Biochemical and Biophysical Research Communications 436 (2013) 578–584

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

MicroRNA-214 provokes cardiac hypertrophy via repression of EZH2

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ARTICLE INFO

Article history:
Received 18 May 2013
Available online 29 May 2013

Keywords:
MiRNAs
miR-214
EZH2
Cardiac hypertrophy

ABSTRACT

Micro RNAs are small, non-coding RNA molecules that regulate gene expression via either translational inhibition or mRNA degredation. Enhancer of zeste homolog 2 (EZH2)-mediated hypertrophic signaling is a major regulatory response to hypertrophic stimuli. In this study, we constructed AAC rat models and PE-induced hypertrophic cardiomyocytes. We demonstrated that miR-214 relative levels were upregulated, whereas EZH2 was downregulated in both vivo and vitro models. Further, one conserved base-pairing site in the EZH2 3′-untranslated region (UTR) was verified. Mutation of the site in the EZH2 3′-UTR completely blocked the negative effect of miR-214 on EZH2, suggesting that EZH2 is a direct target for miR-214 regulation. Using a gain-of-function approach, incorporating the lentivirus constructed miR-214 and its sponge, we demonstrated that miR-214 significantly regulated endogenous levels of EZH2 gene expression; whereas, changes in the expression of the Sine oculis homeobox homolog gene were induced by an adrenergic receptor agonist in the AAC rat model. Having made this study it is possible to conclude that the negative regulation of EZH2 expression contributed to miR-214-mediated cardiac hypertrophy.

1. Introduction

The heart responds to physiological stimuli, tissue injury and endocrine disorders by undergoing hypertrophic growth to sustain cardiac output [1–3]. Cardiac hypertrophy is initially a functional, adaptive response, but prolonged hypertrophy can lead to heart failure, which is one of the major causes of human death around the world [4]. Cardiac hypertrophy occurs when numerous signalling pathways merge, culminating in well-described transcriptional networks [5–8]. Epigenetic regulation via histone methylation stabilizes transcriptional programs in embryonic progenitors and their differentiated descendants. This regulation is likely to be crucial in establishing and maintaining gene expression and stress responses throughout life. Polycomb complexes might be used to stabilise cardiac gene expression, as they control cell identity and epigenetic memory in other systems [9]. Ezh2, the major histone methyltransferase of Polycomb repressor complex 2 (PRC2), trimethylates histone H3 at lysine 27. Stable Ezh2-mediated repression of Six1 in differentiating embryonic cardiac progenitors is essential for normal cardiac growth and for stress responsiveness in adults. Ezh2 modulates a feed-forward pathway that represses fetal gene expression and that is reinforced by repression of Six1 [10].

Recently, a new gene regulator, microRNA (miRNA), was identified as playing an important role in heart disease, including the stimulation of cardiac hypertrophy. miRNAs are a class of conserved, single-stranded, non-coding RNAs and consist of 18–25 nucleotides [11,12]. They negatively regulate target gene expression through mRNA cleavage or translation repression. Cardiac-specific deletion of an RNase III endonuclease Dicer, which is essential for miRNA maturation, induced sudden death and mild cardiac remodeling in juvenile mice, and produced cardiac dysfunction in adult mice, along with the induction of hypertrophic marker genes [13]. Recent studies have also indicated that several miRNAs, including miR-133 [14–19], miR-208 [20,21] and miR-18 [22], actively participate in cardiomyocyte hypertrophy. These miRNAs are supposed to regulate cardiac hypertrophy by controlling cardiac growth, conduction and calcium-dependent signaling. In addition, miR-214 was also proven to upregulate miRNA in hypertrophy during several miRNA array tests [10,23,24]. A recent study discovered a potential regulatory role for micro-RNA-214 (miR-214) in cardiac hypertrophy. It was demonstrated that an overexpression of miR-214 resulted in pathological cardiac growth

Abbreviations: EZH2, enhancer of zeste homolog 2; PRC2, polycomb repressor complex 2; miR-214, microRNA-214; UTR, untranslated region; Six1, sine oculis homeobox homolog 1; Lenti-miR-214, lentivirus-miR-214; Lenti-spg, lentivirus-miR-214 sponge; Acta1, actin alpha 1; Myh7, beta-myosin heavy-chain gene; Nppa, natriuretic peptide A.

