Cardiac-specific mindin overexpression attenuates cardiac hypertrophy via blocking AKT/GSK3β and TGF-β1–Smad signalling

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Aims	Mindin is a secreted extracellular matrix protein, an integrin ligand, and an angiogenesis inhibitor, other examples of which are all key players in the progression of cardiac hypertrophy. However, its function during cardiac hypertrophy remains unclear. This study was aimed to identify the effect of mindin on cardiac hypertrophy and the underlying mechanisms.
Methods and results	A significant down-regulation of mindin expression was observed in human failing hearts. To further investigate the role of mindin in cardiac hypertrophy, we used cultured neonatal rat cardiomyocytes with gain and loss of mindin function and cardiac-specific *Mindin*-overexpressing transgenic (TG) mice. In cultured cardiomyocytes, mindin negatively regulated angiotensin II (Ang II)-mediated hypertrophic growth, as detected by [^{3}H]-Leucine incorporation, cardiac myocyte area, and hypertrophic marker protein levels. Cardiac hypertrophy *in vivo* was produced by aortic banding (AB) or Ang II infusion in TG mice and their wild-type controls. The extent of cardiac hypertrophy was evaluated by echocardiography as well as by pathological and molecular analyses of heart samples. Mindin overexpression in the heart markedly attenuated cardiac hypertrophy, fibrosis, and left ventricular dysfunction in mice in response to AB or Ang II. Further analysis of the signalling events *in vitro* and *in vivo* indicated that these beneficial effects of mindin were associated with the interruption of AKT/glycogen synthase kinase 3β (GSK3β) and transforming growth factor (TGF)-β1–Smad signalling.
Conclusion	The present study demonstrates for the first time that mindin serves as a novel mediator that protects against cardiac hypertrophy and the transition to heart failure by blocking AKT/GSK3β and TGF-β1–Smad signalling.
Keywords	Mindin • Hypertrophy • Remodelling • Signal transduction • AKT

1. Introduction

Heart failure (HF)—a debilitating disease with high rates of mortality and morbidity—is increasing in prevalence. The pathogenesis underlying HF is complex but often relates to cardiac remodelling, which involves myocyte hypertrophy, foetal programme re-expression, and phenotypic changes of the extracellular matrix (ECM).[1–3] The ECM not only provides a structural, chemical, and

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mechanical substrate that is essential in cardiac function but also responds to pathophysiological signals. ECM proteins are coupled to the cell through their transmembrane receptors (the integrins) and the binding of ECM proteins to their receptors results in intracellular signalling. The downstream signalling molecules include focal adhesion kinase (FAK); small GTP-binding proteins such as Rho, Rac, and Ras; protein kinase C; mitogen-activated protein kinases; and the phosphatidylinositol 3-kinase/AKT pathway, all of which are important for the initiation/propagation of cardiac hypertrophy.[4–6] Therefore, modulation of ECM-evoked intracellular signalling in the heart may provide a novel approach for preventing cardiac remodelling and progression to HF.

Mindin (spondin 2), a secreted ECM protein that belongs to the mindin/F-spondin family, comprises N-terminal FS1 and FS2 (named for F-spondin) domains and C-terminal thrombospondin type 1 repeats (TSR). The FS domain mediates integrin binding, which is required for inflammatory cell recruitment and T-cell priming.[7–10] The TSR domain recognizes pathogen-associated molecular patterns and initiates innate immune responses.[9,11,12] In addition, mindin has been shown to promote the outgrowth and adhesion of embryonic hippocampal neurones and to inhibit angiogenesis.[13,14] Despite its demonstrated roles in infectious diseases and cancer,[10,12,15,16] little is known about whether mindin is involved in cardiovascular disease. Mindin is highly expressed in the heart,[11] suggesting a possible function for mindin in heart disease. The role of mindin in cardiac hypertrophy, however, has not yet been studied. Given that mindin is an ECM protein, an integrin ligand, and an angiogenesis inhibitor, other examples of which are all key players in cardiac hypertrophy, we hypothesized that mindin may be involved in the cardiac remodelling process. In the present study, we used cultured neonatal rat cardiomyocytes with gain and loss of mindin function, and cardiac-specific *Mindin*-overexpressing transgenic (TG) mice to investigate the role of mindin in cardiac hypertrophy induced by pressure overload or angiotensin II (Ang II) stimulation and the related molecular mechanisms. We show for the first time that mindin acts as a novel anti-hypertrophic ECM protein that prevents maladaptive remodelling and the transition to HF by blocking AKT/glycogen synthase kinase 3β (GSK3β) and transforming growth factor (TGF)-β1−Smad signalling.

2. Methods

2.1 Animal models

All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee at Renmin Hospital of Wuhan University, China. All surgeries and subsequent analyses were performed in a blinded fashion.

Full-length human mindin cDNA was cloned downstream of the cardiac myosin heavy chain (MHC) promoter. TG mice were then produced by microinjection of the α-MHC-mindin construct into fertilized mouse embryos (C57BL/6 background). Five independent transgenic lines were established and studied. TG mice were identified by PCR analysis of tail genomic DNA. Functional data and gene expression levels were analysed in pairs of α-MHC-mindin (TG) and non-transgenic (WT) male littermates ranging in age from 7 to 8 weeks.

Male TG and WT littermate control mice aged 8–10 weeks were subjected to aortic banding (AB) or a sham operation as described previously.[17,18] Mice were anaesthetized with sodium pentobarbital (Sigma, 80 mg/kg, ip), and horizontal skin incision was made at the level of 2−3 intercostals space. The descending aorta was isolated, and a 7-0 silk suture was snared and pulled back around the aorta. A bent 26-gauge needle (for 25.5−27.5 g) or 27-gauge needle (for 23.5−25.5 g) was then placed next to the aorta, and the suture was tied snugly around the needle and the aorta. After ligation, the needle was quickly removed, the chest and skin were closed, and the mice were allowed to recover. Sham-operated mice underwent the same procedure without constriction. The adequacy of anaesthesia was monitored during the surgical procedures using lack of the pedal withdrawal reflex, slow constant breathing, and no response to surgical manipulation. Buprenorphine (0.1 mg/kg, sc) was administered for post-operative analgesia.

