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Puerarin Attenuates Cardiac Hypertrophy Partly Through Increasing Mir-15b/195 Expression and Suppressing Non-Canonical Transforming Growth Factor Beta (Tgfβ) Signal Pathway

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Previous studies demonstrated that puerarin has therapeutic effects on cardiac hypertrophy. This study aimed to explore whether the effect of puerarin on attenuating cardiac hypertrophy is related to regulation of microRNAs (miRNAs) and the transforming growth factor beta (TGFβ) signal pathway.

Material/Methods: The therapeutic effect of puerarin was assessed using an angiotensin (Ang) II-induced heart hypertrophy model in mice. The primary cardiomyocytes were used as an *in vitro* model. MiR-15 family expression was quantified using qRT-PCR analysis. The expression of the genes involved in canonical and non-canonical TGFβ signal pathways was measured using qRT-PCR and Western blot analysis. *In vitro* cardiac hypertrophic features were assessed by quantifying cardiac hypertrophic genes and measurement of cell surface, protein synthesis, and total protein content.

Results: Puerarin attenuated cardiac hypertrophy and increased miR-15b and miR-195 expression in the mouse cardiac hypertrophy model and in primary cardiomyocytes. It suppressed both canonical and non-canonical TGFβ signal pathways, partially through miR-15b and miR-195. Puerarin reduced mRNA expression of cardiac hypertrophic genes, reduced cell surface area, and lowered the rate of protein synthesis and the total protein content induced by Ang II. Knockdown of endogenous miR-15b and miR-195 partly abrogated these effects. Knockdown of endogenous p38, but not Smad2/3/4, presented similar effects as miR-15b.

Conclusions: Puerarin administration enhances miR-15b and miR-195 expression in an Ang II-induced cardiac hypertrophy model, through which it suppresses both canonical and non-canonical TGFβ signal pathways at the same time. However, the effect of puerarin on attenuating cardiac hypertrophy is mainly through the non-canonical TGFβ pathway.

MeSH Keywords: Cardiomyopathy, Hypertrophic • MicroRNAs • Pueraria • Transforming Growth Factor beta1

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Background

Cardiac hypertrophy refers to an increase in the size and mass of the heart, which is a result of adaptive response to increased cardiac load. It is characterized by enlargement of myocytes by the addition of contractile proteins [1]. Timely treatment of pathological cardiac hypertrophy helps prevent or postpone the progression of heart failure [2].

Puerarin (7,4-dihydroxyisoflavone-8β-glucopyranoside) is a major natural compound extracted from a famous traditional Chinese medicine, kudzu root (7). It has been approved by the State Food and Drug Administration in China for clinical therapy of cardiovascular and other diseases [3]. Recent studies found that puerarin can attenuate pressure overload-induced cardiac hypertrophy by activation of autophagy [4–6]. Puerarin treatment significantly blocks pressure overload-induced activation of PI3K/Akt signaling and c-JNK signaling, which are related to the development of cardiac hypertrophy, fibrosis, and apoptosis [4]. It can also inhibit angiotensin II (Ang II)-induced cardiac hypertrophy through the redox-sensitive ERK1/2, p38, and NF-κB pathways [7]. However, as a natural compound, puerarin may take part in multiple signal pathways, activating or inhibiting multiple target proteins directly or indirectly. It is necessary to explore whether other mechanisms are involved in its effect of attenuating cardiac hypertrophy.

Transforming growth factor β (TGFβ) is a cytokine, the expression and activity of which is significantly increased during cardiac remodeling [8]. In cardiac tissues, activation of the canonical TGFβ signal pathway (TGFβ/Smad) and non-canonical TGFβ signal pathway (TGFβ/TGFβ-activated-kinase 1 (TAK1)p38) are both related to the development of myocyte hypertrophy [8]. A previous study showed that miR-15 family has multiple targets in both canonical and non-canonical TGFβ signal pathways [9]. Through modulating expression of the target genes, the miR-15 family exerts a critical role in regulating cardiac hypertrophy and fibrosis [9]. A recent study showed that the therapeutic effect of puerarin might be partially achieved through modulating miRNAs expression [10]. Therefore, we tried to determine whether the effects of puerarin on attenuating cardiac hypertrophy are related to its regulation of miRNAs and the TGFβ signal pathway.

