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MicroRNA-92b-3p suppresses angiotensin II-induced cardiomyocyte hypertrophy via targeting HAND2

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ABSTRACT

Aims: The pathological cardiac hypertrophy will develop into heart failure, which has no effective treatment currently. Previous studies have proved that microRNAs (miRNAs) participate in the development of cardiac hypertrophy and regulate the pathological progress. In this study, we want to investigate the role of microRNA-92b-3p (miR-92b-3p) in cardiomyocyte hypertrophy and the mechanisms involved.

Materials and methods: Neonatal mouse ventricular cells (NMVCs) were isolated from the hearts of 1–3-d-old newborn C57BL6 mice. The isolated NMVCs were induced hypertrophic phenotype by Angiotensin-II (Ang-II) and the cell size was examined by FITC-phalloidin staining assay. The expression of miR-92b-3p was determined by quantitative real-time PCR (qRT-qPCR). MRNA and protein level of β-MHC, ACTA1 and HAND2 in NMVCs transfected with miR-92b-3p mimic and inhibition were assessed by RT-qPCR assay and western blot assay, respectively. Dual luciferase assay was used to verify the interaction between miR-92b-3p and the 3′-untranslated region (UTR) of HAND2 gene.

Key findings: MiR-92b-3p and HAND2 were significantly increased in Ang-II-induced NMVCs. Overexpression of miR-92b-3p can ameliorate Ang-II-induced cardiomyocyte hypertrophy. MiR-92b-3p negatively regulated HAND2 expression at the transcriptional level. Both miR-92b-3p mimic and HAND2 siRNA could efficiently inhibit Ang-II-induced hypertrophy in mouse cardiomyocytes.

Significance: MiR-92b-3p inhibits Ang-II-induced cardiomyocyte hypertrophy via targeting HAND2.

1. Introduction

Distinct forms of injury or stress, such as hypertension, valve disease, and myocardial infarction will give rise to the pathological cardiac hypertrophy, and the prolonged pathological cardiac hypertrophy will develop into heart failure, which has no cure currently [1,2]. It is well known that different signal pathways are involved in the pathological hypertrophy by different stimuli [3]. MicroRNAs (miRNAs) are endogenous 20–23-nucleotide noncoding RNAs that play important regulatory roles in multicellular organisms and likely influence the transcription of many protein-coding genes [4]. Accumulating studies reveal that miRNAs aberrantly expressed and play important roles in the hypertrophic myocardium during the development of cardiac hypertrophy [5]. Recent evidence indicates that microRNA-16 (miR-16), -30a, and -451, for instance, could attenuated the overload-induced upregulation of hypertrophy-related genes and decreased the cardiomyocyte surface area [6–8]. Conversely, overexpression of miR-142 and miR-328 were sufficient to induce the increase of the surface area of cardiomyocytes and enhanced the up-regulation of hypertrophy-related genes [9,10]. Previous studies had revealed that miR-92b-3p regulated myocyte-specific enhancer factor 2C (MEF2C) and MEF2D levels for homeostasis in Drosophila muscle development and cardiac hypertrophy, respectively [11,12]. However, it remains elusive that the role and the mechanism of miR-92b-3p in cardiac hypertrophy.

Heart and neural crest derivatives-expressed 2 (HAND2) and HAND1, are typical basic Helix-Loop-Helix (bHLH) transcription

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Table 1
Primers used in RT-qPCR assay.

Gene	Sequence (5′-3′)
ACTA1	Forward: 5′-ACT GGG ACG ACA TGG AGAAG-3′ Reverse: 5′-GGA AGC ATA GAG GGA CAGCA-3′
β-MHC	Forward: 5′-GAC CAG ATG AAT GAG CACCG-3′ Reverse: 5′-TCC TCC AGT TGC CTC TTGAG-3′
HAND2	Forward: 5′-GAC TCA GAG CAT CAA CAGCG-3′ Reverse: 5′-CTC CTC TTT CAC GTC GGTCT-3′
GAPDH	Forward: 5′-CAA GAA GGT GGT GAA GCAGG-3′ Reverse: 5′-CCA CCC TGT TGC TGT AGCC-3′
miR-92b-3p	RT, GTCGTATCCAGTGCGTGTCGTGGAGT CGGCAATTGCACTGGATACGACGGAGGCCG Forward: 5′-GTCCGCTATTGCACTCGTCCCGGCCTCC-3′ Reverse: 5′-GTGCGTGTCGTGGAGTC-3′
U6	RT, GTCGTATCCAGTGCGTGTCGTGGAGT CGGCAATTGCACTGGATACGAC Forward: 5′-GTCCGCGTGCTCGCTTCGGCAGC-3′ Reverse: 5′-GTGCGTGTCGTGGAGTC-3′