To confirm the role of mindin in cardiac hypertrophy, the experiments were repeated in an Ang II infusion model, which was established as described previously.[17,19] Mice were anaesthetized with 1.5% isoflurane to implant osmotic minipumps. After loading with Ang II (Sigma), osmotic minipumps (Alzet model 2004; Alza Corp.) were primed at 37°C in normal saline overnight, and then implanted subcutaneously in the dorsal region of mice to obtain a delivery rate of 2.0 mg/kg/day over the course of 4 weeks. Osmotic minipumps containing saline solution were implanted in the control mice.

2.2 Echocardiographic and haemodynamic evaluation

Echocardiography was performed on anaesthetized (1.5% isoflurane) mice, using a MyLab 30CV ultrasound (Biosound Esaote Inc.) with a 10 MHz linear array ultrasound transducer, as previously described.[17,18] The left ventricle (LV) was assessed in both parasternal long-axis and short-axis views at a frame rate of 120 Hz. End-systole and end-diastole were defined as the phases in which the smallest and largest areas of the LV, respectively, were obtained. LV end-systolic diameter (LVESD) and LV end-diastolic diameter (LVEDD) were measured from the LV M-mode tracing with a sweep speed of 50 mm/s at the mid-papillary muscle level.

The invasive haemodynamic measurements were performed in anaesthetized (1.5% isoflurane) mice using cardiac catheterization. A microtip catheter transducer (SPR-839, Millar Instruments, and Houston, TX, USA) was inserted into the right carotid artery and advanced into the LV. After stabilization for 15 min, the pressure signals and heart rate were recorded continuously with an ARIA pressure-volume conductance system coupled with a Powerlab/4SP A/D converter and then stored and displayed on a personal computer as described previously.[17,18] Mice were euthanized by cervical dislocation 4 weeks post-operatively. Hearts, lungs, and tibiae of the mice were dissected and weighed or measured to compare the heart weight (HW)/body weight (BW) (mg/g), HW/tibial length (TL) (mg/mm), and lung weight (LW)/BW (mg/g) ratios in the different groups.

2.3 Histological analysis

Hearts were excised, placed immediately in 10% potassium chloride solution to ensure that they were stopped in diastole, washed with saline solution, and placed in 10% formalin. Hearts were sectioned transversely close to the apex to visualize the left and right ventricles. Several sections (4−5 μm thick) were prepared and stained with haematoxylin-eosin (HE) for histopathology or Picrosirius red (PSR) for collagen deposition and then visualized by light microscopy. To determine the cross-sectional area of myocytes, HE-stained sections were used. A single myocyte was measured using an image quantitative digital analysis system (Image-Pro Plus 6.0). Between 100 and 200 myocytes in the LVs were outlined in each group. From the PSR-stained sections, LV collagen volume fraction was calculated as the area stained by PSR divided by the total area. The

quantitative analysis of histological images was performed in a blinded fashion.

2.4 Recombinant adenoviral vectors, cultured neonatal rat cardiac myocytes, and fibroblasts

To over-express mindin, we used replication-defective adenoviral vectors encompassing the entire coding region of the *Mindin* gene under the control of the cytomegalovirus promoter. A similar adenoviral vector encoding the *GFP* gene was used as a control. To knock down mindin expression, three rat shmindin constructs were obtained from SABiosciences (KR43670G). Next, we generated three Ad-shmindin adenoviruses and selected the one that produced a significant decrease in mindin levels for further experiments. Ad-shRNA was the non-targeting control. We infected cardiac myocytes or fibroblasts with Ad-mindin, Ad-green fluorescent protein (GFP), Ad-shmindin, or Ad-shRNA at a MOI of 100, which resulted in transgene expression without toxicity in 95–100% of the cells.

Neonatal (1–2-day-old) Sprague-Dawley rats were killed by swift decapitation, and their hearts were used for the isolation and cultures of neonatal rat cardiac myocytes and fibroblasts, as described previously.[20,21] The details for cell culture are provided in Supplementary material online, *Methods*. For cell infection, cardiac myocytes or cardiac fibroblasts were cultured at a density of 1×10^6 cells/well in 6-well plates and exposed to 2×10^8 pfu each of virus in 1 mL of serum-free medium for 24 h. The cells were then washed and incubated in serum-containing medium for 24 h. Additional treatments are described in the figure legends.

2.5 [³H]-Leucine incorporation and surface area

[³H]-Leucine incorporation was measured as described previously.[20,21] Briefly, cardiac myocytes were infected with different adenoviruses for 24 h and subsequently stimulated with Ang II (1 μM, Sigma) and co-incubated with [³H]-Leucine (1 μCi/mL, MP Biomedical) for the indicated time. At the end of the experiment, the cells were washed with Hanks' solution, scraped from the well, and treated with 10% trichloroacetic acid at 4°C for 60 min. The precipitates were then dissolved in NaOH (1 N) and subsequently counted with a scintillation counter. For surface area measurements, the cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS), permeabilized in 0.1% Triton X-100 in PBS, and stained with α-actinin (Sigma) at a dilution of 1:100 using standard immunofluorescence staining techniques.

2.6 Real-time quantitative RT-PCR

Total RNA was extracted from the frozen human tissues using TRIzol (Invitrogen, 15596-026). cDNA was synthesized from 2 μg RNA of each sample using the Transcriptor First Strand cDNA Synthesis Kit (Roche, 04896866001). To examine the relative mRNA expression of mindin, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), quantitative RT-PCR analysis was performed using the LightCycler 480 SYBR Green 1 Master Mix (Roche, 04707516001) and the LightCycler 480 QPCR System (Roche). Details are provided in Supplementary material online, *Methods*.

2.7 Western blotting

Western blotting was conducted to determine (i) protein levels of mindin, hypertrophic markers (ANP, BNP, β-MHC), and fibrotic markers [connective tissue growth factor (CTGF), collagen I, collagen III, TGF-β1]; (ii) the activation state of AKT/GSK3β/mammalian target of rapamycin (mTOR)/forkhead box O (FOXO) signalling; and (iii) the activation state of TGF-β1−Smad signalling. Quantification of western blots was performed by Odyssey infrared imaging system (Li-Cor Biosciences) to detect protein expression. The specific protein expression levels were normalized to either GAPDH for the total cell lysate and cytosolic proteins or to lamin-B1 for nuclear proteins. For further details, please see Supplementary material online, *Methods*.