Material and Methods

Animals and protocols

This animal study was approved by the Research Committee of Binzhou People's Hospital. All animal-based studies followed the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health). Eight-week-old male, healthy and specific pathogen-free C57BL/6J mice weighing 19–21 g were purchased from the Experimental Animal Center of Shandong University. The mice were randomized into 3 groups (n=10/group): a sham group that only received phosphate-buffered saline (PBS) infusion; an Ang II (Sigma-Aldrich, St Louis, CA, USA)-infused group; and a puerarin group that received intragastric (ig) administration of puerarin (Shandong Fangming Pharmaceutical Co.; Heze, China) at a dosage of 100 mg/kg/d dissolved in sterile water. The Ang II and puerarin groups both received Ang II dissolved in PBS with 10 μmol/L acetic acid at a dose of 2.5 μg/kg/min using a subcutaneously implanted minipump (model 2002, Alza, Mountain View, CA, USA) for 15 days. Puerarin was administered for 15 consecutive days after the surgery. The mice were housed in specific-pathogen-free (SPF) conditions with a 12-h light/12-h dark cycle and with free access to drinking water and food.

Preparation and cultivation of primary cardiomyocytes

Primary ventricular cardiomyocytes were prepared from neonatal C57BL/6J mice, according to methods described in a previous study [11]. Briefly, ventricular myocardium was minced and digested with 0.125% trypsin without EDTA. The dissociated cells were suspended in DMEM/F12 containing 12.5% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), centrifuged, and pooled. The cells were enriched for cardiomyocytes by differential adhesion for 60 min and were plated in culture plates at a density of 1.0×10^5 cells/cm^2. We added 100 μmol/L BrdU to the cell culture to inhibit the proliferation of non-myocytes. The purity of the cardiomyocytes was identified using anti-sarcomeric, α-actinin, and FITC immunohistochemistry. After 3 days of incubation in the medium, the culture medium was changed to DMEM/F12 containing 0.1% fetal bovine serum (FBS). Before infection or treatment with 100 μmol/L puerarin, the cardiomyocytes were cultured in serum-free medium for 24 h. The cells were stimulated with 1 μmol/L Ang II for 48 h, after pretreatment with or without puerarin or infection with the lentiviral particles.

Reagents and cell transfection

The pLV-miR-15b and pLV-miR-195 expression plasmid, the pLV-miR-15b and pLV-miR-195 locker plasmid, and the lentiviral packaging vector mix were purchased from Biosettia (San Diego, CA, USA). The lentiviral pLV-miR expression vector or the pLV-miR locker vectors were produced by contransfection with helper plasmids mixture into HEK-293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The primary cardiomyocytes were infected with the viral supernatants containing lentiviral vectors with the presence of 8 μg/ml Polybrene (Sigma-Aldrich, St Louis, MO, USA). The ready-to-use Smad2/3 and Smad4 shRNA lentiviral particles (sc-37239-V and sc-29485-V) and p38 shRNA lentiviral particles (sc-29434-V) were purchased from Santa

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Cruz Biotech (Santa Cruz, CA, USA). The primary cardiomyocytes were directly infected with the viral particles with the presence of 8 μg/ml Polybrene according to the manufacturer's protocol (Sigma-Aldrich).

Morphological and histological examination

After the treatments, mice were sacrificed and the excised hearts were weighed after the blood vessels and all the epicardial fat were removed. After gross dissection, the ventricles were sectioned into slices with 2–3 mm thick and then immersed in a formalin solution for over 1 week. Then, the slices were dehydrated in an ascending series of ethanol, cleared in xylene, embedded in paraffin, and sectioned at 5-μm intervals in a coronal orientation. Then the sections were deparaffinized and stained with hematoxylin and eosin (H&E) for conventional histopathologic evaluation.

QRT-PCR analysis of expression of miRNAs and TGF-β signal pathway members

Total RNA from the tissue and cell samples were isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The expression of all miR-15 family members were quantified using the TaqMan microRNA Reverse Transcriptase kit (Applied Biosystems, CA, USA) and TaqMan probes (Applied Biosystems), according to the manufacturer's recommended protocol.

To quantify the expression of TGF-β signal pathway members, including TGFBR1, TGFBR2, TGFBR3, Smad2, Smad3, Smad4, Smad7, TAK1, and p38, cDNA was obtained by reverse transcription using a First Strand Synthesis kit (Invitrogen). The mRNA levels of these genes were quantified using qRT-PCR with the gene-specific primers (Table 1) and Power SYBR Green PCR Master Mix. GAPDH served as the endogenous control. All PCR was performed using an ABI 7000 cycler.

Western blot analysis of Smad family members and p38

Total protein from tissues was extracted by using RIPA buffer and the protein concentration was measured using Pierce BCA protein assay (Thermo Scientific, Rockford, lL, USA). Then the protein samples were separated on 10% SDS PAGE gel and transferred onto nitrocellulose membranes for conventional Western blot analysis. The membranes were incubated with primary antibodies (all purchased from Abcam), including anti-Smad2 (ab33875), anti-Smad3 (ab40854), anti-Smad4 (ab40759), anti-Smad7 (ab124890), anti-TAK1 (ab109526), and anti-p38 (ab7952), separately. Membranes were washed and incubated with corresponding HRP-labeled secondary antibodies. Protein signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Table 1. Primer sequences for qRT-PCR.

