

MicroRNA-206 Downregulates Connexin43 in Cardiomyocytes to Induce Cardiac Arrhythmias in a Transgenic Mouse Model



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Background

MicroRNAs (miRNAs) are critical modulators of various physiological and pathological processes, but their role in cardiac arrhythmias remains yet to be completely understood. Connexin43 (Cx43) is an important cardiac gap junction protein and a potential target of miR-206, and downregulation of Cx43 induces ventricular tachyarrhythmias.

Methods

We investigated the effects of miR-206 overexpression on the adult mouse heart and in cardiac arrhythmias. Luciferase activity assay was employed to validate Cx43 as a direct target of miR-206. Expression of Cx43 was measured in cardiac muscle cell line HL-1 securely expressing miR-206. An inducible miR-206 overexpression mouse model was established to evaluate the in vivo effect of miR-206 on Cx43 expression and cardiac rhythm.

Results

MiR-206 directly recognised 3'-untranslated region of Cx43 mRNA to inhibit its expression in HL-1 cells. Induction of miR-206 in the adult mouse heart suppressed Cx43 expression, particularly in the atria and ventricle. Importantly, miR-206 overexpression also induced abnormal heart-rate and PR interval, and shortened life-span in the experimental mice.

Conclusions

In cardiomyocytes, miR-206 is a upstream regulator of Cx43, and its overexpression downregulates Cx43 to induce abnormal heart-rate and PR interval.

Keywords

MicroRNA-206 • Connexin43 • Cardiomyocyte • Cardiac arrhythmias • Atrial fibrillation

Introduction

Cardiac arrhythmias can be categorised into supra-ventricular and ventricular arrhythmias based on the arrhythmic site of origin [1]. Besides location, arrhythmias can also be categorised into bradyarrhythmias and tachyarrhythmias based on the arrhythmic rate. Arrhythmias can be caused by various factors such as ion channel dysfunctions and/or

structural changes, and all of these factors pose as major contributors to morbidity and mortality of patients. Among all arrhythmias, atrial fibrillation (AF) is the most common, which has an estimated prevalence of 0.4–1% worldwide and increases to ~8% among aged populations (>80 years) [2]. Arrhythmia causes severe impairments in the left ventricle, and contributes to a major proportion of sudden death among patients. Patients with heart failure also manifest

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various other severe abnormalities of heart rhythm besides ventricular tachyarrhythmias, including abnormal heart-rate and PR interval.

The gap junction, which connects the cytoplasm of adjacent cells, is a key regulator of conduction in the heart [3]. Gap junctions are clustered channels, consisting of two hemichannels, each of which is formed by six connexins (Cxs) [4]. Many Cxs are found to be expressed in cardiomyocytes, while connexin43 (Cx43) is the major Cx expressed in both the ventricle and atria [5–7]. Downregulation of Cx43 gap junction channel in the heart was reported to play an important role in AF pathogenesis, causing slowed conduction of the heart and sudden arrhythmic death [8].

Recent studies have implicated microRNAs (miRNAs) in cardiac diseases, and have supported the important roles of miRNAs in cardiac arrhythmias. MiRNAs are a type of short, noncoding RNAs exhibiting important regulatory functions in gene expression during a variety of physiological and pathological processes [9]. MiRNAs are normally negative regulators, to promote mRNA degradation or inhibit protein translation through complementary base pairing to sequences in the 3' untranslated region (3'-UTR) of their target mRNA transcripts [10]. In this context, miRNAs function as an additional regulatory mechanism over complicated biological pathways, such as cardiac excitation and arrhythmia.

Upon screening through several miRNAs predicted to target Cx43, we have experimentally confirmed miR-206 to be an upstream regulator of Cx43. We, therefore, speculated that miR-206 overexpression, and subsequent Cx43 downregulation, could cause abnormal cardiac rhythm. By establishing a transgenic mouse model with inducible miR-206 overexpression in the cardiomyocytes, we observed loss of Cx43 protein in the hearts of transgenic mice, abnormal heart-rate and PR interval, as well as shortened life span.