2.8 Human heart samples

Samples of human failing hearts were collected from the LVs of dilated cardiomyopathy (DCM) patients undergoing heart transplants. Control samples were obtained from the LVs of normal heart donors. The samples were obtained after informed consent and with approval of the local Ethical Committee (Renmin Hospital of Wuhan University Human Research Ethics Committee, Wuhan, China). The investigation conforms with the principles outlined in the Declaration of Helsinki.

2.9 Statistical analysis

Data are expressed as mean ± SEM. Differences among groups were determined by two-way ANOVA followed by a post hoc Tukey test. Comparisons between two groups were performed using an unpaired Student's *t*-test. A value of $P < 0.05$ was considered significant.

3. Results

3.1 Mindin expression is decreased in human failing hearts

To explore the potential role of mindin in cardiac hypertrophy, we first examined mindin expression in LV myocardium samples from DCM patients undergoing heart transplants because of end-stage HF and those from donors. As shown in *Figure 1A*, mindin was highly expressed in normal donor hearts but was significantly downregulated in all human failing hearts. Importantly, protein levels of foetal genes, such as ANP and BNP, were all markedly elevated in DCM hearts compared with donor hearts. The real-time PCR analysis also showed a decrease in mindin mRNA levels, and an increase in the mRNA levels of foetal genes in the failing DCM hearts (*Figure 1B*). These results strengthen the possible involvement of mindin in cardiac hypertrophy.

3.2 Forced mindin expression attenuates the hypertrophic growth of cultured myocytes

As an ECM protein, mindin may affect both cardiomyocytes and non-myocytes within the heart. To examine the role of mindin specifically in cardiomyocytes, we performed gain- and loss-of-function studies using cultured isolated cardiomyocytes. Cells were serum-starved for 24 h in 0.5% foetal calf serum after infection with Ad-mindin or Ad-shmindin and then treated with 1 μM Ang II for the indicated time. Ad-mindin infection led to a substantial increase in the level of mindin protein in neonatal rat cardiomyocytes (see Supplementary material online, *Figure S1*). Additionally, we screened three Ad-shmindin and found that No. 1 Ad-shmindin markedly inhibited mindin expression in cardiac myocytes (see Supplementary material online, *Figure S1*). Therefore, No. 1 Ad-shmindin was selected for subsequent experiments. Further studies showed that mindin overexpression caused by Ad-mindin infection attenuated Ang II-mediated cardiomyocyte hypertrophy, whereas decreased mindin levels via Ad-shmindin infection augmented hypertrophy, as detected by hypertrophic marker protein levels, [³H]-Leucine incorporation, and cardiac myocyte area (*Figure 2A and B*). These *in vitro* data suggest the inhibitory effect of mindin on cardiomyocyte hypertrophy.
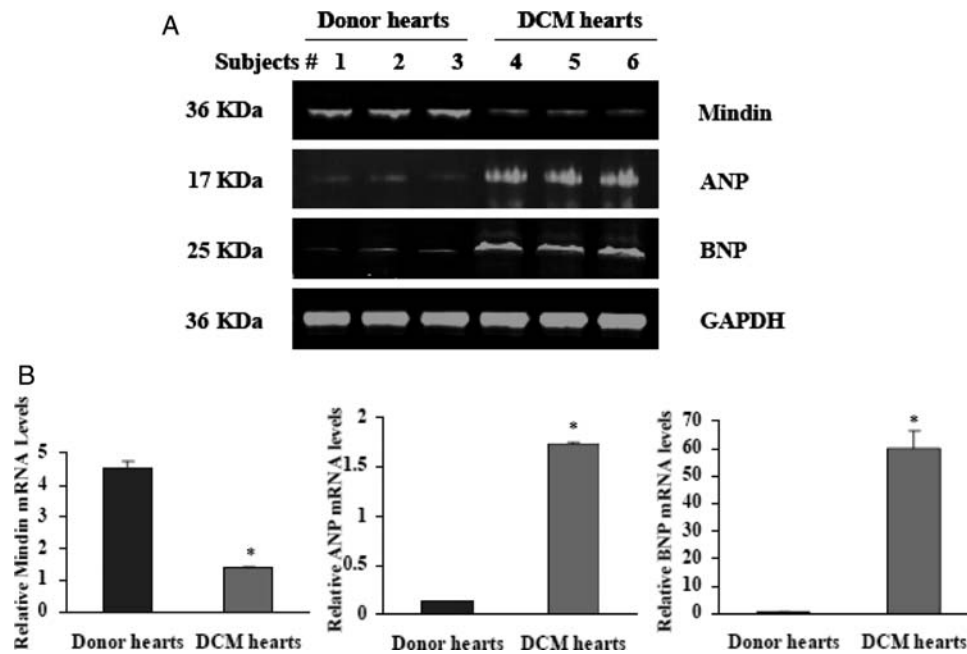
Figure 1 Mindin expression is decreased in human failing hearts. (*A*) Representative western blots of mindin, ANP, and BNP in human failing hearts and donor hearts. (*B*) Real-time PCR analysis of mindin, ANP, and BNP in human failing hearts and donor hearts ($n = 6$). Values represent mean $\pm$ SEM. *$P < 0.01$ compared with donor hearts.

3.3 Generation of mice with cardiac-specific overexpression of mindin

To assess the effect of constitutive human mindin expression on myocardial function, we generated TG mice carrying full-length human *Mindin* cDNA under the control of the α-MHC promoter. Mindin protein levels in various tissues were analysed by western blot analysis using a human-specific anti-mindin antibody. We detected robust expression of human mindin protein in the heart, but not in other organs (*Figure 3A*). Among five established lines of TG mice, the line that expressed the highest levels of human mindin protein in the heart was used for further experiments (*Figure 3A*). Western blot analysis further demonstrated that the expression level of the mouse mindin protein was not modified by expression of the human *Mindin* gene (*Figure 3B*). Under basal conditions, cardiac-specific overexpression of mindin had no significant effect on HW/BW or LW/BW compared with WT mice (see Supplementary material online, *Table S1*), which suggests that mindin plays a negligible role in cardiac development or function under basal conditions.

