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Original Article

Overexpression of microRNA-138 attenuates cardiac hypertrophic response through repression of YAP1

Hui Huang[1], Jie Zeng[1], Yongmei Zhou[1], Rong Yu[2]

Departments of [1]Cardiology, [2]Geriatrics, Sichuan Provincial Academy of Medical Sciences, Sichuan Provincial People's Hospital, Chengdu, China

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Abstract: MicroRNAs (miRNAs) have been identified as important players in the pathogenesis of cardiac hypertrophy. In the present study, we evaluated the potential role of miR-138 in cardiac hypertrophy and the potential molecular mechanisms. We observed that in mice with transverse aortic constriction (TAC), miR-138 expression was remarkably reduced in the hypertrophic hearts. Overexpression of miR-138 attenuated the cardiac hypertrophy *in vivo* and the hypertrophic responses in Ang-II-treated mouse cardiomyocytes *in vitro*. YAP1 was experimentally validated as a direct target of miR-138, and its mRNA and protein levels were increased in Ang-II-treated cardiomyocytes. Therefore, the findings of the present article revealed a novel molecular mechanism for cardiac hypertrophy and indicated miR-138 as a potential therapeutic target for this disease.

Keywords: Cardiac hypertrophy, transverse aorta constriction, microRNA-138, YAP1, cardiomyocyte, angiotensin II

Introduction

Cardiac hypertrophy, the thickening of the myocardium, is a functional, adaptive process to maintain cardiac output in response to chronic pressure or volume overload [1]. It is beneficial in reducing stress placed on the heart. However, prolonged hypertrophy will impair the cardiac function and ultimately lead to heart failure [2]. Cardiac hypertrophy is mainly featured by the reactivation of fetal cardiac genes, the increased rate of protein synthesis, the reorganization of sarcomeric structures, the enlargement of cardiomyocyte size, and the elevated ratio of heart weight/body weight (HW/BW) [3, 4]. Further investigation of the regulatory mechanisms implicated in cardiac hypertrophy would promote the development of novel therapeutic strategies for the treatment of this disease [5].

MicroRNAs (miRNAs) are an abundant class of endogenous, noncoding RNAs, approximately 20-24 nucleotides in length, which negatively regulates gene expression at the post-transcription level through interacting with the 3' untranslated regions (3'-UTRs) of target mRNA [6, 7]. Increasing evidences have showed that, in the cardiovascular system, miRNAs are not only critical for heart and vascular development but also serve an essential role in cardiac pathophysiology, including cardiac hypertrophy [8]. Deregulation of miR-138 has been reported to cause aberrant ventricular cardiomyocyte morphology and cardiac function in zebrafish heart [9], and up-regulation of miR-138 plays a protective role in myocardial adaptation to chronic hypoxia [10, 11]. However, to date, study on the therapeutic potential of miR-138 in cardiac hypertrophy remains to be further explored.

Accordingly, here, we investigated the functional role of miR-138 in cardiac hypertrophy using a TAC mice model and AngII-stimulated cardiomyocytes. We observed that cardiac-specific overexpression of miR-138 protected hearts from TAC-induced cardiac hypertrophy, and attenuated the subsequent heart failure. In addition, miR-138 overexpression protected cardiomyocytes from AngII-induced hypertrophy. Taken together, we identified a previously unknown link between miR-138 and cardiac hypertrophy.

Materials and methods

Recombinant lentivirus construction

MiR-138 sequence was inserted into Pglv3/h1/gfp+puro plasmid vector (Genepharma, Shang-

hai) and driven by H1 promoter (LV-miR-138). The pseudoviral particles were produced using lentivector packaging system (Genepharma, Shanghai). Lentivirus carried negative control sequence (LV-NC) sequence were also purchased from Shanghai GenePharma.

