

# MicroRNA-30b-5p Is Involved in the Regulation of Cardiac Hypertrophy by Targeting CaMKII $\delta$

Juan He, MS, Shan Jiang, MS, Feng-lan Li, MS, Xue-jiao Zhao, MS, Er-fu Chu, MS, Mei-na Sun, MS, Ming-zi Chen, MS, and Hui Li, PhD

**Background:** MicroRNAs (miRNAs) participate in the regulation of cardiac hypertrophy. However, it remains largely unknown as to how miRNAs are integrated into the hypertrophic program. Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) is a hypertrophic signaling marker. It is not yet clear which miRNAs can regulate CaMKII $\delta$ .

**Purpose:** In this study, we identified which miRNAs could regulate CaMKII $\delta$  and how to regulate CaMKII $\delta$ .

**Methods:** Through computational and expression analyses, miR-30b-5p was identified as a candidate regulator of CaMKII $\delta$ . Quantitative expression analysis of hypertrophic models demonstrated significant down-regulation of miR-30b-5p compared with control groups. Luciferase reporter assay showed that miR-30b-5p could significantly inhibit the expression of CaMKII $\delta$ . Moreover, through gain-of-function and loss-of-function approaches, we found miR-30b-5p could negatively regulate the expression of CaMKII $\delta$  and miR-30b-5p was a regulator of cardiac hypertrophy.

**Conclusion:** Our study demonstrates that the expression of miR-30b-5p is down-regulated in cardiac hypertrophy, and restoration of its function inhibits the expression of CaMKII $\delta$ , suggesting that miR-30b-5p may act as a hypertrophic suppressor.

**Key Words:** cardiac hypertrophy, CaMKII $\delta$ , miR-30b-5p, isoproterenol  
(*J Investig Med* 2013;61: 604–612)

Cardiac hypertrophy is a common response to a variety of physiological as well as pathophysiological stimuli, accompanied by maladaptive cardiac remodeling, which leads to heart failure or sudden death.<sup>1</sup> Cardiac hypertrophy induced by sustained pressure-overload is a major risk factor for heart disease and cardiovascular mortality worldwide.<sup>1</sup> Such pathological hypertrophy is regulated by multiple cascades of kinase and phosphatase signaling,<sup>2,3</sup> and a prominent contributor is the Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII).<sup>4,5</sup> CaMKII is a serine/threonine protein kinase with a broad spectrum of substrates.<sup>6,7</sup> The 4 isoforms of CaMKII ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ) are encoded by different genes, which display distinct but overlapping expression patterns.<sup>8</sup> The  $\alpha$  and  $\beta$  isoforms are almost exclusively expressed in the brain, whereas the  $\gamma$  and  $\delta$  isoforms are expressed more ubiquitously. CaMKII $\delta$  and  $\gamma$  are the predominant CaMKII isoforms in the heart with CaMKII $\delta$  displaying the highest level of expression.

From the Department of Biochemistry and Molecular Biology, Harbin Medical University, Harbin, China.

Received April 24, 2012, and in revised form November 6, 2012.

Accepted for publication November 8, 2012.

Reprints: Hui Li, PhD, Department of Biochemistry and Molecular Biology, Harbin Medical University, Harbin, China. E-mail: lihuiteacher@yahoo.com.cn.

Supported by National Natural Science Foundation of China Grants 30771863 and 81172616, and Excellent Academic Leaders Foundation of Harbin Science and Technology Agency of China Grant 2008RFXXS019.

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ISSN: 1081-5589

DOI: 10.2310/JIM.0b013e3182819ac6

Several lines of evidence indicate an important role of CaMKII $\delta$  in pathological cardiac remodeling.<sup>9–11</sup> CaMKII $\delta$  expression and activity are up-regulated in structural heart disease,<sup>9,10</sup> and transgenic overexpression of the nuclear splice variant CaMKII $\delta$ B<sup>12</sup> or the cytosolic splice variant CaMKII $\delta$ C<sup>13</sup> promotes cardiac hypertrophy. Conversely, inhibition of CaMKII activity with a peptide (AC3-I) diminishes pathological cardiac remodeling in response to stress stimuli.<sup>14</sup> However, studies on which miRNAs could regulate CaMKII $\delta$  in cardiac hypertrophy remain relatively few.

MicroRNAs (miRNAs) are a class of small noncoding RNAs that are gene expression regulators affecting mRNA stability or translational efficiency.<sup>15,16</sup> Mature miRNAs negatively regulate their gene targets through complementary sequence pairing to the 3'UTR of mRNA targets by inducing transcript degradation or translational repression.<sup>17</sup> Recent studies show that miRNAs are involved in the pathogenesis of hypertrophy, but their signaling regulations remain to be understood.<sup>18</sup> The hearts can abundantly express miR-30 family members.<sup>18,19</sup> MiR-30b-5p is one of the miR-30 family members. Its sequence is highly conserved from worms to human.<sup>20</sup> At present, the function of miR-30b-5p has been less studied. Through computational and expression analyses, miR-30b-5p was identified as a candidate regulator of CaMKII $\delta$ . Our previous studies have demonstrated miR-30b down-regulation in a model of myocardial injury.<sup>19</sup> Together with these, we hypothesized that miR-30b-5p might be a regulator of CaMKII $\delta$  and may be a regulator of cardiac hypertrophy. The aim of this study was to investigate whether miR-30b-5p was involved in cardiac hypertrophy by regulating CaMKII $\delta$ . Our study revealed that miR-30b-5p was down-regulated in cardiac hypertrophy, and restoration of its function inhibited the expression of CaMKII $\delta$  thereby preventing cellular hypertrophy. These findings suggest that miR-30b-5p may function as a hypertrophic suppressor.