3.4 Forced mindin expression in the heart represses cardiac remodelling in response to pressure overload or Ang II stimulation

To determine whether cardiac overexpression of mindin could reduce the hypertrophic response to pressure overload, WT littermates and TG mice were subjected to AB surgery or sham operation. TG mice demonstrated a significant attenuation of hypertrophy 4 weeks after AB compared with WT mice, as measured by the ratios of HW/BW, HW/TL, and cardiomyocyte cross-sectional area (*Figure 4A*). No significant differences were observed in the sham-operated TG and WT mice. We next assessed the cardiac

function of TG and WT mice by echocardiography. The increases in LV chamber dimensions and wall thickness induced by pressure overload were also markedly attenuated during both systole and diastole in TG mice compared with WT mice (*Table 1*). The histological analyses based on gross and whole-heart examinations and HE staining further confirmed the inhibitory effect of mindin on cardiac hypertrophy after AB (see Supplementary material online, *Figure S2A*). We next examined the potential effect of mindin on hypertrophy mediated by Ang II infusion and found results similar to those observed with AB treatment (*Figure 4B*, Supplementary material online, *Figure S2B*, *Table 2*). In addition, the protein levels of foetal genes, including ANP, BNP, and β-MHC, were significantly lower in TG mice than in WT mice at 4 weeks after AB or Ang II infusion (*Figure 4C and D*). These results imply that cardiac mindin plays an important protective role against pressure overload- or Ang II-induced hypertrophy *in vivo*.

3.5 Mindin suppresses AKT/GSK3β/mTOR/ FOXO signalling *in vivo* and *in vitro*

AKT signalling pathway has previously shown to play an important role in cardiac hypertrophy.[17,18,22] To investigate the molecular mechanisms by which mindin mediates its anti-hypertrophic effect, we examined the activation state of AKT and its downstream targets, including GSK3β, mTOR, forkhead box O3A (FOXO3A), and forkhead box O1 (FOXO1). As expected, both AB and Ang II induced a significant increase in the phosphorylated levels of AKT, GSK3β, mTOR, FOXO3A, and FOXO1 in WT hearts. However, the hypertrophic-stimulus-induced phosphorylation of these signalling molecules was almost completely blocked in TG hearts (*Figure 5A*). To further investigate the inhibitory effect of mindin on AKT/GSK3β/mTOR/FOXO signalling, we exposed cultured neonatal rat cardiomyocytes infected with Ad-mindin, Ad-GFP, Ad-shmindin, or
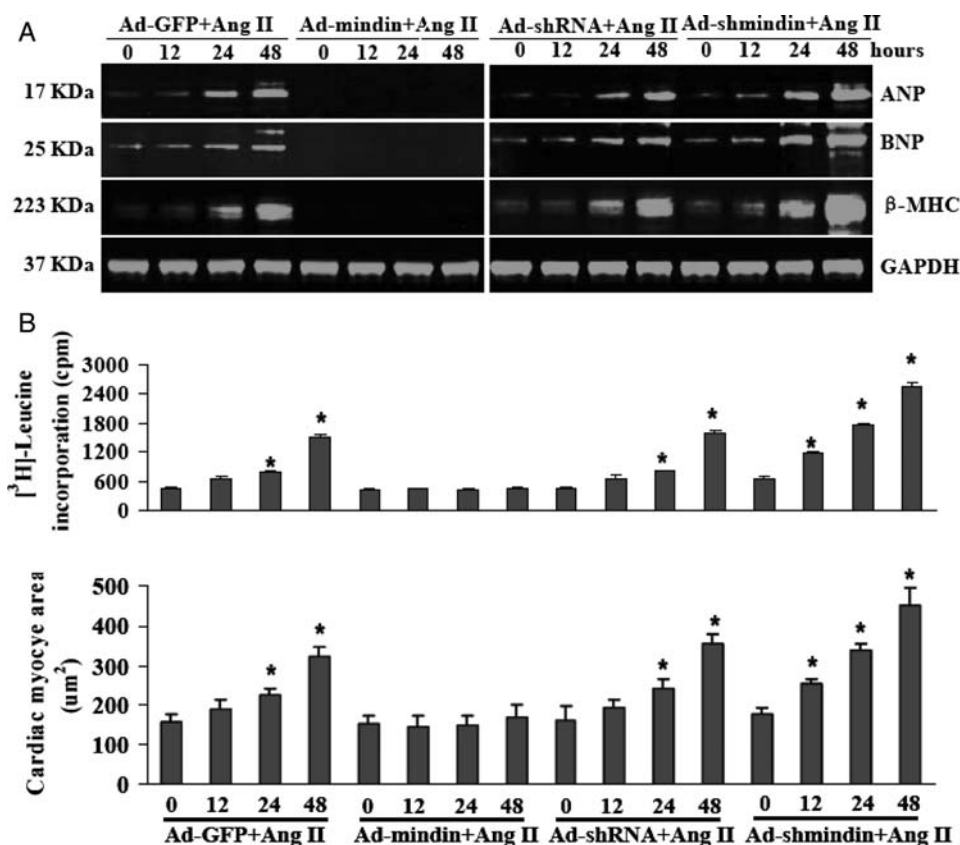
Figure 2 Forced mindin expression attenuates the hypertrophic growth of cultured myocytes. (*A*) Representative western blots for ANP, BNP, and β-MHC after treatment with Ang II for the indicated times in neonatal rat cardiac myocytes infected with different adenoviruses. Reproducible results were obtained in three independent experiments. (*B*) Effects of mindin on [^{3}H]-Leucine incorporation and cardiac myocyte area induced by Ang II treatment at the indicated time points. Cardiomyocytes were infected with Ad-GFP, Ad-mindin, Ad-shRNA, or Ad-shmindin for 24 h and then incubated with 1 μM Ang II for the indicated times. Values represent mean ± SEM. *$P < 0.01$ compared with the Ad-GFP + Ang II group at time 0.
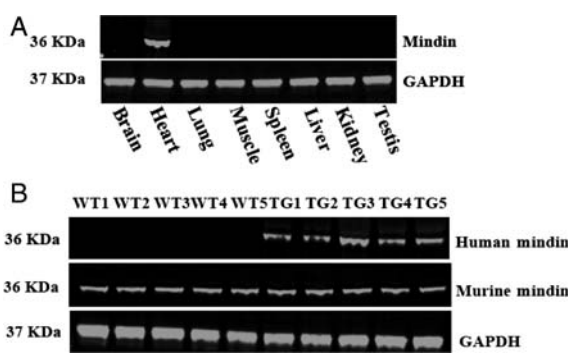