Gene	Primer sequences
TGFBR1	F: 5'-GCTGTGAAGCCTTGAGAGTAATGG-3' R: 5'-GATGCCTTCCTGTTGACTGAGTTG-3'
TGFBR2	F: 5'-TGTCTGTGGATGACCTGGCTAA-3' R: 5'-TTCTAGGACTTCTGGAGCCATGT-3'
TGFBR3	F: 5'-TGGGGTCTCCAGACTGTTTTT-3' R: 5'-CTGCTCCATACTCTTTTCGGG-3'
Smad2	F: 5'-AAGCCATCACCACTCAGAATTG-3' R: 5'-CACTGATCTACCGTATTTGCTGT-3'
Smad3	F: 5'-AGGGGCTCCCTCACGTTATC-3' R: 5'- CATGGCCCGTAATTCATGGTG-3'
Smad4	F: 5'-CATTTCCAATCATCCTGCTCC-3' R: 5'-CCACGTATCCATCAACAGTAAC-3'
Smad7	F: 5'-GGCCGGATCTCAGGCATTC-3' R: 5'-TTGGGTATCTGGAGTAAGGAGG-3'
TAK1	F: 5'-ATCAGTCAACACCCATCATTGAAG-3' R: 5'-GGGCATTGTAAGCTCTTGAATGTG-3'
P38 (p38α)	F: 5'-AAGACTCGTTGGAACCCCAG-3' R: 5'-TCCAGTAGGTCGACAGCCAG-3'

Cell surface area

The surface area of the cardiomyocytes was measured according to methods introduced in a previous study [12]. Briefly, cell images were captured by a microscope with a digital camera (Olympus IX-81, Olympus, Japan). The cell surface area was analyzed using Image-Pro Plus 7.0 (Olympus, Tokyo, Japan). One hundred cells were randomly selected in 3 wells of different treatments.

Measurement of [^{3}H] leucine incorporation and protein content assay

[^{3}H] leucine incorporation was measured according to the method introduced in a previous study [13]. Briefly, cells were cultured in 24-well plates in serum-free medium for 24 h. After the indicated treatment, the cells were pulsed with 1 μCi/mL of [^{3}H] leucine (Amersham Biosciences, Piscataway, USA) for 4 h before harvest. After washing 3 times with PBS, the cells were treated with 5% trichloroacetic acid for 30 min. Finally, cells were solubilized in 500 μL of 1 mol/L NaOH and neutralized using 0.5 mol/L HCl. Then, an aliquot was taken to determine the level of incorporated radioactivity using the Beckman LS 3801 liquid scintillation counter (Beckman, Fullerton, USA). The total protein content per well was measured with a Modified Lowry protein assay kit (Pierce, Rockford, IL, USA).

Statistical analysis

Data analysis was performed using SPSS16.0 statistical software (SPSS, Chicago, USA). One-way ANOVA was performed to compare means of multiple group experiments. Group

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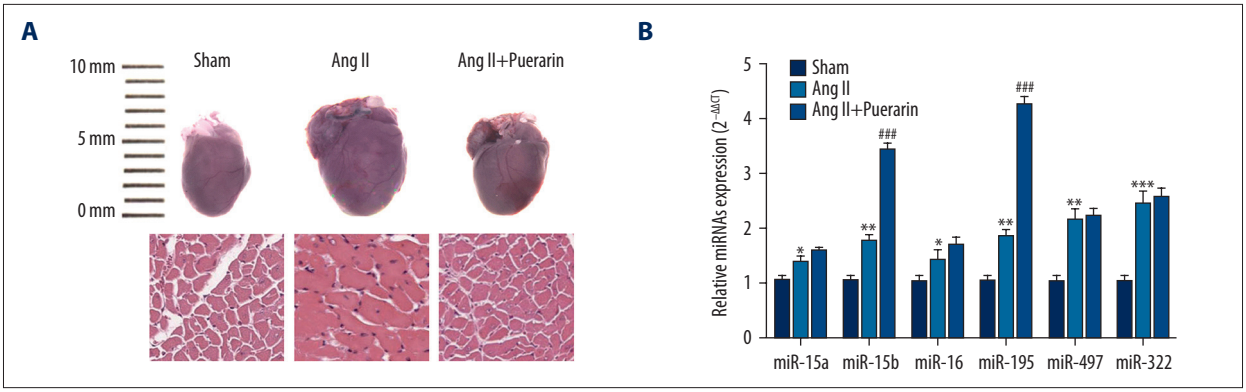

Figure 1. Puerarin attenuates cardiac hypertrophy and increases miR-15b and miR-195 expression. (**A**) Gross specimens (up panel) and the hearts and representative images of cross-sections of the left ventricles stained with HE (original magnification ×200) (down panel) in the sham, Ang II-infused, and puerarin groups. (**B**) qRT-PCR analysis of miR-15 family expression in the ventricular tissues of the 3 groups after indicated treatment. * Comparison with Sham group; # comparison with Ang II group. * and # p<0.05, ** and ## p<0.01, *** and ### p<0.001.

comparison was conducted using unpaired *t* tests. A 2-tailed P<0.05 was considered statistically significant.

