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Original Article

Overexpression of microRNA-26b attenuates angiotensin II-induced cardiac hypertrophy through inhibition of autophagic responses

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Abstract: Although microRNA-26b (miR-26b) has been previously identified as a regulator of cardiac hypertrophy, the specific mechanism remains elusive. Cardiac hypertrophy was induced by thoracic aortic constriction (TAC) in mice. Four weeks after surgery, the cardiac hypertrophy mice model was successfully established. In addition, a cell model of hypertrophy was also established based on angiotensin II (AngII)-induced neonatal mouse ventricular cardiomyocytes. We observed that miR-26b was markedly down-regulated in hypertrophic myocardium tissues and hypertrophic cultured cardiomyocytes, whereas administration of miR-26b mimics suppressed hypertrophic phenotype of cultured cardiomyocytes. Additionally, it was shown that overexpression of miR-26b attenuated autophagic responses in hypertrophic cardiomyocytes, which was confirmed by reducing Beclin-1 expression and the light chain 3(LC3)-II/LC3-I ratio. Taken together, our study provides substantial evidence that upregulation of miR-26b expression might be a potential effective therapeutic strategy to attenuate cardiac hypertrophy.

Keywords: Cardiac hypertrophy, microRNA-26b, autophagy, thoracic aortic constriction, angiotensin II

Introduction

Cardiac hypertrophy, broadly defined as an increase in heart mass, is an adaptive response to a wide array of intrinsic and extrinsic stimuli, which is featured by enlarged cardiomyocyte size, highly organized sarcomeres, increased protein synthesis and re-activation of fetal genes [1]. Although cardiac hypertrophy is initially compensatory to diverse stimuli, prolonged cardiac hypertrophy eventually leads to arrhythmias, heart failure and sudden death [2, 3]. Currently, it remains one of the prominent causes of morbidity and mortality around the world [4]. Therefore, it is of critical importance to develop more efficient novel therapeutic strategies for patients with cardiac hypertrophy.

MicroRNAs (miRNAs) are endogenous small noncoding RNAs with 19-24 nucleotides in length, which post-transcriptionally modulate gene expression by base pairing to 3' untranslated region of targeted mRNAs [5]. Indeed, a large fraction (approximately 60%) of the genome is modulated by miRNAs [6]. It is widely considered that miRNAs serve critical roles in diverse cellular processes, including cell proliferation, differentiation, apoptosis and development [7], and their dysregulated expression is associated with multiple human cardiovascular diseases, including cardiac hypertrophy [8, 9]. The regulatory roles of miRNAs in cardiac hypertrophy have triggered tremendous interest in further exploring their potential as novel therapeutic targets.

The sequences of miR-26a and miR-26b are similar, and their binding sites are identically conserved. Although the down-regulation of miR-26a have been well documented in both hypertrophy rat models and cultured cardiomyocytes [10]; however, to date, there is no report clearly disclosing the regulatory role of miR-26b in cardiac hypertrophy and its biological significance. Herein, we sought to test the hypothesis that miR-26b might be implicated in the etiology of cardiac hypertrophy, and to determine

the underlying molecular behavior of miR-26b in cardiac hypertrophy.

Materials and methods

Animal experiments

In vivo pressure overload-induced cardiac hypertrophy was achieved by thoracic aortic constriction (TAC) as previously described [11]. Sixteen eight-week-old C57BL/6J male mice, obtained from Shanghai Laboratory Animal Center Co. Ltd., were subjected to TAC operation or sham operation (n=8 per group). All animals were anesthetized and placed on a heating pad during operation. For TAC, after thoracotomy, the transverse aorta between the right innominate and left common carotid arteries was dissected and constricted with a 6-0 nylon suture by ligating the aorta against a 27-gauge needle, which was later removed. Mice subjected to a sham operation were considered as controls. All animal experimental protocols were carried out in accordance with the relevant guidelines and regulations and with the approval of the Animal Care and Use Committee of Sichuan Provincial People's Hospital.

