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Modulation of cardiac fibrosis by Krüppel-like factor 6 through transcriptional control of thrombospondin 4 in cardiomyocytes

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Aims Krüppel-like factors (KLFs) are a family of transcription factors which play important roles in the heart under pathological and developmental conditions. We previously identified and cloned *Klf6* whose homozygous mutation in mice results in embryonic lethality suggesting a role in cardiovascular development. Effects of KLF6 on pathological regulation of the heart were investigated in the present study.

Methods and results Mice heterozygous for *Klf6* resulted in significantly diminished levels of cardiac fibrosis in response to angiotensin II infusion. Intriguingly, a similar phenotype was seen in cardiomyocyte-specific *Klf6* knockout mice, but not in cardiac fibroblast-specific knockout mice. Microarray analysis revealed increased levels of the extracellular matrix factor, thrombospondin 4 (TSP4), in the *Klf6*-ablated heart. Mechanistically, KLF6 directly suppressed *Tsp4* expression levels, and cardiac TSP4 regulated the activation of cardiac fibroblasts to regulate cardiac fibrosis.

Conclusion Our present studies on the cardiac function of KLF6 show a new mechanism whereby cardiomyocytes regulate cardiac fibrosis through transcriptional control of the extracellular matrix factor, TSP4, which, in turn, modulates activation of cardiac fibroblasts.

Keywords Fibrosis • Angiotensin II • Krüppel-like factor • Thrombospondin • Cardiomyocyte

1. Introduction

Fibrosis is a hallmark pathological feature of end-stage organ damage.[1] Persistent pathological stimulation promotes a plethora of responses including inflammatory cell infiltration, destruction of parenchymal cell structure, excess extracellular matrix (ECM) deposition by myofibroblasts, and lack of tissue reconstruction. These responses, in turn, provide for a vicious cycle, which subsequently leads to fibrosis and organ failure.[2] Cardiac fibrosis is initiated by direct injury (e.g. ischaemia and viral infection), haemodynamic insult (e.g. hypertension and pressure-overload), in addition to neurohormonal stimulation (e.g. angiotensin II, Ang II).[3] A milieu of cells such as cardiomyocytes, cardiac fibroblasts, inflammatory cells, and endothelial cells, in addition to bioactive molecules such as chemokines/cytokines and extracellular matrix factors, are involved in this response.[4] Deciphering the underlying mechanisms of this process has proven difficult in part due to the

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complicated cell-to-cell hierarchy and interactions that are involved.[5] Recent progress has been made to understand the origin of myofibroblasts by fate mapping; however, mechanisms and regulation of cell-to-cell interaction and communication within the heart and its constituent cells in cardiac fibrosis remain unclear.[6,7] A better understanding of regulatory and mechanistic processes is important as they would provide direct therapeutic targets for modulation by drugs and/or antibody therapy in the prevention and treatment of end-organ damage.

Krüppel-like factors (KLFs) are transcription factors that play diverse roles in tissue remodelling in response to pathological stimuli such as pressure-overload and neurohormonal factors (e.g. Ang II).[8–11] KLF6 has been reported to regulate the hepatic stellate cell that plays a central role in liver fibrosis,[12] and hepatic cell-specific deletion of this factor has been reported to show reduced severity of liver lipofibrosis.[13] In the cardiovasculature, homozygous mutation of *Klf6* results in embryonic lethality with diminished yolk sac vasculature, suggesting involvement in cardiovascular development.[14] We therefore hypothesized that KLF6 might regulate fibrosis of the heart and investigated roles of KLF6 in regulating pathological cardiac fibrosis in the present study, and find that KLF6 in cardiomyocytes regulates cardiac fibrosis through transcriptional control of the extracellular matrix (ECM) factor, thrombospondin 4 (TSP4), in response to Ang II stimulation. Through our studies, we identify a new intercellular regulatory mechanism whereby cardiomyocytes modulate cardiac fibroblast functions and the fibrotic/remodelling response.

2. Methods

2.1 Animals

Mice were housed in temperature-controlled rooms with a 12-h light/12-h dark cycle. All care and experimental procedures of animals were in accordance with the guidelines for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH Publication, eighth edition, 2011) and subjected to prior approval by the local animal protection authority in the University of Tokyo Ethics Committee for Animal Experiments (reference no. P10-040).

Heterozygous deletion of KLF6 (*Klf6*$^{+/-}$) in C57BL/6J mice was generated as previously described.[15] To generate cardiomyocyte- and cardiac fibroblast-specific *Klf6* knockout mice, *Klf6*$^{flox/-}$ and *Klf6*$^{flox/flox}$ mice were, respectively, cross-bred with *αMHC-Cre* and *Postn-Cre* mice. *Tsp4* knockout mice were obtained from The Jackson Laboratory and further back-crossed with C57BL/6J mice.

2.2 Ang II induced cardiac fibrosis

To induce cardiac fibrosis, mice were subjected to continuous Ang II infusion as described previously.[9] Briefly, mice (8- to 10-week-old males) were anaesthetized with intraperitoneal administration of xylazine (5 mg/kg) and ketamine (100 mg/kg). Ang II (Wako, Osaka, Japan) dissolved in 0.15 mol/L of NaCl and 1 mmol/L of acetic acid solution was subcutaneously administered by an osmotic pump (Alzet model 2002, Alza Corp., Mountain View, CA, USA) at a rate of 3.2 mg/kg/day for 3–14 days to 8- to 10-week-old mice. After 14 days of Ang II treatment, mice were sacrificed via cervical dislocation under isoflurane (Baxter, Deerfield, IL, USA) anaesthesia (1.5%), and hearts were immediately harvested for further experiments.

2.3 Pressure-overload induced by a transverse aortic constriction model

Transverse aortic constriction (TAC) was performed as described previously.[10] Briefly, mice (8- to 10-week-old males) were anaesthetized with intraperitoneal administration of xylazine (5 mg/kg) and ketamine (100 mg/kg). The mice were then intubated and ventilated with a tidal volume of 0.4 mL room air at 100 breaths/min. After mid-sternal thoracotomy, the transverse portion of the aortic arch (between the innominate and left common carotid arteries) was ligated by a 10.0-silk suture tied firmly three times against a 26-gauge blunted needle. The thoracic cavity was closed by sternum and skin sutures. Mice were sacrificed via cervical dislocation under isoflurane anaesthesia (1.5%), 14 days after the operation and hearts were immediately harvested for further experiments.

2.4 Echocardiographic analysis

Mice were lightly anaesthetized with 3% inhaled isoflurane and set in a supine position. Two-dimensional (2D) M-mode and Doppler echocardiography was performed with a Vevo2100 Imaging System (Visual Sonics, Inc., Toronto, Canada) equipped with an 18–38 MHz MicroScan^{TM} transducer. The left ventricle (LV) at the papillary muscle level was imaged in 2D mode in the parasternal short-axis view. LV diastolic posterior wall thickness (PWd), interventricular septal thickness at end-diastole (IVSd), LV diastolic dimension (LVDd), and LV end-systolic dimension (LVDs) were measured. LV fractional shortening and ejection fraction were calculated with equipped software.

2.5 Histological analysis and immunohistochemistry

Hearts from mice were embedded in paraffin, and 5 μm thick sections were prepared for Masson's trichrome (MA) staining and immunostaining. Digital images of MA-stained heart were used for measurement of fibrotic area with both Photoshop software (7.0, Adobe, Mountain View, CA, USA) and Image J software (NIH, http://rsbweb.nih.gov/ij/). For immunohistochemistry, after deparaffinization and blocking, mouse heart sections were incubated with KLF6 antibody (Santa Cruz Biotechnology or Biolegend). Secondary antibodies conjugated with horseradish peroxidase (Dako) and 3,3′-diamiobenzidine (DAB; Dako) were used to confirm labelling and counterstained with haematoxylin.

