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MiR-221 Promotes Cardiac Hypertrophy In vitro Through the Modulation of p27 Expression

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ABSTRACT

Cardiac hypertrophy has been known as an independent predictor for cardiovascular morbidity and mortality. Molecular mechanisms underlying the development of heart failure remain elusive. Recently, microRNAs (miRs) have been established as important regulators in cardiac hypertrophy. Here, we reported miR-221 was up-regulated in both transverse aortic constricted mice and patients with hypertrophic cardiomyopathy (HCM). Forced expression of miR-221 by transfection of miR-221 mimics increased myocyte cell size and induced the re-expression of fetal genes, which were inhibited by the knockdown of endogenous miR-221 in cardiomyocytes. The TargetScan algorithm-based prediction identified that p27, a cardiac hypertrophic suppressor, is the putative target of miR-221, which was confirmed by luciferase assay and Western blotting.

In conclusion, our results demonstrated that miR-221 regulated cardiomyocyte hypertrophy probably through down-regulation of p27, suggesting that miR-221 may be a new intervention target for cardiac hypertrophy. J. Cell. Biochem. 113: 2040–2046, 2012.

KEY WORDS: CARDIAC HYPERTROPHY; microRNA; miR-221; p27

Cardiac hypertrophy is an adaptive mechanism that preserves cardiac ejection performance by normalizing wall stress in response to hemodynamic stress or myocardial injury. Even with normal cardiac function, the cardiomyocytes undergo phenotypic changes that include an increase in the size of the cardiomyocytes. The changes in cellular phenotype are preceded and accompanied by a return to the fetal gene program [Heineke and Molkentin, 2006]. Cardiac hypertrophy to progress over time can depress cardiac function, known as " decompensation of hypertrophy," and clinically results in the heart failure syndrome [Mudd and Kass, 2008], so cardiac hypertrophy has long been known as an independent risk factor for cardiac morbidity and all-cause mortality [Levy et al., 1990; Frey and Olson, 2003]. Molecular mechanisms underlying the development of heart failure remain

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elusive. At present, the mechanism-specific therapies have not been developed. Much effort is centered on the identification of signals and pathways leading to pathological hypertrophy for future rational drug design in heart failure.

Several signaling pathways underlying cardiac hypertrophy have been studied during the past decades [Olson, 2004; Heineke and Molkentin, 2006; Barrick et al., 2007]. Key regulators of these pathways have been shown to impact significantly on the development and progression of cardiac hypertrophy [Rohini et al., 2010]. For example, the tumor suppressor p27 has been identified as a vital repressor of cardiac hypertrophy [Hauck et al., 2008]. p27-knock-out mice has been found to develop age-dependent cardiac hypertrophy and a significant increase in heart/body weight ratio, in left ventricular end diastolic and systolic dimensions, while fractional shortening is reduced in response to transverse aortic constriction (TAC). Therefore, p27 is important in preventing pressure overload–induced deterioration of cardiac function.

Recently, microRNAs (miRs) were found to be regulators of cardiac hypertrophy [Care et al., 2007; van Rooij et al., 2007; Wang et al., 2010; Shieh et al., 2011; Xu et al., 2011]. miRs are highly conserved, short non-coding RNAs that regulate gene expression in post transcriptional processes [Bartel, 2004]. By binding to the 3′UTR in target mRNAs, miRs regulate target gene expression via the repression of translation of or by breaking down mRNA directly [Bagga et al., 2005; Yekta et al., 2004]. Up to now, at least 10 miRs have been identified to play a regulatory role in the process of cardiac hypertrophy [Care et al., 2007; Callis et al., 2009; Lin et al., 2009; Li et al., 2010; Wang et al., 2010, 2011; da Costa Martins et al., 2010; Hua et al., 2011; Shieh et al., 2011; Xu et al., 2011]. Nevertheless, previous reports and microarray data results of miRs in cardiac hypertrophy have implied that several other miRs are dysregulated in cardiac hypertrophy, but their roles in cardiac hypertrophy have yet to be elucidated [Cheng et al., 2007; Sayed et al., 2007; Tatsuguchi et al., 2007].

