

Fibulin-2 is essential for angiotensin II-induced myocardial fibrosis mediated by transforming growth factor (TGF)- β

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Fibrosis is an ominous pathological process in failing myocardium, but its pathogenesis is poorly understood. We recently reported that loss of an extracellular matrix (ECM) protein, fibulin-2, protected against ventricular dysfunction after myocardial infarction (MI) in association with absence of activation of transforming growth factor (TGF)- β signaling and suppressed upregulation of ECM protein expression during myocardial remodeling. Here we investigated the role of fibulin-2 in the development of myocardial hypertrophy and fibrosis induced by continuous pressor-dosage of angiotensin II (Ang II) infusion. Both wild type (WT) and fibulin-2 null (Fbln2KO) mice developed comparable hypertension and myocardial hypertrophy by Ang II infusion. However, myocardial fibrosis with significant upregulation of collagen type I and III mRNA was only seen in WT but not in Fbln2KO mice. Transforming growth factor (TGF)- β 1 mRNA and its downstream signal, Smad2, were significantly upregulated in WT by Ang II, whereas there were no Ang II-induced changes in Fbln2KO, suggesting fibulin-2 is necessary for Ang II-induced TGF- β signaling that induces myocardial fibrosis. To test whether fibulin-2 is sufficient for Ang II-induced TGF- β upregulation, isolated Fbln2KO cardiac fibroblasts were treated with Ang II after transfecting with fibulin-2 expression vector or pretreating with recombinant fibulin-2 protein. Ang II-induced TGF- β signaling in Fbln2KO cells was partially rescued by exogenous fibulin-2, suggesting that fibulin-2 is required and probably sufficient for Ang II-induced TGF- β activation. Smad2 phosphorylation was induced just by adding recombinant fibulin-2 to KO cells, suggesting that extracellular interaction between fibulin-2 and latent TGF- β triggered initial TGF- β activation. Our study indicates that Ang II cannot induce TGF- β activation without fibulin-2 and that fibulin-2 has an essential role in Ang II-induced TGF- β signaling and subsequent myocardial fibrosis. Fibulin-2 can be considered as a critical regulator of TGF- β that induces myocardial fibrosis.

Laboratory Investigation (2016) 96, 773–783; doi:10.1038/labinvest.2016.52; published online 25 April 2016

Heart failure is a progressive lethal disease that is a major cause of morbidity and mortality in the modern world. Myocardial remodeling is a pathological process of the myocardium responsible for geometric changes in the ventricular chamber and resulting functional deterioration. A number of studies have implicated that angiotensin II (Ang II), a major bioactive peptide of the renin-angiotensin-aldosterone system (RAAS), has a central role in maintaining cardiovascular homeostasis¹ and that its dysregulation results in pathological remodeling, especially myocardial fibrosis.^{2,3} Progressive fibrosis results in increased ventricular stiffness contributing to diastolic dysfunction and increased arrhythmogenesis.⁴ Beneficial effects of Ang

II inhibition have been reported widely in experimental animal studies^{5,6} and clinical settings.^{7,8} Ang II-induced ventricular remodeling is a widely used animal model to study pathogenesis of heart failure, but its biological effects are diverse and widely modulated by many other factors.

Transforming growth factor (TGF)- β is a multi-functional peptide growth factor with a vital role in the regulation of cell proliferation, differentiation, inflammation, angiogenesis, and wound healing, as recently reviewed.^{9,10} Several lines of evidence support an essential role of excessive TGF- β in pathogenesis of maladaptive remodeling and heart failure.^{11–15} TGF- β serves as a downstream pro-hypertrophic and

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Received 4 January 2016; revised 14 March 2016; accepted 20 March 2016

pro-fibrotic growth factor induced by Ang II.^{16,17} TGF- β has been trialed as a therapeutic target for progressive ventricular remodeling,^{14,18–21} but the results were inconsistent.^{20,22,23} Although excessive TGF- β is deleterious to stressed myocardium, baseline TGF- β is essential to preserve myocardial integrity and structure.¹² Complete elimination of this essential growth factor may not be altogether benign. TGF- β activation is regulated at multiple different sites including within intracellular signaling pathways and through extracellular interactions. In the extracellular milieu, several matricellular proteins have been known to modulate TGF- β activation and have been recently highlighted as major contributors of the pathogenesis of ventricular remodeling.^{24,25}

We recently reported that genetic deletion of fibulin-2, an ECM protein, attenuated ventricular dysfunction and significantly improved survival following experimental myocardial infarction (MI) in the mouse model.²⁶ The same fibulin-2-deficient mice failed to develop suppressor Ang II-induced myocardial hypertrophy in conjunction with suppressed TGF- β mRNA upregulation and its downstream signaling, suggesting that fibulin-2 is necessary for Ang II-induced TGF- β signaling and myocardial hypertrophy.²⁷ Collectively, these studies suggest that fibulin-2 assumes a unique role in mediating TGF- β activation in the ECM to enhance myocardial remodeling. We also found that fibulin-2 appeared to mediate Ang II-induced myocardial fibrosis, but the underlying mechanism of how fibulin-2 facilitates fibrosis was not fully studied. Here we introduce an experimental model that induces myocardial fibrosis by chronic infusion of pressor dosage of Ang II that also causes systemic hypertension and investigate how fibulin-2 deficiency prevents development of myocardial fibrosis, an ominous pathological process that aggravates ventricular remodeling and heart failure.

MATERIALS AND METHODS

Animals

Adult male mice, 12–20 weeks old, were used in this study. The Fbln2KO mice were maintained on the C57BL/6 genetic background after backcrossing for 10 generations as described elsewhere.²⁸ Age-matched WT C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal procedures in this study were performed in adherence with the National Institutes of Health Guidelines on the Use of Laboratory Animals and have been approved by the Institutional Animal Care and Use Committee (IACUC) of Nemours/Alfred I. duPont Hospital for Children.

