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ORIGINAL RESEARCH

KLF12 Aggravates Angiotensin II-Induced Cardiac Remodeling in Male Mice by Transcriptionally Inhibiting SMAD7

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BACKGROUND: Adverse left ventricular remodeling and subsequent heart failure remain a major cause of patient morbidity and mortality worldwide. The KLF family of transcription factors plays crucial roles in heart injury. KLF12 (Krüppel-like factor 12) is a transcription factor that regulates multiple disease processes, although the specific role of KLF12 in cardiac remodeling remains unclear.

METHODS AND RESULTS: In our study, we observed a significant upregulation of KLF12 expression in remodeling hearts. The increased expression of KLF12 primarily originated from cardiac fibroblasts during the fibrotic response induced by angiotensin II. To investigate the effects of KLF12, we performed RNA-seq and found that KLF12 overexpression significantly upregulated the cardiac remodeling associated pathway. Hence, we generated adult mice with cardiac fibroblast-specific overexpression of KLF12 using lentivirus or miRNA (miR-1/133TS) technology. Compared with control mice, KLF12-miR1/133TS transfected mice exhibited exacerbated cardiac remodeling and function. Mechanistically, we discovered that KLF12 directly binds to the promoter of Smad7, leading to the activation of the TGF-β (transforming growth factor beta)-Smad3 pathway.

CONCLUSIONS: In conclusion, KLF12 promoted the development of angiotensin II-induced cardiac remodeling in male mice. Targeting KLF12 may be a promising therapeutic approach to treat cardiac remodeling.

Key Words: angiotensin II ■ cardiac remodeling ■ KLF12 ■ SMAD7

Adverse changes in the structure and function of the left ventricle, leading to the development of heart failure, continue to be a significant contributor to patient illness and death on a global scale.[1] Following cardiac injury, cardiac fibrosis is triggered to repair the damaged tissue. Nevertheless, the abnormal buildup of extracellular matrix proteins and collagen in the myocardium disturbs the natural architecture of the heart muscle, advancing cardiac dysfunction and influencing the clinical trajectory and outcomes of patients with heart failure.[2] Cardiac hypertrophy initially arises as a compensatory response of the myocardium to mechanistic stress provoked by various cardiac disorders. However, pathological cardiac hypertrophy, stemming from persistent stress, is characterized by structural and functional alterations in the heart and stands as an independent risk factor for the development of heart failure.[3,4] Currently, there is a lack of evidence-based therapies that demonstrate significant efficacy against fibrotic diseases, primarily due to the unclear understanding of the underlying mechanisms of cardiac remodeling.

Angiotensin II (Ang II) mediates cardiac remodeling by inducing hypertrophy and fibrosis through a number

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CLINICAL PERSPECTIVE

What Is New?

- Our observation reveals that KLF12 (Krüppel-like factor 12) regulate cardiac remodeling in angiotensin II/transverse aortic constriction-induced heart failure, which directly binds to the promoter of *Smad7*, leading to the activation of the TGF-β (transforming growth factor beta)-Smad3 pathway.

What Are the Clinical Implications?

- The present study provided evidence that KLF12 regulate cardiac remodeling and targeting KLF12 might be a potential strategy for cardiac remodeling.
- Developing KLF12 inhibitors to achieve the transition from research to clinical application.

Nonstandard Abbreviations and Acronyms

Ang II	angiotensin II
CFs	cardiac fibroblasts
KLF12	Krüppel-like factor 12

of signaling pathways, including TGF-β (transforming growth factor beta)/Smad signaling pathway.[5,6] TGF-β is the best-known fibrogenic cytokine described in fibrotic diseases, including heart fibrosis.[7–9] The TGF-β/Smad3 pathway has a pivotal role in cardiac remodeling by activating fibroblasts to produce extracellular matrix in injured heart tissue.[10,11] Upon activation of TGF-β receptors, intracellular mediators known as Smad2/3 become phosphorylated and activated. This activation subsequently leads to the induction of extracellular matrix protein synthesis.[12] To finely control the propagation of TGF-β signaling, various negative feedback loops and multiple layers of regulation exist. One of these loops is accomplished by the transcriptional activation of inhibitory (I)-SMAD7.[13] Lan and coworkers verified that Smad7 plays a protective role in Ang II-induced cardiac remodeling via mechanisms involving the Sp1-TGF-β/Smad-NF-kB-miR-29 regulatory network.[14] Besides, disruption of the interaction between Smad7 and the TGF-β type I receptor has been shown to alleviate the progression of fibrosis.[15]

A number of experimental studies have demonstrated that transcription factor exerts favorable actions on cardiovascular disease.[16,17] Du et al found that cardiac fibroblast (CF)-specific activating transcription factor 3 protects against heart failure by suppressing MAP2K3-p38 signaling.[18] Meanwhile, findings from Loughrey et al verified that runx1 deficiency protects against adverse cardiac remodeling after myocardial infarction.[19] Moreover, our previous findings also proved that IRX2 (Iroquois Homeobox 2) and Tsp40 exert critical roles in cardiac injury and dysfunction under the context of different cardiac stimuli.[20,21] The Krüppel-like family (KLF) of transcription factors encompasses a wide range of biological processes, including proliferation, differentiation, migration, and pluripotency.[22,23] KLF12 (Krüppel-like factor 12) is a transcriptional inhibitor that binds to target gene promoters.[24] KLF12 plays a significant role in governing gene expression during the processes of tumor proliferation and invasion. It exerts extensive control over the activation or repression of genes involved in these cancer-related events.[25,26] Numerous studies have identified that the KLF family plays a critical role in diseases associated with the heart. Drosatos et al verified that cardiomyocyte KLF5 promotes de novo ceramide biosynthesis and contributes to eccentric remodeling in ischemic cardiomyopathy.[27] Moreover, Yu et al identified that knockdown of KLF11 attenuates hypoxia/reoxygenation injury via JAK2/STAT3 (Janus kinase 2/signal transduction and activator of transcription 3) signaling in h9c2.[28] Accordingly, Jain et al demonstrated that KLF4 regulates pressure-induced cardiac hypertrophy.[29] However, there are no available data about the role of KLF12 in cardiac injury. The present study tries to decipher the potential role and molecular basis of KLF12 in cardiac remodeling.

METHODS

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animals and Treatments

Male C57BL/6 mice aged 8 to 10 weeks were obtained from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China). All animal experiments were approved by the Animal Care and Use Committee of Renmin Hospital, Wuhan University, and conducted in accordance with the Guidelines for Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85–23, revised 1996). These experiments were permitted by the Animal Care and Use Committee of Renmin Hospital, Wuhan University, and permitted by the Animal Care and Use Committee of the Wuhan University Renmin Hospital. The mice were housed in a specific pathogen-free barrier system and provided with free access to a standard laboratory chow diet. A cardiac remodeling model was established using long-term infusion of Ang

II, following the protocols described in our previous studies.[20,30] Briefly, after anesthetization with 2% isoflurane, 8- to 10-week-old male mice were implanted with an osmotic minipump (ALZET; Durect Corp) with Ang II (1000 ng/kg/min, H1705, Bachem, Bubendorf, Switzerland) for 12 weeks. For the control group, the age matched mice were infused with the same volume of saline.

Transverse aortic constriction operation was induced as previously described.[31,32] Briefly, mice aged 8 to 10 weeks were anesthetized by intraperitoneal injection of 3% pentobarbital sodium and the adequacy of anesthesia was assessed by the pedal withdrawal reflex, breathing. The surgery involved exposing the left chest through a small incision known as a ministernotomy. The thoracic aorta was located at the second intercostal space and narrowed using a 27-gauge needle and a 7-0 silk suture. Following this procedure, the needle was taken out, and the chest cavity was then closed. Throughout the operation, the patient's body temperature was regulated using a warming pad. The sham group mice underwent a same procedure but were not ligated. Echocardiography was performed 6 weeks after surgery. At the end of the study, mice were euthanized by performing cervical dislocation under deep anesthesia with 3% pentobarbital sodium or 2.5% isoflurane.

Cells and Treatments

Adult mouse CFs were isolated as in our previous studies.[33,34] In brief, adult mice aged 6 to 8 weeks were anesthetized using 2% isoflurane and the hearts were excised and mounted on a Langendorff apparatus. After washing away the blood with Hanks' Balanced Salt Solution, the hearts were perfused with hyaluronidase and collagenase II (2 mg/mL) for 25 minutes. After digestion, differential attachment technique was used to selectively remove other cells and CFs. Subsequently, CFs was cultured in DMEM/F12 (#51445 C, Gibco) supplemented with 10% fetal bovine serum for 48 hours. To synchronize the cells before the experiment, the medium was replaced with serum-free DMEM/F12 for 12 hours. Subsequently, CFs was treated with 1 μmoL/L Ang II for 24 hours to induce fibrotic phenotype.