MiR-221 has been identified in heart and is up-regulated in a mouse model of TAC, implying that miR-221 is involved in cardiac hypertrophy [Sayed et al., 2007; Tatsuguchi et al., 2007]. However, these results were based on microarray data and have not yet been experimentally and clinically validated. In this study, we aimed to explore experimentally whether miR-221 regulates cardiac hypertrophy and which its target is in cardiomyocytes.

MATERIALS AND METHODS

PATIENTS WITH HYPERTROPHIC CARDIOMYOPATHY (HCM) AND THE TAC MOUSE MODEL

The human study was approved by the ethic committee of Beijing FuWai hospital in accordance with the Helsinki declaration. All the patients were asked to provide written consent. The left ventricle tissues were obtained from obstructive hypertrophic cardiomyopathy (HCM) patients who conducted myocardiectomy. Eight non-hypertrophic control hearts were obtained from donors from accidents with no history of cardiac diseases. Medical informed consents were obtained from their relatives. The samples were immediately snap-frozen and stored in liquid nitrogen until further use. All protocols for tissue acquirement and processes carried out on the tissues were performed in compliance with institutional guidelines for human research.

All animal study protocols were approved by the institutional Animal Care and Use Committee. The pressure overload model was obtained through TAC on wild male C57BL/6 mice, as described [Rockman et al., 1991]. Sham-operated mice were served as control to TAC model.

PRIMARY CARDIOMYOCYTE CULTURE

Cardiomyocytes were isolated from newborn Wistar rats aged 1–2 days. In brief, the dissected left ventricles were washed and minced in HEPES-buffered saline solution. The tissues were then dispersed in HEPES-buffered saline solution containing 0.06% collagenase (Worthington Biologicals, Lakewood, NJ) and incubated at 37°C. After dissociation, the cells were centrifuged and then resuspended in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. This was followed by differential preplating to enrich the cardiomyocytes. The enriched cardiomyocytes were then plated at a density of 10^6 cells/ml in DMEM with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM bromodeoxyuridine (BrDu). The media were replaced with a serum-free maintenance medium after a 48-h incubation period; the cells were incubated for another 24 h before any treatment.

QUANTITATIVE REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION (qRT-PCR) ANALYSIS

RNA was isolated using a mirVana miR isolation kit (Ambion, Austin, TX). RNA quality was evaluated by the 260–280 nm optical density ratios. The quantification of miR-221 was conducted by qRT-PCR using a TaqMan miRNA assay (Applied Biosystems, Carlsbad, CA) and normalized with U6 (Applied Biosystems). To assess fetal gene expression levels, the SYBR green method was applied. QRT-PCR for the fetal genes was performed using PrimeScript RT Master Mix Perfect Real Time (Takara Bio, Inc., Shiga, Japan). Real-time PCR was performed using the ABI 7900HT Sequence Detection System (Applied Biosystems), according to the manufacturer's protocols. The amount of target RNA was normalized to the amount of endogenous control glyceraldehyde 3-phosphate dehydrogenate (GAPDH). Fetal gene expression quantities were compared using the relative Ct method, as described by Schmittgen and Livak [2008].

The following forward and reverse primers were used:

ANP: 5′-GGGCTCCTTCTCCATCAC-3′, 5′-CGGCATCTTCTCCTC-CAG-3′; BNP: 5′-AGAACAATCCACGATGCAGAAG-3′, 5′-AAA-CAACCTCAGCCCGTCACA-3′; ACTA1: 5′ TCA GGC GGT GCT GTC TCT CT 3′, 5′ TCC CCA GAA TCC AAC ACG A 3′; GAPDH: 5′-CTCTACCCACGGCAAGTTC-3′, 5′-GCCAGTAGACTCCACGACA-TA-3′.

miRNA MIMIC OR ANTAGOMIR TRANSFECTION IN CARDIOMYOCYTES

After serum starving for 24 h, miR-221 mimic or antagomir (Gene-Pharma Co. Shanghai, China) was transfected into cardiomyocytes using Lipofectamine 2000 (Invitrogen, CA), according to the manufacturer's protocol. The transfected concentration was 100 nM.