Chronic Subcutaneous Ang II Infusion

Under isoflurane anesthesia (1.5%), a mini-osmotic pump (ALZET, model 2004; ALZACorp, Palo Alto, CA) filled with either pressor dose of Ang II (2 μ g/kg/min; Sigma-Aldrich, St Louis, MO)²⁹ or normal saline was inserted underneath the skin. Body weight (BW), systolic blood pressure (SBP), and

heart rate (HR) were measured before, 2 weeks, and 4 weeks after Ang II infusion. After 4 wk of Ang II infusion, all mice were euthanized with CO₂ inhalation. The weight of the left ventricle was measured, and the ratio of left ventricular weight (LVW) to the BW was calculated as an index of LV hypertrophy.

Echocardiography

Under isoflurane anesthesia (1.5%), M-mode echocardiography was performed before, 2 weeks, and 4 weeks after the initiation of Ang II infusion (Vevo770 System, VisualSonics, Toronto, Canada). Diastolic measurements of inter-ventricular septum (IVSd), LV internal dimension (LVIDd), LV posterior wall thickness (LVPWd), and systolic measurement of LVIDs were obtained. The LV shortening fraction (% LVSF) was calculated as below:

$$\%SF = [(LVIDd - LVIDs)/LVIDd] \times 100(\%)$$

Histopathology and Immunohistochemistry

The LV myocardium was stored in 10% formalin/PBS or frozen medium (OCT) for routine histology (hematoxylin and eosin (H&E) and Masson's trichrome staining) or immunohistochemistry, respectively. Serial 8- μ m-thick cryosections of frozen specimens were prepared for immunohistochemistry. The detailed protocol for immunostaining has been described elsewhere.³⁰ Primary antibody used was anti-fibulin-2.³¹ Alexa Fluor 546 goat anti-rabbit IgG antibody (Invitrogen Corporation, Carlsbad, CA) was used for secondary antibody. The specimens were observed under a light microscope or epifluorescent microscope (Olympus, Japan). The area of fibrosis in Masson's trichrome staining was measured by ImageJ and was represented as percentage of total myocardial area. Average myocyte cross-sectional area in H&E slides was also measured by ImageJ.

Quantitative Real-Time RT-PCR

Total RNA was extracted from the LV myocardium using TRI Reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The cDNA was synthesized from 2 μ g of total RNA by oligo(dT)-primed reverse transcription s(RT) (iScript cDNA synthesis kit, Bio-Rad Laboratories, Hercules, CA). Real-time reverse transcription-polymerase chain reaction (qRT-PCR) was performed with MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories). Collagen type I (Col I), collagen type III (Col III), fibulin-2, TGF- β 1, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and β -myosin heavy chain (β -MHC) were studied. The expression for each gene was determined as the relative expression or cycle threshold (Ct) of the gene of interest to the expression of GAPDH by the following formula:

$$\Delta Ct = 2^{[(Ct_{GAPDH}) - (Ct_{\text{gene of interest}})]}$$

Primers for qRT-PCR are listed in online Supplementary Table 1.

Table 1 Physiological parameters during pressor dosage of Ang II infusion (2 µg/kg/min) over 4 weeks

	Before (0 wk)				2 wks				4 wks			
	WT-Sh	WT-Ang II	KO-Sh	KO-Ang II	WT-Sh	WT-Ang II	KO-Sh	KO-Ang II	WT-Sh	WT-Ang II	KO-Sh	KO-Ang II
BW (g)	30.4±2.2	30.3±1.2	28.7±3.1	30.9±2.4	29.5±2.3	28.5±1.0	29.0±3.1	28.4±2.7	29.7±2.2	28.4±1.2	28.9±3.0	27.7±2.1
HR (beats/min)	633±82	558±94	605±113	590±132	662±95	559±70	687±42	588±69	661±55	580±58	652±87	642±83
SBP (mmHg)	100±11	104±9	105±13	107±8	112±8	158±19 ^{ab}	109±11	161±11 ^{ab}	120±10	170±12 ^{ab}	121±7	162±12 ^{ab}

^a*P*<0.05 compared with sham.

^b*P*<0.05 compared with 0 day.

Western Blotting Analysis

Myocardial tissue was homogenized with RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA), and protein concentration of the supernatants was determined by Pierce BCA Assays (Pierce Biotechnology, Rockford, IL). Protein lysates (50 µg) were electrophoresed on a 14% sodium dodecyl sulfate (SDS)-polyacrylamide gel and then electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). Primary antibodies used were against phospho-Smad2, Smad2, phospho-ERK1/2, ERK1/2, phospho-p38MAPK, p38MAPK, β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA), and fibulin-2. Blots were incubated with horseradish-labeled secondary antibodies (anti-mouse IgG or anti-rabbit IgG), and signals were detected using Pierce ECL Western Blotting Substrate (Pierce Biotechnology). Membrane was exposed to Fuji radiograph film, and signal intensities were analyzed with the ImageJ software.

Cell Culture

Cardiac fibroblasts (CFs) were isolated from ventricular myocardium of Fbln2KO after repeated digestion with collagenase IV (100 U/ml)/trypsin (0.6 mg/ml) (Invitrogen, Carlsbad, CA) solution. The CFs were resuspended in DMEM with 20% FBS (Mediatech, Herndon, VA) and 1% penicillin and streptomycin (Invitrogen) and were plated on laminin-coated 60-mm dishes (BD Biosciences, Franklin Lakes, NJ). After the first passage, CFs were transfected with fibulin-2 expression plasmid in Rc/CMV vector³¹ and empty Rc/CMV vector (control) by Nucleofactor Technology (Lonza, Walkersville, MD). In another experiment, recombinant fibulin-2³¹ was plated on the laminin-coated plates at two different concentrations (0.5 and 2.5 µg/ml as a final concentration of culture medium) before CFs were seeded. Then, CFs were incubated in serum-free medium for 24 h, followed by Ang II (1 × 10⁻⁷ M) treatment for 24 h. The CFs were then harvested.

Statistical Analysis

All values in the text and figures are presented as mean ± s.d. unless specified otherwise. All data were subjected to one-way ANOVA followed by Bonferroni correction for *post hoc t*-test.

Western blot densities were analyzed with the Kruskal–Wallis test followed by Dunn's *post hoc* test. *P*-values of less than 0.05 were considered statistically significant.