Results

Puerarin attenuates cardiac hypertrophy and increases miR-15b and miR-195 expression

After 15 days of treatment with Ang II, the mice showed increased heart size compared with the sham group (Figure 1A, up panel). By performing H&E staining, the left ventricular tissues of Ang II-infused mice also presented evident features of cardiac hypertrophy (Figure 1A, down panel). However, these pathological changes were significantly attenuated by long-term treatment with puerarin at a dose of 100 mg/kg (Figure 1A). Previous studies showed that the therapeutic effect of puerarin might be partially achieved through modulating miRNAs expression [10] and that the miR-15 family might play an important role in the development of cardiac hypertrophy [9]. Therefore, we explored whether puerarin could affect the expression of this miRNA family. Through qRT-PCR analysis using the ventricular tissue samples, we observed that Ang II significantly promoted the expression of all miR-15 family members (Figure 1B). Administration of puerarin further enhanced the expression of miR-15b and miR-195 (Figure 1B). These results suggest that puerarin has a therapeutic effect on cardiac hypertrophy and can enhance miR-15b and miR-195 expression.

Puerarin suppresses both canonical and non-canonical TGFβ signal pathways partially through miR-15b and miR-195

Activation of the TGFβ in the heart promotes the development of fibrosis and hypertrophy [9]. Considering the vital role of TGFβ in cardiac hypertrophy, we then studied whether puerarin can suppress the canonical and non-canonical TGFβ signal pathways. The miR-15 family members have the same 'seed' sequence and can target multiple genes in the TGF-β signal pathway [9]. Through performing qRT-PCR and Western blot analysis based on ventricular tissues, we observed that puerarin substantially alleviated Ang II-induced high expression of canonical TGFβ members, including Smad2, Smad3, and Smad4, and non-canonical TGFβ member p38 at both mRNA and protein levels (Figure 2A, 2B). However, it increased Smad7 expression (Figure 2A, 2B). The primary cardiomyocytes treated with puerarin had significantly enhanced expression of some miR-15 family members, including miR-15a, miR-15b, miR-16, and miR-195. Among them, miR-15b and miR-195 had a greater than 4-fold increase in expression (Figure 2C). To further verify the regulative effect of miR-15b and miR-195 on the TGFβ signal pathway, the primary cardiomyocytes were first infected with miR-15b/195 expression or inhibition lentiviral particles (Figure 2D, 2E). Overexpression of miR-15b or miR-195 significantly decreased the expression of Smad2, Smad3, Smad4, Smad7, and p38 (Figure 2F). In contrast, knockdown of endogenous miR-15b or/and miR-195 significantly promoted the expression of these genes (Figure 2G). These results confirmed that the expression of Smad2, Smad3, Smad4, Smad7, and p38 can be modulated by miR-15b and miR-195, which is consistent with a recent study [9]. However, knockdown of endogenous miR-15b and miR-195 only partially antagonized the effect of puerarin in suppressing Smad2, Smad3, and Smad4 expression. It has no effect on puerarin-mediated Smad7 increase (Figure 2H). These results suggest that puerarin suppresses the expression of Smad2, Smad3, Smad4, and p38 at least partially through increasing miR-15b and miR-195, but there might be other inducing effects of puerarin in promoting Smad7 expression. Since Smad7 is a negative regulator of the canonical TGFβ signal pathway, these findings suggest