2.6 Immunofluorescent staining

For visualizing KLF6 localization in primary cultured rat neonatal cardiomyocytes and cardiac fibroblasts, anti-rabbit KLF6 antibody (Biolegend) and anti-mouse Troponin T antibody (Thermo Fischer) were used as primary antibodies, and then followed by corresponding secondary antibodies: Alex Fluor 488 or 635 (Life Technologies) was used for fluorescent labelling. Nuclear staining was performed with Hoechst33258 after the final series of washes. Finally, the specimen was mounted with Fluorescent Mounting Medium (Dako) and then visualized with a laser confocal microscope (LSM 510, Zeiss).

2.7 Microarray analysis

Mouse genome-wide gene expression analysis was performed with the Affymetrix Mouse Gene 1.0 ST Array. Whole heart RNA was extracted from wild-type and *Klf6*$^{+/-}$ mice. Total RNA was extracted by using the RNeasy fibrous tissue kit (Qiagen) and quantified. Microarray analyses were performed in duplicate from independent mice according to the standard Affymetrix Genechip protocol. Genes exhibiting significantly different expression levels in wild-type and *Klf6*$^{+/-}$ mice hearts were selected (>2.0-fold change).

2.8 Isolation of neonatal cardiomyocytes and non-myocytes

Neonatal ventricles from 1-day-old Sprague–Dawley rats euthanized by decapitation were separated and minced in ice-cold balanced salt solution as described in the Neonatal Cardiomyocyte Isolation System according to the manufacturer's instructions with minor modifications (Worthington Biochemical). To isolate cardiac fibroblasts and cardiomyocytes, minced

heart was incubated in a balanced salt solution containing 300 U/mL of collagenase type 2 for 30–45 min at 37°C with agitation. The digestion steps were repeated 10 times, and the collected cell suspension was mixed with 10% volume of chilled FCS and then pelleted by centrifugation. The pellet was dissolved in chilled FCS and kept at 4°C for 12 h. Differentiation of myocytes from non-myocytes was performed by the discontinuous Percoll gradient method.[16] Balanced salt solutions containing 40.5 or 58.5% of Percoll (Sigma-Aldrich) were prepared. The cardiac cell suspension was placed onto the layer of 58.5% of Percoll solution. After centrifugation (1200 *g* at room temperature for 30 min), the cardiomyocytes gathered in the interface layer, and the cardiac fibroblasts migrated to the layer of 40.5% of Percoll solution. The purified cardiomyoctes were then passed onto gelatin-coated 12-well culture plates at a density of 2×10^5cells/well in Medium 199 (Gibco and Life Technologies) supplemented with 10% FCS. Preserved angiotensin receptor expression in isolated cardiomyocytes was confirmed by RNA and protein expression levels (see Supplementary material online, *Figure S6A* and *B*).

2.9 RNA extraction and real-time PCR

For RNA extraction and purification, we used the RNeasy mini Kit for primary cultured cells, the RNeasy Fibrous Tissue Kit for heart samples, and the RNeasy Plus Micro Kit (all Qiagen) for FACS-sorted cells according to the manufacturer's protocols. First-strand cDNA was synthesized from 1 μg (tissue and cultured cells) or 30 ng (FACS-sorted cells) of total RNA, random primers, and SuperScript III Reverse Transcriptase (Invitrogen). Real-time PCR was performed with a LightCycler 480 SYBR Green I Master (Roche Applied Science) in a LightCycler 480 instrument according to the manufacturer's instructions (Roche Applied Science). The expression level of each gene was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (Gapdh), which served as an endogenous internal control. The sequences of the PCR primers were as follows: *Gapdh*, forward 5′-AGGTCGGTGTGAACGGATTTG-3′ and reverse 5′-TGTAGACCATGTAGTTGAGGTCA-3′; *Klf6*, forward 5′-TATCTTCAGGATGAGCCCTGCTAC-3′ and reverse 5′-AGACTTCACCAATGGGATCAGAGG-3′; *Tsp4*, forward 5′-TGGAAGGACTCCAGGAATGT-3′ and reverse 5′-TCATAAAAGCGCACCCTGA-3′; transforming growth factor-β1 (*Tgfb1*), forward 5′-CGTTACAGTGTTTCTGCCACCT-3′ and reverse 5′-AGACGAAGCACACTGGTCCAGC-3′; connective tissue growth factor (*Ctgf*), forward 5′-CTAAGACCTGTGGGATGGGC-3′ and reverse 5′-CTCAAAGATGTCATTGTCCCC-3′; collagen-1 (*Col1a*), forward 5′-TGGAGACAGGTCAGACCTG-3′ and reverse 5′-TATTCGATGACTGTCTTGCC-3′; matrix metalloproteinase 9 (*Mmp9*), forward 5′-ATCTCTTCTAGAGACTGGGAAGGAG-3′ and reverse 5′-AATAAAAGGTCAGAATCCACCCTAC-3′; smooth muscle α-actin (*Acta2*), forward 5′-AGCTGTTTTCCCATCCATTG-3′ and reverse 5′-GCGCTTCATCACCCACGTAG-3′.

2.10 Western blot analysis

Mouse heart specimens were homogenized with T-PER protein extraction buffer (Thermo Scientific) containing a protease inhibitor cocktail (Roche) and phosphatase inhibitors (Roche). Cultured cells were homogenized with M-PER protein extraction buffer (Thermo Scientific) containing a protease inhibitor cocktail (Roche) and phosphatase inhibitors (Roche). Protein concentration was assayed by the BCA protein assay kit (Pierce), and 5 or 10 μg of the protein was resolved by 10 or 12% of NuPAGE gels (Invitrogen) and then transferred to the polyvinylidene difluoride membrane (Invitrogen). The blot was probed with primary antibodies against KLF6 (Santa Cruz Biotechnology or Biolegend) and TSP4 (R&D). Membranes were washed and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). The blot for GAPDH served as an internal control for protein loading. Labelling bands were detected by ECLplus (Thermo Scientific).

2.11 Preparation of plasmid constructs and recombinant adenoviruses

The KLF6 expression vector pCAG-KLF6 was obtained by inserting the KLF6-coding cDNA into pCAGMS vector.[9] The adenoviral KLF6 construct was constructed as previously described.[17] Primary cultured cardiomyocytes from neonatal rats were infected with adenoviral KLF6 or backbone adenovirus at 100 m.o.i. for 12 h with serum-free Medium 199. The cells were then stimulated with Ang II (10 μM) in fresh medium for 12 h, and the cells were subjected to western blot.

2.12 Co-transfection reporter assay

Co-transfection reporter assay was performed as previously described. Primary cultured rat neonatal cardiomyocytes (1.25×10^5 cells/well in 24-well plates) were transfected with 200 ng of pGL3 luciferase reporter vector containing the *Tsp4* promoter region construct and 200 ng of pCAG-KLF6 and Lipofectamine 2000 (Invitrogen).[10] Luciferase activity was measured with a luciferase assay system (Promega) and then normalized to the protein concentration in each cell lysate. Assays were done in duplicate, and expression levels of KLF6 were confirmed by immunoblotting with anti-KLF6 antibody (Santa Cruz Biotechnology).