Animal model

All experiments and animal care procedures were approved by the Animal Care and Use Committee of Sichuan Provincial People' Hospital. Male C57BL/6 mice (SPF grade, 8 weeks, 22-25 g), obtained from Shanghai Laboratory Animal Center Co. Ltd. (Shanghai, China), were housed at 25°C with 12/12-h light/dark cycles and allowed free access to standard lab-chow and water. After 1 week of adaptation period, mice were anesthetized, and transverse aortic constriction (TAC) was performed to induce the cardiac hypertrophy of mice (n=30). Briefly, a 6-0 tie was employed to tie two circles around the abdominal aorta by a 27-gauge needle, after which the needle was removed and the chest were closed. Among them, 20 mice were intramyocardially injected with lentivirus containing either miR-138 sequence (LV-138, n=10) or negative control sequence (LV-NC, n=10) immediately after TAC. The mice in the sham group (n=10) only received thoracotomy and pericardiotomy. Four weeks later, the mice were anesthetized, and the cardiac functions were analyzed. Then, the mice were killed, and hearts were gathered for further evaluation.

Echocardiography

At four weeks after operation, mice were anaesthetized and body temperature was maintained by placing the mice on a heating pad (37°C). Echocardiography was conducted using a 30-MHz probe interfaced with a Vevo-770 high-frequency ultrasound system (VisualSonics, Toronto, Canada). Left ventricular anterior wall thickness at diastole (LVAWd), left ventricular posterior wall thickness at diastole (LVPWd), fractional shortening (FS) and ejection fraction (EF) were measured.

Histological analysis

Excised hearts were weighed, fixed by immersion in 4% polyformaldehyde, embedded in paraffin, sectioned into 4-μm thickness slides and stained with hematoxylin and eosin (H&E). Cardiomyocytes were chosen from each cross section, and cross sectional area (CSA) of cardiomyocytes was measured using Image Pro-Plus version 6.0 image analysis software (Media Cybernetics Inc., Rockville, MD, USA) in 10 randomly chosen points from each cross section.

Cardiomyocytes culture and cell models for hypertrophy

Neonatal mouse cardiomyocytes were isolated from ventricles of 1-day-old newborn C57BL6 mice. In brief, ventricles were washed, and cut into small pieces, and digested with trypsin and Type II collagenase at 37°C. Then the cells were subjected to centrifugation, followed by differential preplating to remove nonmyocytes and enrich cardiomyocytes. The purified cardiomyocytes were cultured in DMEM supplemented with vitamin B12, $NaHCO_3$, and 10% FBS for 48 hours. After infected with lentivirus for 72 hours, AngII (1 μmol/L and 10 μmol/L) or DMSO was added to the cells. After 12 h incubation, cardiomyocytes were collected and lysed for the further processing.

RNA extraction and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA samples from cardiac tissues and cultured cardiomyocytes were isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). To quantify miRNA expression, 1 μg of total RNA was reverse-transcribed into cDNA using the TaqMan MicroRNA Reverse Transcription kit (Takara, Japan) and a stem-loop RT primer. To quantify mRNA expression, 1 μg of total RNA was reverse-transcribed into cDNA using the PrimeScript RT reagent kit (Takara). Next, qRT-PCR was performed with the resulting RT product, SYBR Green Dye (Invitrogen) and specific primers on an Applied Biosystems 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). U6 or GAPDH was used as a normalization control. The primers used are listed in **Table 1**. The qRT-PCR results were analyzed using the $2^{-\Delta\Delta CT}$ method [12].

Protein extraction and western blot

Cardiomyocytes were collected and lysed using RIPA Lysis buffer (Beyotime, China). The protein concentration was measured using a Pierce BCA protein assay kit (Thermo Scientific,

Table 1. The sequences of primers used for qRT-PCR

Gene name	Primer sequences
miR-138-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGGCCT
U6-RT	AACGCTTCACGAATTTGCGT
miR-138 Forward primer	AGCTGGTGTTGTGAATC
miR-138 Reverse primer	GTGCAGGGTCCGAGGT
U6 Forward primer	CTCGCTTCGGCAGCACA
U6 Reverse primer	AACGCTTCACGAATTTGCGT
YAP1 Forward primer	TTTCGGCAGGCAATACGGA
YAP1 Reverse primer	CTGCTCCAGTGTAGGCAACT
GAPDH Forward primer	GTGAAGGTCGGAGTCAACG
GAPDH Reverse primer	TGAGGTCAATGAAGGGGTC

Rockford, IL, USA). Equivalent quantity of protein was loaded and separated by SDS-PAGE. Then all the separated protein was electrotransferred onto polyvinylidene fluoride (PVDF) membranes. The membrane was incubated with the antibodies of interest. Proteins were scanned and detected by enhanced chemiluminescence (Bio-Rad Laboratories, Hercules, CA, USA) using a ChemiDoc MP system (Bio-Rad Laboratories). Image J software was used to analyze densitometric results of band. Protein levels of GAPDH were used as loading controls.

