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Overexpression of miR-135b attenuates pathological cardiac hypertrophy by targeting CACNA1C

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ABSTRACT

Background: Cardiac hypertrophy is a serious factor underlying heart failure. Although a large number of pathogenic genes have been identified, the underlying molecular mechanisms of cardiac hypertrophy are still poorly understood. MicroRNAs are a class of small non-coding RNAs which regulate their target genes at the post-transcriptional level. L-type calcium channels play important role in hypertrophic signaling pathways, and CACNA1C is encoded by L-type calcium channels. Here, we hypothesize that the overexpression of miR-135b can attenuate hypertrophy by targeting CACNA1C.
Methods: We test the functional involvement of miR-135b in cardiac hypertrophy model. In order to evaluate the effect of miR-135b in cardiac hypertrophy, miR-135b mimic, miR-135b agomir and α-MHC-miR-135b transgenic mice were used for the overexpression of miR-135b. Luciferase reporter assays were used to testify the binding of miR-135b to the CACNA1C 3′UTR.
Results: Our results revealed that in a pathological cardiac hypertrophy model, the expression of miR-135b was clearly downregulated. Hypertrophic marker genes were upregulated after the knockdown of miR-135b *in vitro*, while the overexpression of miR-135b attenuated hypertrophy. These results suggested that miR-135b may weaken hypertrophic signals. We then explored the mechanism of miR-135b in hypertrophy and identified that CACNA1C was a target gene for miR-135b. The overexpression of miR-135b attenuated cardiac hypertrophy by targeting CACNA1C.
Conclusions: Our studies revealed that miR-135b is a critical regulator of cardiomyocyte hypertrophy. Our findings may provide a novel strategy for the treatment of cardiac hypertrophy.

1. Introduction

Pathological cardiac hypertrophy represents a serious risk for cardiovascular disease. Initially, cardiac hypertrophy is considered to be an adaptive response to maintain normal cardiac function. However, continuous hypertrophic growth is associated with heart failure and sudden death [1–3] and can also increase interstitial fibrosis and arrhythmia [4]. Pathological cardiac hypertrophy is a complicated disease derived from primary cardiac diseases such as hypertension, valvular heart disease and coronary artery disease [5]. While a range of pathways and targets have been discussed as potential treatments, pathological cardiac hypertrophy inevitably still leads to heart failure, it is necessary to identify molecules and novel targets that may regulate cardiac hypertrophy.

MicroRNAs are a class of small non-coding RNAs which regulate their target genes at the post-transcriptional level [6,7]. Research is now beginning to elucidate the role of miRNAs in cardiac development and function [8,9]. miR-135 is associated with a highly conserved mammalian gene and plays a key role in many serious disease conditions [10–13]. In skeletal muscle development, miR-135 is known to act as a potential regulator of myogenesis [14]. Furthermore, miR-135 is also considered to be a key regulator of myocardial ischemia/reperfusion

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injury [15]. Eight weeks after transverse aortic constriction (TAC) in wild type mice, the expression of miR-135 was shown to be upregulated compared with miR-1 [16]. However, little is known about the function and regulatory mechanism of miR-135 in cardiac hypertrophy.

Calcium ion (Ca^{2+}) influx through L-type Ca^{2+} channels (LTCCs) in cardiac myocytes is the initiating event in the excitation-contraction coupling process. This process is also involved in intracellular signaling and the gene regulatory events that underlie cardiac hypertrophy and disease [17]. In addition to regulating cardiomyocyte contraction, mutations in the genes encoding the LTCC are also associated with various types of inherited arrhythmia [18]. However, the relationship between miR-135 and LTCCs remains unknown.

In the present study, we investigated the function of miR-135b in cardiac hypertrophy and explored LTCC-related mechanisms. The overexpression of miR-135b reduced cardiomyocyte hypertrophy in response to Ang II stimulation. We generated α-MHC-miR-135b transgenic mice that were able to antagonize the pressure overload induced by transverse aortic constriction (TAC). Furthermore, we found that CACNA1C was a potential target for miR-135b. These findings provide new insights in our understanding of the pathogenesis of cardiac hypertrophy.

2. Materials and methods

2.1. Ethics statement

The study was approved by the ethics committee of Harbin Medical University, all experimental animals received humane care. All institutional and national guidelines for the care and use of laboratory animals were followed.

2.2. Primary culture of neonatal mouse cardiomyocytes

Neonatal mice cardiomyocytes were isolated from 1 to 3 days old C57BL/6 mice hearts. Cells were suspended in DMEM (Hyclone, USA) with 10% FBS, and cultured in humidified incubator (95% air-5% CO_2) for 48 h. Cardiomyocytes were incubated with 100 nmol/L Ang II for 48 h. The serum-free medium containing Ang II was changed every 24 h. For immunofluorescence, monoclonal antibody against sarcomeric α-actinin (Sigma, USA) was added at dilutions of 1:200. Immunofluorescence was examined under a fluorescence microscope (Zeiss, Germany).

2.3. Gene transfection

MiR-135b mimic, negative control (NC) miRNA, AMO miR-135b and AMO-NC were commercially synthesized by RiboBio Co., Ltd. (Guangzhou, China). Cells were transfected Using X-treme GENE siRNA transfection reagent (Sigma, USA) according to the manufacturer's instruction.

2.4. Pressure overload-induced cardiac hypertrophy in vivo

Briefly, the chest of C57BL/6 mouse was opened and the thoracic aorta was identified. A 5-0 silk suture was placed around the transverse aorta and tied around a 26-gauge blunt needle which was subsequently removed. The chest was closed and the animals were kept ventilated until recovery of autonomic breath.

