

MicroRNA-1 suppresses cardiac hypertrophy by targeting nuclear factor of activated T cells cytoplasmic 3

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Abstract. Cardiomyocyte hypertrophy is a threat to human health due to the probability of sudden heart failure-induced mortality. Previous studies have demonstrated that nuclear factor of activated T cells cytoplasmic 3 (NFATC3) is important in the process of cardiomyocyte hypertrophy. However, the molecular mechanism underlying the alteration in the expression levels of NFATC3 during cardiomyocyte hypertrophy has remained to be fully elucidated. In order to shed light on the molecular mechanism, the present study employed several approaches, including the measurement of the cell surface area, analysis of the protein/DNA ratio, western blot analysis and a Luciferase reporter assay using isolated rat cardiomyocytes as model. The results showed that expression of *microRNA-1* (*miR-1*) was reduced in patients diagnosed with cardiac hypertrophy and rat cardiomyocytes treated with pro-hypertrophic stimuli. The increase in the expression of *miR-1* was able to inhibit the hypertrophic remodeling of cardiomyocytes. The suppression of *miR-1* was sufficient to induce cardiomyocyte hypertrophy, and further experiments confirmed that NFATC3 was a target of *miR-1* in cardiomyocytes. Forced expression of NFATC3 inhibited the protective activity of *miR-1* against hypertrophic stimuli in the cardiomyocytes. These findings provided clarification of the regulatory signaling pathway underlying cardiac hypertrophy, and provided evidence that targeting the *miR-1*/NFATC3 pathway may be a promising strategy for the prevention and treatment of heart hypertrophy.

Introduction

Cardiomyocyte hypertrophy is a compensatory heart response towards harmful stimuli, and patients with cardiomyocyte

hypertrophy usually have a poor prognosis, with the onset of heart systolic and diastolic dysfunction, and ultimately sudden heart failure-induced mortality (1). In order to develop novel therapeutic strategies, the molecular pathways underlying cardiomyocyte hypertrophy require further elucidation.

MicroRNAs (miRNAs; miRs) belong to a group of 20-22 nucleotide-long non-coding RNAs, which suppress the expression of target mRNAs by binding their 3'-untranslated region (UTR) in a miRNA recognition element (MRE)-dependent mechanism (2). Accumulated evidence has demonstrated that miRNA is closely associated with the initiation and progression of cardiomyocyte hypertrophy (3). *miR-1* has been demonstrated to suppress cardiac hypertrophy by decreasing the expression levels of calmodulin and Mef2a (4). The anti-hypertrophic effect of *miR-1* may also be associated with the insulin-like growth factor signaling pathway (5), the twinfilin-1 cytoskeleton regulatory protein (6), NCX1 and Anxa5 proteins (7). The introduction of *miR-1* was demonstrated to reverse the pathological remodeling induced by pro-hypertrophic stimuli (8). Isoproterenol (ISO) is a β -adrenoceptor activator, which has been confirmed to exert cardioprotective effects by promoting the expression of *miR-1* (9). Notably, cardiac *miR-1* abundance can be indirectly detected by evaluating the serum expression levels of fatty acid binding protein 3 in patients with cardiomyocyte hypertrophy, suggesting that *miR-1* is a promising diagnostic biomarker for susceptibility to cardiomyocyte hypertrophy (10). These findings indicate that *miR-1* is an attractive epigenetic factor regulating cardiac hypertrophy. However, the downstream effectors of *miR-1* have not been examined in full.

Nuclear factor of activated T cells cytoplasmic 3 (NFATC3) is important in the initiation and progression of cardiomyocyte hypertrophy. Calcineurin is a serine/threonine protein phosphatase, which dephosphorylates NFATC3, resulting in its nuclear localization, and these events lead to the pathological remodeling of hypertrophic cardiomyocytes (11,12). However, the regulatory pathways located upstream of NFATC3, particularly miRNAs targeting this pro-hypertrophic factor, remain to be fully elucidated.

The present study aimed to investigate the possible molecular associations between *miR-1* and NFATC3 during the response of cardiomyocytes in the presence of pro-hypertrophic stimuli. The present study may enhance the current

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understanding of the molecular mechanisms associated with cardiac hypertrophy.

