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miR-145 inhibits isoproterenol-induced cardiomyocyte hypertrophy by targeting the expression and localization of GATA6

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ABSTRACT

Excessive βAR stimulation is an independent factor in inducing pathological cardiac hypertrophy. Here, we report miR-145 regulates both expression and localization of GATA6, thereby protecting the heart against cardiomyocyte hypertrophy induced by isoproterenol (ISO). The protective activity of miR-145 was associated with down-regulation of ANF, BNP and β-MHC expression, a decreased rate of protein synthesis, inhibited cardiomyocyte growth and the modulation of several signaling pathways including ERK1/2, JNK and Akt-GSK3β. The anti-hypertrophic effect was abrogated by exogenous over-expression of transcription factor GATA6 which was further identified as a direct target of miR-145. In addition, GSK3β antagonists, LiCl and TDZD8, restored the nuclear accumulation of GATA6, which was attenuated by miR-145 Finally, we observed a dynamic pattern of miR-145 expression in ISO-treated NRCMs and in the hearts of TAC mice. Together, our results identify miR-145 as an important regulator in cardiac hypertrophy.
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1. Introduction

Cardiac hypertrophy is a common pathology in many cardiovascular diseases. Long-term, excessive β-adrenergic receptor (βAR) stimulation is an independent factor in inducing pathological cardiac hypertrophy [1]. In spite of the wide clinical use of β-receptor blockers, the mechanisms by which the complex network of signaling pathways downstream of the βAR are regulated require further study to identify more specific therapeutic targets [2]. GATA family transcription factors are important in regulating the development, differentiation and growth of many cells and tissues [3,4]. They share a domain consisting of two adjacent zinc fingers that specifically recognizes nucleotide sequence elements that conform to the consensus 5′-(A/T)GATA(A/G)-3′ [4]. GATA4 and GATA6 are two well-established pro-hypertrophic transcription factors. Over-expression of GATA4 and/or GATA6 has been found to induce pathological hypertrophy in vitro and in vivo [5,6]. The downstream transcription targets of GATA4 and GATA6 include ANF, BNP and β-MHC, which are expressed in the fetal heart and are reactivated in the hypertrophied heart [7]. The continuous and abundant GATA factor expression in the heart throughout the lifespan implies the existence of a strict regulatory mechanism that restricts GATA-mediated transcription activation in the normal heart.

Here, we report a novel mechanism of GATA6 regulation that is mediated by a small non-coding RNA, miR-145. We demonstrate that miR-145 attenuates hypertrophy of cardiomyocytes induced by the βAR agonist isoproterenol (ISO) by suppressing the expression and nuclear translocation of GATA6. miR-145 suppresses GATA6 expression by targeting its 3′UTR and reduces the nuclear accumulation of GATA6 through inhibition of the Akt-GSK3β pathway. Finally, we show that the expression of miR-145 is dynamically regulated in both ISO-treated cardiomyocytes and in the hearts of mice that have undergone transverse aortic constriction (TAC). These results suggest a role for miR-145 in protecting the heart against βAR agonist-induced pathological hypertrophy.

2. Materials and methods

2.1. Mice

Eight- to twelve-week-old male C57BL/6J mice were purchased from the Animal Center of Yangzhou University (Yangzhou, China)

Abbreviations: βAR, β-adrenergic receptor; ISO, isoproterenol; ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; β-MHC, beta-myosin heavy chain; GATA6, GATA binding protein 6; GSK3β, glycogen synthase kinase 3 beta; NRCMs, neonatal rat ventricle myocytes; TAC, transverse aortic constriction; ERK, extracellular regulated protein kinase; JNK, c-Jun N-terminal kinase; mTOR, mammalian target of rapamycin; p70S6K, p70-ribosomal S6 kinase; GAPDH, glyceraldehyde phosphate dehydrogenase; Crm-1, chromosome maintenance region 1; ILK, integrin linked kinase; UTR, untranslated region

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and maintained in the Animal Laboratory Center of Drum Tower Hospital (Nanjing, China) on a 12 h light/dark cycle (lights off at 19:00) with food and water available *ad libitum*. Cardiomyocytes were obtained from 1-day-old Sprague–Dawley rats purchased from the Animal Center of Nanjing Medical University (Nanjing, China). All of the animal experiment protocols were approved by the Institutional Animal Care and Use Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School.

2.2. Transverse aortic constriction (TAC)

Thirty-two male C57BL/6J mice were randomly divided into four groups of eight mice each, as follows: sham 1 wk group, TAC 1 wk group, sham 4 wk group and TAC 4 wk group. Essentially, surgical procedures were performed as previously described [8]. Briefly, a partial ligation (constriction) of the aortic arch was introduced between the right innominate artery and the left common carotid artery using a 27-gauge needle as a guide. The sham operation was performed likewise, but the aorta was not ligated. The success of the procedure was confirmed by measuring both left ventricle function and the thickness of the inter-ventricular wall by echocardiography before the animal was sacrificed.

2.3. Primary culture of neonatal rat cardiac myocytes (NRCMs)

The generation of a miR-145 adenovirus (Ad-mir-145) and the isolation of cardiomyocytes from 1-day-old Sprague–Dawley rats were performed as previously described [9]. Adenoviruses were used to transduce cells at the indicated multiplicity of infection (MOI). The cells were further transfected with plasmid constructs and, at 48 h after transfection, were stimulated for 24 h with 10 μM ISO (Sigma, St. Louis, USA). Lithium chloride (LiCl, Sigma, St. Louis, USA) was diluted with phosphate buffered saline (PBS) and added to the cells at a final concentration of 10 mM 2 h before harvesting; 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD8, Sigma, St. Louis, USA) with dimethyl sulfoxide (DMSO) and added at concentration of 10 μM, 12 h before harvesting. The control cells were added with the same volume PBS or DMSO.