factors, both of which belong to the Twist family [13]. HAND2 is one of the four cardiac transcription factors, GATA4, HAND2, MEF2C and T-box transcription factor 5 (TBX5), which can cooperatively reprogram non-myocytes and cardiac fibroblasts into beating cardiac-like myocytes for heart repair [14]. More important, recent evidence indicates that HAND2 was sufficient to increase the cell area of cardiomyocytes and the expression of the hypertrophic genes in vitro. Forced expression of HAND2 could promote cardiac hypertrophy and develop left ventricular dilatation and induce the upregulation of cardiac genes, such as natriuretic peptides atrial natriuretic factor (ANP), brain natriuretic peptide (BNP) and β-myosin heavy chain (β-MHC) in vivo. And it was also reported that HAND2 was regulated by miR-25 to induce cardiac hypertrophy under the control of calcineurin/Nfat signaling [15]. In this study, we will investigate the role of miR-92b-3p in cardiomyocyte hypertrophy and the mechanism involved.

2. Materials and methods

2.1. Primary culture of mouse ventricular cardiomyocytes and treatments

As described previously, neonatal mouse ventricular cells (NMVCs) were isolated from the hearts of 1–3-d-old newborn C57BL6 mice (License number SCXK (YUE) 2004-0011, Department of Experimental Animal Research Center, Sun Yat-sen University, Guangzhou, China) [16]. The newborn mice were killed by cervical dislocation. Isolated NMVCs were plated onto 12-well plates and maintained for 48 h in DMEM/F-12 supplemented with 10% FBS (Gibco, New York, NY), then incubated with 10^{-8} M Ang-II for 48 h to induce the hypertrophic phenotype. Cells were transfected with 100 nM scramble or miR-92b-3p mimic, or 100 nM HAND2 siRNA through oligofectamine reagent (Invitrogen, Carlsbad, CA). MiR-92b-3p mimic [12] and HAND2 siRNA [17] were synthesized by Ribobio (Guangzhou, China), with the following sequences:

miR-92b-3p mimic: 5′-UAUUGCACUCGUCCCGGCCUCC-3′;
HAND2 siRNA: 5′-CCTTCAAGGCGGAGATCAAdTdT-3′ (sense).

2.2. FITC-phalloidin staining

Cultured NMVCs were washed with Phosphate Buffered Saline (PBS) for two times then fixed in 3.7% formaldehyde and permeabilized in 0.1% Triton X-100 for 10 min at room temperature, respectively. Monolayers were subsequently washed in blocking solution (Sigma-Aldrich, USA) and incubated with FITC-phalloidin (10 μg/ml, Sigma-Aldrich) for 40 min at 37 °C. Monolayers were then washed again, post-fixed with 3.7% formaldehyde. Leica SP5 confocal microscope (Leica, Mannheim, Germany) was used to gain confocal micrographs and the cell size (total area) was quantified using MiVnt imaging software (Weiyu, Zhuhai, China).

2.3. Quantitative miRNA and mRNA measurements

The reverse-transcription quantitative PCR (RT-qPCR) was performed following the manufacturer's protocol (Takara, Japan). RT-qPCR for miR-92b-3p was performed on cDNA generated from 0.5 μg total RNA and U6 small RNA was used for data normalization, followed by the operation steps: 95 °C for 20 s, 44 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 25 s. The mRNA level of GAPDH was used as an internal control for mRNA expression analyses, with following program steps: 94 °C for 5 min, 39 cycles of 94 °C for 25 s, 59 °C for 25 s, and 72 °C for 25 s. PCR and analyses were performed with the ViiA7 Quantitative PCR System (Applied Biosystems, Carlsbad, CA). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of miR-92b-3p and coding genes [18]. The PCR primers for miR-92b-3p, U6 and coding genes used in this study are shown in Table 1.

