Original Paper

Overexpression of miR-19b Impairs Cardiac Development in Zebrafish by Targeting *ctnnb1*

Mengmeng Li Xiaoshan Hu Jingai Zhu Chun Zhu Shasha Zhu Xuehua Liu
Jing Xu Shuping Han Zhangbin Yu

State key Laboratory of Reproductive Medicine, Department of Pediatrics, Nanjing Maternity and Child Health Care Hospital Affiliated to Nanjing Medical University, Nanjing, China

Key Words

miR-19b • Zebrafish • Cardiac development • *ctnnb1*

Abstract

Background: MicroRNAs are broadly accepted as crucial regulators of cardiovascular development, and dysregulation of their expression has been linked to cardiac disease. MicroRNA cluster miR-17-92 has been implicated in cardiac development and function, yet its defined mechanisms of action in this context are uncertain. Here, we focused on miR-19b, a key component of the miR-17-92 cluster proven to induce cardiomyocyte proliferation *in vitro*. We aimed to identify the biological significance of miR-19b in cardiac development and its underlying molecular mechanism of action *in vivo*. **Methods:** We micro-injected zebrafish embryos with different concentrations (0, 2, 4 and 8 μ M) of miR-19b mimics or a negative control, and assessed the embryo malformation rate, mortality rate, hatching rate and heart abnormalities at 72 hours post-fertilization (72 hpf). **Results:** We found that overexpression of miR-19b impacted left-right symmetry and cardiac development of zebrafish embryos, characterized by pericardial edema, slower heart rate and cardiac looping defects in a dose-dependent manner. Moreover, several important signaling molecules in the Wnt signaling pathway were abnormally expressed, suggesting that overexpression of miR-19b induces the inhibition of the Wnt signaling pathway by directly targeting *ctnnb1*. Interestingly, the deformed cardiac phenotype was partially rescued by treatment with the GSK3 β inhibitor lithium chloride. **Conclusion:** Our findings suggest that miR-19b regulates laterality development and heart looping in zebrafish embryos by targeting *ctnnb1*.

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Introduction

Congenital heart disease accounts for nearly one-third of all major congenital anomalies, representing a considerable health problem [1]. Disturbances in cardiac left-right (LR) patterning signaling pathway(s) are thought to be a major contributor toward the development of these anomalies [2]. It is widely accepted that the formation of the mature vertebrate heart involves the complex orchestration of gene expression. Numerous genes are known to be critical for cardiac morphogenesis, although their exact functions and their integration with other cardiac regulators are poorly understood [3]. A newly recognized regulator of cardiovascular development and function are microRNAs (miRNAs) [4]. MiRNAs are a class of ~22-nucleotide non-coding RNAs that regulate the expression of protein-coding genes post-transcriptionally [5, 6]. More than 1,000 human miRNAs have been identified; however, the biological functions of many of them remain unknown.

It was recently reported that miR-19b is sufficient and required for neonatal cardiomyocyte proliferation *in vitro* [7]. This finding is consistent with the view that miR-19b is a key component of the miR-17-92 cluster that induces cell proliferation and oncogenic growth [8]. These studies suggest that miR-19b has a role in cardiovascular development and maintenance of the cardiovascular system; however, whether overexpression of miR-19b can induce developmental abnormalities and cardiac LR asymmetric defects has not been explored *in vivo*.

Cardiac development is a complicated and elaborate biological process that goes through a series of important morphological events, including cell specification, migration and differentiation [9]. This process involves a number of signaling pathways, such as the aryl hydrocarbon receptor (AhR), Wnt and retinoic acid (RA) signaling pathways [10–12]. Recent studies have revealed that Wnt signaling has a complex array of functions in cardiovascular development and heart morphogenesis, expanding the role for these pathways beyond cell specification and differentiation, and indicating that Wnt activity is a key regulator of cardiac stem/progenitor cell self-renewal and differentiation [13]. It has also been established that Wnt/ β -catenin signaling plays an important role in Kupffer's vesicle (KV) development and cardiac LR patterning [14]. Thus, we hypothesized that overexpression of miR-19b can induce developmental abnormalities and cardiac LR asymmetric defects *in vivo* through the Wnt signaling pathway.