Figure 3 Generation of mice with cardiac-specific overexpression of mindin. (*A*) Representative blots for human mindin from various tissues of TG mice, as indicated. (*B*) Representative blots for transgenic mindin and endogenous mindin levels in heart tissue from five lines of both TG and WT mice.

Ad-shRNA to 1 μM Ang II. As shown in *Figure 5B*, Ang II-stimulated AKT/GSK3β/mTOR/FOXO phosphorylation was attenuated by infection with Ad-mindin and promoted by infection with Ad-shmindin.

Taken together, these results suggest that mindin may suppress cardiac hypertrophy through the inhibition of AKT/GSK3β/mTOR/FOXO signalling *in vivo* and *in vitro*.

3.6 Forced mindin expression attenuates fibrosis in response to pressure overload or Ang II stimulation

Fibrosis is another classical feature of pathological cardiac hypertrophy and is characterized by the accumulation of collagen. To further investigate the mechanism by which mindin inhibits cardiac hypertrophy, we examined the ability of mindin to inhibit fibrosis. To determine the extent of fibrosis, paraffin-embedded slides were stained with PSR, and the staining was analysed quantitatively. As shown in *Figure 6A and B*, marked perivascular and interstitial fibrosis and an increased LV collagen volume were present in the WT mice subjected to AB or Ang II infusion. Importantly, cardiac fibrosis was markedly attenuated in TG mice compared with their WT littermates (*Figure 6A and B*). Subsequent analyses of protein expression levels of known mediators of fibrosis, including CTGF, collagen I, collagen III, and TGF-β1, revealed a blunted response in TG mice (see Supplementary material online, *Figure S3A*). These data together indicate an anti-fibrotic effect of mindin.
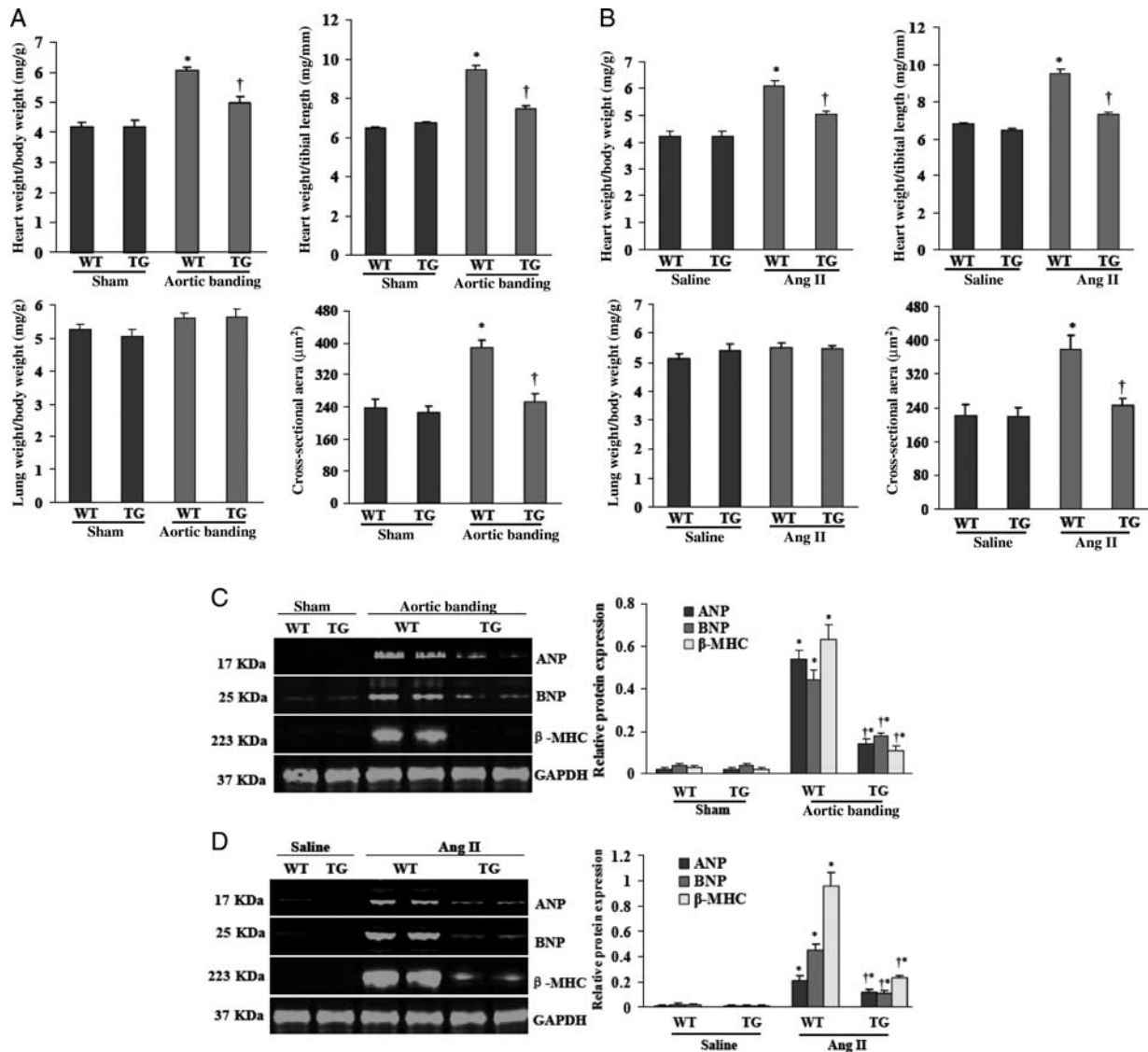
Figure 4 Forced mindin expression in the heart produces resistance to cardiac remodelling in response to pressure overload or Ang II stimulation. (*A* and *B*) Statistical results for the HW/BW, HW/TL, and LW/BW ratios as well as myocyte cross-sectional areas (*n* = 100 cells per group) 4 weeks after AB surgery (*A*) or Ang II infusion (*B*) in WT and TG mice (*n* = 5–6 mice per group). (*C* and *D*) Protein expression levels of ANP, BNP, and β-MHC 4 weeks after AB surgery (*C*) or Ang II infusion (*D*) in WT and TG mice (*n* = 4). Left, representative blots; right, quantitative results. *P < 0.01 for WT/sham or WT/saline values; †P < 0.01 for WT/AB or WT/Ang II after AB or Ang II infusion.

