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Interference of TGF-β1/Smad7 signal pathway affects myocardial fibrosis in hypertension

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Abstract: Hypertension can cause myocardial fibrosis, during which tumor growth factor-beta 1 (TGF-β1) can facilitate myocardial cell proliferation and transition towards myofibroblast (MFB). Smad7 is a negative regulator of TGF-β1/Smads signal pathway. This study used hypertension rat model to investigate the regulatory role of TGF-β1/Smad7 signal pathway in myocardial fibrosis. Rat renal hypertension model was established to test collagen volume fraction, myocardial hydroxyl proline content and COL1A1 expression as well as the expression of TGF-β1 and Smad7. The expressions of TGF-β1, Smad7, COLA1A1 and α-SMA at certain generations (P2, P4 and P6) were measured in cultured human cardiac fibroblast (HCF) during differentiation towards MFB differentiation. P6 generation HCF was transfected with pIRES2-EGFP-Smad7 and pIRES2-EGFP-blank followed by measuring expressions of TGF-β1, Smad7, COLA1A1 and α-SMA. Hydroxyl-proline content and collagen volume fraction were compared between Ad-NC and Ad-Smad7 injection groups. Hypertensive rats had significantly elevated collagen volume fraction, hydroxyl proline contents, and expression of COLA1 and TGF-β1 than Sham group, whilst Smad7 expression was lower. With increased cell passage, HCF showed gradually increased TGF-β1, COL1A1 and α-SMA expression, plus decreased Smad7 expression. Over-expression of Smad7 remarkably decreased COLA1 and α-SMA expression in HCF. Tail vein injection of Ad-Smad7 decreased both hydroxyl proline and collagen volume fraction. Elevated TGF-β1 expression and decreased Smad7 expression are found in fibrotic myocardial tissues of hypertensive rats. Over-expression of Smad7 inhibits differentiation of HCF towards MFB cells, thus decreasing myocardial fibrosis in hypertensive rats.

Keywords: TGF-β1, Smad7, hypertension, myocardial fibrosis, human cardiac fibroblast.

INTRODUCTION

Myocardial fibrosis (MF) is a pathological damage caused by various cardiovascular diseases, among which hypertension is a major reason (Liu *et al*., 2014 and Wu *et al*., 2016). MF is also an important pathological change and basic feature of hypertensive cardiac reconstruction, as well as a common and severe complication of hypertension (Sowers 2007). Abundant deposition of cardiac extracellular matrix (ECM) plus increased collagen content for over-deposition, disrupted ratio of all collagen subtypes, irregular spatial arrangement can further cause decreased cardiac relaxation property and compliance, increased stiffness, weakened diastolic/systolic functions, eventually leading to cardiac diseases such as myocardial hypertrophy, heart failure, and abnormal cardiac rhythm. Therefore, inhibition of MF or cardiac reconstruction may provide major goals and treatment measures for hypertension to improve cardiac functions and to prevent heart failure (Hlavackova *et al*., 2011).

Transforming growth factor-β1 (TGF-β1) is one regulatory factor closely correlated with MF (Wang *et al*., 2016 and Lei *et al*., 2011). Elevated contents and activation of TGF-β1 is the common pathway and intermediate bridge of MF caused by various factors, and is believed to be an MF facilitating factor with direct roles (Zhang 2014). TGF-β1 can facilitate cardiac fibroblast (CFB) proliferation and its transition toward myofibrtoblast (MFB) via regulating a series of signal transduction molecules, and can facilitate synthesis and deposition of collagen and fibronectin in extracellular matrix (ECM0, thus accelerating MF occurrence and progression (Zhang 2016 and Li *et al*., 2016). Smads is the only cytosolic signal transduction protein with signal transduction function on TGF-β receptor kinase, and plays critical roles in TGF-β1/Smads signal transduction and regulation (Li *et al*., 2012 and Sethi *et al*., 2011).