Luciferase reporter gene assay

To examine whether miR-138 regulates the expression of YAP1, a dual luciferase GP-Check2 reporter plasmid (Shanghai GenePharma Co., Ltd.) was used to generate a reporter plasmid harboring the 3'-UTR of YAP1. The YAP1 3'-UTR containing the binding sequence with miR-138 was amplified from mice genomic DNA and cloned into the GP-Check2 plasmid, which was considered the wild type (WT) plasmid. In addition, the GP-Check2 plasmid with a mutated targeting fragment served as the mutant type (MUT) plasmid. Briefly, cardiomyocytes were co-transfected with the WT/MUT vector containing YAP1 3'-UTR and miR-138 mimics or control oligonucleotide (miR-NC). The cells were harvested 24 h after transfection, and the luciferase activity was measured using a dual luciferase reporter assay kit (Promega Corporation, Madison, WI, USA).

Statistical analysis

All experiment data were analyzed statistically by GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) and were expressed as mean ± standard deviation (SD). Comparisons between two groups were performed through an unpaired Student's *t*-test. The correlation between variables was assessed by Pearson's correlation analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

MiR-138 expression is decreased in cardiac hypertrophy

To identify the expression of miR-138 in cardiac hypertrophy, we established TAC mice model. One mouse in TAC group died during the experiment. There were no differences of body weight among groups (data not shown), but the heart-to-body weight ratios (HW/BW) and left ventricle-to-body weight ratios (LVW/BW) were remarkably elevated in TAC mice model (**Figure 1A**). Echocardiographic measurement showed that eight weeks after the operation, the TAC mice developed a significant cardiac hypertrophy, including the increased LVAWd (left ventricular anterior wall thickness at diastole) and LVPWd (left ventricular posterior wall thickness at diastole), and the decreased EF (ejection fraction) and FS (fractional shortening) (**Table 2**). Measurement of CSA of cardiomyocytes in H&E-stained LV sections indicated that TAC remarkably enlarged the cardiomyocytes size (**Figure 1B**). Moreover, western blotting analysis showed increased expression of ANP, a hypertrophic marker [13], in the TAC mice (**Figure 1C**). All of these data suggested that the TAC procedures effectively induced cardiac hypertrophy in these mice. qRT-PCR analysis showed that miR-138 expression was significantly reduced in the cardiomyocytes of TAC