2.13 Chromatin immunoprecipitation assays

Chromatin immunoprecipitation assays were performed as previously described with rat neonatal cardiomyocytes stimulated with Ang II (10 μM) for 24 h prior to crosslinking for 10 min with 1% formaldehyde.[10] Chromatin was sheared by sonication to an average size of 200–1000 bp (Covaris). Immunoprecipitation was performed with anti-KLF6 antibody (Santa Cruz Biotechnology) and rabbit IgG antibody (Santa Cruz Biotechnology). PCR amplification of the *Tsp4* promoter region spanning KLF-binding elements was performed using the following primers: forward: 5′- TCCGTTGTGGTCCTCTCTGCCAT-3′ and reverse 5′- CGCTTTATGGTCCAGCCACCCG-3′.

2.14 Cardiac fibroblast modulation by TSP4

Modulation of cardiac fibroblast activity by matricellular TSP4 protein was evaluated as previously described with minor modification.[18] Briefly, six-well culture plates were coated with 0.1% gelatin (Wako) or 50 μg/mL of mouse recombinant TSP4 (R&D systems) for 20 h at 4°C and then blocked with 0.5% polyvinylpyrrolidone (Sigma) for 1 h at room temperature. Primary cultured cardiac fibroblasts from neonatal rats (1.5×10^5/well) were seeded with serum-free Medium 199 (Gibco). Twelve hours after passage, the cells were stimulated with Ang II (10 μM) for 24 h and were harvested for detection of mRNA expression levels.

2.15 Integrin inhibition assay

Integrin-blocking effect was assayed according to a previously described method.[18] Cardiac fibroblasts from wild-type mice were pretreated for 20 min at 37°C with blocking antibodies to integrins and plated on recombinant TSP4 -coated plates then stimulated with Ang II (10 μM) for 24 h. Integrin-blocking antibodies: rabbit anti-integrin $\beta 3$ (Biolegend), rat anti-$\alpha 5\beta 1$, anti-$\alpha 4$ (Chemicon), and anti-αM (clone M1/70, Biolegend).

2.16 Statistical analysis

All data are presented as mean ± SD. Difference between two groups was analysed by the Welch's *t* test. Comparisons between multiple groups were done using one- or two-way ANOVA followed by a *post hoc* Bonferroni test. A *P*-value of <0.05 was considered significant.

3. Results

3.1 *Klf6* haploinsufficiency results in reduced cardiac fibrosis and preserved cardiac function

Heterozygous deletion of *Klf6* (*Klf6*$^{+/-}$) showed, in the heart, markedly decreased fibrotic deposition in response to Ang II overload (*Figure 1A*, left panels), and decreased interstitial fibrosis compared with that of wild-type mice (quantitative analysis, *Figure 1B*), whereas perivascular fibrosis showed no apparent difference (*Figure 1A*, right panels). These results were also confirmed by biochemical analysis (see Supplementary material online, *Figure S6D*). Additionally, haemodynamic analysis showed blood pressure-independent fibrotic effects by Ang II stimulation (see Supplementary material online, *Figure S1A–C*). Furthermore, heart weight after Ang II stimulation was increased in the wild-type group compared with that of *Klf6*$^{+/-}$ mice (*Figure 1C*). Functionally, the heart of *Klf6*$^{+/-}$ mice exhibited lower end-diastolic pressure (see Supplementary material online, *Figure S1D* and *E* and Supplementary material online, *Table S1*), preserved left ventricular geometries, and systolic and diastolic function by echocardiographic examination after Ang II treatment (*Figure 1D* and *E*, and see Supplementary material online, *Figure S2A* and *E*). Reduced cardiac fibrosis of *Klf6* knockout mice was accompanied by decreased expression levels of fibrotic regulatory genes such as *Tgfb1*, *Ctgf*, and *Col1a* (*Figure 1F* and see Supplementary material online, *Figure S6C*). Moreover, pressure-overload by TAC to wild-type and *Klf6*$^{+/-}$ mice did not show difference in cardiac fibrosis (*Figure 1G* and *H*), relative heart weight (*Figure 1I*), haemodynamic analysis (see Supplementary material online, *Figure S1F–H* and Supplementary material online, *Table S1*), or echocardiographic function (see Supplementary material online, *Figure S2D*). To note, Ang II treatment or TAC for 14 days induced significant cardiac fibrosis in wild-type mice. However, attenuated cardiac fibrosis was detected only in Ang II treated, but not in TAC-treated, *Klf6*$^{+/-}$ mice.

At the cellular level, immunohistochemical studies showed that KLF6 was specifically up-regulated in the nuclei of cardiomyocytes but not in those of cardiac fibroblasts under conditions of cardiac fibrosis (*Figure 2A* and *B*). KLF6 was markedly induced in cardiomyocytes but not in cardiac fibroblasts as early as 1 day after Ang II stimulation, and this induction continued during stimulation, thus suggesting that KLF6 may regulate the cardiac fibrotic process not through the cardiac fibroblast but by modulation of cardiomyocytes. Specific expression of KLF6 was further confirmed using cultured cardiomyocytes and fibroblasts in which KLF6 induction was seen after treatment with Ang II in cardiomyocytes but not in fibroblasts (*Figure 2C* and *D*). Collectively, these results show that cardiomyocyte KLF6 participates in cardiac fibrosis induced by Ang II but not by TAC.

3.2 Cardiomyocyte KLF6 expression is essential for cardiac fibrosis

To further clarify the role and importance of cardiomyocyte-specific expression of KLF6, cell type-specific *Klf6*-deleted mice were generated including cardiomyocyte-specific (*Klf6*$^{flox/-}$;*αMHC-Cre*) and cardiac fibroblast-specific (*Klf6*$^{flox/flox}$;*Postn-Cre*)-deleted mice. Deletion of KLF6 in these conditional knockout mice was confirmed by western blot and RT-PCR analysis after cell separation by flow cytometry (*Figure 3E* and *F*, and see Supplementary material online, *Figure S3A–C*). At baseline, both *Klf6*$^{flox/-}$;*αMHC-Cre* and *Klf6*$^{flox/flox}$;*Postn-Cre* mice showed comparable cardiac function (see Supplementary material online, *Figure S2B* and *C*). Only cardiomyocyte-specific but not the fibroblast-specific *Klf6*-deleted mice showed decreased cardiac fibrotic deposition in response to Ang II stimulation (*Figure 3A–D*) which was reminiscent if not identical to the phenotype observed in the heart of *Klf6*$^{+/-}$ mice. Biochemical analysis also confirmed significantly decreased fibrotic content in *Klf6*$^{flox/-}$;*αMHC-Cre* mouse hearts (see Supplementary material online, *Figure S6D*). Expression levels of fibrotic marker genes (*Tgfb1*, *Ctgf*, and *Col1a*) were also decreased in the hearts of these mice, but neither in those of control nor fibroblast-specific *Klf6*-deleted mice (*Figure 3F* and see Supplementary material online, *Figure S3D*). Functional analysis by echocardiography further confirmed that *Klf6*$^{flox/-}$;*αMHC-Cre* mice showed lack of functional impairment with preserved left ventricular geometries and systolic function (*Figure 3G* and *H*). *Klf6*$^{flox/-}$;*αMHC-Cre* mice did not show difference in systolic blood pressure under Ang II treatment or pressure-overload compared with *Klf6*$^{+/-}$ mice (see Supplementary material online, *Figure S1I* and *J*).

These results showed indispensability of KLF6 expression by cardiomyocytes for cardiac fibrotic progression, and a possible signalling pathway from cardiomyocytes to cardiac fibroblasts in this process.