CARDIOMYOCYTE IMMUNOCHEMISTRY AND CELL SURFACE AREA MEASUREMENT

Cardiomyocytes were plated at a density of 5×10^5 cells/ml. After treatment for 48 h, the cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 1% Triton X-100 in PBS, followed by blocking with 1% BSA in PBS for 30 min at room temperature. They were incubated with Texas red phalloidin (Invitrogen) in PBS for 30 min at room temperature. After washing with PBS, the nuclei were stained with 0.1 μg/ml 4′, 6-diamidino-2-phenylindole in PBS. Immunofluorescence was analyzed using a fluorescence microscope. An average of 150 cardiomyocytes from 30 fields were chosen at random for measurement of cell sizes, as described [Liu and Olson, 2002; Lin et al., 2009]. Briefly, Immunofluorescence images captured from cardiomyocytes stained with Texas red phalloidin (Invitrogen) were opened in ImageJ software (http://rsb.info.nih.gov/ij/). After setting the threshold, areas of 150 cells per group were outlined and bucket-filled in Gimp. Cardiomyocyte area was calculated by use of the ImageJ Particle Analyzer algorithm [Siedlecki et al., 2009]. Where cells were adjacent, care was taken to manually make a clean and minimal-width division of their respective areas. Data from all the fields were combined and analyzed.

PLASMID CONSTRUCTION AND LUCIFERASE ASSAY

The full length 3′UTR of p27 was cloned by standard procedures into the pMIR-Report vector (Ambion, Austin, TX), immediately downstream of the stop codon of the luciferase gene to generate the pMIR-p27-3′UTR luciferase reporter plasmid. Mutagenesis of the pMIR-p27-3′UTR was performed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The two binding sites of miR-221 on the p27 3′UTR were mutated simultaneously (double mutated: DMut) to analyze their functional role. Cells were cotransfected with 2 μg wildtype pMIR-p27-3′UTR (or with mutated Pmir-3′UTR, DMut), 50 pmol miRNA mimics, and 0.01 μg Renilla in 24 plates. Forty-eight hours after transfection, the cells were washed and lysed with Passive Lysis buffer (Promega, Madison, WI), and the luciferase activity was measured using a luminometer (SIRIUS, Pforzheim, Germany) as described previously [Chen et al., 2010].

WESTERN BLOT ANALYSIS

Cardiomyocytes were plated at a concentration of 10^6 cells/ml. After transfection of miR-221 mimic or antagomir for 48 h, the cells were lysated with RIPA buffer (1 × PBS, 1% NP40, 0.1% SDS, 5mM EDTA, 0.5% Sodium Deoxycholate, 1 mM Sodium Orthovanadate, 1% PMSF). After centrifuging at 12,000 rpm for 15 min at 4°C to remove the cell debris, the supernatant was transferred and the protein concentration was assessed using a BCA protein assay kit. The samples were resolved by 10% SDS–PAGE and transferred to nitrocellulose membrane. After blocking with 5% skimmed milk at room temperature for 1 h, the membrane was incubated with p27 antibody (Cell Signaling, Danvers, MA, dilution 1:1,000) or GAPDH (Cell Signaling, dilution 1:3,000) at 4°C overnight. After washing, fluorescence-conjugated rabbit secondary monoclonal antibody (Cell Signaling, dilution 1:1,000) was added and incubated at room temperature for 1 h. Proteins were detected using the AP-NBT/BCIP system.

STATISTICS

All data are expressed as mean ± SE. Two-tailed unpaired Student's *t*-test was used for statistical comparisons. *P*-value <0.05 was considered to be significant.

RESULTS

EXPRESSION OF miR-221 INCREASED IN THE LEFT VENTRICULAR TISSUE OF TAC MICE AND IN HCM PATIENTS

Data of the echocardiographic parameters of TAC mice at 2 and 9 weeks were provided in the supplementary material (Fig. S1 and Fig. S2) to show that our TAC hypertrophic models were successfully established. Moreover, as ejection fraction of TAC mice at 2 weeks was remained unchanged, data of foetal gene expression of our TAC mice at 2 weeks was also provided in supplementary material, serving as the molecular evidence that our 2-week TAC mice were undergoing pathological hypertrophy (Fig. S3).