2.4. Western blot analysis

Protein samples were extracted from NMVCs. The amount of 40 μg protein was separated using 10% SDS-PAGE, transferred onto a polyvinylideneuoride (PVDF) membrane. The PVDF was then incubated with anti-β-MHC antibody (1:1000, Aviva Systems Biology, CA, USA), anti-ACTA1 antibody (1:5000, Proteintech, Wuhan, China), anti-HAND2 antibody (1:500, Abcam, Cambridge, MA, USA), respectively. Membranes were then washed with TBST for 15 min and incubated with a secondary antibody (1:5000, Santa Cruz, CA, USA) for 1 h at room temperature. An anti-GAPDH antibody (1:2000, Santa Cruz) was used to detect the level of GAPDH as an internal control. Protein was visualized using the ECL Plus detection system (GE Healthcare, Waukesha, WI).

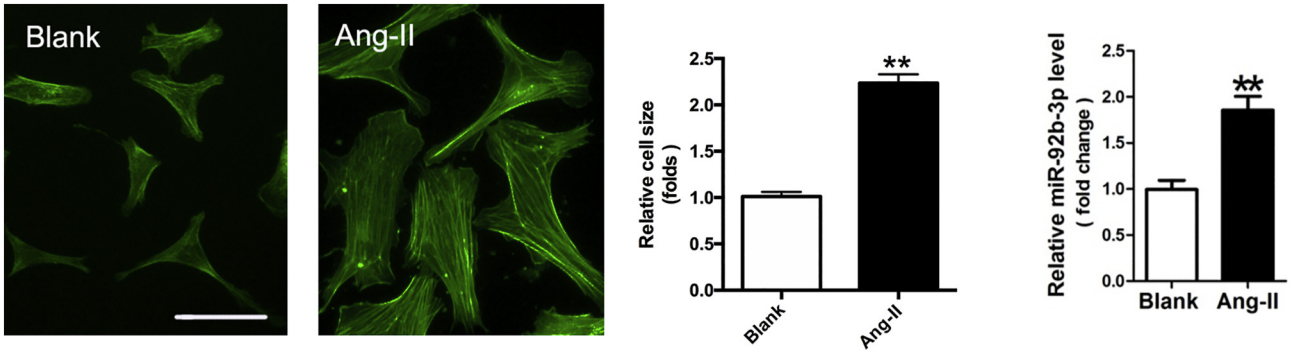

Fig. 1. Up-regulation of miR-92b-3p in the hypertrophic cardiomyocytes. FITC-phalloidin staining assay and detection of miR-92b-3p by RT-qPCR assay in Ang-II-treated cardiomyocytes. The scale bar was 50 μm. Data are shown as mean ± sem, $*p < 0.05$, $**p < 0.01$ vs. blank. N = 3.