2.4. Generation of the GATA6 over-expression construct and transfection of cardiomyocytes

A plasmid containing the coding sequence of rat GATA6 (NCBI Reference Sequence: NM_019185) was prepared by cloning the indicated sequence into the pCMV-FLAG-2 vector (Sigma, St. Louis, USA). Effectene transfection reagent (Qiagen, Cat. No. 301425) was used to transfect NRCMs with the plasmid. A rat miR-145 inhibitor and an miRNA inhibitor control were purchased from RiBo Bio (Guangzhou, China).

2.5. Luciferase reporter assay

The firefly luciferase cDNA was fused with the rat GATA6 3′UTR (from NCBI Reference Sequence: NM_019185) containing either the seed sequence for miR-145 (nucleotides 1602-1609 of the GATA6 cDNA) or a mutated form of this seed sequence (5′-AACTCCAA-3′) [10]. The two resulting fusion constructs were cloned into the pGL3 promoter luciferase reporter vector (Promega, Madison, USA). Preconfluent H9c2 cells were transduced with Ad-LacZ or Ad-miR-145 for 24 h, and the cells were then transfected with 300 ng of the firefly luciferase reporter plasmid (pGL3-luc-GATA6-3′UTR or pGL3-luc-GATA6-3′UTR mut) and 20 ng of the Renilla luciferase reporter plasmid pRL-RSV (Promega, Madison, USA) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, USA). Cell lysates were assayed for luciferase activity 48 h after transfection using the Luciferase Assay System (Promega, Madison, USA) according to the manufacturer's instructions. All of the assays were performed in duplicate, and three biological replicates were assayed per condition.

2.6. Quantitative real-time PCR

Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Two micrograms of total RNA were reverse transcribed in a total volume of 20 μl, using MMLV reverse transcriptase kit (Promega, Madison, USA). Real-time PCR was performed using SYBR green (Bio-Rad, Hercules, USA) fluorescence to detect miR-145 expression as previously described [9]. Complementary DNA was synthesized using a miR-145-specific stem-loop primer with the following sequence: 5′-CTCAACTGGTGTCGTGGAGTCGG CAATTCAGTTGAGAGGGATTC-3′. The quantitative polymerase chain reaction was performed using the following primers: forward, 5′-CGCGCTCGAGCCCAGAGCAATAAGCCACAT-3′; reverse, 5′-GGTGTCG TGGAGTCGGCAATTCAGTTGAG-3′. The primers corresponding to the small nuclear RNA U6 (U6) gene were: forward, 5′-CTCGCTT CGGCAGCACA-3′; reverse, 5′-AACGCTTCACGAATTTGCGT-3′. The U6 reverse primer was also used in the reverse transcription of U6. The PCR conditions used were as follows: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The samples were run in duplicate, and RNA preparations from three independent experiments were assayed. Each real-time PCR reaction consisted of 2 μl RT product, 10 μl SYBR Green PCR Master Mix, and 500 nM each of the forward and reverse primers. Reactions were carried out on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, USA). The fold change in the expression of each gene was calculated using the $2^{\Delta\Delta ct}$ method with U6 as an internal control. The primers used to assay the expression of the rat ANF, BNP, β-MHC and GAPDH genes are listed in Table 1. GAPDH served as an internal control gene for these assays.

2.7. Western blotting analysis

Total protein lysates were assayed for protein expression using anti-ANF (Millipore, Billerica, USA), anti-GATA6 (Cell Signaling Technology, Danvers, USA), anti-ERK, anti-p-ERK (T204/Y202), anti-JNK, anti-p-JNK (T183), anti-Akt, anti-p-Akt (S473), anti-p-Akt (T308), anti-mTOR, anti-p-mTOR (S2448), anti-GSK3β, anti-p-GSK3β (S9), anti-GATA4, anti-p70S6K, anti-p-p70S6K (T389), anti-Lamin B1, anti-C/EBP-β, anti-p53 and anti-GAPDH (Bioworld, Dublin, USA) antibodies. Nuclear and cytoplasmic lysates were prepared to evaluate the nuclear translocation of GATA6 and GATA4. GAPDH served as an internal control in the expression assays performed on the total protein lysates and on the cytoplasmic lysates, while Lamin B1 served as an internal control in assays performed on the nuclear lysates.

2.8. Immunofluorescence

Cells were cultured in chambers (Millipore, Billerica, USA) at a density of 1×10^5 cells/chamber and transduced with Ad-miR-145 or Ad-LacZ. Either the miR-145 inhibitor or the control inhib-

Table 1
Real-time PCR primer sequences.

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
ANF	ATGGGCTCCTTCTCCATCAC	TCTTCGGTACCGGAAGCTG
BNP	TTGGGCAGAAGATAGACCGGAT	GGTCTTCCTAAAACAACCTCA
β-MHC	GCAGCTTATCAGGAAGGAATAC	CTTGCGTACTCTGTCACTC
GAPDH	TCTACATGTTCCAGTATGACTC	ACTCCACGACATACTCAGCACC

itor was introduced into the cells by transfection 48 h before the cells were treated with 10 μM ISO. LiCl (10 mM) or TDZD8 (10 μM) were used to treat the cells at 2 or 12 h before harvesting, respectively. The cells were then fixed in 4% paraformaldehyde and incubated with anti-alpha-sarcomeric actin (Abcam, Hong Kong) and/or anti-GATA6 (Cell Signaling Technology, Danvers, USA) antibodies at room temperature for 2 h, after which the cells were incubated for 1 h with FITC-conjugated goat anti-rabbit antibody (Millipore, Billerica, USA). The cell nuclei were stained with DAPI (5 μg/ml). The cells were examined by confocal microscopy.

2.9. S^{35} cysteine incorporation

The cells were incubated in serum-free DMEM for 12 h before the addition of 1 μCi/ml [^{35}S]-cysteine (Amersham Bioscience, USA) in the presence of 10 μM ISO. After a further 12 h incubation, the cells were washed twice with ice-cold PBS and fixed with ice-cold 10% TCA (Wako Pure Chemical Industries, Japan). The TCA-insoluble fraction was resolved with 1 M NaOH, and the radioactivity of the solution was then determined using a liquid scintillation counter (Bioscan, Triathler, USA).

