Smad7 inhibits angiotensin II-induced hypertensive cardiac remodelling

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1. Introduction

Hypertensive heart disease is a major global health problem and a considerable cause of cardiovascular morbidity and mortality.1 Hypertensive cardiac remodelling is characterized by hypertrophy, fibrosis, and inflammation, which can lead to left ventricular (LV) dysfunction and heart failure.2–4 Angiotensin II (Ang II), a mediator of the renin–angiotensin system, plays an important part in the pathogenesis of hypertensive heart disease.3–5 Beyond its known haemodynamic effect, Ang II mediates cardiac remodelling by inducing hypertrophy, fibrosis, and inflammation through numbers of signalling pathways, including nuclear factor κB (NF-κB) and TGF-β/Smad signalling pathways.3–6 It is now well recognized that Ang II mediates cardiovascular fibrosis via TGF-β-dependent and -independent Smad signalling, which is negatively regulated by Smad7, a downstream inhibitory Smad in TGF-β signalling.7–9

In addition, Smad7 has also been shown to inhibit inflammation by blocking activation of NF-κB signalling pathway.10 Thus, Smad7 acts as a negative regulator for both TGF-β/Smad and NF-κB signalling pathways.11

Increasing evidence shows that drugs targeting Ang II and its type 1 receptor are widely used for the treatment of hypertensive heart disease.12 In addition, functional blocking of TGF-β with a neutralizing antibody is capable of attenuating cardiac remodelling in hypertensive animal model.13 suggesting that targeting the Ang II–TGF-β axis may represent a novel therapy for hypertensive cardiac disease. We have recently reported that mice lacking Smad3 are protected against Ang II-induced cardiac inflammation, fibrosis.14 This also suggests that blockade of downstream TGF-β/Smad signalling may have therapeutic potential for hypertensive cardiopathy. As Smad7 is an important inhibitor of both TGF-β/Smad and NF-κB signalling and has been shown to inhibit

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Keywords

Ang II • Smad7 • Fibrosis • Inflammation • miR-29

Aims

Smad7 plays a negative regulatory role in many inflammatory diseases, but its effect on hypertensive disease remains unknown. The present study tested the hypothesis that overexpression of Smad7 may have therapeutic potential for angiotensin II (Ang II)-mediated hypertensive cardiac remodelling.

Methods and results

Hypertensive heart disease was induced in mice by subcutaneous infusion of Ang II for 28 days and treated with Smad7 by a non-invasive ultrasound-microbubble-mediated inducible Smad7 gene transfer. We found that cardiac Smad7 was largely reduced in the hypertensive and overexpression of cardiac Smad7 protected against the fall in the left ventricular (LV) ejection fraction (EF), an increase in LV mass, and cardiac inflammation and fibrosis such as up-regulation of pro-inflammatory cytokines (IL-1β, TNF-α) and fibrotic markers (collagen I, α-SMA), and infiltration of CD3+ T cells and F4/80+ macrophages. Further studies revealed that inactivation of the Sp1-TGF-β/Smad3-mediated cardiac fibrosis and NF-κB (NF-κB, nuclear factor κB) pathways and prevention of cardiac miR-29 loss were mechanisms by which overexpression of Smad7 inhibited Ang II-mediated cardiac remodelling. Importantly, we also found that treatment with Smad7 when hypertensive cardiopathy established at day 14 halted the progression of cardiac injury by blunting the fall of EF and an increase in LV mass, and blocking TGF-β/Smad3-mediated cardiac fibrosis and NF-κB-driven inflammation.

Conclusion

Smad7 plays a protective role in Ang II-induced cardiac remodelling via mechanisms involving the Sp1-TGF-β/Smad3-NF-κB-miR-29 regulatory network. Thus, Smad7 may be a novel therapeutic agent for hypertensive cardiovascular diseases.
fibrosis and inflammation in a number of animal models, including unilateral ureteral obstruction nephropathy, autoimmune crescentic glomerulonephritis, and diabetic kidney disease.\textsuperscript{15–17} In the present study we tested the hypothesis that Smad7 may have therapeutic potential for hypertensive cardiac disease.