Echocardiography and histological analysis

Four weeks after TAC, transthoracic echocardiographic analysis was performed to evaluate the heart function of mice using a 30-MHz probe interfaced with a Vevo-770 high-frequency ultrasound system (VisualSonics® Vevo770®, VisualSonics Inc., Toronto, Canada). All assessments were averaged for at least three separate cardiac cycles. Then these mice were euthanized, and their hearts were surgically removed to determine the heart weight (HW) and left ventricular weight (LVW). Then the heart to body weight ratio (HW/BW) and left ventricle weight to body weight ratio (LVW/BW) were obtained. For histological analysis, the ventricles were excised, fixed with 4% formaldehyde, dehydrated and then embedded in paraffin wax, sectioned at 4-6 μm thickness and stained with hematoxylin and eosin (H&E). The cross-sectional area (CSA) of cardiomyocytes was determined using Image Pro-Plus version 6.0 image analysis software (Media Cybernetics Inc., Rockville, MD, USA).

Cardiomyocytes culture and transfection

Cardiomyocytes were isolated from the hearts of 1-day-old newborn C57BL6 mice and were digested by trypsin and collagenase type II. Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) with 10% FBS (Gibco). Furthermore, cardiomyocytes were treated with 1 μmol/L AngII (Sigma, Saint Louis, MO, USA) for inducing hypertrophy. After 12 h, miR-26b mimics (miR-26b) and miR-26b mimics control (miR-NC) were respectively transfected into cardiomyocytes using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 48-h post-transfection, quantitative RT-PCR was performed to evaluate the transfection efficacy.

Quantitative RT-PCR

Total RNA was extracted and isolated from cardiac tissues and cultured cardiomyocytes using TRIzol Reagent (Invitrogen). Purity and integrity of the RNA samples were analyzed by an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The RNA was than reverse-transcribed to complementary DNA (cDNA) using the TaqMan MicroRNA Reverse Transcription kit (Takara, Japan). Quantitative RT-PCR was performed with MiRNA Q-PCR Detection Kit (GeneCopoeia, Rockville, MD, USA) on an Applied Biosystems 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The levels of the universal small nuclear RNA U6 were used to normalize the expression levels. The miR-26b stem-loop RT primer used was: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACCTAT-3'; miR-26b 5'-TTCAAGTAATTCAGG-3' (sense), and 5'-GTGCAGGGTCCGAGGT-3' (antisense); while that of U6 stem-loop RT primer was: 5'-AACGCTTCACGAATTTGCGT-3'; U6 5'-CTCGCTTCGGCAGCACA-3' (sense), and U6 5'-AACGCTTCACGAATTTGCGT-3' (antisense). Data are presented as a relative amount using the calculation of $2^{-\Delta\Delta Ct}$ [12]. Experiments were performed in triplicate.

Western blotting

Protein was extracted from cardiac tissues and cultured cardiomyocytes using RIPA buffer (Beyotime, Shanghai, China) supplemented with protease inhibitors. Equal amounts of protein samples (40 μg) were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore Corporation, Billerica, MA, USA). The membrane was subsequently blocked with 10% skim milk in TBST solution for