Materials and methods

Patients and samples. Heart tissue samples were collected from patients succumbed to cardiac hypertrophy ($n=15$; age, 40–86 years; males, $n=7$; females, $n=8$) and patients who succumbed to conditions other than cardiac hypertrophy as controls ($n=15$; age, 32–77 years; males, $n=9$; females, $n=6$) at the First Affiliated Hospital of Xinxiang Medical University (Weihui, China). Fresh tissues were obtained with written informed consent from all patients according to protocols approved by Ethical Review Board in the First Affiliated Hospital of Xinxiang Medical University (Weihui, China).

Cardiomyocyte isolation and culture. The experimental animal protocol of the present study was approved by the ethics committee of the First Affiliated Hospital of Xinxiang Medical University (Weihui, China). Cardiomyocytes were obtained from 8-week-old Wistar rats ($n=9$; male; weight, 4–6 g; purchased from Jinfeng Experimental Animal Company, Jinan, China) kept in ventilated housing with 50–60% humidity at 18–22°C following previously described procedures (13). Briefly, the hearts were harvested and homogenized with HEPES (Sigma-Aldrich)-buffered saline following sacrifice of the animals by CO₂ aspiration. The heart tissue samples were subsequently treated with 1.2 mg/ml pancreatin and 0.14 mg/ml collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) in HEPES-buffered saline. The cells were then resuspended in Dulbecco's modified Eagle's medium/F-12 (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 5% heat-inactivated horse serum (Invitrogen Life Technologies), 0.1 mM ascorbate (Sigma-Aldrich), insulin-transferrin-sodium selenite medium (Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin and 0.1 mM bromodeoxyuridine (all from Sigma-Aldrich) at 1×10^6 cells/ml prior to being plated at 5×10^5 cells/well and cultured at 37°C for 1 h. Cells were treated with ISO (10 µM; Sigma-Aldrich) and Aldomet (Aldo; 1 µM; Sigma-Aldrich) for the indicated durations (0, 1, 2, 4 or 8 h).

Analysis of *miR-1* expression using RT-qPCR. Total RNA was extracted from the coronary tissue samples using TRIzol™ reagent (Sigma-Aldrich, St Louis, MO, USA). The RT reaction was performed using the TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. qPCR was performed using TaqMan® 2X Universal PCR Master mix (Applied Biosystems) on a CFX96™ Real-Time PCR Detection system (Bio-Rad Laboratories, Hercules, CA, USA) supplied with analytical software. The reactions were incubated in an 9700 Thermocycler (Applied Biosystems) in a 96-well plate for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then held at 4°C. The primers are as follows: *miR-1*, GAGTTGCCTGACTTCT, and TTCAACAGGCCTAGTA (Thermo Fisher, Grand Island, NY, USA); β -myosin heavy chain (β -MHC), CTGGCACCGTGGACTACAAC, and CGCACAAAGTGAGGATAGGGT (Sangon Biotech,

Shanghai, China). The $\Delta\Delta CT$ method was used to quantify the abundance of *miRNA* or *mRNA*.

Adenoviral construction and infection. A recombinant adenovirus expressing the constitutively active form of NFATC3 (Ad-NFATC3) was obtained from Dr Michael C. Naski (Department of Pathology, University of Texas Health Science Center, San Antonio, TX, USA) (14). An adenoviral vector containing enhanced green fluorescent protein (Ad-EGFP), was provided by Dr Liu (Medical College, Qingdao University, Qingdao, China) (15) using a TCID50 Quick Determination kit (Jimei Biotech, Shanghai, China), and used as a negative control. The propagation and titration of the adenoviruses was performed as previously described (16). The adenoviruses were added to the cultures 48 h prior to the experiments at a multiplicity of infection of 10 and incubated at 37°C for 3 h, following which the adenoviruses were removed by replacing with fresh culture medium.

Measurement of cell surface area. The cell surface area of the experimental cells was determined based on the fluorescent staining of F-actin in the cardiomyocytes, as previously described (17). Briefly, following the indicated treatments, the cardiomyocytes were fixed with 4% paraformaldehyde and treated with 0.1% Triton X-100. The cells were then incubated overnight with fluorescent phalloidin-tetramethylrhodamine conjugate (Sigma-Aldrich) at room temperature, prior to being visualized using a Zeiss LSM 510 META laser confocal microscope (Zeiss, Oberkochen, Germany). A total of 100 cells were counted in 30–50 randomly-selected fields. The size of the area outlined by F-actin was determined using ImageJ software (version 1.48u; National Institutes of Health, Bethesda, MD, USA). The data are expressed as the mean \pm standard deviation.