3.3 *Tsp4* is a direct target of KLF6 and has cardioprotective properties

To identify the factor(s) that originate in cardiomyocytes to mediate cardiac fibrosis, we analyzed genome-wide gene expression profiles for the whole heart between wild-type mice and *Klf6*$^{+/-}$ mice under Ang II stimulation (*Table 1*). Expression levels of candidate factors were screened for genes encoding secreted proteins whose expression levels were differently changed between the wild-type and knockout mice by RT-PCR analysis. As TSP4 has been reported to prevent excess cardiac fibrosis and dysfunction by pressure-overload,[19,20] we hypothesized that increased *Tsp4* expression (3.613 up-fold change) might be involved in attenuated cardiac fibrosis in *Klf6*$^{+/-}$ mice under Ang II stimulation (*Table 1*). We therefore proceeded to test whether *Tsp4* is a target gene of KLF6. mRNA and protein levels of TSP4 were markedly up-regulated in the hearts of both *Klf6*$^{+/-}$ (*Figure 4A* and *C*) and *Klf6*$^{flox/-}$;*αMHC-Cre* mice (*Figure 4B* and *D*) after stimulation. Overexpression of KLF6 in cardiomyocytes resulted in decreased TSP4 expression levels in cell lysate and also attenuated secretion into medium (*Figure 4E*). Immunostaining for TSP4 showed this protein to be localized in the interstitial space of the wild-type heart with Ang II stimulation; however, increased expression levels in cardiomyocytes were recognized in the *Klf6*$^{flox/-}$;*αMHC-Cre* mice (see Supplementary material online, *Figure S4C* and *D*). Primary cultured cardiomyocytes also expressed TSP4 after Ang II treatment (see Supplementary material online, *Figure S4E*). At the molecular level, the *Tsp4* promoter contains four putative KLF-binding motifs (Sp1/Egr-1) at −290 bp from the transcriptional start site (see Supplementary material online, *Figure S4A*). Chromatin immunoprecipitation experiments showed direct recruitment of KLF6 to this region under Ang II stimulation (*Figure 4F*). Furthermore, reporter analysis of the *Tsp4* promoter showed dose-dependent repression by KLF6 overexpression (*Figure 4G*), and mutational analysis of the *Tsp4* promoter region revealed that this effect was attributed to the Sp1 site most proximal to the transcription start site (see Supplementary material online, *Figure S4B*). KLF6, therefore, appears to be a negative regulator of *Tsp4* transcription.

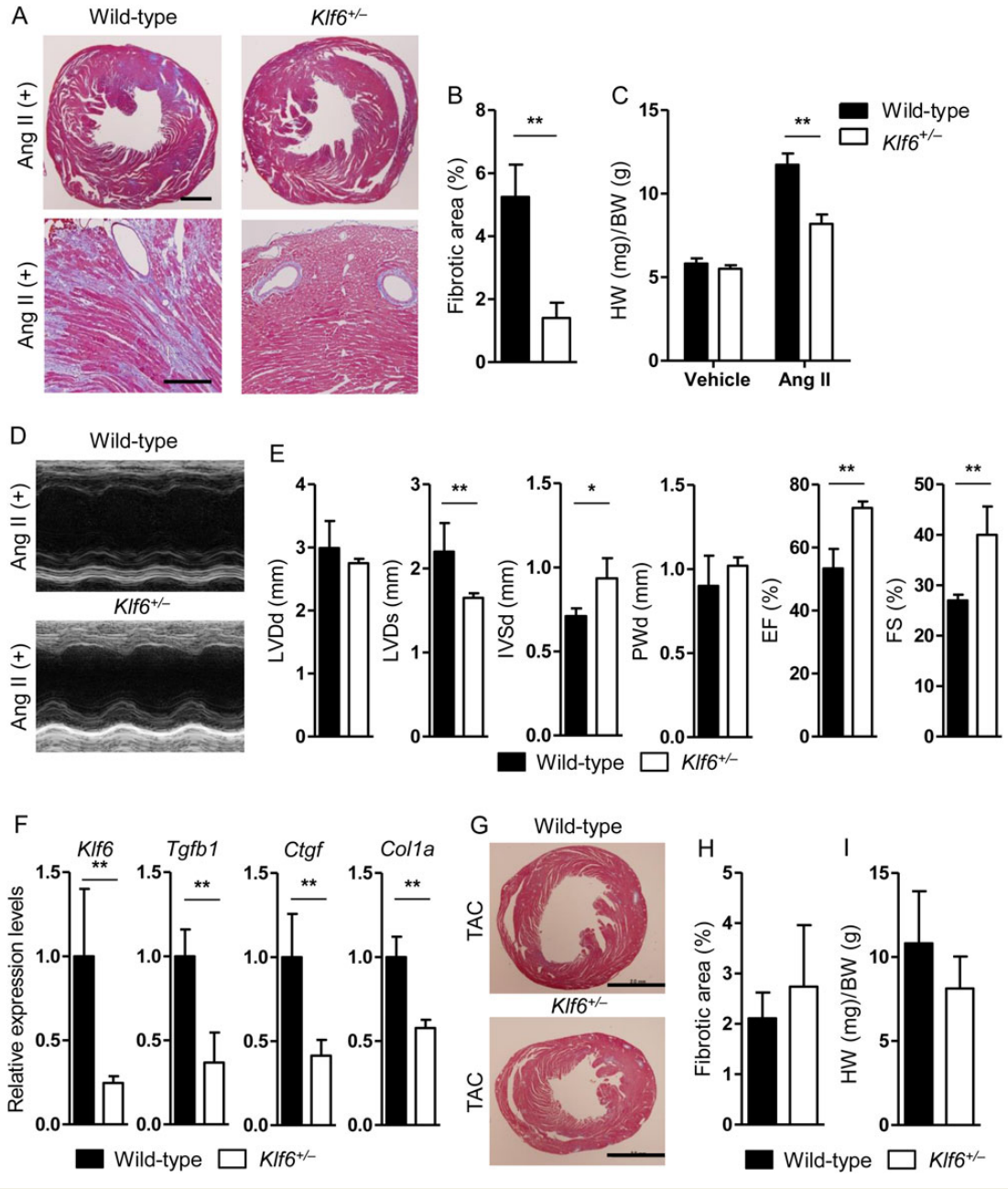

Figure 1 *Klf6* haploinsufficiency resulted in decreased cardiac fibrosis and preserved cardiac function. (*A* and *B*) *Klf6*$^{+/-}$ mice showed markedly decreased interstitial cardiac fibrosis compared with wild-type mice after continuous Ang II stimulation for 14 days, and heart samples were stained with Masson's trichrome. Representative figures are shown. Scale bar: 2.0 mm (upper panels), 500 μm (lower panels). (*C*–*E*) *Klf6*$^{+/-}$ mouse hearts showed preserved left ventricular function and size, whereas wild-type mouse hearts showed decreased function and dilated left ventricular size by continuous Ang II infusion. (*F*) Whole heart mRNA was subjected to quantitative RT-PCR. *Klf6*$^{+/-}$ mouse hearts showed decreased expression levels of *Tgfb1*, *Ctgf*, and *Col1a*. (*G*) Representative figures of wild-type and *Klf6*$^{+/-}$ mouse hearts subjected to pressure-overload (TAC) and stained with Masson's trichrome. Scale bar: 2.0 mm. (*H* and *I*) There was no significant difference between wild-type and *Klf6*$^{+/-}$ mouse hearts in cardiac fibrosis and cardiac hypertrophy by TAC. HW, heart weight; BW, body weight. Data are presented as mean ± SD. $n = 3$–6 per group. *$P < 0.05$, **$P < 0.01$.

