next aimed to specifically analyze the *Sox9* containing fraction of ECs after 1 week of TAC. We identified 9 different clusters (Fig. 6A), of which all except clusters #7 and 8 showed high expression of the endothelial cell markers *Cdh5* and *Pecam1* (fig. S20A). *Sox9* induction by TAC was mainly found in ECs of cluster #5, which was markedly expanded by TAC in comparison to sham mice and expressed genes related to the immune system, cell proliferation, and extracellular matrix organization (Fig. 6B, fig. S20B-C). ECs from Cluster #5 from TAC treated *Sox9*$^{fl/fl}$ mice, which were identified as venule ECs (fig. S21), showed induction of fibrillar collagens (such as *Col1a1*, *Col3a1,* encoding for collagen type I alpha 1 chain and collagen type III alpha 1 chain, respectively), mesenchyme, and matrix organizers (like *Lox,* lysyl oxidase), and the signaling molecule *Ccn2*. Upregulation of these genes was strongly attenuated in *Sox9*$^{EC-KO}$ mice upon TAC (Fig. 6C). *Sox9* expression in these

cardiac ECs of *Sox9*$^{fl/fl}$ mice showed co-expression with both *Tgfbr2* (transforming growth factor beta receptor 2) and *Vcam1* (vascular cell adhesion molecule 1), which were highly enriched to Cluster #5, both on RNA and protein levels in immunofluorescence staining (fig. S20C and S22A-E).

Deletion of endothelial *Sox9* during pre-established cardiac pressure-overload halts the progression of left ventricular dysfunction

To test a potential therapeutic role of endothelial *Sox9* deletion in pre-established disease, we induced the EC-specific loss of *Sox9* 1.5 weeks after TAC surgery and monitored the mice for 4.5 additional weeks (Fig. 6D). All mice developed a similar degree of cardiac hypertrophy and LV dysfunction after 1.5 weeks, but delayed endothelial deletion of *Sox9* attenuated further disease progression as shown by reduced cardiac hypertrophy, maintained systolic function, and a tendency towards ameliorated cardiac fibrosis (Fig. 6E-F, fig. S23A-E, Data file S2J). Using single cell RNA sequencing, we aimed to investigate whether this was caused by a reversion of the transcriptome switch seen before (fig. S24A-B). Indeed, using the identified cluster markers, we re-identified the former Cluster #5 as new Cluster #9 and #11, which both displayed reduced abundance of mesenchymal genes (*Ccn2*, *Col3a1*) after 3 weeks of TAC, and 2 weeks after *Sox9* deletion in delayed *Sox9*$^{EC\text{-}KO}$ mice vs. littermates (Fig. 6G-H, fig. S24C-25B). This indicated that pro-fibrotic gene-expression was partially reversed by delayed endothelial *Sox9* deletion. We also found a general decline in cell cycle-related gene-expression (cluster #10) at 3 weeks versus 1 week after TAC in both genotypes, which is in line with our previous findings (*9*).

Endothelial SOX9 directly targets *Ccn2* transcription

To identify direct transcriptional targets of SOX9, we assayed for transposase-accessible chromatin using sequencing (ATAC sequencing) of isolated cardiac ECs from *Sox9*$^{EC\text{-}OE}$ mice 4 weeks after initiation of *Sox9* overexpression. We found that the set of GO terms associated with genes localized within opened chromatin regions identified by ATAC sequencing was similar to that previously seen in RNA sequencing in these ECs (fig. S26A-B). Intersecting ATAC and RNA sequencing in ECs from *Sox9*$^{EC\text{-}OE}$ revealed genes that were induced by SOX9 in both DNA accessibility and mRNA expression (Fig. 7A). These genes belonged to the GO-terms (among others) chondrocyte differentiation, extracellular matrix organization, and positive regulation of I-kappaB kinase/NF-kappaB signaling. Examples are *Col2a1* and *Ccn2*. In addition, we intersected genes with opened chromatin in ATAC sequencing of cardiac ECs from *Sox9*$^{EC\text{-}OE}$ mice with genes expressed in *Sox9*-expressing ECs in Cluster #5 (from Fig. 6A). We found an overlap of 202 genes, which belonged to the GO classes transforming growth factor beta receptor signaling pathway, cartilage development and positive regulation of inflammatory response, and contained genes like *Adamts1* (ADAM metallopeptidase with thrombospondin type 1 motif 1), *Ccn2*, *Cdk19* (cyclin dependent kinase 19) and *Igfbp3* (insulin like growth factor binding protein 3) (Fig. 7B). The promotor regions of genes from Cluster #5 were highly enriched for SOX9 motifs, in contrast to Cluster #1 (Fig. 7C), which is located opposite to #5 in the UMAP plot (Fig. 6A). Hence, we performed cleavage under targets and tagmentation (CUT&TAG) sequencing of cardiac ECs from *Sox9*$^{EC\text{-}OE}$ mice to detect direct SOX9-binding DNA sequences. We then intersected SOX9-bound DNA regions with both opened chromatin regions and SOX9-dependent gene-expression in these cells or with the genes enriched in Cluster #5 from Fig. 6A. We found that the overlapping genes belonged to cell differentiation, extracellular matrix organization, and inflammatory response regulation GOs (fig. S26C-D). By this approach, we identified (among others) *Ccn2*, encoding for connective tissue growth factor

(CTGF), as a direct Sox9 target in ECs (Fig. 7D, fig. S26E) with SOX9 binding to a formerly identified promotor region in the *Ccn2* gene locus (GSE69109) (*42*).