RESULTS

Physiological Effects of Pressor Dosage of Ang II Infusion

All mice survived Ang II treatment for 4 weeks. The BW, HR, and SBP were measured before treatment and 2 weeks, and 4 weeks after Ang II infusion (Table 1). The BW did not change in either WT or Fbln2KO over 4 weeks. Ang II-induced hypertension (40–50 mm Hg increase compared with sham) was noted in both WT and Fbln2KO at 2 weeks and 4 weeks, but there was no significant difference between WT and Fbln2KO. The HR did not change significantly among the four groups. Echocardiogram revealed that the increase in LV wall thickness after Ang II treatment was comparable between WT and Fbln2KO, but there was no change in LV cavity size (LVIDd) and/or LV systolic function (%LVSF) by Ang II treatment in either group (Figure 1a). The LVW/BW ratio increase by Ang II treatment was 32.5% in WT and 34.5% in Fbln2KO mice when compared with sham mice, respectively, consistent with the echocardiographic findings (Figure 1b). Pressor dosage of Ang II infusion resulted in the same degree of LV hypertrophy in both WT and Fbln2 KO without change in systolic function or ventricular chamber size. Fibulin-2 deletion did not attenuate the degree of Ang II-induced myocardial hypertrophy.

Development of Myocardial Fibrosis was Markedly Reduced in Fbln2KO Mice

The degree of myocardial fibrosis was assessed by Masson's Trichrome staining (Figure 2a). In WT with Ang II treatment, there was significant increase in fibrosis (blue color) in both perivascular and interstitial area in WT, whereas the degree of fibrosis did not increase by Ang II in Fbln2KO mice. Quantitative assessment of fibrotic area showed that Ang II-treated WT had significantly increased fibrosis compared with the rest of the groups (Figure 2c). With Ang II treatment, spatial expression of fibulin-2 protein was increased in the interstitial area in WT when compared with sham; there was no fibulin-2 expression in

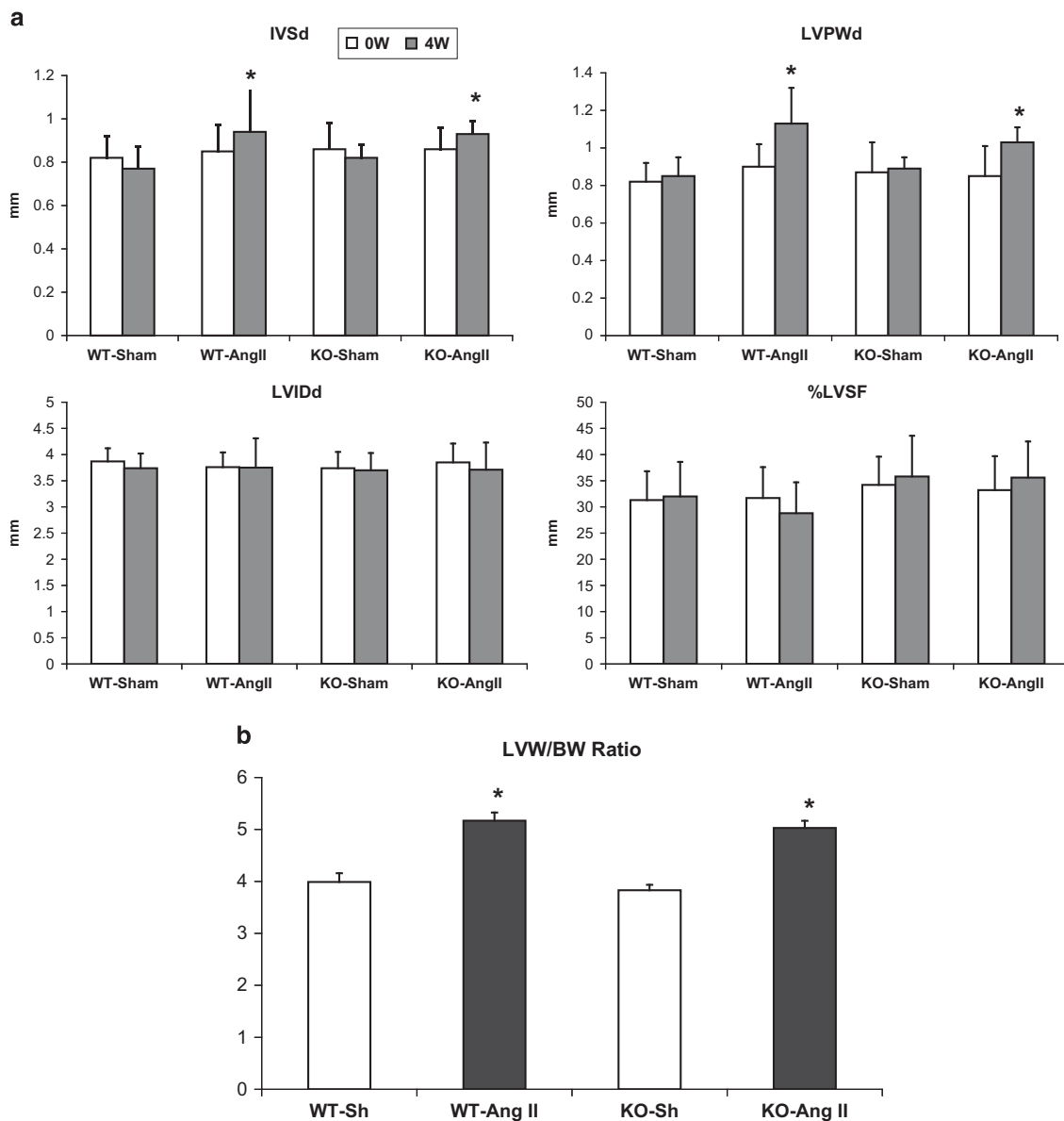


Figure 1 (a) Echocardiographic parameters at 0 wk and 4 wk after pressor dosage of Ang II infusion. Significant but comparable increase in IVS and LV wall thickness was seen in both WT and KO (Fbln2KO). There was no significant change in LV chamber size (LVIDd) or LV systolic function (%LVSF) after Ang II treatment in either group ($n=10$ in all four groups). * $P<0.05$ compared with sham. IVSd, interventricular septal wall thickness in diastole; LVPWd, left ventricular posterior wall thickness in diastole; LVIDd, left ventricular internal diameter in diastole; %LVSF, % left ventricular shortening fraction. (b) LVW (left ventricular weight) /BW (body weight) ratio. There was significant but comparable increase of LVW/BW in the Ang II-treated groups, both WT and Fbln2KO, when compared with sham groups. Data are shown as mean \pm s.d.