Analysis of Cardiac Fibroblast Proliferation

CFs isolated from adult mice were infected for 4 hours with adenovirus carrying Klf12 or a control construct. After 24 h infection, they were cultured in DMEM/F12 medium for 3 days with or without 5% fetal bovine serum. Subsequently, CF was treated with Ang II for 3 days. MTT assay was used to evaluate the proliferation of CFs.

RNA Sequencing

Mice with fibroblast-specific overexpression of KLF12 were subjected to Ang II infusion for 12 weeks. After that, the total RNA was extracted and the quality of the extracted total RNA samples was detected with the RNA 6000 Nano Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA). RNA-sequencing (RNA-seq) and subsequent analyses were performed by Bioyi Biotechnology Co., Ltd (Wuhan, China). Briefly, Sequencing libraries were prepared using the DNBSEQ T7 platform (BGI, Wuhan, China) according to standard procedures. Initially, raw reads underwent filtering with FASTP to eliminate sequences containing adapters, those with an N proportion exceeding 10%, and bases with a proportion >50%, followed by quality assessment via FastQC to derive clean reads. Subsequently, these clean reads were aligned to the GRCm38/mm10 mouse genome using HISAT2. For the identification of differentially expressed genes, DESeq2 was employed to compute fragments per kilobase million values for all mapped genes. differentially expressed genes exhibiting a fold change >2 and an adjusted *P* value <0.05 were classified as significant differentially expressed genes.

Chromatin Immunoprecipitation Polymerase Chain Reaction

Chromatin immunoprecipitation-polymerase chain reaction (ChIP-PCR) was performed according to our previous study.[20] Briefly, ChIP-PCR was conducted on mouse CFs to assess the binding of KLF12 to the promoter region of Smad7. For ChIP, a rabbit polyclonal antibody specific to KLF12 or its respective IgG isotype control was used. ChIP-PCR was performed using primers (forward 5′- AGTGGCGGGTGCCGGAGGTCTT-3′; reverse 5′- GCTCCGGGCTCGGGTTTCTA –3′) spanning the KLF12 binding site in the Smad7 promoter. The negative primers sequences that do not contain the KLF12 motif region –2kb upstream from the SMAD7 promoter region for ChIP-PCR: forward 5′- CTGAAGACATTAGGAGGCCAAC-3′; reverse 5′-CCCACCATTACAGGCAG –3′. The negative primers sequences that do not contain the KLF12 motif region +2kb downstream from the SMAD7 promoter region for ChIP-PCR: forward 5'-CAAGGCAGTCCGAGGTG –3′; reverse 5'-GCTGCCGCTCCTTGAGTTTC-3′.

Echocardiography and Hemodynamics

Echocardiography and hemodynamic assessments were conducted following the protocols described in our previous studies.[35,36] Briefly, mice cardiac functional parameters were recorded using the Vevo 3100

High-Resolution Preclinical Imaging System (FUJIFILM VisualSonics). Invasive hemodynamic parameters were collected using a 1.4F Millar catheter transducer (SPR-839; Millar Instruments) and analyzed using the PVAN data analysis software.

More supplemental methods can be found in in Data S1, and the primary antibodies for Western blot are listed in Table S1, the primer sets used for PCR are listed in Table S1.

Statistical Analysis

All data were expressed as mean±SD and analyzed using GraphPad Prism (version 8.0). The Shapiro–Wilk test was used to evaluate the normal distribution of the data. The differences between the 2 groups were compared using the unpaired Student *t* test. Single-factor ANOVA and Tukey post hoc test were used to compare groups. $P<0.05$ was considered statistically significant. All values are reported as mean±SD.

RESULTS

KLF12 is Upregulated in Failing Heart

To investigate the potential involvement of KLF12 in failing heart, we initially analyzed the KLF12 expression in human heart samples obtained from donors with dilated cardiomyopathy. These dilated cardiomyopathyhearts displayed cardiac fibrosis, as evidenced by elevated levels of α-SMA (alpha smooth muscle actin; Figure S1A). As shown in Figure 1A and 1B, the protein and mRNA level of KLF12 were significantly elevated in the dilated cardiomyopathy heart compared with the control. To further explore whether KLF12 was implicated in the pathogenesis of cardiac fibrosis, we

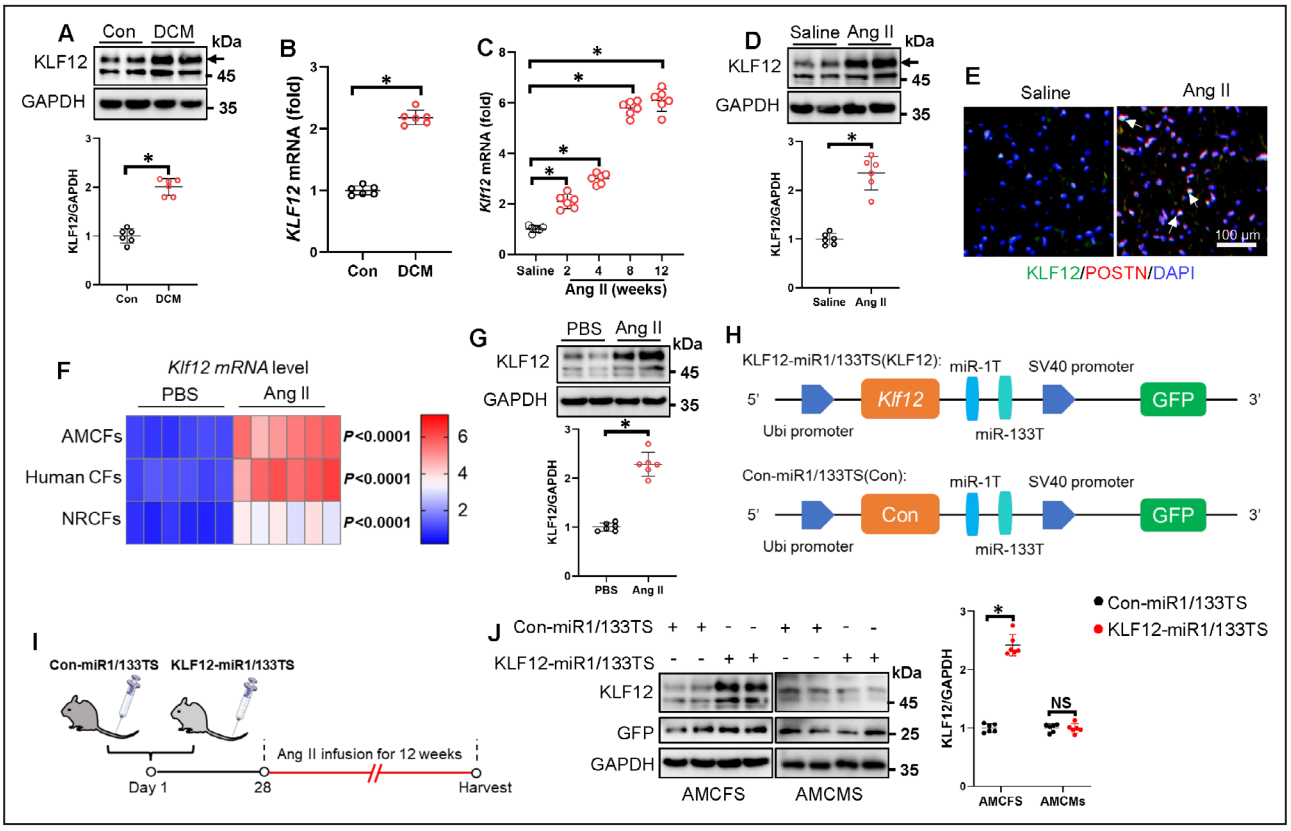

Figure 1. KLF12 is upregulated in failing heart.