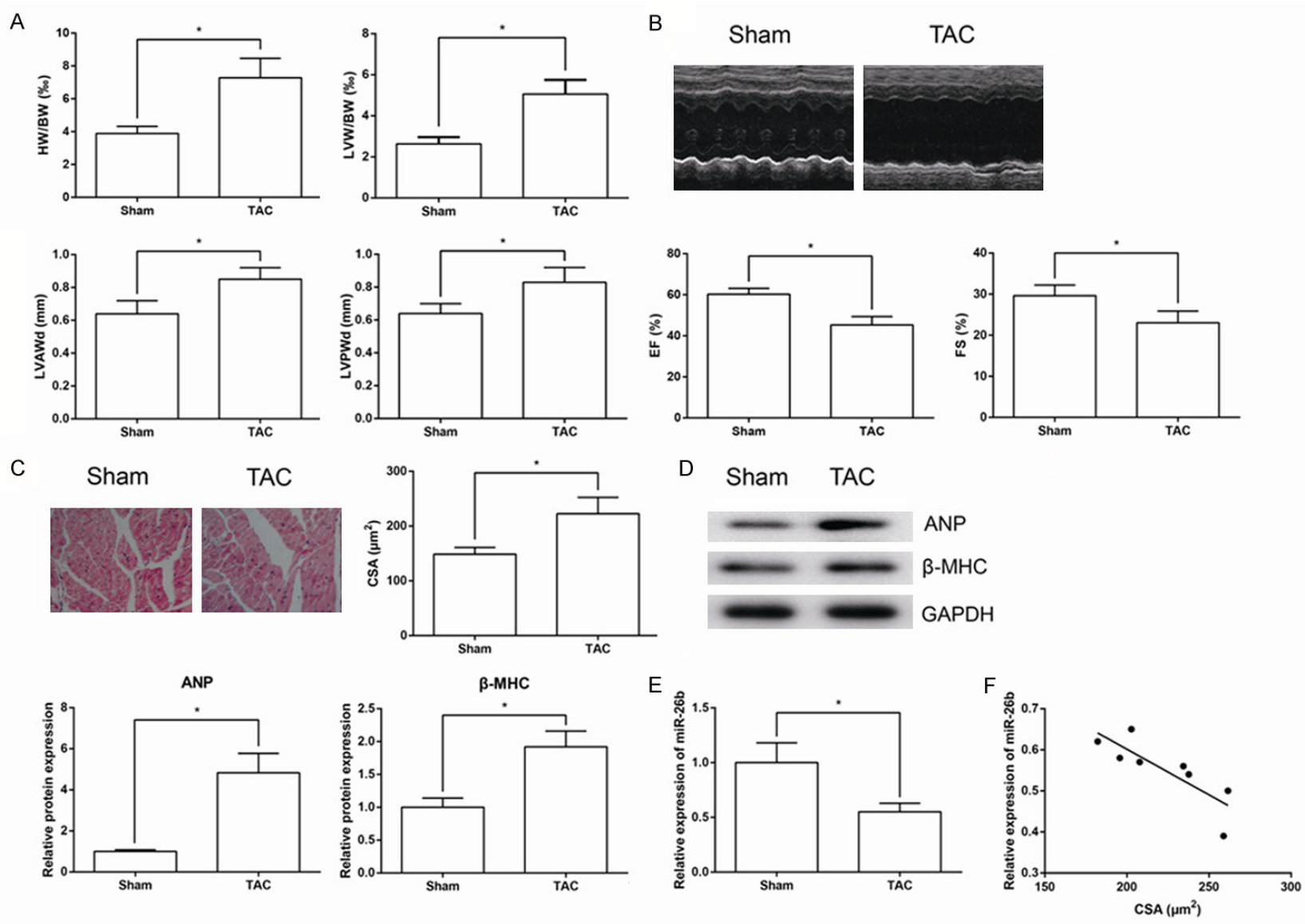
A
HW/BW (‰)
LVW/BW (‰)
LVAWd (mm)
LVPWd (mm)
EF (%)
FS (%)
Sham
TAC
B
Sham
TAC
C
Sham
TAC
CSA (μm²)
D
Sham
TAC
ANP
β-MHC
GAPDH
ANP
β-MHC
Relative protein expression
E
Relative expression of miR-26b
F
Relative expression of miR-26b
CSA (μm²)

Figure 1. MiR-26b is down-regulated in the heart tissues of TAC mice. A: The ratio of heart weight to body weight (HW/BW) and the ratio of left ventricle weight to body weight (LVW/BW) were measured. B: The echocardiographic parameters of left ventricles were measured. C: The cross-sectional area (CSA) of cardiomyocytes in the HE-stained LV sections was measured. D: Protein levels of ANP and β-MHC in the myocardial tissues were detected by Western blot analysis. E: MiR-26b levels in the myocardial tissues were detected by quantitative RT-PCR. F: The correlation of miR-26b levels and CSA of cardiomyocytes was analyzed. Data are presented as mean ± SD. *P<0.05 was considered to be statistically significant.