Materials and Methods

Cell Line

Cardiac muscle cell line HL-1 was maintained in culture flasks coated with 5 μ l/mL fibronectin and 0.02% gelatin. Cells were kept at 37 °C in 5% CO₂ and 95% humidity in Claycomb medium (Sigma, MO, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mM norepinephrine, 100 μ g/mL streptomycin and 100 U/mL penicillin. Once the culture reached confluence and the hearts were beating, 5 \times 10⁵ cells were subsequently plated in 6-well plates coated with fibronectin/gelatin [11].

MicroRNA assays

MiR-206 expression was measured with TaqMan Advanced miRNA Assay Kit (mmu481645_mir, Applied Biosystems, Waltham, MA, USA) according to manufacturer's protocol. For stable expression of miR-206, the MISSION Lenti miR-206 mimic (HLMIR0364; Sigma-Aldrich, Burlington, MA, USA) and negative control (NCLMIR001; Sigma-Aldrich, USA) were

packaged into lentivirus following manufacturer's instructions, which were then used to establish stable cell lines.

Luciferase Assay

3–5 \times 10⁵ cells were transfected with 1 μ g pMT01 (control vector), or Cx43 3'-UTR reporter (GeneCopoeia, Rockville, MD, USA), which contain both renilla and firefly luciferase reporters, respectively, by Superfect (Qiagen, Valencia, CA, USA). Luciferase activity was assessed using the Dual Luciferase Reporter Assay (Genecopia) with renilla luciferase activity as internal normalisation.

Quantitative Real-Time PCR Analysis

Total RNA was isolated with TRIzol assay (Invitrogen, Carlsbad, CA, USA) following manufacturer's protocols. Isolated RNA was then reversed transcribed using ABI TaqMan RT kit (Applied Biosystems, USA) and used as template in PCR reactions performed in BioRad CFX96 Thermocycler (BioRad, Hercules, CA, USA), using SSo Advanced SYBR Green MasterMix (Bio-Rad, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used to normalise gene expression. Primer sequences are provided as follows: Cx43 forward 5'-TCC TGG GTA CAA GCT GGT CAC TGG-3', reverse 5'-GCT GCT GGC TCT GCT GGA AGG-3'; GAPDH forward 5'-TGA CAA GCT TCC CAT TCT CG-3', reverse 5'-GTG AAG GTC GGT GTG AAC G-3'.

Western Blot

Mouse hearts were collected and lysates of protein samples were prepared using previously established method [12]. 20 μ g of samples were separated by 10% SDS-PAGE, followed by transferring onto a nitrocellulose membrane (GE Healthcare, NJ, USA). Antibodies used in the study were as follows: Cx43 (Cell Signaling, Danvers, MA, USA), HRP-coupled goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA).

Inducible miR-206 Overexpression in Transgenic Mice

All animal procedures have been approved by and carried out following the Animal Care and Use Committee of The Affiliated Wuxi No.2 People's Hospital of Nanjing Medical University. Genomic region of miR-206 precursor was amplified using the mouse genomic DNA, which was then cloned into a plasmid containing the tetracycline responsive promoter (TetO). The construct, hereby named TetO-miR206, was then injected into the CD-1 embryo pronuclei, which were implanted into pseudo-pregnant recipient female mice. TetO-miR206 founder mice were mated with CD-1 mice to expand for at least seven generations on the CD-1 genetic background. Non-transgenic littermates (termed "control mice") were compared with transgenic littermates with the genotype α MHC-tTA/TetO-miR206 (termed " α MHC-miR206 mice").

Electrocardiogram (ECG) Monitoring

For electrocardiogram (ECG) monitoring, both groups of mice, anaesthetised with isoflurane, were implanted with telemetry transmitters (ETA-F10; DSI) in the back with leads tunnelled to

the left lower and right upperthorax. Heart-rate and PR interval were evaluated by Ponemah Physiology Platform (DSI, Data Science, Beijing, China) from 24-hour long monitoring.

