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Puerarin Attenuates Transforming Growth Factor Beta-1 Induced Hypertrophic Responses and Smad Proetin Upregulation in Neonatal Rat Cardiomyocytes

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Abstract Recent studies have suggested that puerarin may attenuate cardiac hypertrophy in mice; however, the underlying mechanism remains unclear. To investigate the role of puerarin in transforming growth factor beta 1 (TGF-β_1)-induced cardiac hypertrophy and the underlying mechanisms. Primary neonatal rat cardiomyocytes (NRCMs) were isolated form the heart of neonatal Wistar rats (1- to 2-day-old) and treated with different doses of puerarin (0,0.1,1or 5 g/L) in the presence of TGF-β_1 (3 μg/L) to investigate the effect of puerarin on TGF-β_1-induced hypertrophic changes and Smad protein alterations. RNA and protein biosynthesis in NRCMs were evaluated by synpropidium iodide staining and [^{3}H]-leucine incorporation, respectively. Gene expression alterations were determined using quantitative real-time PCR and Western blot analysis. Compared with Smad2 knockdown, puerarin treatment (1 g/L) exhibited similar but stronger effects in abrogating TGF-β_1-induced RNA and protein biosynthesis as well as fetal gene upregulation in NRCMs. In addition, puerarin treatment (1 g/L) could remarkably reverse TGF-β_1-induced upregulation of Smad protein expression in NRCMs, suggesting that deactivation of the Smad signaling is possibly involved in the antihypertrophic role of puerarin. Our data suggest that puerarin may protect cardiomyoctyes against TGF-β_1-induced hypertrophic responses and Smad signaling activation, providing basic dosage information and possible targets for puerarin treatment in animal models.

Keywords: *cardiomyocyte hypertrophy, transforming growth factor-β_1, the Smad signaling*

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1. Introduction

Cardiac hypertrophy refers to increases in the heart size and mass resulting from adaptive responses to increased hemodynamic stress. It is characterized by enlargement of cardiomyocytes, enhancement of protein synthesis, and reactivation of fetal genes like atrial natriuretic factor (ANF) and β-myosin heavy chain (β-MHC) in cardiomyocytes. Pathological cardiac hypertrophy is an independent risk factor for cardiovascular morbidity and mortality, leading to cardiac dysfunction, heart failure, and even sudden death [1,2,3]. Currently, cardiac hypertrophy therapy is focused on blocking the hypertrophic signaling pathways [4], including sarcomeric Z-disc and its associated proteins that drive mechanical stress-induced signal transduction, the insulin-like growth factor-1 and transforming growth factor beta (TGF-β) signaling that are responsible for the eutrophic myocyte growth, and the G-protein-coupled receptors signaling that is linked to the progression to heart failure [5,6]. Exploring therapeutic agents that target the molecules involved in these pathways would generate effective remedies for patients with cardiac hypertrophy.

Puerarin(7,4′-dihydroxyisoflavone-8β-glucopyranoside), a major active ingredient in the Chinese medicine *Pueraria Radix* isolated from the kudzu root (*Pueraria lobota*), exhibits therapeutic effects on various disorders, including hypertension [7], myocardial ischemia [8], diabetes mellitus [9], and cerebral ischemia [10], due to its antioxidant [11], antiplatelet [12], antiinflammatory [13],

antiarrhythmic [14], and antidiabetic [15] properties [16]. Recent studies have suggested that puerarin can effectively inhibit cardiac function impairments, cardiac fibrosis, heart size increase, and fetal gene upregulation in angiotensin II- or pressure overload-induced hypertrophy in mice [17,18,19,20,21]. The antihypertrophic effect of puerarin has been linked to the blockade of phosphoinositide 3-kinase (PI3K), c-Jun N-terminal kinase (JNK), extracellular signal–regulated kinases (ERK), p38 mitogen-activated protein kinase (MAPK), and nuclear factor-kappa B (NF-κB) signaling pathways. Although the TGF-β/Smad signaling has also been shown to promote cardiac hypertrophy development [22,23,24,25], little is known about its involvement in the antihypertrophic effect of puerarin.