2.5. Real-time PCR

Total RNA for 500 ng was reverse transcribed to cDNA using Reverse Transcription Master Kit (Toyobo, Japan) according to the manufacturer's instructions. Real-time PCR was performed on ABI 7500 fast system (Applied Biosystems, USA) using SYBR Green I (Toyobo, Japan). GAPDH served as an internal control. The relative quantification of gene expression was determined using the $2^{-\Delta\Delta CT}$ method.

2.6. Western blot

The suspension was subjected to 10% acrylamide gel electrophoresis (SDS-PAGE) followed by electroblotting to a polyvinylidene difluoride membranes (Roche Applied Science, Prague, Czech Republic). After blocking with 5% (w/v) BSA dissolved in TBST for 2 h, the membranes were incubated at 4 °C overnight with primary antibodies of CaV1.2 (Alomone, Israel) and GAPDH (ZSGB-BIO, China), followed by incubation with HRB labeled goat anti-mouse IgG or anti-rabbit IgG (1:1000) (ZSGB-BIO, China) for 1 h. Western blotting bands were quantified using Quantity One software. GAPDH served as an internal control.

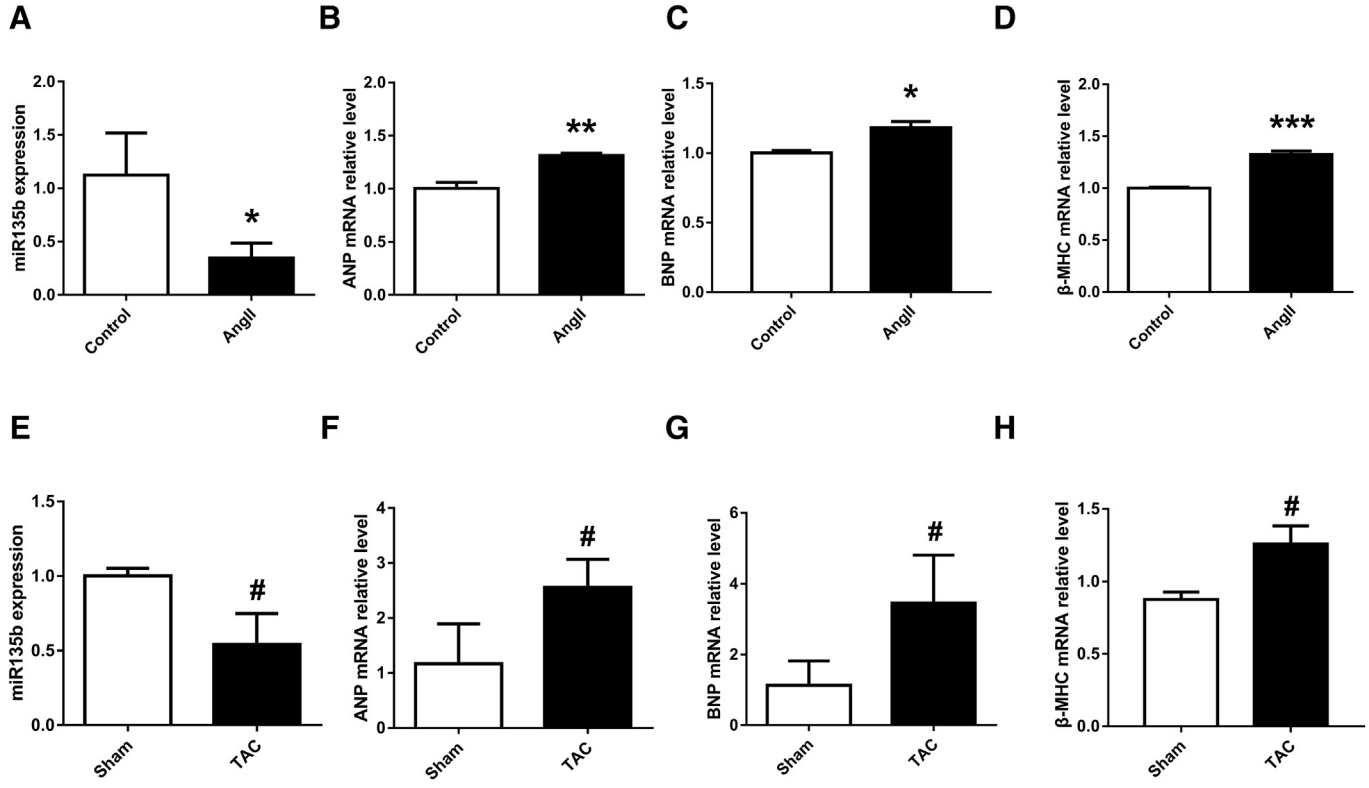

Fig. 1. Downregulation of miR-135b in a model of pathological cardiac hypertrophy *in vivo* and *in vitro*. (A) Reduction of miR-135b expression levels in hypertrophic cardiomyocytes. The mRNA expression of ANP (B), BNP (C) and β-MHC (D) was increased in Ang II-treated cardiomyocytes. (E) Reduction of miR-135b expression levels in an *in vivo* model. The expression of ANP (F), BNP (G) and β-MHC (H) was increased in mice treated with transverse aortic constriction. $^{*}P < 0.05$ *versus* Control, $^{**}P < 0.01$ *versus* Control, $^{***}P < 0.001$ *versus* Control; $n = 3$. $^{\#}P < 0.05$ *versus* Sham; n = 3–6.

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