Statistical Analysis

Values were presented as mean \pm standard deviation (SD). Two-tailed Mann-Whitney U test was used for comparing means between the two groups. P values <0.05 were considered to be statistically significant.

Results

MiR-206 Inhibits Cx43 Expression Via its 3'-untranslated Region (UTR)

We examined the sequence of Cx43 mRNA, using the miRanda algorithm [13] to identify miRNAs that could potentially target Cx43 mRNA. MiR-206 was identified as a high confidence candidate (Figure 1A). In order to establish direct targeting between miR-206 and Cx43 mRNA 3'-UTR, wild type (Cx43-wt) targeting sequence of miR-206 on 3'-UTR of Cx43 mRNA, and the mutated version (Cx43-mut), were cloned at the downstream of the luciferase open reading frame (ORF) (Figure 1B). Luciferase constructs were transfected into cardiac muscle cell line HL-1 [11] securely

expressing miR-206 mimic (Figure 1C), followed by luciferase activity assay. We observed that the activity of Cx43-wt construct was greatly reduced in cells overexpressing miR-206, whereas activity of Cx43-mut construct was unaltered under the same conditions (Figure 1D).

As expected, in HL-1 cells overexpressing miR-206, expressions of both Cx43 mRNA and protein were greatly inhibited (Figure 2A and B). Next, expression of Cx43 was re-introduced into HL-1 cells securely expressing miR-206 mimic, using a construct containing Cx43 ORF independent of its 3'-UTR. Expression of this Cx43 construct readily restored both the mRNA and protein to their original levels in the absence of miR-206 (Figure 2A and B). Taking results in Figures 1 and 2 together, we concluded that, in HL-1 cells, miR-206 functioned to inhibit Cx43 expression via its 3'-UTR.

Induction of miR-206 in the Adult Mouse Heart Suppresses Cx43 Expression

An inducible miR-206 overexpression mouse model was established (see Materials and Methods). Breeding pairs and their offspring were fed with doxycycline-containing drinking water until weaning, at which time doxycycline was removed from drinking water (Figure 3A). Non-transgenic littermates were termed control mice, while transgenic littermates

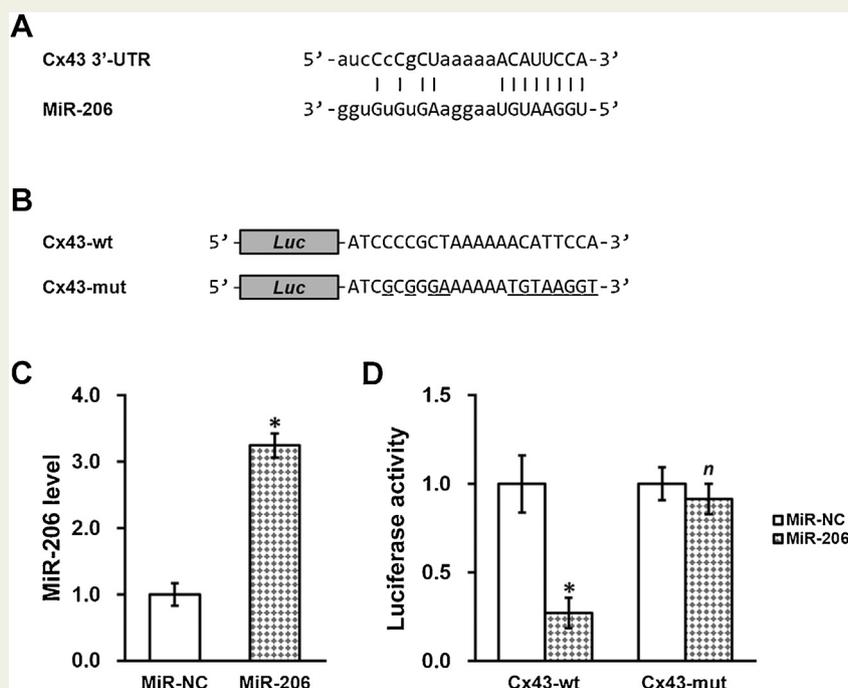


Figure 1 MiR-206 directly recognises 3'-UTR of Cx43 mRNA.