In the present study, we isolated primary neonatal rat cardiomyocytes (NRCMs) from the heart of neonatal Wistar rats to explore the role of puerarin in TGF-β_1-induced hypertrophic changes and the underlying mechanism. Our results suggest that deactivation of the Smad signaling might be involved in the protective role of puerarin against TGF-β_1-induced hypertrophic changes in NRCMs, providing basic dosage information and possible targets for puerarin treatment in animal models.

2. Materials and Methods

2.1. Isolation, Culture, and Treatment of NRCMs

Neonatal Wistar rats (1-to2-day-old) were obtained from the Medical Laboratory Animal Center of Sun Yat-Sen University (Guangzhou, China). All animal care and procedures were approved by the Ethics Committee of Guangzhou Medical University (Guangzhou, China). All animal experiments were performed in accordance with the guidelines for the use of laboratory animals of Guangzhou Medical University (EC Directive 86/609/EEC).

Neonatal Wistar rats were euthanized via hypothermia. The hearts were immediately excised, and the ventricles were dissected. NRCMs were isolated using 0.25% trypsin (Gibco, Maryland, USA) and purified as previously described [26]. The cells were resuspended in Dulbecco's modified Eagles's medium (DMEM, Gibco) containing 20% fetal calf serum and 1% penicillin-streptomycin and plated in tissue culture dishes for 2 h to remove noncardiomyocyte cells. In the first part the isolated NRCMs were cultured in DMEM supplemented with 10% FBS and 0.1 mM bromide oxy-uridine and were seeded at a low density (1×10^5 cells/mL) for TGF-β_1 treatment or a high density (1×10^6 cells/mL) for other experiments. After a 24-h starvation, NRCMs were treated with TGF-β_1 (0.1, 1, 3, or 10 μg/L; Calbiochem, San Diego, CA, USA), followed by analyses described below.

Then in the second part Puerarin (0.1, 1, or 5 g/L; Great Sun Biochemical Pharmaceutical Co., Ltd., Guangzhou, China), or Smad2siRNA (0.6, 1.0, or 1.4 μL; Invitrogen, Carlsbad, CA, USA) were individually or in combination treated to NRCMs with TGF-β_1 3μg/L, followed by analyses described below.

2.2. SiRNA Transfection

NRCMs (10^6 cells/well) were plated in a 6-well plate on day 1 and transfected with different amounts of Smad2siRNA (0.6, 1.0, or 1.4 μL) on day 4 using Lipofectamin^{TM}2000 (Invitrogen) according to the manufacturer's protocol. At 24 h after transfection, cells were treated with TGF-β_1 (3 μg/L) alone or in combination with puerarin (1 g/L). Cells were collected at 24 h after treatment for gene expression detection.

2.3. Propidium iodide (PI) Staining for Cellular RNA

Cellular RNA was stained with RNA-sensitive fluorescence PI (Thermo Fisher Scientific, MD, USA) after DNase treatment [27,28]. Briefly, the NRCMs were treated with TGF-β_1 (0, 0.1, 1, 3, or 10 μg/L) in the first part, or TGF-β_1 3μg/L in combination with puerarin (0.1, 1, or 5 g/L) for 24 h in the second part, followed by Hanks' balanced salt solution (HBSS) rinses and 75% ethanol fixation for 60 min. The ethanol-fixed cells were rinsed with HBSS and incubated in a solution containing DNase (1 mg/mL), sucrose (0.25 mol/L), $MgCl_2$ (5 mmol/L), and Tris-HCL (20 mmol/L, pH 6.5) at 36 ℃ for 40 min. After incubation, 1 mL of HBSS containing PI (0.05 mg/mL) was added to each well and incubated for 30 min. The RNA contents were determined by measuring the fluorescent signals in 5 randomly selected fields in each well using a fluorescence conversion microscope (1500X/CC; Leica, Germany).