To test this hypothesis, we used a zebrafish model injected with miR-19b mimics and assessed cardiac developmental phenotypes and changes in the Wnt signaling pathway, in order to identify the potential molecular mechanism that contributes to the effect of overexpression of miR-19b on cardiac development. We chose zebrafish as our experimental model because they are an attractive and widely used vertebrate model for studying cardiovascular development. Cardiovascular development in the zebrafish is well defined and has been shown to be morphologically and physiologically similar to that of mammals [15, 16]. Additionally, severe defects in the heart do not lead to immediate lethality as in many vertebrate models, and even in the total absence of blood circulation they receive enough oxygen by passive diffusion to survive and continue to develop in a relatively normal fashion for several days, thereby allowing a detailed analysis of animals with severe cardiovascular defects [17]. Moreover, early-stage cardiac development of zebrafish is similar to that of the human heart in many respects, such as migration of cardiac precursor cells towards the central line, heart tube formation, early chamber formation and the looping process [18, 19]. Thus, studies in the zebrafish embryo can facilitate the characterization of cardiac defects and their underlying molecular mechanisms.

Materials and Methods

Animal husbandry

The Tübingen strain zebrafish used in this study were housed in the zebrafish facility of the Model Animal Research Center (MARC), Nanjing University in accordance with an Institutional Animal Care and

Use Committee (IACUC)-approved protocol. Embryos were obtained from the natural spawning of wild-type (WT) adults, and were grown at $28^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in embryo medium as previously described [20]. Morphological features were used to determine the embryonic developmental stage, as described by Kimmel et al. [21]. Embryos older than 24 h post-fertilization (hpf) were incubated in 0.003% phenylthiourea to inhibit pigment formation.

MiRNA mimics and microinjection

The miR-19b mimic (5'-UGU GCA AAU CCA UGC AAA ACU GA-3') and standard negative control (NC; 5'-UUC UCC GAA CGU GUC ACG UTT-3') were purchased from Genepharma (Shanghai, China), and were dissolved in diethyleneglycol carbonate at $20\text{ }\mu\text{m}$. Injection of 2, 4 or $8\text{ }\mu\text{m}$ of the mimic or NC into single-to-four-cell-stage zebrafish embryos was performed using back-filled fine borosilicate glass capillary needles. Following microinjection, embryos were incubated at $28^{\circ}\text{C} \pm 1^{\circ}\text{C}$, and solutions were changed and degenerating embryos removed daily.

Hatching rate, malformation rate, lethality and observation of zebrafish heart development

After microinjection, we determined the hatching rate at 72 hpf, and malformation and lethality rates at 24, 36, 48, 72 and 96 hpf, observed the heart-specific phenotype at 36, 48 and 72hpf. Embryos were photographed with an Olympus DP71 digital camera (Olympus, Tokyo, Japan) to observe the heart-specific phenotype, and the digital images were processed using Adobe Photoshop software CS5 software (Adobe Systems, San Jose, CA, USA).

Measurement of heart rate

To measure embryonic heart rate, embryos were anesthetized in Tricaine (Sigma, St. Louis, MO, USA). The stock tricaine solution (pH 7) was made as follows: 400 mg tricaine powder, 97.9 ml double-distilled water and 2.1 ml of 1 mol/l Tris (pH 9). To anesthetize the fish, 400 μl tricaine stock solution was diluted in 8 ml of clean tank water. The anesthetized embryos were then transferred to a recording chamber perfused with modified Tyrode's solution (136 mmol/l NaCl, 5.4 mmol/l KCl, 0.3 mmol/l NaH_2PO_4 , 1.8 mmol/l CaCl_2 , 1 mmol/l MgCl_2 , 10 mmol/l HEPES and 5 mmol/l glucose, pH 7.3) at different time points. The heart rate was calculated by counting the number of sequential contractions, beginning and ending at the end diastole. This was conducted under a dissecting microscope in 20-s intervals.

Preparation of RNA probes for in situ hybridization

RNA probes for in situ hybridization were generated for the following genes: atrial myosin heavy chain (amhc), ventricular myosin heavy chain (vmhc), cardiac myosin light chain-2 (cmlc2), natriuretic peptide precursor A (nppa), notch homolog 1b (notch1b), bone morphogenetic protein 4 (bmp4), catenin (cadherin-associated protein), beta 1 (ctnnb1), GATA-binding protein 4 (gata4), forkhead box j1a (foxj1a) and lefty2 (lft2). The probes were amplified from cDNA generated from embryos at 120 hpf and subcloned into pGEM-T easy vectors (Takara, Japan). These plasmids were then linearized and transcribed using a MAXIscript In Vitro Transcription Kit (Ambion, USA). Digoxigenin (DIG)-labeled single-strand RNA probes were prepared using a DIG RNA labeling kit (Roche, USA) according to the manufacturer's instructions, and stored at -80°C for future use. The in situ hybridization primer sequences, restriction sites and direction of probe synthesis are listed in Table 1.