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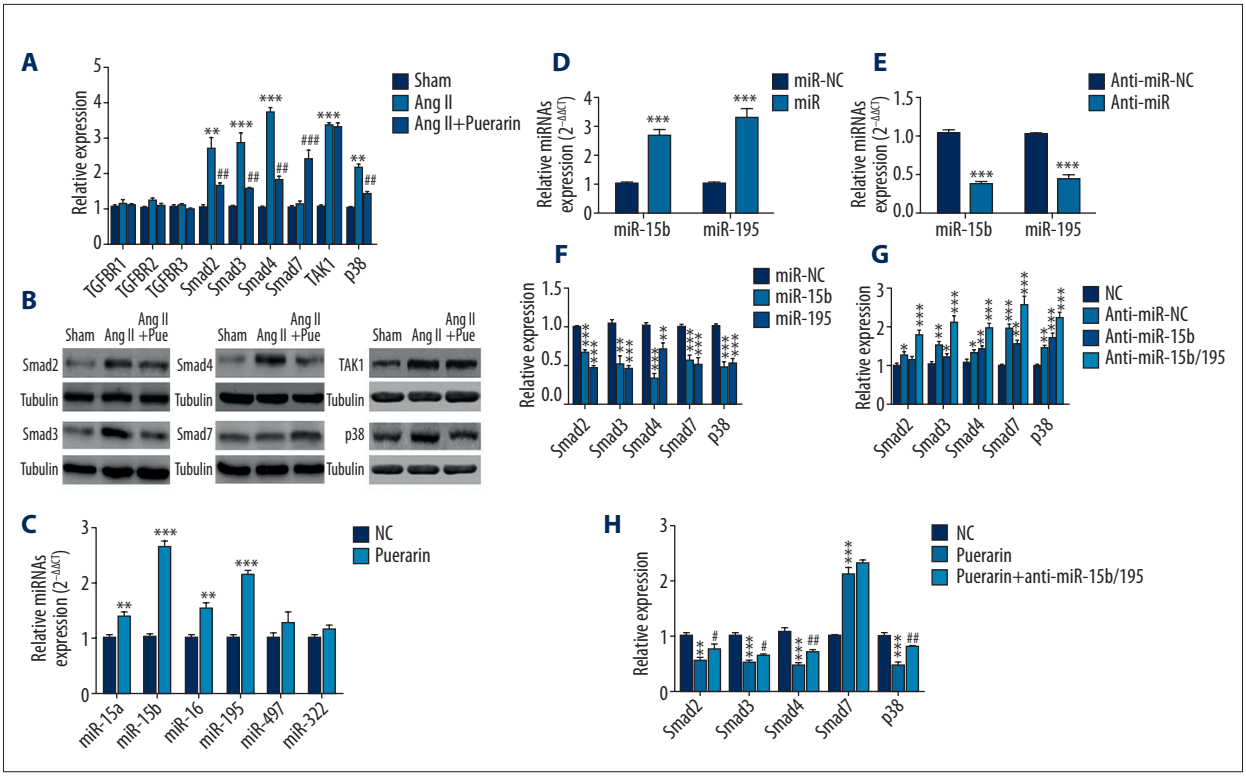

Figure 2. Puerarin suppresses the canonical and non-canonical TGFβ signal pathways partially through miR-15b and miR-195. (**A**) QRT-PCR analysis of the TGFβ receptors (TGFBR1, TGFBR2 and TGFBR3), canonical (Smad2, Smad3, Smad4, and Smad7) and non-canonical TGFβ signal members (TAK1 and p38) in the ventricular tissues in the sham, Ang II-infused, and puerarin groups. (**B**) Western blot analysis of Smad2, Smad3, Smad4, Smad7, TAK1, and p38 expression in the groups indicated in Figure A. (**C**) QRT-PCR analysis of miR-15 family expression in the primary cardiomyocytes after treatment with puerarin. (**D, E**) QRT-PCR analysis of miR-15b and miR-195 expression in the primary cardiomyocytes after infection with the pLV-miR-15b and pLV-miR-195 expression (**D**) or the pLV-miR-15b and pLV-miR-195 locker lentiviral particles (**E**). (**F, G, H**) QRT-PCR analysis Smad2, Smad3, Smad4, Smad7, and p38 expression in the primary cardiomyocytes with miR-15b or miR-195 overexpression (**F**) or miR-15b or miR-195 knockdown (**G**) or after treatment with puerarin with or without knockdown of endogenous miR-15b and miR-195 (**H**). * Comparison with NC group; # comparison with puerarin group. * and # p<0.05, ** and ## p<0.01, *** and ### p<0.001.

that puerarin can generally inhibit both canonical and non-canonical TGFβ signals.

Puerarin attenuates cardiac hypertrophy partially through miR-15b and miR-195

Cardiac hypertrophy is characterized by substantially increased expression of the hypertrophic marker genes and increases in protein synthesis and cell size [14]. Therefore, we decided to study the involvement of miR-15b and miR-195 in cardiac hypertrophy. Puerarin treatment significantly attenuated increased expression of the hypertrophic marker genes (atrial natriuretic peptide [(ANP], B-type natriuretic peptide [BNP], and β-myosin heavy polypeptide [MHC]) induced by Ang II (Figure 3A). Puerarin treatment also alleviated increasing cell surface area (Figure 3B), [^{3}H]leucine incorporation (Figure 3C), and total protein content (Figure 3D) induced by Ang II. However, knockdown of endogenous miR-15b and miR-195 partly cancelled the protective effects of puerarin (Figure 3A–3D). These results suggest that puerarin attenuates cardiac hypertrophy partially through miR-15b and miR-195.