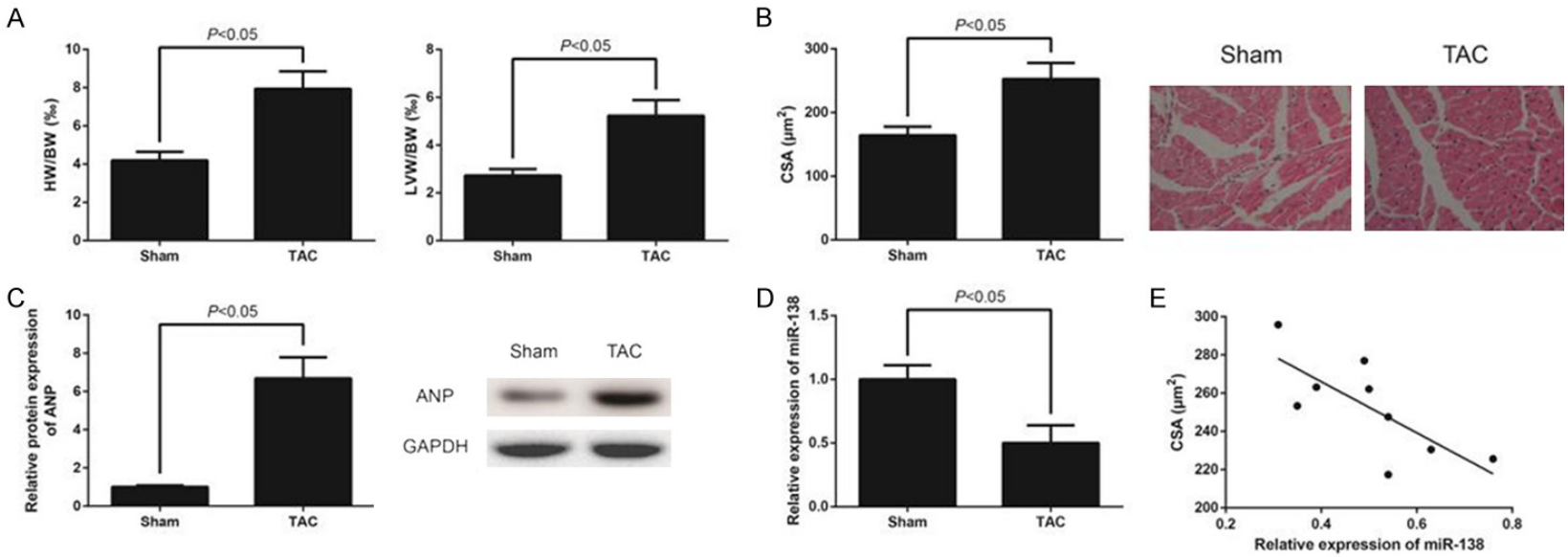

Figure 1. MiR-138 expression is decreased in cardiac hypertrophy. A: The heart-to-body weight ratios (HW/BW) and left ventricle-to-body weight ratios (LVW/BW) were measured. B: H&E stained LV sections of mice. Cross sectional area (CSA) of cardiomyocyte was measured. C: ANP protein expression was detected by western bolt analysis. D: miR-138 expression was investigated by qRT-PCR analysis. The data are expressed as mean ± SD, n=9-10/group. Student's *t*-test was used with **P*<0.05. E: The correlation of miR-138 expression and CSA of cardiomyocytes in TAC mice was detected by Pearson's correlation analysis (r=-0.743, *P*=0.022).

Table 2. Echocardiographic analysis of left ventricles

Group	LVAWd (mm)	LVPWd (mm)	EF (%)	FS (%)
Sham	0.63±0.04	0.66±0.04	61.3±1.9	30.5±2.2
TAC	0.88±0.05	0.85±0.08	44.8±3.8	23.5±2.7
LV-miR-NC	0.86±0.06	0.84±0.09	42.6±4.3	22.9±2.9
LV-miR-138	0.69±0.06	0.75±0.07	57.2±4.6	28.6±3.1

LVAWd, left ventricular anterior wall thickness at diastole; LVPWd, left ventricular posterior wall thickness at diastole; EF, ejection fraction; FS, fractional shortening.

mice (**Figure 1D**), and miR-138 expression showed a high inverse correlation with CSA of cardiomyocytes of TAC mice (r=-0.743, *P*=0.022; **Figure 1E**), indicating that miR-138 might be implicated in the development of cardiac hypertrophy and affect the heart functions *in vivo*.

Lentiviral vector-mediated miR-138 overexpression inhibits cardiac hypertrophy in vivo

To further confirm whether miR-138 is involved in cardiac hypertrophy, we generated a miR-138-overexpressing recombinant lentivirus (LV-miR-138). Compared with LV-miR-NC treated mice, LV-miR-138 treated mice had evidently higher expression levels of miR-138 in the heart but not in other organs, including kidney, liver and spleen (Data not shown). The HW/BW and LVW/BW ratios were obviously reduced in LV-miR-138 treated mice (**Figure 2A**). As shown in **Table 2**, cardiac hypertrophy was remarkably attenuated in LV-miR-138 treated mice, including the reduced LVAWd and LVPWd, and the elevated EF and FS. Cardiomyocytes size as determined in H&E-stained LV sections was significantly reduced in LV-miR-138 treated mice (**Figure 2B**). The expression of ANP was also evidently decreased in LV-miR-138 treated mice (**Figure 2C**). Taken together, these results indicated that overexpression of miR-138 significantly attenuated TAC-induced cardiac hypertrophy.