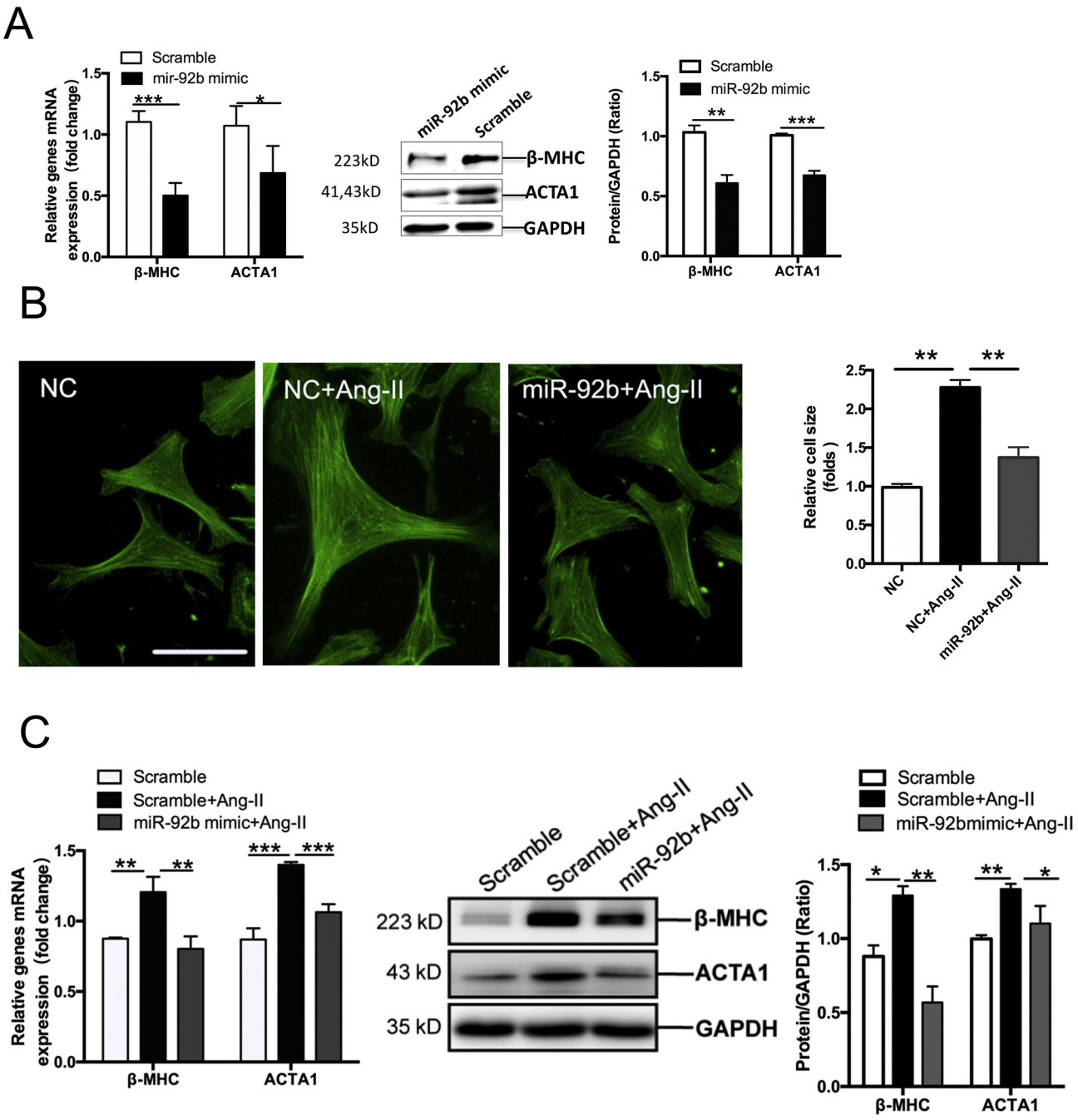

Fig. 2. MiR-92b-3p attenuates Ang-II-induced cardiomyocyte hypertrophy. (A) β-MHC and ACTA1 mRNA and protein expression in mouse cardiomyocytes transfected with miR-92b-3p mimic by RT-qPCR and Western blot assay, respectively. (B) FITC-phalloidin staining assay of Ang-II-treated NMVCs. The scale bar was 50 μm, $*p < 0.05$, $**p < 0.01$. N = 3. (C) β-MHC and ACTA1 mRNA and protein expression in Ang-II-induced NMVCs with transfected of miR-92b-3p mimic. Data are shown as mean ± sem, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. N = 3.

2.5. Dual luciferase assay for HAND2 target identification

According to the method described in our previous report [19], we constructed the recombinant luciferase reporter plasmid containing the potential miR-92b-3p binding site sequence of HAND2 gene in this study. Using a site-directed mutagenesis kit (TransGen, Beijing, China), the miR-92b-3p binding site sequence was changed to construct a recombinant luciferase reporter plasmid containing the mutant potential miR-92b-3p binding sequence. We then co-transfected human embryonic kidney (HEK) 293 cells (3×10^5 cells per well in the 12-well plate) with 200 ng of recombinant luciferase reporter plasmid, 50 nM miR-92b-3p mimic, and 20 ng of pRL-TK plasmid as an internal control (Promega, Madison, WI). After transfection for 24 h, activities of firefly luciferase (FL) and Renilla luciferase (RL) were obtained. The relative ratio of the FL/RL was used to determine the suppression of HAND2 by miR-92b-3p.

