MicroRNA-29c affects zebrafish cardiac development via targeting Wnt4

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Abstract. As a single cardiac malformation, ventricular septal defect (VSD) is the most common form of congenital heart disease. However, the precise molecular mechanisms underlying VSD are not completely understood. Numerous microRNAs (miRs/miRNAs) are associated with ventricular septal defects. miR-29c inhibits the proliferation and promotes the apoptosis and differentiation of P19 embryonal carcinoma cells, possibly via suppressing Wnt4 signaling. However, to the best of our knowledge, no *in vivo* studies have been published to determine whether overexpression of miR-29c leads to developmental abnormalities. The present study was designed to observe the effect of miRNA-29c on cardiac development and its possible mechanism *in vivo*. Zebrafish embryos were microinjected with different doses (1, 1.6 and 2 μmol) miR-29c mimics or negative controls, and hatchability, mortality and cardiac malformation were subsequently observed. The results showed that in zebrafish embryos, miR-29c overexpression attenuated heart development in a dose-dependent manner, manifested by heart rate slowdown, pericardial edema and heart looping disorder. Further experiments showed that overexpression of miR-29c was associated with the Wnt4/β-catenin signaling pathway to regulate zebrafish embryonic heart development. In conclusion, the present results demonstrated that miR-29c regulated the lateral development and cardiac circulation of zebrafish embryo by targeting Wnt4.

Introduction

As the first functional organ during embryonic development, the heart must maintain a high level of efficiency during the whole life cycle of the organism, from the early formation of the primitive cardiac tube to the formation of the chamber, and even in the later stages of development (1). Cardiac development involves numerous genes regulated by multiple signaling pathways. Any genetic dysregulation may lead to abnormal cardiac development and congenital heart disease (CHD) (2). In previous decades, novel molecular technologies have focused on the molecular mechanism of cardiac development. It has been found that Nkx2 homeobox 5 (3), T-box transcription factor 5 (4) and GATA binding protein 4 (5) are closely related to heart development and function. However, the gene regulatory network for heart development remains to be elucidated.

MicroRNAs (miRs/miRNAs) are endogenous small RNAs harboring 20-24 nucleotides. miRNAs interact with the 3'-untranslated region (UTRs) of specific RNA targets to promote homologous mRNA degradation, inhibit protein translation and regulate gene expression at the post-transcriptional level (6). Previous data have reported that miRNAs play a critical role in various biological processes, including cell proliferation, cell cycle, apoptosis (7), differentiation (8), cell migration and intracellular signal transduction (9,10). Previous research has confirmed that miRNAs are involved in the regulation of normal heart development (11). The miR-1/miR-133 cluster is the most abundant small RNA expressed in the heart (12), and is the first and most widely studied miRNA among all heart development-specific miRNAs (1). miR-1 overexpression resulted in thinning of the ventricular wall and embryo death at embryonic day 13.5 (E13.5) (13), whereas deficiency of miR-1-2 led to embryo death at E15.5 due to poor ventricular myocyte proliferation (12). Additionally, miR-133a was revealed to negatively regulate cardiac myocyte proliferation (14,15). Furthermore, miR-1 promoted the differentiation of embryonic stem cells into cardiomyocytes during *in vitro* studies. However, to investigate the molecular mechanisms underpinning these processes, more regulatory miRNAs need to be studied.

Previously, it has been reported that miR-29c expression in fetal CHDs was significantly upregulated (16,17), which suggested that miR-29c may be another cardiac development-specific miRNA. Further research revealed that miR-29c could promote the differentiation and apoptosis, and inhibit the proliferation of cardiomyocyte-like cells, possibly by suppressing Wnt4 signaling in P19 embryonal carcinoma cells (17,18). The present study aimed to observe the mechanism of miRNA-29c in cardiac development.

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Zebrafish have only one atrium and one ventricle. A previous study reported that the embryonic cardiac development of zebrafish is similar to that of mammals (19). Zebrafish have a shorter growth cycle compared with mammals. Zebrafish embryos are transparent, and the heart is located on the ventral side; hence early-stage cardiac physiology can be easily observed. Consequently, zebrafish are commonly used to model cardiovascular diseases. In the present study, using a zebrafish model injected with miR-29c mimics, the effect of miR-29c overexpression on embryonic heart development and the activation of Wnt4 signaling pathway were observed. The present study demonstrated that miR-29c regulated the lateral development and cardiac circulation of zebrafish embryo by targeting Wnt4.

Materials and methods

Zebrafish feeding. According to protocols approved by the Research Ethics Committee of Jiangsu Taizhou People's Hospital (Taizhou Clinical Medical School of Nanjing Medical University), Female zebrafish were reared in the zebrafish installation of the Model Animal Research Center, Nanjing Medical University. As previously described (20), the embryos obtained from the natural oviposition of wild-type (WT) adults were cultured in embryo medium, as previously described (21), at 28.5˚C. As previously described (22-24), embryonic development was staged based on morphological characteristics. The zebrafish embryo appears in a transparent form, but after ~24 h of development, it begins to generate melanin, which hinders *in vivo* observation and signal detection with fluorescence. Therefore, it is necessary to inhibit the generation of melanin to maintain the transparency of zebrafish embryo, thereby reducing errors in the results. Thus, the embryos were incubated with 0.003% 1-phenyl-2-thiourea (Sigma-Aldrich; Merck KGaA) at 8 h post-fertilization (hpf) to prevent melanocyte development.

miR-29c mimic synthesis and microinjection. The present study was divided into three groups: wild-type (WT), experimental (miR-29c mimics) and negative control (NC) groups. The miR-29c mimics (5'-UGACCGAUUUCUCCUGGUGUUC-3') and NC (5'-UUCUCCGAACGUGUCACGUTT-3') were synthesized by Shanghai GenePharma Co., Ltd., and dissolved in 20 μm diethylpyrocarbonate. Using a capillary needle filled with fine borosilicate glass on the back, miR-29c mimics (1, 1.6 and 2 μM) or equivalent concentrations of NC was injected into single to four-cell-stage embryos, respectively. The injected embryos were cultured in a constant temperature incubator at 28.5˚C for future use. The interval between transfection and subsequent experimentation was at least 24 h. Degenerated zebrafish embryos were removed at any time and the solutions were replaced daily.