### 2. Methods

#### 2.1 Mouse model of Ang II-induced hypertension

Hypertension was induced in mice (CD-1 background, male, aged 8–10 weeks, 30–35 g) by subcutaneous infusion of Ang II at a dose of 1.46 mg/kg per day for 14 or 28 days via osmotic minipumps (Model 2004, ALZA Corp., Palo Alto, CA, USA) as previously described.\textsuperscript{14,15} Mice were euthanized by cardiac blood collection under anaesthesia with ketamine (80 mg/kg) and xylazine (15 mg/kg) intraperitoneally at days 14 and 28 after Ang II infusion. Systolic blood pressure was measured in a conscious mouse by the non-invasive tail-cuff method using the CODA blood pressure system (Kent Scientific, Torrington, CT, USA) following the manufacturer’s instruction. LV tissues were collected for real-time PCR, immunohistochemistry, and western blot analysis. The experimental procedures were approved by the Animal Ethics Committee of The Chinese University of Hong Kong.

#### 2.2 Ultrasound-mediated gene transfer of inducible Smad7 gene-bearing microbubbles into the mouse heart

Following the protocol as previously described,\textsuperscript{15} a mixture of doxycycline-regulated pTRE-Flag-M2Smad7-expressing plasmids was prepared and transferred into the mouse heart using the non-invasive ultrasound-microbubble-mediated technique. Briefly, pTRE-Flag-M2Smad7 and Tet-on plasmids (100 μg/mouse) were mixed with Sonovue (Bracco Diagnostics, Princeton, NJ, USA) in a 1:1 ratio (volume:volume). Then the mixture (400 μL) was injected into mice via tail vein, followed by ultrasound treatment (2 W/cm²) by placing the ultrasound probe on the chest skin over the heart with a plus-wave output for a total of 5 min with 30 s intervals. After ultrasound treatment, 200 μg/mL of doxycycline (Sigma, St Louis, MO, USA) was injected intraperitoneally, followed by the addition of doxycycline in the daily drinking water (200 μg/mL) for the entire experimental period. Control animals had the same protocol but received the Tet-on/pTRE empty vectors daily drinking water (200 μg/mL of doxycycline (Sigma, St Louis, MO, USA)). The LV tissues were lysed and extracted for western blot analysis (2 W/cm²) by placing the ultrasound probe on the chest skin over the heart with a plus-wave output for a total of 5 min with 30 s intervals. After ultrasound treatment, 200 μg/mL of doxycycline (Sigma, St Louis, MO, USA) was injected intraperitoneally, followed by the addition of doxycycline in the daily drinking water (200 μg/mL) for the entire experimental period. Control animals had the same protocol but received the Tet-on/pTRE empty vectors daily drinking water (200 μg/mL of doxycycline (Sigma, St Louis, MO, USA)). The LV tissues were lysed and extracted for western blot analysis (2 W/cm²) by placing the ultrasound probe on the chest skin over the heart with a plus-wave output for a total of 5 min with 30 s intervals. After ultrasound treatment, 200 μg/mL of doxycycline (Sigma, St Louis, MO, USA) was injected intraperitoneally, followed by the addition of doxycycline in the daily drinking water (200 μg/mL) for the entire experimental period. Control animals had the same protocol but received the Tet-on/pTRE empty vectors daily drinking water (200 μg/mL of doxycycline (Sigma, St Louis, MO, USA)).

#### 2.3 Echocardiography

 Transthoracic echocardiography was conducted in all experimental mice before and at day 14 or 28 after Ang II infusion. Echocardiography was performed using a Vevo770 high-resolution ultrasound imaging system (VisualSonics, Inc., Toronto, Canada) with an RMV 707B scanhead (30 MHz) (VisualSonics, Inc.) after lightly anesthetizing with ketamine (50 mg/kg) and xylazine (50 mg/kg) intraperitoneally. The LV ejection fraction (LVEF = [(LVDD^2 − LVSD^2)/LVDD^2] × 100%) and LV mass (mg) = 1.055 × [(ILV + LVDD + LVVP^2) − LVDD^2] were calculated.