TGF-β1 induces collagen synthesis via activation of a number of transcription factors, including Smads.[19] To further elucidate the molecular mechanisms underlying the anti-fibrotic effects of mindin, we assessed the regulatory role of mindin on activation of the Smad cascade. TG mice showed lower Smad 2/3 phosphorylation and lower nuclear translocation of Smad 2/3 when compared with WT mice (see Supplementary material online, *Figure S3B*). We then infected cardiac fibroblasts with Ad-mindin, Ad-shmindin or their respective controls and treated them with 10 ng/mL TGF-β1 for specific periods. Western blot analyses revealed significant phosphorylation of Smad2 and translocation of Smad2/3 but no significant alterations in the total protein levels of Smad2 after TGF-β1 treatment (see Supplementary material online, *Figure S3C*). However, Ad-mindin infection almost completely suppressed Smad2

phosphorylation and Smad2/3 nuclear translocation (see Supplementary material online, *Figure S3C*). Importantly, Ad-shmindin infection enhanced the effects of TGF-β1 (see Supplementary material online, *Figure S3C*). These findings suggest that mindin blocks fibrosis by disrupting TGF-β1–Smad signalling.

4. Discussion

Cardiac hypertrophy is an adaptive response to mechanical loading and neuro-hormonal signals that initially maintains or even increases cardiac output. With persistent stress, however, compensatory hypertrophy can evolve into a decompensated state associated with profound changes in thermodynamic performances, foetal reprogramming, extracellular remodelling, as well as contractile and

Table 1 Echocardiographic parameters in mindin TG mice and WT littermates at 4 weeks after sham operation or AB

Parameter	Sham-WT mice	Sham-TG mice	AB-WT mice	AB-TG mice
Number	9	8	9	8
BW (g)	28.20 ± 0.76	29.44 ± 0.74	28.61 ± 0.43	27.41 ± 0.53
HR (b.p.m)	481.00 ± 9.96	502.52 ± 8.96	508.46 ± 11.45	546.85 ± 14.46
LVEDD(mm)	3.65 ± 0.04	3.59 ± 0.05	4.30 ± 0.06*	3.78 ± 0.03**
LVESD(mm)	1.62 ± 0.02	1.64 ± 0.04	2.41 ± 0.04*	1.86 ± 0.03**
PWT(mm)	0.61 ± 0.01	0.68 ± 0.02	0.86 ± 0.02	0.75 ± 0.02
IVSD (mm)	0.56 ± 0.01	0.63 ± 0.01	0.85 ± 0.02*	0.79 ± 0.02
FS (%)	55.70 ± 0.55	54.21 ± 1.52	43.95 ± 1.11*	50.89 ± 0.57**

All values are mean ± SEM.
BW, body weight; HR, heart rate; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; PWT, posterior wall thickness; IVSD, left ventricular septum diastolic; FS, fractional shortening.
*$P < 0.05$ vs. WT sham operation.
**$P < 0.05$ vs. WT AB after 4 weeks AB.

Table 2 Echocardiographic parameters in WT and TG mice at 4 weeks after Ang II or saline infusion

Parameter	Saline-WT mice	Saline-TG mice	Ang II-WT mice	Ang II-TG mice
Number	9	8	9	8
BW (g)	29.34 ± 0.53	28.31 ± 0.75	28.87 ± 0.58	26.92 ± 0.70
HR (b.p.m.)	491.92 ± 10.04	504.97 ± 8.37	507.13 ± 11.55	499.31 ± 10.15
LVEDD(mm)	3.67 ± 0.05	3.71 ± 0.01	4.36 ± 0.05*	3.84 ± 0.01**
LVESD(mm)	1.61 ± 0.03	1.65 ± 0.01	2.39 ± 0.10*	1.82 ± 0.02**
PWT(mm)	0.60 ± 0.02	0.65 ± 0.01	0.87 ± 0.02	0.76 ± 0.01
IVSD (mm)	0.53 ± 0.02	0.64 ± 0.01	0.84 ± 0.01	0.71 ± 0.01
FS (%)	55.93 ± 0.90	55.48 ± 0.34	45.31 ± 1.90*	52.78 ± 0.51**

All values are mean ± SEM.
*$P < 0.05$ vs. WT Saline infusion.
**$P < 0.05$ vs. WT Ang II infusion after 4 weeks Ang II infusion.

diastolic dysfunction.[1−3] The molecular mechanisms that mediate the transition from compensated hypertrophy to decompensated HF remain elusive. In the present study, we identify mindin (a secreted cardiac ECM protein) as an intrinsic anti-hypertrophic factor that prevents maladaptive remodelling and the transition to HF.

The major new findings of this study are as follows: (i) mindin expression is significantly decreased in human failing hearts that display severe cardiac hypertrophy; (ii) mindin negatively regulates hypertrophic growth of cultured myocytes; (iii) cardiac-specific over-expression of mindin markedly attenuates cardiac hypertrophy, fibrosis, and left ventricular dysfunction in mice in response to AB or Ang II infusion; and (iv) mindin regulates AKT/GSK3β and TGF-β1 signalling pathways. Together, we demonstrate for the first time that mindin plays a critical role in attenuating both ventricular remodelling and cardiac hypertrophy.