2.10. Statistics

All data are presented as the means ± S.E.M. from at least three independent experiments. Statistical analysis with Student's *t* test was performed using SPSS software. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Over-expression of miR-145 inhibited ISO-induced hypertrophy in cardiomyocytes

In a previous study, we found that miR-145 regulates the mitochondrial apoptotic pathway in cardiomyocytes [9]. Additionally, results from miRNA microarray analysis of healthy people and end-stage heart failure patients have shown that miR-145 expression is significantly up-regulated in the latter group [12]. These findings led us to ask whether miR-145 plays a role in ISO-induced hypertrophy in cardiomyocytes. We first examined miR-145 expression in NRCMs treated with either Ad-miR-145 or the miR-145 inhibitor. As expected, miR-145 expression increased after infection with Ad-miR-145 and decreased after transfection of the miR-145 inhibitor. Both of these effects were dose-dependent (Fig. 1A). We next examined the effect of miR-145 upon: (1) the expression of ANF, BNP and β-MHC, (2) the rate of protein synthesis, and (3) cell size. Real-time PCR assays showed that, following ISO treatment, the levels of transcripts corresponding to the ANF, BNP and β-MHC genes declined in a dose-dependent manner in Ad-miR-145-transduced cardiomyocytes compared with Ad-lacZ-transduced cardiomyocytes, whereas transfection of cells with the miR-145 inhibitor did not decrease the levels of these transcripts (Fig. 1B). The ^{35}S incorporation assay also identified a marked decrease in the rate of protein synthesis in cells transduced with Ad-miR-145 and treated with ISO as compared with that in cells treated with ISO alone. Down-regulation of miR-145 by the miR-145 inhibitor did not affect the high ^{35}S uptake observed in ISO-treated cardiomyocytes (Fig. 1C). Lastly, confocal microscopy showed that ISO-treated cells typically presented with enlarged cytoplasmic regions and strong actin staining. Over-expression of miR-145 in these cells resulted in reductions in both the size of the cytoplasmic regions observed and the intensity of the actin staining. The size of the cells treated with the miR-145 inhibitor and ISO was comparable to that of the cells treated with ISO alone (Fig. 1D and E).

3.2. miR-145 suppressed ISO-induced cardiomyocyte hypertrophy by targeting the transcription factor GATA6

Given that miR-145 expression protects cardiomyocytes against ISO-induced hypertrophy, we next asked how miR-145 functions in the hypertrophic process and whether it has any direct functional targets. We searched for the predicted targets of miR-145 in the miRDB database (http://mirdb.org/miRDB/) and found that GATA6 is a potential target for this microRNA (Fig. 2A). Accordingly, we constructed luciferase reporter plasmids containing the 3′UTR region of the rat GATA6 gene carrying either an unmutated or a mutated putative miR-145 binding site (Fig. 2A) [10]. After overexpression of miR-145 in H9c2 cells, luciferase activity of the construct carrying the unmutated 3'UTR of GATA6 decreased significantly, but the similar effect was not observed with the luciferase fusion construct carrying the mutated 3'UTR of GATA6 (Fig. 2B). We performed western blotting analysis to examine the expression of endogenous GATA6 in NRCMs transduced with Ad-miR145. As shown in Fig. 2C, over-expression of miR-145 markedly suppressed endogenous GATA6 expression in a dose-dependent manner in the absence of ISO, whereas the expression of GATA4 showed no obviously dose-dependent change (Fig. 2C). Importantly, increasing the expression of GATA6 in NRCMs (Fig. 2D) in the presence of ISO totally or partially abrogated the effects of miR-145 on fetal gene expression (Fig. 3A), on the rate of ^{35}S uptake (Fig. 3B), and on cell size (Fig. 3C and D), which suggests that miR-145 exerts its regulatory effects by targeting GATA6.

3.3. miR-145 inhibited the Akt-GSK3β pathway

We next investigated the effects of miR-145 on several important hypertrophic signaling pathways. Over-expression of miR-145 was found to increase the phosphorylation of ERK and JNK at 24 h after ISO stimulation (Fig. 4A). While the phosphorylation of Akt at T308 was unaffected by miR-145 over-expression, its phosphorylation at S473 was significantly down-regulated (Fig. 4B). In addition, the levels of phosphorylation of several pathway components downstream of p-Akt (S473), namely the phosphorylation of mTOR (at S2448), p70S6K (at T389) and GSK3β (at S9), were down-regulated by miR-145 over-expression when assayed at 24 h after ISO treatment. The protein level of ANF was also altered by miR-145 and/or ISO in a manner similar to that observed for p-Akt (S473) (Fig. 4B). Moreover, we found that the expression levels of both GATA6 and GATA4 were markedly decreased by miR-145 in the presence of ISO treatment (Fig. 4B).

3.4. miR-145 attenuated GATA6 nuclear translocation by inhibiting GSK3β phosphorylation

Given that GATA4 and GATA6 are expressed abundantly in the heart throughout life, it is likely that their sub-cellular localizations and transcriptional activities are strictly regulated. Because we have shown that GATA6 is a functional target of miR-145, we were interested to know whether miR-145 expression affects the sub-cellular localization of GATA6 in ISO-treated cardiomyocytes. As shown in Fig. 5A, ISO stimulation caused nearly all of the GATA6 protein to localize to the nucleus, while overexpression of miR-145 partly restored the normal cytoplasmic pattern of GATA6 localization. The results of the western blotting further demonstrated that ISO-induced GATA6 nuclear translocation was significantly inhibited by miR-145 (Fig. 5C). In contrast, cells treated with the miR-145 inhibitor displayed an abnormal nuclear distribution of GATA6 (Fig. 5A and C).