Analysis of the protein/DNA ratio. The evaluation of the protein/DNA ratio in the experimental cardiomyocytes was performed, as described in a previous study (13). Salmon sperm, perchloric acid and KOH were purchased from Shanghai Sangon Biotech. Hoechst dye and human serum albumin were purchased from Sigma-Aldrich.

Western blot analysis. The procedures used to perform western blotting assays were those previously described (15). Briefly, the total protein was extracted from the cells using PER Mammalian Protein Extraction reagent (200 µl for 1×10^6 cells; Thermo Fisher Scientific) and the protein concentration was determined using the bicinchoninic acid assay (Invitrogen Life Technologies). A total of 20 µg protein per lane was separated by 10% SDS-PAGE and subsequent transfer onto 0.45-µm nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were then blocked with 5% fat-free dry milk and incubated with the indicated primary antibodies (Abs), including GAPDH (D16H11) XP® rabbit monoclonal Ab (cat no. 5174; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) and NFATc3 mouse monoclonal Ab (cat no. sc-8405; 1:500, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), overnight at 4°C. After three washes with phosphate-buffered saline containing Tween 20, membranes were incubated with goat anti-rabbit and anti-mouse antibodies

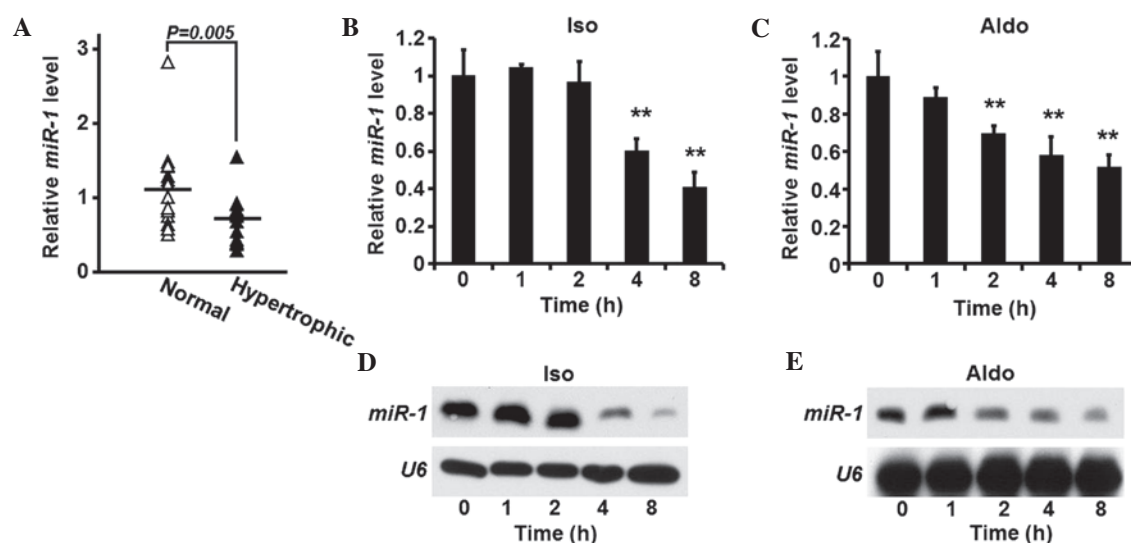


Figure 1. *miR-1* was suppressed in the heart tissue samples of patients with cardiac hypertrophy, and its expression levels were also decreased in rat cardiomyocytes treated with ISO or Aldo. (A) Expression levels of *miR-1* were determined using RT-qPCR analysis in the cardiac tissue samples of patients with cardiac hypertrophy (n=15) and healthy volunteers (n=15). Rat cardiomyocytes were treated with (B) ISO (10 μM) or (C) Aldo (1 μM). At the indicated time-points, the expression levels of *miR-1* were quantified in the cells using RT-qPCR analysis. (D and E) Expression levels of *miR-1* were further quantified by western blot analysis in the rat cardiomyocytes following treatment. Values are expressed as the mean ± standard deviation of three independent experiments. **P<0.01 vs. 0 h. *miR-1*, microRNA; Iso, isoproterenol; Aldo, Aldomet; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

(cat. nos. ZDR-5306 and ZDR-5307, respectively; 1:5,000; ZSGB-BIO, Beijing, China) for 1 h. Finally, the blots were visualized using SuperSignal West Dura Extended Duration substrate (Thermo Fisher Scientific, Inc.). ImageJ software was used for quantification of the blots.