SOX9-dependent endothelial mesenchymal activation does not lead to complete endothelial-to-mesenchymal transition

5 We next aimed to address whether adult ECs activate only a transient mesenchymal-endothelial state or whether they fully differentiate towards mesenchymal cells. Thus, we crossed *Cdh5*-driven *Sox9*$^{\text{EC-OE}}$ with mT/mG lineage-tracing mice resulting in tracking *Sox9*-overexpressing ECs in vivo (fig. S27A-C). Merging of staining for endothelial markers (CD31 and VE-Cadherin, respectively) and GFP indicated that recombined *Sox9*$^{\text{EC-OE}}$ cells did not lose their 10 endothelial identity during mesenchymal activation, as virtually 100% of them remained positive for CD31 or VE-Cadherin (Fig. 8A-C, fig. S27D-F). Additionally, we used transcriptome data as generated by single cell RNA sequencing from *Sox9*-overexpressing ECs in Fig. 3A and analyzed co-expression profiles of non-contaminated ECs (subtracting Clusters #6, pericytes; #8, immune cells; #10, fibroblasts; and #11, non-EC nerve cells). We quantified expression of 15 endothelial markers (*Pecam1*, *Cdh5*, or both) together with *Sox9* or mesenchymal gene expression. Indeed, overexpression of *Sox9* induced the expression of *Ccn2* or *Fn1* in ECs that co-expressed one or both endothelial markers (fig. S27G). These results indicated that endothelial SOX9 overexpression caused mesenchymal gene activation in ECs, but endothelial identity remained largely preserved.

20 **Endothelial SOX9 triggers fibroblast activation**

We next investigated whether ECs, upon mesenchymal activation, activate fibroblasts in addition to producing ECM. We co-cultured HUVECs with or without adenoviral *Sox9* (ad*Sox9*) overexpression with primary cardiac fibroblasts in a two-chamber culture (fig. S28A). Fibroblasts that were co-cultured with ad*Sox9* treated ECs showed increased endogenous *Sox9* 25 expression, enhanced expression of different collagens, ECM remodeling genes, the myofibroblast marker *Acta2* (alpha smooth muscle actin) as well as augmented migratory capacity (Fig. 8D-E). Because we identified *Ccn2*, a known mediator of fibroblast activation and ECM secretion, as a direct target of SOX9, we aimed to test whether inhibition of *Ccn2* would mitigate the SOX9-mediated fibroblast activation by ECs. Co-culture of fibroblasts with ECs 30 pre-treated with si*CCN2* and ad*Sox9* (compared to siCtrl + ad*Sox9*) indeed showed that the induction of ECM genes and the enhanced migratory capacity in fibroblasts was partially inhibited when *CCN2* was silenced in co-cultured ECs overexpressing *Sox9* (fig. S28B-C). To investigate cardiac fibroblast behavior upon EC *Sox9* overexpression in vivo, we analyzed hearts of *Sox9*$^{\text{EC-OE}}$ mice in a time course experiment (fig. S28D). Immunostaining revealed that 35 *Sox9*$^{\text{EC-OE}}$ mice displayed an increased number of PDGFRα-expressing fibroblasts 30 days after overexpression of *Sox9* in ECs, indicating that EC SOX9 triggered fibroblast expansion. On the other hand, ECM deposition was observed early (10 days) after initiation of endothelial *Sox9* overexpression, suggesting that ECs contribute to ECM before fibroblast expansion, but the amount of matrix production by each cell type could not be quantified by this approach (fig. 40 S28E-F). In order to further analyze fibroblast activation in parallel to mesenchymal activation of ECs, we characterized both cell types with cardiac non-myocyte single cell RNA sequencing, which identified different clusters of cardiac cell types (Fig. 8F, fig. S29A-B). Both, the analysis of representative genes [*Ccn2*, *Col1a1*, and *Aspn* (asporin) as mesenchymal genes; *Bcl2l1* (Bcl2 like 1) as anti-apoptotic gene; *Irgm1*(immunity-related GTPase family M member 1), and *Ifit2* 45 (interferon induced protein with tetratricopeptide repeats 2) as inflammatory genes; as well as *Tpx2* (TPX2 microtubule nucleation factor), and *Cdk1* (cyclin dependent kinase 1) as

proliferative genes] and cluster-based analysis of GO terms revealed individual mesenchymal activation patterns in both endothelial cells and fibroblasts. Both cell types exerted early expression of mesenchymal and inflammatory genes upon endothelial *Sox9* overexpression, while ECs specifically showed an early activation of cell cycle genes. In addition, angiogenesis-related genes were mainly downregulated in ECs in the early phase of *Sox9* overexpression (Fig. 8G, fig. S30A-B). These results indicated that SOX9-triggered endothelial mesenchymal activation led to matrix production by ECs themselves, but also induced the paracrine activation of fibroblasts in vivo (fig. S30C). The paracrine activation of fibroblasts and their subsequent enhanced migration, matrix synthesis and proliferation will amplify the profibrotic effect, which is initiated by a subset of *Sox9*-expressing endothelial cells.