GSK3β is an important negative regulator of GATA4 translocation, and we hypothesized that the sub-cellular localization of GATA6 might also be affected by GSK3β. If so, the inhibition of

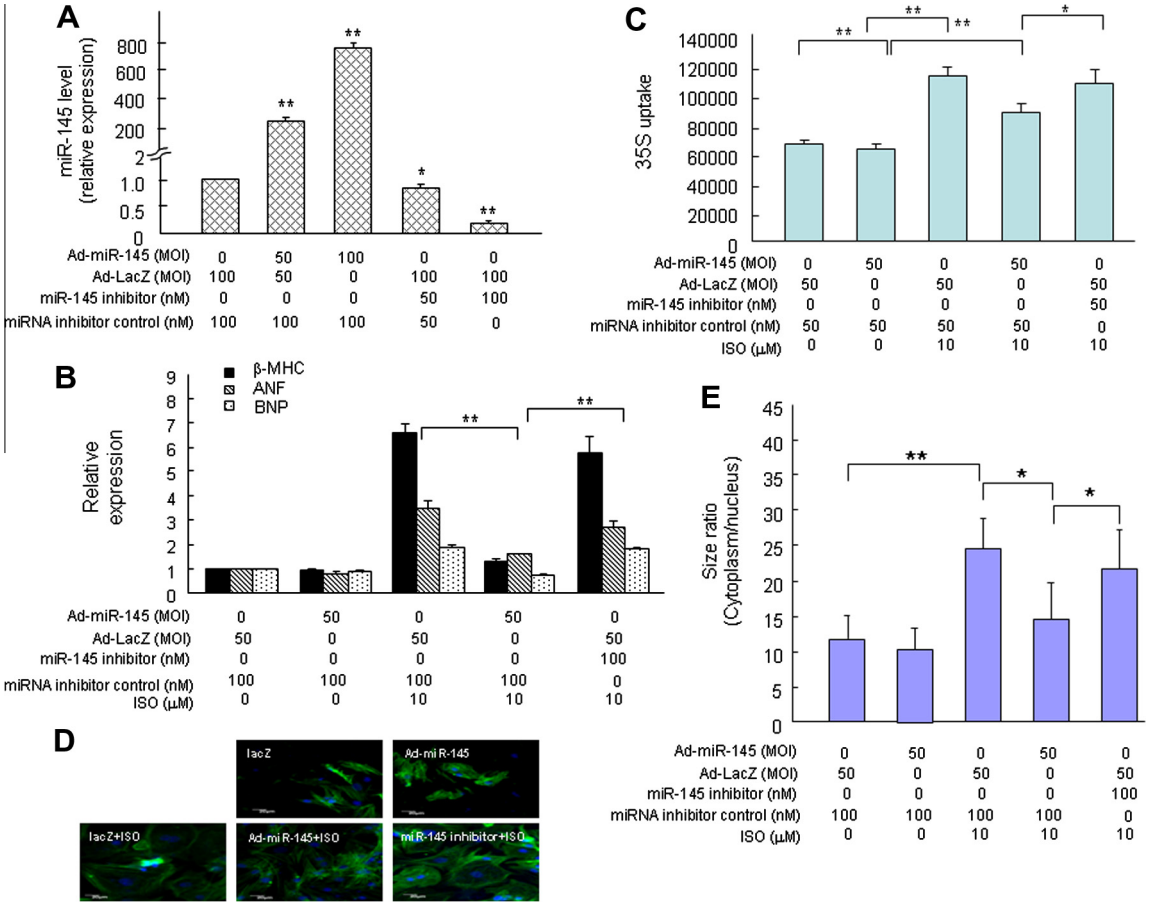

Fig. 1. miR-145 inhibited ISO-induced hypertrophy in cultured NRCMs. (A) Stem-loop real time PCR was used to detect changes in miR-145 levels in NRCMs after infected with Ad-miR-145 or transfected with miR-145 inhibitor at the MOI or nM indicated for 48 h. (B) Quantitative real time PCR was used to measure the expression of the fetal genes ANF, BNP and β-MHC in cells following the indicated treatments. (C) The rate of ^{35}S uptake was measured using a liquid scintillation counter. (D) Confocal microscopy images show the changes in cell size in the indicated groups. The scale bar represents 20 μm. (E) The average ratio of cell size to nucleus size was calculated for 50 randomly chosen cells from each group in (D). The data are presented as the means ± S.E.M. Statistical significance was determined by Student's *t* test. *, $P < 0.05$; **, $P < 0.01$.