To clarify the effect of secreted TSP4 from cardiomyocytes on cardiac fibroblasts under Ang II -administered conditions, murine cardiac fibroblasts were treated with recombinant murine TSP4 and further cultured with Ang II stimulation for 24 h. Expression levels of fibroblast activation markers (*Mmp9*, *Tgfb1*, *Ctgf*, and *Col1a*) were markedly decreased in the TSP4 group (*Figure 5A*). We further carried out experiments using transfer of conditioned medium. Wild-type cardiac fibroblasts were cultured with medium enriched by wild-type or *Tsp4KO* cardiomyocytes. Incubation with medium from *Tsp4KO* cardiomyocytes showed higher activation, and this effect was also confirmed by co-culture of cardiac fibroblasts with *Tsp4KO* cardiomyocytes (*Figure 5B* and see Supplementary material online, *Figure S4F*). Suppression of cardiac fibroblast activation by TSP4 was thought to be due partly to negative regulation of FAK phosphorylation through

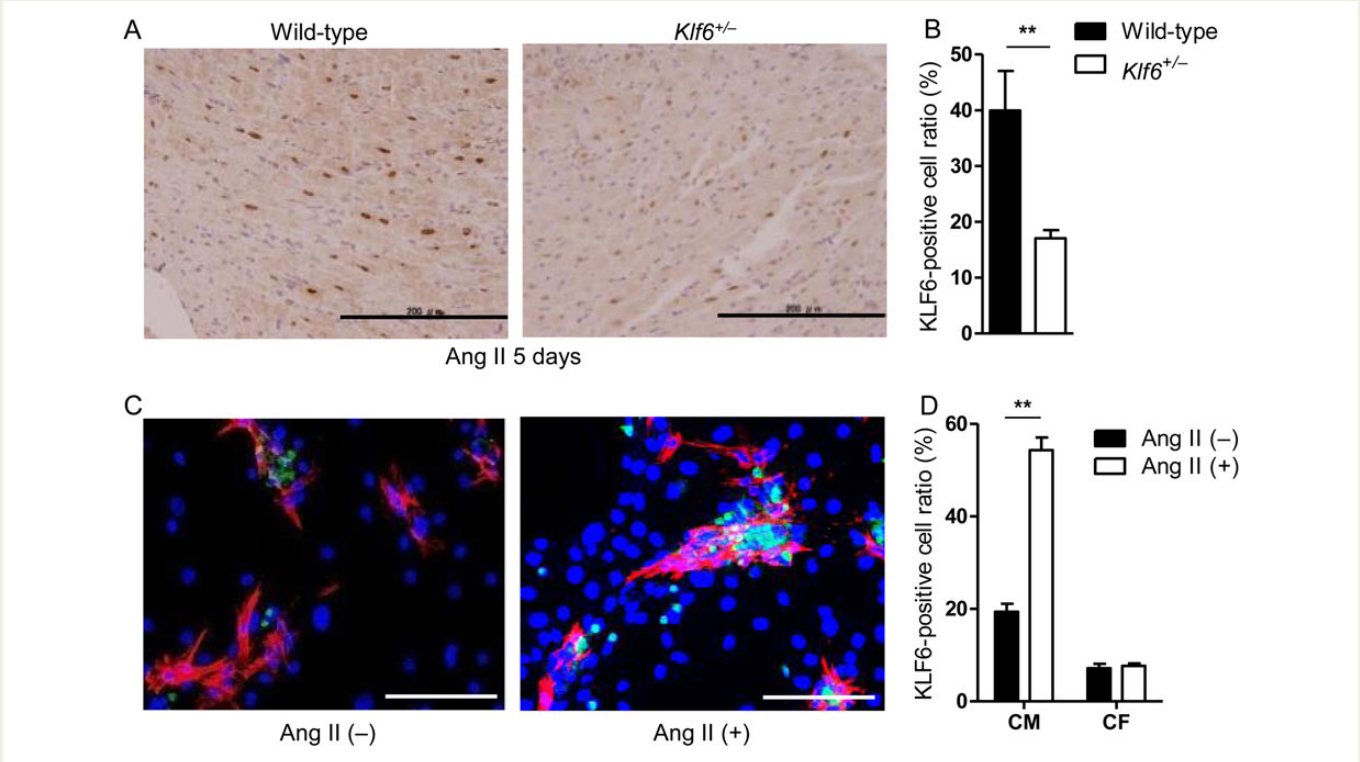

Figure 2 KLF6 expression increased in cardiomyocytes, but not in cardiac fibroblasts, after Ang II stimulation. (*A* and *B*) Immunohistochemistry showed KLF6 up-regulation (brown stained) in cardiomyocytes of wild-type, but not in *Klf6*$^{+/-}$ mice, after Ang II stimulation. Scale bar: 200 μm. Note that KLF6 was up-regulated in stimulated myocardium, specifically in the nuclei of cardiomyocytes. (*C* and *D*) Primary cultured rat neonatal cardiomyocytes and cardiac fibroblasts were stimulated by Ang II and subjected to immunofluorescent staining. After stimulation, KLF6 (green) was selectively expressed in cardiomyocytes. Green fluorescence, KLF6; red fluorescence, Troponin T. Scale bar: 50 μm. Data are presented as mean ± SD. $n = 3–4$ per group. $^{**}P < 0.01$.

the integrin pathway (*Figure 5C* and *D*). KLF6 deletion in the myocardium did not affect apoptosis, while proliferation of non-cardiomyocyte cells was reduced. This was thought to be due to reduced cardiac fibroblast activity (see Supplementary material online, *Figure S5A–C*).

Taken together, our results showed that KLF6 in cardiomyocytes modulates cardiac fibrosis induced by Ang II stimulation through negative transcriptional control of TSP4 expression.

4. Discussion

The present study suggests that KLF6 in cardiomyocytes plays a pivotal role in progression of cardiac fibrosis through modulation of the ECM protein, thrombospondin 4 (TSP4), to regulate fibroblast activation under Ang II stimulation. Our findings may provide new physiological/pathological aspects in cell-to-cell communication between cardiomyocytes and fibroblasts in the heart.

Both whole body and cardiomyocyte-specific deletion of KLF6 resulted in decreased cardiac fibrosis accompanied by preserved cardiac function and increased expression levels of TSP4. Of interest, a phenotype of aggravated fibrosis has been reported in *Tsp4* knockout mouse heart under pressure-overload.[19,20] Although their results are based on TAC treatment and ours are on Ang II treatment, cardioprotective effects of TSP4 against cardiac fibrosis are consistent. Several humoral factors such as TGFβ*1*,[21–24] FGFs,[25,26] and gp130/IL6 family factors[27–30] have been reported to mediate cardiac intracellular regulation. In the present study, we showed that the ECM protein, TSP4, is centrally involved in intercellular regulation of cardiac fibrosis through the cardiomyocyte to fibroblast, at least through actions of KLF6.

KLF transcription factors are known to be involved in a wide spectrum of cardiac pathologies ranging from heart development[31] to remodelling[32] and in response to internal and/or external stimuli.[8] Disruption of KLF13 results in perinatal lethal abnormalities of heart development,[33] and KLF15 deletion exhibits eccentric cardiac hypertrophy in response to pressure-overload.[34] KLF5 has also been shown to be expressed specifically in the cardiac fibroblast, and to regulate cardiomyocyte hypertrophy through IGF-1 secreted from cardiac fibroblasts.[10] In the present study, we showed that KLF6 in cardiomyocytes regulates activation of cardiac fibroblasts through ECM protein secretion. Others have shown that cardiomyocyte-specific KLF4 promotes enhanced cardiomyocyte hypertrophy in response to β-adrenergic stimulation.[35] These accumulating data indicate the importance of understanding the role of KLFs in a single-cell manner to decipher cell-to-cell regulation in cardiac pathologies. Our findings add to our understanding of the collective and cooperative roles that the KLF-related network plays in the cardiac stress response.