MiR-138 overexpression attenuates AngII-induced cardiomyocytes hypertrophy in vitro

We further evaluated the anti-hypertrophic role of miR-138 in cultured cardiomyocytes *in vitro*. 72 h after infection, the level of miR-138 was remarkably enhanced in LV-miR-138 treated cardiomyocytes (Data not shown). We found that miR-138 expression was obviously decreased in AngII-treated cardiomyocytes in a dose-dependent manner, compared with that in the controls (**Figure 3A**), whereas overexpression of miR-138 obviously inhibited 1 μmol/L AngII-induced hypertrophy in LV-miR-138 treated cardiomyocytes compared with LV-miR-NC treated cardiomyocytes (**Figure 3B**). Also, in the presence of 1 μmol/L AngII, ANP expression was markedly reduced in miR-138 overexpressing cardiomyocytes compared to LV-miR-NC treated cardiomyocytes (**Figure 3C**). These findings revealed that miR-138 overexpression restored cardiomyocyte phenotype under hypertrophic stimuli.

MiR-138 targets YAP1 expression in cardiomyocytes

Then, we investigated the molecular mechanism which miR-138 inhibited the cardiac hypertrophy. By scanning the target gene of miR-138 through a sequence alignment analysis, we found that 3'-UTR of YAP1 mRNA contains a complimentary binding site for the seed sequence of miRNA-138 (**Figure 4A**). To confirm the correlation between miR-138 and YAP1, we detected the expression of YAP1 in hypertrophic cardiomyocytes. As expected, YAP1 mRNA and protein expression were markedly enhanced in 1 μmol/L AngII-treated cardiomyocytes, and overexpression of miR-138 noticeably down-regulated YAP1 mRNA and protein expression *in vitro* (**Figure 4B**, **4C**). Furthermore, to clarify whether miR-138 suppress the expression of YAP1 directly, we decided to confirm this assumption using luciferase reporter assay. As shown in **Figure 4D**, the activity of the luciferase reporter gene linked to the 3'UTR of WT-YAP1 was reduced with the presence of miR-138, while that of MUT-YAP1 did not obviously change.

Discussion

Despite the great development of novel therapeutic approaches, cardiovascular disease remains the leading cause of death and morbidity around the world. Cardiac hypertrophy is considered as a risk factor of heart failure and other cardiovascular diseases [14]. A number of recent studies have indicated that several miRNAs, including miR-150 [15], miR-26a [16], miR-155 [17] and miR-101b [18], actively par-