2.6. Statistical analysis

Results were expressed as means ± sem. In each experiment, all determinations were performed at least in triplicate. Differences between two experimental groups were compared using the independent sample *t*-tests and the ANOVA test was applied to comparisons among multiple groups. All statistical analyses were performed in SPSS 20.0

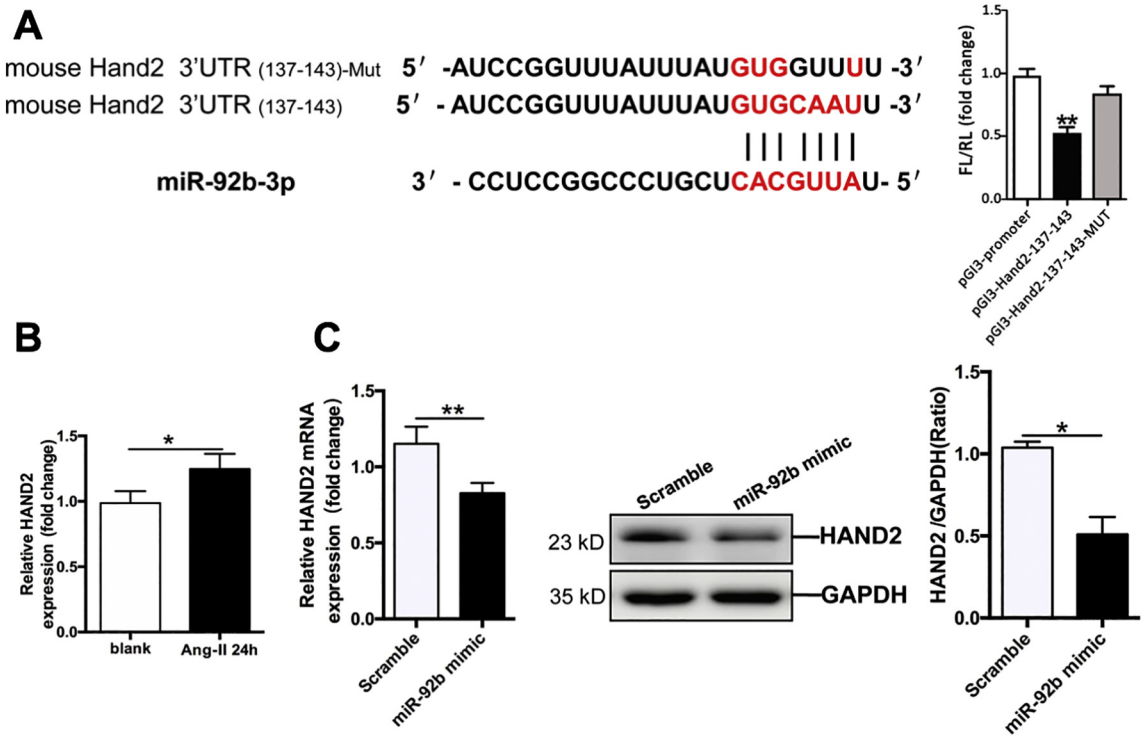

Fig. 3. Identification of HAND2 as a target of miR-92b-3p. (A) Predicted miR-92b-3p seed sequence matches to the 3′-UTR of HAND2 mRNA. The seed sequence of miR-92b-3p is AUUGCAC, and the complementary nucleotide sequences are in red. Data are shown as mean ± sem, $^{**}p < 0.01$ vs pGl3-promoter vector control, N = 3. (B) HAND2 mRNA expression in the Ang-II-induced cell model of cardiac hypertrophy. (C) Detections of HAND2 mRNA and protein expression in mouse cardiomyocytes transfected with miR-92b-3p mimic by RT-qPCR and western blot assay, respectively. Data are shown as mean ± sem, $^{*}p < 0.05$, $^{**}p < 0.01$. N = 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Statistical Package Program for Mac (SPSS Inc., IL, USA). A value of two-sided $p < 0.05$ was considered statistically significant.