Whole-mount in situ hybridization

In situ hybridization using riboprobes specific for vmhc, amhc, cmlc2, nppa, notch1b, bmp4, ctnnb1, gata4, foxj1a and lft2 was performed as previously described [22–24]. All embryos were fixed in 4% paraformaldehyde at 4°C for 12–16 h, before serial dehydration through graded methanol solutions (25%, 50%, 75% and 100%) and storage at -20°C for at least 30 min. Hybridization was performed overnight at 65°C in 50% formamide buffer with DIG-labeled RNA anti-sense probes. After being washed, embryos were treated in blocking solution for 1–4 h. Hybridization was detected using an anti-DIG-AP Fab fragment and nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Roche, USA) staining. The control and experimental embryos were processed in parallel. In situ images were captured using an Olympus DP71 digital camera (Olympus, Tokyo, Japan).

Table 1. Description of probes used for in situ hybridization

Probe name	Amplification primers	Restriction sites	Transcription direction
vmhc	F: CTCCTGGTGCAATGGAGAATC R: TTCAGCTCAGAGCATTTCGTCC	sall	T7
amhc	F: AAGCATTCGCTCGTGGACT R: CATCCAGTGTCTGCTGGT	NcoI	SP6
cmlc2	F: CTCTTCCAATGTCTTCTCC R: TATTTCCAGCCACGTCTA	Sall	T73
nppa	F: ATGGCCGGGGGACTAATTC R: CCGCGTATTGCAGCTAACC	SacI	T7
notch1b	F: GGCCAAACATGTGAGGTG R: GCTGTATCTTGTCCGCT	SacII	SP6
bmp4	F: TGCCAAGTCTCTACTGGGAG R: CGTGATTGGTGGAGTTGAG	SacII	SP6
ctnnb1	F: AGTCTGACCTGATGGAGCT R: GCTGGTGCACCATCACAG	sall	T7
lft2	F: CCACAGCGATCTCACTCA R: CCATTTCAGCCGCATAAC	NcoI	SP6
foxj1a	F: ATGCTCTCCATGAGTTCCATGGA R: CGGGACTCACGCTCAGGT	ApaI	SP6
gata4	F: GCTCGTGGAGAATAATCGC R: TGCGTTTATGCCAGAATCAG	NcoI	SP6

Quantification of gene expression by real-time quantitative reverse transcription PCR

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to examine the relative expression level of *ctnnb1*, using β -actin as a reference gene. Total RNA was extracted from 60 embryos using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for each assay. RNA was reverse-transcribed, and PCR was performed using the SYBR Green method following the manufacturer's protocol in an ABI 7500 detection system (Applied Biosystems, Foster City, CA). Real-time PCR conditions were as follows: 95°C for 10 s followed by 40 cycles of 94°C for 5 s, and 72°C for 30 s with a final extension of 7 min at 72°C. Primers used for qRT-PCR were as follows: *ctnnb1*, forward primer 5'-ATG ACC TGG AAC GAG ACT GG-3' and reverse primer 5'-ATA ACC TCC GGA ATG GAA GG-3'; and β -actin, forward primer 5'-CAA CAG AGA GAA GAT GAC ACA GAT CA-3' and reverse primer 5'-GTC ACA CCA TCA CCA GAG TCC ATC AC-3'. Relative quantification of each gene was performed by the comparative CT method. The transcript level of the target gene was normalized to the mRNA level of β -actin according to standard procedures.

Stem-loop RT-PCR

Stem-loop RT-PCR was performed as previously described [25, 26]. Total RNAs were reversely transcribed using the miR-19b RT primer 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGACTGGATACGACTCAGTT-3' and the U6 RT primer 5'-AAAAATATGGAGCGCTTCACG-3'. The PCR primers were listed as follows: for U6, forward primer 5' – TTGGTCTGATCTGGCACATATAC-3' and reverse primer 5'-AAAAATATGGAGCGCTTCACG-3'; for miR-19b, forward primer 5' TCCATGCAAACTGAGTTCG-3' and reverse primer 5'-GTGCAGGGTCCGAGGT-3'.