(A) Predicted miR-206 targeting sequence on the 3'-UTR of Cx43 mRNA. (B) Wild type (Cx43-wt) targeting sequence of miR-206 on 3'-UTR of Cx43 mRNA, and the mutated version (Cx43-mut), were cloned at the downstream of the luciferase open reading frame (Luc). (C) Levels of miR-206 in HL-1 cells were examined after stable expression of negative control miR (miR-NC) or miR-206 mimic, respectively. (D) Luciferase activities of Cx43-wt or Cx43-mut constructs in HL-1 cells were examined after stable expression of negative control miR (miR-NC) or miR-206 mimic, respectively. Values are mean \pm SD from three independent experiments. * $p < 0.05$, ⁿ not significant, compared to miR-NC.

Abbreviations: 3'-UTR, 3' untranslated region; Cx43, Connexin43.

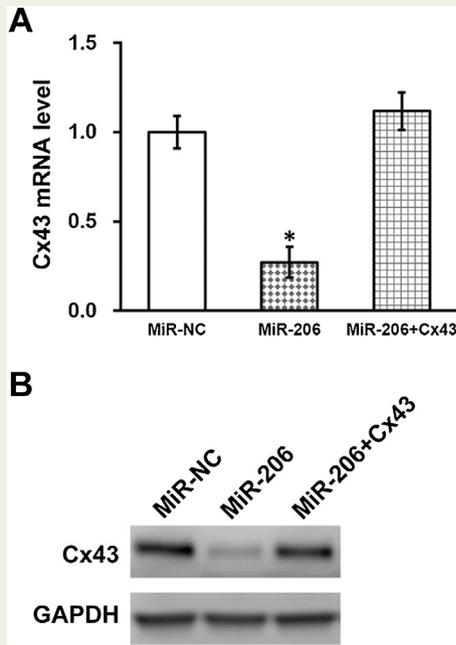


Figure 2 MiR-206 inhibits Cx43 expression via its 3'-UTR.

HL-1 cells were transfected with negative control miR (miR-NC) or miR-206 mimic, respectively, following expression of Cx43 independent of its 3'-UTR (miR-206 + Cx43). (A) mRNA and (B) protein levels of Cx43 were then subjected to RT-PCR or Western blot analyses, respectively. Values are mean \pm SD from three independent experiments. * $p < 0.05$, compared to both miR-NC and miR-206 + Cx43.

Abbreviations: 3'-UTR, 3' untranslated region; Cx43, Connexin43; RT-PCR, reverse transcription polymerase chain reaction.

genotyped α MHC-tTA/TetO-miR206 were termed α MHC-miR206 mice. We confirmed that miR-206 was greatly upregulated in the transgenic hearts at 6 and 12 weeks after doxycycline removal from drinking water, and the overexpression was more pronounced at week 12 (Figure 3B).

Connexin43 has already been shown as a direct target of miR-206 in vitro in HL-1 cells, we therefore verified whether this miR-206/Cx43 targeting cascade also existed in vivo in the adult mouse heart. We examined the mRNA and protein levels of Cx43 in heart lysates of both control and α MHC-miR206 mice at weeks 6 and 12 after removal of doxycycline from drinking water, and observed significantly reduced Cx43 expression in the α MHC-miR206 mouse hearts, compared to control mice, at both time points (Figure 4A and B). It was also noted that the inhibitory effect on Cx43 expression was greater at week 12 than week 6 in α MHC-miR206 mice.