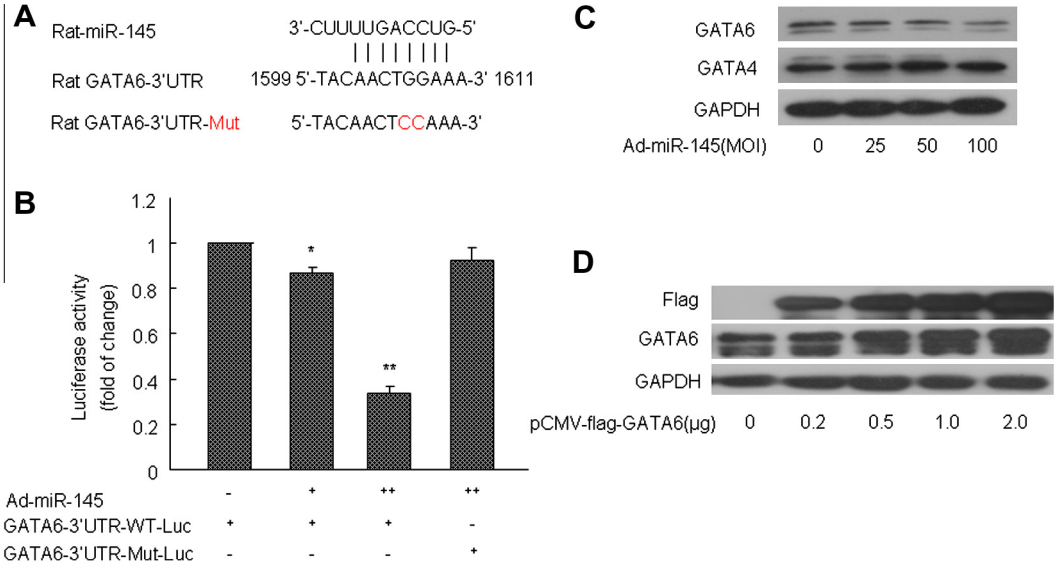

Fig. 2. miR-145 targeted the 3′UTR of GATA6. (A) The putative binding site for miR-145 in rat GATA6 (upper sequence) and the corresponding mutated seed sequence used in this study (lower sequence) are shown. (B) Luciferase assays were performed in H9c2 cells using 300 ng pGL3-GATA6-3′UTR-Luc or pGL3-GATA6-3′UTRmut-Luc. Cells were transfected with different doses of Ad-miR-145 (+, 50 MOI; ++, 100 MOI). The luciferase activity was normalized for transfection efficiency using the corresponding Renilla luciferase activity. The data are presented as the means ± S.E.M. Statistical significance was determined using Student's *t* test. *, $P < 0.05$; **, $P < 0.01$. (C) Western blotting analysis of GATA6 and GATA4 expression in total protein lysates of NRCMs transduced with different doses of Ad-miR-145. (D) Western blotting analysis of flag tag and GATA6 expression in total protein lysates of NRCMs transfected with different doses of pCMV-Flag-GATA6 (0, 0.2, 0.5, 1.0, 2.0 μg). GAPDH served as an internal control.

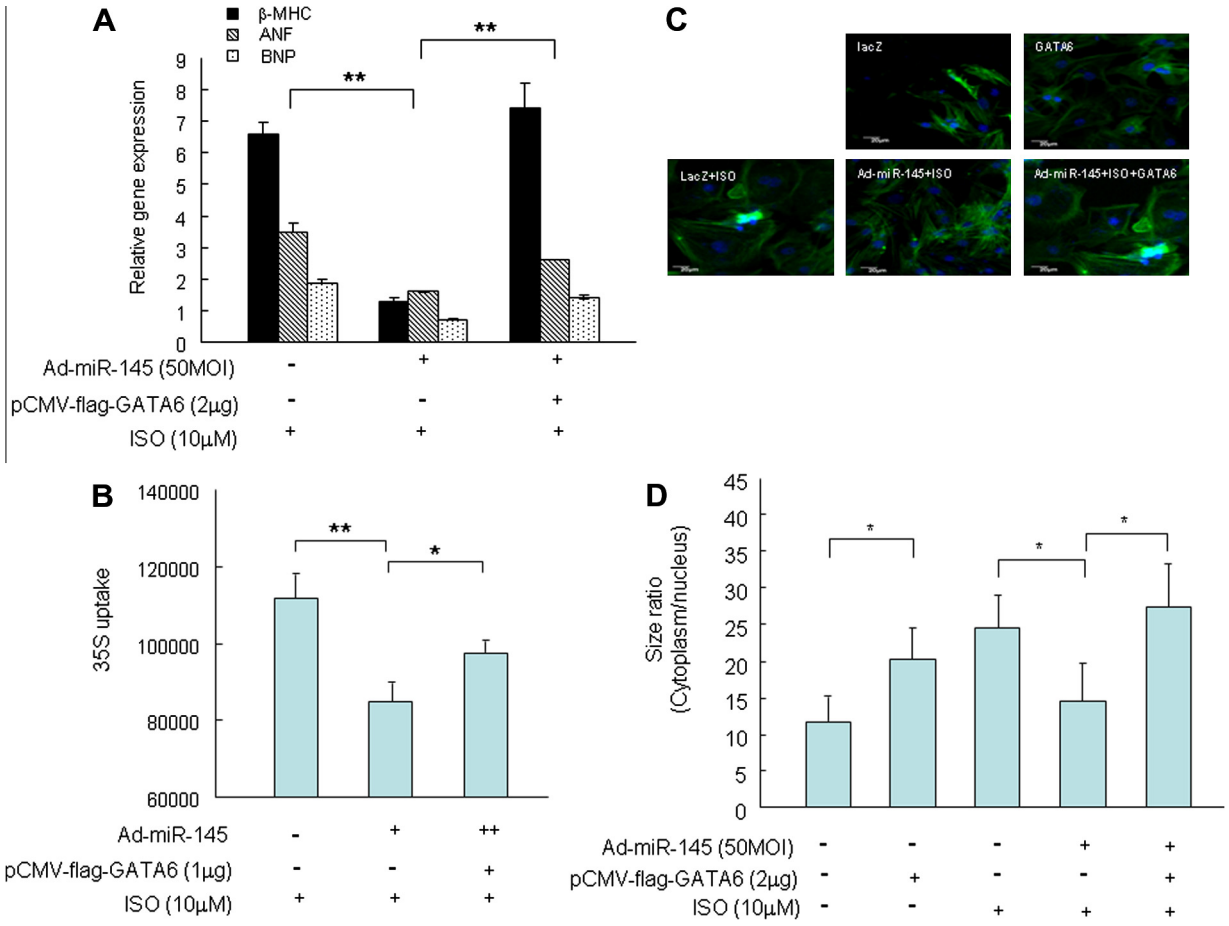

Fig. 3. Over-expression of GATA6 abrogated the protective effects of miR-145. (A) Quantitative real time PCR analysis of fetal gene expression in the indicated samples. (B) The rate of ^{35}S uptake under different experimental conditions is shown. ^{35}S uptake was measured using a liquid scintillation counter. (C) Confocal microscopy images showing the size of cells in each indicated group. (D) The average ratio of cell size to nucleus size was calculated for 50 cells randomly chosen from different fields of view. The data are presented as the means ± S.E.M. Statistical significance was determined using Student's *t* test. *, $P < 0.05$; **, $P < 0.01$.

GATA6 nuclear translocation by miR-145 could be at least partly explained by miR-145-mediated inhibition of the Akt-GSK3β pathway. To test this hypothesis, we employed two structurally different GSK3β inhibitors: lithium chloride (LiCl) and 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD8). Western blotting showed that treatment of cells with LiCl or TDZD8 alone increased the nuclear localization of GATA6 and GATA4 (Fig. 5D). Through a combination of confocal microscopy and western blotting analysis, we found that either LiCl or TDZD8 treatment abrogated the inhibitory effect of miR-145 on GATA6 nuclear translocation in the presence of ISO (Fig. 5B and D). These results suggest that miR-145 attenuates GATA6 nuclear accumulation through inhibition of GSK3β phosphorylation.