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0006-291X
http://dx.doi.org/10.1016/j.bbrc.2013.05.079

and heart failure in transgenic mice [25]. However, the pathological miR-214 signaling of cardiac hypertrophy has still not been elucidated.

In this study, we explored the relationship between miR-214 and the EZH2-dependent signaling pathway. This study demonstrated that EZH2 levels were negatively regulated by miR-214. Treatment with miR-214 resulted in striking decreases in EZH2 mRNA and protein expression, coincident with increases in cardiomyocyte hypertrophy, which was induced by the application of an adrenergic receptor agonist. We suggest that silencing of EZH2, which stimulates Six1, could be one of the miR-214-mediated mechanisms that provoke cardiac hypertrophy.

2. Materials and methods

2.1. Construction of plasmids and site-directed mutagenesis

The precursor sequence for miR-214 was amplified by PCR using human genomic DNA as a template, and the PCR products were cloned into the pGL3-promoter vector (Invitrogen) to generate miRNA expression plasmids. For the construction of the luciferase reporter plasmid, the full-length 3′-UTR of EZH2 was amplified from rat genomic DNA by PCR and was inserted into the 3′-UTR of the firefly luciferase gene. The mutated 3′-UTR luciferase reporter plasmids were generated by site-directed mutagenesis using platinum Taq DNA Polymerase High Fidelity, according to the manufacturer's instructions (Invitrogen).

2.2. Luciferase assay

HEK293T cells were transfected with luciferase reporter plasmids and the miRNA expression plasmid, and a Renilla luciferase plasmid was cotransfected as an internal control. Cells were harvested 24 h after transfection. Luciferase activity was measured with a dual luciferase reporter assay kit (Promega) on a luminometer [19], as described previously.

2.3. Western blot analysis

Western blotting was performed. Protein lysates were run on SDS–PAGE gels and transferred to PVDF membranes [15,26,27]. Antibodies against EZH2 and Six1 were purchased from Abcam Co. Antibody against GAPDH was obtained from CST Co.

2.4. Rat cardiac hypertrophy model

Left-ventricle hypertrophy was induced in 150- to 180-g male Sprague–Dawley rats by abdominal aorta constriction (AAC), as described previously [16]. Briefly, the rats were temporarily anesthetized with chloral hydrate (0.2 mg/g), followed by exposure of the abdomen, and then the suprarenal abdominal aorta was isolated and tightened with a 4-0 nylon suture against a 24-gauge needle. After removing the needle, the incision was closed. A control group underwent a sham operation involving all the procedures except for aorta constriction. After surgery, each rat was administered penicillin twice daily for the first 3 days. The rats were sacrificed 21 days after surgery [28]. All of the animal protocols were approved by the Institute of Health Sciences Institutional Animal Care and Use Committee.

2.5. Cardiomyocyte immunochemistry and cell surface area analysis

Cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 in PBS, followed by blocking with 5% goat serum in PBS for 1 h at room temperature. The cells were then incubated with anti-actinin antibody (Epitomics) at a 1:250 dilution overnight. After washing with PBS three times, the second antibody, coupled with Alexa Fluor 488 donkey (Invitrogen), was added to the cells. The nuclei were stained with propidium iodide (PI). After washing, the slides were mounted using a fluorescent mounting medium. Cell surface area was analyzed using Image-Pro Plus software (Media Cybernetics). Images were obtained using an Leica confocal microscope (CTR MIC) [29].

2.6. Statistical analysis

All of the experiments were performed at least three times. The data are expressed as means ± SEMs and were analyzed by ANOVA and post hoc analysis or by *t*-test as appropriate. A p value of 0.05 or less was considered significant [30,31].