In situ hybridization probe preparation. Probes of the following genes were synthesized for *in situ* hybridization: Ventricular myosin heavy chain (vmhc), atrial myosin heavy chain (amhc), natriuretic peptide precursor A (nppa), cardiac myosin light chain-2 (cmlc2), bone morphogenetic protein 4 (bmp4), notch homolog 1b (notch1b), fli-1 proto-oncogene ETS transcription factor a (fli1a). The hybridization probes were amplified from the cDNA generated from the embryos at 120 h post-fertilization. cDNA was synthesized from 500 ng RNA using a reverse transcriptase kit (Vazyme Biotech Co., Ltd.) with the following temperature protocol: 25˚C for 5 min, followed by 50˚C for 15 min and 85˚C for 5 min (25). Then, cDNA was subcloned into a pGEM-T plasmid vector (Takara Bio, Inc.). The plasmid vector was linearized and transcribed using the Ambion® MAXIscript® T7 *In Vitro* Transcription kit (Ambion; Thermo Fisher Scientific, Inc.). The T3 RNA polymerase was purchased from Ambion (Thermo Fisher Scientific, Inc.). Single-strand RNA probes were prepared using a Digoxigenin RNA Labeling kit, according to the manufacturer's instructions (Roche Diagnostics). The template was removed using 2 U/l ribonuclease-free DNase I (Ambion; Thermo Fisher Scientific, Inc.) for 15 min at 37˚C. The RNase inhibitor used was purchased from Ambion (Thermo Fisher Scientific, Inc.). Digoxigenin-labeled probes (DIG-dUTP) were frozen at -80˚C. The primer sequences and restriction sites are shown in Table I.

Observation of malformation rate, lethality, hatch rate and zebrafish cardiac phenotypes. Following injection, the malformation rate and lethality at 24, 48, 72 and 96 hpf, and the hatch rate at 72 hpf were measured with an Olympus SZ61 dissecting microscope as previously described (25,26). The cardiac phenotypes of the embryos at 48 and 72 hpf were observed. Cardiac-specific phenotypes were imaged with a digital camera (Olympus DP71; Olympus Corporation). Adobe Photoshop CS5 (Adobe Systems, Inc.) was used to process the digital images.

Measurement of heart rate. Every 20 sec, the heart rate was recorded as the number of consecutive contractions during one diastole phase under a light dissecting microscope (MVX10; Olympus Corporation; magnification, x8) every 20 sec. The method is briefly described as follows (26): Tricaine stock solution (Sigma-Aldrich; Merck KGaA) was prepared for anesthesia using 97.9 ml double-distilled water, 400 mg tricaine powder and 2.1 ml of 1 mol/l Tris (pH 9). Subsequently, 400 μl tricaine (4 mg/ml) was diluted in 8 ml double-distilled water. Following anesthesia, the embryos were transferred to a recording chamber filled with modified Tyrode's solution. The heart rates of the embryos were then measured at different time points as previously described (25).

Histological staining. Zebrafish were euthanized in a mixture of ice and water for 20 min and then placed in a refrigerator at -20˚C. Cessation of the heartbeat was used to confirm death. To observe histological changes, zebrafish embryos were fixed with 4% paraformaldehyde at 4˚C overnight and embedded in paraffin. The embryos were sectioned consecutively at 5-μm thickness. Embryonic tissues were stained with hematoxylin (1 min) and eosin (5 sec) at room temperature. Photomicrographs were taken using an Olympus DP71 digital camera (Olympus Corporation) under a polarizing light microscope (Olympus BX51; Olympus Corporation; magnification, x200). At least three consecutive sections were selected to ensure that the section contained the embryonic heart structures of atrioventricular canal (AVC), and/or leaflets of the atrioventricular valve.

Table I. Description of probes used for *in situ* hybridization.

Probe name	Amplification primers	Restriction sites	Transcription direction
vmhc	Forward: CTCCTGGTGCAAAGAATC Reverse: TTCAGCTCAGAGTGGCATTCGTCC	*Sal*I	T7
amhc	Forward: AAGCATTCGCTCGTGGACT Reverse: CATCCAGTGTCTGCTGGT	*Nco*I	SP6
bmp4	Forward: TGCCAAGTCCTACTGGGAG Reverse: CGTGATTGGTGGAGTTGAG	*Sac*II	SP6
cmlc2	Forward: CTCTTCCAATGTCTTCTCC Reverse: TATTTCCAGCCACGTCTA	*Sal*I	T73
notch1b	Forward: GGCCAAACATGTGAGGTG Reverse: GCTGTATCTTGTGCCGCT	*Sac*I	SP6
nppa	Forward: ATGGCCGGGGGACTAATTC Reverse: CCGCGTATTGCAGCTAACC	*Sac*I	T7
has2	Forward: ACGACACTGTTCGGCATTT Reverse: CAGCGGGTTTGTTGGTTG	*Apa*I	SP6
Fli1a	Forward: GTCTTATGATGCTGTACGGAGG Reverse: CCATCTTCGAGTGCAGTTCAAG	*Nco*I	SP6

vmhc, ventricular myosin heavy chain; amhc, atrial myosin heavy chain; bmp4, bone morphogenetic protein 4; cmlc2, cardiac myosin light chain-2; notch1b, notch homolog 1b; nppa, natriuretic peptide precursor A; has2, hyaluronan synthase 2; fli1a, fli-1 proto-oncogene ETS transcription factor a.