Mammalian cytosolic Smads protein can be further divided into receptor activation type (R-Smads), in which Smad2 and Smad3 plays major roles; co-mediator type (Co-Smads), also named as Smad4 and inhibitor Smads protein (I-Smads), which mainly includes Smad6, Smad7, both of which can block the binding of R-Smads onto TβR-I to suppress R-Smads phosphorylation, or inhibit the binding between R-Smads and Co-Smads to block cytosolic signal transduction process of TGF-β1 (Loboda *et al*., 2016 and Li *et al*., 2009).

As Smad7 is a negative regulator of TGF-β1/Smads signal pathway (Zhou *et al*., 2014 and Gao *et al*., 2013), the enhancement of its expression and function may weaken

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MF. However, the role of TGF-β1/Smad7 signal pathway in the development and pathogenesis of MF remains poorly understood. Through establishing hypertensive rat model, this study aims to investigate the effect of TGF-β1/Smad7 signal pathway on MF, in order to identify whether targeting TGF-β1/Smad7 signaling is beneficial for the treatment of MF.

Table 1: Hydroxy proline content and CVF test results

	SBP (mmHg)	Hydroxyl-proline (μg/mg)	CVF (%)
Sham group	116.5±14.6	38.2±1.4	1.23±0.11
Hypertension group	193.2±17.3*	81.2±2.9*	4.59±0.31*

Note: *, p<0.05 compared to Sham rats.

Table 2: Hydroxyl-proline content and CVF results between two groups of rats

	Hydroxyl-proline (μg/mg)	CVF (%)
Ad-NC injection	83.3±3.1	5.23±0.28
Ad-Smad7 injection	51.6±2.2*	3.26±0.11*

Note: *, p<0.05 compared to Ad-NC injection group.

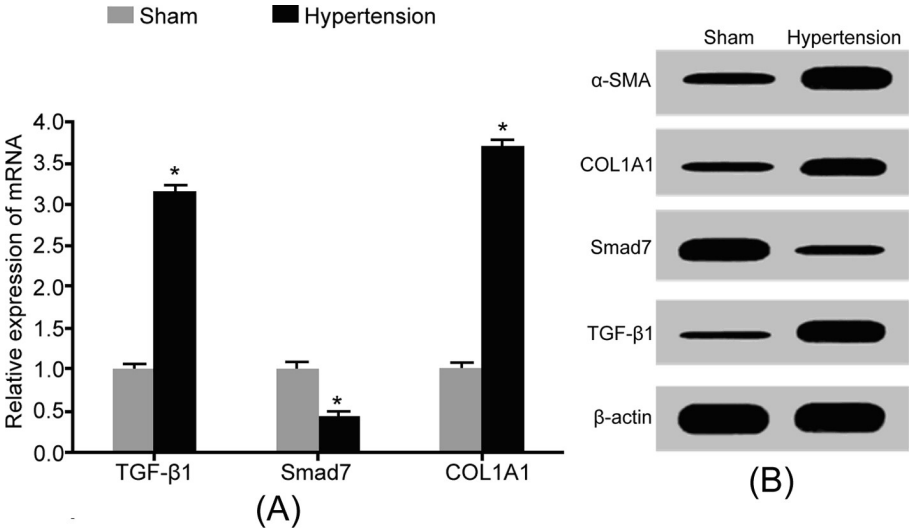

Fig. 1: Significant MF of hypertensive rats, plus elevated TGF-β1 expression and lower Smad7 expression. (A) qRT-PCR for myocardial tissue mRNA expression (B) Western blot for myocardial tissue protein expression. *, p<0.05 compared to Sham group.