Treatment of the miRNA mimics. The *miR-1* and control mimics were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The cells were transfected with either 300 nM control mimics or 300 nM *miR-1* mimics. The experiments described above were performed 48 h post-transfection.

Luciferase assay. To investigate whether NFATC3 is a target of *miR-1*, two recombinant vectors were constructed to express luciferase under the regulation of the 3'-UTR of NFATC3 mRNA, containing either wild-type *miR-1* MREs or mutant MREs (pMIR-REPORT-NFATC3-wt and pMIR-REPORT-NFATC3-mut, respectively). At 48 h post-transfection with Lipofectamine® 2000 (Invitrogen Life Technologies), lysis buffer (Promega Corporation, Madison, WI, USA) was added to the cell culture, and the expression of luciferase was detected using a Dual-Luciferase Reporter system (Promega Corp.), according to the manufacturers' instructions.

Statistical analysis. Statistical significance was calculated using Students' *t*-test. All statistical analyses were performed using SPSS software version 19.0 (International Business Machines, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-1 is suppressed in heart tissue samples of patients with cardiac hypertrophy. Heart tissue samples were obtained from

patients suffering from cardiomyocyte hypertrophy (n=15). In addition, cardiac tissue samples obtained from donors without heart diseases, which served as controls (n=15). The expression levels of *miR-1* were quantified in the tissue samples, and compared between the two groups. The expression of *miR-1* was significantly suppressed in the hypertrophic heart tissue samples, compared with the control group (Fig. 1A).

Expression of miR-1 is reduced in rat cardiomyocytes following pro-hypertrophic treatment. As *miR-1* is aberrantly expressed in human hypertrophic heart tissue samples, the expression levels of *miR-1* were also investigated in rat cardiomyocytes. Isolated rat cardiomyocytes were treated with ISO or Aldo, and the expression levels of *miR-1* were quantified. The expression levels of *miR-1* gradually decreased following each treatment (Fig. 1B and C). Western blotting was used to confirm the reduction in the expression levels of *miR-1* induced by ISO or Aldo (Fig. 1D and E).

miR-1 attenuates hypertrophic responses in rat cardiomyocytes in vitro. As the expression levels of *miR-1* were found to be reduced in the hypertrophic heart tissue samples, the potential changes in the expression levels of *miR-1* were investigated, in order to determine whether the changes in expression levels affected the hypertrophic response of the cardiomyocytes. Synthetic miRNA mimics were then used to restore the expression of *miR-1* in the cardiomyocytes pre-treated with ISO or Aldo (Fig. 2A and B). Restoration of the expression of *miR-1* was demonstrated to reduce the cell surface area of the cardiomyocytes (Fig. 2C and D). In addition, the ratio of protein to DNA was decreased when *miR-1* was overexpressed (Fig. 2E and F), and the mRNA expression levels of β-MHC were also reduced (Fig. 2G and H). These results suggested that *miR-1* suppression is required for the pro-hypertrophic activity of ISO and Aldo.