Discussion

Organ fibrosis is a key driver of many chronic diseases, including systolic and diastolic heart failure, and liver and lung fibrosis. Various stimuli, such as permanent cardiac afterload during hypertension or valvular disease, pulmonary cell damage, or hepatic lipid accumulation, each initiate fibrosis that accelerates organ damage and dysfunction. Endothelial mesenchymal activation (and in some cases full EndoMT) has been observed in the development of several diseases associated with fibrosis, such as in cancer (*43*), renal fibrosis (*44*), liver fibrosis (*45*), wound scarring (*11*), myocardial (*9, 12*) and vascular disease (*13*). Especially in the heart, full EndoMT does not occur; instead only a transient state has been found (transient endothelial-mesenchymal activation as typical feature of endothelial-mesenchymal plasticity (*12, 46*)), in which ECs upregulate a set of mesenchymal genes. Among these genes are several collagen and extracellular matrix related genes. In other organs (such as the kidney) (*44*), full EndoMT might take place, whereby ECs completely transition to a fibroblast cell fate, but some controversy still exists (*47*). Although endothelial-mesenchymal activation and/or EndoMT were widely observed in different diseases, whether it is an epiphenomenon or a key contributor to fibrosis and organ dysfunction remained unknown.

Our results indicate that endothelial *Sox9* is induced in response to pathological EC stimulation by the EndoMT activator TGF-β in human venous and cardiac microvascular ECs in vitro and by different disease stimuli (including pressure overload, high fat diet, and toxins) in vivo. TGF-β is a master regulator of fibrosis in many organs and was suggested to induce *Sox9* expression in chondrocytes and fibroblasts by transcriptional and post-transcriptional mechanisms, although its effect on endothelial *Sox9* was so far not reported (*48, 49*). Interestingly, *Sox9* was mainly upregulated after TAC in one cluster of cardiac ECs that expressed extracellular matrix genes as well as *Vcam1* and *Tgfbr2*, rendering these ECs more responsive to TGF-β. This implies a specific fibrogenic subset of ECs that was similarly identified in single cell sequencing of human hearts (ECFB cluster, see above) and had been previously suggested to exist in mice (*50*).

Endothelial *Sox9* ablation appeared to be more efficient compared to untargeted, broad TGF-β inhibition or the recently reported endothelial deletion of the histone demethylase *Jmjd2b,* which reduced matrix-producing ECs, but this was not sufficient to improve cardiac function in a model of myocardial infarction (*5*).

In addition to direct synthesis of ECM upon SOX9 induction by ECs, at least equally important might be SOX9-dependent paracrine interaction of ECs with fibroblasts and other cells, for example via growth factors such as CTGF (encoded by the *Ccn2* gene), which we identified as direct target gene of SOX9 in ECs. Intriguingly, *Sox9* overexpression in ECs triggered an increased expression of *Sox9*, fibrosis and myofibroblast genes as well as an increased migratory

capacity in co-cultured fibroblasts, which was in part ameliorated by a concomitant *Ccn2* knock-down in ECs. A similar, EC derived CTGF-dependent paracrine crosstalk towards fibroblasts was previously observed in cardiac ischemia/reperfusion injury and skin fibrosis (*51, 52*). CTGF was also shown to be critical in deteriorating organ function and fibrosis in heart (*53*), lung (*54*), and liver (*55*). The induction of inflammatory genes (for example, *Cxcl10*, C-X-C motif chemokine ligand 10, *Lgals3,* galectin 3) by endothelial SOX9 caused increased recruitment of leukocytes in the heart, which reportedly contribute to tissue fibrosis by releasing paracrine factors (such as TGF-β) (*56*). Cell-wide analysis of mesenchymal activation following endothelium-restricted Sox9 overexpression, however, suggested that both phenomena (matrix production by ECs and by EC-activated fibroblasts) contribute to organ fibrosis. Importantly, the paracrine activation of fibroblasts leading to their enhanced migration, proliferation and matrix production will amplify the effect of the *Sox9*-expressing fibrogenic ECs and this might explain the high anti-fibrotic efficiency of endothelial targeted *Sox9* deletion. Other paracrine effects triggered by endothelial SOX9 included organ growth in heart, lung, liver and spleen, most likely through the release of growth factors (such as CTGF, IGFBP2), but also through matrix related proteins such as Fibronectin, which induces cardiomyocyte growth (*9*).

The vasculature, especially the endothelium, has recently come into focus as therapeutic target (*57, 58*). EC directed therapy might especially be needed in organs with limited regenerative capacity, such as the heart, where fibrosis is irreversible in advanced disease (*59*). In the future, strategies could be developed that enable SOX9 downregulation in ECs, for example via endothelial-targeted gene therapy.