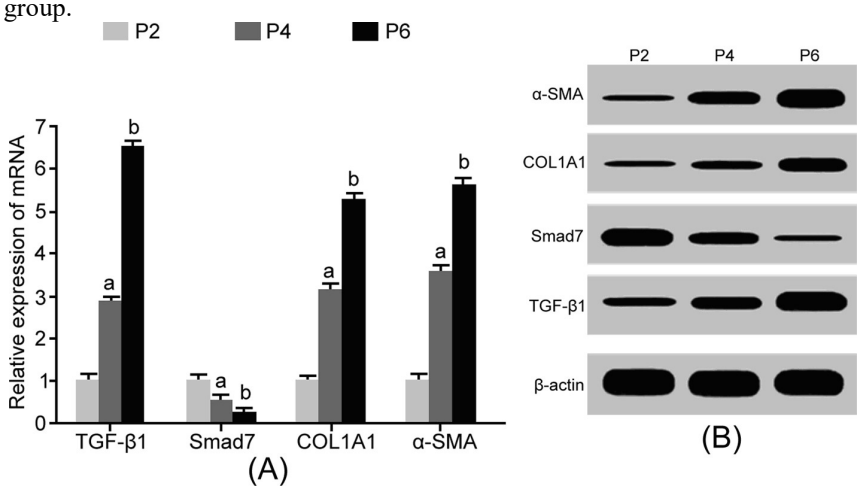

Fig. 2: Elevated TGF-β1 expression and lower Smad7 expression during HCF differentiation. (A) qRT-PCR for mRNA expression; (B) Western blot for protein expression. a, p<0.05 comparing between P4 and P2; b, p<0.05 comparing between P6 and P4.

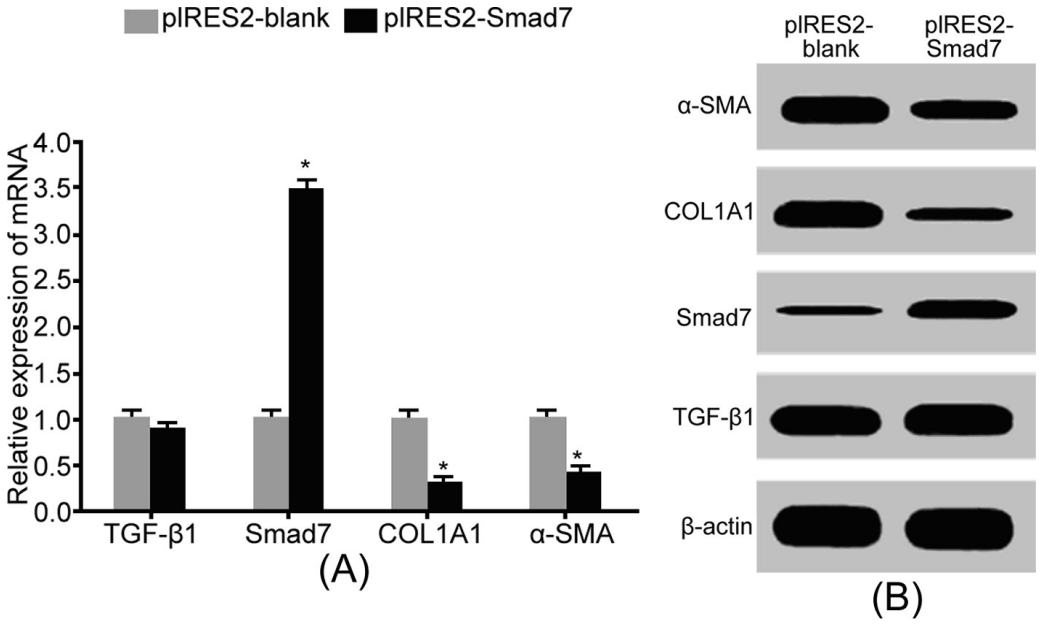

Fig. 3: Smad7 up-regulation inhibited HCF differentiation and weakened collagen synthesis potency. (A) qRT-PCR for mRNA expression (B) Western blot for protein expression. *, $p<0.05$ comparing between pIERS2-Smad7 and pIRES2-blank

MATERIALS AND METHODS

Major reagent and material

Human cardiac fibroblast (HCF) and Fibroblast Medium-2 were purchased from ScienCell (US). Opti-MEM was purchased from Gibco (US). RNA extraction kit Rneasy MiNi Kit and fluorescent quantitative kit QuantiTect SYBR Green RT-PCR Kit were purchased from Qiagen (Germany). Transfection reagent Lipofectamine 2000 was purchased from Invitrogen (US). Rabbit anti-Smad7 and β-actin were purchased from Santa Cruz (US). Rabbit anti-type I collagen (COL1A1) was purchased from CST (US). Mouse anti-TGF-β1, anti-α-smooth muscle actin (α-SMA), and HRP labelled secondary antibody were purchased from Abcam (US). Hydroxyl proline test kit was purchased from Jiancheng Bio (China). pIRES2-EGFP-blank empty plasmid and Smad7 over-expression plasmid pIRES2-EGFP-Smad2 were purchased from Yantuo Biotech (China). Adenovirus vector for overexpression of CDS region of Smad7 gene (Ad-Smad7) and negative control Ad-NC were purchased from Jikai Bio (China). Poly-L-lysine was purchased from Corning (US).