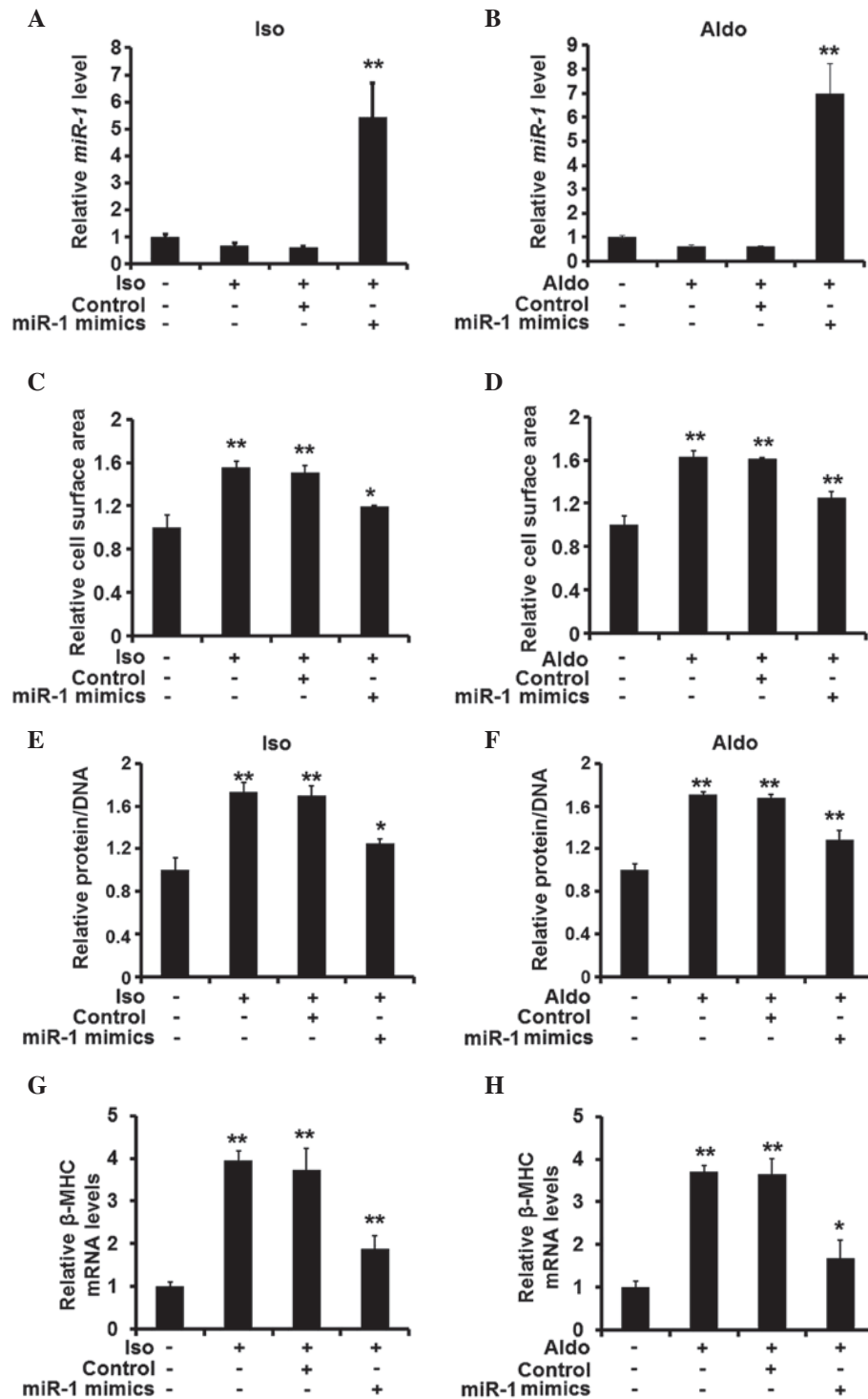


Figure 2. *miR-1* attenuates the hypertrophic response of rat cardiomyocytes *in vitro*. The rat cardiomyocytes were treated with (A) ISO (10 μ M) or (B) Aldo (1 μ M) following 48 h treatment with the control or *miR-1* mimics (30 nM). After 4 h, the expression of *miR-1* was detected in the cells using reverse transcription-quantitative polymerase chain reaction. The (C and D) cell surface area, (E and F) ratio of protein to DNA and (G and H) mRNA expression levels of β -MHC were evaluated in rat cardiomyocytes treated with ISO (10 μ M) or Aldo (1 μ M). Values are expressed as the mean \pm standard deviation of three independent experiments. * P <0.05; ** P <0.01 vs. untreated group. *miR-1*, microRNA; Iso, isoproterenol; Aldo, Aldomet; MHC, β -MHC, β -myosin heavy chain.