Whole mount in situ hybridization. The whole *in situ* hybridization was performed using the whole *in situ* hybridization kit for zebrafish embryos (Nanjing YSY Biotech Company, Ltd.) according to the manufacturer's protocol. Riboprobes were used for confirming the sequences of amhc, vmhc, nppa, cmlc2, bmp4, notch1b, appa, hyaluronan synthase 2 (has2), fli-1 proto-oncogene ETS transcription factor a (fli1a) and versican a (vcana) as previously described (22-24). The detailed procedure of the whole mount *in situ* hybridization was performed as previously described (23,25,26). Embryos were immobilized in 4% paraformaldehyde for 12-16 h at 4˚C, dehydrated continuously by graded methanol solution and stored at -20˚C for at least 30 min. The antisense probe (1 ng/μl) of digoxigenin-labeled RNA was hybridized overnight in 50% formamide buffer at 65˚C. The non-specific binding probes were washed in the SSC solution (cat. no. S6639; Sigma-Aldrich; Merck KGaA). Following washing with SSC, the embryos were immersed in blocking solution (10% sheep serum, 70% MAB and 0.1% Tween; Roche Diagnostics) at room temperature for 1 h. Subsequently, the embryos were incubated in blocking solution containing 1/5,000 anti-Digoxigenin-AP Fab fragment (Roche Diagnostics) at 4˚C overnight. Hybridization was detected using nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate and anti-DIG-AP Fab fragment staining. The experimental and control embryos were treated concurrently. The results of *in situ* hybridization were observed under a light dissecting microscope (Olympus Corporation; magnification, x8) and recorded using the RetigaExit Fast 1394 CCD camera (Olympus Corporation). The images were processed with Adobe Photoshop CS5 Extended software (Adobe Systems, Inc.) for contrast and light intensity processing.

Reverse transcription-quantitative PCR (RT-qPCR). The relative expression levels of Wnt4 and β-catenin were examined by RT-qPCR and normalized to the reference gene β-actin. Total RNA was extracted from at least 60 embryos for each measurement using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized from 500 ng RNA using a reverse transcriptase kit (Vazyme Biotech Co., Ltd.) with the following temperature protocol: 25˚C for 5 min, followed by 50˚C for 15 min and 85˚C for 5 min. qPCR was performed using SYBR-Green (Vazyme Biotech Co., Ltd.) on an ABI 7500 detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The following primer pairs were used for qPCR: Wnt4 forward, 5'-TGTGCAAACGGAACCTTGAG-3' and reverse, 5'-ATGCCCTTGTCACTGCAAAG-3'; β-catenin forward, 5'-AGACAGCTCGTTGTACTGCT-3' and reverse, 5'-GTGTCGTGATGGCGTAGAA-3'; and β-actin forward, 5'-CAACAGAGAGAAGATGACACAGATCA-3' and reverse, 5'-GTCACACCATCACCAGAGTCCATCAC-3'. The following thermocycling conditions were used for qPCR: Initial denaturation (95˚C, 10 sec), followed by 40 cycles of amplification and quantification (94˚C, 5 sec and 72˚C, 30 sec) with a final extension (7 min, 72˚C). Relative gene expression levels were quantified based on the cycle quantification (Cq) values and normalized to the reference gene β-actin. Gene expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (27).

Table II. Mortality rates (%) of zebrafish embryos.

Group	Time, hpf			
	24	48	72	96
WT	7.11	13.12	14.26	14.59
Control (1 μM)	7.18	13.42	14.67	14.93
miR-29c (1 μM)	18.52	26.74	28.81	31.36
Control (1.6 μM)	8.26	16.42	16.76	16.69
miR-29c (1.6 μM)	21.19[a]	25.21[a]	32.62[a]	39.95[a]
Control (2 μM)	9.51	15.52	17.66	17.99
miR-29c (2 μM)	40.61[a]	58.62[a]	89.26[a]	100.00[a]

[a]P<0.05 vs. WT and control group. miR, microRNA; WT, wild-type; hpf, hours post-fertilization.

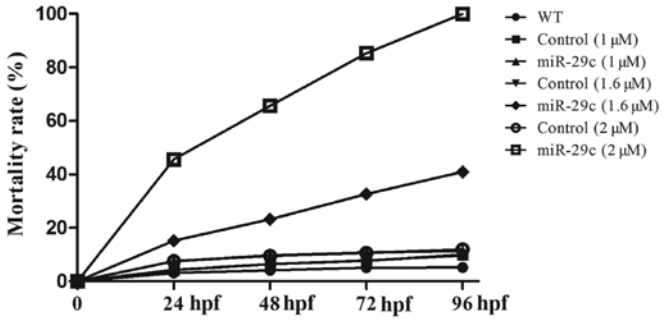

Figure 1. Effect of miR-29c overexpression on mortality. Compared with the WT and control groups, the mortality rate of miR-29c mimic-injected embryos ranged between 18.52-100%. The hatchability was significantly reduced. The data suggested that with increasing miR-29c concentration, the mortality of zebrafish increased, showing a dose-dependent trend. WT, wild-type; miR, microRNA; hpf, hours post-fertilization.

Stem-loop RT-qPCR. Stem-loop RT-PCR was performed to examine the relative expression levels of miR-29c following microinjection. miRNA levels were measured using the BulgeLoop™ miRNA qPCR Primer Set (Vazyme Biotech Co., Ltd.) according to the manufacturer's instructions. The following primer pairs were used for PCR: U6 forward, 5'-TTGGTCTGATCTGGCACATATAC-3' and reverse, 5'-AAAAATATGGAGCGCTTCACG-3' and miR-29c forward, 5'-GCCTAGCACCATTTGAAATCG-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3'. Relative miR-29c expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to the reference gene U6. Stem-loop RT-qPCR was performed according to the aforementioned RT-qPCR protocol.

Statistical analysis. Quantitative data from three independent experiments were expressed as the mean ± SD. Statistical analyses were performed using SPSS 24.0 software (IBM Corp.). Categorical data were tested using χ^2 and Fisher's exact tests. For comparison of multiple rates, the Scheffe post hoc test was performed following the chi-squared test. Measurement data were analyzed with one-way ANOVA followed by Student-Newman-Keuls post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Table III. Hatch rate of zebrafish embryos at 72 h post-fertilization.