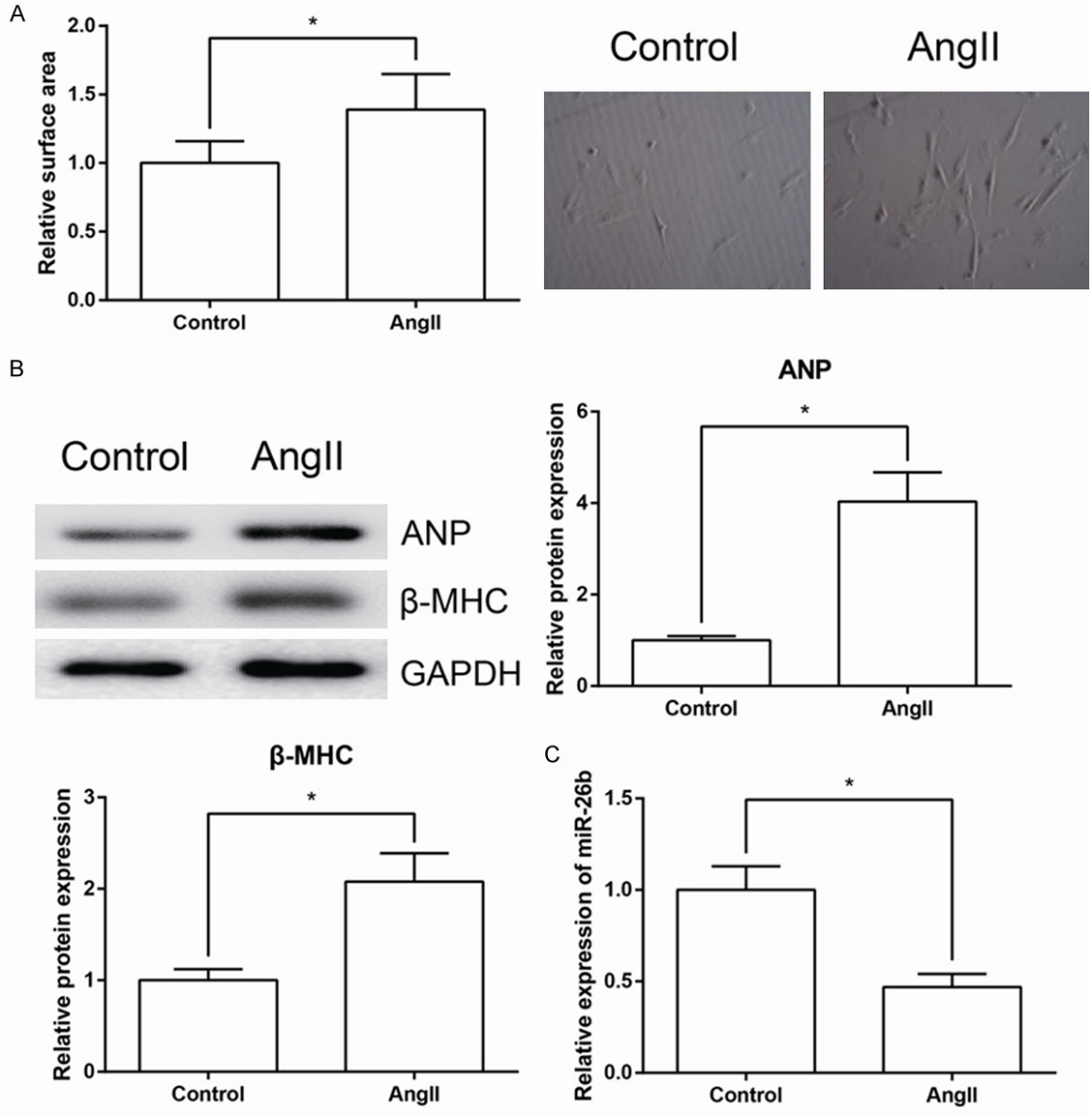

Figure 2. MiR-26b is down-regulated in the AngII-induced hypertrophic cardiomyocytes. A: The surface area of AngII-treated cultured mouse cardiomyocytes was determined. B: Protein levels of ANP and β-MHC in cultured mouse cardiomyocytes were detected by Western blot analysis. C: MiR-26b levels in cultured mouse cardiomyocytes were detected by quantitative RT-PCR. Data are presented as mean ± SD. *P<0.05 was considered to be statistically significant.