To determine the potential involvement of miR-221 in cardiac hypertrophy, we performed qRT-PCR analysis to examine the expression levels of miR-221 in the left ventricular tissue of the TAC mouse models and in the HCM patients. As indicated in Figure 1, increased expression levels of miR-221 were observed in hypertrophic and heart failure mouse hearts induced by 2-week and 9-week

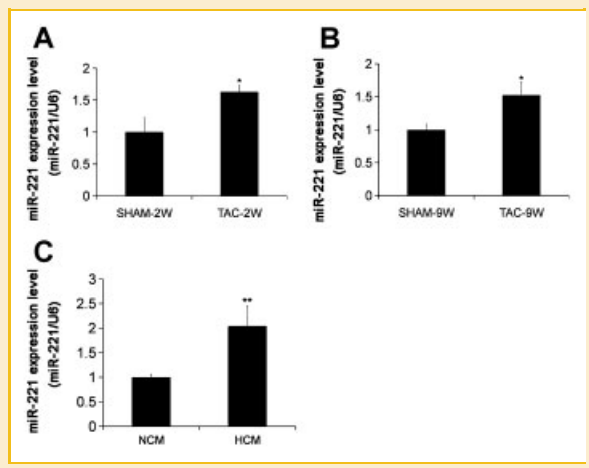

Fig. 1. miR-221 expression in cardiac hypertrophy. A: qRT-PCR analysis and relative expression values of miR-221 in sham-operated and transverse aortic constricted (TAC) mice at 2 weeks (SHAM-2W and TAC-2W, n = 4 mice per group). *P*-value indicates comparison to the sham group (B) qRT-PCR analysis and relative expression values of miR-221 in sham-operated and TAC mice at 9 weeks (SHAM-9W and TAC-9W, n = 6 mice per group). *P*-value indicates comparison to the Sham group. C: qRT-PCR analysis and relative expression values of miR-221 in humans with hypertrophic cardiomyopathy. Relative expression values of miR-221 were obtained by normalization with U6. Expression in the ventricles was measured in patients with hypertrophic cardiomyopathy and in normal controls (n = 4 for the disease samples; n = 8 for the control samples). NCM, control samples; HCM, hypertrophic cardiomyopathy samples. Data represent mean ± SE based on a minimum of three experiments. $^{*}P<0.05$, $^{**}P<0.01$.