Mindin expression was significantly down-regulated in human failing hearts. Based on our finding that transgenic overexpression of mindin in the heart attenuated AB- or Ang II-induced hypertrophy and fibrosis, the reduced expression of endogenous mindin observed in human failing hearts is likely maladaptive. These results strongly suggest that mindin plays an important role in protecting the heart against a maladaptive response to stress. This is consistent with

some studies showing that other ECM proteins, such as osteopontin and melusin, are required to sustain compensatory cardiac hypertrophy in response to chronic pressure overload and prevent the transition towards HF.[23,24] Notably, unlike osteopontin and melusin, which regulate hypertrophy induced only by mechanical stimuli, mindin can modulate hypertrophy induced by both mechanical and humoural stimuli, indicating a more potent function of mindin in hypertrophy.

As an ECM protein, mindin can interact with cardiomyocytes and other cardiac cell types to modulate the remodelling process. Indeed, using both isolated cardiomyocytes and fibroblasts, we observed that mindin overexpression or sh-RNA knockdown of mindin-regulated critical cell signalling pathways related to cardiomyocyte hypertrophy and fibrosis. To investigate the molecular mechanism by which mindin mediates its anti-hypertrophic effects on cardiomyocytes, we examined AKT signalling, a pivotal contributor to the development of cardiac hypertrophy.[25−30] AKT phosphorylation in response to hypertrophic stimuli was significantly reduced in *Mindin* TG mice. In accordance with our findings, F-spondin (another member of the mindin/F-spondin family) has been shown to inhibit the activation of AKT when human umbilical vein endothelial cells (HUVECs) on vitronectin are stimulated with vascular
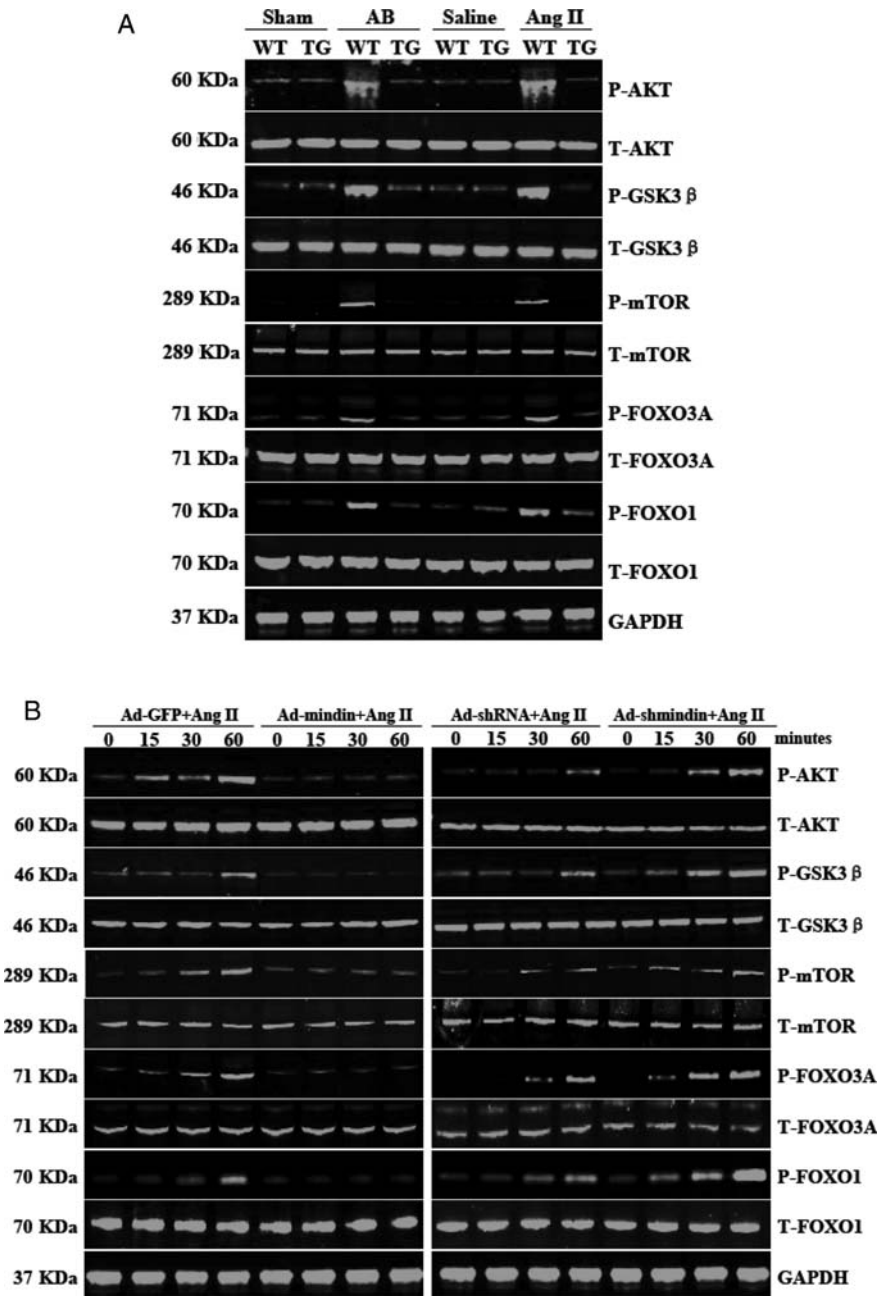
Figure 5 Mindin suppresses AKT/GSK3β/mTOR/FOXO signalling *in vivo* and *in vitro*. (*A*) Representative blots for AKT, GSK3β, mTOR, FOXO3A, and FOXO1 phosphorylation as well as their total protein expression levels 4 weeks following AB surgery or Ang II infusion in WT and TG ($n = 4$). (*B*) Representative blots for AKT, GSK3β, mTOR, FOXO3A, and FOXO1 phosphorylation and their total protein expression levels after treatment with Ang II for the indicated times in neonatal rat cardiomyocytes infected with Ad-GFP, Ad-mindin, Ad-shRNA, or Ad-shmindin. Reproducible results were obtained in three independent experiments.

endothelial growth factor (VEGF).[31] AKT promotes hypertrophy in part through its inhibitory effects on GSK3β (a negative regulator of calcineurin/nuclear factor of activated T cells signalling and hypertrophy), FOXO transcription factors (which promote the transcription of atrophy-related genes), and mTOR (a critical regulator of protein synthesis necessary for hypertrophy). Consistent with the observed decrease in AKT activity, hypertrophic stimuli resulted in decreased levels of phosphorylation of GSK3β^{Ser9} and FOXO transcription factors at AKT phosphorylation sites (reducing their anti-hypertrophic effects), as well as decreased activation of mTOR in TG mice

compared with WT mice. Mindin similarly attenuated AKT signalling, cell size, and protein synthesis in isolated cardiomyocytes, which indicates that mindin acts directly on cardiomyocytes.