2 h and incubated with primary antibodies at 4°C overnight. After washing, the membrane was incubated with corresponding HRP-conjugated secondary antibodies at room temperature for 1 h. The proteins of interest were visualized using ECL (Pierce, Rockford, IL, USA) and

quantified using the ImageJ software (NIH, Bethesda, USA). Protein expression was normalized to levels of GAPDH expression.

Statistical analysis

The statistical analysis were performed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, Calif, USA), and the results are expressed as the mean ± standard deviation (SD). Differences between experimental groups were analyzed with the unpaired Student's *t*-test. A value of $P<0.05$ was considered statistically significant.

Results

MiR-26b is down-regulated in the heart tissues of TAC mice and AngII-induced hypertrophic cardiomyocytes

We first detected the status of miR-26b expression in our TAC mouse models. Four weeks after operation, all mice were alive, and we found the ratio of heart weight to body weight (HW/BW) and the ratio of left ventricle weight to body weight (LVW/BW) were noticeably increased in the TAC group (**Figure 1A**). The echocardiography assessment indicated that, compared with the sham group, the LVAWd and LVPWd were increased in the TAC group, whereas the EF and FS were remarkably decreased (**Figure 1B**). Besides, analysis of HE-stained LV sections demonstrated that the cross-sectional area (CSA) of cardiomyocytes following TAC operation was evidently augmented compared with the sham group (**Figure 1C**). The protein levels of ANP and β-MHC, the gene markers of cardiac hypertrophy, were significantly elevated in the rat hearts subjected to TAC operation (**Figure 1D**), indicating that the cardiac hypertrophy mice model induced by pressure-overload was successfully established. Furthermore, we observed that miR-26b expression was remarkably decreased in the heart tissues of TAC group compared with that in the sham group (**Figure 1E**), and miR-26b expression was negatively correlated with the CSA of cardiomyocytes in the TAC group (**Figure 1F**).

In vitro cultured cardiomyocytes were treated with AngII for 12 h to induce hypertrophic phenotype. The surface area of cardiomyocytes treated with AngII was significantly increased than that of the controls (**Figure 2A**). Moreover, as shown in **Figure 2B**, the protein levels of ANP and β-MHC were moderately increased in the AngII-treated cardiomyocytes, indicating that the hypertrophic cardiomyocytes were successfully induced in the present study. Furthermore, the levels of miR-26b were reduced in the hypertrophic cardiomyocytes induced by 1 μmol/L AngII (**Figure 2C**).

Overexpression of miR-26b attenuates cardiomyocyte hypertrophy in vitro

To examine whether miR-26b can regulate cardiomyocyte hypertrophy, after AngII treatment, cardiomyocytes were transfected with miR-26b mimics for 48 h to overexpress miR-26b. Quantitative RT-PCR was performed to determine the expression of miR-26b. As shown in **Figure 3A**, transfection with miR-26b mimics-up-regulated the levels of miR-26b in the AngII-treated cardiomyocytes. Importantly, overexpression of miR-26b further reduced the protein levels of ANP and β-MHC in the AngII-treated cardiomyocytes (**Figure 3B**), and reduced the surface area of the when compared to that of the cardiomyocytes with AngII treatment only (**Figure 3C**).