Fbln2KO mice. Significant increase in mRNA levels of collagen type I (Col I) and collagen type III (Col III) by Ang II was seen in WT, whereas there was no significant increase by Ang II in Fbln2KO mice (Figure 3). These results indicated that the development of Ang II-induced myocardial fibrosis was significantly attenuated by the absence of fibulin-2. Myocyte cross-sectional area was equally increased by Ang II treatment in both WT and Fbln2KO, consistent with LVW/BW (Figure 2b and d). However, β -MHC showed no change by Ang II in either

group. Although Ang II-induced myocardial hypertrophy was comparable between WT and Fbln2KO, ANP and BNP mRNA were both significantly higher in Fbln2KO than WT (Figure 3).

The Activation of Ang II-induced TGF- β Signaling Pathway was also Attenuated in Fbln2KO Mice

Ang II treatment significantly increased TGF- β 1 mRNA levels in WT but not in Fbln2KO, suggesting fibulin-2 is required for Ang II-induced TGF- β 1 mRNA upregulation.

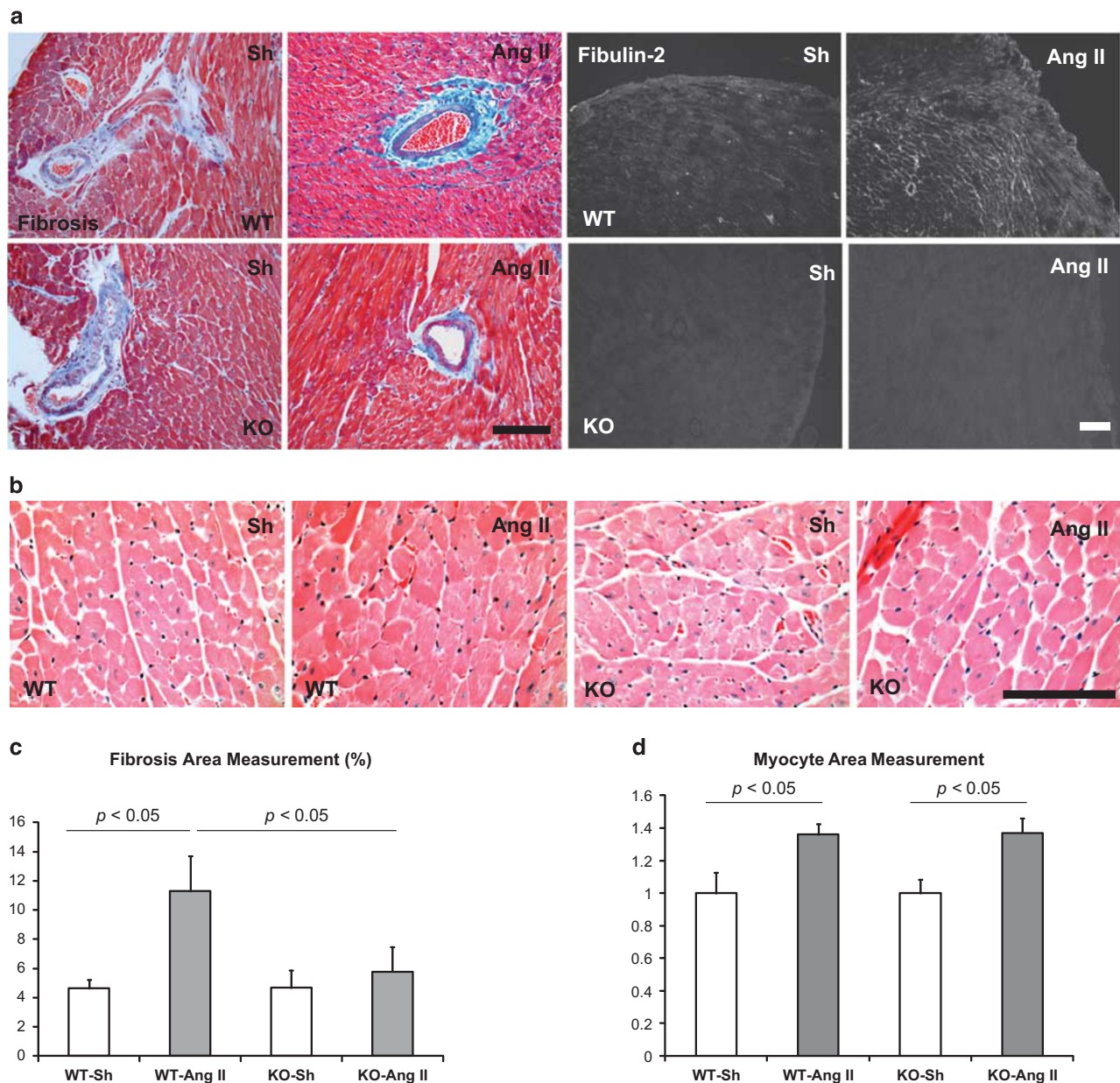


Figure 2 Development of myocardial fibrosis (a) and hypertrophy (b) after Ang II infusion. (a) Masson's trichrome staining (left 4) and fibulin-2 localization by immunohistochemistry (right 4). WT developed significant perivascular and interstitial fibrosis by Ang II infusion, whereas Ang II-induced fibrosis was almost completely abolished in KO (Fbln2KO). Fibulin-2 protein expression was significantly increased after Ang II infusion in WT (diffuse interstitial localization). Magnification bar = 100 μ m. (b) High magnification of left ventricular myocardium by hematoxylin–eosin (H&E) staining. Magnification bar = 100 μ m. (c) Quantification of myocardial fibrosis by measuring fibrotic area (blue color after Masson's Trichrome staining) using Image J software. Y axis indicates % area of fibrosis. Ang II-treated WT showed significant myocardial fibrosis compared with WT sham or Ang II-treated KO. Sample size: WT-sham ($n=3$), WT-Ang II ($n=5$), KO-sham ($n=3$), and KO-Ang II ($n=5$). (d) Myocyte cross-sectional area was measured by ImageJ. Average area of WT sham was arbitrarily set at 1. Sample size: $n=4$ in all groups. Data are shown as mean \pm s.d.