TSP4 has been reported to exert a cardioprotective role under pressure-overload,[36,37] and *Tsp4* knockout mice show accelerated cardiac fibrosis under such conditions.[19] Additionally, intracellular TSP4 located in the endoplasmic reticulum (ER) has been reported

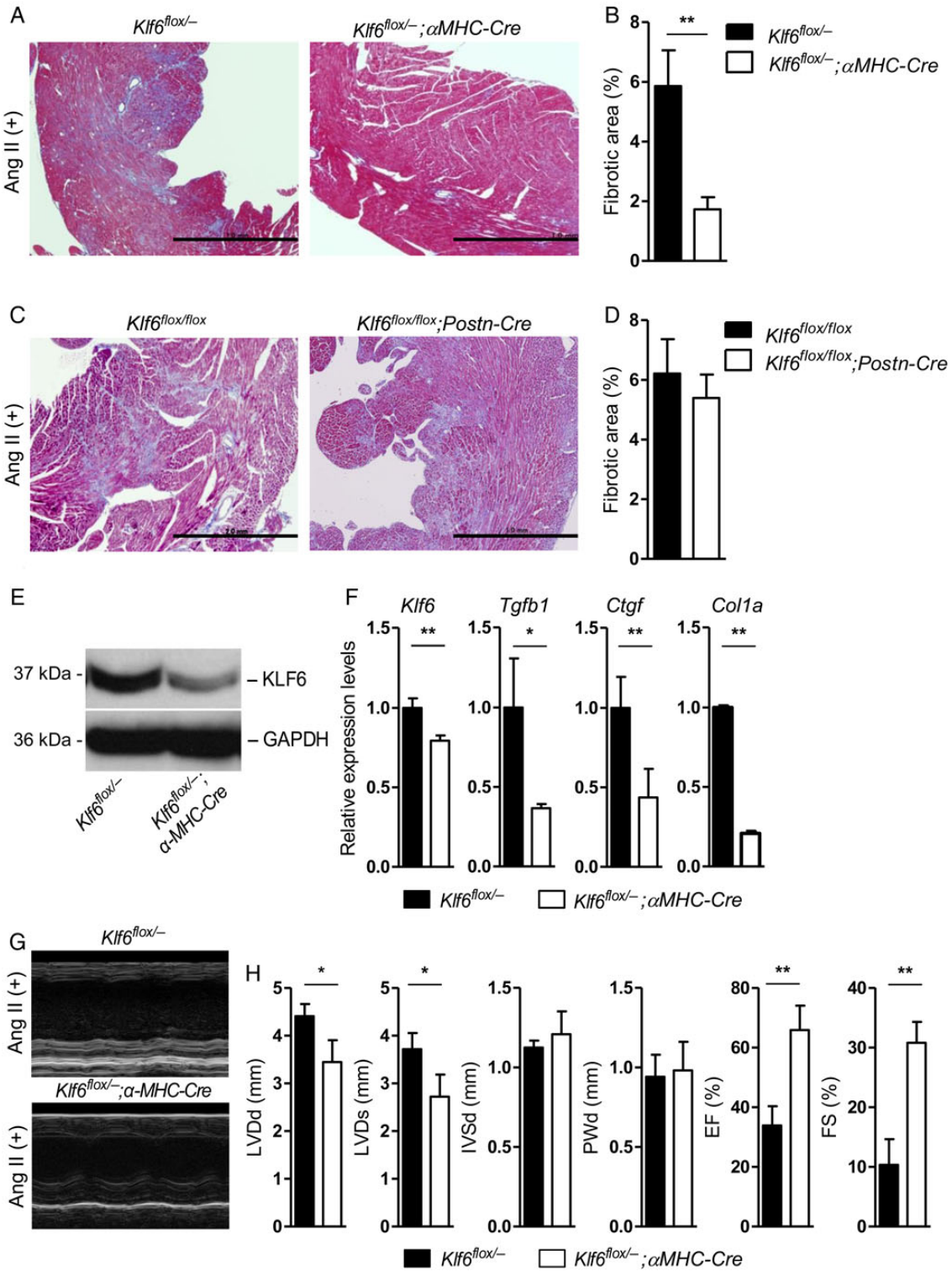

Figure 3 Expression of KLF6 in cardiomyocytes is essential for cardiac fibrosis. (*A*–*D*) Conditional deletion of *Klf6* in the cardiomyocyte (*Klf6*$^{flox/-}$;*αMHC-Cre*) and cardiac fibroblast (*Klf6*$^{flox/flox}$;*Postn-Cre*). Both mice were subjected to Ang II stimulation for 14 days, and cardiac fibrosis was analysed. *Klf6* deletion in cardiomyocytes showed significantly less fibrosis than that in the cardiac fibroblast-specific *Klf6*-deleted mice in response to Ang II stimulation. Cardiac fibrosis area was calculated by fibrotic area/heart area, and then compared with control mice. Scale bar: 1.0 mm. (*E* and *F*) *Klf6* protein and mRNA expression levels were significantly decreased in cardiomyocyte-specific *Klf6*-deleted mice heart. (*F*) Whole heart mRNA was subjected to quantitative PCR. Cardiomyocyte-specific *Klf6*-deleted mouse hearts also showed decreased expression levels of *Tgfb1*, *Ctgf*, and *Col1a*. (*G* and *H*) *Klf6*$^{+/-}$ mouse hearts showed preserved left ventricular function and size, whereas wild-type mouse hearts showed decreased function and dilated left ventricular size by continuous Ang II infusion. Data are presented as mean ± SD. $n = 3$–5 per group. $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 1 Microarray data analysis

Genbank no.	Gene description	Gene symbol	Fold change	Regulation
BC080666	Cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	Cilp	4.961	Up
D13664	Periostin, osteoblast-specific factor	Postn	4.923	Up
BC015260	FK506-binding protein 5	Fkbp5	4.408	Up
BC023373	Angiopoietin-like 7	Angptl7	3.866	Up
AF102887	Thrombospondin 4	Tsp4	3.613	Up
AF180805	Microfibrillar-associated protein 5	Mfap5	2.727	Up
M64086	Serine (or cysteine) peptidase inhibitor, clade A, member 3N	Serpina3n	2.704	Up
BC014722	Secreted frizzled-related protein 2	Sfrp2	2.689	Up
BC051649	Elastin	Eln	2.653	Up
BC002043	Cyclin-dependent kinase inhibitor 1A (P21)	Cdkn1a	2.648	Up
BC116846	Synaptotagmin XII	Syt12	2.616	Up
BC042422	Thrombospondin 1	Tsp1	2.581	Up
BC034888	Asporin	Aspn	2.522	Up
M65142	Lysyl oxidase	Lox	2.383	Up
BC031758	Metallothionein 2	Mt2	2.381	Up
BC131907	Collagen triple helix repeat containing 1	Cthrc1	2.366	Up
BC022666	Microfibrillar-associated protein 4	Mfap4	2.354	Up
L38990	Glucokinase	Gck	2.262	Up
AF033530	Cartilage oligomeric matrix protein	Comp	2.177	Up
BC055077	Collagen, type V, alpha 2	Col5a2	2.171	Up
X04684	Tissue inhibitor of metalloproteinase 1	Timp1	2.158	Up
BC058275	Platelet-derived growth factor receptor-like	Pdgfrl	2.129	Up
AB021861	Mitogen-activated protein kinase kinase kinase 6	Map3k6	2.128	Up
BC064779	Fibromodulin	Fmod	2.116	Up
BC019946	Activating transcription factor 3	Atf3	2.116	Up
BC061695	Pleiotrophin	Ptn	2.115	Up
BC025860	Tumour necrosis factor receptor superfamily, member 12a	Tnfrsf12a	2.099	Up
BC119198	Actin-binding Rho-activating protein	Abra	2.098	Up
BC020152	Integrin, beta-like 1	Itgbl1	2.096	Up
BC054782	Heat shock protein 1A	Hspa1a	2.058	Up
Z47205	Zinc finger and BTB domain containing 16	Zbtb16	2.044	Up
BC061100	Myosin, light polypeptide 7, regulatory	Myl7	2.041	Down
BC002076	Mesenchyme homeobox 2	Meox2	2.028	Down
U88568	Frizzled-related protein	Frzb	2.023	Up
BC068150	Nuclear receptor subfamily 4, group A, member 3	Nr4a3	2.004	Up