2.4. [^{3}H]-leucine Incorporation

[^{3}H]-leucine incorporation was measured as previously described [28]. NRCMs were seeded at a density of 10^6 cells/well in 24-well plates and cultured overnight. After a 24- h starvation, NRCMs were treated with different concentrations of TGF-β_1 (0, 0.1, 1, 3, or 10 μg/L) alone or TGF-β_1 3μg/L in combination with puerarin (0.1, 1, or 5 g/L) for 24 h and incubated with one microcurie per-milliliter [^{3}H]-leucine (Chinese Atomic Research Institute, Beijing, China) in leucine-free medium for additional 12 h. The radioactivity incorporated into the trichloroacetic acid precipitable component was determined by β-scintillation counting.

2.5. Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen) and subjected to qRT-PCR with an Access RT-PCR system (Invitrogen). The qRT-PCR reactions were carried out using a Quanti-Tect^{TM} SYBR Green PCR Kit (Qiagen, Hilden, Germany). The PCR primers were as follows: sense primer for ANF, 5′-AAA GCA AAC TGA GGG CT-3′; antisense primer for ANF, 5′-GGG ATC TTT TGC GAT CT-3′; sense primer for β-MHC, 5′-TGC AGT

TAA AGG TGA AGG C-3′; antisense primer for β-MHC, 5′-CAG GGC TTC ACA GGC AT-3′; sense primer for Smad2, 5′-GGG AAG TGT TTT GCC GAG TG-3′; antisense primer for Smad2,′3′-TAG GGT GGT CCG ACA TTG GA-5′; sense primer for GAPDH, 5′- CGG AGT CAA CGG ATT TGG TGG TAT-3′; antisense primer for GAPDH, 5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′.

2.6. Western Blot Analysis

NRCMs were lysed in lysis buffer (Cell Signaling Technology, Danvers, MA, USA) on ice.After centrifugation for 2 min at 12,000 *g*, the protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were transferred to PVDF membranes. The membranes were blocked by 3% BSA in TBST (10 mm Tris, 0.1 M NaCl, 0.1% Tween-20, pH 7.4) for 1.5 h. Following the incubation with monoclonal anti-Smad2, anti-pSmad2 (Ser465/467), or anti-Smad2/3 antibody (Cell Signaling Technology), the membranes were washed 3 times for 10 min with TBST and then incubated with appropriate horseradish peroxidase-labeled secondary antibodies (1:1000; Cell Signaling Technology) for 1 h at room temperature. Following three 10-min washes with TBST, the protein bands were detected by chemiluminescence (Sigma, St. Louis, MO, USA).

2.7. Statistical Analysis

Data are presented as mean ± standard deviation. Statistical analysis was performed using Analysis of Variances (ANOVA) with SPSS 18.0 software (IBM Corporation, Armonk, NY, USA). Levene's tests for equal variances were applied before one-way ANOVAs. When equal variances were assumed, a one-way ANOVA with Bonferroni *post- hoc* testing was used for multiple group comparison. If equal variances were not assumed, Tamhane's T2 test was used for multiple comparison tests. All tests were two-tailed. Differences with a *P* value less than 0.05 were considered statistically significant.

3. Results

3.1. Dosage Optimization of TGF-β_1 to Induce Hypertrophic Responses in NRCMs

To optimize the dose of TGF-β_1 to induce hypertrophic responses, we stimulated NRCMs with TGF-β_1 for 24 h. As shown in Figure 1A–G, 3 μg/L of TGF-β_1 resulted in significantly increased PI fluorescence intensity and [^{3}H]-leucine incorporation in NRCMs compared with all other groups ($P < 0.01$ vs. other groups). Similar results were observed in the mRNA levels of fetal genes ANF and β-MHC (Figure 1H and 1I). Therefore, 3 μg/L TGF-β_1 that generates the maximum effect in inducing hypertrophic responses in NRCMs was applied in the following experiment.