Phosphorylation of Smad2, a downstream maker of TGF- β downstream canonical pathway, was significantly increased in WT by Ang II treatment, but there was no change in Fbln2KO (Figure 4). Protein expression of fibulin-2 was increased in WT by Ang II, which is consistent with the finding of immunohistochemistry (Figure 2) and qRT-PCR (Figure 3).

There was no significant changes in phosphorylation in p38MAPK, JNK, or TAK1, markers for noncanonical pathways, by Ang II in either WT or Fbln2KO (data not shown). The study suggests that fibulin-2 is required for Ang II-induced TGF- β 1 mRNA upregulation and its downstream Smad signaling.

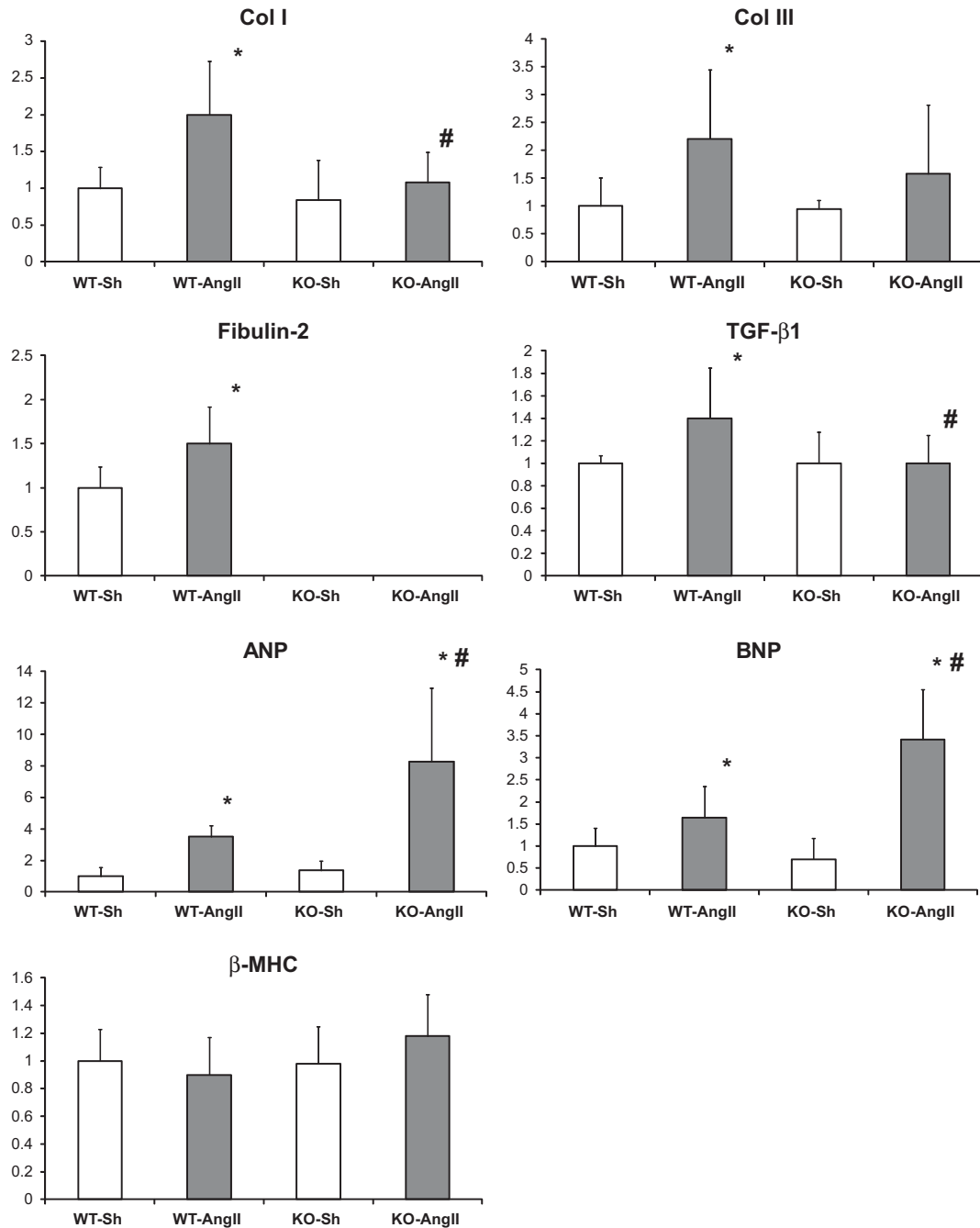


Figure 3 Myocardial mRNA expression of selective remodeling genes by real-time RT-PCR (qRT-PCR). Col I (collagen type I), Col III (collagen type III), fibulin-2, TGF-β1, ANP, BNP, and β-MHC were studied. Col I, Col III, and TGF-β1 were significantly increased by Ang II in WT but not as significantly in Fbln2KO. Ang II-induced TGF-β1 increase was abolished in Fbln2KO. On the contrary, the Ang II-induced increase in ANP and BNP was significantly higher in Fbln2KO when compared with WT. There was no significant change in β-MHC after Ang II treatment in either WT or KO. Data are shown as mean ± s.d. **P* < 0.05 compared with sham (Sh). #*P* < 0.05 compared with WT. Sample size: *n* = 10 in all four groups.