Although we reveal a contribution of both ECs and fibroblasts, our study has limitations. To what degree reduced fibrosis upon *Sox9* deletion is the consequence of reduced endothelial matrix production versus reduced fibroblast activation remains unclear and might vary between disease models and organs. Moreover, as we show profibrotic intercellular crosstalk it is highly likely that additional factors are involved upstream and downstream of SOX9, which remain to be identified. In addition, it could be that inflammatory cells take part in the pro-fibrotic crosstalk in addition to fibroblasts and ECs.

Therefore, we reveal in this study that ECs play a so far overlooked role as critical upstream inducer of fibrosis during pathological stress and therefore become a primary target cell for therapeutic interventions to inhibit excessive fibrosis and to counteract organ dysfunction.

Materials and Methods

Study design

All experiments were carried out in at least 3 biological replicates.
Sample size was chosen as a result of previous experience regarding data variability in similar models and experimental set-ups. No statistical method was used to predetermine sample size. Mice were allocated to the different experimental groups due to genotyping results and randomly assigned to different treatments. The investigators were blinded for mouse genotype and treatment during surgeries, echocardiography, cardiac catheterization, organ weight determination and all histological and immunofluorescence quantifications. Animals found dead in the first days after intervention or found dead during the ongoing study with unknown reason of death were excluded from the entire study. Two animals from prospective TAC groups were

excluded from the entire analysis due to ambiguous carotid artery Doppler imaging. No animal was excluded from the study post-hoc.

Methods summary

Transgenic mice were created that overexpress SOX9 specifically in endothelial cells upon Tamoxifen administration by crossing mice harboring an inactive *Sox9* allele in the genomic *Hprt* locus, which is activated upon recombination, with *Cdh5*-CreER mice (EC-specific Cre-driver). For EC-specific loss-of-function studies, mice with loxP sites in the genomic *Sox9* locus were bred with *Cdh5*-CreER mice. Tamoxifen-injected Cre$^{-/-}$ littermates with appropriate loxP sites served as controls in all experiments. Lineage tracing was conducted by crossing *Hprt*$^{flox\text{-}Sox9\text{-}flox}$×*Cdh5*-CreER mice with mT/mG mice whose cell-wide expression of membrane-associated tandem-dimer Tomato switches to GFP in recombined cells. Murine stress models to induce organ fibrosis included the following: transverse aortic constriction; administration of NOS inhibitor L-NAME in combination with high fat diet; intra-tracheal bleomycin injection; and choline-deficient, amino acid-defined diet. Longitudinal and end-point phenotyping in treated and sham-treated wildtype and transgenic groups comprised: Cardiac, vessel, lung and abdominal echography; dobutamine induced cardiac stress; left-ventricular catheterization; tail cuff-based non-invasive blood pressure measurement; pulse oximetry; glucose tolerance testing; plasma sampling; and optical tissue clearing. Organs were harvested at study endpoint for further processing including: RNA isolation; protein isolation; MACS-based cell isolation; histologic and fluorescence staining. In-depth transcriptome analyses were performed utilizing bulk or single cell RNA sequencing of isolated endothelial cells or isolated non-myocytes. Analyses of transcriptional regulation was conducted using ATAC and SOX9 CUT&TAG sequencing. Human cells were cultured according to the distributor's protocols. Cellular co-culture was conducted in connected chambers. Cellular siRNA-mediated gene silencing, adenoviral infection, TGF-β stimulation; RT-qPCR; Western Blotting; and tissue staining were conducted following standard protocols. GraphPad Prism 8, R version 4, and Microsoft Excel 365 were used for statistical analysis.

A detailed Materials and Methods description is provided in the supplementary document.

Statistical analysis

For statistical analysis GraphPad Prism software (version 8, GraphPad Software Inc.) was used. Data are shown as mean ± standard error of the mean (s. e. m.) unless noted otherwise. The exact number of biological replicates (number of mice, samples or cell culture dishes) is indicated in the figure legends. All experiments were carried out in at least 3 biological replicates. Data were analyzed by column or grouped analysis as appropriate. Before column (1-factor) analysis, normality was tested using Shapiro-Wilk test (for $n \leq 4$), Kolmogorov-Smirnov test (for $n \geq 5$), or Anderson-Darling test (for repeated-measurement analysis). If the normality test was passed, an unpaired, two-tailed Student´s t-test was used to determine statistical significance when two groups were compared. More than two groups were compared with 1-way ANOVA (when differing by one factor) or 2-way ANOVA (when differing by 2 factors) and Fisher's LSD post-hoc test. Two groups were compared over time with paired, unpaired or mixed-model 2-way ANOVA as applicable and Fisher's LSD post-hoc test. More than two groups when characterized by two factors were compared over time with paired, unpaired or mixed-model 3-way ANOVA as applicable and Fisher's LSD post-hoc test. Time analyses with non-matching groups and incomplete time-group relations were analyzed with 1-way ANOVA and Fisher's LSD post-hoc test in combination with 3-way ANOVA under the exclusion of the incomplete timepoint. Not normally distributed data were tested with Mann-Whitney test (when comparing

two groups) or Kruskal-Wallis test with uncorrected Dunn's post-hoc test (when comparing three or more groups). Each statistical test used is indicated in the Figure legend. Statistics of ANOVA are supplied by degrees of freedom (df) in the numerator (dfn) and in the denominator (dfd), depicted as df="dfn":"dfd". All tests were performed two-sided unless noted otherwise. A p-value of <0.05 was considered statistically significant.