Whole heart RNA was obtained from wild-type and *Klf6*$^{+/-}$ mice after Ang II stimulation and then subjected to microarray analysis (fold change: *Klf6*$^{+/-}$ vs. wild-type, Ang II (+), $n = 2$).

to alleviate cardiac damage by enhancing adaptation and protection to ER stress,[38] suggesting cell protective effects in both intracellular and extracellular conditions. The role of TSP4 in regulating pathological remodelling responses in the heart has therefore been the attention of recent interest, and our study identifies a new functional role to this protein.[39] To note, increased expression levels of secreted factors other than Tsp4 (*Table 1*) were also seen in the heart of *Klf6*-deleted mice, including periostin which is also known to be a cardioprotective matricellular protein necessary for maintaining fibroblast integrity.[40,41] As KLF6 is also known as an activator of the TGF-β signalling pathway,[8] we confirmed modulated TGF-β secretion and signalling (see Supplementary material online, *Figure S6E–G*). KLF6 therefore likely modulates cardiac fibrosis through a multitude of mechanisms and factors with further combinatorial regulation. Intriguingly, in liver fibrosis, down-regulation of KLF6 in stellate cells results in activation of fibrogenic genes, and hepatocyte-specific depletion did not alter fibrogenic gene expression or the extent of fibrosis.[42] Accumulating data therefore collectively suggests that KLF6 regulates tissue fibrosis in a context-dependent and cell type-specific manner in each tissue/organ with possible cross-talk regulation which will be addressed in future investigations.[43,44]

In conclusion, we demonstrated that KLF6-mediated signalling from cardiomyocytes to fibroblasts regulated cardiac fibrosis. In this signalling pathway, KLF6 regulated expression levels of the matricellular protein, thrombospondin 4 (TSP4), which further deactivated cardiac fibroblasts. As cardiac fibrosis is a central regulatory process in heart failure as well as in autoimmune disease and malignancies,[1] a better understanding of the underlying mechanisms of the fibrotic process has been anxiously awaited with expectations that this would lead to the development of specific therapies for fibrotic organ damage which is presently considered irreversible. KLF6 is likely a pivotal regulator of the fibrotic response, and further investigation of