#### 2.4 Immunohistochemistry

 Immunohistochemistry was performed in paraffin sections using a microwave-based antigen retrieval method.\textsuperscript{19} The antibodies used in this study were as follows: collagen I (Southern Biotech, Inc., Birmingham, AL, USA), α-SMA (R&D, Minneapolis, MN, USA), TNFα, IL-1β, TGF-β1, and phospho-smad2/3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-NF-κB/p65 (Cell Signaling Technology, Beverly, MA, USA), CD3+ T cells (Abcam, Cambridge, UK), macrophages (F4/80+) (Serotec, Oxford, UK). All slides (except sections stained with antibodies against α-SMA, phospho-Smad2/3, and phospho-NF-κB/p65) were counterstained with haematoxylin. The percentage of positive staining for collagen I, α-SMA, TNFα, IL-1β, TGF-β1 was measured by using a quantitative image analysis system (Image-Pro Plus 6.5, Media Cybernetics, Silver Spring, MD, USA), whereas positive cells per square millimetre for CD3, F4/80, phospho-smad2/3, and phospho-p65 were counted under a ×20 power field of microscope in five random areas of LV tissues using a 0.25 mm² graticule fitted in the eyepiece of the microscope as previously described.\textsuperscript{14}

#### 2.5 Real-time PCR

 Total RNA was extracted from LV tissues and real-time PCR analysis was performed using Bio-Rad iQ SYBR Green supermix with Opticon2 (Bio-Rad, Hercules, CA, USA) as previously described.\textsuperscript{14} Primers used for the detection of mRNA expression of collagen I, α-SMA, TGF-β1, IL-1β, TNFα, and GAPDH were described previously.\textsuperscript{14,15}

 In addition, miR-29b expression was detected by real-time PCR using the TaqMan microRNA Assay (Applied Biosystems, Foster City, CA, USA) with small nuclear RNA U6 as an endogenous control for normalization as previously described.\textsuperscript{20} The reaction specificity was confirmed by melting curve analysis. The ratio against housekeeping gene GAPDH for individual mRNA was calculated and expressed as mean ± standard errors.

#### 2.6 Western blot analysis

 The LV tissues were lysed and extracted for western blot analysis as described previously.\textsuperscript{14}

 Briefly, after blocking nonspecific binding with 5% BSA, membranes were incubated overnight at 4°C with primary antibodies against phospho-p65 (ser276), phospho-IκBα (ser32), and IκBα (Cell Signaling), p65, phospho-smad2/3, Smad2/3, Smad7, and Sp1 (Santa Cruz), phospho-Smad2/3, and phospho-p65 were counted under a ×20 power field of microscope in five random areas of LV tissues using a 0.25 mm² graticule fitted in the eyepiece of the microscope as previously described.\textsuperscript{14}

#### 2.7 Statistical analyses

 Data obtained from this study were expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA followed by Newman–Keuls multiple comparison test from GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

### 3. Results

#### 3.1 Overexpression of Smad7 prevents Ang II-induced cardiac dysfunction, fibrosis, and inflammation

 We first examined Smad7 gene transfection efficiency and levels of cardiac Smad7 expression in the hypertensive heart. An inducible Flag-tagged M2Smad7 gene was transferred into the normal mouse heart.
at the same time of Ang II infusion by the ultrasound-microbubble-mediated gene therapy technique. Immunohistochemistry detected that Smad7 protein was highly expressed in the normal mouse heart, but lost at day 28 after Ang II infusion (Figure 1A). In contrast, ultrasound treatment resulted in higher levels of Smad7 gene transfer and cardiac Smad7 expression as demonstrated by almost all cardiac myocytes, interstitial fibroblasts, and vascular cells expressing the Flag-M2 protein (a tagged Smad7 transgene protein) and Smad7 protein (Figure 1A). Examination by echocardiography revealed that overexpression of Smad7 protected against Ang II-induced cardiac functional injury by inhibiting an increase in LV mass and the fall in the LVEF in response to Ang II infusion, although no effect on systolic blood pressure was notable (Figure 1B and C). Analysis with immunohistochemistry, western blot, and real-time PCR showed that Ang II infusion caused a moderate to severe cardiac fibrosis and inflammation in both untreated and control-treated animals by a marked up-regulation of both fibrotic markers (collagen I, α-SMA) and pro-inflammatory cytokines (IL-1β and TNF-α), and an increase in numbers of CD3+ T cells and