3. Results

3.1. EZH2 is decreased in hypertrophic rat hearts and in PE-induced hypertrophic cardiomyocytes

Previous studies have linked miR-214 to cardiac hypertrophy, and the expression level of miR-214 was dynamically and inconsistently regulated during the early process [10,23,24]. To address this issue, we verified the changes in cardiac miR-214 expression in a SD rat cardiac hypertrophy model generated by abdominal aorta constriction (AAC). After constriction for 21 days, the ratio of heart weight to body weight was significantly increased, and the hearts showed apparent hypertrophic growth, compared with the hearts of the sham-operated rats (Fig. 1A and B). Several hypertrophic markers, including the β-myosin heavy chain (Myh7), skeletal muscle α-actin (Acta1) and atrial natriuretic peptide (Nppa), were also examined. As expected, Acta1, Myh7 and Nppa were upregulated (Fig. 1C). Using real-time PCR analysis, the level of miR-214 in the hypertrophic left ventricle was increased by 160%, compared with levels in the sham controls (Fig. 1D). To investigate whether the repression of EZH2 by miR-214 played a pathological role in this process, we detected the expression of EZH2 in hypertrophic left ventricles. Real-time PCR analysis showed that the mRNA level of EZH2 was decreased ($P < 0.05$, Fig. 1E). Additionally, the protein level of EZH2 was decreased as determined by western blot analysis (Fig. 1F). In addition this, we induced primary cultured neonatal rat cardiomyocyte hypertrophy *in vitro* by stimulation with phenylephrine (PE). After 48 h of treatment, the cardiomyocytes developed hypertrophy, evidenced by their increased cell surface area (Fig. 2A and B). Real-time PCR analysis revealed that the expression of miR-214 in hypertrophic cardiomyocytes was increased by 130% compared to that in the controls ($P < 0.05$; Fig. 2C). In agreement with the increase in miR-214, the endogenous EZH2 protein level was downregulated in PE-induced hypertrophic cardiomyocytes (Fig. 2D). These results suggest that EZH2 was derepressed by miR-214 during the pathogenesis of cardiac hypertrophy.

3.2. MiR-214 targets the 3′-UTR of EZH2

We validated the miRNA-mRNA hybridization structures and free energies between microRNA seed sequences and mRNA sequences by RNAhybrid. The EZH2 3′ UTR mutant had 3 different bases, which are C–G,G–A,C–A (Fig. 3A). The chimeric vector, luciferase-EZH2 3′-UTR, carries a constitutively activated promoter, which can express luciferase as a reporter (Fig. 3B). The effect of miR-214 on luciferase expression can be represented by the changes in luciferase activity. We co-transfected this vector with the miR-214 mimic into myogenic 293T cells. As indicated in Fig. 3C, the introduction of miR-214 mimic decreased luciferase