Luciferase assay

To generate luciferase miR-19b target reporters, Oligonucleotides corresponding to a portion of the 3'-untranslated region (UTR) of *ctnnb1* were synthesized, annealed and cloned downstream of the luciferase gene in the pGL3-promoter vector. The sequence identity was confirmed by automatic sequencing. Oligo sequences were as follows. *Ctnnb1* 3'UTR forward primer: 5'-TCT AGA ATA CAA TGA CTT TTT AGC TG-3'; *Ctnnb1* 3'UTR reverse primer: 5'-TCT AGA TTA GCC AAG TCT CCA ACC-3'. The embryos were divided into two groups: one group was microinjected with 60 ng pGL3-promoter *ctnnb1* 3'UTR at the single-to-four-cell stage, and another group was microinjected with 60 ng pGL3-promoter *ctnnb1* 3'UTR and 4 μ m miR-19b mimic. Nine hours after microinjection, embryos were harvested, and the relative luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and a luminometer (FLUOstar Omega, BMG LABTECH, Ortenberg, Germany), with Firefly luciferase data normalized to Renilla luciferase.

Lithium chloride treatment

Lithium chloride (LiCl) treatment was carried out as previously described [27].

Table 2. Mortality rates of zebrafish embryos injected with miR-19b mimics. * Compared with the WT group; P < 0.05

groups	Time(hpf)				
	24hpf	36hpf	48hpf	72hpf	96hpf
WT	7.00%	11.00%	13.00%	14.00%	14.00%
NC(2 μ m)	8.00%	11.00%	14.00%	14.00%	14.00%
miR-19b mimic(2 μ m)	19.00%*	23.00%*	25.00%*	29.00%*	30.00%*
NC(4 μ m)	8.00%	12.00%	16.00%	16.00%	16.00%
miR-19b mimic(4 μ m)	21.00%*	27.00%*	29.00%*	32.00%*	33.00%*
NC(8 μ m)	9.00%	12.00%	15.00%	17.00%	17.00%
miR-19b mimic(8 μ m)	50.00%*	78.00%*	98.00%*	100.00%*	100.00%*

Table 3. Hatching rates of zebrafish embryos at 72 hpf after injection with miR-19b mimics. * Compared with the WT group; P < 0.05

	n	hatching number	hatching rate (%)
WT	86	85	98.84
NC(2 μ m)	86	85	98.84
miR-19b mimic(2 μ m)	69	65	94.20
NC(4 μ m)	84	82	97.62
miR-19b mimic(4 μ m)	68	59	86.76*
NC(8 μ m)	83	82	98.80
miR-19b mimic(8 μ m)	0	0	—

Fig. 1. Overexpression of miR-19b increased the mortality rate of zebrafish in a dose-dependent manner. The effect of miR-19b mimic injection on zebrafish mortality is shown. The mortality rates ranged from 19% to 100% in embryos injected with miR-19b mimics. The data shows that with an increasing concentration of miR-19b mimic, the mortality rate of zebrafish was increased, indicating specific dose-dependent effects.

Statistical analysis

Statistical analyses were performed using the χ^2 test and Fisher's exact test for categorical data, or a one-way analysis of variance (ANOVA; SPSS version 17.0; IBM Corporation: Armonk, NY, USA) for measurement data. A P-value less than 0.05 was considered statistically significant.

Results

Lethality and hatching rates of miR-19b mimic-injected zebrafish embryos

To characterize the effect of miR-19b overexpression during zebrafish development, we evaluated the effect of increasing concentrations of miR-19b mimic on mortality and hatching rates at different development stages (24, 36, 48, 72 and 96 hpf). Our results show that the lethality rates were increased significantly, which ranged from 19% to 100% in miR-19b mimic-injected embryos, compared with wild-type (WT) embryos and NC-injected embryos (Table 2). The hatching rates in miR-19b mimic-injected embryos decreased when compared with the rates in the WT and NC groups (Table 3). This data shows that with increasing miR-19b concentration, the mortality rates of zebrafish were increased and the hatching rates declined, indicating specific dose-dependent effects (Fig. 1). These findings demonstrate

Fig. 2. Overexpression of miR-19b leads to embryonic defects in zebrafish developmental morphology. Representative images of WT (A, B and C), NC (D, E and F), and mir-19b mimic-injected embryos (G, H and I) are shown. MiR-19b-injected embryos displayed severe pericardial edema. All images are at a magnification of 3.2 times. Red arrows: pericardial edema.

Fig. 3. Overexpression of miR-19b increased the malformation rates of zebrafish in a dose-dependent manner. The effect of miR-19b mimic injection on zebrafish malformations is shown. The rates of malformation ranged from 40.74% to 100% in embryos injected with miR-19b mimics. The data shows that with increasing concentrations of miR-19b mimic, the malformation rates of zebrafish were increased, indicating specific dose-dependent effects.

that overexpression of miR-19b can retard the development of zebrafish embryos and lead to the death of zebrafish embryos and larvae.

