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# MicroRNA-214 provokes cardiac hypertrophy via repression of EZH2 ☆

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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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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