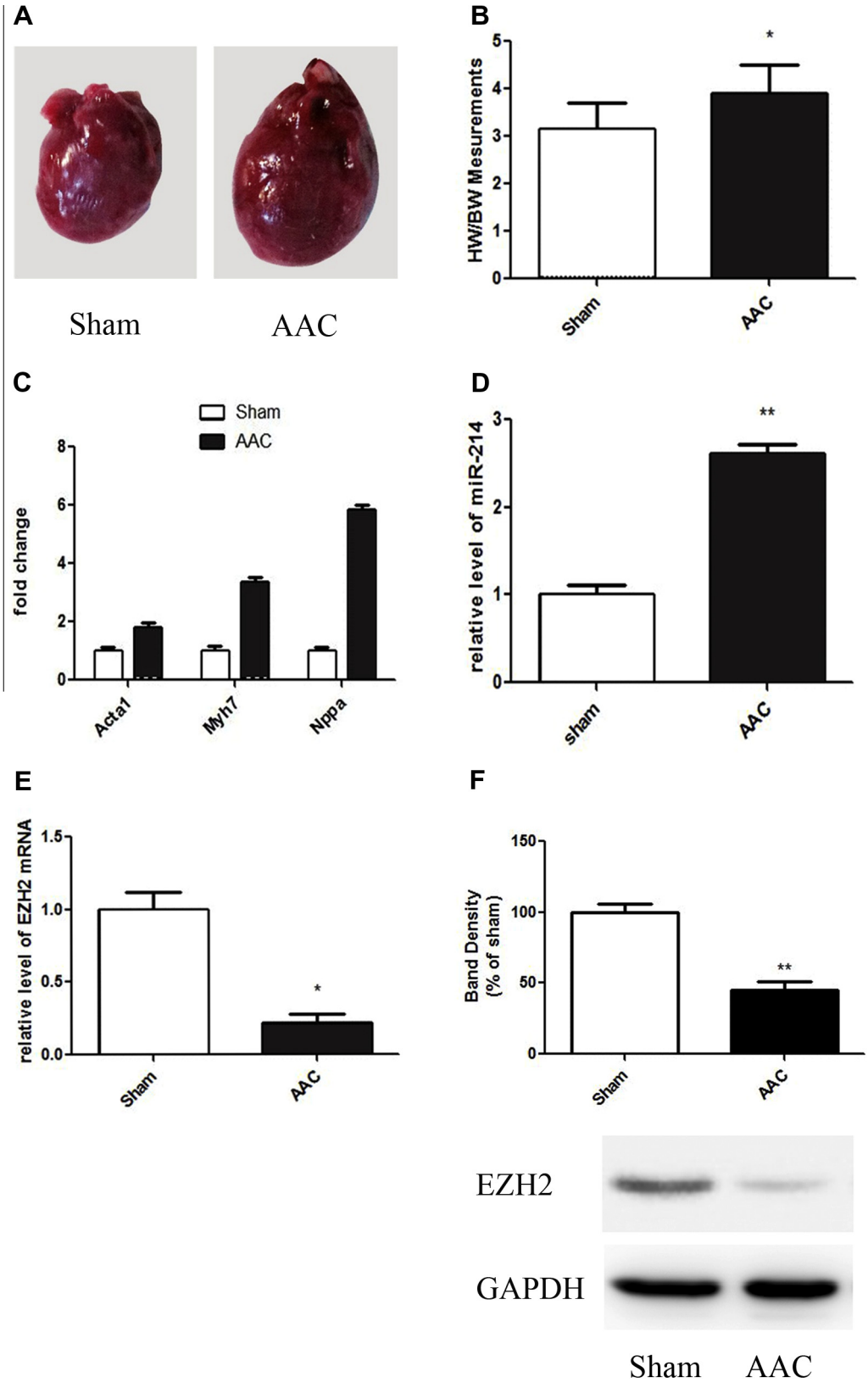

Fig. 1. EZH2 is decreased in hypertrophic rat hearts in contrast to upregulation of miR-214. SD rats were subjected to sham operations or AAC for 21 days, and then their hearts were analyzed. (A) Representative hearts from rats after sham operation or AAC. Scale bar: 10 μm. (B) Heart weight-to-body weight ratio of SD rats ($n = 8$) was significantly greater than that of controls ($n = 8$). (C) Real-time PCR analysis of the expression of Acta1, Myh7, and Nppa in sham and AAC rat hearts ($n = 8$ for sham group; $n = 8$ for AAC group). (D) The expression of miR-214 level was obviously increased in AAC rat hearts. (E) The mRNA level of EZH2 was downregulated in AAC rat hearts. (F) EZH2 was also downregulated as determined by western blot analysis and semiquantified by Image J in each group. The data shown represent three independent experiments. $^{*}P < 0.05$ vs. sham; $^{**}P < 0.01$ vs. sham.