Silencing of miR-1 is sufficient to initiate cardiomyocyte hypertrophy. Following establishment of the role of *miR-1* in the hypertrophic response of rat cardiomyocytes, the present study investigated the effects of *miR-1* silencing on the cardiomyocytes. A synthetic inhibitor specific for *miR-1* was used to reduce the expression levels of endogenous *miR-1* (Fig. 3A). Following treatment with the inhibitor, the surface area of

the cardiomyocytes increased significantly (Fig. 3B), and the protein/DNA ratio (Fig. 3C) and expression of β -MHC also increased (Fig. 3D). These results suggested that silencing of the expression of *miR-1* induced cardiomyocyte hypertrophy.

miR-1 targets NFATC3 in rat cardiomyocytes. As *miR-1* is important in suppressing cardiomyocyte hypertrophy,

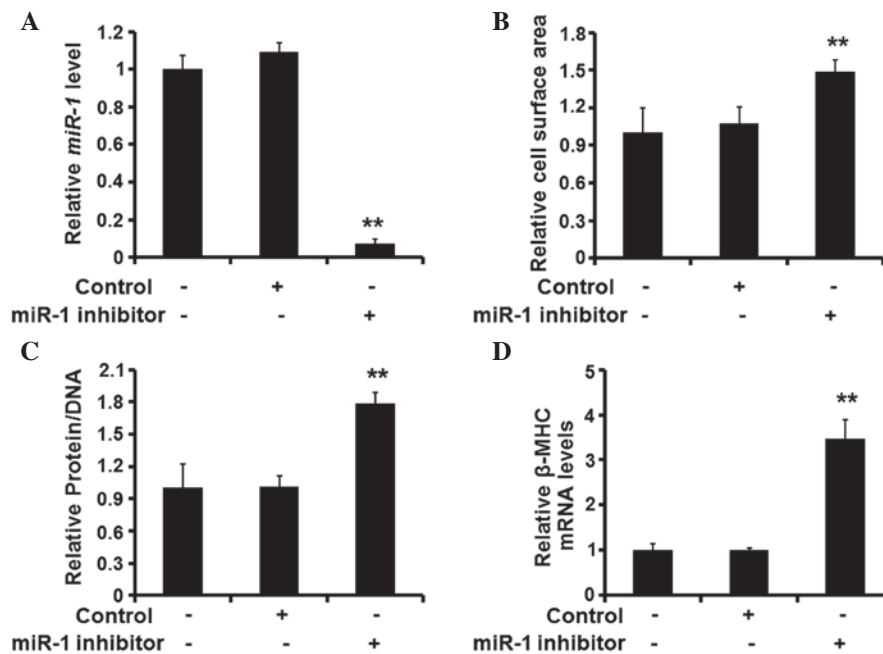


Figure 3. *miR-1* silencing is sufficient to initiate cardiomyocyte hypertrophy. Rat cardiomyocytes were treated with control or *miR-1* inhibitor (10 nM). After 48 h, the (A) expression levels of *miR-1* were quantified in the cells using reverse transcription-quantitative polymerase chain reaction. The (B) cell surface area, (C) ratio of protein and DNA and (D) mRNA expression levels of β -MHC were evaluated in the rat cardiomyocytes treated with the control or *miR-1* inhibitor (10 nM). Values are expressed as the mean \pm standard deviation of three independent experiments. ** $P < 0.01$ vs. mock group. *miR-1*, microRNA; Iso, isoproterenol; β -MHC, β -myosin heavy chain.

the present study investigated its underlying molecular mechanisms. Potential targets of *miR-1* were screened using the online database, TargetScan (<http://www.targetscan.org/>). NFATC3, a putative transcription factor that enhances hypertrophic response by promoting the expression of myocardin (18), was among the predicted targets of *miR-1*. One copy of *miR-1* MRE was located within the 3'-UTR of NFATC3 mRNA (Fig. 4A). There was an inverse association between the expression levels of *miR-1* and NFATC3 in the human heart tissue samples (Fig. 4B). The expression levels of NFATC3 increased and decreased in the rat cardiomyocytes following *miR-1* silencing and overexpression, respectively (Fig. 4C and D). A luciferase reporter assay demonstrated that the suppression of endogenous *miR-1* increased the expression levels of luciferase by pMIR-REPORT-NFATC3-wt (Fig. 4E), whereas increased expression of *miR-1* reduced the expression levels of luciferase by pMIR-REPORT-NFATC3-wt, but not pMIR-REPORT-NFATC3-mut (Fig. 4F). These results suggested that *miR-1* targeted and suppressed the expression of NFATC3.