A, Cardiac KLF12 expression detected by Western blot in human (Con, n=6; DCM, n=6). **B**, Relative *KLF12* mRNA level (n=6). **C**, Relative *Klf12* mRNA level in mice (n=6). **D**, Cardiac KLF12 expression detected by Western blot in mice (n=6). **E**, Representative images of KLF12 (green), POSTN (red) in 12-week-Ang II-infused heart samples. Nuclei were stained with DAPI (blue). Hearts were subjected to immunofluorescence staining to detect the cellular location of KLF12 (scale bar=100 μm, n=6). **F**, Relative *Klf12* mRNA level (n=6). **G**, Cardiac KLF12 expression detected by Western blot in CFs (n=6). **H**, Schematic representation of KLF12 expression cassette in the lentivirus vectors engineered to overexpress exogenous KLF12 or negative control in CFs expression mir-1a-3p and mir-133a-3p. **I**, Protocol diagram for the study design. **J**, Representative Western blot images and statistical results (n=6). All data are expressed as the mean±SD, and analyzed using 1-way ANOVA followed by Tukey post hoc test. *$P<0.05$ vs the matched group. AMCF indicates angiotensin II-infused adult mouse cardio fibroblast; AMCM, adult mouse cardiac myocytes; Ang II, angiotensin II; CF, cardio fibroblast; Con, control; DCM, dilated cardiomyopathy; GFP, green fluorescent protein; KLF12, Krüppel-like factor 12; NRCF, neonatal rat cardio fibroblast; NS, not significant; and POSTN, periostin.

established a murine model of cardiac fibrosis using Ang II, which is the key driver of the response that leads to cardiac fibrosis.[37] As depicted in Figure S1B and S1D, The mRNA levels of fibrosis markers, such as *Col1 Ctgf* and α-*Sma*, were increased in Ang infusion-induced fibrotic hearts. Specifically, the mRNA level of Klf12 was significantly elevated in the hearts subjected to Ang II infusion (Figure 1C). Consistent with the mRNA expression, Western blotting analysis indicated an increase in KLF12 protein expression in mice with Ang II infusion (Figure 1D). Besides, we detected KLF12 expression level in other mouse models associated with fibrosis, such as ischemic injury and doxorubicin-induced cardiotoxicity. As shown in Figure S1E and S1F, the expression of KLF12 was upregulated after 4 weeks of doxorubicin administration; however, KLF12 expression did not change significantly after 24 hours of ischemia-reperfusion injury. To determine the cellular origin of the upregulated KLF12 in fibrotic hearts, we isolated and fractionated endothelial cells, cardiomyocytes, macrophages, and CFs from hearts treated with Ang II for 12 weeks. CFs contributed largely to KLF12 production but not endothelial cells, cardiomyocytes and macrophages (Figure S1G). Immunofluorescence staining for KLF12 and cardiac α-actinin (a marker for cardiomyocytes) revealed that KLF12 staining in the heart was very weak at baseline, intensifying after long-term Ang II infusion (Figure S1H). In agreement with this finding, immunofluorescence staining for KLF12 and POSTN (periostin, a marker for CFs) revealed POSTN+/KLF12+ CFs were almost absent in saline, but significantly increased after long-term Ang II infusion (Figure 1E) Next, the KLF12 protein expression was compared between CFs isolated from mice infused with Ang II and those from mice infused with saline. The results demonstrated a notable upregulation of KLF12 protein in CFs from Ang II-treated mice compared with those from saline-treated mice. (Figure S1I) Consistent with these findings, the mRNA expression of *Klf12* was significantly elevated in Ang II-infused adult mouse CFs, human CFs, and neonatal rat CFs compared with those treated with PBS (Figure 1F). Congruously, isolated mouse CFs incubated with Ang II showed significantly increased KLF12 protein expression (Figure 1G). To investigate the impact of CF KLF12 in Ang II-induced heart failure, we implemented a miRNA-assisted/lentivirus-mediated system, following a previously reported methodology (Figure 1H).[18,38] This system effectively enhanced the presence of KLF12-miR1/133TS transfected CFs while disrupting KLF12 overexpression in cardiomyocytes (Figure 1I and 1J and Figure S1J). To further investigate whether the expression of other *Klf* genes is influenced by KLF12-miR1/133TS transfection in CFs, we evaluated the mRNA levels of various *Klf* genes in freshly isolated mouse CFs derived from KLF12-miR1/133TS transfected mice. No significant differences in the expression of *Klf3*, *Klf8*, *Klf9*, *Klf10*, *Klf13*, *Klf14*, and *Klf16* were observed between CFs isolated from KLF12-miR1/133TS transfected mice and those from Con-miR1/133TS transfected mice. (Figure S1K).

Fibroblast-Specific Overexpression of KLF12 Aggravates Cardiac Remodeling in Mice

In order to further validate the involvement of KLF12 in Ang II associated heart failure, RNA-seq was performed between KLF12-miR1/133TS transfected hearts and Con-miR1/133TS transfected hearts after Ang II administration. Compared with Con-miR1/133TS transfected hearts, 2612 genes were upregulated and 1722 genes were downregulated in the hearts of mice transfected with KLF12-miR1/133TS (Figure 2A and Figure S2A). Reactome analysis of differentially expressed genes showed that some signaling pathways were closely related to cardiac remodeling (Figure S2B). Therefore, based on these findings, we hypothesized that KLF12 has an impact on Ang II-induced cardiac remodeling. The injection of Ang II resulted in the increase of systolic blood pressure in mice, but there was no significant difference between the control group and the group pretreated with KLF12 carrier. (Figure S2C). The heart rate was unchanged in KLF12-miR1/133TS transfected mice and controls following Ang II infusion (Figure S2D). As depicted in Figure S2E, KLF12-miR1/133TS transfected mice exhibited deteriorated fibrotic remodeling upon Ang II infusion, as indicated by increased mRNA levels of fibrotic markers, including collagen 1 (*Col1*), *Col3*, connective tissue growth factor (*Ctgf*), and α-*Sma*. Immunoblotting results further confirmed that the protein levels of α-SMA and CTGF (connective tissue growth factor) induced by Ang II infusion were exacerbated by fibroblast-specific overexpression of KLF12 in mice (Figure S2F and S2G). Further analyses upon Masson staining revealed that overexpression of KLF12 in CFs led to increased production and deposition of collagen. (Figure 2B). Furthermore, upon Ang II infusion, KLF12 overexpression exhibited a notable exacerbation of cardiac enlargement compared with the controls (Figure 2C) Accordingly, mice with Ang II infusion exhibited increased heart weight-to-tibia length ratio and heart weight-to-body weight ratio (Figure 2D and 2E). Mice pretreated with KLF12 vectors exhibited a significantly hypertrophic heart, as evidenced by the increased cross-sectional area of cardiomyocytes (Figure 2F and 2G). Consistently, the reactivation of fetal gene programs in the heart induced by Ang II was further intensified by the fibroblast-specific overexpression of KLF12, as evidenced by the elevated mRNA levels of *Anp*, *Bnp*, and β-*Mhc*, as well as the decreased mRNA level of α-*Mhc* (Figure 2H and Figure S2H). These