Generation of renal hypertension

Adult male SD rats (6~8 weeks age, body weight 220±20g) were purchased from Qingdao University (Shandong China). Rats were anesthetized by intraperitoneal injection of pentobarbital sodium. An incision was made to expose the abdominal cavity, with separation of abdominal aorta superior of bilateral renal artery. 1.0 suture was used to ligate abdominal aorta along with 7G needles along the direction of abdominal aorta. The needle was then extracted, and abdominal cavity was closed after smooth blood flow of abdominal aorta. Sham group used rats with abdominal exposure but without ligation. 6 weeks after surgery, model RBP1 rat heart rate monitor was used to measure systolic blood pressure (SBP) in rat tail artery. Those rats with higher than 150mmHg SBP indicated successful generation of hypertension model.

For the study of Smad7 interference, hypertensive rats were further divided into two group: Ad-NC injection group received 50μL 1 X 10^{10} PFU/mL Ad-NC adenovirus once per week for 6 consecutive weeks, and Ad-Smad7 injection group which received 50μL 1 X 10^{10} PFU/mL Ad-Smad7 adenovirus via tail veins once per week for 6 consecutive weeks. Rats were used for all experiments and all procedures were approved by the Animal Ethics Committee of the Affiliated Hospital of Qingdao University.

Cell culture and grouping

HCF cells were inoculated in culture dish with poly-L-lysine coating, with the addition of Fibroblast Medium-2 in a 37°C chamber with 5% CO_2. After paving the dish, cells were passed at 1:5 ratios. Those cells at log-growth phase with satisfactory status were used for experiments. Cultured HCF cells were collected after plating at passage 2 (P2), P4 and P6 followed by measuring the expression of TGF-β1 and Smad 7. 6^{th} generation of HCF cells were sub-divided into two transfection groups: pIRES-EGFP-blank group and pIRES2-EGFP-Smad7 group. After 72h continuous incubation, over-expression efficiency and related indexes were measured.

qRT-PCR for gene expression assay

Quanti Tech SYBR Green RT-PCR Kit was used to test gene expression by qRT-PCR using RNA extracted by Rneasy MiNi Kit. In a 20μL qRT-PCR system, there were 10μL 2XQuantiTect SYBR Green RT-PCR Master Mix, 1.0μL of forward and reverse primer (0.5μm/L), 2μg template RNA, 0.5μL Quanti Tect RT Mix, and ddH_2O. Reverse transcription conditions were: 50°C for 30min. PCR conditions were: 95°C pre-denature for 15min, followed by 40 cycles each containing 94°C denature for 15s, 60°C annealing for 30s and 72°C elongation for 30s. Gene expression was examined on Bio-Rad CFX96/CFX connect real-time fluorescent quantitative PCR cycler. β-actin was also used as an internal control.

Western Blot for protein expression

SDS lysis buffer was used to lyse cells by 5min boiling. After protein quantification by BCA method, 50μg samples were separated in SDS-PAGE and were then transferred to PVDF membrane, which was blocked in 5% defatted milk powder at room temperature incubation. Primary antibody (TGF-β1 at 1:500, Smad7 at 1:300, COL1A1 at 1:400, α-SMA at 1:400, β-actin at 1:800) was added for 4°C overnight incubation. After PBST rinsing for three times, HRP conjugated secondary antibody (1:5000 dilution) was added for 60min incubation. The membrane was rinsed in PBST for three times and incubated using ECL method. After dark exposure and development, the film was scanned and analyzed.