Suppression of NFATC3 is required for the anti-hypertrophic effects of *miR-1*. As NFATC3 was identified as a novel target of *miR-1*, the present study aimed to establish the role of NFATC3 in the hypertrophic process of *miR-1*. An adenoviral vector expressing NFATC3 was used to increase the expression levels of NFATC3 in the rat cardiomyocytes (Fig. 4G). The overexpression of NFATC3 eradicated the effects of *miR-1* restoration in cardiomyocytes treated with ISO, evidenced by an increased cell surface area (Fig. 4H), protein/DNA ratio (Fig. 4I) and expression of β -MHC (Fig. 4J). These results suggested that the suppression of NFATC3 is required for the anti-hypertrophic effects of *miR-1*.

Discussion

Although *miR-1* is a closely associated with the progression of cardiomyocyte hypertrophy, the molecular mechanisms underlying its effects in heart disease remains to be fully elucidated. The present study identified NFATC3 as a novel target of cardiac hypertrophy-associated miRNA. The regulation of NFATC3 by *miR-1* was associated with the putative recognition of binding sites within the 3'-UTR region of target mRNA molecules. However, *miR-1* may exert its regulatory effects on NFATC3 through other mechanisms, including the suppression of NFATC3 inhibitory modulators.

To the best of our knowledge, the present study established for the first time a direct association between two well-known cardiac hypertrophy-associated genes, *miR-1* and NFATC3. However, the molecular events, which underlie the aberrant expression of NFATC3 in cardiomyocyte hypertrophy remain to be fully elucidated, although its pro-hypertrophic function has been well-established in previous studies (18,19). In addition, the present study provided further clarification of the molecular signaling pathway responsible for the high expression levels of NFATC3 during the hypertrophic process.

Although *miR-1* was identified as a negative regulator of the expression of NFATC3 in the cardiomyocytes in the present study, other miRNAs have also been found among the predicted regulators of NFATC3. *miR-122* has been demonstrated to be an important factor for the development of hepatic tissues, and in the physiological and pathological processes of the liver (20,21), which is a potential regulator of NFATC3. Therefore, it is important to investigate the association between these potential epigenetic regulators and NFATC3. The mapping of a complete regulatory network of NFATC3 is likely to contribute to an improved understanding