Effect of miR-19b overexpression on gross morphology

To select a suitable concentration for future work, the effects of increasing concentrations of miR-19b mimic on malformation rates at different stages (24, 36, 48, 72 and 96 hpf) were evaluated. The malformation rate was defined as the ratio of embryos and larvae with gross morphological deformities, including small size and pericardial edema (Fig. 2). Quantification of these data showed that the malformation rate of zebrafish embryos and larvae injected with miR-19b increased in a dose-dependent manner (Fig. 3). Specifically, the malformation rates ranged from 40.74% to 100% in miR-19b mimic-injected embryos compared with WT and NC-injected embryos (Table 4). After injection with 4 μm miR-19b mimic, the phenotypes were visible. No embryos survived to 72 and 96 hpf after an injection of 8 μm miR-19b mimic.

Effect of miR-19b overexpression on heart morphology

Based on our assessment of the effect of miR-19b on gross morphology, we chose a concentration of 4 μm, whose overexpression effect verified by stem-loop RT-PCR (Fig. 4),

Table 4. Malformation rates of zebrafish embryos at different time points after injection with miR-19b mimics. * Compared with the WT group; P < 0.05

	Time(hpf)				
	24hpf	36hpf	48hpf	72hpf	96hpf
WT	2.15%	3.37%	3.45%	3.49%	3.49%
NC(2 μ m)	2.17%	3.37%	4.65%	4.65%	4.65%
miR-19b mimic(2 μ m)	40.74%*	45.45%*	46.67%*	47.89%*	48.57%*
NC(4 μ m)	3.26%	3.41%	3.57%	3.57%	3.57%
miR-19b mimic(4 μ m)	79.75%*	83.56%*	84.51%*	85.29%*	85.07%*
NC(8 μ m)	3.30%	4.55%	4.71%	4.82%	4.82%
miR-19b mimic(8 μ m)	100%*	100%*	100%*	—	—

Fig. 4. Expression of miR-19b is up-regulated in miR-19b mimic injected zebrafish. Stem-loop RT-PCR validation of miR-19b expression in WT, miR-19b mimic injected and NC injected zebrafish at 3, 9, 12, 24 and 48 hpf. Data are representative of three independent experiments; data are expressed as the means $\pm$ SD; n = 80 zebrafish embryos per group. *p < 0.05.

Fig. 5. Overexpression of miR-19b leads to embryonic defects in zebrafish cardiac morphology. Representative images (6.3 times magnification) of the lateral view of WT (A, B and C), NC (D, E and F), and miR-19b mimic injected embryos (G, H, and I) are shown. In WT and NC embryos, the ventricle and atrium largely overlap with each other; however, the ventricles of miR-19b mimic-injected embryos were positioned anterior to the atrium with little overlap. Instead of the normal looped and S-shaped hearts observed in the control group, the heart chambers of miR-19b mimic-injected embryos were string-like and elongated. V: ventricle; A: atrium.

to investigate its effect on cardiac development. In normal cardiac development, the linear heart tube has formed at 24 hpf, and the tube lies along the anterioposterior axis, with the atrial end to the left of the midline. Subsequently, visibly distinct ventricular and atrial chambers have formed by 30 hpf, and the heart undergoes looping morphogenesis by 36 hpf, with functional valves formed by 48 hpf [17]. At 48 hpf, the early cardiac development

Fig. 6. Overexpression of miR-19b decreased heart rates in zebrafish embryos and larvae. The effect of injection with miR-19b on heart rate is shown. The heart rate was determined in embryos and larvae at 36 (A), 48 (B), 72 (C) and 96 hpf (D). The heart rate gradually increased with development; however, it was reduced in miR-19b mimic-injected embryos when compared with controls.

and posterior circulation function has completed; thus, we chose the time points 36, 48 and 96 hpf to assess cardiac development. As shown in Fig. 5, obvious abnormalities in heart morphology were observed in miR-19b-injected embryos, including severe pericardial edema and cardiac looping defects. Instead of the normal looped and S-shaped hearts that we observed in the WT and NC-injected embryos, the heart chambers from miR-19b-injected embryos were string-like and elongated. In the WT or NC group, the ventricle and atrium overlapped and were not distinguishable in the lateral view. In contrast, ventricles of the treated embryos were positioned anterior to the atrium, so the chambers could be easily distinguished with little overlap. These results indicated that miR-19b caused abnormal heart tube development in zebrafish.