MiR-15b attenuates cardiac hypertrophy through the non-canonical TGFβ pathway

Since puerarin can modulate both canonical and non-canonical TGFβ pathways by increasing miR-15b and miR-195 expression, we then studied which pathway is involved in its therapeutic effect on cardiac hypertrophy. Overexpression of miR-15b attenuates cardiac hypertrophy in terms of hypertrophic marker genes (Figure 4A), cell surface area (Figure 4B), [^{3}H]leucine incorporation (Figure 4E), and total protein content (Figure 4G). Knockdown of endogenous Smad2/3/4 using shRNA lentiviral particles could not exert therapeutic effects as miR-15b (Figure 4B, 4D, 4F, 4H). However, knockdown of endogenous p38 partly alleviated Ang II-induced cardiac hypertrophic features,

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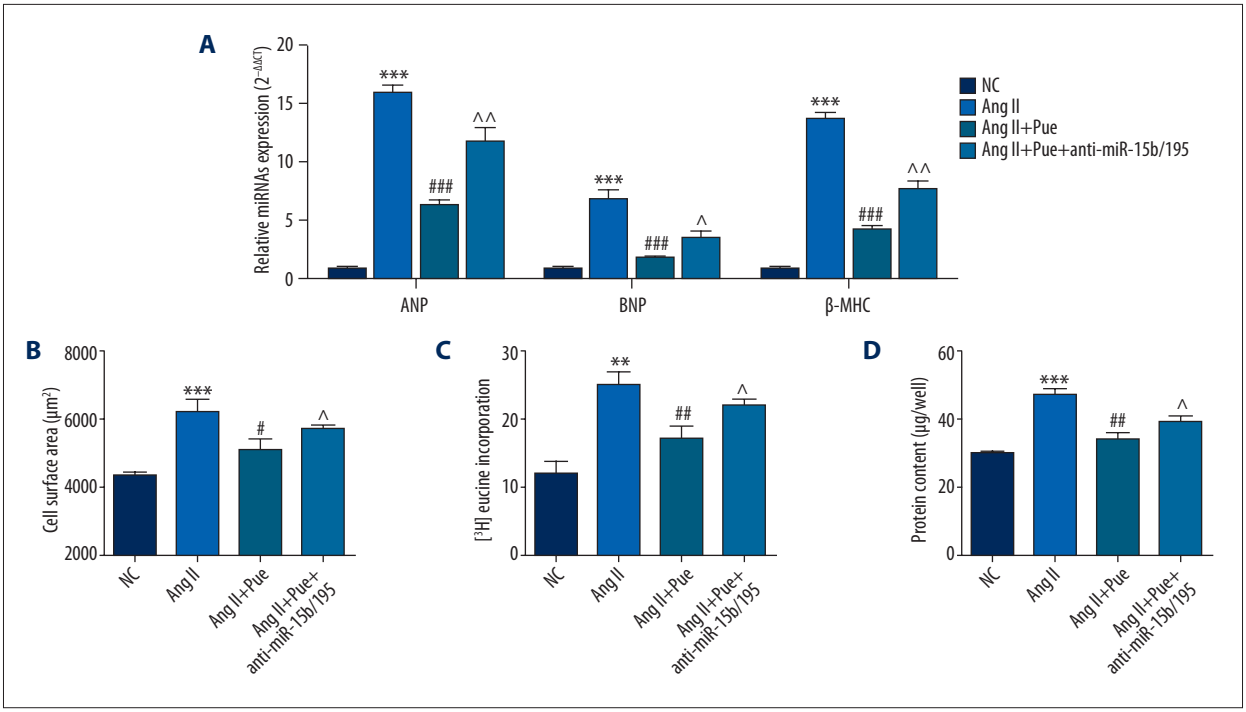

Figure 3. Puerarin attenuates cardiac hypertrophy partially through miR-15b and miR-195. (**A**) QRT-PCR analysis of mRNA expression of ANP, BNP, and β-MHC in the primary cardiomyocytes after indicated treatments. (**B–D**) Measurement of cell surface area (**B**), the rate of protein synthesis (**C**), and the total protein content (**D**) in the cardiomyocytes after indicated treatments. * Comparison with NC group; # comparison with Ang II group; ^ comparison with Ang II+Pue group. *, # and ^ p<0.05, **, ## and ^^ p<0.01, ***, ### and ^^^, p<0.001. Pue: puerarin.

which were similar to miR-15b overexpression (Figure 4B, 4D, 4F, 4H). These results suggest that miR-15b attenuates cardiac hypertrophy through the non-canonical TGFβ pathway, but not through the canonical TGFβ pathway.