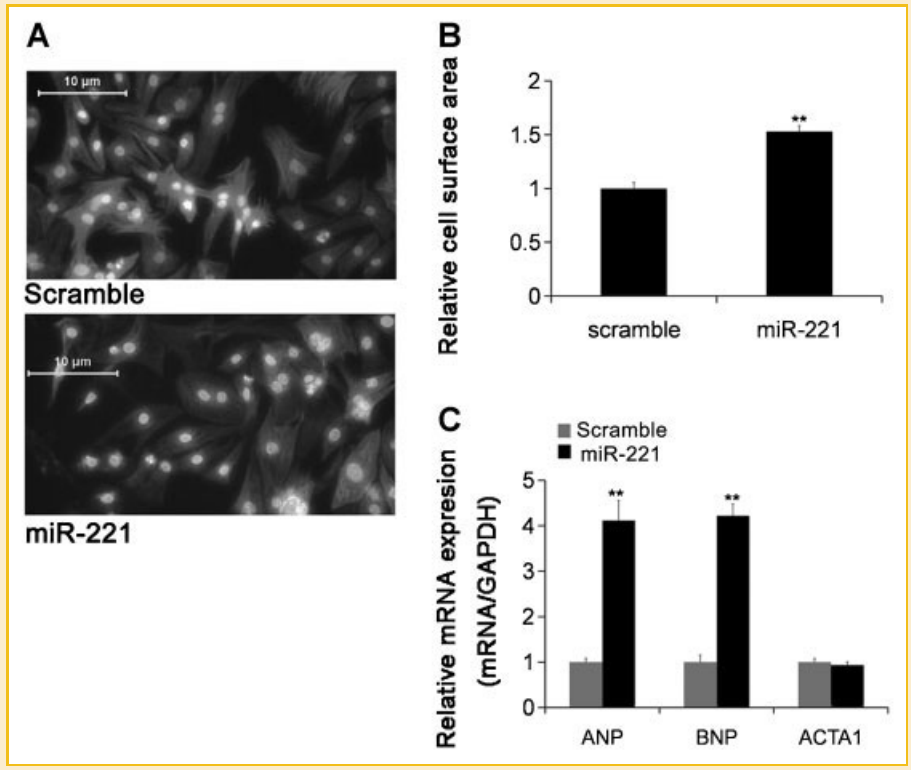

Fig. 2. Up-regulation of miR-221 induced cardiomyocyte hypertrophy. A: Representative fluorescence images produced using cardiomyocyte immunochemistry showing that the up-regulation of miR-221 in cardiomyocytes significantly increased myocyte size. Scale bar 10 μM. B: Quantitative analysis of cardiomyocyte cell size. About 150 cells that were immunostained with red phalloidin were randomly chosen from each treatment for surface area measurement using ImageJ software. The results are presented as relative cell area compared with the controls. C: qRT-PCR analysis of the hypertrophic marker genes ANP, BNP, and ACTA1 using total RNA isolated from cardiomyocytes treated with miR-221 or with the scramble, glyceraldehyde 3-phosphate dehydrogenate (GAPDH) was used as the reference gene. Data represent mean ± SE based on a minimum of three experiments.** $P < 0.01$ compared with the scrambles.

thoracic aortic banding (1.5-fold and 1.6-fold, respectively; $P < 0.05$). Moreover, the expression level was up-regulated twofold in patients with HCM. These results confirmed that miR-221 expression levels consistently increased through the different stages of cardiac hypertrophy, as well as in the different types of cardiac hypertrophy stimulated by both extrinsic and intrinsic stress, suggesting that miR-221 is a universal participator in cardiac hypertrophy.

OVEREXPRESSION OF miR-221 INDUCED CARDIOMYOCYTE HYPERTROPHY

To analyze the effects of miR-221 overexpression in vitro, we forced the expression of miR-221 in primary neonatal rat cardiomyocytes by transfection with miR-221 mimics. After 48 h transfection, we measured the parameters of cardiomyocyte hypertrophy, including cell size enlargement and fetal gene reactivation. As shown in Figure 2B, the mean cardiomyocyte size increased by 53%. Fetal genes, atrial natriuretic factor (ANP) and b-type natriuretic protein (BNP) were up-regulated 3.1-fold and 3.2-fold, respectively (Fig. 2C), suggesting that overexpression of miR-221 in cardiomyocytes was sufficient to provoke cardiomyocyte hypertrophy. No significant alteration was observed in the expression of skeletal muscle alpha-actin (ACTA1).

ATTENUATION OF miR-221 INHIBITED THE EXPRESSION OF A CARDIAC HYPERTROPHIC MARKER

To assess the functional consequences of silencing endogenous miR-221 in vitro, we transfected miR-221 antagomir in primary neonatal rat cardiomyocytes. Inhibition of endogenous miR-221 produced no significant change in cardiomyocyte size. However, miR-221 inhibition resulted in significantly decreased fetal gene re-expression. In a representative experiment, ANP and ACTA1 were down-regulated by 30 and 50%, respectively. BNP expression was also found to be decreased, but not significantly (Fig. 3). These results showed that inhibition of miR-221 in vitro might attenuate the hypertrophic response.

p27 IS THE TARGET OF miR-221 IN CARDIOMYOCYTES

To explore the mechanism by which miR-221 regulated cardiomyocyte hypertrophy, we searched for the targets of miR-221 using the TargetScan algorithm. Bioinformatic analysis led to the identification of p27, p57, DYRK1A, PAK1, and RGS6 as potential targets of miR-221 (Fig. 4A). Of these potential targets, p27 has been reported earlier to be translationally inhibited by miR-221 in tumor cells [Yang et al., 2011]. p27 is expressed in adult cardiomyocytes and down-regulated in response to hypertrophic stimuli in vivo [Li and Brooks, 1997]. Furthermore, p27-knockout mice developed age-