Effect of miR-19b overexpression on cardiac function

The development of the cardiac circulation in zebrafish is completed by 48 hpf, so the heart rate was examined at 48, 72 and 96 hpf to evaluate the effects of overexpression of miR-19b on cardiac contraction. As shown in Fig. 6, the mean heart rates at 36 hpf were 145.1 ± 2.6 beats per minute (bpm), 148.5 ± 4.9 bpm and 78.5 ± 3.9 bpm for WT, NC-injected and 4 μ m miR-19b mimic-injected embryos, respectively ($n = 50$). At 48 hpf, the mean heart rates were 158.1 ± 4.6 bpm, 158.5 ± 4.9 bpm and 88.5 ± 3.9 bpm for WT, NC-injected and 4 μ m miR-19b mimic-injected embryos, respectively ($n = 50$). At 72 hpf, the mean heart rates were 204 ± 6.4 bpm, 200.8 ± 6.2 bpm and 170 ± 9.3 bpm for WT, NC-injected and 4 μ m miR-19b mimic-injected embryos, respectively ($n = 50$). At 96 hpf, the mean heart rates were 208.5 ± 3.7 bpm, 206.7 ± 3.5 bpm and 177.8 ± 3.4 bpm for the WT, NC-injected and 4 μ m miR-19b mimic-injected embryos, respectively ($n = 50$). These data demonstrated that heart rate was significantly decreased by miR-19b overexpression. Conversely, the ventricles and atria of control embryos exhibited vigorous, rhythmic contractions, ensuring circulation throughout the body. In the miR-19b mimic-injected embryos, irregular and weak contractions were

Fig. 7. Overexpression of miR-19b alters the expression of cardiac chamber marker genes. Representative images (8 times magnification) of 48 hpf embryos showing the expression of vmhc (A, B, C and D), amhc (E, F, G and H) and cmlc2 (I, J, K and L) by in situ hybridization are shown. After the heart tube has formed, leftward movement of the heart (jogging) is followed by rightward bending of the ventricle (D-looping) in WT embryos (A, E and I) and NC-injected embryos (B, F and J). In contrast, miR-19b mimic-injected embryos exhibited multiple LR defects, including reversed looping (L-looping; D, H and L) and no looping (C, G and K). In the bottom right-hand corner, the number of embryos examined with the representative expression/total number of embryo are presented. Black dashed lines: the midline of the body axis; red arrow: ventricle; blue arrow: atrium.

observed readily. Taken together, these data indicate that overexpression of miR-19b leads to a remarkable change in the contractile function of the heart.

Overexpression of mir-19b alters the expression of amhc, vmhc and cmlc2

To further investigate the effect of miR-19b overexpression on cardiac chamber and atrioventricular canal development at the molecular level, the expressions of amhc, vmhc and cmlc2 were examined at 48 hpf by whole-mount in situ hybridization. Normally, amhc is expressed in the atrium, vmhc is expressed in the ventricle, and cmlc2 is expressed throughout the heart. Compared to control embryos, embryos injected with miR-19b mimic showed an abnormal expression pattern of vmhc, amhc and cmlc2, with LR defects, including reversed looping and no looping, resulting in abnormal atrial development (Fig. 7). Based on this data we hypothesized that overexpression of mir-19b could impair cardiac chamber development; however, the expression of atrioventricular canal marker genes was not changed in embryos injected with miR-19b (Fig. 8). Taken together, this data demonstrates that overexpression of mir-19b can induce significant negative effects on cardiac chamber development, but not on heart valve development.

Overexpression of mir-19b alters the Wnt signaling pathway in zebrafish

In order to better understand the mechanism underlying overexpression of mir-19b on cardiac development, we studied the Wnt signaling pathway, which is divided

Fig. 8. Overexpression of miR-19b has no effect on the expression of atrioventricular canal marker genes. Representative images (8 times magnification) of 48 hpf embryos showing the expression of *nppa* (A, B, C and D), *notch1b* (E, F, G and H) and *bmp4* (I, J, K and L), as assessed using in situ hybridization, are shown. MiR-19b mimic-injected embryos (C, D, G, H, K and L) exhibited no significant difference in expression level and scope, except for changes in expression position due to LR defects when compared with WT embryos (A, E and I) and NC-injected embryos (B, F and J). In the bottom right-hand corner of each image, the number of embryos examined with the representative expression/total number of embryos are presented. Red arrow: atrioventricular canal.