![Figure 1](https://academic.oup.com/cardiovascres/article-abstract/99/4/665/264319) Overexpression of cardiac Smad7 attenuates Ang II-induced cardiac dysfunction. (A) Ultrasound-mediated transgenic (exogenous) and total Smad7 expression. The transferred exogenous Smad7 is detected by the antibody to Flag-M2, whereas total Smad7 protein expression is shown by positive staining with anti-mouse Smad7 antibody. Note that ultrasound mediates high Smad7 transfection rate and total cardiac Smad7 expression by almost all cardiac cell types, thereby preventing a loss of cardiac Smad7 in response to Ang II infusion at day 28. An example of Flag-M2 and Smad7 expressing myocytes and interstitial fibroblasts (arrowheads) is illustrated in the insert area (×400). (B) Representative echocardiography at day 28 after Ang II infusion. Results show that overexpression of Smad7 protects against Ang II-increased LV mass and a fall of LVEF. (C) Effect of Smad7 overexpression on systolic blood pressure in response to Ang II infusion. Data represent the mean ± SEM for a group of eight mice with Smad7 or control treatment and for a group of six normal mice. *P < 0.05, **P < 0.001 vs. saline; ***P < 0.001 vs. empty vector control (EV). Magnification: × 200.
macrophages (Figure 2 and Supplementary material online, Figures S1–S3). In contrast, ultrasound-mediated Smad7 overexpression markedly inhibited all parameters of cardiac fibrosis and inflammation (Figure 2 and Supplementary material online, Figures S1–S3).

3.2 Smad7 has therapeutic potential for established hypertensive cardiac disease

To explore whether Smad7 has therapeutic effect on hypertensive cardiac remodelling, we transferred Smad7 into the established hypertensive heart at day 14 after Ang II infusion. Again, Smad7 treatment from day 14 onwards did not alter Ang II-induced high blood pressure, but largely attenuated Ang II-increased LV mass and the deterioration of LVEF when compared with control-treated animals (Figure 3). In addition, real-time PCR, western blot, and immunohistochemistry detected that Smad7 overexpression was capable of blocking Ang II-induced cardiac fibrosis (both collagen I and α-SMA expression) and inflammation including IL-1β and TNF-α expression and T-cell and macrophage accumulation (Figure 4 and Supplementary material online, Figure S4 and S5). Nevertheless, Smad7 overexpression did not reverse

3.3 Blockade of Sp1-TGF-β/Smad-NF-κB pathways and prevention from the cardiac miR-29b loss are mechanisms by which Smad7 protects against Ang II-induced hypertensive remodelling

We next investigated the mechanisms by which overexpression of Smad7 protects against Ang II-induced cardiac inflammation and fibrosis. We found that Ang II infusion significantly up-regulated cardiac Smad ubiquitination regulatory factor 2 (Smurf2), resulting in the degradation of cardiac Smad7, which was prevented by overexpression of Smad7 (Figure 5A and B). Indeed, endogenous cardiac Smad7 protein became almost undetectable after Ang II infusion, which was restored by ultrasound-mediated Smad7 gene transfer as identified by Flag-M2 protein expression (exogenous Smad7) and immunoreactive Smad7-positive cells within the heart tissue (Figure 1A). Further studies showed that overexpression of cardiac Smad7 inhibited activation of TGF-β/Smad signalling as demonstrated by suppressing up-regulation of TGF-β1 at both mRNA and protein levels and phosphorylation of Smad2/3 (Figure 5C–E). Smad7 overexpression also attenuated activation of NF-κB signalling as illustrated by lowering phosphorylated IkBα and NF-κB/p65 in the hypertensive heart (Figure 6A and B). Similarly, the therapeutic effect of Smad7 overexpression on cardiac fibrosis and inflammation in the established hypertensive heart was also associated with the inhibition of both TGF-β/Smad and NF-κB signalling pathways (Supplementary material online, Figure S6).