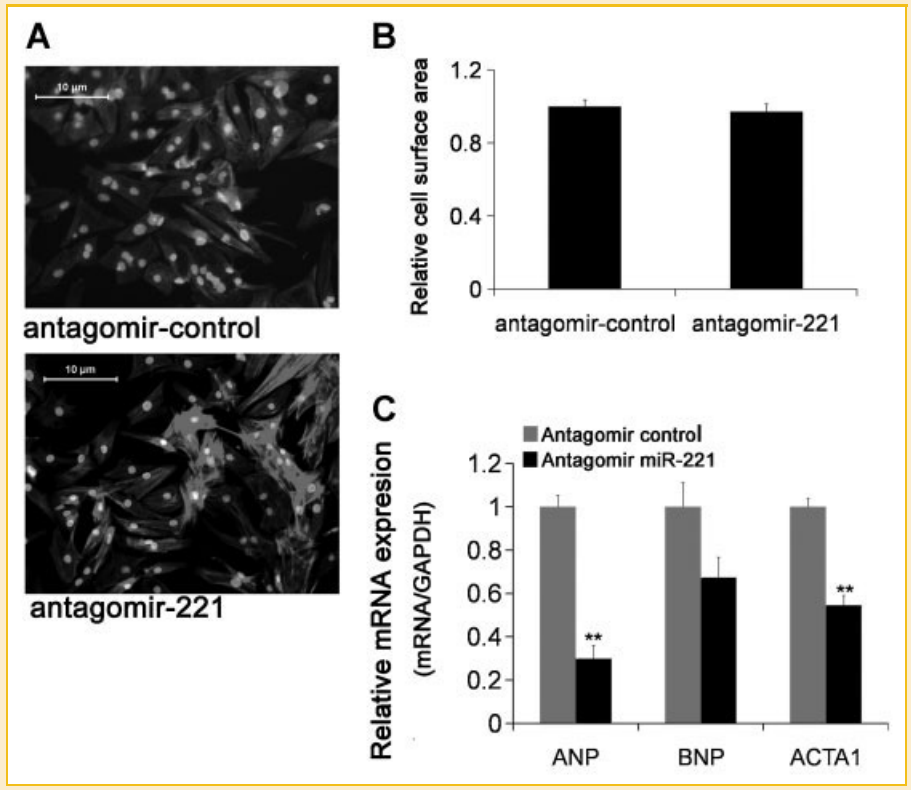

Fig. 3. Inhibition of miR-221 in cardiomyocyte. A: Representative fluorescence images produced using immunochemistry with inhibition of miR-221 in cardiomyocytes. Scale bar 10 μM. B: Quantitative analysis of cardiomyocyte cell size. About 150 cells that were immunostained with red phalloidin were randomly chosen from each treatment for surface area measurement using ImageJ software. The results are presented as relative cell area compared with the controls. C: qRT-PCR analysis of the hypertrophic marker genes ANP, BNP, and ACTA1 using total RNA isolated from cardiomyocytes treated with antagomir-221 or with the antagomir-control, GAPDH was used as the reference gene. Data represent mean ± SE based on a minimum of three experiments.$^{**}P < 0.01$ compared with the antagomir-controls.

dependent cardiac hypertrophy and showed exaggerated hypertrophic responses to aortic banding [Hauck et al., 2008]. Our bioinformatic analysis showed that p27 mRNA 3′UTR regions contained two conserved "seed" sequences complimentary to the miR-221 sequence (Fig. 4B). This evidence and our results strongly support that p27 may be regulated by miR-221 in cardiomyocytes. To test this hypothesis, we conducted western blotting to analyze p27 protein levels in primary neonatal rat cardiomyocytes with transfection of miR-221 mimic or antagomir. As indicated in Figure 4D, miR-221 mimic overexpression caused a significant decrease in the level of the p27 protein. This suggested that p27 is a target for miR-221 in cardiomyocytes. To further examine this interaction, we performed luciferase reporter assays in HeLa cells. As indicated in Figure 4C, cotransfection of miR-221 with wildtype pMIR-p27-3′UTR resulted in a significant decrease in luciferase activity. This decrease was restored by cotransfection of miR-221 with constructs containing mutated 3′UTR sequences. These results demonstrated that p27 was a real target of miR-221 in cardiomyocytes.