Overexpression of miR-26b inhibits autophagy in AngII-induced hypertrophic cardiomyocytes

Basal autophagy is essential to maintaining cellular homeostasis in the heart [13]. To investigate the protective mechanism of miR-26b in cardiac hypertrophy, the expression of autophagy-related proteins, including LC3-II and Beclin-1, was examined. Western blot analysis suggested that TAC operation caused an elevation in the LC3-II/LC3-I ratio and Beclin-1 expression in the mouse heart tissues (**Figure 4A**). The autophagic flux was also detected in AngII-induced hypertrophic cardiomyocytes. As expected, we found a significantly increased cardiomyocyte autophagy following AngII treatment, as indicated by the increased LC3-II/LC3-I ratio and up-regulated Beclin-1, and these effects were significantly inhibited by overexpression of miR-26b (**Figure 4B**).

Discussion

The critical role of miRNAs in physiological and pathological processes has promoted intensive research into miRNAs as miRNA-based

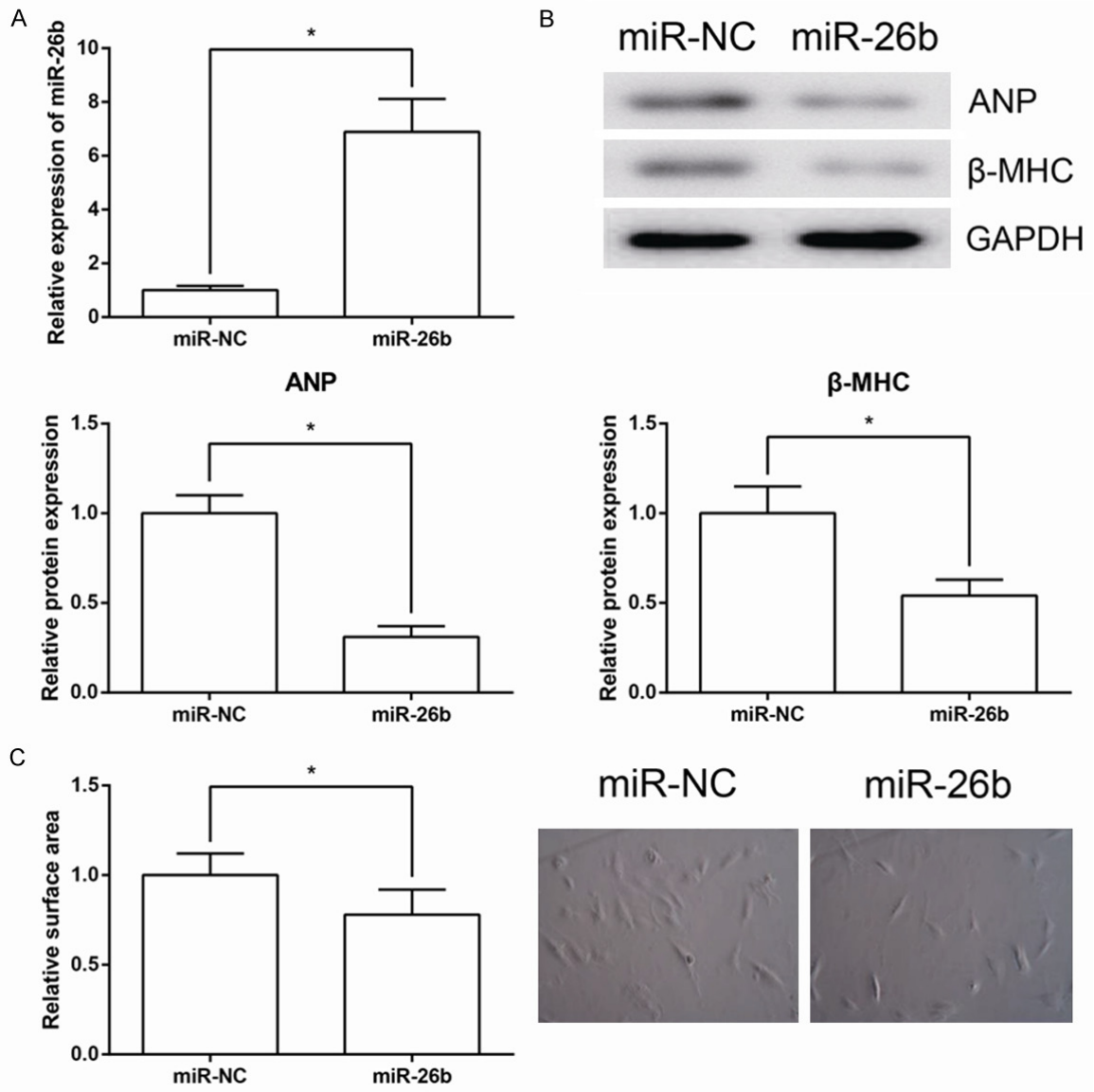

Figure 3. Overexpression of miR-26b attenuates cardiomyocyte hypertrophy in vitro. A: MiR-26b levels in cultured mouse cardiomyocytes after transfection were detected by quantitative RT-PCR. B: Protein levels of ANP and β-MHC in cultured mouse cardiomyocytes after transfection were detected by Western blotting analysis. C: The surface area of cultured mouse cardiomyocytes was determined after transfection with miR-26b mimics. Data are presented as mean ± SD. *P<0.05 was considered to be statistically significant.

therapeutic strategies for the treatment of cardiovascular diseases. In cardiac hypertrophy, several aberrantly expressed miRNAs have been identified. For example, miR-218 expression is reduced in response to myocardial hypertrophy induced by TAC and in cardiomyocytes treated with isoprenaline [14], and miR-297 promotes cardiomyocyte hypertrophy by inhibiting the expression of sigma-1 receptor [15]. Nevertheless, more miRNAs in the progression of cardiac hypertrophy still require further exploration. Here we demonstrated that miR-26b has a key role in cardiac hypertrophy through regulating autophagic responses.

Although miR-26b in the heart has been found to inhibit the development of hypertrophy in the previous studies [16, 17], the expression pattern and functions of miR-26b in cardiac hypertrophy remain to be further elucidated. In this study, we established a mouse model of cardiac hypertrophy using TAC method and found that miR-26b is down-regulated in the myocardial tissues of TAC mice. This antihypertrophic