Assay for myocardial hydroxyl proline levels

100mg myocardial tissues were homogenized and digested. After hydrolyzation, the supernatant was collected to measure hydroxyl proline content using spectrometry method following manual instruction. Hydroxyl proline content (μg/mg) = [(absorbance value of test tube – absorbance value of empty tube)/(absorbance value of standard tubes - absorbance value of empty tube)] X standard contents (5μg/mL) X [hydrate total volume (10mL) / tissue wet weight (mg)].

Assay for collagen volume fraction

Myocardial tissues were collected from transacted sections in middle segment of left ventricles, and were fixed in 4% paraformaldehyde, followed by dehydration and paraffin embedding. 5μm slices were prepared for antigen specific VG staining, under which collagen was marked as red. BI-2000 medical image analysis system was used to differentiate collagen and non-collagen by gray values. Collagen volume fraction (CVF) was calculated as collagen area against the summation of myocardial and mesenchyme tissues.

STATISTICAL ANALYSIS

SPSS 18.0 software was used for data analysis. Measurement data were presented as mean ± standard deviation (SD), whilst enumeration data were presented as percentage. Comparison of enumeration data between groups was performed by chi-square test. Comparison of measurement data between groups was performed by one-way analysis of variance (ANOVA). A statistical significant was defined when $p<0.05$.

RESULTS

Hypertensive rats had significant MF, elevated TGF-β1 and decreased Smad7 expression

Compared to Sham group, hypertension group showed significantly elevated systolic pressure in tail artery, indicating successful establishment of hypertension model (table 1). Hypertension model showed remarkably elevated collagen in myocardial mesenchyme and vascular peripheral tissues, with visible scar tissues and more collagen in muscular cleft. VG staining showed significantly higher CVF in hypertensive rats than sham group (table 1). Hydroxy-proline test revealed significantly higher proline contents in myocardial tissues of hypertension model rats than sham group (table 1). qRT-PCR results showed significantly higher TGF-β1 and COL1A1 mRNA expression in hypertension model rats than those in sham group, whilst Smad7 mRNA expression was lower (fig. 1A). Western blot results also showed significantly elevated TGF-β1 and COL1A1 protein expression, plus lower Smad7 protein expression in hypertension model group compared to those in sham group (fig. 1B). These results indicated significant MF in hypertension rats and possible roles of TGF-β1 and Smad7 in MF.

Elevated TGF-β1 expression and lower Smad7 expression during HCF differentiation

HCF can undergo the differentiation process toward myofibroblast (MFB) under *in vitro* culture conditions. The specific protein marker for MFB, α-SMA, has elevated expression level with extended cell passage. Expression level of COL1A was also increased with cell passage, indicating potentiated collagen synthesis potency during the differentiation of HCF toward MFB (fig. 2A and 2B). During cell differentiation, TGF-β1 expression was gradually increased whilst Smad7 expression was decreased (fig. 2A and 2B).

Smad7 up-regulation inhibited HCF differentiation and weakened collagen synthesis potency

Transfection of Smad7 over-expression plasmid in HCF cells significantly elevated Smad7 mRNA and protein expression, decreased COL1A1 and α-SMA mRNA/protein expression, without significant effects on TGF-β1 expression (fig. 3A and 3B). These results showed that over-expression of Smad7 significantly decreased collagen synthesis and MFB differentiation potency of HCF.

Tail vein injection of smad7 decreased hydroxyl-proline content and CVF in hypertension model rat myocardial tissues

Comparing to Ad-NC injection group, Ad-Smad7 injection significantly decreased CVF in hypertension rats (table 2). Assay for hydroxyl-proline content showed significantly lower contents in Ad-Smad7 injection group than that in Ad-NC injection group (table 2). These results indicated that over-expression of Smad7 decreased MF severity of hypertension rats.

DISCUSSION

Cardiac reconstruction as induced by hypertension is an independent risk factor of cardiovascular complications. It increases the incidence of acute myocardial infarction (AMI), acute/chronic heart failure, cardiac shock and other cardiovascular incidences by 6~8 folds (James 2017 and Messerli *et al*., 2017). MF is a fibrosis change in myocardial tissues and mesenchyme tissues, and eventually leads to myocardial hypertrophy, heart failure and irregular heart rhythm (Istratoaie *et al*., 2016 and Gyongyosi *et al*., 2017). Therefore, inhibition of MF or cardiac reconstruction might be beneficial for treating hypertensive diseases.