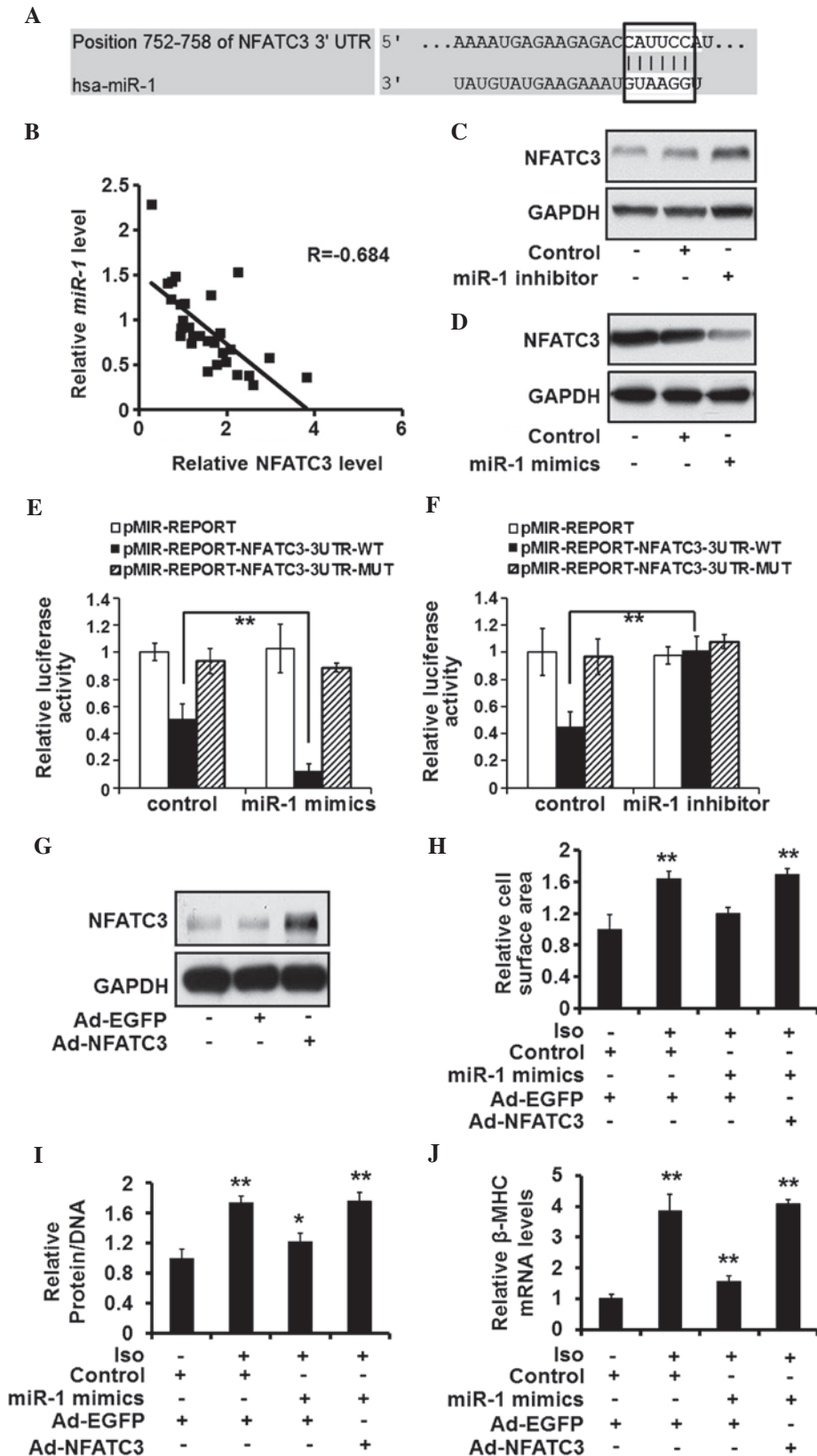


Figure 4. *miR-1* targets NFATC3 and NFATC3 suppression is required for the anti-hypertrophic effects of *miR-1*. (A) Putative *miR-1* seed sequence within the 3'-UTR of NFATC3 mRNA is highlighted. (B) Correlation between the expression of *miR-1* and NFATC3, determined using Pearson's analysis. (C and D) Protein expression levels of NFATC3, quantified using western blotting in rat cardiomyocytes treated with control, *miR-1* mimics (30 nM) or inhibitor (30 nM). (E and F) Expression levels of luciferase induced by pMIR-REPORT, pMIR-REPORT-NFATC3-3'UTR-WT, or pMIR-REPORT-NFATC3-3'UTR-MUT were estimated in the rat cardiomyocytes treated with control, *miR-1* mimics (30 nM) or inhibitor (30 nM). (G) ISO (10 μ M)-stimulated rat cardiomyocytes were treated with control or *miR-1* mimics (10 nM), and Ad-EGFP or Ad-NFATC3 (multiplicity of infection, 10). Protein expression of NFATC3 was detected by western blotting. (H) Cell surface area, (I) protein/DNA ratio and (J) mRNA expression of β -MHC were evaluated in the rat cardiomyocytes exposed to the same treatment. Values are expressed as the mean \pm standard deviation of three independent experiments. * $P < 0.05$; ** $P < 0.01$ vs. Ad-EGFP and Control-transfected group. *miR-1*, microRNA; Iso, isoproterenol; NFATC3, nuclear factor of activated T cells cytoplasmic 3; EGFP, enhanced green fluorescent protein; UTR, untranslated region; Ad, adenoviral vector.

of the molecular signaling pathways, which are associated with the formation and progression of cardiomyocyte hypertrophy.

In addition to the examination of the molecular mechanisms underlying the effects of *miR-1*, the present study examined the clinical expression profile of *miR-1* in patients with cardiac hypertrophy. The results revealed that *miR-1* was overexpressed in the cardiac tissue samples of patients with cardiac hypertrophy. Previously, elevated serum miRNA levels have been demonstrated to be of useful diagnostic and prognostic value in patients suffering from various diseases, including cancer and heart disease (22,23). Further investigations are required in order to determine whether the serum levels of *miR-1* can be used for diagnosis and prognosis of patients with cardiac hypertrophy.

In conclusion, the present study demonstrated that *miR-1* suppressed the expression of NFATC3 by targeting its MRE within its 3'-UTR region, and the overexpression of *miR-1* may result in the aberrant expression of NFATC3 in hypertrophic cardiomyocytes. These data may contribute to a better understanding of the molecular mechanisms underlying cardiac hypertrophy, and provide evidence supporting the targeting of *miR-1*/NFATC3 as a promising therapeutic strategy for heart diseases.

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