Supplementary Materials

Materials and Methods

Figs. S1 - S30

Tables S1 - S8

Movies S1 - S2

Data files S1 - S5

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Data and materials availability: All data associated with this study are present in the paper or supplementary materials. All sequencing data generated during this study are available in the Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through the GEO series accession number GSE198041 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE198041). The following genome assembly was used for alignment: mm10, GRCm38 - mm10 - Genome - Assembly - NCBI (nih.gov) .

Figure Captions

Fig. 1. Organ fibrosis is associated with increased endothelial SOX9 in mice and humans.

(**A**) Endothelial *Sox9* mRNA levels following TAC (normalized to *Hprt1, hypoxanthine phosphoribosyltransferase 1* expression), different dietary regimens inducing diastolic heart failure (normalized to *Gapdh, glyceraldehyde-3-phosphate dehydrogenase* expression), bleomycin injection (normalized to *Hprt1* expression), and CDAA diet (normalized to *Gak*, cyclin G associated kinase, *Srp72*, signal recognition particle 72, and *Mrpl46, mitochondrial ribosomal protein L46* expression) as indicated; *p<0.05, ****p<0.0001, Student's t-test, n=4/6 (Control, Ctrl/TAC), n=5/7 (Ctrl/BLM), n=5/5 (Ctrl/CDAA), #p<0.05 vs. each other group, 1-way ANOVA with Fisher's LSD post-hoc test, n=5/7/7/7 (HFD/L-NAME experiment, order of

n-number per group as in columns from left to right). (**B**) Violin plots for the expression level of *SOX9* in healthy vs. HFrEF EC and ECFB clusters (log2 transformed and normalized UMI counts) in human single-nuclei RNA-sequencing as filtered in (*35*); **p<0.01, ****p<0.0001, adjusted p-values based on Bonferroni correction using all genes of the dataset to compare the expression in violin plots as calculated with the Seurat function "FindAllMarkers” using “bimod” as the statistical test. (**C**) Immunohistochemical staining of diseased human cardiac tissue and correlation of SOX9 expression with the degree of organ fibrosis (fibrotic score), arrows indicate $Sox9^{+}$ cells; a full list of human samples is included in the supplement; scale bars: 10 µm (IHC), 100 µm (Sirius red); underlined, italic digits display significant Pearson correlation coefficient, n=20; all data are expressed as mean ± s.e.m.; all tests were conducted two-sided.

Fig. 2. Increased endothelial *Sox9* expression promotes fibrosis in vivo.

(**A**) Experimental design of targeted endothelial *Sox9* overexpression in vivo. (**B**) Representative Sirius red fibrosis staining of organs as indicated; scale bars: 1000 µm (upper), 200 µm (lower). (**C**) Quantification of fibrotic lesions; *p<0.05, **p<0.01, Student’s t-test, n=11/9 (heart), n=7/5 (lung), n=14/13 (liver), n=9/9 (spleen, order of n-number per group as in columns from left to right). (**D**) Representative cardiac sections of 3-D microscopy of fibrotic vessels following optical tissue clearing. Arrows indicate cross-sectioned vessels with perivascular fibrosis; scale bar: 200 µm. (**E-F**) Analysis of cardiac function in $Sox9^{EC\text{-}OE}$ mice by echocardiography: (**E**) Global longitudinal strain (GLS); $^{‡‡‡}p_{interaction}<0.001$ $Sox9^{EC\text{-}OE}$ vs. Ctrl, 2-way ANOVA, df=5:90, arrows indicate first appearance of significant differences in Fisher’s LSD post-hoc test, p<0.05, n=9/11 (order of n-number per group as in legend from top to bottom). (**F**) LV fractional shortening (%LV-FS); $^{‡‡‡}p_{interaction}<0.001$, $^{‡‡‡‡}p_{interaction}<0.0001$ $Sox9^{EC\text{-}OE}$ vs. Ctrl, 2-way ANOVA, df=9:158 (LV-FS), arrows indicate first appearance of significant differences in Fisher’s LSD post-hoc test, p<0.05, n=9/11. (**G**) Analysis of pulmonary function in $Sox9^{EC\text{-}OE}$ mice: Blood oxygenation (%SpO_2) $^{‡‡‡‡}p_{interaction}<0.0001$ $Sox9^{EC\text{-}OE}$ vs. Ctrl, 2-way ANOVA, df=5:58 (SpO_2), arrows indicate first appearance of significant differences in Fisher’s LSD post-hoc test, p<0.05, n=7/7/6/6/4/7 early to late time points (Ctrl)/ 5/5/7/7/4/5 ($Sox9^{EC\text{-}OE}$); all data are expressed as mean ± s.e.m.; all tests were conducted two-sided; regularly dashed lines with offset symbol indicate non-paired analyses.