Group	n	Hatch number	Hatch rate, %
WT	87	85	97.70
Control (1 μM)	87	85	97.70
miR-29c (1 μM)	68	64	94.11
Control (1.6 μM)	85	83	97.65
miR-29c (1.6 μM)	68	58	85.29[a]
Control (2 μM)	84	81	96.42
miR-29c (2 μM)	38	10	26.32[a]

[a]P<0.05 vs. WT and control group. miR, microRNA; WT, wild-type.

Table IV. Malformation rate (%) of zebrafish embryos at different time points.

Group	Time, hpf			
	24	48	72	96
WT	2.08	2.35	2.52	2.86
Control (1 μM)	2.36	2.72	2.86	3.46
miR-29c (1 μM)	30.08[a]	34.44[a]	46.40[a]	48.45[a]
Control (1.6 μM)	3.25	3.89	3.92	4.56
miR-29c (1.6 μM)	58.75[a]	78.69[a]	89.92[a]	96.89[a]
Control (2 μM)	3.35	3.78	4.92	5.69
miR-29c (2 μM)	67.38[a]	89.75[a]	98.20[a]	100.00[a]

[a]P<0.05 vs. WT and control group. miR, microRNA; WT, wild-type; hpf, hours post-fertilization.

Results

Effects of miR-29c overexpression on mortality and hatchability. The effects of miR-29c mimics (1, 1.6 and 2 μM) on the mortality and hatch rate at different stages of embryonic development (24, 48, 72 and 96 hpf) were assessed. The lethal rate of miR-29c mimic-injected embryos ranged between 18.52-100% (Table II), which was significantly higher (1.6 and 2 μM miR-29c mimic concentrations) compared with WT and NC embryos. Compared with the WT and NC groups, the hatchability of miR-29c mimic-injected embryos (1.6 and 2 μM miR-29c mimic concentrations) was significantly reduced (Table III). The data indicated that with the increase of miR-29 mimic concentration, the mortality increased, and hatchability decreased, showing a dose-dependent trend (Fig. 1). The results suggested that miRNA-29c overexpression may hinder development and increase the mortality of zebrafish embryos.

Effects of miR-29c overexpression on the morphology of zebrafish embryos. The malformation rate is the proportion of zebrafish embryos and larvae with obvious morphological abnormalities, such as pericardial edema and shrinkage. The

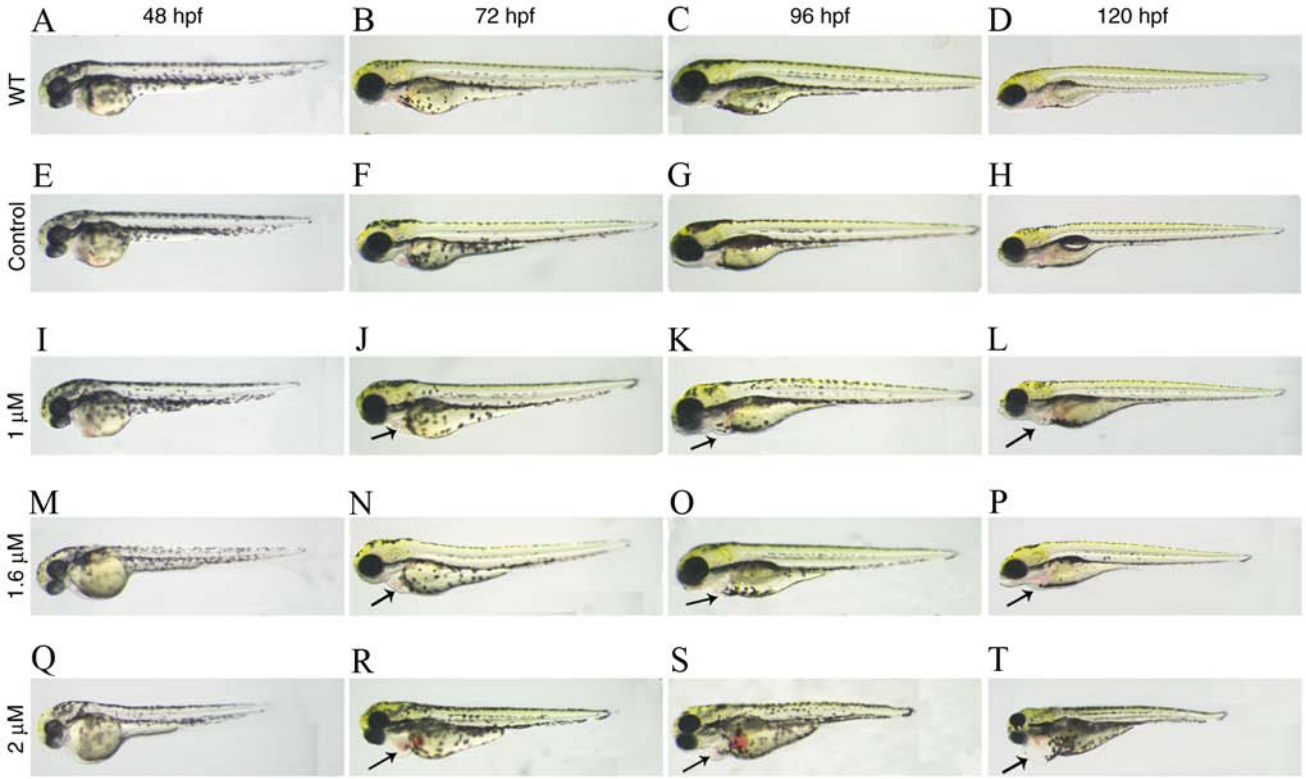

Figure 2. Effects of miR-29c overexpression on the morphology of zebrafish embryos. Representative pictures (magnification, x3.2) of (A-D) WT, (E-H) control and (I-T) miR-29c mimic-injected embryos. Embryos injected with miR-29c mimics showed severe pericardial edema (black arrow). WT, wild-type; miR, microRNA; hpf, hours post-fertilization.