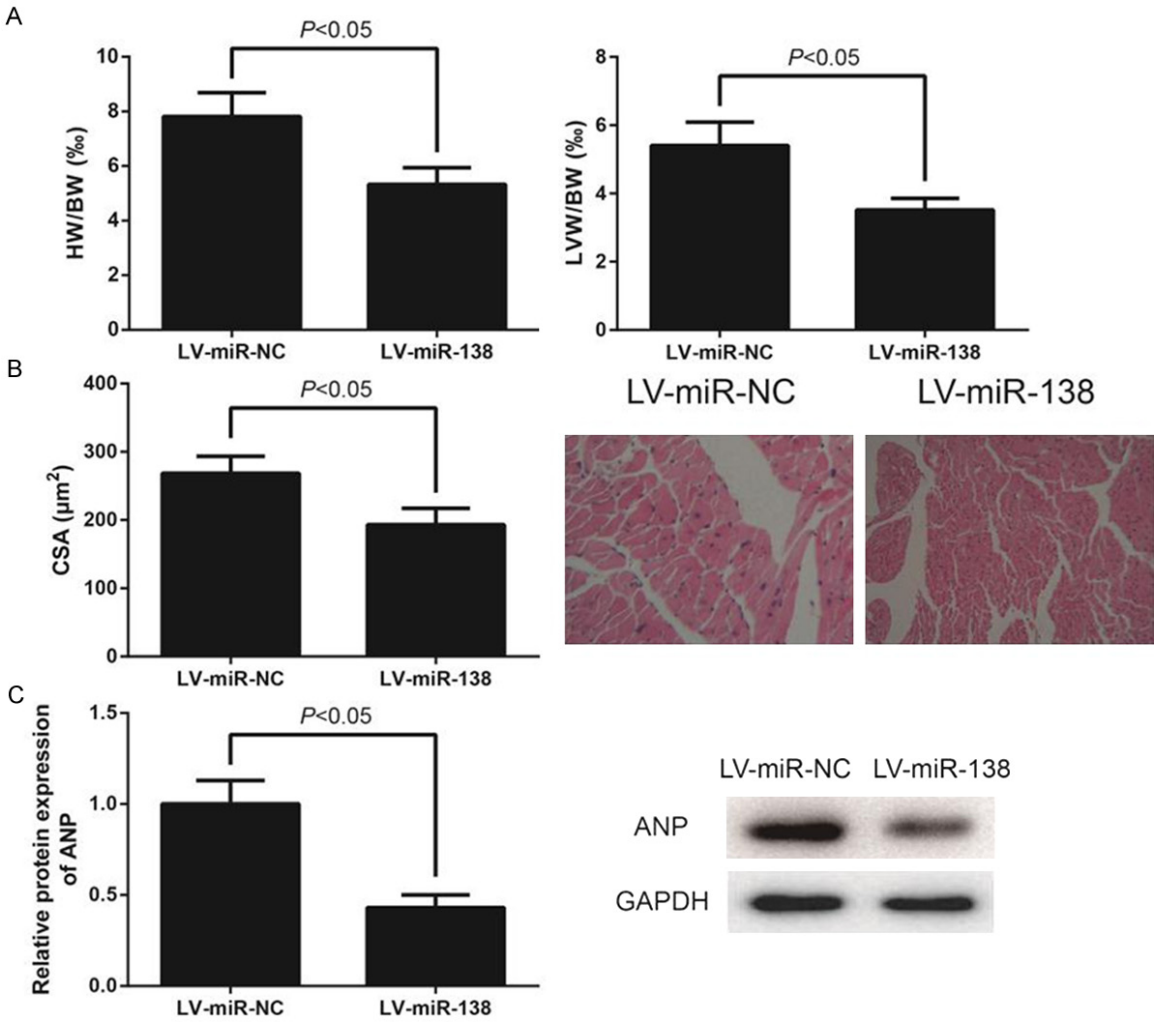

Figure 2. Lentiviral vector-mediated miR-138 overexpression inhibits cardiac hypertrophy in vivo. A: The heart-to-body weight ratios (HW/BW) and left ventricle-to-body weight ratios (LVW/BW) were measured. B: H&E stained LV sections of mice. Cross sectional area (CSA) of cardiomyocyte was measured. C: ANP protein expression was investigated by western bolt analysis. The data are expressed as mean ± SD, n=10/group. Student's *t*-test was used with **P*<0.05.

ticipate in cardiomyocyte hypertrophy and consequent heart failure. In this study, we have, for the first time, demonstrated that miR-138 was down-regulated in hypertrophic cardiomyocytes *in vivo* and *in vitro*.

The biological function of miR-138 has been so far mainly studied in relation to cancer [19-21], but it was recently demonstrated that miR-138 is also involved in cardiovascular diseases [22, 23]. As estimated by the heart weight/body weight ratio, cardiomyocyte area and echocardiographic measurements, cardiac hypertrophy by TAC for four weeks was successfully established in mice in this study. Moreover, enforced expression of miR-138 through recombinant lentivirus inhibited the hypertrophy of TAC-operated mouse hearts.

Cardiomyocytes are extremely sensitive to hypertrophic stimuli. AngII plays a critical role in cardiac growth, resulting in increases in cardiac fibrosis, remodeling and dysfunction [24]. In cell-based studies, we provided an evidence for miR-138 to have protective effects against AngII-induced cardiomyocyte hypertrophy, similar to animal data. Ovexpression of miR-138 remarkably attenuated all AngII-induced cardiac hypertrophic responses. Taken together with our *in vivo* and *in vitro* findings, we found that miR-138 displayed a protective role against AngII-induced cardiac hypertrophy.