DISCUSSION

The present study is the first report that miR-221 promotes cardiomyocyte hypertrophy through the modulation of p27 expression and the first to suggest that miR-221 might be a useful new intervention target for cardiac hypertrophy.

The TAC mouse model is a classical model of cardiac hypertrophy induced by extrinsic pressure overload. The sudden onset of blood pressure achieved with TAC caused an approximately 50% increase in left ventricular mass within 2 weeks [Patten and Hall-Porter, 2009]. The increased mass represents the hypertrophic stage and progresses to the decompensated stage by 9 weeks [Barrick et al., 2007]. In the present study, we showed that miR-221 expression increased 1.6-fold in a 2-week TAC mouse model and 1.5-fold in a 9-week TAC mouse model, suggesting that miR-221 may be involved in the throughout development and progression of cardiac hypertrophy. In addition, the up-regulation of miR-221 expression in both the hypertrophic and decompensated stages may account for the mechanism by which the heart transitions from the hypertrophic stage to heart failure stage in response to stimuli. On the other hand, HCM is known to be caused mainly by gene dysfunction [Maron, 2002] which represents another source of hypertrophy induced by intrinsic stimulus. In the present study, we found a twofold up-regulation of miR-221 expression level in HCM patients. In summary, the up-regulation of miR-221 in both TAC mice and HCM patients suggested that miR-221 may universally participate in different types of cardiac hypertrophy.

In the present study, for the first time, we found that p27 is a target of miR-221 in cardiomyocytes, supporting that the