3.5. miR-145 expression was altered in ISO-treated cardiomyocytes and in TAC mice

To investigate whether the expression level of miR-145 is correlated with cardiomyocyte hypertrophy in vitro and in vivo, we analyzed the level of mature miR-145 in NRCMs at different time points after treatment with ISO. The quantitative real-time PCR results showed that the expression of miR-145 increased in response to ISO treatment and reached a peak at 4 h following administration of the drug, after which time it declined gradually to an approximately basal level (Fig. 6A). The level of GATA6 protein, as determined by western blotting, was found to be increased dramatically at the 8-hour and 12-hour timepoints and was decreased slightly at 24 h after ISO stimulation (Fig. 6B). We also discovered elevated miR-145 expression in mice that had undergone the TAC

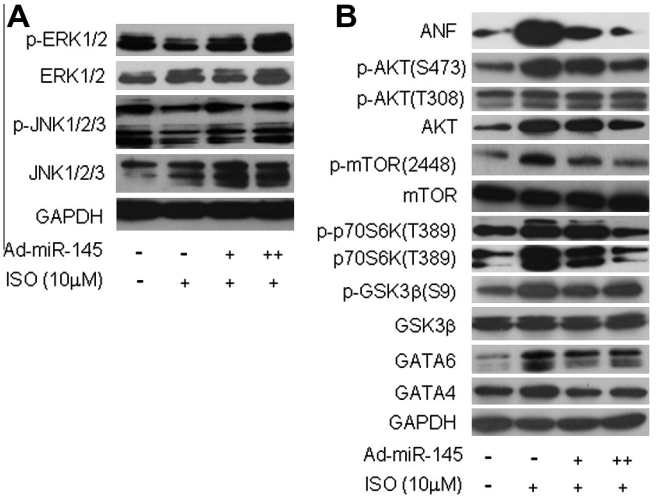

Fig. 4. miR-145 regulated hypertrophic pathways. Cells were transduced with gradient doses of Ad-miR-145 (+, 50 MOI; ++, 100 MOI) for 48 h and then treated with 10 μM ISO for 24 h. (A) Western blotting analysis of the levels of p-ERK, ERK, p-JNK and JNK in total protein lysates. (B) Western blotting analysis of the levels of the indicated proteins in total protein lysates. GAPDH served as an internal control in both experiments.

