Department of Gynaecology and Obstetrics[1], Guangdong General Hospital (Guangdong Academy of Medical Science); Department of Cardiac Surgery[2], Guangdong Cardiovascular Institute, Guangzhou, China.

Long noncoding RNA SNHG6 contributes to ventricular septal defect formation via negative regulation of miR-101 and activation of Wnt/β-catenin pathway

Yanping Jiang[1,#], Jian Zhuang[2,#], Yanyan Lin[1], Xiaoyun Wang[1], Jianguo Chen[1], Fengzhen Han[1,*]

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**Corresponding author: Fengzhen Han, Department of Gynaecology and Obstetrics, Guangdong General Hospital (Guangdong Academy of Medical Science), NO.106 Zhongshan Second Road, Yuexiu District, Guangzhou, Guangdong 510080, China.*
fengzhenhan06@126.com
[#]First co-authors

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This study aimed to investigate the role and regulatory mechanism of small nucleolar RNA host gene 6 (SNHG6), a long noncoding RNA, in the formation of ventricular septal defect (VSD). The expression of SNHG6 in fetal cardiac tissues with VSD, mouse heart embryo development and the differentiation of P19 cells into cardiomyocytes were determined. Moreover, the effect of aberrant expression of SNHG6 on P19 cell proliferation, cell cycle, apoptosis and differentiation was further analyzed to explore the role of SNHG6 in affecting myocardial development. Furthermore, the regulatory mechanism between SNHG6 and miR-101 as well as between SNHG6 and activation of Wnt/β-catenin pathway was investigated. SNHG6 was upregulated in fetal cardiac tissues with VSD, and decreased in the embryonic development of mice and differentiation of P19 cells into cardiomyocytes. Overexpression of SNHG6 inhibited P19 cell proliferation and induced apoptosis, as well as promoted cell differentiation into cardiomyocytes. Furthermore, SNHG6 could negative regulate the expression of miR-101, and the effects of SNHG6 on the modulation of P19 cell function were through negative regulation of miR-101. In addition, overexpression of SNHG6 activated Wnt/β-catenin pathway, which was reversed after overexpression of SNHG6 and miR-101 synchronously. Our study reveals that SNHG6 may contribute to VSD formation via negative regulation of miR-101 and activation of Wnt/β-catenin pathway. SNHG6 may constitute a potential therapeutic target in this disease.

1. Introduction

Congenital heart disease is a common birth defect including cardiovascular malformations and contributes significantly to infant mortality (Bruneau 2008; Gücer et al. 2005; Van et al. 2011). Ventricular septal defect (VSD) represents the most common congenital heart malformation, comprising approximately 40% of congenital heart disease (Ampie and El-Amin 2015; Penny and Vick 2011). However, the aetiology of VSD has not been fully understood.

Long noncoding RNA (lncRNA) is a type of noncoding RNA over 200 nucleotides (nt) in length and lacks of apparent protein coding capability (Kung et al. 2013). lncRNA can participate in a variety of biological and pathological processes, including cardiovascular diseases (Lorenzen and Thum 2016; Thum and Condorelli 2015; Uchida and Dimmeler 2015). For instance, overexpression of lncRNA TUC40- reduced cardiomyocyte induction and differentiation, inhibited proliferation, and promoted apoptosis, suggesting that TUC40- may serve as a potential pathologic factor for VSD (Li et al. 2017). LncRNA-uc.167 expression is found significantly upregulated in heart tissues of VSD and overexpression of uc.167 inhibited proliferation and promoted apoptosis in embryonic myocardial cells, confirming that uc.167 may contribute to VSD development potential of VSD (Song et al. 2016). Despite this, studies about lncRNA in the cardiovascular field are rare. Exploration of the function of crucial lncRNAs will further deepen our understanding of the mechanisms underlying heart development.

Small nucleolar RNA host gene 6 (SNHG6) is a recently identified cancer-related lncRNA that has been confirmed to modulate the development of many human cancers, such as hepatocellular carcinoma (Birgani et al. 2018; Chang et al. 2016), gastric cancer (Kai et al. 2017), and osteosarcoma (Zheng et al. 2018). Nevertheless, whether SNHG6 is involved in the development of cardiovascular diseases is largely unknown. In the present study, the expression of SNHG6 in fetal cardiac tissues with VSD, mouse heart embryo development and the differentiation of P19 cells into cardiomyocytes was respectively determined. Moreover, the effect of aberrant expression of SNHG6 on P19 cell proliferation, cell cycle, apoptosis and differentiation was further analyzed to explore the role of SNHG6 in affecting myocardial development. Furthermore, the regulatory mechanism between SNHG6 and miR-101 as well as between SNHG6 and activation of Wnt/β-catenin pathway was investigated. The findings of this study will provide a new insight for better understanding of the pathogenesis of VSD and congenital heart disease.