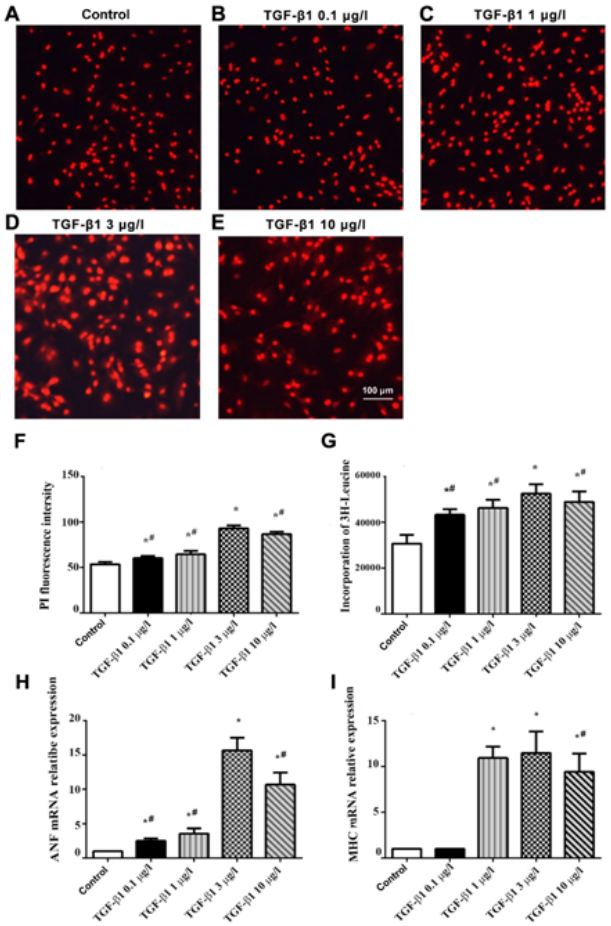

Figure 1. Dosage optimization of TGF-β_1 to induce hypertrophic responses in primary neonatal rat cardiomyocytes (NRCMs). NRCMs were seeded at a density of 10^6 cells/well in 24-well plates and cultured overnight. After a 24- h incubation in serum-free medium, NRCMs were stimulated with 0, 0.1, 1, 3, or 10 μg/L of TGF-β_1 for 24 h. (A–F) Cellular RNA was stained with RNA-sensitive fluorescence probe propidium iodide (PI) after DNase treatment. Representative images are shown in (A–E). Scale bar: 100 μm. The fluorescent signals were measured in five randomly selected fields in each well (F). (G) Protein synthesis was evaluated by measuring the incorporation of [^{3}H]-leucine. The radioactivity of [^{3}H]-leucine incorporated into the trichloroacetic acid precipitable material was determined by β-scintillation counting. The mRNA levels of atrial natriuretic factor (ANF) (H) and β-myosin heavy chain (β-MHC) (I) were determined using quantitative real-time PCR (qRT-PCR). GAPDH was used as an internal control. Data are expressed as mean ± standard deviation (SD). $^*P < 0.05$ vs. control; $^\#P < 0.05$ vs. 3 μg/L TGF-β_1 (n=7).

3.2. TGF-β_1 Activates the Smad Signaling in NRCMs

Because the TGF-β/Smad signaling promotes cardiac hypertrophy [22,23,24,25], we hypothesized that puerarin might protect the heart from TGF-β_1-induced hypertrophy by suppressing the Smad signaling. To test our hypothesis, we explored whether TGF-β_1 can induce the Smad signaling in NRCMs. As shown in Figure 2, TGF-β_1 (3 μg/L) significantly induced upregulation of mRNA and protein expression of Smad2, pSmad2, and total Smad2/3 in NRCMs starting at 30, and 15 min and peaking at 1 h, 2 h, and 30 min, respectively, after treatment, indicating a chronological activation of the Smad signaling in NRCMs exposed to TGF-β_1.

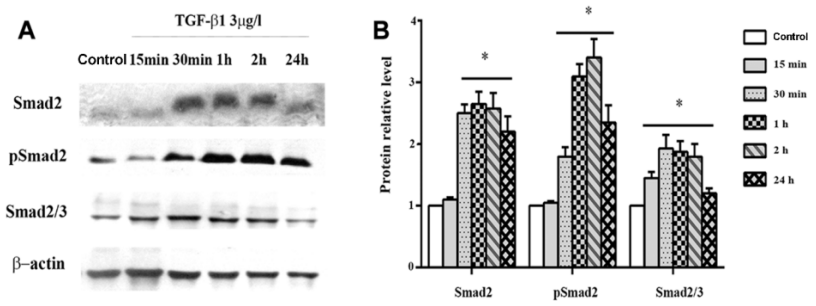

Figure 2. Smad protein expression in TGF-β1-stimulated NRCMs. NRCMs were treated with TGF-β1 (3 μg/L) for 15 min, 30 min, 1 h, 2 h, or 24 h. (A) The protein expressions of Smad2, phospho-Smad2 (pSmad2; Ser465/467), and total Smad2/3 were determined using Western blot analysis. (B) Quantification of (A). β-actin was used as an internal control. Data are expressed as mean ± SD. *P < 0.05 vs. control (n=7)