Since Sp1, a ubiquitous transcription factor, is required for Ang II-induced fibrosis and inflammation,21,22 we examined levels of Sp1 in Ang II-induced hypertensive heart and found that Ang II infusion markedly increased cardiac Sp1, which was inhibited by overexpression of Smad7 (Figure 6C).

Because loss of miR-29b has been shown to be associated with cardiac fibrosis and is negatively regulated by both TGF-β/Smad3 and NF-κB-YY1,20,23,24 we examined whether overexpression of Smad7 has effect on cardiac miR-29b expression in the hypertensive heart. As shown in Figure 6D, real-time PCR revealed that cardiac miR-29b was largely decreased in Ang II-infused mice. In contrast, overexpression of Smad7 prevented the loss of cardiac miR-29b (Figure 6D).

4. Discussion

A large body of evidence has demonstrated that Ang II is an important mediator in cardiac fibrosis, inflammation, and cardiac dysfunction.1–5 The current study added new evidence for a protective role and therapeutic potential of Smad7 in Ang II-induced hypertensive cardiac disease. We found that overexpression of cardiac Smad7 inhibited Ang II-induced cardiac remodelling and had therapeutic potential for hypertensive cardiopathy. Blockade of Sp1-TGF-β/Smad-NF-κB signalling pathways and prevention of the loss of cardiac miR-29b were key mechanisms by which Smad7 inhibited Ang II-mediated cardiopathy. Consistent with the previous observation that decreased cardiac Smad7 contributes to cardiac fibrosis,25,26 the present study showed that cardiac Smad7 expression was markedly reduced in response to Ang II infusion, resulting in the development of cardiac fibrosis and inflammation accompanied by impaired cardiac function including an increase in LV mass and reduction of LVEF. Ang II-induced loss of cardiac injury when compared with day 14 before treatment (Figure 4 and Supplementary material online, Figure S4 and S5).
cardiac Smad7 may be Smurf2-dependent because Smurf2 is an E3 ligase that specifically binds and degrades Smad7.9 Once Smad7 is lost, Ang II-induced activation of Smad3 via both TGF-β-dependent and independent pathways is enhanced,7,8 which results in enhanced Smad3-mediated fibrosis and inflammation in obstructive and diabetic nephropathy and that overexpression of Smad7 is able to block NF-κB-dependent inflammation by inducing IκBα, an inhibitor of NF-κB, or preventing it from degradation in a number of experimental models and in vitro.10,15–17,31

The present study added new evidence that blockade of NF-κB activation may be a mechanism through which Smad7 suppresses Ang II-induced cardiac inflammation.

Down-regulation of Sp1 pathway may be an additional mechanism by which Smad7 inhibited Ang II-mediated fibrosis and inflammation. Sp1 is required for Ang II-induced fibrotic and inflammatory response.21,32–35

It has been demonstrated that Sp1 can interact with both Smad3 and enhanced NF-κB signalling in obstructive and diabetic nephropathy and that overexpression of Smad7 is able to block NF-κB-dependent inflammation by inducing IκBα, an inhibitor of NF-κB, or preventing it from degradation in a number of experimental models and in vitro.10,15–17,31

The present study added new evidence that blockade of NF-κB activation may be a mechanism through which Smad7 suppresses Ang II-induced cardiac inflammation.