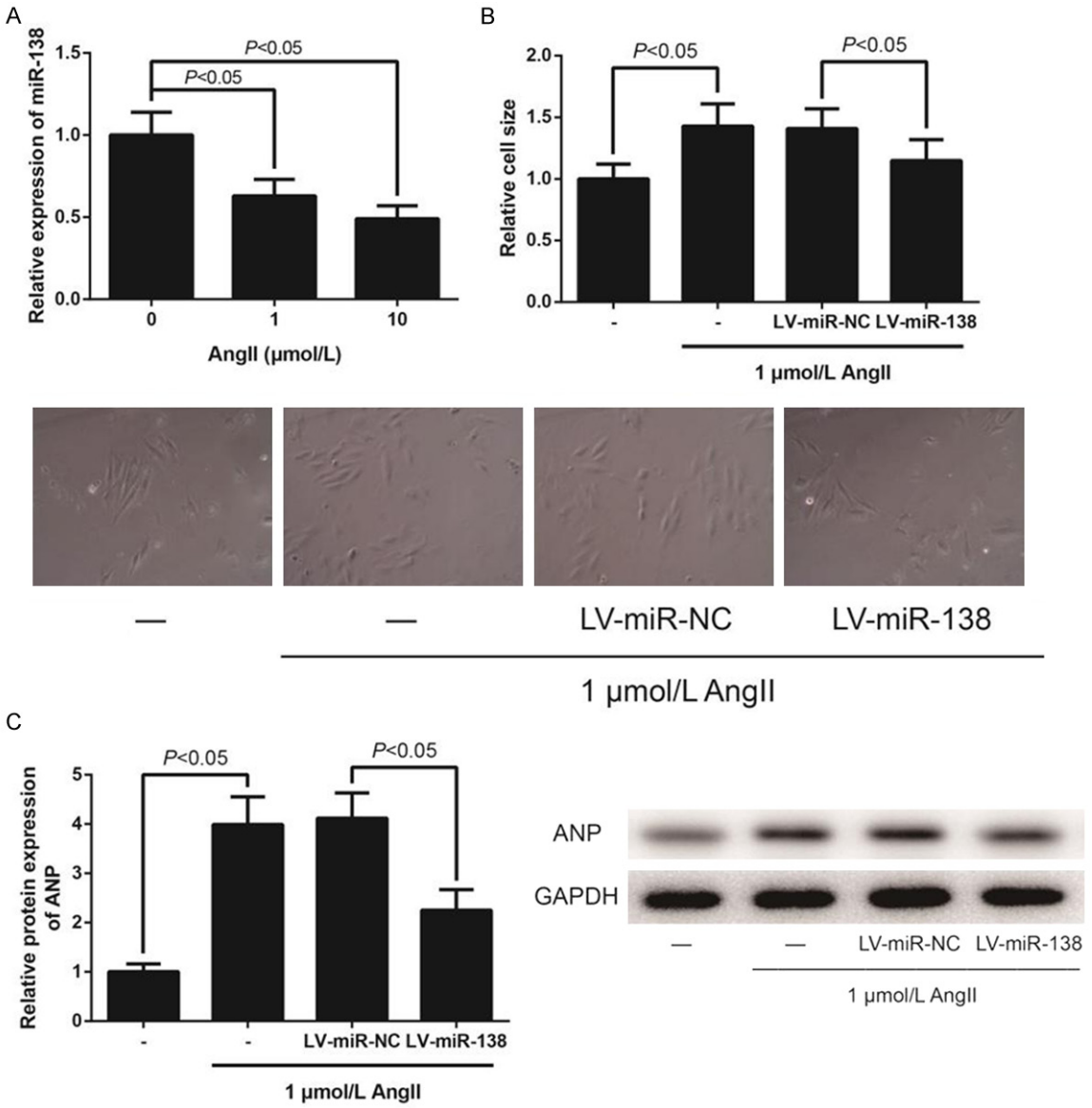

Figure 3. miR-138 overexpression attenuates AngII-induced cardiac hypertrophy. A: miR-138 expression was detected by qRT-PCR analysis in the AngII-treated cardiomyocytes. B: Relative cell size of cardiomyocytes was detected. C: ANP protein expression was investigated by western bolt analysis. The data are expressed as mean ± SD. Student's *t*-test was used with **P*<0.05.

In a cell, miRNAs exert their biological functions via binding to the mRNA 3'-UTR of the target gene to block its expression [25] Several target genes of miR-138 have been identified and verified in previous studies. In the present study, to determine the mechanism by which miR-138 exerts its function in cardiac hypertrophy, we performed bioinformatic analysis and luciferase reporter assays and identified YAP1 as a direct target gene of miR-138. Yes-associated protein 1 (YAP1) is a downstream nuclear effector of the Hippo signaling, which is a highly conserved regulator of organ size, tissue repair and cell fate in vertebrates [26]. Moreover, as the core component of Hippo signaling, YAP1 was recently found to promote the development and progression of various human diseases, including cardiac hypertrophy. Heterozygous YAP1 deletion has been reported to attenuate compensatory cardiomyocyte hypertrophy in mice with chronic myocardial infarction [27]. A recent study demonstrated that YAP upregulates miR-206, which, in turn, plays a critical role in mediating the effect of YAP upon cardiomyocyte hypertrophy [28]. In the present study, we verified that miR-138 might exert its