The mechanism by which mindin specifically blocks AKT signalling remains unknown. As a ligand for integrins, mindin may alter integrin signalling complexes to regulate AKT activation. In general, integrin signalling is essential for both normal cardiac function and compensatory hypertrophy,[32,33] and therefore, it is unlikely that mindin inhibits integrin signalling *per se*. Mindin may modulate integrin signalling or a specific integrin complex in a manner that specifically regulates AKT.
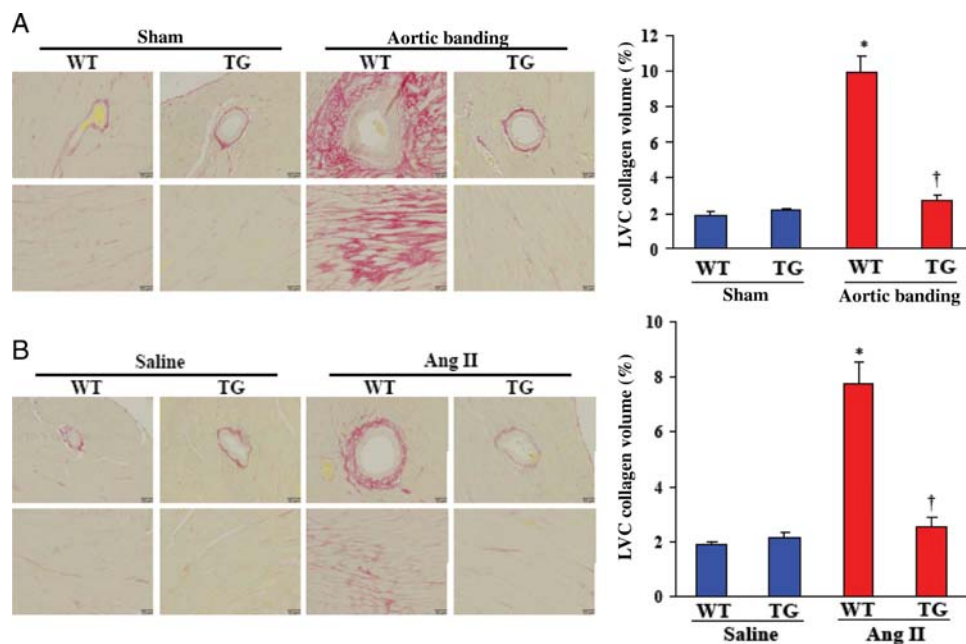
Figure 6 Forced mindin expression attenuates fibrosis in response to pressure overload or Ang II stimulation. (*A* and *B*) Histological sections of the LVs of WT and TG mice (n = 6) were stained with PSR at 4 weeks after AB surgery (*A*) or Ang II infusion (*B*). Left, representative images; right, quantitative results. Scale bar: 32 μm. Fibrotic areas of the histological sections were quantified using an image analysis system. *$P < 0.01$ for WT/ sham or WT/saline values; $^{†}P < 0.01$ for WT/AB or WT/Ang II after AB or Ang II infusion.

F-spondin, for instance, blocks integrin αvβ3 and inhibits tyrosine phosphorylation of FAK and activation of AKT in VEGF-stimulated HUVECs.[31] Further experiments are needed to determine the molecular signalling mechanism by which mindin regulates AKT. It is worthy to note that AKT pathway is a member of totally non-specific pathways, a change in AKT pathway is likely to be an indicator much more than a true determinant and a pharmacological target. Further studies are called for to establish a more specific target responsible for the anti-hypertrophic effect of mindin.

Cardiomyocyte enlargement is a feature of both physiological and pathological hypertrophy, whereas fibrosis is a classical feature of pathological hypertrophy.[34] The present study reveals for the first time that mindin blocks cardiac fibrosis and attenuates the expression of several fibrotic mediators induced by chronic pressure overload or Ang II stimulation. To elucidate the mechanisms underlying the inhibitory effect of mindin on fibrosis, we analysed key components of the TGF-β1–Smad signalling pathway, which plays an important role in the progression of fibrosis.[35–37] Our data demonstrate that mindin abrogates TGF-β1 expression, Smad2 phosphorylation, and Smad2/3 translocation in hypertrophied hearts, thus inhibiting fibrosis. In isolated cardiac fibroblasts, mindin also blocked the phosphorylation of Smad2 and the nuclear translocation of Smads 2 and 3 in response to TGF-β1, which suggests that mindin inhibits the TGF-β1–Smad signalling pathway both upstream and downstream of TGF-β1 expression. In addition to its effects on fibrosis, TGF-β1–Smad signalling has also been shown to induce cardiomyocyte apoptosis,[38,39] another common feature of pathological hypertrophy. The strong inhibition of TGF-β1–Smad signalling may be a mechanism by which mindin attenuates maladaptive responses to cardiac stress (i.e. fibrosis and apoptosis). Thus, our results suggest that mindin attenuates cardiomyocyte enlargement through AKT while concomitantly reducing fibrosis through the inhibition of TGF-β1–Smad signalling.

In conclusion, our present work provides the first evidence that cardiac mindin reduces cardiac remodelling in response to hypertrophic stimuli via inhibition of the AKT/GSK3β and TGF-β1–Smad signalling pathway. We propose that targeting of the mindin signalling may develop novel promising strategies for the treatment of cardiac hypertrophy and HF.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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