With the observed reduction in Cx43 protein levels in whole heart lysates, we next examined its expression levels in different heart sections, namely the atria and ventricle, 12 weeks after doxycycline withdrawal. As expected, Cx43 expression was repressed throughout the atria and ventricles of α MHC-miR206 mice, compared to control mice (Figure 5A

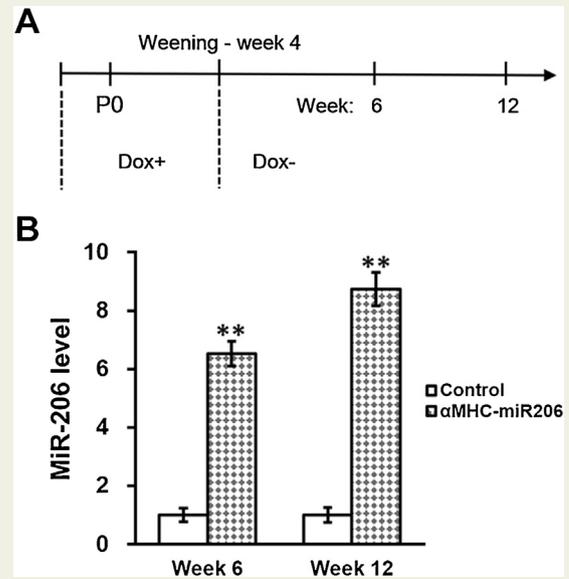


Figure 3 Induction of miR-206 in adult mouse heart. (A) Schematic outline of doxycycline (Dox) treatment and study time points. (B) Relative miR-206 levels in transgenic heart at 6 and 12 weeks after doxycycline removal (Dox-) from water supply. Values are mean \pm SD ($n = 6$). ** $p < 0.01$ * $p < 0.05$, compared to control.

and B). Taken together, the above results clearly demonstrated that miR-206 overexpression in the adult heart caused marked downregulation in Cx43 expression.

MiR-206 Overexpression in the Adult Heart Induces Cardiac Arrhythmias

Since the loss of Cx43 gap junction channels in the heart was reported to induce ventricular tachyarrhythmias [8], we next hypothesised that miR-206 mediated downregulation of Cx43 in the adult mouse heart could also contribute to cardiac arrhythmias. Heart-rate and PR interval were monitored for 24 hours at week 12 after doxycycline removal. We observed a significantly reduced heart-rate in α MHC-miR206 mice compared to control mice (Figure 6A). Furthermore, the PR interval was also significantly longer in α MHC-miR206 mice than control mice (Figure 6B). Last, we have monitored the survival of mice for a period of 16 weeks after doxycycline removal from water supply, and found control mice displayed a significantly higher survival rate than α MHC-miR206 mice (Figure 6C). The observed abnormalities in the above two key cardiac rhythmic parameters, as well as shortened life span, supported our hypothesis that miR-206 overexpression in adult hearts could induce cardiac arrhythmias.

Discussion

Recent investigations have implicated miRNAs in regulating gap junction proteins, suggesting a network of miRNAs is critically involved in cardiac rhythm. In myocarditis and myocardial infarction mouse models, miR-1 was able to

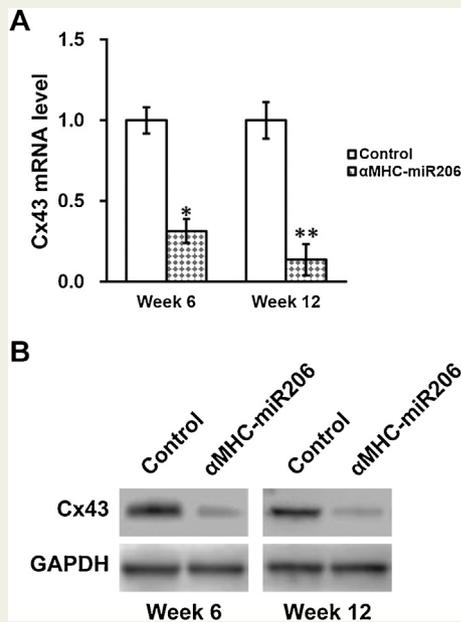


Figure 4 MiR-206 in adult mouse heart suppresses Cx43 expression.