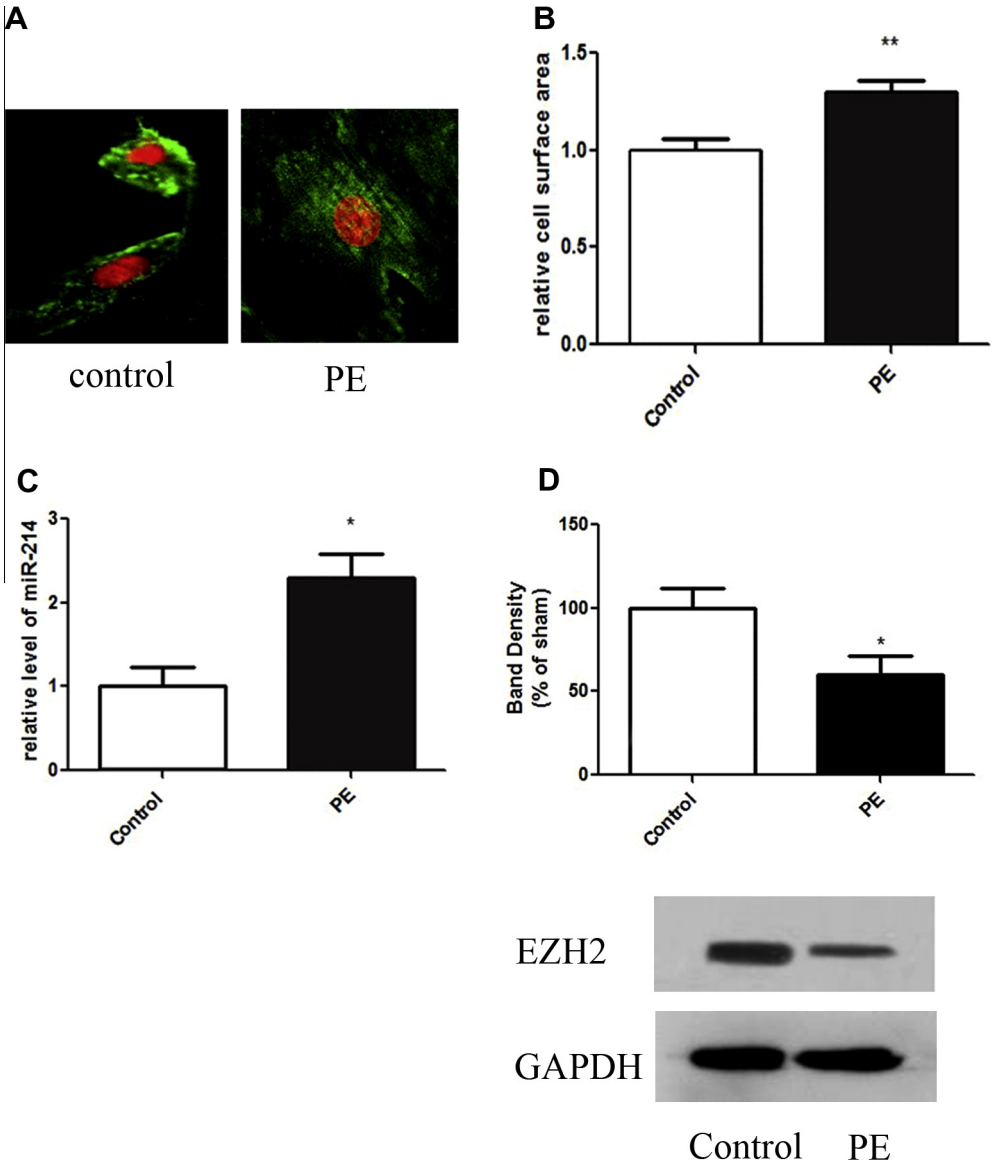

Fig. 2. EZH2 is downregulated in hypertrophic cardiomyocytes. (A) α-actinin staining shows hypertrophy of neonatal cardiomyocytes treated with 100 μM phenylephrine (PE) for 48 h. Representative confocal images are shown. Scale bar: 10 μm. (B) Quantitative analysis of cardiomyocyte size. Approximately 200 cells immunostained with anti-α-actinin antibody were randomly chosen from each treatment for surface area measurement. (C) The expression level of miR-214 was increased in PE-treated cardiomyocytes analyzed by real-time PCR. (D) EZH2 was downregulated in PE-induced hypertrophic cardiomyocytes as determined by western blot analysis and semiquantified. $^{*}P < 0.05$ vs. control.

activity by 60%, compared with the cells treated with a control mimic ($P < 0.05$). In a parallel experiment, the inhibitory effect of miR-214 on cells transfected with the mutant reporter vector (the putative 3′ UTR targeting site was mutated) was completely eliminated, as evidenced by high luciferase activity (Fig. 3C). These results confirmed the bioinformatics prediction that the 3′-UTR of EZH2 would be targeted by miR-214.

3.3. Overexpression of miR-214 by infection with lentivirus induces cardiomyocyte hypertrophy

To investigate the possible effects of miR-214 on cardiomyocyte hypertrophy, we infected neonatal rat cardiomyocytes with Lenti-miR-214 and examined the miR-214 expression levels. The Lenti-vector was used as a control. The infection of cardiomyocytes with Lenti-miR-214 induced a 33-fold increase in miR-214 over the endogenous level, as determined by real-time PCR analysis (Fig. 4A). To test further whether the increase in miR-214 was sufficient to induce cardiac hypertrophy, we constructed a lentivirus overexpressing miR-214 (Lenti-miR-214) and infected the neonatal cardiomyocytes. After infection for 72 h, the cell size, as measured by cell surface area, was increased with Lenti-miR-214 infection compared with Lenti-vector infection. Overexpression of miR-214 induced an the increase in cell size as measured by cell surface area (Fig. 4B and C). These results indicate that miR-214 overexpression in cardiomyocytes affects the morphology and structure of the cells, perhaps through the regulation of the cytoskeleton. To determine the effect of miR-214 overexpression on hypertrophic cardiomyocytes at the molecular level, the hypertrophic markers Acta1, Myh7 and Nppa were examined. Expression of all these genes was significantly increased in Lenti-miR-214-infected cells (Fig. 4D). These results indicate that miR-214 overexpression could result in cardiomyocyte hypertrophy. To establish whether the silencing of endogenous miR-214 would impair hypertrophy *in vitro*, we generated a Lentiviral vector in which a 3′-UTR with tandem miR-214-binding sites was linked to the