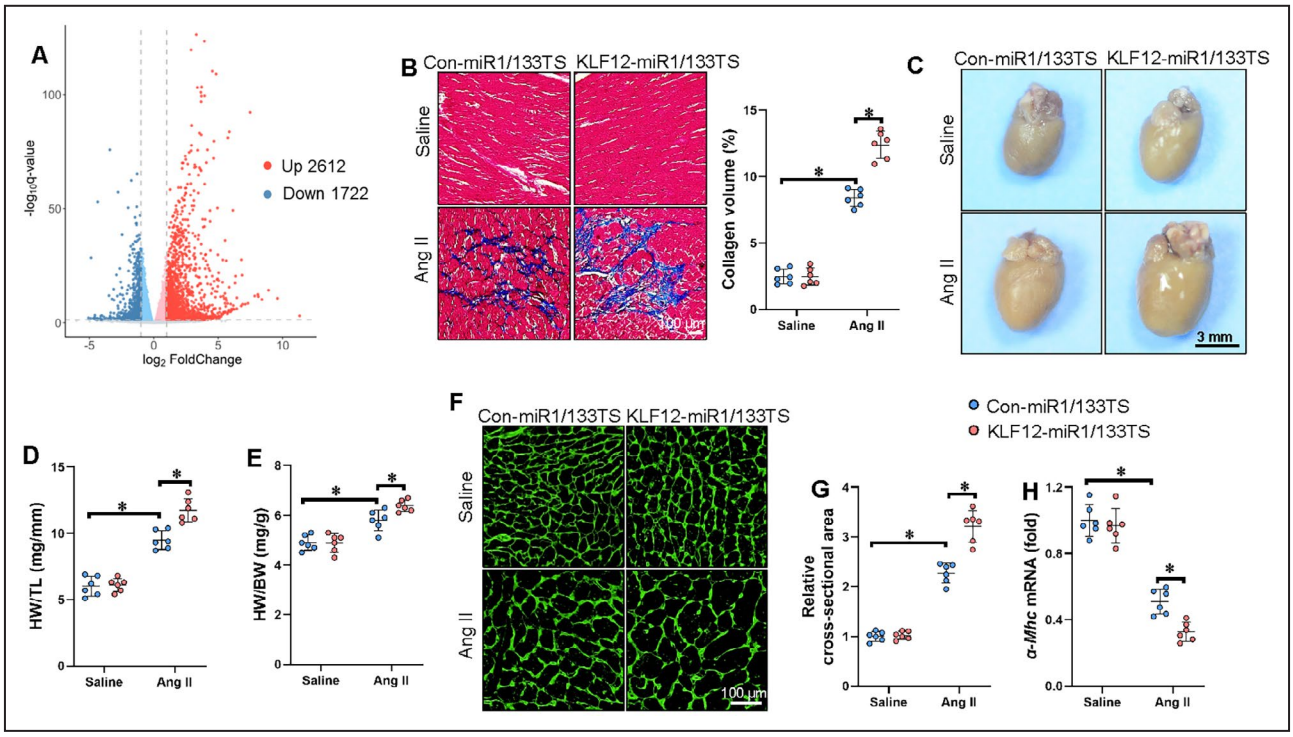

Figure 2. Fibroblast-specific overexpression of KLF12 aggravates cardiac remodeling in mice.
A, The volcano map of RNA-sequencing data set. **B**, Representative images of Masson and quantitative results of fibrotic area (scale bar=100 μm, n=6). **C**, Representative image of the whole mount of the mouse heart (scale bar=3 mm, n=6). **D**, Heart weight-to-tibia length in mice (n=6). **E**, Heart weight-to-body weight () in mice (n=6). **F** and **G**, Representative images of WGA and quantitative results of cell area (scale bar=100 μm, n=6). **H**, Relative α-*Mhc* mRNA levels in murine hearts (n=6). All data are expressed as the mean±SD, and analyzed using 1-way ANOVA followed by Tukey post hoc test. **P*<0.05 vs the matched group. Ang II indicates angiotensin II; HW/BW, heart weight-to-body weight; HW/TL, heart weight-to-tibia length; KLF12, Krüppel-like factor 12; and WGA, wheat germ agglutinin.

findings indicated that the elevated expression of KLF12 in CFs exacerbates cardiac remodeling in mice infused with Ang II.

Next, we investigated whether KLF12 overexpression in fibroblasts affects cardiac function. As shown in Figure 3A through 3D, fibroblast-specific KLF12 overexpression worsened Ang II-induced cardiac dysfunction, as demonstrated by decreased ejection fraction and increased left ventricular end-diastolic size and left ventricular interdiastolic septal thickness. In addition to Ang II infusion, transverse aortic constriction is another widely studied model of cardiac remodeling in mice.[32,39] To rule out the possibility that the proremodeling effect of KLF12 is limited to Ang II-induced cardiac fibrosis, we further investigated whether specific overexpression of KLF12 in CFs exacerbates stress-overloading induced remodeling in mice (Figure 3E). Mice with fibroblast-specific overexpression of KLF12 and subjected to pressure overload exhibited more severe cardiac hypertrophy, as evidenced by a further increase in the heart weight-to-tibia length ratio and cross-sectional area of cardiomyocytes (Figure 3F through 3H). Besides, mice overexpressing KLF12 showed a moderate increase in the area of fibrosis in response to pressure overload (Figure 3I and 3J). The decreased ejection fraction and increased left ventricular end-diastolic size were further intensified following KLF12 overexpression in response to pressure overload. (Figure 3K and 3L). Collectively, fibroblast-specific overexpression of KLF12 deteriorated cardiac remodeling in mice.

KLF12 is Required for Cardiac Fibroblast-to-Myofibroblast Conversion

Subsequently, we examined the direct impact of KLF12 overexpression on the phenotype of CFs. CFs were isolated from adult mouse hearts, and KLF12 overexpression was achieved through adenovirus transduction. As depicted in Figure S3A and S3B, infection of CFs with *Ad-Klf12* significantly increased the expression of KLF12. KLF12 upregulation in CFs significantly increased α-*Sma* expression and other fibrotic markers in in the presence of Ang II (Figure 4A). Immunofluorescence analysis also demonstrated that the overexpression of KLF12 significantly elevated the

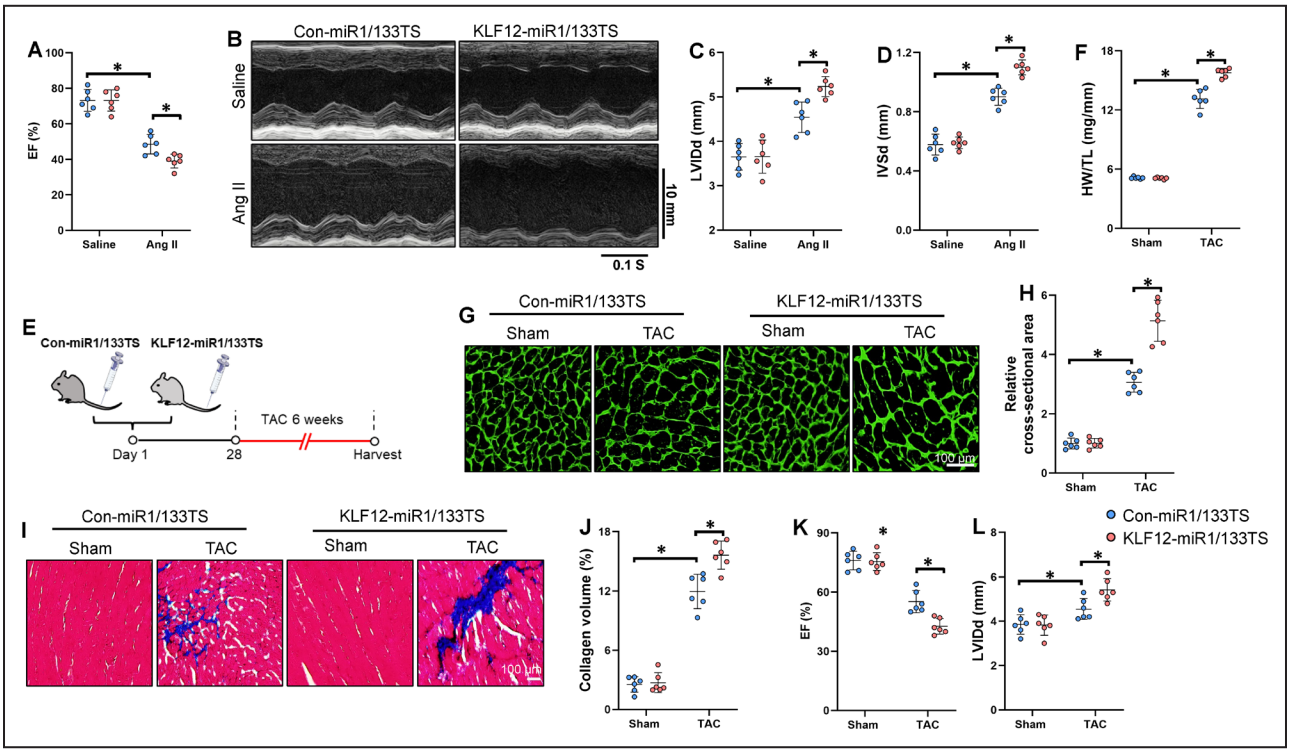

Figure 3. Fibroblast-specific overexpression of KLF12 aggravates cardiac dysfunction.
A, Cardiac function was presented as EF (n=6). **B**, Representative M-mode echocardiography was recorded (scale bar=10 mm, n=6). **C** and **D**, Cardiac function was analyzed by transthoracic echocardiography and presented as LVIDd and IVSd (n=6). **E**, Protocol diagram for the study design. **F**, The ratio of HW/TL (n=6). **G** and **H**, Representative images of WGA and quantitative results of cell area (scale bar=100 μm, n=6). **I** and **J**, Representative images of Masson and quantitative results of fibrotic area (scale bar=100 μm, n=6). **K** and **L**, Cardiac function was analyzed by transthoracic echocardiography and presented as EF and LVIDd (n=6). All data are expressed as the mean±SD, and analyzed using 1-way ANOVA followed by Tukey post hoc test. *P<0.05 vs the matched group. Ang II indicates angiotensin II; EF, ejection fraction; HW/TL, heart weight-to-tibia length; IVSd, interventricular septum thickness; KLF12, Krüppel-like factor 12; LVIDd, left ventricular end-diastolic size; TAC, transverse aortic constriction; and WGA, wheat germ agglutinin.