## MATERIALS AND METHODS

### Animals

Wistar rats (180–210 g) were obtained from Vital River Laboratories (Peking, People's Republic of China). All animal experimental protocols complied with the "Guide for the Care and Use of Laboratory Animals" published by the United States National Institutes of Health. The study was approved by the ethics committees of Harbin Medical University for the Care and Use of Laboratory Animals. All animals were housed at the animal care facility of Harbin Medical University at 25°C with 12/12-hour light/dark cycles and allowed free access to normal rat chow and water throughout the study period. Rats were randomly assigned to different treatment groups.

### Animal Experiment

Pressure overload was induced by abdominal aorta constriction (AAC) in Wistar rats according to the method described previously.<sup>21</sup> Forty-five rats were randomly assigned to 3 experimental groups: In group 1 (n = 15), sham-operated

animals served as controls. In group 2 (n = 15), cardiac hypertrophy was induced by AAC. In group 3 (n = 15), normal Wistar rats served as controls.

Cardiac hypertrophy was induced by AAC according to the method described previously.<sup>21</sup> Briefly, rats were anesthetized with 10% chloral hydrate at a dose of 0.3 mL/100 mg by intraperitoneal injection. The animal should be carefully monitored from the point of view of body temperature, respiratory rate, circulation, airway problems, and injury from sharp objects for the adequacy of anesthesia. For the adequacy of anesthesia, the body temperature, respiratory rate, circulation, airway problems, and injury from sharp objects of the animals should be carefully monitored. And then the retroperitoneum was entered at 2 cm left lumbar vertebrae under the costal arch through a small incision. The abdominal aorta was isolated above the renal artery crotch and constricted by a 4-0 silk suture ligature tied against a 22-gauge needle. The needle was removed to form a constriction of 0.7 mm in diameter. Sham-operated rats underwent a similar surgical procedure without aorta constriction. The animals were divided into sham receiving oral administration of saline and AAC rats with saline.

### miRNA Target Prediction

CaMKII $\delta$  genome miRNA targets were predicted by RNAhybrid 2.2 (bibiserv.techfak.uni-bielefeld.de/mahybrid) and miRanda 3.2a (www.microrna.org), we predicted that miR-30b-5p was an important candidate regulator of CaMKII $\delta$ . The potential binding site was selected by choosing the complementary base sequences with minimum free energy.<sup>22,23</sup> Among the candidates, miR-30b-5p has the lowest minimum free energy.

### Neonatal Rat Cardiomyocyte Isolation and Culture

Neonatal rat cardiomyocytes were isolated and cultured, as previously described.<sup>24,25</sup> All experimental protocols were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Science, National Research Council 1996), as approved by the Animal Resource Center at Harbin Medical University (Harbin, China). Neonatal rat pups (1–3 days old) were killed by decapitation with sharp scissors and hearts were isolated and placed in cold PBS solution. After the hearts were collected, they were rinsed with PBS 2 to 3 times to remove debris. The hearts were minced with scissors and placed in a small volume of cold Dulbecco modified Eagle medium (DMEM). Heart tissues were placed in a 35-mm petri dish with 2 mL of cold DMEM, after which the minced tissues were transferred to a 15-mL centrifuge tube and 3 mL of trypsin (0.15% wt/vol) were added to the 15-mL falcon tube. Falcon tube was agitated for 5 minutes at 37°C to homogenize tissue. After homogenization, supernatant was removed to a new 15-mL falcon tube and 6 mL of DMEM medium, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin solution were added to the new 15-mL falcon tube. Then repeat the previously mentioned steps, until the tissues digest completely. After the final digestion, all supernatant fractions were combined and filtered to a 50-mL falcon tube using cell strainer with 40  $\mu$ m nylon mesh (BD, USA). Then the colatus liquid were centrifuged at 1000 rpm for 10 minutes at room temperature. The supernatant was discarded and the pellet was blended in fresh DMEM medium by the pipette. Cells were plated at a density of  $(1.8\text{--}2.0) \times 10^6$  cells per plate in a 60-mm petri dish. Two hours later, we remove the culture medium to another 60-mm petri dish and continue to foster. After 24 hours, the

medium was changed. These cells can be used for subsequent experiments after being cultured for 48 hours.

### Cell Culture and Treatment

Neonatal rat cardiomyocytes were cultured in DMEM supplemented with 10% FBS and penicillin-streptomycin (100 IU/mL) in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. To test an experimental hypertrophic condition, cells were serum starved for 18 hours in DMEM containing 1% FBS and treated with 50- $\mu$ m isoproterenol (ISO) for 48 hours.

### Immunofluorescence Microscopy

Neonatal rat cardiomyocytes grown on glass coverslips were fixed with 4% paraformaldehyde in PBS at room temperature. Fixed cells were rinsed with PBS and permeabilized with PBS containing 0.1% Triton X-100 for 30 minutes. Permeabilized cells were rinsed with PBS and incubated with 1% BSA in PBS for 1 hour at room temperature. Then cells were incubated with anti- $\alpha$ -sarcomeric actin ( $\alpha$ -SCA) antibody (Sigma, USA; 1:100 dilution overnight at 4°C). After washing 3 times with PBS, the primary antibody was detected with FITC-conjugated goat-antirabbit IgG antibody (Invitrogen, USA; 1:50 dilution for 2 hours at room temperature) and the nuclei were costained with DAPI (Beyotime, Shanghai, People's Republic of China). The coverslips were then washed and mounted on glass slides. Fluorescent images were obtained using an Olympus BX51 microscope.