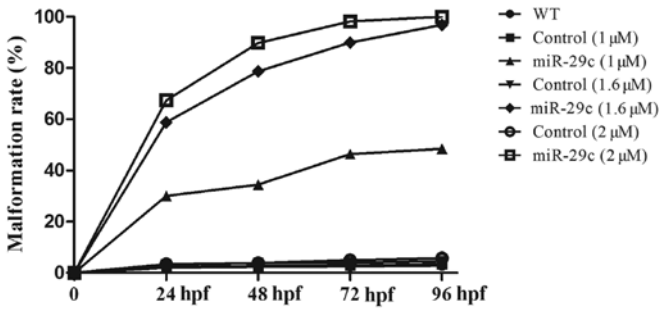

Figure 3. Effects of miR-29c overexpression on the malformation rate. The malformation rate of miR-29c mimic-injected embryos ranged between 30.08-100%, which was higher compared with WT and control groups. The data showed that the malformation rate of zebrafish embryos increased in a dose-dependent manner following miR-29c mimic injection. WT, wild-type; miR, microRNA; hpf, hours post-fertilization.

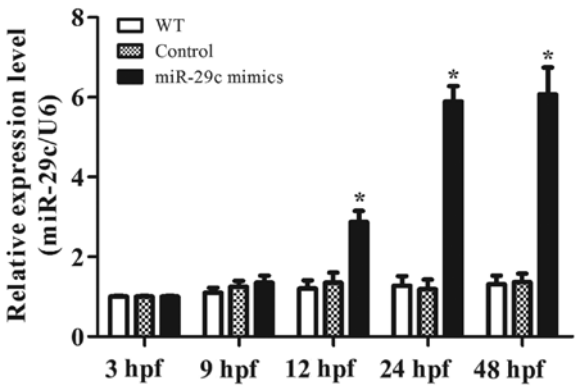

Figure 4. Verification of miR-29c overexpression. Stem-loop reverse transcription-quantitative PCR was performed to verify the expression levels of miR-29c in the miR-29c mimics, WT and control groups at 3, 9, 12, 24 and 48 hpf. The data are expressed as the mean ± SD (n=86 zebrafish embryos per group). *P<0.05 vs. control. WT, wild-type; miR, microRNA; hpf, hours post-fertilization.

effect of increasing concentrations of miR-29c mimics on the malformation rate was evaluated at different stages (24, 48, 72 and 96 hpf) (Fig. 2). Quantitative analysis indicated that the malformation rate of zebrafish embryos injected with miR-29c mimics increased in a dose-dependent manner (Fig. 3). In particular, the malformation rate of miR-29c mimic-injected embryos ranged between 30.08-100%, which was significantly higher compared with NC and WT groups (Table IV). The phenotype was observed after injection of 1 μM and 1.6 μM miR-29c mimic. Following injection of 2 μM miR-29c mimic, no embryos survived to 96 hpf. Therefore, 1.6 μM miR-29c mimic was chosen for further experiments.

Effects of miR-29c overexpression on heart rate and cardiac function. A dose of 1.6 μM miR-29c mimic or 1.6 μM NC was chosen to explore the effects of miR-29c overexpression on the average heart rate (AHR) and cardiac function. RT-qPCR was used to confirm miR-29c overexpression (Fig. 4). At ~16 hpf, the cardiac progenitor cells of zebrafish begin to differentiate; cardiac precursor cells reach the midline and bind to each other and the cardiac cone extends. At ~24 hpf, the cardiac cone gradually grows into a linear cardiac tube, then curls to the left to form the anatomical atrium, ventricle and atrio-

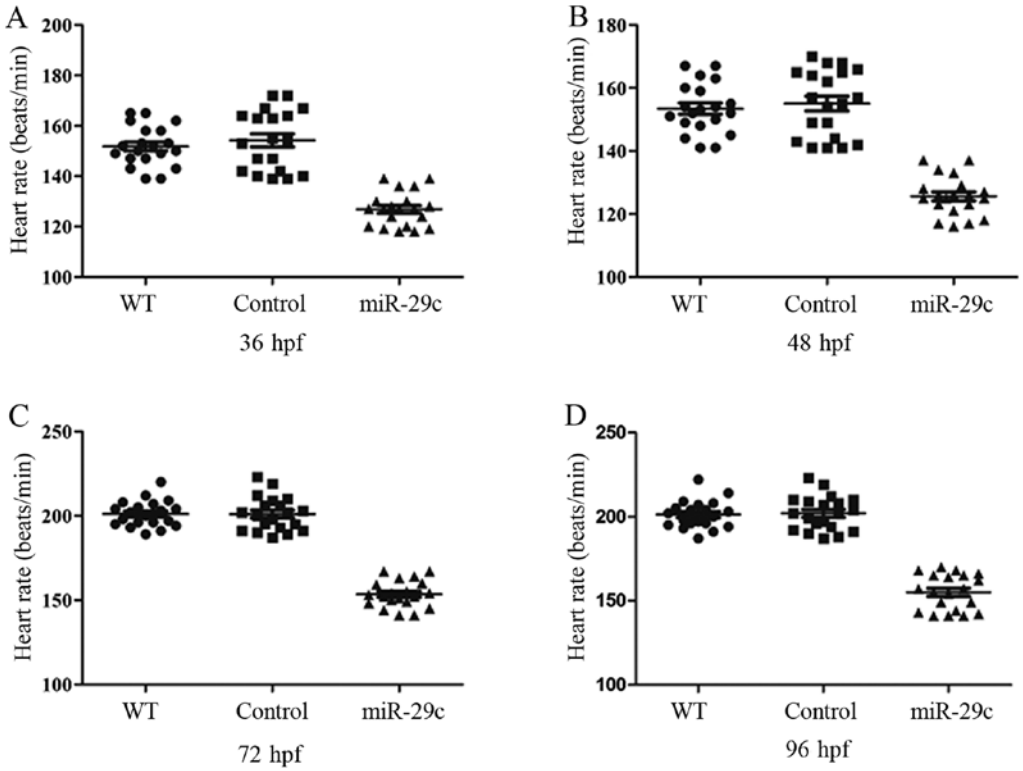

Figure 5. miR-29c overexpression decreases the AHR of zebrafish embryonic hearts. The AHRs were examined in the embryos and larvae at (A) 36, (B) 48, (C) 72 and (D) 96 hpf. The AHRs increased as the heart developed. AHRs were reduced in the miR-29c mimics injection group compared with the WT and control groups. AHR, average heart rate; WT, wild-type; miR, microRNA; hpf, hours post-fertilization.