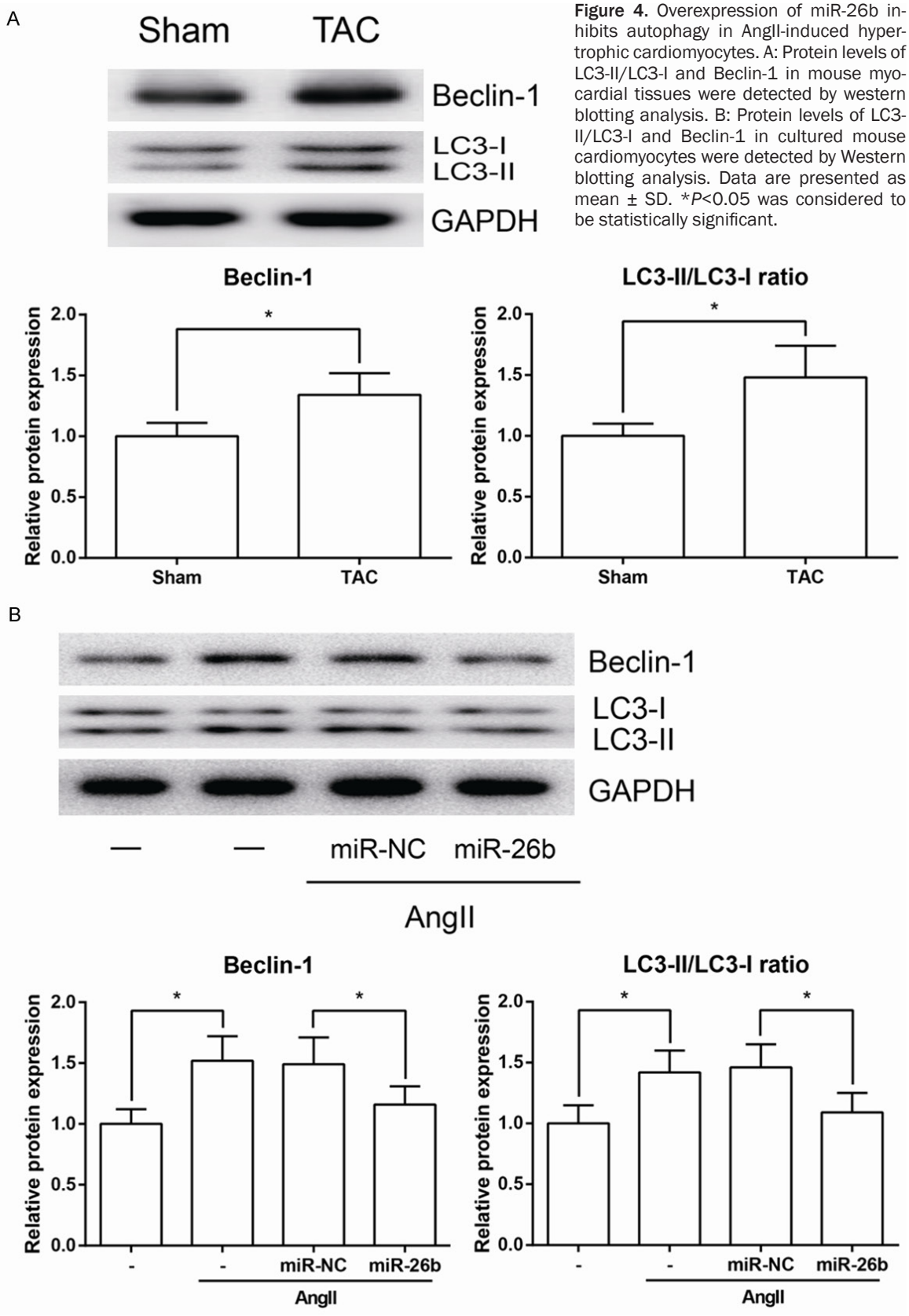

Figure 4. Overexpression of miR-26b inhibits autophagy in AngII-induced hypertrophic cardiomyocytes. A: Protein levels of LC3-II/LC3-I and Beclin-1 in mouse myocardial tissues were detected by western blotting analysis. B: Protein levels of LC3-II/LC3-I and Beclin-1 in cultured mouse cardiomyocytes were detected by Western blotting analysis. Data are presented as mean ± SD. **P*<0.05 was considered to be statistically significant.

effect of miR-26b was further investigated in vitro. As a critical growth factor, AngII is certain to induce cardiac hypertrophy [18], and we found that miR-26b is also down-regulated in AngII-induced hypertrophic cardiomyocytes. As expected, miR-26b overexpression through transfection of miR-26b mimics could reverse the enlarged surface area of cultured cardiomyocytes induced by AngII.

How does miR-26b lead to antihypertrophy? Recent evidence suggests that the cellular autophagic process is closely related to a variety of cardiovascular diseases [19], including cardiac hypertrophy. Autophagy is an evolutionary conserved and dynamic cellular process in which cytoplasmic materials are sequestered into double-membrane vesicles and delivered to the lysosomes where they are degraded and recycled [20]. The critical role of autophagy was also frequently observed during the process of cardiac hypertrophy [21-23]. We found that autophagy was remarkably increased in TAC-treated mouse hearts and AngII-induced hypertrophic cardiomyocytes, and these data were comparable with those studies done by Huang and their colleagues [24]. Besides, overexpression of miR-26b obviously inhibits autophagy in the hypertrophic cardiomyocytes. A recent study also found that miR-26b inhibited autophagy in prostate cells by down-regulation of ULK2 expression [25].

In summary, through establishing and studying animal models and cell lines, we have revealed that miR-26b was significantly down-regulated in hypertrophic myocardium tissues and hypertrophic cultured cardiomyocytes. Mechanistic analysis showed that overexpression of miR-26b attenuates cardiac hypertrophy through inhibition of autophagic responses. These findings provide a better understanding of the molecular mechanisms underlying cardiac hypertrophy and identify novel potential therapeutic targets for cardiac hypertrophy in the near future.

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Disclosure of conflict of interest

None.

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