α-SMA fluorescence intensity in Ang II-treated CFs (Figure 4B and 4C). More important, our findings revealed that the upregulation of KLF12 in CFs contributed to cell proliferation, as assessed by the incorporation of 5-ethynyl-2′-deoxyuridine and a higher proliferative rate (Figure 4D and 4E). CFs were isolated from adult mice, infected with Ad-*Klf12* or Ad-*Gfp*, and then stimulated with Ang II for 24 hours to induce a fibrotic phenotype. Then, a conditioned medium was collected from these myofibroblasts and subjected to neonatal rat cardiomyocytes for 24 hours in the absence or presence of Ang II, and then the cell area of the cardiomyocytes was detected with anti-α-actinin immunofluorescence staining (Figure 4F). As shown in Figure 4G and 4H, conditioned medium derived from CFs infected with Ad-*Klf12* significantly exacerbated Ang II-induced cardiomyocyte hypertrophy in neonatal rat cardiomyocytes. We then determined whether KLF12 deficiency could provide protection against Ang II-induced transdifferentiation of fibroblasts into myofibroblasts in vitro. We silenced KLF12 in CFs. Two independent small interfering RNAs against KLF12 (si*Klf12* and si*Klf12#*) were used to confirm the specificity and exclude any off-target effects (Figure S3C). KLF12 silence alleviated the Ang II induced transdifferentiation of fibroblasts into myofibroblasts, which was reflected by decreased mRNA levels of α-*Sma* and *Col1* (Figure S3D and S3E). Furthermore, knockdown of KLF12 resulted in a decrease in cell proliferation, as indicated by a reduction in the incorporation of 5-ethynyl-2′-deoxyuridine and a lower proliferative rate (Figure S3F and S3G). Subsequently, we assessed cell proliferation in mice. Our findings demonstrated that the upregulation of KLF12 in CFs contributed to cell proliferation, as evidenced by the significant increase in vimentin production and elevated levels of Ki67 in the heart tissues (Figure S3H). Immunofluorescence results further revealed that KLF12 overexpression in CFs prominently promoted the colocalization of vimentin and Ki67 (Figure 4I). Additionally, we detected cell apoptosis by TUNEL staining as suggested, as shown in Figure S3I, upregulation of KLF12 in CFs did not affect cell apoptosis in Ang II-stimulated hearts. These

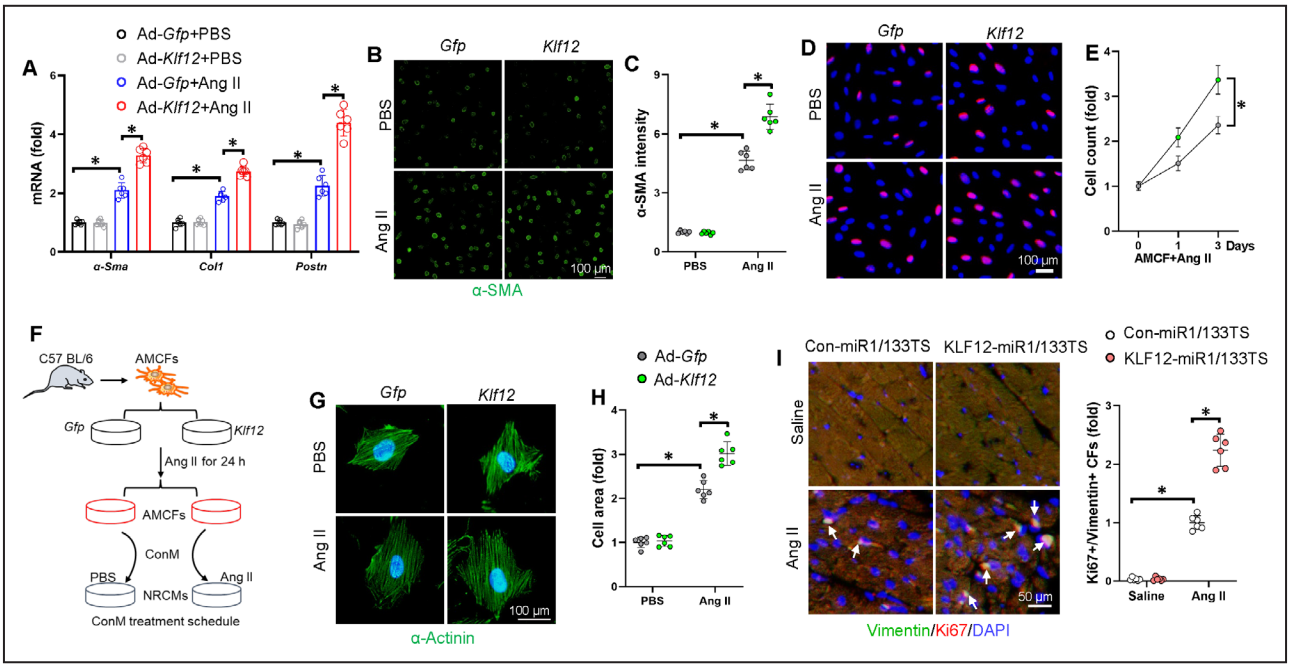

Figure 4. KLF12 is required for cardiac fibroblast-to-myofibroblast conversion.
A, Relative *α-Sma, Col1*, and *Postn* mRNA levels in CFs (n=6). **B** and **C**, Immunofluorescence staining of α-SMA (green) and quantitative results (scale bar=100 μm, n=6). **D**, Representative images of 5-ethynyl-2′-deoxyuridine (red). Nuclei were stained with DAPI (blue). (scale bar=100 μm, n=6). **E**, The proliferative rate of CFs (n=6). **F**, The treatment schedule of CFs-derived ConM on NRCMs. **G**, Representative images of α-actinin (green). Nuclei were stained with DAPI (blue). (scale bar=100 μm, n=6). **H**, The quantitative results of cell area (n=6). **I**, Representative images of Ki67 (red) and Vimentin (green) in heart samples. Nuclei were stained with DAPI (blue). Hearts were subjected to immunofluorescence staining to detect the fibroblast proliferation in heart tissue (scale bar=100 μm, n=6). All data are expressed as the mean±SD, and analyzed using 1-way ANOVA followed by Tukey post hoc test. **P*<0.05 vs the matched group. α-SMA indicates alpha smooth muscle actin; AMCF, angiotensin II-infused adult mouse cardio fibroblast; Ang II, angiotensin II; CF, cardio fibroblast; ConM, conditioned medium; KLF12, Krüppel-like factor 12; and NRCM, neonatal rat cardiomyocytes.

data implied that KLF12 accelerated the proliferation and activation of CFs.

KLF12 Exacerbates Cardiac Remodeling Via Transcriptionally Inhibiting Smad7

The TGF-β-Smad pathway has been implicated as a major contributor to tissue fibrosis in various organ systems.[40] We have previously discovered that the TGF-β pathway plays crucial roles in cardiac hypertrophy and fibrosis associated with Ang II infusion and ischemia–reperfusion.[38,41] Therefore, we first assessed the expression of proteins associated with the TGF-β-Smad pathway in CFs subjected to Ang II infusion. As shown in Figure 5A through 5D, overexpression of KLF12 in Ang II-infused CFs resulted in an increase in p-SMAD3 levels, whereas there was no significant change observed in the levels of t-Smad3, Smaad4, and TGF-β1. Quantitative PCR analysis further confirmed that the upregulation of KLF12 did not influence the expression levels of t-SMAD3, SMAD4, and TGF-β1 in CFs (Figure S4A through S4C). Activation of Smad3 is a pivotal driver of fibrosis, and it is well established that TGF-β3, PDGF (platelet-derived growth factor), and plasminogen activator inhibitor-1 are capable of activating Smad3.[42–44] Therefore, our initial investigation aimed to determine whether KLF12 overexpression could modulate the levels of TGF-β3, PDGF, and plasminogen activator inhibitor-1. However, no substantial alterations were observed in the levels of TGF-β3, PDGF, and plasminogen activator inhibitor-1 in Ang II-infused CFs (Figure S4D through S4G). Smad6 and Smad7 function as inhibitory Smads (I-Smads) and play a role in suppressing the activation of the TGF-β pathway.[45] It is widely acknowledged that Smad7 plays a negative regulatory role in mediating cardiovascular fibrosis induced by Ang II.[46–48] As depicted in Figure 5E through 5I, in Ang II-infused CFs, the upregulation of KLF12 resulted in a significant decrease in Smad7 levels, whereas no significant changes were observed in the levels of Smad6. KLF12 acted as a transcriptional factor by specifically binding to the CACCC motif of target genes.[49] Sequence analysis identified a highly conserved unfolded protein response element (UPRE) consensus sequence, a well-accepted KLF12 binding site, in the Smad7 promoter (Figure 5J). Subsequently, we conducted a luciferase assay using mouse CFs.