3. Results

3.1. Up-regulation of miR-92b-3p in the hypertrophic cardiomyocyte

A cell model of hypertrophy was established in NMVCs induced by Ang-II. The FITC-Phalloidin staining results revealed that the cell size of cardiomyocytes was significantly increased in the hypertrophic cardiomyocytes induced by Ang-II treatment. At the same time, our RT-qPCR results showed that the expression of miR-92b-3p was markedly upregulated in the NMVCs in response to Ang-II treatment (Fig. 1).

3.2. MiR-92b-3p attenuate Ang-II-induced cardiac hypertrophic phenotype

We increased the level of miR-92b-3p in NMVCs through transfecting miR-92b-3p mimic. MiR-92b-3p could significantly inhibited β-MHC and ACTA1 mRNA and protein expressions in NMVCs compared with the scramble control miRNA (Fig. 2A). To further confirm the effects of miR-92b-3p on the cardiac hypertrophic phenotype in NMVCs, we established a cell model of Ang-II-induced cardiomyocyte hypertrophy, followed by the transfection of miR-92b-3p mimic. As expected, the result of FITC-phalloidin staining assay revealed that miR-92b-3p mimic could significantly decrease the cell size of Ang-II-treated NMVCs (Fig. 2B). Consistently, the increases of β-MHC and ACTA1 mRNA and protein expression in Ang-II-treated NMVCs could be reversed by miR-92b-3p (Fig. 2C).

3.3. Verification of HAND2 as a target gene of miR-92b-3p

Analysis of the databases Mirdb (www.mirdb.org) and TargetScanVert (www.targetscan.org) showed that HAND2 is a potential target gene of miR-92b-3p. The matching position for miR-92b-3p within 3′-UTR of the targeted mRNAs is shown in red words (Fig. 3A). More importantly, the dual luciferase assay for HAND2 target identification showed that miR-92b-3p significantly attenuated the luciferase activity (Fig. 3A). Our results showed that HAND2 mRNA expression was significantly increased in Ang-II-treated mouse cardiomyocytes (Fig. 3B). In addition, both of the RT-qPCR and western blot results revealed that miR-92b-3p markedly reduced the HAND2 mRNA and protein expression in NMVCs (Fig. 3C).

3.4. MiR-92b-3p and HAND2 siRNA attenuate the hypertrophic phenotype in cardiomyocytes

MiR-92b-3p mimic and HAND2 siRNA were transfected into NMVCs, followed by FITC-phalloidin staining assay and the determining of the expressions of hypertrophy-related genes. Results of FITC-phalloidin staining indicated that miR-92b-3p mimic and HAND2 siRNA could efficiently abolish the increase of cell size of Ang-II-induced NMVCs (Fig. 4A). Consistently, the Western blot results showed that the up-regulations of β-MHC, ACTA1 and HAND2 by Ang-II in NMVCs could be reversed by miR-92b-3p mimic and HAND2 siRNA, respectively (Fig. 4B).

4. Discussion

In this study, we found that miR-92b-3p significantly increased in hypertrophic cardiomyocytes, accompanied by the increased expression of HAND2. Our data revealed that miR-92b-3p could attenuate Ang-II-induced cardiomyocyte hypertrophy with downregulating the hypertrophy related genes. We also found that miR-92b-3p negatively regulates HAND2 expression by targeting the 3′-UTR of HAND2 mRNA. And both miR-92b-3p mimic and HAND2 siRNA could inhibit Ang-II-induced hypertrophy in mouse cardiomyocytes.