Addition of Exogenous Fibulin-2 to Fbln2KO CFs Rescued Ang II-induced TGF-β Signaling

We have previously shown that fibulin-2 is required for Ang II-induced TGF-β upregulation and ECM protein upregulation in isolated CFs.^{26,32} In this study, fibulin-2-deficient CFs were rescued by either transfecting fibulin-2

expression vector or adding recombinant mouse fibulin-2 to the culture plates before treating with Ang II. By transfecting fibulin-2 expression vector, fibulin-2 protein was synthesized by Fbln2KO CFs. Ang II treatment increased phosphorylated Smad2 (Figure 5a). By adding recombinant fibulin-2 in Fbln2KO cells, Ang II treatment increased

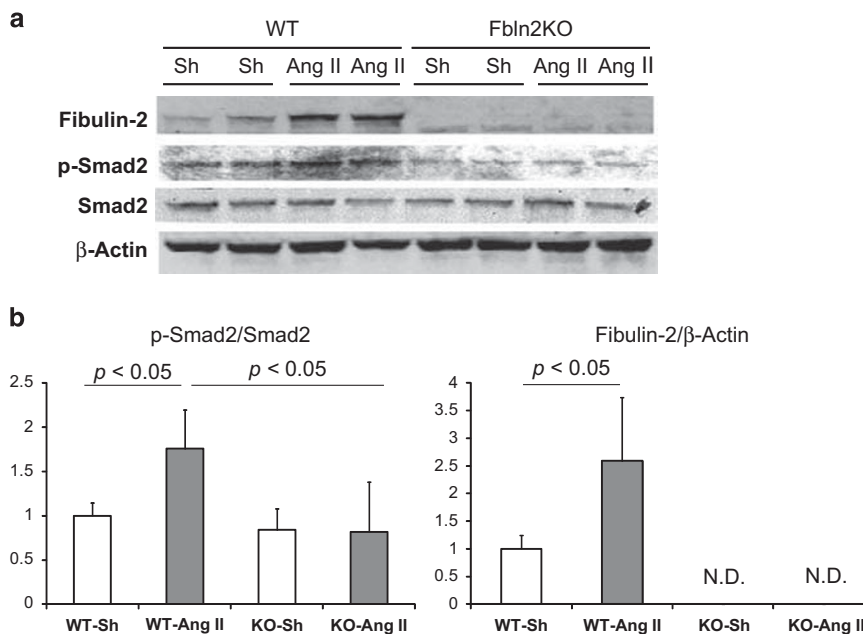


Figure 4 Protein expression by western blots. (a) Fibulin-2 protein expression and Smad2 phosphorylation were significantly increased with Ang II treatment in WT, whereas there was no significant change in these protein expressions in Fbln2 KO hearts. (b) Quantification of protein expression. Sample size: $n=4$ in all groups.

TGF- β downstream noncanonical signaling markers ERK1/2 and p38MAPK but did not upregulate Smad2 (Figure 5b). However, Smad2 phosphorylation was increased just by adding recombinant fibulin-2. These findings suggest that fibulin-2 is, in part, sufficient for Ang II-induced TGF- β signaling responsible for ECM remodeling.

DISCUSSION

Our current study demonstrated that the absence of fibulin-2 attenuated Ang II-induced myocardial fibrosis but not myocardial hypertrophy. Fibulin-2 was not required for Ang II-induced systemic hypertension. In this model, the mouse LV myocardium was exposed to both direct pharmacological effects of Ang II and the pressure overload (40–50 mmHg) secondary to hypertension. With this sequence of experiments, we have demonstrated that fibulin-2 is essential for upregulation of TGF- β mRNA and subsequent signaling in developing myocardial fibrosis.

Fibulin-2 Deficiency did not Prevent Hypertension or Hypertrophy but Attenuated Myocardial Fibrosis

In the current study, we focused on induction of myocardial fibrosis by infusing high-dose Ang II (2 $\mu\text{g}/\text{kg}/\text{min}$) for 4 weeks, which is a 10-fold higher dosage than the dose of subpressor used in the previous study.²⁷ High-dose Ang II infusion is reported to induce hypertension via direct vasoconstriction and resulting LV hypertrophy and myocardial fibrosis in WT mice.²⁹ In our study, the degree of hypertension and myocardial hypertrophy were comparable between WT and Fbln2KO, but myocardial fibrosis was seen

only in WT and was almost abolished in Fbln2KO mice (Figure 2). There was no LV dilatation or LV systolic dysfunction in either group after high-dose Ang II infusion, as demonstrated by echocardiography (Figure 1). The attenuated myocardial fibrosis in Fbln2KO by Ang II is associated with lack of upregulation of TGF- β 1 mRNA and its downstream signal, phosphorylated Smad2. Decreased upregulation of Col I and Col III mRNA expression in Fbln2KO may be attributed to lack of TGF- β activation. Smad-dependent pathways are a central pathway for TGF- β -induced myocardial fibrosis.³³ Our results were similar to other experiments with Smad3KO mice in response to Ang II infusion.³⁴

In our current model, a comparable degree of myocardial hypertrophy was induced in Fbln2KO without TGF- β activation, suggesting that pressure overload and other Ang II-induced TGF- β -independent pathways may be responsible for hypertrophy in both groups, such as interleukin-6 (IL-6) and nitric oxide (NO).³⁵ Enhanced TGF- β signaling has a principal role in inducing myocardial fibrosis. This is in agreement with the previous studies in which TGF- β inhibition by TGF- β neutralizing antibody resulted in attenuation of myocardial fibrosis but no significant change in myocardial hypertrophy in a pressure overload model.^{15,36} Absence of fibulin-2 did not allow Ang II-induced TGF- β 1 upregulation, suggesting Ang II cannot directly induce TGF- β mRNA expression without fibulin-2. Of note, Smad2 activation was increased only by adding recombinant fibulin-2 to isolated Fbln2KO CFs, suggesting extracellular interaction between fibulin-2 and latent TGF- β complex