### Measurement of Hypertrophic Growth in Cultured Cardiomyocytes

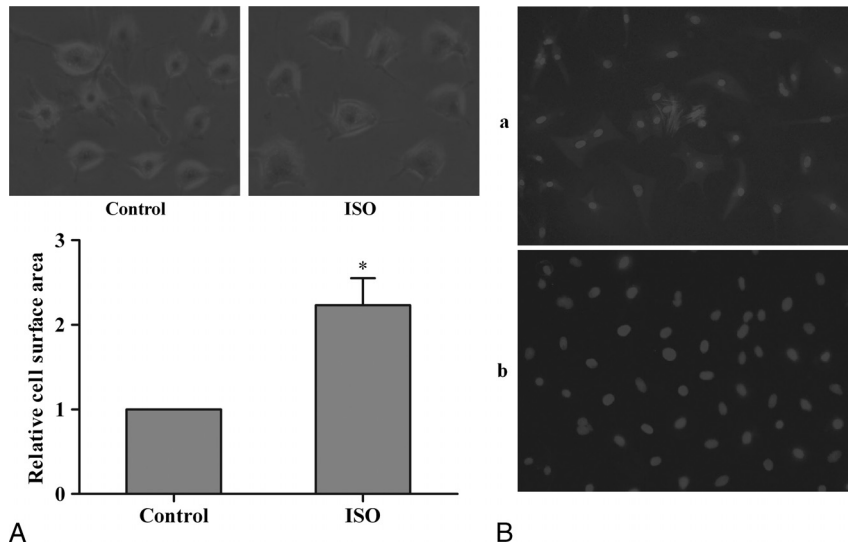
Neonatal cardiomyocyte cultures prepared as described previously were maintained in the appropriate culture medium. Images were obtained using an Olympus DP-71 digital camera attached to an Olympus BX51 microscope. Ten random photographs were taken from each sample and surface area was determined by at least 5 cells from each photograph using Image Pro Plus 6.1 software (Mediacybernetics). In each experiment, areas of cells were averaged and normalized to the control value. The data shown represent the image analysis from 5 or 6 independent experiments.

### Real-Time Polymerase Chain Reaction

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. For quantification of the miR-30b, 2  $\mu$ g total RNA was reverse-transcribed and amplified by using the mirVana qRT-PCR miRNA Detection Kit (Ambion) according to the manufacturer's guidelines. The primers for U6 small nuclear RNA (an endogenous internal control) were purchased from Ambion. Real-time polymerase chain reactions (PCRs) for U6 from each sample were performed in parallel. Quantitative miRNA expression data were analyzed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA).

### Nucleotides and Plasmids

The necessary miRNAs, miR-30b-5p mimics and control miRNAs, were synthesized by Ribobio Co Ltd (Peking, People's Republic of China). Anti-miR-30b-5p oligonucleotide (AMO-miR-30b-5p), an oligo-RNA (5'-AGCUGAGUGUAGGAUGUUACA-3') with a 2'-O-methoxyethyl group, was synthesized by Ribobio. pMIR was used as a basis to design and construct plasmids containing the 3'UTR sequence of CaMKII $\delta$  genome and a reporter gene, enhanced green fluorescence protein, which was designated as pMIR-CaMKII $\delta$ . The enhanced green fluorescence protein-coding sequence was amplified via PCR from pMIR-CaMKII $\delta$  with the sense primer 5'-CGACGCGTGAAGTTGTGAAGGTTCTACG-3'