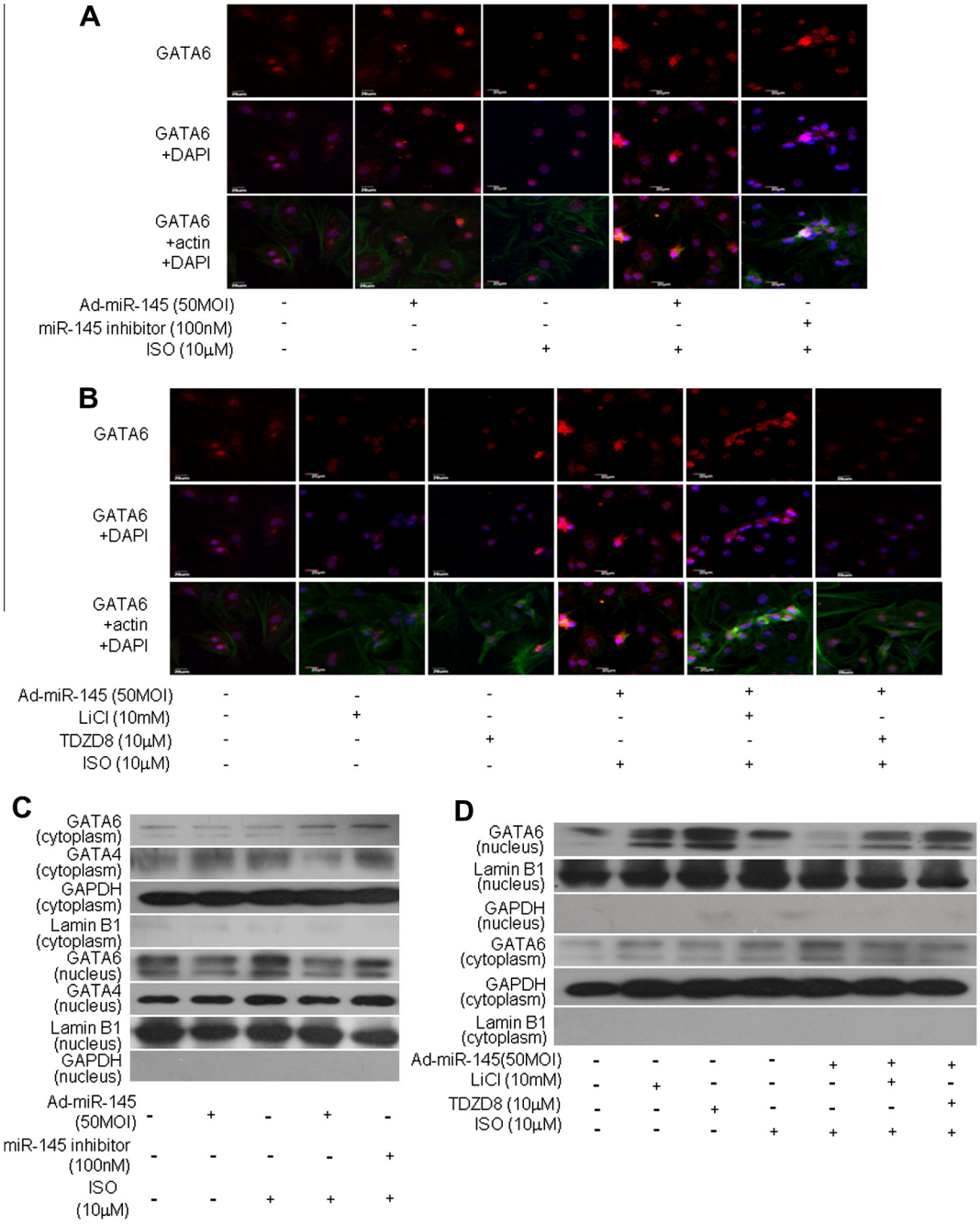

Fig. 5. miR-145 inhibited the nuclear accumulation of GATA6 through regulation of GSK3β. Cells were transduced with 50 MOI Ad-miR-145 before being treated with 10 μM ISO for 24 h. Before harvesting, cells were treated with 10 mM LiCl for 2 h or 10 μM TDZD8 for 12 h. The control cells were treated with the same volume PBS and/or DMSO. (A) and (B) Confocal microscopy images show the sub-cellular localization of GATA6 (red) relative to the nucleus, stained by DAPI (blue), and the cytoplasm, stained by sarcomeric actin (green). The upper row of panels shows GATA6 staining only. The middle row shows the merged images of GATA6 and DAPI staining, while the lower panels show the merged GATA6, DAPI and sarcomeric actin images. (C) and (D) Western blotting was performed to detect the levels of GATA4 and GATA6 in the isolated nuclear and cytoplasmic lysates. Lamin B1 and GAPDH served as internal positive controls for the nuclear and cytoplasmic lysates, respectively.

procedure. This was observed at an early stage after the procedure (1 week). However, this level had declined by 4 week after TAC to a level comparable to that found in the sham group (Fig. 6C). Given that miR-145 was previously shown to be regulated by transcription factor p53 and C/EBP-β, we detected both protein levels in ISO-treated cardiomyocytes. Western blot showed that while p53 was up-regulated and maintained at a high level in response to ISO stimulation, C/EBP-β was significantly up-regulated from 4 to 24 h after ISO was introduced (Fig. 6D). These results provide further evidence of a correlation between miR-145 expression and cardiomyocyte hypertrophy.

4. Discussion

microRNAs have been implicated in various physiological and pathological processes in the cardiovascular system [11]. In the present study, we have demonstrated that miR-145 regulates ISO-induced cardiomyocyte hypertrophy by modulating the expression and localization of the transcription factor GATA6.

Cardiac hypertrophy is a complicated process. While the final stage of hypertrophy is accompanied by compromised cardiomyocyte function, the very first stage is often seen as an adaptive strategy in stressed cardiomyocytes. The borderline between

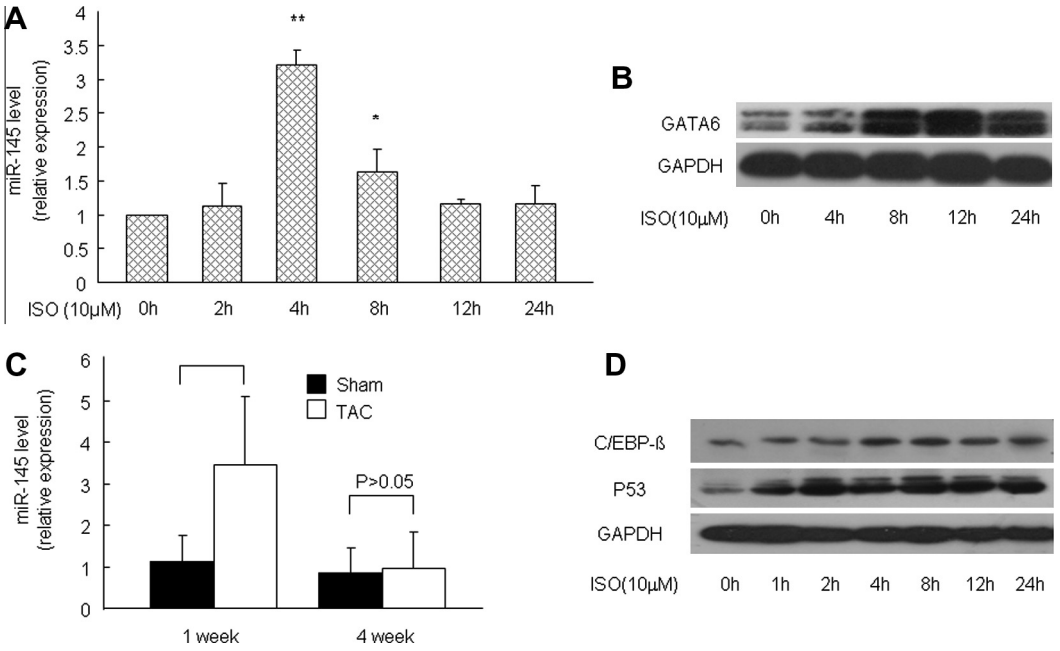

Fig. 6. miR-145 expression was altered in ISO-treated NRCMs and in the cardiac tissues of TAC mice. (A) Stem-loop real time PCR analysis of the expression level of miR-145 in cardiomyocytes (NRCMs) at various time points after treated with 10 μM ISO. (B) Western blotting analysis of the level of GATA6 in whole cell lysates from cells treated with 10 μM ISO for the indicated time courses. (C) Stem-loop real time PCR analysis of the expression of miR-145 in ventricle tissues from mice at 1 or 4 week after TAC or sham operation (n = 8 in each group). The data are presented as the means ± S.E.M. Statistical significance was determined using Student's t test. (D) Western blotting analysis of the levels of p53 and C/EBP-β at different time point after ISO treatment, GAPDH served as internal control. *, $P < 0.05$; **, $P < 0.01$.

physiological and pathological hypertrophy has so far remained ambiguous. A previous genome-wide profiling study of miRNAs in healthy people and in patients with end-stage heart failure found that miR-145 was significantly up-regulated in the latter group [12]. In our study, we also observed a significant elevation of miR-145 expression in the hearts of mice 1 week after they had undergone the TAC procedure (Fig. 6C). Based on these findings, we have concluded that miR-145 is involved in the regulation of pathological hypertrophy. However, the mechanisms by which miR-145 functions in the stages of physiological and pathological hypertrophy require further investigation through in vivo experiments.