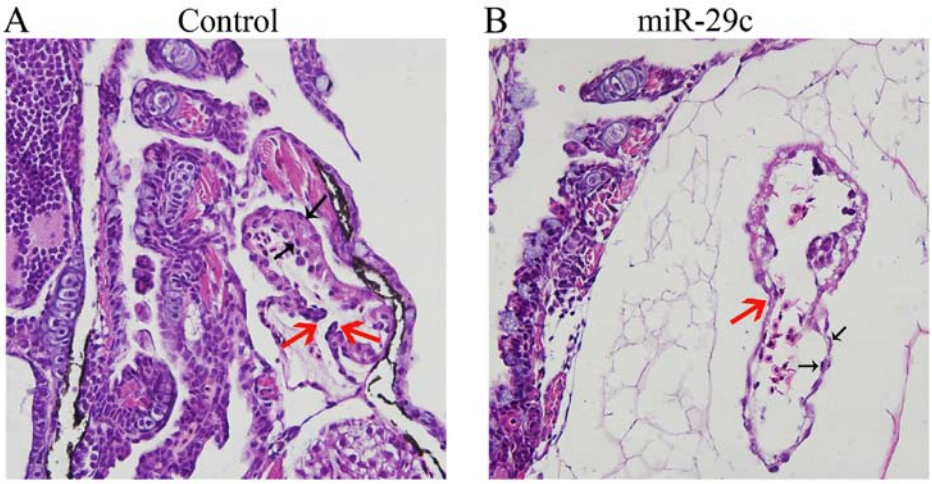

Figure 6. Overexpression of miR-29c affects the histological structure of zebrafish embryonic hearts. Embryonic heart tissue sections at 120 h post-fertilization were obtained following injection of (A) 1.6 μM NC or (B) 1.6 μM miR-29c mimics into fertilized zebrafish eggs. Red arrows indicate the atrioventricular junction and black arrows indicate the myocardium. An obvious valvular lobule, normal myocardium and normal endocardial layer can be observed in the embryo injected with 2 μM NC. Overexpression of miR-29c resulted in a lobular defect of the atrioventricular valve, thinning of the myocardium and fewer endocardial cells (magnification, x200). miR, microRNA; NC, negative control.

ventricular valve. The heart development of zebrafish embryo is completed within 48 hpf (28). Therefore, the heart rate at 36, 48, 72 and 96 hpf were next measured to assess the effect of miR-29c overexpression on heart contraction. At 36 hpf, the AHR was 148.8±6.6 beats per minute (bpm) in the WT group, 147.3±7.9 bpm in the NC group and 126.8±4.9 bpm in 1.6 μM miR-29c mimic injection group. At 48 hpf, these AHR changed to 158.6±7.6 bpm, 154.7±5.8 bpm and 125.6±3.8 bpm. At 72 hpf, the AHRs were 202.5±8.4 bpm, 202.6±9.4 bpm and 164153.5±7.6 bpm. At 96 hpf, the AHRs increased to 206.2±9.5 bpm, 204.7±8.2 bpm and 155.5±9.8 bpm, respectively (n=20; Fig. 5). The aforementioned data indicated that the AHR significantly reduced following miR-29c mimic injection. The atria and ventricle of control embryos exhibited rhythmic and robust contraction, which ensured circulation in the whole body. By contrast, weak and irregular contractions