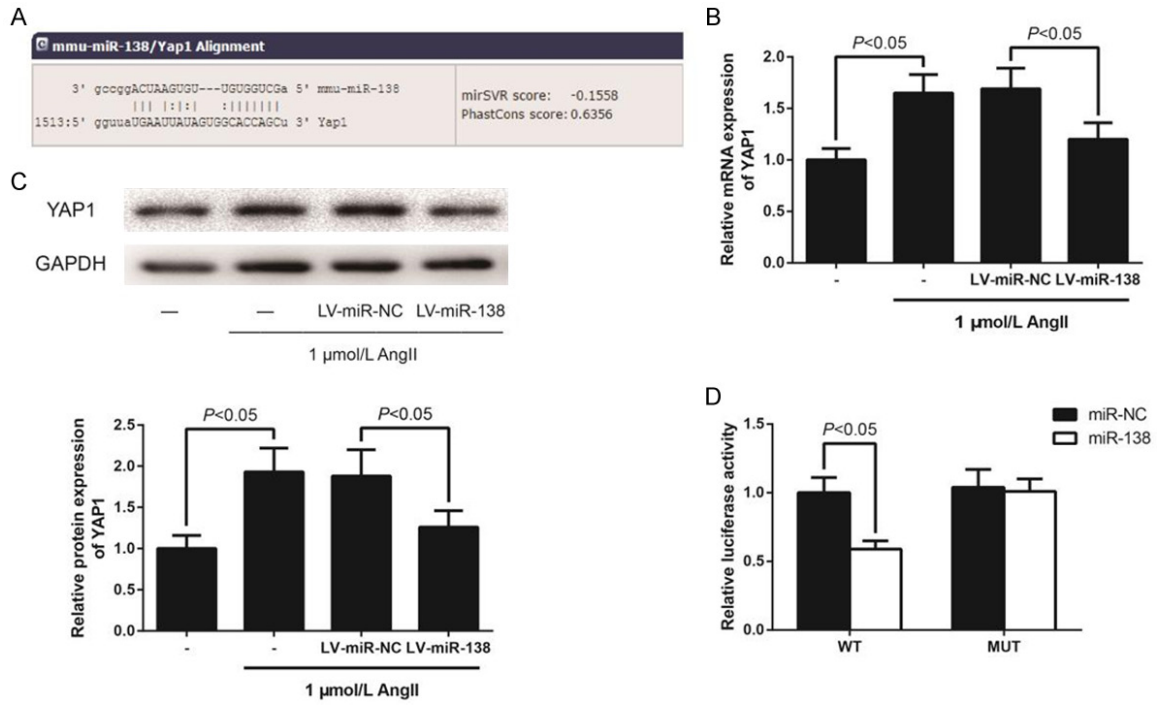

Figure 4. MiR-138 targets YAP1 expression. A: Bioinformatic analysis suggested that mmu-miR-138 could target the 3'-UTR of YAP1 mRNA. B: YAP1 mRNA expression was detected by qRT-PCR analysis. C: YAP1 protein expression was detected by western blot analysis. D: Luciferase reporter assay showed that delivery of miR-138 could inhibit the luciferase activity of the reporter containing the 3'-UTR of YAP1 mRNA. The data are expressed as mean ± SD. Student's *t*-test was used with **P*<0.05.

anti-hypertrophic function through targeting 3'UTR of YAP1.

Taken together, this study provides *in vivo* and *in vitro* evidences that miR-138 may attenuate early compensatory cardiac hypertrophy via repression of YAP1 expression. These results suggested that cardiac hypertrophy could be overcome by targeting miR-138 or the Hippo signaling in a disease-specific manner.

Disclosure of conflict of interest

None.

Address correspondence to: Rong Yu, Department of Geriatrics, Sichuan Provincial Academy of Medical Sciences, Sichuan Provincial People's Hospital, Chengdu 610072, China. E-mail: yurong_happy@126.com

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