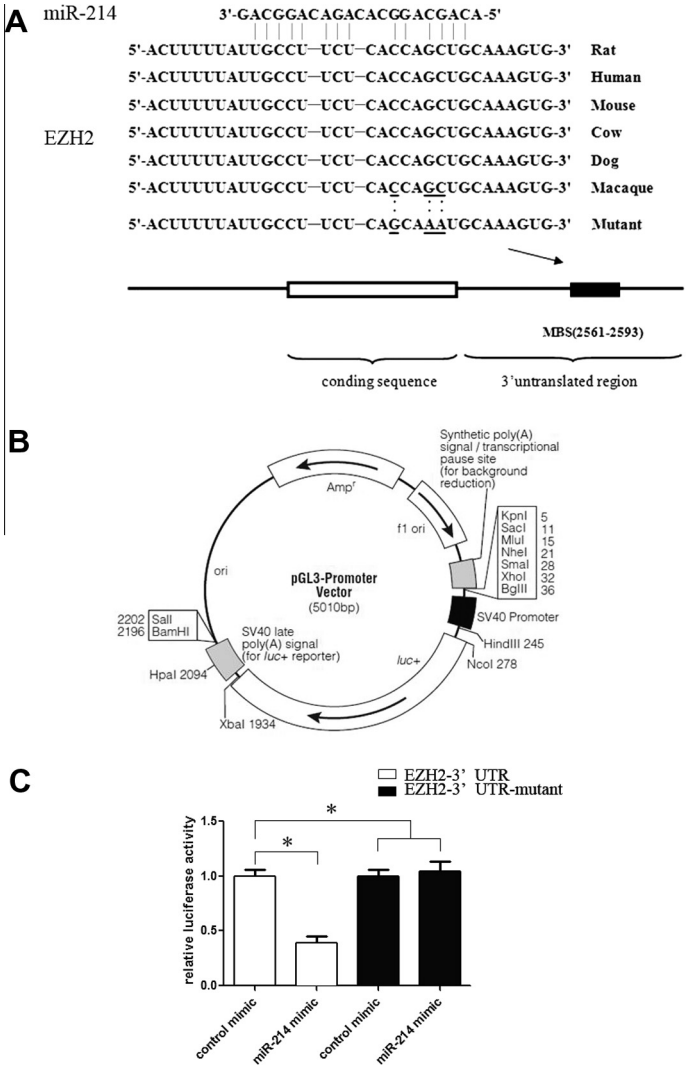

Fig. 3. Analysis of the EZH2 3′-UTR by luciferase activity assay. (A) Alignment of rat miR-214 sequence with the 3′-UTRs of EZH2 among different species. The absolute position of the miR-214 binding site in rat EZH2 mRNA and EZH2 3′-UTR mutant are shown. MBS:miR-214 binding site. (B) Luciferase-EZH2 3′-UTR carries pGL3-promoter vector. (C) Addition of the miR-214 mimic attenuated EZH2 3′-UTR reporter gene activity. Mutation of miR-214 putative target site blocked the suppressive effect of miR-214 on the target, suggesting EZH2 as a miR-214 target gene. $^{*}P < 0.05$ vs. control mimic.

enhanced green fluorescent protein (EGFP) reporter gene. The complementary sequence acts as a sponge to sequester endogenous miR-214. Infection of Lenti-sponge (Lenti-spg) silenced miR-214. Neonatal cardiomyocytes were infected with Lenti-miR-214 or Lenti-vector for 72 h, followed by treatment with PE for an additional 48 h. This demonstrated that miR-214 decreased 7-fold with the infection of Lenti-spg (Fig. 4E). Importantly, infection with Lenti-spg significantly reduced the cell size by PE stimulation (Fig. 4F and G) and inhibited the expression of hypertrophic markers (Fig. 4H). These results indicate that silencing of endogenous miR-214 upregulated the expression of EZH2 and attenuated cardiomyocyte hypertrophy. Collectively, these results suggest that elevated levels of miR-214 induced cardiac hypertrophy. However, blocking the function of miR-214 directly inhibited cardiac hypertrophy, which might have occurred through derepression of EZH2. Our results thus support the notion that miR-214 would induce hypertrophic activity.