Expressions of mRNA (A) and protein (B) of Cx43 were examined in hearts of control and in α MHC-miR206 mice at 6 and 12 weeks after doxycycline removal from water supply. Values are mean \pm SD (n = 6). ** p < 0.01, * p < 0.05, compared to control. Abbreviations: Cx43, Connexin43.

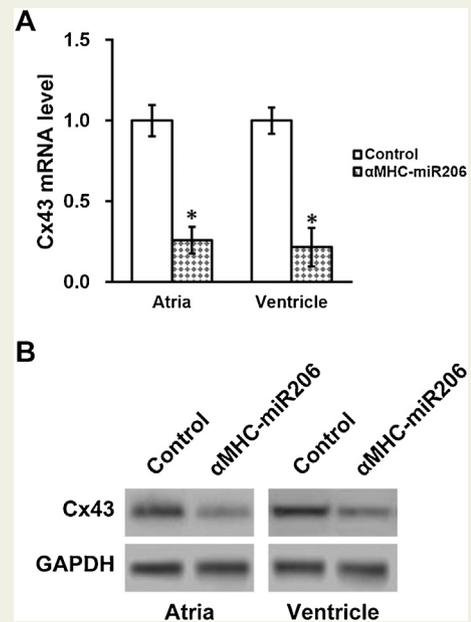


Figure 5 Loss of Cx43 in the atria and ventricle of α MHC-miR206 mice.

At week 12 after doxycycline removal from water supply, expressions of mRNA (A) and protein (B) of Cx43 were examined in atria and ventricle of control and in α MHC-miR206 mice. Values are mean \pm SD (n = 6). * p < 0.05, compared to control. Abbreviations: Cx43, Connexin43.

repress Cx43 expression [14,15]. In addition, the miR-17/92 cluster was also implicated in arrhythmias and cardiomyopathy. Cx43 was also reported to be a direct target of miR-19a/b, which suppressed Cx43 in transgenic animal hearts [16]. In the current study, we have presented results indicating that miR-206 is yet another upstream regulator of Cx43 in cardiomyocytes, and its overexpression causes abnormal heart-rate and PR interval, which can be attributed to

downregulated Cx43, an essential gap junction protein. Our results are consistent with the arrhythmogenic myocardial substrate reported in the loss of Cx43 models, therefore also point to the complicated nature of molecular pathways regulating heart expression of Cx43. Gap junction channels are indispensable for intercellular communication and amplification of electrical signals across the heart. In advanced stages of heart disease, Cx expression and intercellular signal

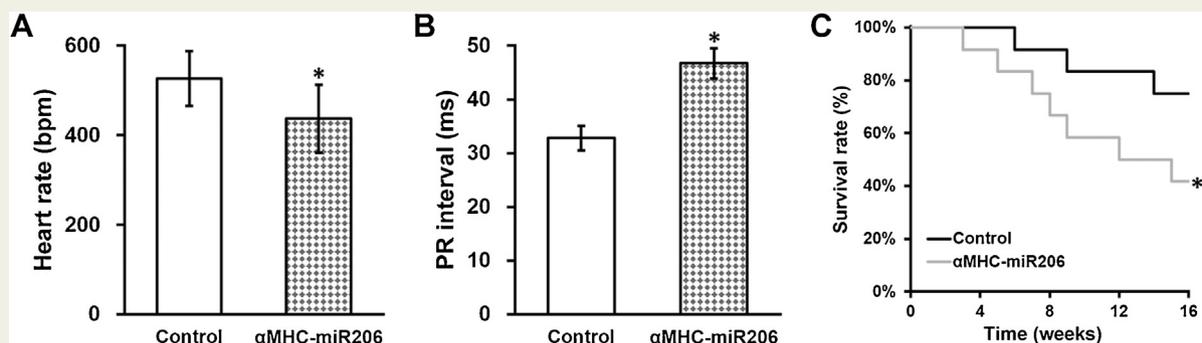


Figure 6 Heart-rate and PR interval in control and α MHC-miR206 mice.