Although we demonstrated in our study that miR-145 and GATA6 was both up-regulated at the early stage of ISO stimulation (Fig. 6A and B), the mechanisms seem to be different. Previous study identified that the transcription of miR-145 is positively regulated by p53 and negatively regulated by C/EBP-β [13,14]. We found that the protein level of p53 increased during the whole course of ISO treatment, while that of C/EBP-β elevated only since 4 h after ISO was added. Our result suggests that during the first 4 h of ISO stimulation, the positive regulation of miR-145 by p53 dominated the negative regulation by C/EBP-β, resulting in an increase in miR-145 level with a restricted up-regulation of GATA6 (Fig. 6A and B); however in the later stage, the effect of p53 counteracted that of C/EBP-β, thus resulting in a stabilized level of miR-145 with a dramatically up-regulated GATA6 (Fig. 6A and B). Nevertheless, the complex mechanisms regarding the dynamic changes of miR-145 and GATA6 in response to ISO treatment indeed warrant further studies.

Our results also showed that miR-145 regulates the nuclear translocation of GATA6 through GSK3β (Fig. 5B and D). GSK3β has attracted a great deal of attention in the field of cardiovascular research. Studies have shown that it plays cardioprotective roles in ischemia preconditioning and cardiac hypertrophy [15–17]. Notably, GSK3β was shown to directly phosphorylate GATA4 and stimulate Crm-1-dependent nuclear export of GATA4 [17]. Crm-1 is a versatile nuclear transport receptor that recognizes the leucine-rich nuclear export signal (NES) sequence. Earlier reports suggested that phosphorylation of GATA4 by GSK3β exposes the NES sequence of GATA4 and thus commits the protein to nuclear export [17]. In the case of rat GATA6, we also found a conserved leucine-rich amino acid sequence (LPGLPYL) that is very likely to be the NES sequence. Furthermore, a previous study demonstrated that the nuclear export of GATA6 also depends upon Crm-1 [18]. These findings, coupled with the fact that phosphorylation of GSK3β at Ser9 and Ser21 typically leads to its inactivation [17], made it reasonable for us to hypothesize that miR-145-mediated attenuation of the nuclear accumulation of GATA6 is at least partially due to the ability of miR-145 to inhibit GSK3β phosphorylation. In the presence of ISO, GSK3β is phosphorylated and inactivated by its upstream regulator Akt, and the removal of this negative constraint from GATA transcription factors would likely result in their nuclear accumulation and activation. In our study, we showed that treatment of cells with LiCl or TDZD8 caused marked nuclear accumulation of GATA6 (Fig. 5A and B); thus, we have verified that GSK3β regulates GATA6. However, whether there is a direct protein-protein interaction between GSK3β and GATA6 and whether GSK3β phosphorylates GATA6 in a manner similar to GATA4 are questions that require further research.

When we analyzed the localization of GATA6 by confocal microscopy and western blotting, we found that even untreated cardiomyocytes contain a considerable amount of nuclear GATA6 (Fig. 5A). Although miR-145 expression dramatically reduced the nuclear levels of GATA6, it only modestly increased the cytoplasmic level of the protein (Fig. 5A). We hypothesize that this was due to proteasome-mediated degradation of GATA6 in the cytoplasm. Earlier reports identified a cAMP-dependent, proteasome-driven mechanism of GATA6 proteolysis that acts upon GATA6 proteins that are exported from the nucleus [18,19]. This process seems to be activated by JNK [18]. Consistent with this, we observed an increase in JNK phosphorylation in response to miR-145 (Fig. 4A). This may be another mechanism by which miR-145 regulates GATA6 expression.

Compared with GATA6, GATA4 has been more intensively studied in the cardiovascular field. For a long time, the apparent "functional redundancy" of GATA4 and GATA6 has kept GATA6 out of the spotlight [5,6]. However, in recent years, it has been noted that GATA6 is both necessary and sufficient to regulate the cardiac hypertrophy process [5]. In our study, we did not observe any notable changes in GATA4 expression in response to miR-145 alone (Fig. 2c). Meanwhile, over-expression of GATA6 was sufficient to offset the anti-hypertrophic effects of miR-145 (Fig. 3A–D). These observations collectively show that GATA6 is an independent functional target of miR-145 in ISO-induced cardiomyocyte hypertrophy.

Long-term βAR stimulation is characterized by activation of the PI3K-Akt pathway [20]. Two sites of phosphorylation on Akt, T308 and S473, are both linked with βAR stimulation [20]. Selectivity in the regulation of these two phosphorylated isoforms has only been reported in a few studies, and the mechanisms of their regulation remain poorly understood. Agouni et al. reported that, in renal carcinoma, the parathyroid hormone-related protein activates integrin-linked kinase (ILK), which then selectively phosphorylates (or facilitates the phosphorylation of) Ser473 of Akt [21]. Coincidentally, another recent study found that ILK, the key cardiac sensor of hypertrophic stress, is one of the targets of miR-145 [22]. This is consistent with our finding that miR-145 favors to inhibit the phosphorylation of Akt at S473 (Fig. 4B). In addition to ILK, miR-145 might also target other signaling molecules in the PI3K-Akt pathway, such as p70S6K [10]. The p70S6K protein is thought to play important roles in cardiac hypertrophy, apoptosis and angiogenesis. In our study, we found that the levels of total p70S6K and phosphorylated p70S6K were both down-regulated by miR-145 (Fig. 4B). It would therefore be interesting to determine whether miR-145 is functionally involved in all these processes.

In summary, we identified a novel post-transcriptional regulatory mechanism for βAR agonist-induced cardiomyocyte hypertrophy. We also demonstrated a link between GATA6 and the Akt-GSK3β pathway. The facts that miR-145 modulates multiple hypertrophic signaling pathways and that its expression level is dynamically altered during the development of hypertrophy strongly support the notion that miR-145 is a critical regulator of cardiomyocyte hypertrophy and encourages further investigation of the role of miR-145 in physiological and/or pathological hypertrophy and the transition between them.

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