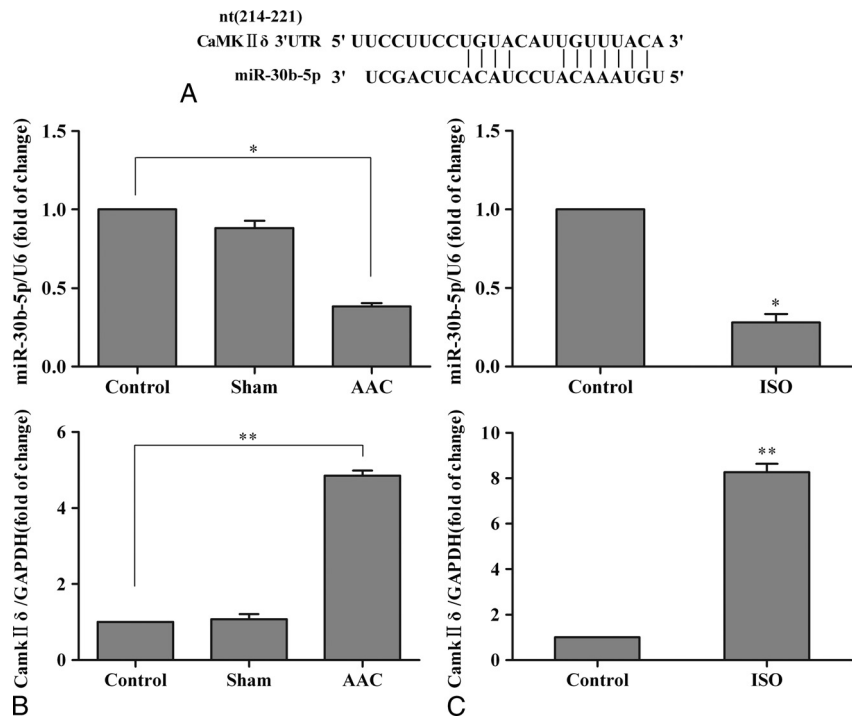


**FIGURE 1.** A, Induction of cardiomyocyte hypertrophy by ISO. Cultured neonatal rat cardiomyocytes were maintained in 1% serum media for 18 hours and treated with ISO (50  $\mu$ m) for 48 hours. The cellular hypertrophy was assayed by cell surface area measurement. Cell size was analyzed using Image Pro Plus software, and the values represent the relative area  $\pm$  S.E., original magnification,  $\times 200$ . In this experiment, areas of cells were averaged and normalized to the control value. Mean values from 5 experiments are shown. \* $P < 0.05$ . B, Neonatal rat cardiomyocytes were stained with anti- $\alpha$ -SCA antibody and FITC-conjugated antibody, respectively, as described under "Methods." Coverslips were mounted on a slide and analyzed by fluorescence microscopy. The primary antibody of "a" was  $\alpha$ -SCA antibody and the primary antibody of "b" was H<sub>2</sub>O. Irregular graphics,  $\alpha$ -SCA; approximate round, nucleus (DAPI); merge,  $\alpha$ -SCA/H<sub>2</sub>O + DAPI. Original magnification,  $\times 200$ .

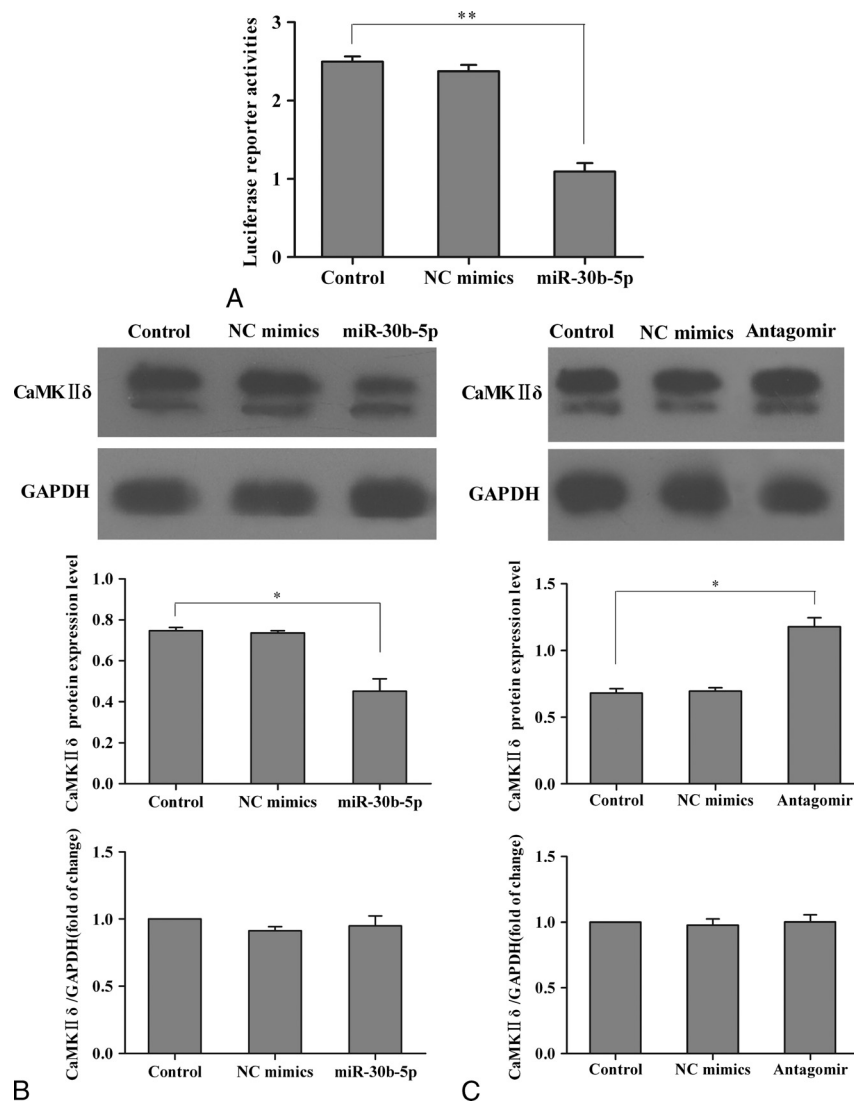
and the antisense primer 5'-CCCAAGCTTCTTGGAGTATTGCG GAGT-3'. The successful construction of the plasmid was confirmed by restrict digestion and sequencing.

**Luciferase Reporter Assay**

A total of  $1 \times 10^6$  293T cells were cultured in 6-well plates in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C and



**FIGURE 2.** A, Diagram of seed sequence of miR-30b-5p matched the 3'UTR of the CaMKII $\delta$  gene. "nt" stands for nucleotide. B, The relative expression levels of miR-30b-5p and CaMKII $\delta$  in rat's heart by AAC. Data represent fold-change in expression of 6 replicates, \* $P < 0.05$ , \*\* $P < 0.01$ . C, The relative expression levels of miR-30b-5p and CaMKII $\delta$  in cultured neonatal rat cardiomyocytes by ISO stimulus. Data represent fold-change in expression of 6 replicates, \* $P < 0.05$ , \*\* $P < 0.01$ .