into the canonical and non-canonical pathways. The canonical Wnt pathway (or Wnt/ β -catenin pathway) is the pathway in which Wnt binds to and activates the frizzled seven-transmembranespan receptor. Next, axin is removed from the “destruction complex”, and β -catenin moves into the nucleus, binds to LEF/TCF transcription factor and activates gene transcription [28, 29]. Recent studies have suggested that Wnt signaling plays a critical role in the embryonic development of a variety of organisms, and that the functions of Wnt signaling can be divided into one of the following categories: axis patterning, cell fate specification, cell proliferation and cell migration [30]. In zebrafish, Wnt signaling has been shown to play a key role in KV development and cardiac asymmetry [14]. Thus, we analyzed the expression of *lft2*, *foxj1a* and *gata4*, and found them to be abnormally expressed in embryos injected with miR-19b mimics (Fig. 9). Specifically, the expression position of *lft2* was altered in mir-19b mimic-injected embryos, while *foxj1a* expression in dorsal forerunner cells (DFCs) was markedly downregulated and *gata4* expression was expanded caudally and laterally. These results indicate that overexpression of mir-19b alters the Wnt signaling pathway in zebrafish, contributing to abnormal cardiac development.

*MiR-19b directly targets *ctnnb1**

The protein product of *ctnnb1* is β -catenin, one of the canonical Wnt signaling molecules. Recent studies have shown that knockdown of *ctnnb1* in the whole embryo, or specifically

Fig. 9. Overexpression of miR-19b altered *lft2*, *foxj1a* and *gata4* expression. Representative images of *lft2* expression in the cardiac field are shown using a dorsal view of 22-somite-stage embryos, with the anterior end at the top. The expression position of *lft2* was altered in miR-19b mimic-injected embryos (C), compared with WT (A) and NC-injected (B) embryos. Representative images of *foxj1a* expression in DFCs are also shown. *Foxj1a* expression in DFCs was severely downregulated in miR-19b-overexpressing zebrafish embryos (F), compared with WT (D)

and NC-injected (E) embryos. Representative images of *gata4* expression are presented. *Gata4* expression was restricted to the rostral Anterior lateral plate mesoderm (ALPM) in WT embryos (G) and in embryos injected with NC (H); however, its expression was expanded caudally and laterally in embryos injected with miR-19b (I). The asterisk indicates alteration of *gata4* expression.

in DFCs, interrupts normal asymmetry of the heart [31]. To further investigate this potential target of miR-19b, we generated a pGL3-promoter *ctnnb1* 3'UTR miR-19b target reporter construct (Fig. 10C). We found that luciferase activity was decreased after microinjection of pGL3-promoter *ctnnb1* 3'UTR and miR-19b mimic compared with microinjection of pGL3-promoter *ctnnb1* 3'UTR (Fig. 10D), indicating that miR-19b directly targets *ctnnb1*. Moreover, we measured the expression level of *ctnnb1* using qPCR and in situ hybridization and confirmed that the expression level of *ctnnb1* is negatively correlated with miR-19b expression (Fig. 10A and B). These results suggest that overexpression of miR-19b induces the inhibition of the Wnt signaling pathway by directly targeting *ctnnb1*.

Effects of miR-19b overexpression on zebrafish heart development are partially rescued by activation of the Wnt signaling pathway

As shown in the data mentioned above, we hypothesized that inhibition of Wnt signaling plays an important role in the effects of miR-19b overexpression on zebrafish embryonic development, especially cardiac development. In order to test this hypothesis, we examined whether activating the Wnt signaling pathway could rescue the miR-19b mimic-injected embryos. This was achieved using LiCl, a GSK3 β inhibitor, to temporarily achieve activation of Wnt/ β -catenin signaling [32]. We assessed the effect of LiCl on miR-19b-induced cardiac anomalies morphologically and by in situ hybridization (Fig. 11). We found that treatment with LiCl partially rescued the cardiac defects caused by overexpression of mir-19b, including pericardial edema, heart looping defect and cardiac asymmetry. We also

Fig. 10. MiR-19b directly targets *cttnb1*. A representative image of *cttnb1* expression in 48 hpf embryos determined by in situ hybridization is shown (A). The expression in the miR-19b mimic-injected embryos was decreased when compared with WT and NC-injected embryos. Similarly, real-time PCR showed the expression level of *cttnb1* at 24 and 48 hpf was significantly downregulated in the miR-19b mimic-injected embryos. A schematic of the luciferase vector-expressing *cttnb1* 3'UTR construct is shown (C). Luciferase assay with *cttnb1* 3'UTR of WT, NC and miR-19b mimic-injected embryos is presented (D). ** $P < 0.01$; *** $P < 0.001$.

found that the effects of overexpression of miR-19b on zebrafish heart development were partially rescued by LiCl through appropriate activation of the Wnt signaling pathway.