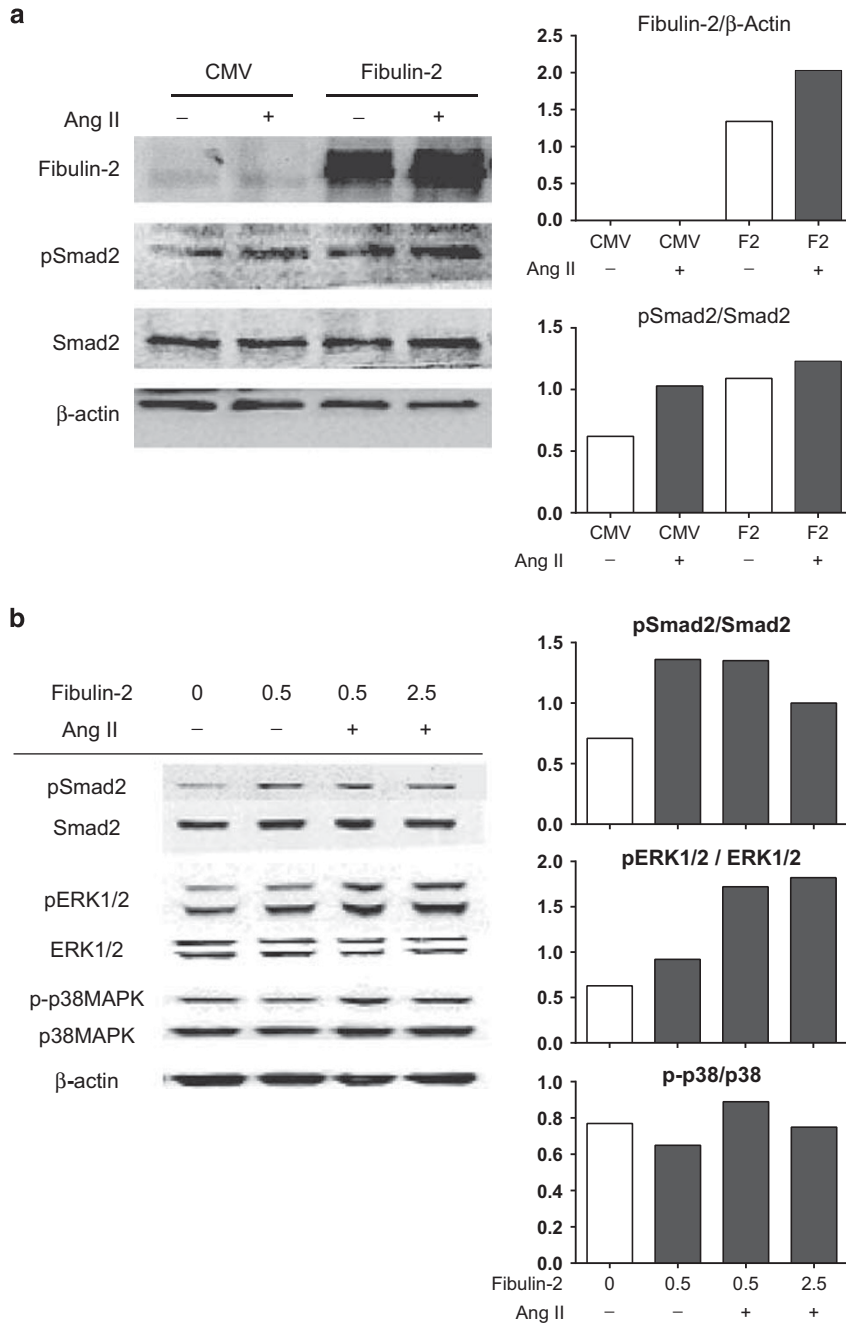


Figure 5 The effects of introducing exogenous fibulin-2 in Fbln2KO cardiac fibroblasts (CFs) for Ang II treatment. **(a)** Either Rc/CMV vector or fibulin-2 expression vector was transfected in CFs before Ang II treatment. Western blots showing fibulin-2 expression and Smad2 phosphorylation. Fibulin-2 transfection successfully introduced fibulin-2 on Fbln2KO CFs, in which Ang II slightly increased Smad2 phosphorylation. **(b)** Recombinant mouse fibulin-2 was added to Fbln2KO CFs in two different concentrations; 0.5 and 2.5 μ g/ml. Smad2 phosphorylation was increased only by adding recombinant fibulin-2. ERK1/2 showed increased phosphorylation after adding both recombinant fibulin-2 and Ang II. Quantification of protein expression were shown in the graphs for both **a** and **b**.

(Figure 5b). Fibulin-2 appears to mediate TGF- β -induced TGF- β synthesis/activation or TGF- β autoinduction.

Interestingly, we found that upregulation of ANP and BNP mRNA was significantly higher in Fbln2KO than in WT by Ang II despite a similar degree of hypertrophy between WT and Fbln2KO. Although natriuretic peptides are upregulated

in pressure overload-induced hypertrophy (transverse arch constriction or TAC model), they are also known to counteract against TGF- β in its pro-fibrotic properties.³⁷ The synthesis of natriuretic peptides is induced not only by mechanical stretch but also directly by Ang II.³⁸ The upregulation of natriuretic peptides may counteract the