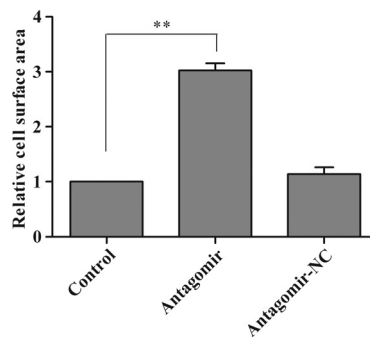
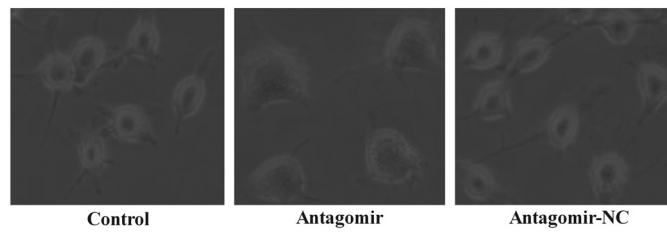
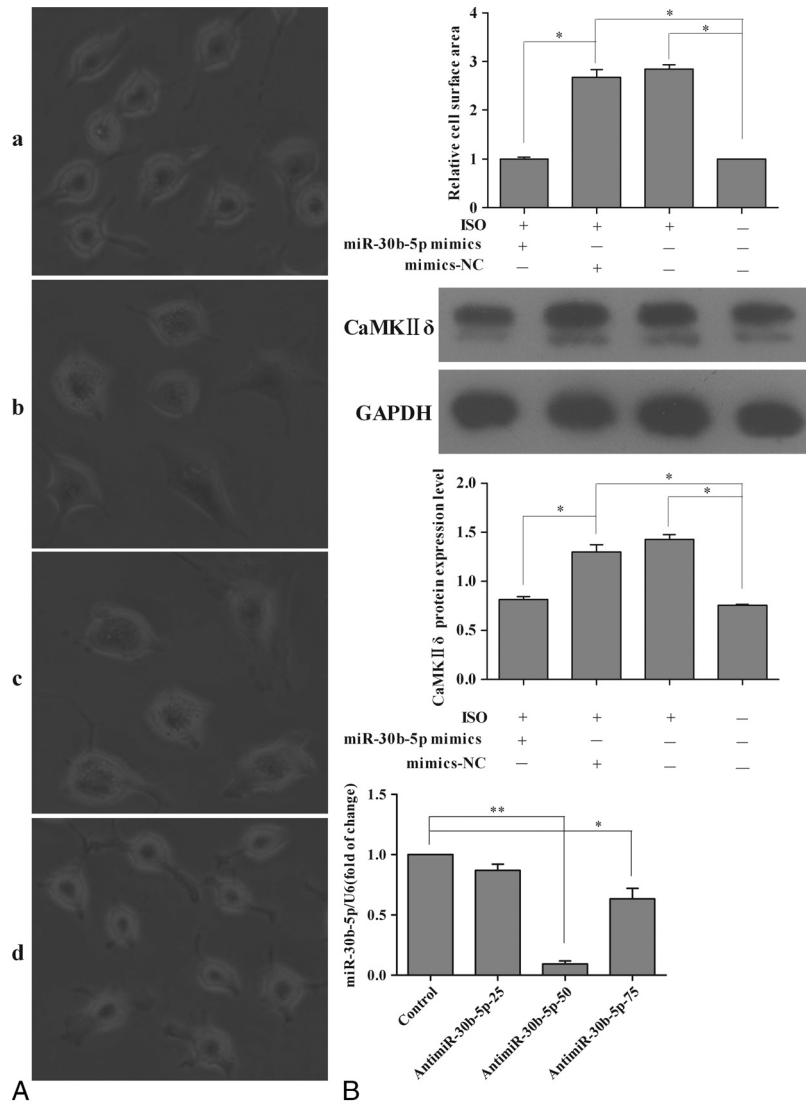


**FIGURE 3.** A, Luciferase reporter activities showing the interaction between miR-30b-5p and CaMKII $\delta$ . 293T cells were transfected with miR-30b-5p mimics and the constructs of the CaMKII $\delta$ 3'UTR or transfected with NC mimics and the constructs of the CaMKII $\delta$ 3'UTR. NC refers to scramble oligonucleotides. Forty-eight hours after transfection, cells were harvested.  $n = 6$ ,  $n$  stands for 6 replicates,  $**P < 0.01$ . B, The expression levels of CaMKII $\delta$  after overexpression miR-30b-5p. Cultured neonatal rat cardiomyocytes were transfected with miR-30b-5p mimics. Twenty-four hours after transfection, we detected the mRNA expression levels of CaMKII $\delta$ ; and 48 hours after transfection, we detected the protein expression levels of CaMKII $\delta$ . NC refers to scramble oligonucleotides. GAPDH served as a loading control. Data derived from 6 independent experiments,  $*P < 0.05$ . C, The expression levels of CaMKII $\delta$  after knock-out miR-30b-5p. Cultured neonatal rat cardiomyocytes were transfected with miR-30b-5p antagomir. Twenty-four hours after transfection, we detected the mRNA expression levels of CaMKII $\delta$  and 48 hours after transfection, we detected the protein expression levels of CaMKII $\delta$ . Antagomir refers to oligonucleotides antisense of miR-30b-5p and NC mimics refers to control oligonucleotides of antagomir. GAPDH served as a loading control. Data derived from 6 independent experiments,  $*P < 0.05$ .

transfected with 5 nmol/L miR30b-5p mimics or miR30b-5p mimics control together with 1.2  $\mu$ g of a luciferase reporter plasmid (pMIR) carrying a firefly luciferase complementary DNA driven by CaMKII $\delta$  binding sites (Clontech, Mountain View, CA) and 1.2  $\mu$ g of a galactosidase control reporter plasmid (pMIR; Promega, Madison, WI). After incubation for 24 hours, luciferase and  $\beta$ -galactosidase activities were measured using an assay kit for luciferase and  $\beta$ -galactosidase, respectively, according to the manufacturer's instructions (Promega). Luciferase activities were determined and normalized to  $\beta$ -galactosidase activity.