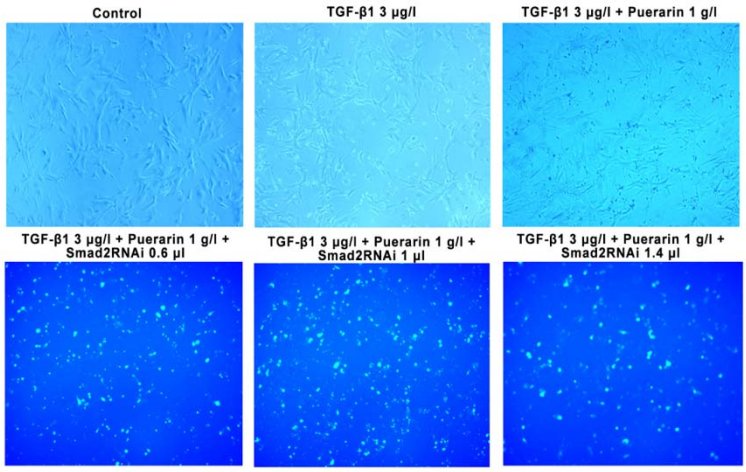

Figure 3. NRCMs were transfected with different amounts of Smad2siRNA. Images were acquired at 24 h after transfection using a fluorescence microscope. Representative images are shown

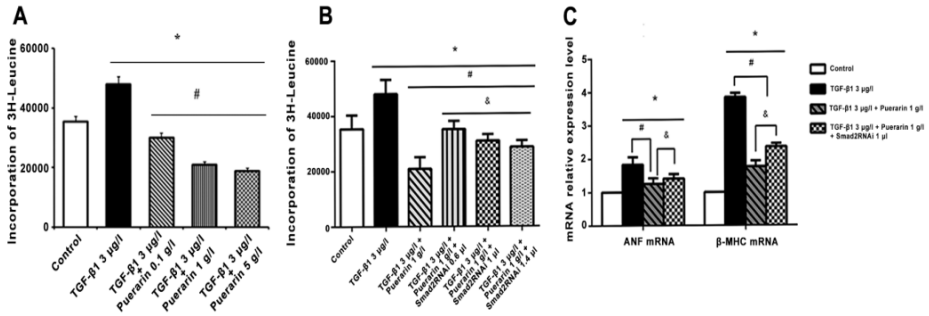

Figure 4. Puerarin abrogated TGF-β1-induced hypertrophic response in NRCMs. (A) NRCMs were treated with different doses of puerarin (0.1, 1, or 5 g/L) for 24 h in the presence of TGF-β1 (3 μg/L). Protein synthesis in NRCMs was evaluated by measuring [^{3}H]-leucine incorporation. (B) NRCMs were transfected with different amounts of Smad2siRNA (0.6, 1, or 1.4 μL). After 24 h of transfection, untransfected cells were treated with TGF-β1 (3 μg/L) individually or in combination with puerarin (1 g/L) for 24 h, and Smad2siRNA-transfected NRCMs were treated with TGF-β1 (3 μg/L) for 24 h. Protein synthesis was evaluated by measuring [^{3}H]-leucine incorporation. (C) NRCMs were transfected with 1 μL of Smad2siRNA. After 24 h of transfection, untransfected cells were treated with TGF-β1 (3 μg/L) individually or in combination with puerarin (1 g/L) for 24 h, and Smad2siRNA -transfected cells were treated with TGF-β1 (3 μg/L) for 24 h. The mRNA levels of ANF and β-MHC were determined using qRT-PCR. GAPDH was used as an internal control. Data are expressed as mean ± SD. *P<0.05vs.control; #P<0.01vs.TGF-β_1 group; &P<0.05vs. puerarin group (n=6)