At week 12 after doxycycline removal from water supply, heart-rate (A) and PR interval (B) in control and α MHC-miR206 mice were monitored, respectively. Values are mean \pm SD (n = 6). (C) Survival of control and α MHC-miR206 mice (n = 12 each group) were monitored for a period of 16 weeks after doxycycline removal from water supply. * p < 0.05, compared to control.

exchanges are reduced and normal gap junction channel distribution is disrupted. These pathological processes have been widely reported to occur during lethal arrhythmias through the origination of proarrhythmic substrates [17,18]. Various Cxs are expressed in cardiomyocytes, while Cx43 is the major type of Cx expressed in both the atria and ventricle [5–7].

We have established a conditional transgenic mouse model to show that miR-206 overexpression in adult cardiomyocytes causes abnormal heart-rate and PR interval in the atria and ventricle of adult heart. Our results have demonstrated that loss of Cx43, a direct target of miR-206, may contribute to the above symptoms. MiR-206 has been previously reported to modulate intrinsic cardiac autonomic nerve remodelling by regulating SOD1 [19]. Downregulation of endogenous miR-206 in cardiomyocytes attenuated Yes-associated protein-induced cardiac hypertrophy and survival [20]. Considering miRNAs are pleiotropic, downstream effects of dysregulated miR-206 are likely mediated by various target genes. In the context of this study, in adult mouse cardiomyocytes, we observed that miR-206 overexpression strongly repressed Cx43 expression, and loss of Cx43 in the miR-206 overexpressing transgenic mice contributed to abnormal heart-rate and PR interval. Our current observations, together with previous reports [19,20], strongly indicate that miR-206 is implicated in various cardiac diseases by targeting different downstream genes. In addition, it was previously reported that cardiac knockout of Cx43 caused slowed conduction of the heart and sudden arrhythmic death [8], which is consistent with the abnormal heart-rate, as well as shortened life span, observed in the current study.

Besides reduced heart-rate, increased PR interval was also observed in the miR-206 transgenic mice. Cx43 expression is greatly suppressed inside the AV node, which only increases after the compact portion of the AV node [21]. Prolonged PR interval is likely attributed to the inhibition of Cx43 in this portion of the conduction system, although our data cannot rule out the involvement of a secondary mechanism.

Our study, as well as others, has suggested that remodeling of gap junctions reduces intercellular coupling and contributes to the re-entrant arrhythmia pathogenesis. Atrial and ventricular arrhythmias are complex pathological processes, and believed to arise from multiple determinant factors. To date, we haven't uncovered the electrophysiological mechanisms underlying miR-206 involvement in arrhythmias, although cellular uncoupling as a result of Cx43 gap junction channel disruption could reveal ectopic foci and/or enhance early afterdepolarisations [22]. In a chimeric focal Cx43 loss in the myocardium murine model, focal uncoupling sites were correlated with elevation of spontaneous ectopic incidences, likely caused by uncovering inherent automaticity or disruption of the conduction wave front which led to wave breaks [23]. Other unknown effects of miR-206 on potential targets that have not yet been identified could also contribute to gap junction remodelling.

In conclusion, we hereby report for the first time that, miR-206 directly recognises 3'-UTR of Cx43 mRNA to inhibit its

expression in HL-1 cells. Induction of miR-206 in the adult mouse heart suppressed Cx43 expression, particularly in the atria and ventricle. Importantly, miR-206 overexpression also induced abnormal heart-rate and PR interval, as well as shortened life span in the experimental mice. MiR-206 is a novel upstream regulator of Cx43, and its overexpression downregulates Cx43 in cardiomyocytes to contribute to abnormal heart-rate and PR interval, and shortened life-span. It is worth noting that, since miRNAs are pleiotropic, overexpressing miR-206 is likely to cause effects other than those observed in the current study, which certainly warrants further investigations.

Conflicts of Interest

The authors declare no conflicts of interest.

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None.

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