### Western Blot Analysis

Western blot analysis was performed according to standard procedures. Total protein was isolated from cultured neonatal rat cardiac myocytes, and 20  $\mu$ g of protein samples was resolved by sodium dodecyl sulfate poly-acrylamide gel electrophoresis and transferred onto a PVDF membrane. The membrane was blocked for 2 hours in 5% nonfat milk in Tris-buffered saline and 0.1% Tween-20 (TBS-T) and then incubated with primary antibody against CaMKII $\delta$  (1:1000; Santa Cruz, CA), or GAPDH (1:1000; Zhongshanjinqiao, China) as an internal control overnight at 4°C, followed by incubation with



C

horseradish peroxidase-conjugated secondary antibody (1:8000; Zhongshanjinqiao) for 2 hours at room temperature. Antibody binding was detected by enhanced chemiluminescence. After washing 3 times with TBS-T, the membrane was developed using an enhanced chemiluminescent detection system. Signal intensities were quantitated using the Gel Image Analysis System (Kodak, Shanghai, China).

### miR-30b-5p Silencing

Chemically modified antagomir complementary to miR-30b-5p were used to inhibit its expression. Chemically modified oligonucleotides were used as a negative control (antagomir-NC). All of the bases were 2'-OMe modified. Antagomir oligonucleotides were synthesized and purified with high-performance liquid chromatography by Ribobio Co Ltd. Cells were transfected with antagomir or antagomir-NC using lipofectamin 2000 (Invitrogen). The added quantity of antagomir is 50 nM.

### Statistical Analysis

Statistical analysis was performed using the software SPSS 18.0 (SPSS, Chicago, IL). The normality of distribution was assessed using the Kolmogorov-Smirnov test. Data with a normal distribution are presented as mean (SD). The significance of difference between groups was estimated with the Student *t* test and  $\chi^2$  test. A value of  $P < 0.05$  was considered to be statistically significant.

## RESULTS

### Induction of Cardiac Hypertrophy in Both Cultured Neonatal Rat Cardiomyocytes and the Intact Adult Heart

We established a cardiac hypertrophy model that shows cardiac dysfunction at chronic stage by performing an AAC. Cardiac hypertrophy was induced by AAC according to the results described previously.<sup>21</sup> The animals were divided into control, sham, and AAC groups. After 10 weeks, AAC-induced cardiac hypertrophy was assessed by echocardiography and morphology analyses.

To verify the ISO-induced cellular hypertrophy in cultured neonatal rat cardiomyocytes, the cell surface area measurement was performed as described in Materials and Methods. As demonstrated in Figure 1A, cultured neonatal rat cardiomyocytes were treated with ISO and the cell size was significantly increased by 2.3-fold. Neonatal rat cardiomyocytes were isolated using the differential velocity adherent method and by this way almost all of the cells we got were cardiomyocytes. In support of this, immunofluorescence staining demonstrated the purity of cells we got (Fig. 1B).

### The Expression of miR-30b-5p Is Down-regulated in Cardiac Hypertrophy

Previous studies suggested that CaMKII $\delta$  is associated with cardiac hypertrophy.<sup>16</sup> Nevertheless, the miRNAs that regulate the expression of CaMKII $\delta$  during cardiac hypertrophy remain largely unknown. Guided by the prediction algorithms, we identified that the seed sequence of miR-30b-5p matched the 3'UTR of the CaMKII $\delta$  mRNA (Fig. 2A). But the prediction result needed verification, so we detected the expression levels of CaMKII $\delta$  and miR-30b-5p by TaqMan RT-PCR assay in the hypertrophic model. We found that miR-30b-5p expression was significantly decreased (Fig. 2B), whereas CaMKII $\delta$  expression was significantly increased (Fig. 2C). These results indicate that miR-30b-5p may play a role in regulating CaMKII $\delta$ .

### CaMKII $\delta$ Is a Target of miR-30b-5p

To determine whether miR-30b-5p indeed could influence the protein translation of CaMKII $\delta$ , we analyzed CaMKII $\delta$ -3'UTR luciferase activity. We first produced the constructs of CaMKII $\delta$ -3'UTR and synthesized the mimics of miR-30b-5p. Subsequently, we cotransfected with the mimics of miR-30b-5p and the constructs of CaMKII $\delta$ -3'UTR. miR-30b-5p could significantly decrease the luciferase activity of CaMKII $\delta$ -3'UTR (Fig. 3A). We then overexpressed miR-30b-5p in the cardiomyocytes to test if endogenous CaMKII $\delta$  was regulated by miR-30b-5p. CaMKII $\delta$  protein but not mRNA levels were significantly decreased in the presence of miR-30b-5p (Fig. 3B), suggesting that miR-30b-5p predominantly suppresses CaMKII $\delta$  translation. To further confirm the decrease of CaMKII $\delta$  protein levels were regulated by miR-30b-5p, we used the antagomir of miR-30b-5p to test if knockdown of miR-30b-5p could influence the expression levels of CaMKII $\delta$ . CaMKII $\delta$  protein but not mRNA levels were significantly increased in the absence of miR-30b-5p (Fig. 3C). These data indicated that CaMKII $\delta$  was a target of miR-30b-5p.