4. Discussion

It is generally believed that there is an urgent need to develop effective, targeted therapeutic strategies to increase the survival rate for patients with cardiac hypertrophy, which is the most common cause of heart failure [10,32]. Thus, identification of the molecular pathogenesis of cardiac hypertrophy is crucial. Recently, an increasing number of studies have demonstrated that post-transcription regulation of miRNAs frequently occurs in cardiac tissues. Experimental evidence has demonstrated that miRNAs would be ideal biomarkers or therapeutic targets [33]. Previous studies have also found that upregulated miRNAs are common in cardiac hypertrophy [20,28]. A number of studies have created signatures for cardiac hypertrophy, which will help further establish molecular diagnoses and improve prognosis and therapy using miRNAs. However, only a limited number of studies have explored the modulatory function of miRNAs in cardiac hypertrophy. The roles of specific miRNAs in cardiac hypertrophy are still poorly understood.

It is noteworthy that miR-214 is frequently upregulated in pressure-overloaded hypertrophic hearts. Recent studies have also demonstrated that miR-214 displays obvious myocardial expression during heart development. However, miR-214 function in cardiac hypertrophy has not yet been elucidated. In this study, we investigated the physiological functions of miR-214 and the molecular mechanisms by which miR-214 induces cardiac hypertrophy. The physiological and pathological consequences of miR-214 were