Figure 4 Smad7 overexpression suppresses Ang II-induced cardiac fibrosis and inflammation in the established hypertensive heart disease. (A) Western blot and real-time PCR analysis of cardiac collagen I. (B) Western blot and real-time PCR analysis of cardiac α-SMA. (C and D) Cardiac IL-1β and TNF-α mRNA expression detected by real-time PCR. (E and F) Quantitative analysis of cardiac CD3+ T cells and F4/80+ macrophages detected by immunohistochemical staining. Data represent the mean ± SEM for a group of eight mice with Smad7 or control treatment at day 28, for a group of five mice before treatment at day 14, and for a group of six normal mice. *P < 0.05, **P < 0.01, ***P < 0.001 vs. saline; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. empty vector control (EV); †P < 0.05, ††P < 0.01, †††P < 0.001 vs. day 14 disease (D14) before treatment.
NF-κB to play a critical role in fibrosis and inflammation.\textsuperscript{32–35} Therefore, Sp1 may cooperate with both Smad3 and NF-κB in the development of Ang II-induced cardiac fibrosis and inflammation. Therefore, inactivation of the Sp1/Smad3/NF-κB axis in response to Ang II may be a key mechanism through which Smad7 protected against cardiac fibrosis and inflammation.

Interestingly, we also found that prevention of cardiac miR-29 loss may contribute to a protective role of Smad7 in Ang II-mediated cardiac fibrosis and inflammation. Therefore, inactivation of the Sp1/Smad3/NF-κB axis in response to Ang II may be a key mechanism through which Smad7 protected against cardiac fibrosis and inflammation.

Interestingly, we also found that prevention of cardiac miR-29 loss may contribute to a protective role of Smad7 in Ang II-mediated cardiac fibrosis and inflammation. Therefore, inactivation of the Sp1/Smad3/NF-κB axis in response to Ang II may be a key mechanism through which Smad7 protected against cardiac fibrosis and inflammation.

Figure 5 Inactivation of TGF-β/Smad signalling is a key mechanism by which Smad7 protects against Ang II-induced cardiac fibrosis. (A) Western blot analysis of cardiac Smurf2. (B) Western blot analysis of cardiac Smad7. (C) Western blot analysis of phosphorylated Smad2/3 in the hypertensive heart. (D) Immunohistochemical analysis of cardiac TGF-β1 expression. (E) Real-time PCR analysis of cardiac TGF-β mRNA expression. Data represent the mean ± SEM for a group of eight mice with Smad7 or control treatment and for a group of six normal mice.*P < 0.05, ***P < 0.001 vs. saline; #P < 0.05, ##P < 0.01 vs. empty vector control (EV). Magnification: × 200.

Smad3-mediated kidney and lung fibrosis.\textsuperscript{20,36} Our recent study showed that Smad7 inhibits renal fibrosis via altering the Smad3-regulated microRNAs that are related to fibrosis including restored renal miR-29b.\textsuperscript{37} In line with these findings, results from the present study suggested that prevention of Ang II-induced loss of miR-29b via TGF-β/Smad3 and NF-κB-dependent pathways may also be a mechanism by which overexpression of Smad7 inhibits Ang II-induced cardiac remodelling.

The present study also demonstrated that Smad7 may be a therapeutic agent for hypertensive cardiac disease, which is clinically relevant. This was supported by the findings that ultrasound-mediated gene transfer of Smad7 in the established hypertensive cardiac disease was capable of attenuating Ang II-induced progressive cardiac dysfunction and remodelling. Therefore, Smad7 not only exhibits a protective role in Ang II-induced cardiac fibrosis and inflammation, but also exerts a therapeutic potential for hypertensive cardiac disease. This is consistent with...
previous reports on a number of kidney diseases that overexpression of Smad7 attenuates renal inflammation and fibrosis without detectable side-effects as seen in obstructive nephropathy, remnant kidney disease, diabetic nephropathy, and autoimmune nephritis. In summary, Smad 7 plays a protective role in Ang II-mediated cardiac fibrosis and inflammation. The ability of Smad7 to block Ang II-induced hypertensive cardiopathy also reveals a therapeutic potential for Smad7 in chronic cardiac disease. Inhibition of Sp1-TGF-β/Smad3-NF-κB pathways and prevention of loss of miR-29 may be mechanisms by which Smad7 protects against cardiac remodelling.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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