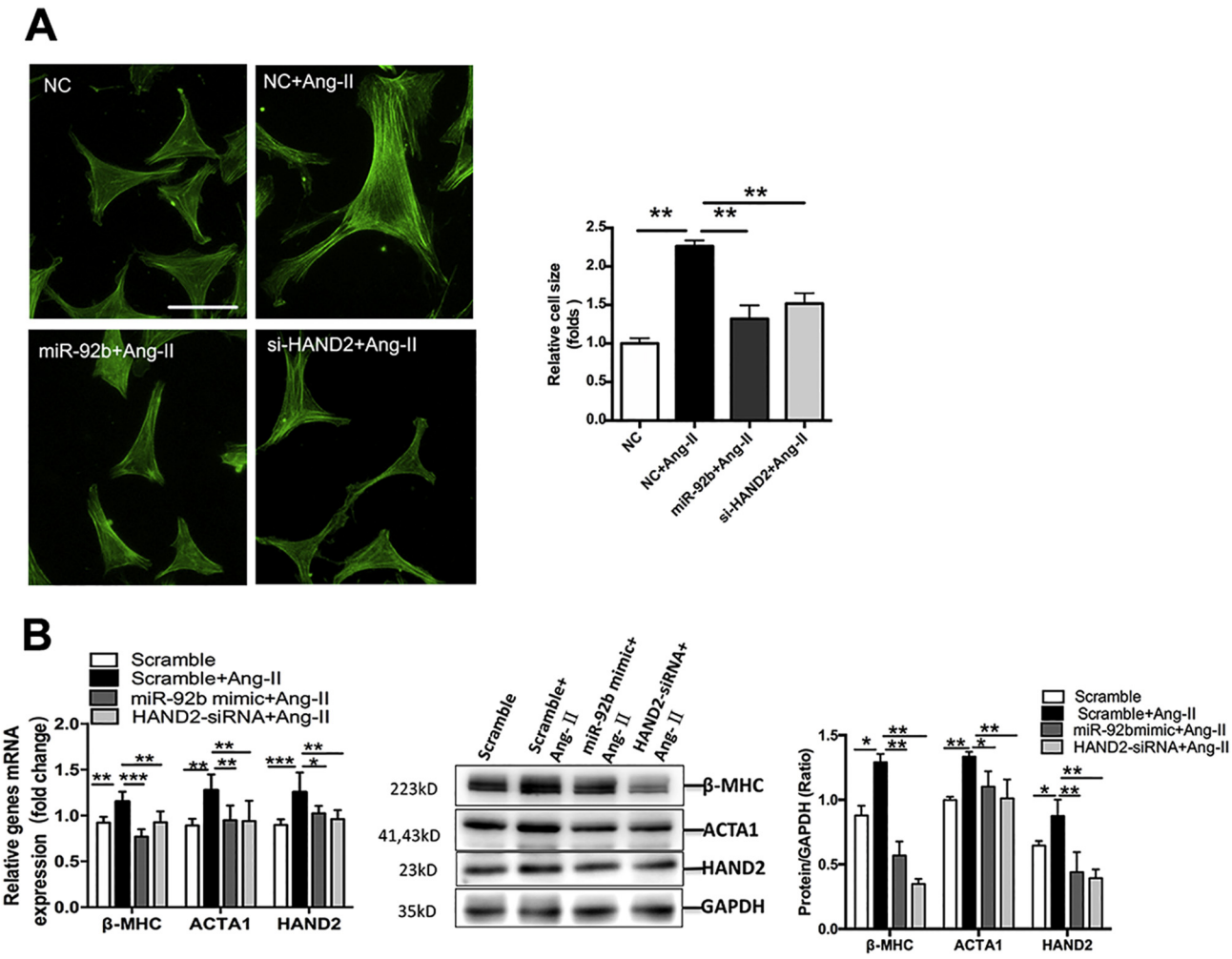

Fig. 4. MiR-92b-3p and HAND2 siRNA inhibit the hypertrophic phenotype in Ang-II-treated cardiomyocytes. (A) FITC-phalloidin staining assay. The scale bar was 50 μm, $*p < 0.05$, $**p < 0.01$. N = 3. (B) β-MHC, ACTA1 and HAND2 mRNA and protein expression in NMVCs were detected by RT-qPCR and western blot assay, respectively. Data are shown as mean ± sem, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. N = 3.