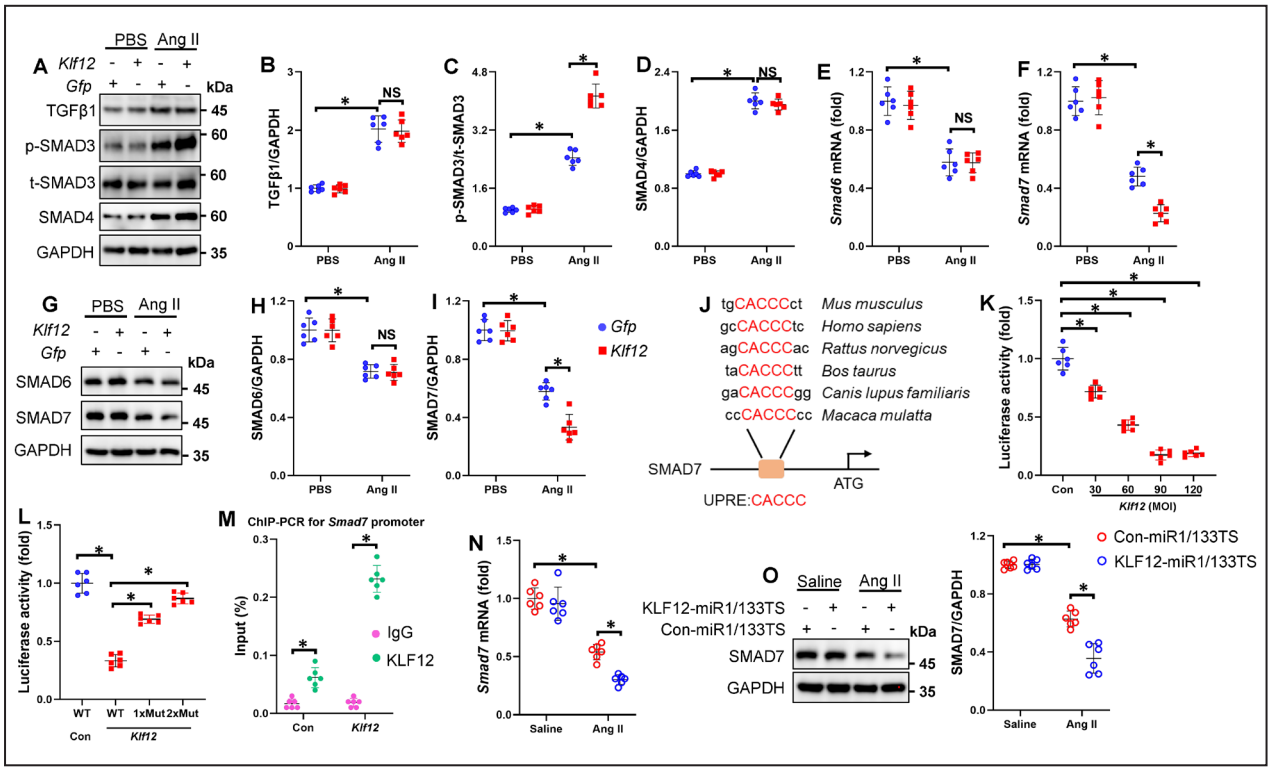

Figure 5. KLF12 exacerbates cardiac remodeling via transcriptionally inhibiting Smad7.
A through **D**, Representative Western blot images and statistical results (n=6). **E**, Relative *Smad6* mRNA levels (n=6). **F**, Relative *Smad7* mRNA levels (n=6). **G** and **I**, Representative Western blot images and statistical results (n=6). **J**, A conserved UPRE was identified in the Smad7 promoter across different species. **K**, Smad7 promoter activity was measured by a luciferase assay (n=6). **L**, Smad7 promoter activity was measured by a luciferase assay (n=6). **M**, Independent ChIP-PCR was performed with Klf12-overexpressing CFs (n=6). **O**, Relative *Smad7* mRNA levels (n=6). **P**, Representative Western blot images and statistical results (n=6). All data are expressed as the mean±SD, and analyzed using 1-way ANOVA followed by Tukey post hoc test. *P<0.05 vs the matched group. Ang II indicates angiotensin II; ChIP-PCR, chromatin immunoprecipitation-polymerase chain reaction; KLF12, Krüppel-like factor 12; NS, not significant; TGF-β, transforming growth factor beta; UPRE, unfolded protein response element; and WT, wild type.

The overexpression of KLF12 led to a significant and dose-dependent reduction in luciferase activity, indicating that KLF12 directly stimulated the transcription of Smad7 (Figure 5K). Notably, when the KLF12 binding site was mutated, the KLF12-mediated downregulation of luciferase activity was completely abolished (Figure 5L). To further support this finding, we performed ChIP followed by PCR analysis using Ang II-treated mouse CFs, and the results demonstrated the enrichment of the Smad7 promoter region within the KLF12 precipitate (Figure 5M). To ensure the specificity of the enrichment of KLF12 observed at the SMAD7 promoter, we designed negative primers that do not contain the KLF12 motif region –2 upstream and +2 kb downstream from the SMAD7 promoter region. As shown in Figure S4H, KLF12 did not enrich at the SMAD7 promoter when the corresponding primer sequences did not contain the KLF12 binding motif. Consistent with these in vitro findings, Smad7 mRNA and protein expression were decreased in KLF12-miR1/133TS transfected mice after Ang II infusion (Figure 5N and 5O). Based on these findings, it can be inferred that Smad7 is a direct transcriptional target of KLF12.

Smad7 Effectively Reverses the Conversion of Cardiac Fibroblasts to Myofibroblasts Induced by KLF12

To investigate the role of Smad7 in the fibrotic response mediated by KLF12, mouse cardiac CFs were infected with an adenovirus carrying Smad7 to achieve overexpression of Smad7. The efficiency of infection was confirmed by Western blot and quantitative PCR (Figure S5A and S5B). As shown in Figure S5C, overexpression of Smad7 reversed the increase of p-Smad3 induced by KLF12. Overexpression of Smad7 effectively eliminated the KLF12-induced transdifferentiation of fibroblasts into myofibroblasts in CFs (Figure 6A and 6B). KLF12 overexpression enhanced the Ang II-induced collagen accumulation, as indicated by the increased mRNA levels of *Col1* and *Postn*. However, this effect of KLF12 was effectively blocked by the upregulation of Smad7 (Figure 6C and 6D). In addition, the increased