Fig. 3. Overexpression of SOX9 in endothelial cells is associated with mesenchymal gene expression.

(**A**) UMAP plot of cardiac ECs upon endothelial SOX9 overexpression analyzed with single-cell RNA-sequencing; n=5,769 total cells with 2,802 (Ctrl)/2,967 ($Sox9^{EC\text{-}OE}$) cells from 3 animals per group. (**B-C**) Representative GO term analysis of identified EC clusters; p-values refer to GO term analysis of GO: Biological process (BP) and GO: Cellular compartment (CC). (**D**) RT-qPCR analysis of selected genes in HUVECs following ad*Sox9* infection (normalized to *18S* expression); numbers indicate p-values >0.05 of specific interest, *p<0.05, **p<0.01, ****p<0.0001, Mann-Whitney test, n=10/11 independently treated cell culture dishes (order of n-number per group as in columns from left to right). (**E**) RT-qPCR analysis of selected genes in HCMECs following ad*Sox9* infection (normalized to *Hprt1* expression); numbers indicate p-values >0.05 of specific interest, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Mann-

Whitney test, n=12/12 independently treated cell culture dishes; all data are expressed as mean ± s.e.m.; all tests were conducted two-sided.

Fig. 4. Endothelial *Sox9* deletion inhibits fibrosis and improves heart function in mice following transverse aortic constriction and a high fat diet + L-NAME regimen.

5 (**A**) Experimental design of TAC in *Sox9*$^{EC\text{-}KO}$ and control mice. (**B**) Morphometric analyses following 2 weeks of TAC; *p<0.05, ***p<0.001, 2-way ANOVA with Fisher's LSD post-hoc test, n=6/6/10/12 (order of n-number per group as in columns from left to right). (**C**) Morphometric analyses following 10 weeks TAC; ns: not significant, 2-way ANOVA with Fisher's LSD post-hoc test, n=6/5/9/6, $^{\#}p_{row}<0.05$, $^{\#\#\#}p_{row}<0.001$ TAC vs. Sham, 2-way ANOVA, 10 df=1:22. (**D**) Representative Sirius red fibrosis staining of cardiac cross-sections 2 or 10 weeks after TAC, resp.; scale bar: 1000 µm. (**E**) Quantification of fibrotic lesions; *p<0.05, **p<0.01, 2-way ANOVA with Fisher's LSD post-hoc test, n=4/4/7/7 (2 weeks), n=4/4/6/6 (10 weeks). (**F**) Echocardiographic analysis of fractional shortening in mice during 10 weeks TAC; *p<0.05, **p<0.01 TAC *Sox9*$^{EC\text{-}KO}$ vs. TAC *Sox9*$^{fl/fl}$, 3-way ANOVA with Fisher's LSD post-hoc test, 15 n=6/5/9/6 (order of n-number per group as in legend from top to bottom), $^{\dagger\dagger}p_{interaction}<0.01$, $^{\dagger\dagger\dagger}p_{interaction}<0.001$ vs. corresponding Sham, 2-way ANOVA, df=5:65 (*Sox9*$^{fl/fl}$), df=5:45 (*Sox9*$^{EC\text{-}KO}$). (**G**) Experimental design of diastolic dysfunction in *Sox9*$^{EC\text{-}KO}$ and control mice. (**H**) Morphometric analysis; *p<0.05, 2-way ANOVA with Fisher's LSD post-hoc test, n=5/5/13/14 (order of n-number per group as in columns from left to right). (**I**) Left-ventricular contractility 20 assessed by echocardiography; 3-way ANOVA with Fisher's LSD post-hoc test, n=5/5/13/14 (order of n-number per group as in legend from top to bottom). (**J**) Hemodynamic assessment of end-diastolic LV pressure; *p<0.05, **p<0.01, 2-way ANOVA with Fisher's LSD post-hoc test, n=3/3/5/5 (order of n-number per group as in columns from left to right). (**K**) Echocardiographic assessment of cardiac compliance and diastolic function; **p<0.01, ***p<0.001, ****p<0.0001, 25 HFD+L-NAME *Sox9*$^{EC\text{-}KO}$ vs. HFD+L-NAME *Sox9*$^{fl/fl}$, 3-way ANOVA with Fisher's LSD post-hoc test, n=5/5/13/14 (order of n-number per group as in legend from top to bottom), $^{\dagger}p_{interaction}<0.05$, vs. corresponding Sham, 2-way ANOVA, df=2:31. (**L-M**) Representative Sirius red fibrosis staining of cardiac cross-sections and quantification of fibrotic lesions; scale bar: 1000 µm;*p<0.05, 2-way ANOVA with Fisher's LSD post-hoc test, n=5/5/9/9 (order of n- 30 number per group as in columns from left to right); all data are expressed as mean ± s.e.m.; all tests were conducted two-sided.

Fig. 5. Deletion of *Sox9* in endothelial cells ameliorates pulmonary and liver fibrosis in mice.