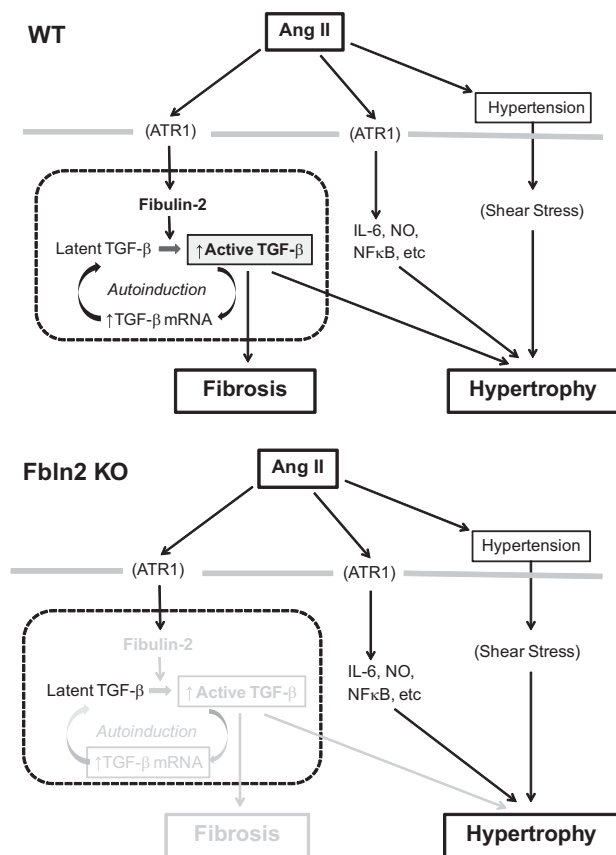


Figure 6 Possible underlying mechanism of how fibulin-2 is involved in TGF- β autoinduction (TGF- β -induced TGF- β synthesis and activation), myocardial hypertrophy, and fibrosis in response to pressor dosage of Ang II. Myocardial hypertrophy was induced predominantly via hypertension-induced shear stress and Ang II receptor type 1 (ATR1)-mediated signaling pathways independent of TGF- β , as presence or absence of fibulin-2 did not alter hypertrophy in this model. Increased fibulin-2 protein expression may enhance TGF- β activation through extracellular interaction that triggers TGF- β autoinduction in WT leading fibrosis, whereas absence of fibulin-2 cannot initiate TGF- β autoinduction. IL-6, interleukin-6; NO, nitric oxide.

progression of fibrosis in Fbln2KO. The mechanism of prominent upregulation of natriuretic peptides in Fbln2KO remains unclear in this study, but a similar finding was reported in osteopontin-deficient mice in which Ang II-induced myocardial fibrosis was attenuated.²⁹ As stated above, the role of natriuretic peptides in our model may be different from that in TAC-induced ventricular remodeling model, in which ANP and BNP upregulation primarily represents the degree of hypertrophy.

Fibulin-2 is Required and Sufficient for Ang II-induced TGF- β Activation

Adding exogenous fibulin-2 in Fbln2KO CFs by transfecting fibulin-2 expression vector or by adding recombinant fibulin-2 protein rescued, in part, Ang II-induced TGF- β signaling in Fbln2KO CFs, suggesting fibulin-2 is also sufficient for Ang

II-induced TGF- β activation, as shown as increased phosphorylation in Smad2 (Figure 5a) and in ERK1/2 (Figure 5b). With the suppressor Ang II infusion model, we demonstrated the fibulin-2-mediated autocrine and paracrine positive-feedback loop involving TGF- β as a central mechanism of Ang II-induced myocardial hypertrophy.²⁷ In our current model, myocardial hypertrophy was induced equally in both WT and Fbln2KO regardless of myocardial TGF- β activation, but fibrosis was almost abolished in Fbln2KO in conjunction with suppressed TGF- β activation. Fibulin-2 had a pivotal role in activating TGF- β and subsequently introducing myocardial fibrosis. Separately, Karakikes *et al.*³⁹ identified fibulin-2 as a direct downstream marker in miRNA treatment (miR-1) that prevented pressure overload-induced pathological remodeling in conjunction with attenuated TGF- β signaling. Fibulin-2 was upregulated in the left ventricular myocardium of endstage heart failure patients in conjunction with significantly enhanced TGF- β bioactivity when compared with control myocardium, suggesting a pathological role of fibulin-2 in exacerbating adverse ventricular remodeling also in humans.⁴⁰ These findings, together with our current results, strongly suggest that fibulin-2 has a critical role in pathological remodeling that can become a potential target for attenuating heart failure.

Fibulin-2 is a Unique Matricellular Protein in Modulating ECM during Ventricular Remodeling

We have proposed that fibulin-2 is a new member of matricellular protein that does not participate in structural integrity of a tissue but is mainly involved in regulating activation of enzymes, proteinases, and growth factors.²⁵ Spatially, fibulin-2 is highly upregulated at the sites of epithelial-mesenchymal transformation during embryonic development, but its expression is restricted to the vascular endothelial basement membrane, cardiac valves, skin basal layers, and periostial membrane, thereafter.^{30,41} Fibulin-2 is upregulated in the tissue repair process in skin wound healing⁴² and MI.²⁶ Loss of fibulin-2 protected against cardiac rupture and ventricular dysfunction, and significantly improved survival after MI by attenuating TGF- β signaling.²⁶ Both MMP-2 mRNA and activation levels were markedly reduced in Fbln2KO when compared with WT during ventricular remodeling after MI.²⁶

The underlying molecular mechanism of how fibulin-2 regulates TGF- β synthesis remains elusive, but it is important to note that Ang II cannot induce TGF- β upregulation without fibulin-2, suggesting that Ang II does not directly induce TGF- β expression without ECM involvement. Ang II initially upregulates fibulin-2, which subsequently activates TGF- β probably by cleaving free TGF- β from the latent complex in ECM. Isolated free TGF- β induces TGF- β synthesis in an autocrine manner, which promotes a positive-feedback loop (autoinduction) (Figure 6). We speculate that secreted fibulin-2 first enhances TGF- β activation by interacting with inactive latent complex in the

ECM and that released free TGF- β can induce autoinduction by autocrine and/or paracrine means.

There are a few limitations in this study. Ang II is known to induce myocardial fibrosis through increased inflammatory process, which is also mediated by TGF- β .³⁴ We have not investigated the involvement of inflammatory cells and their cytokines in this study. In our previous post-MI study, the number of macrophages and neutrophils in the ischemic myocardium were significantly reduced in Fbln2KO mice when compared with WT 3 days after MI.²⁶ We have not examined LV diastolic function, which is an excellent functional marker of myocardial fibrosis and/or hypertrophy. To claim that this is an animal model of pathological myocardial fibrosis, it is critical to assess diastolic function. However, echocardiographic assessment of LV diastolic dysfunction in the mouse model remains controversial.

Our current study suggests that fibulin-2 is essential for Ang II-induced myocardial fibrosis by mediating TGF- β signaling. Without fibulin-2, TGF- β autoinduction does not occur. Targeting fibulin-2 may have potential therapeutic indication not only by preventing TGF- β -mediated myocardial fibrosis, thus attenuating the progression of heart failure in humans, but also by enhancing expression of natriuretic peptides that have antifibrotic properties.⁴³ Further investigation is required to delineate the underlying molecular mechanisms that involve fibulin-2.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

ACKNOWLEDGMENTS

This study was supported by U.S. National Institute of Health (1P20RR020173-01 to TT).

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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