3.3. Puerarin or Knockdown of Smad2 Inhibits TGF-β_1-induced Hypertrophic Responses in NRCMs

Next, we investigated the effect of puerarin or knockdown of Smad2 on TGF-β_1-induced hypertrophic responses in NRCMs. We treated NRCMs with different doses of puerarin in the presence of TGF-β_1 (3 μg/L) or transfected NRCMs with different amounts of Smad2 siRNA (Figure 3) prior to TGF-β_1 exposure. As shown in Figure 4A and 4B, both puerarin and Smad2 siRNA could significantly reverse TGF-β_1-enhanced [^{3}H]-leucine incorporation in NRCMs (all $P< 0.05$), but puerarin (1 g/L) exhibited a higher potency than different amounts of Smad2 siRNA ($P<0.05$). Consistently, puerarin (1 g/L) also significantly countered TGF-β_1-enhanced mRNA expression of ANF and β-MHC, with a greater effect than Smad2 siRNA (Figure 4C; all $P< 0.05$). Taken together, these results suggest that the Smad2 signaling is required for TGF-β_1-induced protein synthesis and fetal gene expression in NRCMs and that puerarin could effectively reverse these effects of TGF-β_1 in NRCMs.

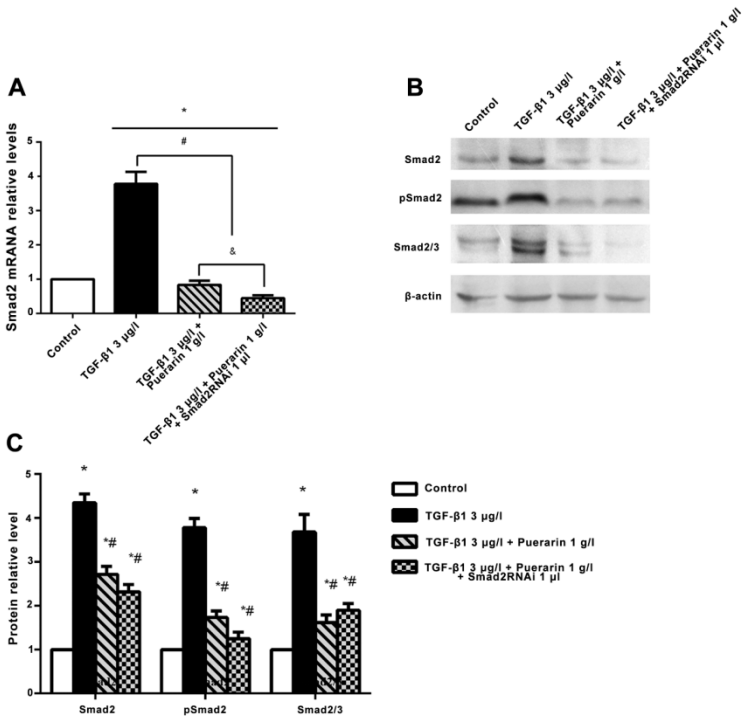

Figure 5. Puerarin attenuated TGF-β_1-induced Smad expression in NRCMs NRCMs were transfected with 1 μL of Smad2siRNA. After 24 h of transfection, untransfected cells were treated with TGF-β_1 (3 μg/L) individually or in combination with puerarin (1 g/L) for 24 h, and Smad2siRNA-transfected cells were treated with TGF-β_1 (3 μg/L) for 24 h. Untreated cells were used as a control. mRNA (A) and protein (B) expression of Smad2, pSmad2, and total Smad2/3 were determined using qRT-PCR and Western blot analysis, respectively. (C) Quantification of (B). Data are expressed as mean ± SD. *P < 0.05 vs. control; #P < 0.01 vs. TGF-β1 group; &P < 0.05 vs. puerarin group (n=6)

3.4. Puerarin Attenuates TGF-β_1-induced Smad Activation in NRCMs

Next, we sought to explore whether puerain can affect Smad protein expression in the presence of TGF-β_1. We determined the mRNA and protein expression of Smad2 in NRCMs exposed to TGF-β_1 alone or in combination with puerarin. As shown in Figure 5A–C, like Smad2 siRNA, puerarin could significantly abrogate TGF-β_1-enhanced expression of Smad2, pSmad2, and total Smad2/3 in NRCMs (all $P< 0.01$ vs. TGF-β_1 group), suggesting that puerarin protects NRCMs against TGF-β_1-induced hypertrophic changes possibly via deactivation of the Smad signaling.