35 (**A**) Experimental design of inducing lung fibrosis with bleomycin (BLM) in *Sox9*$^{EC\text{-}KO}$ mice. (**B**) Morphometric analysis to reveal Fulton´s index (RV weight / (LV weight + S(eptum) weight), and blood oximetry (%SpO_2); bar chart, *p<0.05, ***p<0.001, ****p<0.0001, 2-way ANOVA with Fisher's LSD post-hoc test, n=4/4/10/13 (order of n-number per group as in columns from left to right), time course chart, ****p<0.0001 BLM *Sox9*$^{EC\text{-}KO}$ vs. BLM *Sox9*$^{fl/fl}$, 3-way 40 ANOVA with Fisher's LSD post-hoc test, n=4/4/10/13 (same groups as for Fulton´s index), $^{\dagger}p_{interaction}<0.05$, $^{\dagger\dagger\dagger\dagger}p_{interaction}<0.0001$ vs. corresponding NaCl, 2-way ANOVA, df=2:30 (*Sox9*$^{fl/fl}$), df=2:34 (*Sox9*$^{EC\text{-}KO}$). (**C**) Pulmonary artery flow measurement (PAT/ET) negatively correlating to PA resistance with representative tracings; scale bar: 200 mm s^{-1}; *p<0.05, ***p<0.001,

****p<0.0001, 2-way ANOVA with Fisher's LSD post-hoc test, n=4/4/10/12 (order of n-number per group as in columns from left to right). (**D**) Echocardiographic analysis of right-ventricular failure (depicted as RV fractional shortening, %RV-FS); ***p<0.001, 2-way ANOVA with Fisher's LSD post-hoc test, n=4/4/10/12. (**E-F**) Representative Sirius red fibrosis staining of lung sections and quantification of fibrotic lesions; scale bar: 200 µm, *p<0.05, ***p<0.001, 2-way ANOVA with Fisher's LSD post-hoc test, n=4/4/8/8. (**G**) Experimental design of NASH in *Sox9*$^{\text{EC-KO}}$ mice with 15 W CDAA diet. (**H**) Liver weight; ns: not significant, 2-way ANOVA with Fisher's LSD post-hoc test, n=4/5/10/9, $^{\#\#\#\#}p_{\text{row}}<0.0001$ CDAA vs. Chow, 2-way ANOVA, df=1:24. (**I**) Liver echographic analysis of liver fibrosis, arrow depicts scheme of signal attenuation between upper and lower areal after CDAA administration; scale bar: mm; **p<0.01, ****p<0.0001, 2-way ANOVA with Fisher's LSD post-hoc test, n=4/4/10/9. (**J**) Examination of portal vein diameter and spleen weight as secondary markers of liver damage; scale bar: mm; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, 2-way ANOVA with Fisher's LSD post-hoc test, n=4/5/10/9. (**K-L**) Representative Sirius red fibrosis staining of big liver lobe cross-sections and quantification of fibrotic lesions; scale bar: 100 µm; *p<0.05, **p<0.01, ****p<0.0001, 2-way ANOVA with Fisher's LSD post-hoc test, n=4/4/9/9; all data are expressed as mean ± s.e.m.; all tests were conducted two-sided.

Fig. 6. Endothelial SOX9 mediates mesenchymal activation in distinct endothelial subtypes.

(**A**) UMAP plots of cardiac ECs after 1-week TAC and endothelial *Sox9* deletion or control as revealed by single-cell RNA-sequencing. (**B**) GO term-based characterization of identified clusters in single cell sequencing with a focus on Cluster #5; p-values refer to GO term analysis. (**C**) Violin plots depicting the regulation of selected genes in Cluster #5; adjusted p-values based on Bonferroni correction using all genes of the dataset to compare the expression in violin plots were calculated with the Seurat function "FindAllMarkers" using "bimod" as the statistical test, n=1,918 total cells with 438 (fl/fl)/582 (EC-KO) for sham and for TAC 401 (fl/fl)/497 (EC-KO) cells from 3 animals per group. (**D**) Experimental design of delayed endothelial Sox9 ablation during pre-existing disease. (**E**) Morphometric analyses following TAC in mice as indicated; *p<0.05 between indicated groups, $^{€€}p<0.01$ between indicated time points, 1-way ANOVA with Fisher's LSD post-hoc test, n=4/4 (BL fl/fl and EC-KO)/ 4/4/5/5 (Sham fl/fl/ Sham EC-KO/ TAC fl/fl/ TAC EC-KO 1.5 W)/ 4/4/6/7 (Sham fl/fl/ Sham EC-KO/ TAC fl/fl/ TAC EC-KO 6 W), $^{\dagger\dagger\dagger}p_{\text{row}}<0.001$, $^{\dagger\dagger\dagger\dagger}p_{\text{row}}<0.0001$ vs. corresponding Sham (w/o BL timepoint), 2-way ANOVA, df=1:15 (*Sox9*$^{\text{fl/fl}}$), df=1:16 (*Sox9*$^{\text{EC-KO}}$), $^{\S\S}p_{\text{Sham/TAC}}<0.01$ (w/o BL time point), 3-way ANOVA, df=1:31. (**F**) Echocardiographic analysis in indicated mice; **p<0.01, ***p<0.001, 2-way ANOVA with Fisher's LSD post-hoc test, n=4/4/6/7 (order of n-number per group as in legend from top to bottom), $^{\dagger\dagger\dagger\dagger}p_{\text{interaction}}<0.0001$ vs. corresponding Sham, 2-way ANOVA, df=4:32 (*Sox9*$^{\text{fl/fl}}$), df=4:36 (*Sox9*$^{\text{EC-KO}}$), $^{\S}p_{\text{time}\times\text{Sham/TAC}}<0.05$ (BL to 10 d time point), 3-way ANOVA, df=1:17. (**G**) UMAP plots of cardiac ECs after 1-3 W TAC and endothelial *Sox9* deletion or control as revealed by single-cell RNA-sequencing and illustrated in fig. S24A. Treatment-separated UMAP plots are depicted in fig. S24D. (**H**) Violin plots depicting the regulation of selected genes in Clusters #9 & #11; n=4,493 total cells with 1,334 (1 W sham)/1,866 (1 W TAC)/629 (3 W TAC) cells for fl/fl and 664 (3 W TAC) cells for EC-KO mice from 3 animals