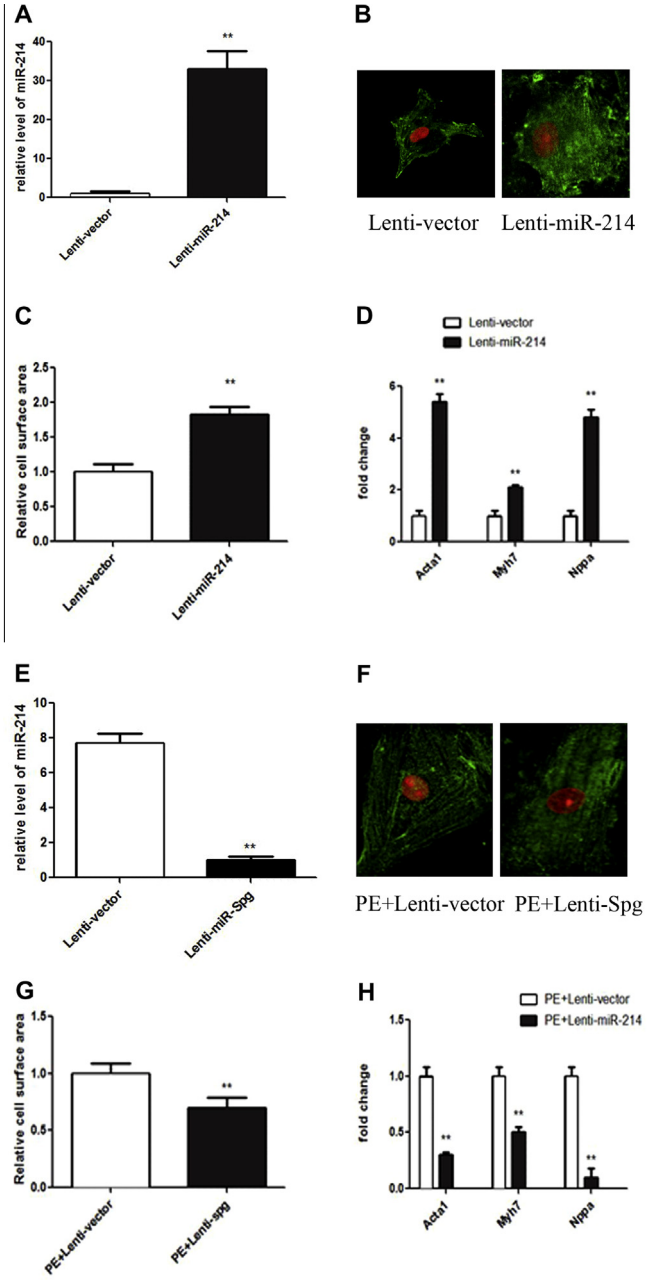

Fig. 4. Effects of overexpression or knockdown of miR-214 on cardiomyocytes. Cardiomyocytes were infected with recombinant Lentivirus expressing miR-214 (Lenti-miR-214) or with an empty vector (Lenti-vector) for 72 h. (A) Overexpression of miR-214 in neonatal rat cardiomyocytes significantly increased expression of miR-214. (B) Lenti-miR-214 increased cell size vs. Lenti-vector. Representative confocal images of α-actinin immunostaining are shown. Scale bar: 10 μm. (C) Quantitative analysis of cardiomyocyte size. (D) miR-214 provoked the expression of the indicated hypertrophic markers in neonatal rat cardiomyocytes by real-time PCR analysis. Neonatal rat cardiomyocytes were infected with recombinant Lentivirus, expressing miR-214 sponge (Lenti-spg) or Lenti-vector, for 72 h and were treated with 100 μM PE for additional 48 h; then, the cells were analyzed. (E) Repression of miR-214 in neonatal rat cardiomyocytes significantly decreased expression of miR-214. (F) α-actinin staining of PE-treated neonatal rat cardiomyocytes indicated that the cell size of Lenti-spg infected cardiomyocytes was reduced. Representative images were obtained by confocal microscope. Scale bar: 10 μm. (G) Quantitative analysis of cardiomyocyte size. (H) The expression of the indicated hypertrophic markers was downregulated in PE-stimulated neonatal rat cardiomyocytes infected with Lenti-spg, compared with cells infected with Lenti-vector by real-time PCR analysis. $^{**}P < 0.01$ vs. Lenti-vector.

elucidated in cardiac hypertrophy using both gain-of-function and loss-of-function approaches. Most importantly, we performed curative experiments to demonstrate that miR-214 inhibition was indeed beneficial for pressure overload-induced cardiac hypertrophy and HF. Previous studies have revealed that a limited number of miRNAs could constitute new therapeutic targets for ischemic heart diseases [34–38], as well as hypertension-induced HF [22,32,39–41]. Our study showed that knockdown of miR-214 by Lentivirus significantly attenuated hypertrophic effects caused by pressure overload, providing a new therapeutic target for pressure overload heart diseases.

In this study, we identified that EZH2 was the target of miR-214 and of the partially mediated cardiac hypertrophy that resulted from miR-214 overexpression. Six1 is a well-known critical regulator during cardiac hypertrophy. MiR-214 sponge inhibits mechanical stress-induced hypertrophy of cultured neonatal rat ventricular cardiomyocytes. Cardiac hypertrophy, induced by pressure overload in heterozygous EZH2-deficient mice, is slightly more pronounced than in wild-type controls. Studies in cardiomyocyte-specific EZH2 knockout mice have shown that EZH2 suppresses cardiac growth and embryonic gene expression, at the same time protecting the heart from oxidative damage. Previous studies have shown that miR-214 represses transcription of developmental regulators in embryonic stem (ES) cells and in committed cell lineages, including skeletal muscle cells (SMC), by targeting EZH2 [42]. In our study, we observed that miR-214 targeted EZH2 in cardiomyocytes. These findings are corroborated by the significant downregulation of EZH2 expression in the 293T cells of co-transfected miR-214, further confirmed by the Lentivirus experiment, which showed that overexpression of miR-214 increased hypertrophic growth. As miR-214 might target hundreds of mRNAs simultaneously, our results demonstrate that EZH2 is an important, but possibly not the only, target gene responsible for cardiac hypertrophy in miR-214-induced cardiac hypertrophy.

In conclusion, our study identified that the miR-214-regulated EZH2 signaling pathway is a regulator of cardiac hypertrophy, suggesting that antagonizing miR-214 might be a new therapeutic entry point for treating cardiac diseases.

Acknowledgments

We are grateful to Dr. Haiyang Xie for valuable help with the cell and animal experiments and to Dr. Xiaobo Yu for advice on the statistical analysis. Sources of funding: This study was supported by a research fund from Zhejiang Natural Science Foundation (Y2100486). The funding agencies had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.079.

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