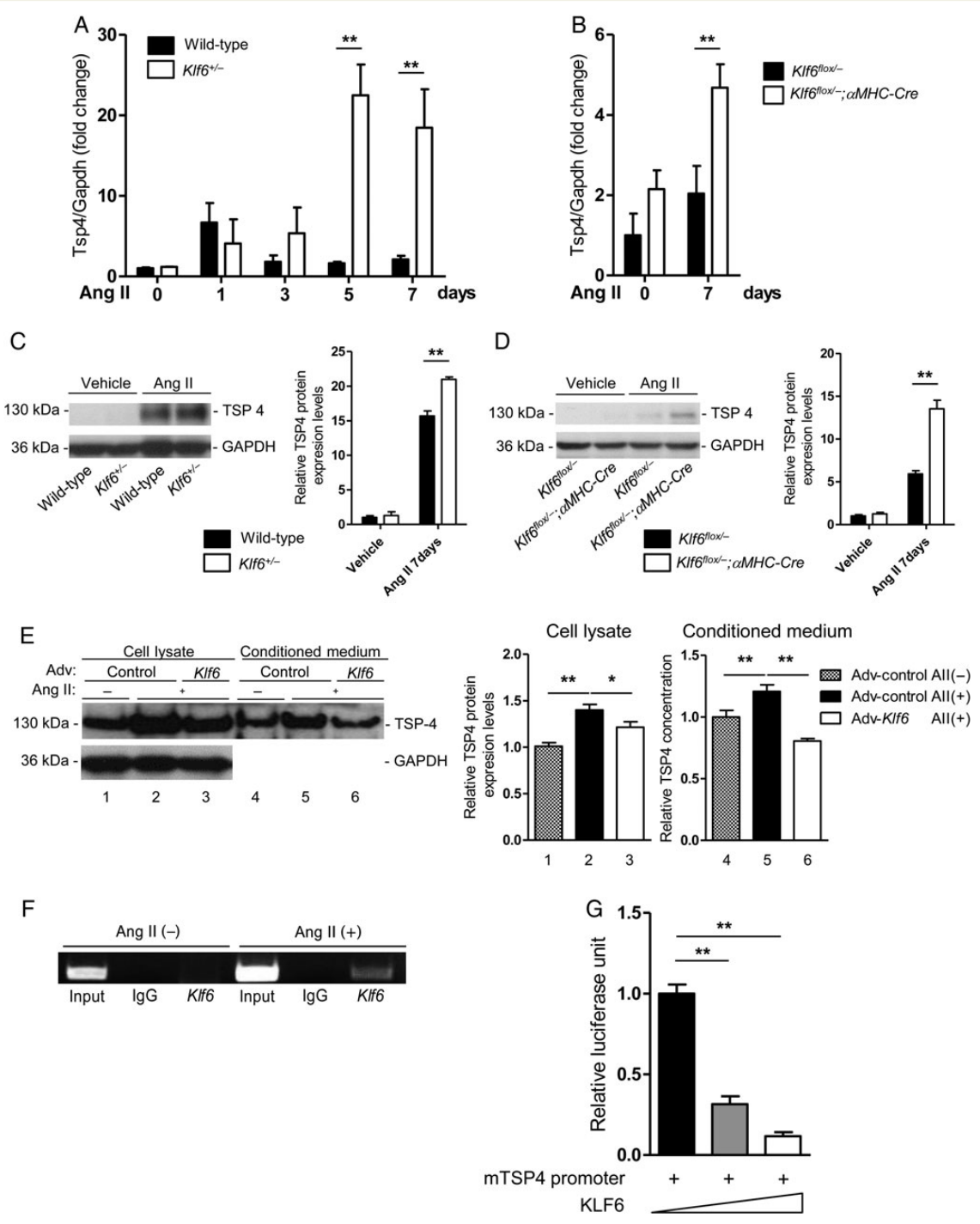

Figure 4 KLF6 suppressed TSP4 expression levels in response to Ang II stimulation. (*A* and *B*) *Tsp4* mRNA expression levels after Ang II stimulation by qRT-PCR. Whole heart samples were normalized by *Gapdh* and then by basal expression levels of wild-type mice. *Klf6*$^{+/-}$ and cardiomyocyte-specific *Klf6*-deleted mice heart showed remarkably increased TSP4 expression levels after Ang II stimulation. (*C*) TSP4 protein expression levels were assayed from wild-type or *Klf6*$^{+/-}$ mouse whole heart samples after Ang II stimulation. Right bar graph shows quantification of TSP4 protein levels normalized by GAPDH. $n = 3$. (*D*) TSP4 protein expression levels were assayed from *Klf6*$^{flox/-}$ or *Klf6*$^{flox/-}$;*αMHC-Cre* mouse whole heart samples after Ang II stimulation. Right bar graph shows quantification of TSP4 protein levels normalized by GAPDH. $n = 3$. (*E*) Primary cultured neonatal rat cardiomyocytes were infected with adenoviral KLF6 and backbone adenovirus at 100 m.o.i. for 12 h. Cells were then stimulated with Ang II (10 μM) for 12 h. Cells and medium were subjected to western blot. GAPDH was used as protein-loading control for cell lysate. Right bar graphs indicate densitometric quantification of TSP4 levels (normalized by GAPDH in the cell lysate). Assays were done in triplicate. (*F*) KLF6 was directly recruited to the *Tsp4* promoter region under Ang II stimulation. Chromatin immunoprecipitation was done with KLF6 antibody, and refined DNA samples were evaluated by PCR using primer pairs for the *Tsp4* promoter region. Input was 5% of the chromatin sample. (*G*) The KLF6 expression vector (pCAG-KLF6) and the *Tsp4* promoter reporter constructs were co-transfected and subjected to evaluation of luciferase reporter activity. Luciferase activity was normalized by protein concentration, and relative activities were calculated. Assays were done in triplicate. Data are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

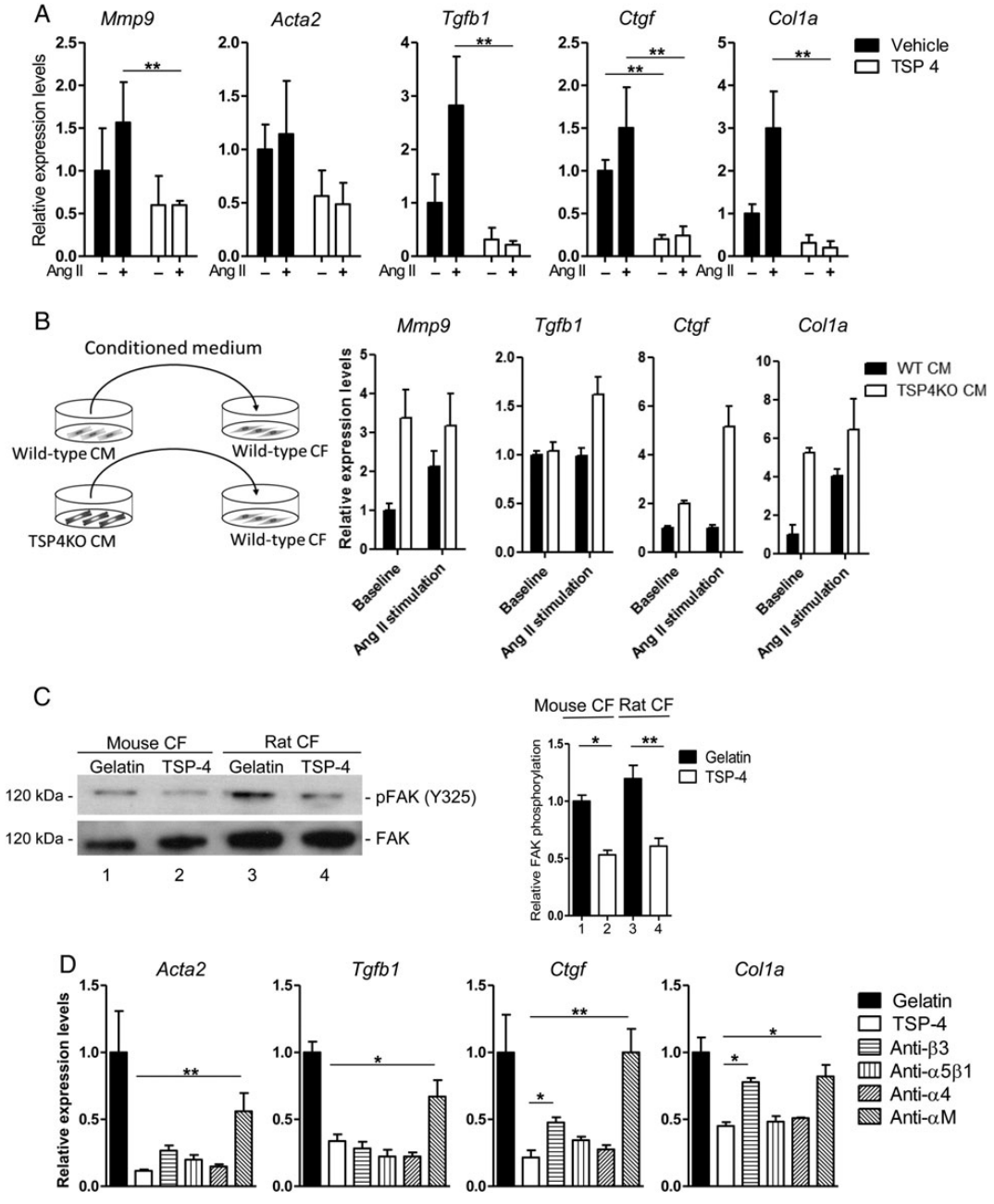

Figure 5 TSP4 negatively regulates cardiac fibroblast activity. (*A*) Murine cardiac fibroblasts (CF) were treated with recombinant murine TSP4 and further cultured with Ang II stimulation for 24 h. Expression levels of CF activation markers (*mmp9*, *acta2*, *tgfb1*, *ctgf*, and *col1a*) were compared with the vehicle group. (*B*) Schematic illustration of conditioned medium transfer assay. Purified cardiomyocytes (CM) from wild-type and *Tsp4KO* mice were incubated with Ang II for 24 h. The conditioned medium was added to culture medium of purified CF from wild-type mice, and the CF was incubated for another 24 h. Bar graphs indicate expression levels of CF activation makers as analysed by qRT-PCR. Two-way ANOVA showed a significant difference in marker gene expression levels between wild-type and *Tsp4KO* CM (*mmp9* $P = 0.0353$, *tgfb1* $P = 0.0148$, *ctgf* $P = 0.0005$, and *col1a* $P = 0.0128$). (*C*) Modulation of CF activity was evaluated using TSP4-coated plates. Primary cultured mouse and rat neonatal CFs were stimulated with Ang II (10 μM) for 24 h, and were harvested for evaluation of protein focal activated kinase (FAK) phosphorylation levels. FAK protein levels were used as a loading control. Right bar graph indicates quantification of FAK phosphorylation levels normalized by FAK and compared with the gelatin-treated group. (*D*) TSP4 exerts intracellular effects via integrin pathways. CF from wild-type mice was pretreated with blocking antibodies to integrins ($\beta3$, $\alpha5\beta1$, $\alpha4$, and αM), then plated on recombinant TSP4-coated plates and stimulated with Ang II (10 μM) for 24 h. Expression levels of CF activation markers (*acta2*, *tgfb1*, *ctgf*, and *col1a*) were analysed by qRT-PCR and compared with the group with no antibodies (TSP4). Data are presented as mean ± SD. $n = 3–4$ per group. $^*P < 0.05$, $^{**}P < 0.01$.

signalling through this molecule in the future will further aid in a better understanding not only of cardiac fibrosis but also of other pathological tissue/organ fibrosis.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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