Discussion

Puerarin is a pure extract from and the major bioactive compound of kudzu root [15]. Previous studies demonstrated that puerarin has therapeutic effects on cardiac hypertrophy. As a natural compound with a relatively complex structure, studies showed that it can attenuate cardiac hypertrophy through multiple pathways. Generally, puerarin can attenuate Ang II-induced cardiac hypertrophy via inhibiting activation of the redox-sensitive ERK1/2, p38 and the NF-κB pathways [7] and inhibiting NADPH oxidase activation and oxidative stress-triggered AP-1 signaling pathways [16]. In addition, puerarin can also attenuate pressure overload-induced cardiac hypertrophy by activation of autophagy [4,5] and can block pressure overload-induced activation of PI3K/Akt signaling and c-JNK signaling, which are related to the development of cardiac hypertrophy, fibrosis, and apoptosis [4]. However, whether other mechanisms are involved in its therapeutic effect on cardiac hypertrophy is not clear. In this study, we confirmed that puerarin can attenuate Ang II-induced cardiac hypertrophy. In addition, we also observed that puerarin enhanced the expression of the miR-15 family, especially miR-15b and miR-195.

A previous study reported that the miR-15 family is up-regulated in the overloaded heart in multiple species, acting as a spontaneous response to alleviate cardiac hypertrophy [9]. In fact, the miR-15 family members share a common seed region, which targets multiple members in both the canonical and non-canonical TGF-β pathways. For example, the miR-15 family can directly target TGFBR1, Smad2, Smad3, Smad7, p38, and endoglin [9]. Among them, TGFBR1, Smad2, Smad3, and endoglin are TGFβ canonical signaling activators, while Smad7 is a signal repressor. Inhibition of miR-15b in a mouse cardiac hypertrophy model aggravated fibrosis and hypertrophy [9]. In cardiac fibroblasts, activation of canonical TGFβ signal promotes the development of fibrosis [17]. In contrast, the heterozygous TGFβ1-deficient mice have attenuated fibrosis in the aging heart [18]. In cardiomyocytes, binding of TGFβ to its receptors leads to activation of the non-canonical pathway, which is associated with phosphorylation of TAK1 and p38, and subsequent activation of multiple transcription factors, such as GATA4, MEF2C, and SRF [19]. Activation of this signal axis can regulate the development of myocyte hypertrophy during cardiac remodeling [20]. Therefore, the miR-15

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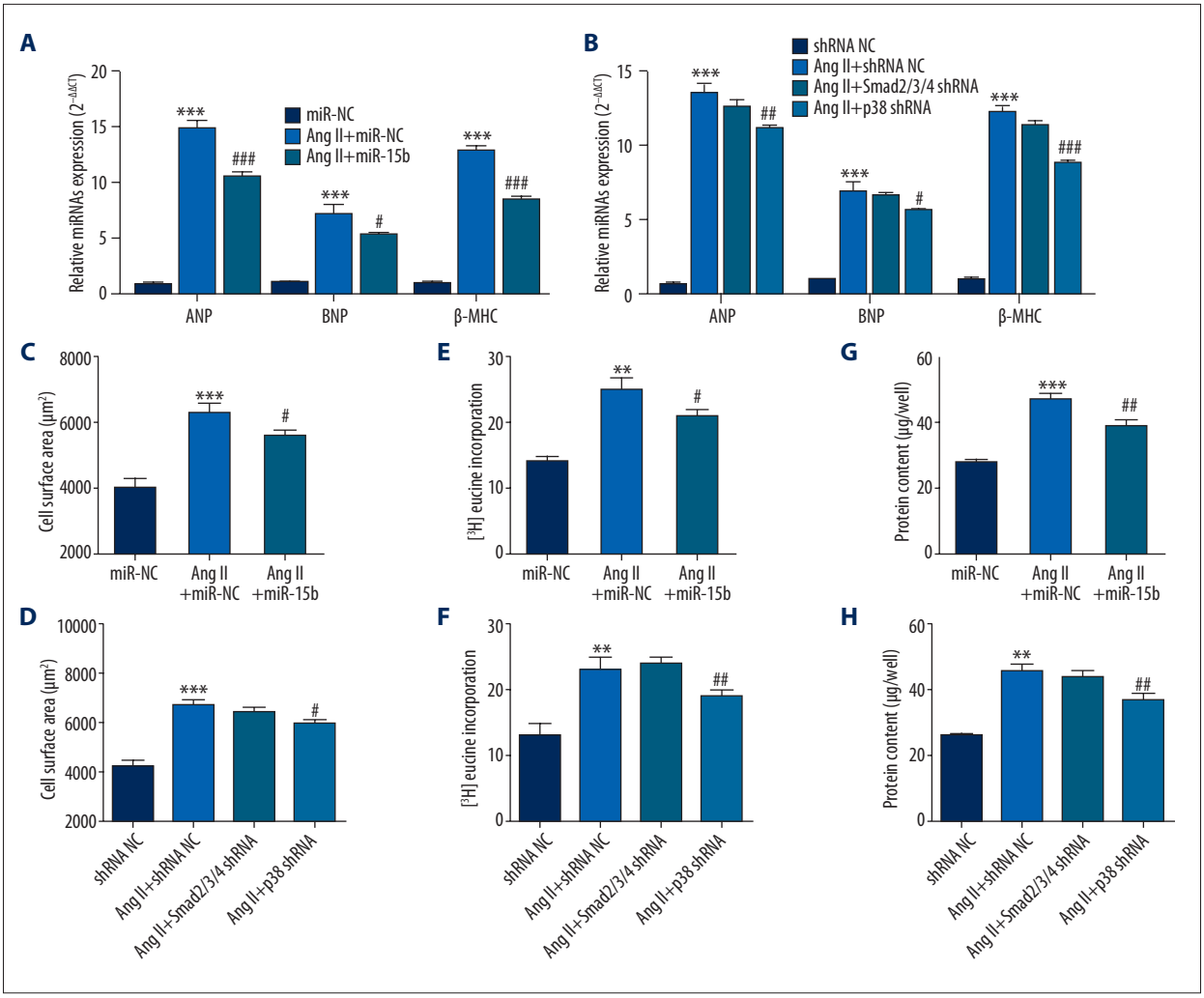