Under hypertension status, tissue loading of myocardial tissues were significantly elevated, and can activate renin-angiotensin-aldosterone system (RAAS) (Yamout *et al*., 2014 and Ferrari 2013), which can increase angiotensin II (Ang II) contents in general circulation and heart tissues (Chaszczewska-Markowska *et al*., 2016). Ang II can up-regulate expression and release of TGF-β1, which further accelerates MF pathogenesis (Zhao *et al*., 2015). TGF-β1 is an important factor in the regulation of MF and its up-regulation or activation are common pathway and intermediate bridge of various factors leading to MF and is believed to play a direct role in facilitating MF (Zhang 2014). TGF-β1 is one potent facilitating factor for synthesis and deposition of collagen and other ECM components, and can significantly facilitate collagen synthesis, stimulate plasminogen activator inhibitor production for suppressing ECM degradation, thus enhancing ECM deposition in myocardial tissues and facilitating MF pathogenesis (Zhang *et al*., 2016). Besides cardiomyocytes, cardiac fibroblast (CFB) is an important cell component in myocardial tissues, and is the major cell type regulating ECM synthesis and degradation. During occurrence of MF, CFB is abnormally activated, with enhanced proliferation and migration potency, and is transformed towards secretory type MFB, with significant enhancement of collagen synthesis and secretion potency, thus playing an important role in facilitating MF. In either of *in vivo* MF process or *in vitro* culture system, CFB itself can secret TGF-β1 in an autocrine manner, and can facilitate self-proliferation and MFB differentiation via a series signal transduction molecule regulation and significantly enhance synthesis and deposition potency of ECM components such as collagen and fibronectin, thus promoting MF occurrence and progression (Zhang 2016).

TGF-β can exert its biological effects only with binding onto specific cell surface receptor. Mammalian cells express three types of TGF-β receptors, including TβR-I, TβR-II and TβR-III. TβR-III lacks endogenous activity without signal transduction function by itself. Activated forms of TβR-I and TβR-II are internal body receptors for responding to TGF-β protein and exerting signal transduction function (Jachec *et al*., 2008 and Zhan *et al*., 2015). Smads is the only identified signal transduction protein family with functions on TβR-I receptor kinase and plays a crucial role in TGF-β1/Smads pathway regulation. There are 8 types of Smads proteins in mammalian cell cytoplasm, and can be divided into three sub-classes: (1) receptor activated Smads protein (R-Smads), which mainly consists of Smad1, Smad2, Smad3, Smad5 and Smad8, among those Smad2 and Smad3 induce TGF-β protein signal transduction; (2) Co-mediating Smads protein (Co-Smads), with only Smad4 in mammalian cells for inducting signal transduction of all TGF-β protein family; and (3) inhibitory Smads protein (I-Smads), which mainly includes Smad6 and Smad7, both of which can block the binding between R-Smads and TβR-I for further inhibition of R-Smads phosphorylation, or inhibition of R-Smads and Co-Smads binding to block the signal transduction pathway of TGF-β1 toward cytoplasm (Loboda *et al*., 2016 and Li *et al*., 2009). As Smad7 is a negative regulatory factor of TGF-β1/Smads signal pathway, we hypothesized that its over expression or functional enhancement might weaken MF process. Therefore, this study thus used hypertensive rat model to investigate the regulatory function of TGF-β1/Smad7 signal pathway in MF.