### miR-30b-5p Is a Regulator of Cardiac Hypertrophy

To understand whether miR-30b-5p plays a functional role in cardiac hypertrophy, we tested whether enforced expression of miR-30b-5p could influence hypertrophy induced by ISO stimuli. We infected the cultured neonatal rat cardiomyocytes with the mimics of miR-30b-5p before ISO treatment. Compared with the control groups, the cell surface areas were not significantly altered and the protein expression of CaMKII $\delta$  was not significantly changed either (Fig. 4A), suggesting that overexpression miR-30b-5p could attenuate cardiomyocytes hypertrophy induced by ISO stimuli. These results demonstrated that miR-30b-5p can protect cardiomyocytes against

**FIGURE 4.** A, Overexpression miR-30b-5p could attenuate cardiomyocytes hypertrophy induced by ISO stimuli. Isolated rat neonatal cardiomyocytes were transfected with miR-30b-5p mimics (a), scramble oligonucleotides (b), H<sub>2</sub>O (c), and untreated (d) at the onset of the experiment. After 24 hours, a, b, and c were all treated with ISO (50  $\mu$ M). Cells were imaged after 48 hours treatment. The cellular hypertrophy was assayed by cell surface area measurement. Cell size was analyzed using Image Pro Plus software, and the values represent the relative area (SE), original magnification,  $\times 200$ . Areas of cells were averaged and normalized to the control value. Mean values from 6 experiments are shown. Western blot analysis of CaMKII $\delta$  protein levels were after cells treated with ISO (50  $\mu$ M) for 48 hours. GAPDH is used to control for protein loading. Data were derived from 6 replicates,  $*P < 0.05$ . B, The suitable concentration of antagomir to reduce miR-30b-5p levels. Isolated rat neonatal cardiomyocytes were transfected with antagomir of miR-30b-5p at the concentration of 25, 50, and 75 nM. After 24 hours, qRT-PCR was used to detect the levels of miR-30b-5p. U6 served as a loading control. Data were derived from 6 replicates,  $*P < 0.05$ ,  $**P < 0.01$ . C, Knockdown miR-30b-5p could result in cardiomyocytes hypertrophy. Isolated rat neonatal cardiomyocytes were transfected with antagomir (50 nM) of miR-30b-5p or NC mimics. Antagomir refers to oligonucleotides antisense of miR-30b-5p and NC mimics refers to control oligonucleotides of antagomir. Cells were imaged after 48 hours transfection. The cellular hypertrophy was assayed by cell surface area measurement. Cell size was analyzed using Image Pro Plus software, and the values represent the relative area (SE), original magnification,  $\times 200$ . Areas of cells were averaged and normalized to the control value. Mean values from 5 experiments are shown.  $**P < 0.01$ .

ISO-mediated cardiac hypertrophy. To further confirm whether miR-30b-5p could be a regulator of cardiac hypertrophy. We used the antagomir of miR-30b-5p to test if knockdown of miR-30b-5p could influence the cell size. We tried antagomir at 25, 50, and 75 nM, and observed that 50 nM could effectively reduce miR-30b-5p levels (Fig. 4B). As shown in Figure 4C, knockdown miR-30b-5p could result in cardiomyocyte hypertrophy. The cell size was increased by detecting the cell surface area. Taken together, these observations demonstrate that miR-30b-5p is a regulator of cardiac hypertrophy.

## DISCUSSION

As miRNAs have been first discovered in the *Caenorhabditis elegans* heterochronic gene *lin-4*,<sup>26</sup> more than 1000 miRNAs have been so far discovered. They play pivotal roles in many physiological and pathological processes, and their levels are altered in response to hypertrophic stimulation.<sup>27,28</sup> Recent studies show that miRNAs are involved in the pathogenesis of hypertrophy; however, their signaling regulations remain to be understood. In this study, we investigated the function and molecular mechanisms of miR-30b-5p in cardiac hypertrophy.

Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) has been shown to be transducer pathological Ca<sup>2+</sup> signals in the heart, and inhibitors directed against these enzymes sustain cardiac function in response to stress.<sup>14,29–32</sup> CaMKII $\delta$  is the major CaMKII isoform in the heart. CaMKII is a serine/threonine protein kinase with a broad spectrum of substrates.<sup>6,7</sup> The 4 isoforms of CaMKII ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ) are encoded by different genes, which display distinct but overlapping expression patterns.<sup>8</sup> The  $\delta$  isoform has been found to be up-regulated during cardiac hypertrophy induced by pressure overload, and overexpression of CaMKII $\delta$  is able to initiate hypertrophic responses.<sup>33,34</sup> At present, the regulators of CaMKII $\delta$  are not clear.