4. Discussion

In the present study, we investigated the effect of puerarin on TGF-β_1-induced hypertrophic changes and Smad protein alterations in NRCMs. Our results showed that puerarin could effectively reverse TGF-β_1-induced hypertrophic changes and significantly abrogate TGF-β_1-induced Smad signaling activation in NRCMs, providing Smad proteins as possible targets for puerarin treatment in animal models.

Cardiac hypertrophy is characterized by enlargement of cardiomyocytes, enhancement of protein synthesis, and reactivation of fetal genes [1,2,3]. Our results showed that TGF-β_1 could significantly promote total RNA and protein synthesis and fetal gene transcription in NRCMs, suggesting a prohypertrophic effect of TGF-β_1 in NRCMs, consistent with previous studies [22,25,29,30]. TGF-β_1 is secreted by cardiac fibroblasts, cardiomyocytes, and endothelial cells, mediating intracellular signaling via the Smad family [31,32]. Our results showed that significant upregulation of total Smad2/3 began at 15 min and peaked at 30 min, whereas that of Smad2 and pSmad2 began and peaked at later time points post TGF-β_1 stimulation, indicating a chronological activation of total Smad2/3, Smad2, and pSmad2 in TGF-β_1-stimulated NRCMs. It has been reported that pharmacological inhibition of the Smad2 signaling can attenuate aortic banding-induced cardiomyocyte hypertrophy and restore cardiac function in mice [33], suggesting that targeting the Smad2 signaling is a promising therapeutic strategy in the treatment of cardiac hypertrophy. Consistently, in the present study, we found that knockdown of Smad2 could effectively reverse TGF-β_1-induced hypertrophic changes in NRCMs, indicating that the Smad signaling is required in TGF-β_1-induced hypertrophic changes. Importantly, puerarin treatment exhibited similar but stronger effects than Smad2siRNA in inhibiting TGF-β_1-stimulated protein synthesis and fetal gene transcription. Based on these findings, we speculate that puerarin might attenuate TGF-β_1-induced hypertrophic responses in NRCMs possibly and partially via suppressing the Smad2 signaling. Furthermore, we found that, like Smad2 siRNA, puerarin significantly abrogated TGF-β_1-enhanced expression of Smad2, pSmad2, and total Smad2/3 in NRCMs, suggesting an involvement of Smad signaling suppression in the antihypertrophic role of puerarin in NRCMs.

Previous studies have suggested other signaling pathways underlying the antihypertrophic effect of puerarin. For example, in spontaneously hypertensive rats, puerarin reduces mRNA expression of TGF-β_1 and Smad3 while increasing that of Smad7 in myocardium [34]. In addition, puerarin retards the progression of cardiac hypertrophy by

blocking the PI3K/Akt and JNK signaling pathways [19]. Our previous study also suggests that puerarin can protect against cardiomyocyte hypertrophy by restoring autophagy through the AMPK/mTOR signaling [35]. These findings collectively suggest that puerarin may ameliorate cardiac hypertrophy via modulating multiple signaling pathways, explaining why puerarin exhibited greater inhibition in TGF-β_1-induced protein synthesis in NRCMs compared with Smad2 knockdown.

In conclusion, our results demonstrated inhibitory effects of puerarin on TGF-β_1-induced hypertrophic responses and Smad signaling activation in NRCMs, providing new insights into the mechanism underlying the antihypertrophic effect of puerarin. Future studies will further investigate the antihypertrophic role of puerarin and the involvement of the Smad signaling in an anrimal model.

Acknowledgments

All authors declare that they have no conflict of interests.

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