per group; all data are expressed as mean ± s.e.m.; all tests were conducted two-sided; regularly dashed lines with offset symbol indicate non-paired analyses.

Fig. 7. SOX9 directly targets *Ccn2* transcription.

(**A**) Venn diagram showing differential regulation of genes from cardiac bulk-EC ATAC-sequencing intersected with cardiac bulk-EC RNA-sequencing following endothelial SOX9 overexpression; the overlap reveals potential direct SOX9-targets and GO terms. Genes used for merging were filtered as depicted in fig. S5A and fig. S26A. Numbers in the Venn diagrams refer to number of genes in the corresponding part of the diagram, p-values refer to significance in GO term analysis. (**B**) Venn diagram showing differential regulation of genes from cardiac bulk-EC ATAC-sequencing following endothelial SOX9 overexpression intersected with Cluster #5 (Sox9-expressing cluster) of cardiac single-cell EC RNA-sequencing following TAC in *Sox9*$^{EC\text{-}KO}$ mice; the overlap reveals potential direct SOX9-targets and GO terms. Genes used for merging were filtered as depicted in fig. S5A and fig. 6A. Numbers in the Venn diagrams refer to number of genes in the corresponding part of the diagram, p-values refer to significance in GO term analysis. (**C**) Bar graph depicting Sox9 motif appearance in Cluster #5 vs. #1 suggesting Sox9 dependent transcription in Cluster #5. Score is calculated as (–log10 of p-value) × (% of target – % of background) with values taken from MACS of Cluster #1 and #5 sequences (related to Fig. 6A). (**D**) Representative genome sequences displayed *via* Integrative Genomics Viewer mapping fold enrichment of RNAseq, ATACseq, and CUT&TAGseq together with published promoter and enhancer regions (GSE69109 (*42*)) for *Ccn2* as potential direct Sox9 target; scale bar: 5 kb.

Fig. 8. Endothelial SOX9 mediates ECM deposition and fibroblast activation via endothelial-to-mesenchymal activation.

(**A-B**) Representative immunofluorescence images of mT/mG lineage tracing in cardiac cross-sections after 4 weeks of endothelial *Sox9* overexpression; arrows indicate GFP$^+$ CD31$^+$ ECs (A) or GFP$^+$ VE-Cadherin$^+$ ECs (B); CD31 and VE-Cadherin are endothelial markers; images of Ctrl groups are displayed in fig. S27D-E; scale bar: 20 µm. (**C**) Quantification of mT/mG lineage tracing in cardiac cross-sections after 4 W of endothelial SOX9 overexpression as displayed in Fig. 8A-B & fig. S27D-E; n=4/4 (order of n-number per group as in columns from left to right). (**D**) RT-qPCR analysis of selected genes in neonatal rat cardiac fibroblasts (NRFB) following co-culture with ad*Sox9*-transfected HUVECs (normalized to *Gapdh* expression); numbers indicate p-values >0.05 of specific interest, *p<0.05, **p<0.01, Mann-Whitney test, n=11/12 independently treated cell culture dishes. (**E**) Scratch migration assay of NRFBs during co-culture with ad*Sox9*-transfected HUVECs; scale bar: 100 µm; *p<0.05, Student's t-test, n=9/9 independently treated cell culture dishes. (**F**) UMAP plot of cardiac non-myocyte cells upon endothelial *Sox9* overexpression analyzed with single-cell RNA-sequencing as depicted in fig. S28D; cell types have been identified as depicted in fig. S29A-B; n=4,207 total cells with 879 (10 d)/507 (20 d)/1,465 (30 d) cells from *Sox9*$^{EC\text{-}OE}$ and 1,356 cells from 30 d Ctrl mice from 3 animals per group. (**G**) Dot plot visualizing induction of representative mesenchymal, inflammatory, and proliferative genes in both endothelial cells and fibroblasts upon endothelial SOX9 overexpression over time; all data are expressed as mean ± s.e.m.; all tests were conducted two-sided.