To further investigate the function of CaMKII $\delta$ , we searched which miRNA could regulate it. Then we found miR-30b-5p. The miR-30 family is highly conserved in vertebrates and it is composed by 6 miRNAs (miR-30a, -30b, -30c-1, -30c-2, -30d, and -30e).<sup>35</sup> The miR-30 family members have the same “seed sequence” in their 5' terminuses, and are abundantly expressed in the heart under physiological condition. miR-30b-5p is a mature form of miR-30b that is coded by chromosome 8 in humans and chromosome 7 in rats. Members of the miR-30 family have been implicated in osteoblast differentiation,<sup>36</sup> adipogenesis,<sup>37</sup> mitochondrial fission,<sup>38</sup> epithelial-to-mesenchymal transition,<sup>39,40</sup> cellular senescence,<sup>41</sup> and cancer.<sup>39,42</sup> A recent study shows that the member miR-30c of miR-30 family is expressed predominantly in fibroblasts and can regulate connective tissue growth factor in myocardial matrix remodeling.<sup>43</sup> Dong et al.<sup>44</sup> studied acute myocardial infarction (AMI) and found that miR-30b-5p was one of the differentially expressed miRNAs in acute myocardial infarction and the expression of miR-30b-5p was up-regulated in the noninfarcted areas of the infarcted heart. Our study is the first to identify and validate CaMKII $\delta$  as a target of miR-30b-5p and to demonstrate the role of miR-30b-5p in cardiac hypertrophic.

It would be interesting to study how miR-30b-5p regulates CaMKII $\delta$ . Previous studies showed that different miRNAs have distinct mechanisms in regulating hypertrophy. For example, miR-133 inhibits hypertrophy through targeting RhoA and Cdc42.<sup>45</sup> miR-208 initiates cardiomyocyte hypertrophy by regulating triiodothyronine-dependent repression of  $\beta$ -MHC.<sup>46</sup> We performed the luciferase assay, and observed that luciferase activity was decreased after cotransfection of miR-30b-5p and a

3'UTR vector containing the CaMKII $\delta$  and miR-30b-5p target sequence. CaMKII $\delta$  protein expression was significantly down-regulated after miR-30b-5p overexpression. While transfected with the antagomir of miR-30b-5p in cultured neonatal rat cardiomyocytes, CaMKII $\delta$  protein expression was significantly up-regulated. These data demonstrate that miR-30b-5p specifically acts on the 3'UTR, via the miR-30b-5p binding site, of CaMKII $\delta$  and indicate that miR-30b-5p is a negative regulator of CaMKII $\delta$ .

In addition to the important role of miR-30b-5p in regulating CaMKII $\delta$ , our studies also identify miR-30b-5p as a key regulatory molecule necessary for cardiac hypertrophy. This is perhaps not entirely surprising, because miRNAs are proposed to fine-tune the expression of targeted mRNAs that produce protein products important for a particular tissue and to reduce transcriptional noise by helping to turn over misexpressed mRNAs.<sup>47–52</sup> By direct or indirect effects, a single miRNA may fine-tune the expression of thousands of genes.<sup>52</sup> In this sense, the precise molecular mechanisms underlying miR-30b-5p mediated regulation of cardiac hypertrophy have yet to be defined. Identification of the hypertrophic factors whose expressions are posttranscriptionally fine-tuned by miR-30b-5p will likely be the key to understanding miR-30b-5p function. Additional studies are needed to test and further define this intriguing possibility. We infected cultured neonatal rat cardiomyocytes with miR-30b-5p mimics before ISO stimuli and found that the cardiomyocytes were not hypertrophic. Overexpression miR-30b-5p was crucial for cytoprotective of cardiomyocyte hypertrophy upon treatment with ISO. Subsequently, we infected neonatal rat cardiomyocytes with the antagomir of miR-30b-5p, and observed that the cardiomyocytes were hypertrophic. These results suggest that miR-30b-5p may function as a regulator of cardiac hypertrophy and restoration of miR-30b-5p expression may represent a novel therapeutic strategy in cardiac hypertrophy.

Cardiac hypertrophy induced by ISO can be controlled by complex molecular mechanisms or signaling pathways. Although the present work shows that CaMKII $\delta$  can be regulated by miR-30b-5p, our results do not exclude the involvements of any other miRNAs and/or pathways that can regulate CaMKII $\delta$  directly or indirectly. CaMKII $\delta$  is one of the calmodulin-dependent enzymes and predominates in the heart. CaMKII $\delta$  plays a key role in cardiac gene expression associated with cardiac hypertrophy and plays a predominant role in Ca<sup>2+</sup>-mediated transcriptional gene regulation.<sup>12,53</sup> In the present, there are few researches on miRNA regulators of CaMKII $\delta$ . Ikeda et al.<sup>54</sup> studied cardiac hypertrophy and found that miR-1 could regulate cardiomyocyte growth responses by negatively regulating CaMKII $\delta$ . Cordes et al.<sup>55</sup> used bioinformatics approach to identify potential miR-145 binding sites in several other positive regulators of smooth muscle proliferation, including CaMKII $\delta$ . They found the putative binding site in CaMKII $\delta$  was validated as a miR-145-repressed target by luciferase and Western analysis in vascular smooth muscle cells. Other miRNAs in the hypertrophic model of ISO as well as their roles in regulating CaMKII $\delta$  remain to be further identified.

miRNAs have reshaped our view of how cardiac gene expression is regulated by adding another layer of regulation at the posttranscriptional level. The results reported in this study clearly established a role for miR-30b-5p in repressing hypertrophic genes via antithetical regulation of CaMKII $\delta$ . These results reveal a model to understanding the molecular regulation of miRNAs in cardiac hypertrophy. We anticipate that overexpression miR-3b-5p may be a viable therapeutic strategy to repress CaMKII $\delta$  expression and might remove some of the maladaptive features of hypertrophy.

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