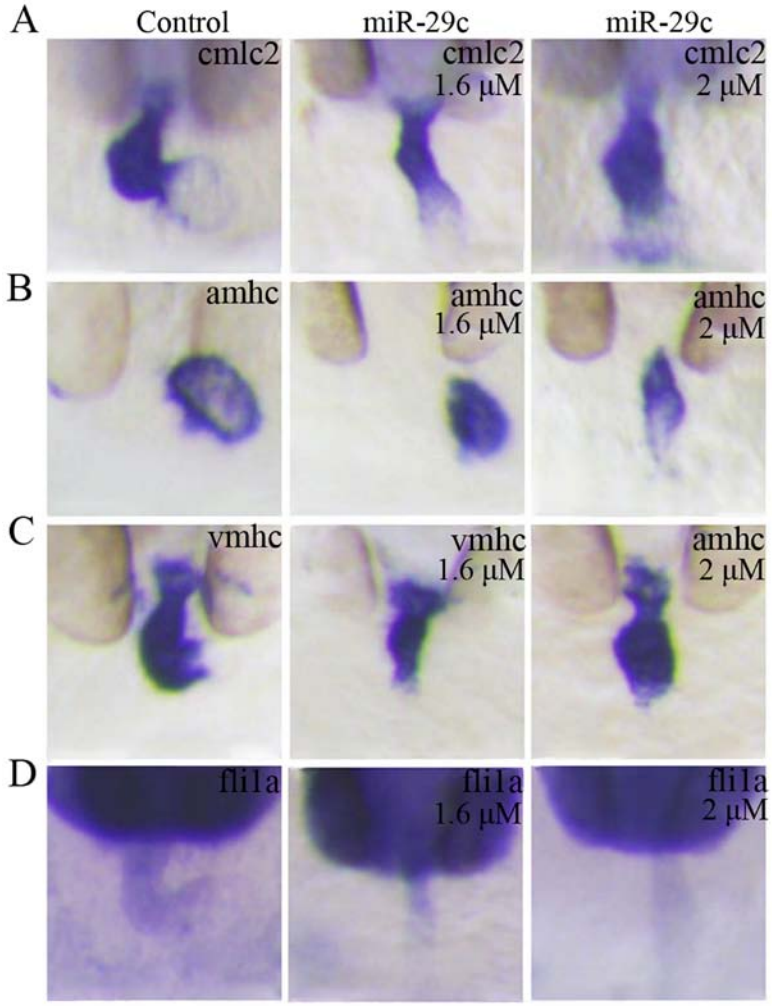

Figure 7. Overexpression of miR-29c affects the cardiac cyclization and the expression of myocardial and endocardial molecular markers in the embryonic hearts of zebrafish. At 48 hpf following injection, abnormal expression of (A) cmlc2, (B) amhc, (C) vmhc and (D) fli1a was observed in the miR-29c mimic injection group (1.6 and 2 μM miR-29c mimic concentrations) compared with the control group (magnification, x8). miR, microRNA; hpf, hours post-fertilization; cmlc2, cardiac myosin light chain-2; amhc, atrial myosin heavy chain; vmhc, ventricular myosin heavy chain; fli1a, fli-1 proto-oncogene ETS transcription factor a; NC, negative control.

were more prominent in the miR-29c mimic injection group. Taken together, the results suggested that miR-29c overexpression could significantly alter cardiac systolic function.

Effects of miR-29c overexpression on the histological structure of zebrafish embryonic heart. Following injection of 1.6 μM NC or 1.6 μM miR-29c mimics into zebrafish fertilized eggs, seven typical embryos were sectioned at 120 hpf. The results showed that the myocardial layer, endocardium and inter atrioventricular valvular lobule of the embryonic heart in the control group developed normally (Fig. 6A). In the 1.6 μM miR-29c mimic injection group, however, pericardial edema presented, as exhibited by cavities outside the heart. The ventricular layer became thinner and the number of endocardial cells decreased (Fig. 6B). The results showed that miR-29c overexpression notably affected zebrafish embryonic cardiogenesis.

Effects of miR-29c overexpression on cardiac cyclization and expression of myocardial and endocardial molecular markers. Under normal conditions, cmlc2 is expressed in the whole heart, vmhc is expressed in the ventricle, amhc is expressed in the atrium, and fli1a is expressed in the endocardium in the embryo heart of zebrafish (22). To study the effects of miR-29c on ventricular and atrioventricular tube development at the molecular level, *in situ* hybridization was performed to detect the expression of amhc, cmlc2, fli1a and vmhc at 48 hpf. Compared with the control group (embryos injected with 2 μM negative control mimics), embryos injected with 1.6 and 2 μM miR-29c mimics displayed abnormal expression patterns of the gene cmlc2, which marks cardiac cyclization, and loss of left-right asymmetry (Fig. 7A), suggesting that miR-29c overexpression altered embryonic cardiac cyclization. The genes amhc (Fig. 7B) and vmhc (Fig. 7C), which mark atrio-genesis, were also abnormally expressed in embryos overexpressing miR-29c, and the atrial expression regions were significantly reduced, suggesting that miR-29c overexpression affected atrial and ventricular genesis in embryonic hearts. Similarly, the expression of fli1a, a marker gene of cardiac endocardio-genesis, was notably downregulated (Fig. 7D) compared with control group, suggesting that miR-29c overexpression affected embryonic cardiac endocardio-genesis.

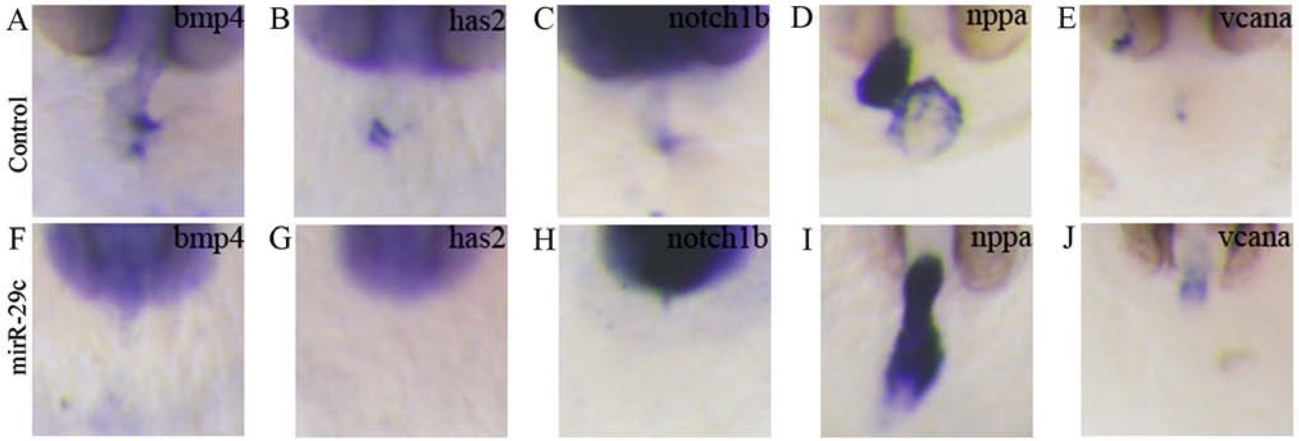

Figure 8. miR-29c overexpression affects the expression of marker genes in zebrafish embryonic heart valves. 1.6 μM miR-29c mimics or 1.6 μM NC were injected into fertilized eggs for 48 hpf, and the zebrafish embryos were hybridized *in situ*. Control embryos were injected with 1.6 μM NC and the expression patterns of cardiac valve precursor marker genes (A) bmp4, (B) has2, (C) notch1b, (D) nppa and (E) vcana were measured. Embryos were injected with 1.6 μM miR-29c mimics and the expression patterns of cardiac valve precursor marker genes (F) bmp4, (G) has2, (H) notch1b, (I) nppa and (J) vcana were measured (magnification, x8). miR, microRNA; hpf, hours post-fertilization; NC, negative control; bmp4, bone morphogenetic protein 4; has2, hyaluronan synthase 2; notch1b, notch homolog 1b; nppa, natriuretic peptide precursor A; vcana, versican a; AVC, atrioventricular canal.