Discussion

The miR-17-92 cluster is required for normal animal development, cell proliferation and tumor growth, with individual members of the cluster appearing to possess distinct functions [33–35]. For example, inhibition of miR-17 after hypoxia-induced hypertension rescued right ventricle hypertrophy [36], whereas miR-18 and miR-19 are downregulated in models of age-related heart failure [37]. These data support a requirement for the strict regulation of the miR-17-92 cluster expression levels to maintain normal heart development and tissue homeostasis. In this study, we focused on miR-19b, which is a key component of the miR-17-92 cluster proven to induce cardiomyocyte proliferation in vitro [7].

The zebrafish heart has a single ventricle and single atrium, which is different from the four-chambered mammalian heart; however, it is well recognized that the early stages of cardiovascular development are very similar between zebrafish and mammals [38]. Thus, we used zebrafish as a model to study the effects of miR-19b overexpression on cardiac development. Our results show that overexpression of miR-19b increased embryonic lethality rate, coupled with a decreased hatching rate of embryos. Furthermore, zebrafish with elevated levels of miR-19b during the early developmental period exhibited gross malformations, such as small size and pericardial edema. Additionally, an abnormal embryonic heart phenotype was observed during early heart development. Specifically, miR-19b mimic-injected embryos

Fig. 11. Treatment with LiCl partly rescues cardiac defects caused by overexpression of miR-19b. Representative images of cardiac morphology (A, C, E, G and I) and *cmlc2* in situ hybridization (B, D, F, H and J) are shown in 48 hpf zebrafish embryos. Mir-19b mimic-injected embryos displayed severe pericardial edema (G) and looping defects (H), which were rescued by treatment with 0.15 μ M LiCl immediately after miR-19b injection (I and J). In contrast, cardiac morphology and looping were normal in WT (A and B), NC-injected (C and D) and 0.15 μ M LiCl-treated embryos (E and F). Red arrow: ventricle; blue arrow: atrium; yellow arrow: pericardial edema.

exhibited abnormal positioning of the ventricle and atrium, defects in cardiac looping and LR asymmetry. In addition to the structural defects described above, a decreased heart rate was observed in miR-19b mimic-injected embryos. However, the trajectory of heart rate was increased in miR-19b mimic-injected embryos. This indicated that overexpression of miR-19b impaired the function of the heart, which exhibited developmental delay. In summary, these results provide experimental confirmation of the hypothesis that early overexpression of miR-19b impacts normal cardiac development, resulting in congenital heart disease.

In addition to characterizing the effect of overexpression of miR-19b on zebrafish embryonic heart development, we also wanted to study its molecular mechanism. It is known that multiple signaling pathways converge to regulate heart development, such as the RA, Wnt, AhR and Bmp signaling pathways [39, 40]. The Wnt signaling pathway has been shown to play an important role in cardiac development. The Wnt proteins are a group of secreted lipid-modified signaling proteins of 350–400 amino acids, which activate various pathways in the cell that can be categorized into canonical and non-canonical Wnt pathways [41]. It has been confirmed that canonical WNT pathway (Wnt/ β -catenin) signaling is required in cardiac LR patterning [14]. Thus, to determine whether the cardiac effects of miR-19b overexpression were associated with the Wnt/ β -catenin pathway, we measured the expression of several key molecules in the canonical Wnt signaling pathway after miR-19b mimic injection. In agreement with our hypothesis, our results indicated that inhibition of the canonical Wnt signaling pathway contributes to cardiac developmental abnormalities in

miR-19b-injected embryos, which was further confirmed by our rescue experiments.

We next found that *ctnnb1* is a functional target of the miR-17-92 cluster. The *ctnnb1* protein product is β -catenin, one of the canonical Wnt signaling molecules, which is known to be important in embryonic development. For example, knockdown of *ctnnb1* in the whole embryo or specifically in DFCs interrupts normal asymmetry of the heart [31]. Our studies link the function of miR-19b and *ctnnb1* in LR asymmetric heart development. Future studies will be important to illustrate how the miR-19b-*ctnnb1* axis participates in cardiac LR asymmetry.

In summary, these studies demonstrate that overexpression of miR-19b is detrimental toward zebrafish embryonic development, particularly with regard to cardiac development. We were able to delineate the molecular mechanisms underlying this effect, leading to the conclusion that overexpression of miR-19b impedes normal heart development in zebrafish through inhibition of the canonical Wnt signaling pathway.

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