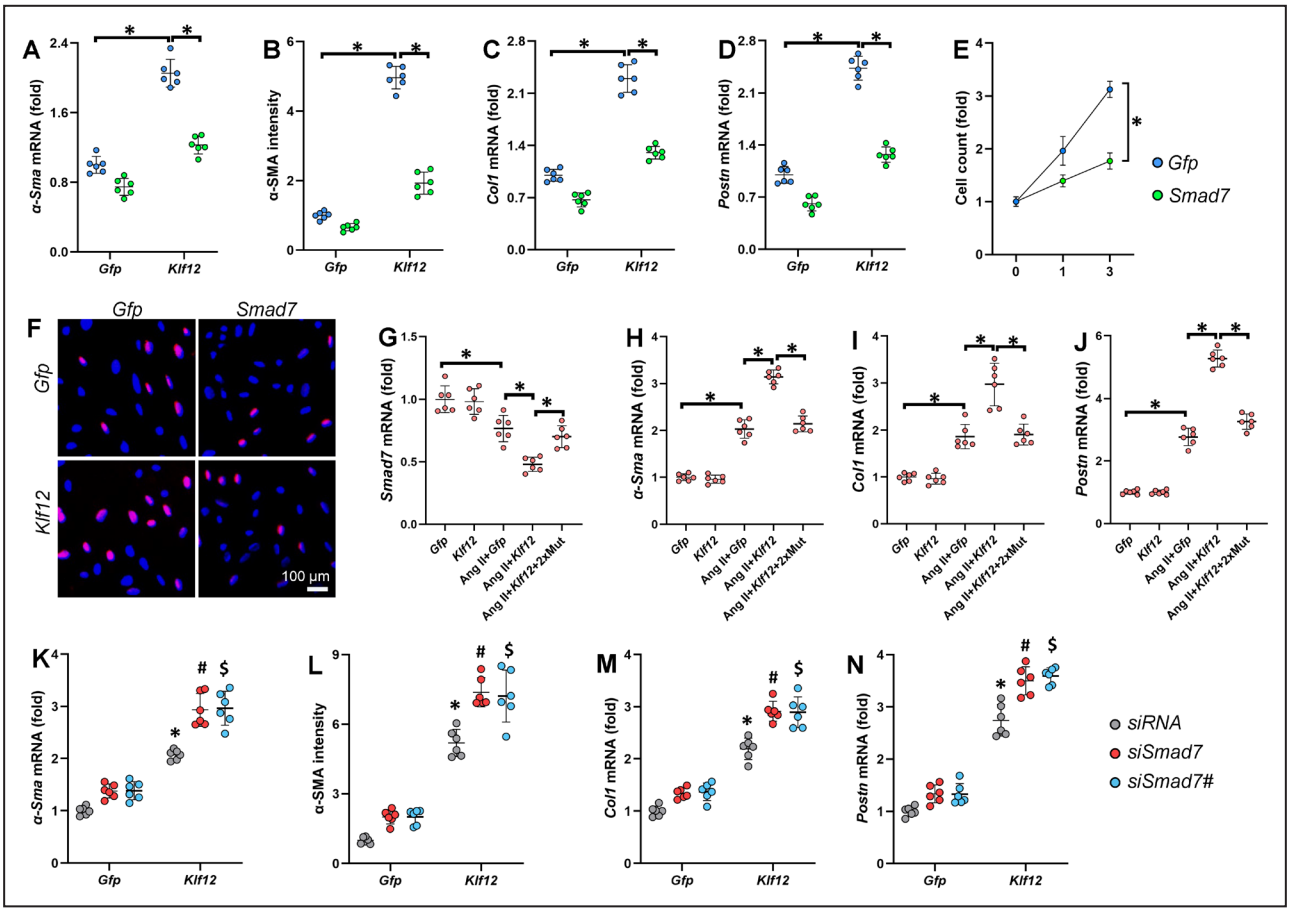

Figure 6. Smad7 effectively reverses the conversion of cardiac fibroblasts to myofibroblasts induced by KLF12.
A, Relative α-*Sma* mRNA levels (n=6). **B**, The quantitative results of α-SMA intensity (n=6). **C**, Relative *Col1* mRNA levels (n=6). **D**, Relative *Postn* mRNA levels (n=6). **E**, The proliferative rate of CFs (n=6). **F**, Representative images of 5-ethynyl-2′-deoxyuridine (red). Nuclei were stained with DAPI (blue). (scale bar=100 μm, n=6). **G** through **J**, Relative *Smad7*, α-*Sma*, *Col1*, and *Postn* mRNA levels (n=6). **K**, Relative α-*Sma* mRNA levels (n=6). **L**, The quantitative results of α-SMA intensity (n=6). **M** and **N**, Relative *Col1* and *Postn* mRNA levels (n=6). All data are expressed as the mean±SD, and analyzed using 1-way ANOVA followed by Tukey post hoc test. **P*<0.05 vs the matched group, $^{\#}P$<0.05 si*Smad7* vs si*RNA*, $^{\$}P$<0.05 si*Smad7*# vs si*RNA*. α-SMA indicates alpha smooth muscle actin; Ang II, angiotensin II; CF, cardio fibroblast; and KLF12, Krüppel-like factor 12.

proliferative capacity of CFs was also abrogated by overexpression Smad7 (Figure 6E and 6F). Moreover, when the binding site of KLF12 was mutated, the ability of KLF12 to transcriptionally inhibit SMAD7 was completely abolished (Figure 6G). Consistent with these findings, after KLF12 binding sites were mutated, KLF12-mediated effect was abrogated, as reflected by α-*Sma*, *Col1*, and *Postn* mRNA levels (Figure 6H through 6J). Subsequently, we investigated whether silencing Smad7 could exacerbate the Ang II-induced transdifferentiation of fibroblasts into myofibroblasts in CFs overexpressing KLF12. We specifically knocked down Smad7 in mouse CFs using 2 independent small interfering RNAs against mouse *Smad7* (si*Smad7* and si*Smad7*#) were selected to avoid off-target effects (Figure S5D and S5E). As shown in Figure 6K and 6L, Smad7 silence significantly promoted Ang II-induced transdifferentiation of fibroblasts into myofibroblasts. Consistently, the deficiency of Smad7 significantly augmented the Ang II-induced collagen accumulation, as indicated by the increased mRNA levels of *Col1* and *Postn* (Figure 6M and 6N).

Overexpression of Smad7 Attenuates Ang II-Induced Cardiac Remodeling and Improved Cardiac Function in KLF12 Overexpression

To ascertain the essential role of Smad7 in KLF12-induced cardiac remodeling, we examined whether the upregulation of Smad7 could rescue the observed pathological phenotype in mice overexpressing KLF12 following Ang II infusion. First, we developed a miRNA-assisted/lentivirus-mediated system to selectively enhance the expression of Smad7 in CFs (Figure S5F through S5H). As anticipated, the overexpression of Smad7 effectively counteracted the pathological phenotype induced by the conditional fibroblast-specific overexpression of KLF12, as evidenced by decreases in the heart weight-to-tibia

length ratio and cardiomyocyte cell area (Figure 7A through 7C). In addition, Smad7 overexpression blocked the reactivation of fetal gene programs post-KF12 upregulation (Figure 7D). Accordingly, Smad7 overexpression interrupted fibrotic remodeling in Ang II-infused hearts, as indicated by Masson staining and the decreased mRNA levels of fibrotic markers (Figure 7D and 7E). Mice with Smad7 overexpression exhibited better cardiac function upon Ang II infusion, as evidenced by the increased ejection fraction and decreased left ventricular end-diastolic size (Figure 7H and 7I). Taken together, these findings provide compelling evidence that the downregulation of Smad7 is responsible for the pathological phenotype observed caused by fibroblast-specific overexpression of KLF12 in mice following Ang II infusion.

DISCUSSION

In this study, we present evidence of a detrimental role played by KLF12 in the progression of cardiac remodeling. Our findings demonstrate that overexpression of KLF12 specifically in fibroblasts worsened 2 distinct heart fibrotic conditions and exacerbated cardiac dysfunction in mice. Mechanistic investigations revealed that KLF12 directly binds to the promoter region of the Smad7 gene, resulting in transcriptional inhibition of SMAD7. These findings underscore the potential for developing therapeutic approaches that target KLF12 to mitigate cardiac remodeling.

KLF12 is a transcriptional inhibitor that binds to target gene promoters.[24] KLF12 belongs to the KLF family, a group of transcriptional regulators involved in various processes, including apoptosis, metastasis, inflammation, and angiogenesis.[50] Previous studies have primarily focused on investigating the role of KLF12 in the context of cancer. Mak and coworkers identified that restoration of KLF12 significantly attenuated anoikis resistance in ovarian cancer cells in vivo and in vitro and KLF12 was the downstream target of miR-141.[51] We validated the role of KLF12 in Ang-II-induced heart failure both in vitro and in vivo, but we did not identify any miRNA associated with the