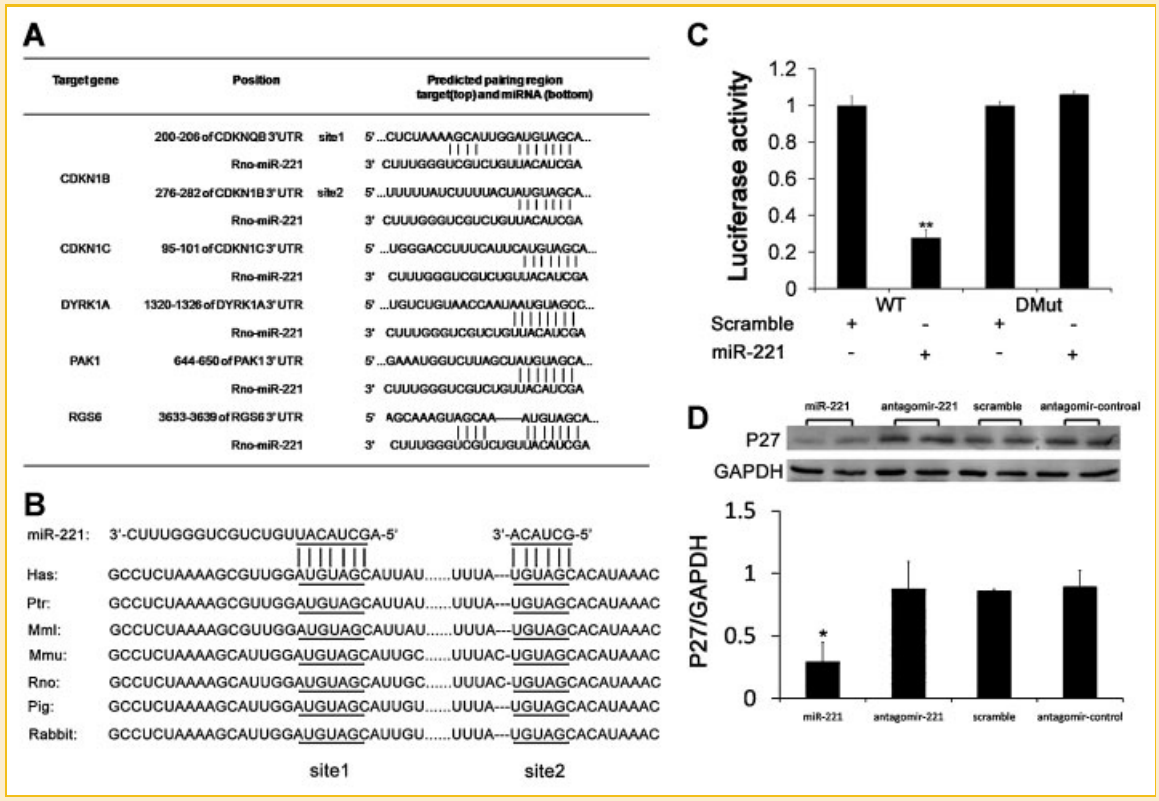

Fig. 4. p27 as a target of miR-221 in cardiomyocytes.A: Potential targets of miR-221 were predicted using the TargetScan algorithm. The predicted targets were CDKN1B (p27), CDKN1C (p57), DYRK1A, PAK1, and RGS6. B: The potential target sites in the 3′ UTR of p27 were conserved in different species. The sequences were aligned using TargetScan. C: Luciferase reporter assays performed by cotransfection of miR-221 mimic with a luciferase reporter gene linked to the p27 3′ UTR, containing either the wild-type (WT) or the mutated miR-221 complementary sites. DMut is the double mutant of the miR-221 complementary sites, site1 and site2. Mean ± SE with a minimum of four experiments per group. D: Western blotting was used to analyze p27 protein levels. GAPDH was used as the endogenous control. $^{*}P < 0.05$, $^{**}P < 0.01$.

up-regulation of miR-221 might be responsible for the down-regulation of p27 during hypertrophy. Considering previous reports that the p27-knockout mice developed age-dependent cardiac hypertrophy and showed exaggerated hypertrophy in response to aortic banding, we deduced that the function of miR-221 in cardiomyocyte hypertrophy might be, at least partially, mediated by the inhibition of p27 expression. However, the relationship between p27 and miR-221 in cardiac hypertrophy remains to be further investigated.

Here, we verified that miR-221 is a regulator of p27 in cardiomyocytes. p27 is a hypertrophic suppressor that is expressed robustly in the heart. Hypertrophic stimulus resulted in the down-regulation of p27, thus contributing to the development of cardiac hypertrophy. However, it is not fully elucidated how p27 is regulated in cardiac hypertrophy. Hauck et al. [2008] have found that hypertrophy induced the proteasomal degradation of p27 through protein kinase CK2-dependent phosphorylation, providing the kinase-based mechanism of p27 regulation in cardiomyocytes. Our results throw the first light on p27 regulation in cardiomyocytes at the epigenetic level.

In the present study, we verified that p27 might be the real target of miR-221 in cardiomyocyte. However, a single miR may have multiple targets. As our bioinformatics analysis indicated, miR-221 has several other targets in cardiomyocytes. While p27 is highly expressed in adult cardiomyocytes, it is down-regulated in response to hypertrophic stimuli in vivo. It is known that p27-knockout mice develop age-dependent cardiac hypertrophy and show exaggerated hypertrophic responses to aortic banding. This evidence prompted us to validate p27 as the authentic target of miR-221 in cardiomyocytes. However, the pro-hypertrophic role of miR-221 in cardiomyocytes reported in this study could also be mediated through other targets. Further studies should explore other targets of miR-221 to fully elucidate the role of miR-221 in cardiac hypertrophy.

Limitations exist in the present work. First, our results showed the pro-hypertrophic effects of miR-221 in baseline cardiomyocyte hypertrophy. How miR-221 responds to the classical stimuli of cardiomyocyte hypertrophy is unknown. Whether miR-221 responds to a specific hypertrophic stimulus or to comprehensive hypertrophic stimuli is also an enigma. Further studies may focus on these problems. Furthermore, although our in vitro study suggests that miR-221 is a regulator of cardiac hypertrophy, its role in vivo remains to be further investigated. Whether the up-regulation of miR-221 could induce cardiac hypertrophy or whether the inhibition of miR-221 could attenuate cardiac hypertrophy in vivo still need to be investigated.

In conclusion, miR-221 is a regulator of cardiomyocyte hypertrophy. It may become a novel potential therapeutic target for cardiac hypertrophy.

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