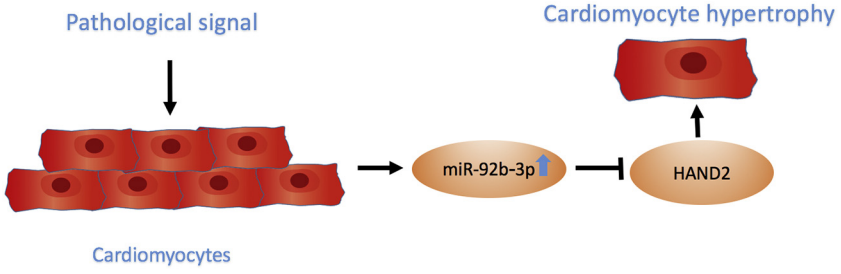

Fig. 5. Schematic diagram of the revealed signaling pathway in regard to the role of miR-92b-3p in cardiomyocyte hypertrophy. Our present study showed that the expression of miR-92b-3p was upregulated in hypertrophic cardiomyocytes induced by Ang-II while overexpression of miR-92b-3p ameliorated the hypertrophic phenotype by targeting HAND2, thus inhibiting the progress of cardiomyocyte hypertrophy. These findings support the scientific hypothesis that forced expression of miR-92b-3p may be a potential target of cardiac hypertrophy prevention and therapy.

In our current study, we observed that miR-92b-3p was obviously up-regulated in Ang-II-treated mouse cardiomyocytes. Furthermore, we found that the expression of hypertrophic gene ACTA1 and β-MHC were reduced in NMVCs transfected with miR-92b-3p mimic. To further investigate the role of miR-92b-3p in cardiomyocyte hypertrophy, we transfected miR-92b-3p mimic into Ang-II-treated mouse cardiomyocytes. As expected, the increases of ACTA1 and β-MHC in mouse hypertrophic cardiomyocytes were reversed by miR-92b-3p, and the increase of cell sizes of the hypertrophic cardiomyocytes was also reversed by miR-92b-3p. Therefore, our data have proved that miR-92b-3p plays a protective role against cardiac hypertrophy. These findings extend and corroborate our pilot studies on miR-92b-3p [12].

HAND2 is a bHLH transcription factor that plays important roles in morphogenesis of tongue [20], development of limb [21–23], and development of the sympathetic nervous system [24,25]. HAND2 is also known as one of the ancient cardiac transcription factors involve in proper development of heart and blood vessels [26,27]. HAND2 promotes cardiomyocyte proliferation and enhance cardiomyocyte production in zebrafish heart [28]. On the one hand, HAND2 is indispensable for morphogenesis of the normal heart and formation of the neural crest-derived aortic arches [29]. On the other hand, HAND2 mRNA and protein expression are upregulated in hypertrophic myocardium of calcineurin transgenic mice (MHC-CnA) and TAC-induced mice, and forced overexpression of HAND2 induces cardiac dilation and the elevated levels of ANP and β-MHC [15]. It is revealed that increased HAND2 expression is able to promote cardiac dilation, cardiomyocyte hypertrophy and contractile defects independently in the myocardium in vivo [15]. Furthermore, the same phenomenon was observed in

neonatal rat cardiomyocytes infected with an adenovirus expressing HAND2, the increased cell size and the up-regulation of the ANP mRNA indicated that overexpression of HAND2 contributes to cardiomyocyte hypertrophy in vitro [15].

Our present study demonstrated that miR-92b-3p inhibits HAND2 at the transcriptional level in cardiomyocytes. In the first place, the in silico prediction indicated that HAND2 was a potential target of miR-92b-3p and the dual luciferase assay revealed that miR-92b-3p specifically bound to the 137–143 site in the 3′-UTR of HAND2. In addition, miR-92b-3p mimic inhibited HAND2 expression at both mRNA and protein levels in mouse cardiomyocytes. Importantly, the upregulations of HAND2 mRNA and protein by Ang-II in cardiomyocytes could be reversed by miR-92b-3p. Moreover, in parallel with the findings with HAND2 siRNA, over-expression of miR-92b-3p decreased the cell size of cardiomyocytes and inhibited the expressions of ACTA1 and β-MHC in Ang-II-induced mouse cardiomyocytes.

In summary, our work demonstrated that miR-92b-3p was upregulated in hypertrophic cardiomyocytes induced by Ang-II and overexpression of miR-92b-3p ameliorated the hypertrophic phenotype. We identified HAND2 as a direct target of miR-92b-3p, and HAND2 mediates the inhibitory effect of miR-92b-3p on cardiomyocytes hypertrophy (Fig. 5). Considering cardiac hypertrophy still has no effective treatment by now, miR-92b-3p may provide some inspiration for hypertrophy prevention and therapy.

Acknowledgements

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