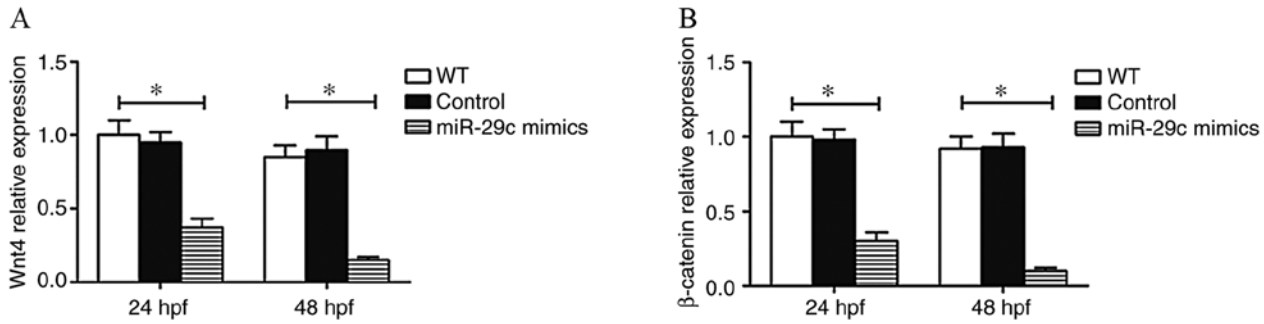

Figure 9. Overexpression of miR-29c involves the Wnt4 signaling pathway. Compared with the WT and negative control groups, the mRNA expression levels of (A) Wnt4 and (B) β-catenin significantly decreased in the miR-29c mimics injection group. *P<0.05. WT, wild-type; miR, microRNA; hpf, hours post-fertilization.

Effects of miR-29c overexpression on the precursor cells of the atrioventricular valve in zebrafish embryos. The embryos were fixed for *in situ* hybridization at 48 hpf. The results showed that compared with the control group (Fig. 8A-E), the expression of bmp4 (Fig. 8F), has2 (Fig. 8G) and notch1b (Fig. 8H) in the atrioventricular pathway diminished at 48 hpf in the miR-29c mimics injection group. In miR-29c overexpression embryos, nppa (Fig. 8I) was heterotopically expressed in the atrioventricular pathway, and highly expressed in the atrium, whereas vcana (Fig. 8J) was not expressed in the atrioventricular pathway, but highly expressed near the ventricular outflow tract. These data suggested that miR-29c overexpression affected the formation of precursor cells of the atrioventricular valves in embryonic hearts.

Effect of miR-29c overexpression on the Wnt4 signaling pathway. The Wnt signaling pathway plays an important role in embryonic development (29). Wnt signaling has been demonstrated to play a pivotal role in cardiac asymmetry and Kupffer's vesicle development in zebrafish (30). Additionally, our previous studies (17,18) found that Wnt4 was the target gene of miR-29c. The present study found that the mRNA expression of Wnt4 and β-catenin decreased in embryos injected with miR-29c mimics (Fig. 9). This suggested that miR-29c overexpression led to abnormal cardiac development in zebrafish by modulating the Wnt4 signaling pathway.

Discussion

As the most common birth defect worldwide, CHD is caused by genetic factors in ~98% cases (31,32). Therefore, exploring the genetic factors leading to CHD is helpful to elucidate CHD etiology. As hypothesized, the present study confirmed the role of miR-29c.

Zebrafish and vertebrates share physiological and morphological commonness in terms of cardiovascular development (33,34). Moreover, unlike that in vertebrates, severe heart malformations do not immediately lead to zebrafish death. Even in the total absence of blood circulation, zebrafish receive enough oxygen by passive diffusion to survive and continue to develop in a relatively normal fashion for several days (28,35). Secondly, zebrafish embryos are transparent, making it convenient to perform microinjections and observe morphological cardiac changes (28). Additionally, early cardiac development in zebrafish embryo is similar to that in

humans, such as the migration of precursor cells to the central line, the formation of cardiac tubes and the formation and circulation of early ventricles (35,36). Zebrafish embryos can be used to model the characteristics and potential molecular mechanisms of cardiac development defects. The present study established a zebrafish model to observe the effects of miR-29c overexpression on embryonic heart development. The data showed that miR-29c overexpression increased embryo mortality and decreased embryo hatchability. In addition, miR-29c overexpression led to left-right asymmetry defects in zebrafish embryos and affected heart development in a dose-dependent manner, manifested by heart rate slowdown, pericardial edema and heart looping disorder during the early stage of development.

The potential molecular mechanisms were further explored. Numerous signaling pathways, such as Wnt, bone morphogenetic protein and retinoic acid, co-regulate cardiac development (37,38). Classical and non-classical Wnt signaling participate in cardiac differentiation in a coordinated manner; mutations in Wnt genes can lead to CHD in several animal models (39). As a member of the Wnt family, Wnt4 plays an important role in regulating cell proliferation, differentiation and apoptosis (29). It has been reported that the Wnt4 signaling (Wnt4/β-catenin) pathway is essential for cardiac left-right patterning (30). Using bioinformatics analysis and luciferase reporter assays, our previous studies verified that Wnt4 was the target gene of miR-29c (17,18). To investigate whether miR-29c regulates cardiac function and phenotype via the Wnt4/β-catenin signaling pathway, the present study detected mRNA expression levels of Wnt4 and β-catenin following miR-29c mimic injection. The expression of Wnt4/β-catenin was downregulated in embryos injected with miR-29c mimics, suggesting that miR-29c may rely on the Wnt4/β-catenin signaling pathway to regulate the development of the zebrafish embryonic heart.

In conclusion, miR-29c regulated zebrafish embryonic heart development via the Wnt4/β-catenin signaling pathway. Inhibiting miR-29c expression may have important practical significance for the prevention, diagnosis and treatment of CHD.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YS and GS designed the study and drafted the manuscript. YS, HL and RC performed the experiments. LZ and GS performed the statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Jiangsu Taizhou People's Hospital (Taizhou Clinical Medical School of Nanjing Medical University).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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