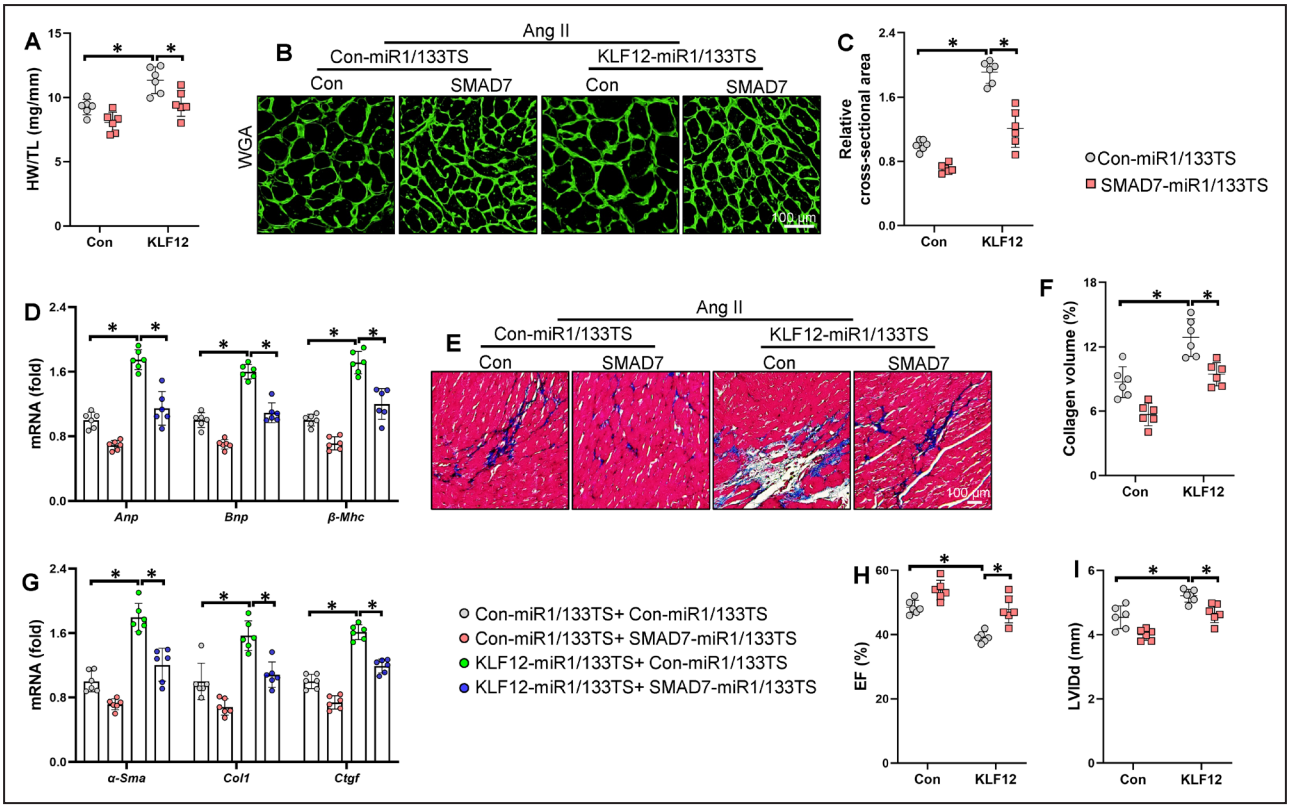

Figure 7. Overexpression Smad7 attenuates Ang II-induced cardiac remodeling and improved cardiac function in with KLF12 overexpression.
A, The ratio of HW/TL (n=6). **B** and **C**, Representative images of WGA and quantitative results of cell area (scale bar=100 μm, n=6). **D**, Relative *Anp*, *Bnp*, and *β-Mhc* mRNA levels (n=6). **E** and **F**, Representative images of Masson and quantitative results of fibrotic area (scale bar=100 μm, n=6). **G**, Relative *Col1*, *α-Sma*, and *Ctgf* mRNA levels (n=6). **H** and **I**, Cardiac function was analyzed by transthoracic echocardiography and presented as EF and LVIDd (n=6). All data are expressed as the mean±SD, and analyzed using 1-way ANOVA followed by Tukey post hoc test. *P<0.05 versus the matched group. Ang II indicates angiotensin II; Con, control; EF, ejection fraction; HW/TL, heart weight-to-tibia length; KLF12, Krüppel-like factor 12; LVIDd, left ventricular end-diastolic size; and WGA, wheat germ agglutinin.

changes in KLF12 expression. Mao et al found KLF12 promoted tumor angiogenesis in small cell lung cancer, where KLF12 transcriptionally inhibits galectin-1 to overcome anti-PD-1 resistance.[52] Similarly, we found that KLF12 exerts a transcriptional inhibitory role in Ang-II-related cardiac remodeling. Additionally, angiogenesis is crucial in cardiovascular diseases, and further research is warranted to investigate whether KLF12 can play a significant role in cardiovascular diseases by influencing neovascularization. On the other hand, some studies report that KLF12 may play a role in transcriptional activation. Sun et al found that KLF12 may transcriptionally activate DVL2 (dishevelled segment polarity protein 2) and promote the sustained activation of the Wnt signaling pathway[53]; Yang and coworkers revealed that KLF12 binds to the PD-L1 promoter and promotes PD-L1 transcription in nonsmall cell lung cancer.[54] As a transcription factor, KLF12 can both activate and inhibit gene expression in different pathological conditions. It is worth further exploring whether KLF12 can exert opposing transcriptional effects in various cardiac pathological models. We have presented, for the first time, the significant role of KLF12 in the heart, laying a solid foundation for subsequent research. It is worthwhile to further explore whether KLF12 also plays a role in renal fibrosis and pulmonary fibrosis. Interestingly, we observed that there is a marked difference in KLF12 expression between neonatal and adult CFs. Neonatal and adult CFs are at different developmental stages. It is possible that KLF12 expression is actively regulated during cardiac development, leading to differences between neonatal and adult stages. Besides, differences in microRNA expression profiles between neonatal and adult CFs may affect KLF12 expression.[55]

TGF-β is the best characterized fibrogenic growth factor.[56,57] TGF-β signals through a series of intracellular effector proteins, the Smads, and through Smad-independent pathways. In vivo studies have demonstrated that Smad3 signaling is critically involved in activation of reparative fibroblasts, inducing extracellular matrix protein synthesis, integrin transcription, and α-SMA expression and contributing to the formation of an organized scar.[58,59] The inhibitory Smads (Smad6/7) serve as endogenous inhibitors of TGF-β signaling, limiting profibrotic responses.[60] Previous studies verified that TGF-β plays a crucial role in the mechanisms by which the KLF family mediates related diseases. Jason and coworkers verified that TGF-β/KLF10 signaling axis regulates atrophy-associated genes to induce muscle wasting in pancreatic cancer.[61] Wen et al revealed a novel mechanism whereby KLF4 mediates the link between TGF-β1-induced gene transcription activation and H3 acetylation during vascular smooth muscle cell differentiation.[62] KLF6 as a key transcription factor required for transactivation of the TGF gene during respiratory syncytial virus infection.[63] KLF2 inhibits TGF-β-mediated cancer cell motility in hepatocellular carcinoma.[64] Ang II induces various pathological changes in the heart, including myocardial remodeling, inflammation, apoptosis, and increased ferroptosis.[65–67] However, we focused on only the phenotype of cardiac remodeling because our sequencing data indicated that KLF12 primarily influences cardiac remodeling. Other members of the KLF family can also influence cardiac remodeling. Cardiomyocyte KLF5 promotes de novo ceramide biosynthesis and contributes to eccentric remodeling in ischemic cardiomyopathy.[27] KLF4 regulates pressure-induced cardiac hypertrophy.[29] In our study, the role of KLF12 in converting CFs to myofibroblasts was demonstrated in cell culture only by overexpressing KLF12 in a CF population. Additional studies using an animal model to inactivate KLF12 in the CF population, along with more comprehensive analysis of CF and myoblast markers, will strengthen the findings. We did not investigate the combined effects of multiple KLF family members on cardiac remodeling. In the future, we plan to explore the potential synergistic effects of multiple KLF family members to determine if they exert a cumulative impact on cardiac remodeling. Cardiac remodeling is indeed a common end point in various cardiac pathological models. It would be a focus of our future research to investigate whether KLF12 also plays a significant role in other cardiac pathological models such as ischemia/reperfusion, myocardial infarction, and doxorubicin-induced heart failure.

CONCLUSIONS

In summary, we explored the effects of KLF12 on Ang II associated remodeling in hearts and uncovered that KLF12 transcriptionally inhibiting SMAD7. Our findings reveal that targeting KLF12 might be a potential strategy for cardiac remodeling. Transcription factor-proteolysis targeting chimeras and transcription factor inhibitors are widely applied in research and clinical applications.[68,69] Considering the key role of cardiac remodeling in heart dysfunction, developing inhibitors targeting KLF12 or degradation-targeting drugs will effectively improve fibrosis following cardiac damage, improving heart function.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Data S1
Tables S1–S2
Figures S1–S5

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