Results of this study showed significantly higher small artery systolic pressure in renal hypertension model rats, indicating successful generation of hypertension. Cardiac mesenchyme has various collagens, among which type I collagen occupies more than 80% and plays an important role in maintaining ventricular wall strength due to strong fibers, stiffness and satisfactory anti-retraction. The overweight of type I collagen, however, can weaken compliance of ventricular wall, and is closely correlated with MF pathogenesis. This study observed significantly higher type I collagen (COL1A1) expression in myocardial tissues of hypertension model compared to sham group, indicating significantly higher collagen synthesis in hypertension rat myocardial tissues, with remarkable MF. Hydroxyl-proline is the major component in collagen and all hydroxyl-proline in animal tissues are exclusively from collagen. Therefore, hydroxyl-proline content in myocardial tissues can represent collagen level for reflecting MF condition. Results of this study revealed significantly higher hydroxyl-proline content in

hypertensive rat myocardial tissues than sham group, demonstrating MF occurrence in hypertensive rat myocardial tissues. We also found that, compared to sham group, hypertensive rats had significantly elevated TGF-β1 protein expression, with decreased Smad7 protein expression. These results indicated possible role of TGF-β1 up-regulation and Smad7 down-regulation in MF of hypertensive rats. Huang *et al* (2014) showed significantly elevated TGF-β1 expression in rat MF myocardial tissues after AMI (Huang *et al*., 2014). Zhang *et al* showed significantly increased TGF-β1 expression in MF rabbit myocardial tissues (Zhang *et al*., 2016). After treatment to relieve MF, TGF-β1 expression in myocardial tissues was remarkably decreased. Shen *et al* found significantly elevated TGF-β1, p-Smad2/3, COL1A1 and α-SMA expression in MF pathogenesis in diabetic rats, plus lower Smad7 expression (Shen *et al*., 2014). Zhao *et al* showed significantly elevated type I and type II collagen in hypertensive rat myocardial tissues, indicating MF features. Mice with MF also showed remarkably higher TGF-β1 and p-Smad3 expression level, but Smad7 protein level was decreased (Zhao *et al*., 2015). In this study, TGF-β1 expression was elevated in myocardial tissues in hypertensive rats with MF, but Smad7 expression was decreased, as similar with Huang *et al* (2014), Shen *et al* (2014) and Zhao *et al* (2015) *in vitro* cultured HCF, this study also observed gradually increased α-SMA expression, which is one specific marker for MFB, indicating differentiation of HCF toward MFB. During such process, TGF-β1 expression and collagen synthesis potency were gradually increased, with lower Smad7 expression, suggesting that down-regulation of suppressing factor Smad7 was facilitating factor in MF process. With Smad7 expression in HCF, cellular expression of α-SMA and COL1A1 was significantly elevated, showing that Smad7 up-regulation could weaken collagen synthesis potency and differentiation toward MFB of HCF cells, indicating that Smad7 might be one inhibitory factor during MF process. Tail vein injection of Ad-Samd7 significantly decreased hydroxyl-proline content and collagen volume fraction in hypertensive rats, indicating that Smad7 up-regulation could weaken MF severity in hypertensive rats. Shen *et al* showed treatment using Shensong Yangxin Capsule (SSYX) significantly weakened MF severity of diabetic rats, plus down-regulation of TGF-β1 and p-Smad2/3, and higher Smad7 expression in myocardial tissues (Zhao *et al.,* 2015). Zhao *et al* (2015) also showed that sesamin ingestion remarkably decreased TGF-β1 or p-Smad3 expression in hypertensive rat myocardial tissues, and increased Smad7 expression, plus weakened collagen deposition or MF severity (Zhao *et al*., 2015). Shen *et al.* (2014) and Zhao *et al* (2015) all observed Smad7 up-regulation after intervention to alleviate MF degree, but without direct observation to confirm the correlation between weakened MF condition after Smad7 over-expression. This study used *in vitro* cultured HCF cells and observed that over-expression of Smad7 could alleviate collagen synthesis or differentiation towards MFB cells, as well as the decreased MF severity in hypertensive rats after Smad7 up-regulation, demonstrating that Smad7 over-expression could suppress MF progression.

CONCLUSION

Increased TGF-β1 and decreased Smad7 expression is found in hypertensive rat MF tissues. Over-expression of Smad7 significantly decreases collagen synthesis potency and differentiation towards MFB cells, and ameliorates MF condition of hypertensive rats, suggesting that it might be a possible inhibitory factor for MF.

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