Figure 4. MiR-15b attenuates cardiac hypertrophy through the non-canonical TGFβ pathway. (**A, B**) QRT-PCR analysis of mRNA expression of ANP, BNP, and β-MHC in the primary cardiomyocytes after knockdown of endogenous miR-15b (**A**) or knockdown of endogenous TAK1 or p38 separately (**B**). (**C–H**) Measurement of cell surface area (**C, D**), the rate of protein synthesis (**E, F**), and the total protein content (**G, H**) in the cardiomyocytes after knockdown of endogenous miR-15b (**C, E, G**) or knockdown of endogenous TAK1 or p38 separately (**D, F, H**). * Comparison with shRNA NC group; # comparison with Ang II+ shRNA NC group. * and # p<0.05, ** and ## p<0.01, *** and ### p<0.001.

family may act as an important regulator of cardiac hypertrophy and fibrosis via inhibiting the TGFβ signal. Based on primary mouse cardiomyocytes, we investigated the effect of puerarin on regulating canonical and non-canonical TGFβ signal members. Puerarin treatment significantly reduced Ang II-induced higher expression of Smad2, Smad3, Smad4, and p38, but significantly increased Smad7. These findings are partly consistent with a previous study that observed that puerarin suppresses Smad3 but increases Smad7 [21]. Therefore, puerarin is an agent that can suppress both canonical and non-canonical TGFβ signals.

In the development of cardiac hypertrophy, previous studies suggested that miRNAs may exert a strong inducing effect. For example, TGF-β1 expression can initiate miR-27b expression, which promotes hypertrophic cell growth. Phenylephrine (PE) treatment can inhibit the hypertrophic cell growth at least partly through suppressing miR-27b [22]. Since puerarin exerts strong effects on promoting miR-15b/195 expression and on inhibiting TGFβ signals, we then studied whether it attenuates cardiac hypertrophy partially through miR-15b and miR-195. Knockdown of endogenous miR-15b and miR-195 partly abrogated the therapeutic effects of puerarin. In addition, by interrupting the canonical and non-canonical TGFβ signals separately, we observed that only p38 shRNA showed an effect similar to that of miR-15b, while Smad2/3/4 shRNA could not alleviate Ang II-induced hypertrophic features. These results suggest that puerarin can attenuate cardiac hypertrophy through

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the non-canonical TGFβ pathway by promoting miR-15b and miR-195. A previous study found that Ang II-induced cardiomyocyte hypertrophy in an *in vitro* model is TAK1- dependent and Smad2/3-independent [23]. This is consistent with our finding that puerarin attenuates cardiac hypertrophy through the non-canonical TGFβ pathway.

Conclusions

Puerarin administration enhances miR-15b and miR-195 expression in an Ang II-induced cardiac hypertrophy model, through which it suppresses the canonical and non-canonical TGFβ signal pathways at the same time. However, the effect of puerarin on attenuating cardiac hypertrophy is exerted mainly through the non-canonical TGFβ pathway.

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