2. Investigations and results

2.1. LncRNA SNHG6 was upregulated in fetal cardiac tissues with VSD, and decreased in the embryonic development of mice and differentiation of P19 cells into cardiomyocytes

To detect whether SNHG6 plays a key role in heart development, we firstly detected the expression of SNHG6 in fetal cardiac tissues with VSD. The results show that SNHG6 expression in fetal cardiac tissues with VSD was significantly higher than that in normal control tissues ($P < 0.01$, Fig. 1A). Moreover, we detected the expression of SNHG6 in mouse heart embryo development.

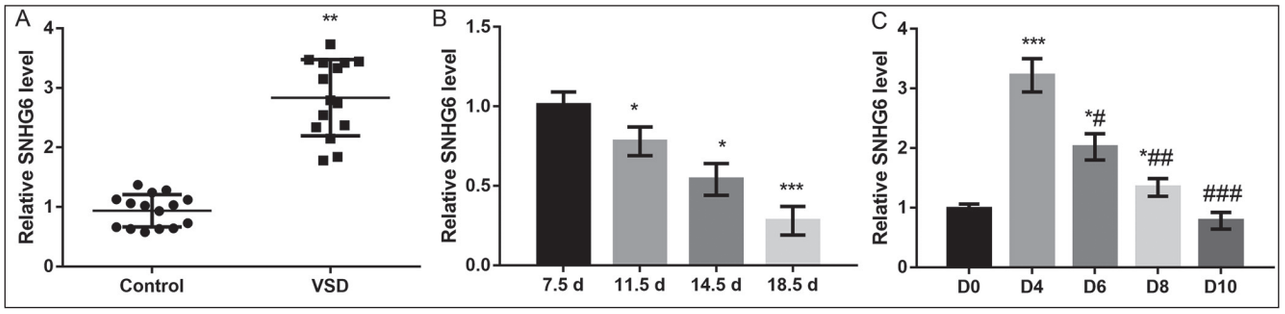

Fig. 1: LncRNA SNHG6 was upregulated in fetal cardiac tissues with VSD (A), and decreased in the embryonic development of mice (B) and differentiation of P19 cells into cardiomyocytes (C). All experiments were repeated three times. The data were presented as mean±standard deviation (SD). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with corresponding control. In Fig. 1C, # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ compared with D4.

The results showed that from embryonic day 7.5 to18.5, the expression levels of SNHG6 were gradually decreased with the increase of embryo development time, and significant difference began to emerge after embryonic day 11.5 compared with embryonic day 7.5 ($P < 0.05$, Fig. 1B). Furthermore, during the induction of P19 cell differentiation into cardiomyocytes, the expression of SNHG6 was analyzed. The results show that compared with day 0, SNHG6 expression reached the peak at day 4 of differentiation ($P < 0.001$), and then significantly decreased with the increase of differentiation time ($P < 0.05$, Fig. 1C).

2.2. Overexpression of SNHG6 inhibited cell proliferation and induced apoptosis

To further detect the role of SNHG6 in cardiomyocyte differentiation, we overexpressed and suppressed the expression of SNHG6 in P19 cells. As shown in Fig. 2A, SNHG6 was successfully overexpressed and suppressed in P19 cells after transfection with pcDNA-SNHG6 and si-SNHG6, and the transfection efficiency was high ($P < 0.01$). After 24, 48 h and 72 h of different transfections, the results of MTT assay showed that overexpression of SNHG6 markedly inhibited cell viability, while suppression of SNHG6 had opposite effects ($P < 0.05$, Fig. 2B). In addition, the results of flow cytometry showed that overexpression of SNHG6 resulted in a remarkable increase in the cell population at G1 phase and obvious decrease at S phase, indicating that overexpression of SNHG6 arrested cell cycle at G1 phase (Fig. 2C). However, suppression of SNHG6 led to a significant increase in the cell population at S phase (Fig. 2C). In addition, we also investigated cell apoptosis after aberrant expression of SNHG6. The results showed that the percentage of apoptotic cells in pcDNA-SNHG6 group was significantly higher than that in pcDNA3.1 group ($P < 0.01$, Fig. 2D). Further western blotting showed that the expression changes of apoptosis-related proteins were in line with the percentage of apoptotic cells in different transfected groups, that was, the expression levels of Bax/Bcl2, cleaved/pro-caspase3, and cleaved/pro-caspase9 were remarkably increased after overexpression of SNHG6. However, suppression of SNHG6 did not exhibit significant effects on cell apoptosis and the expression changes of apoptosis-related proteins.

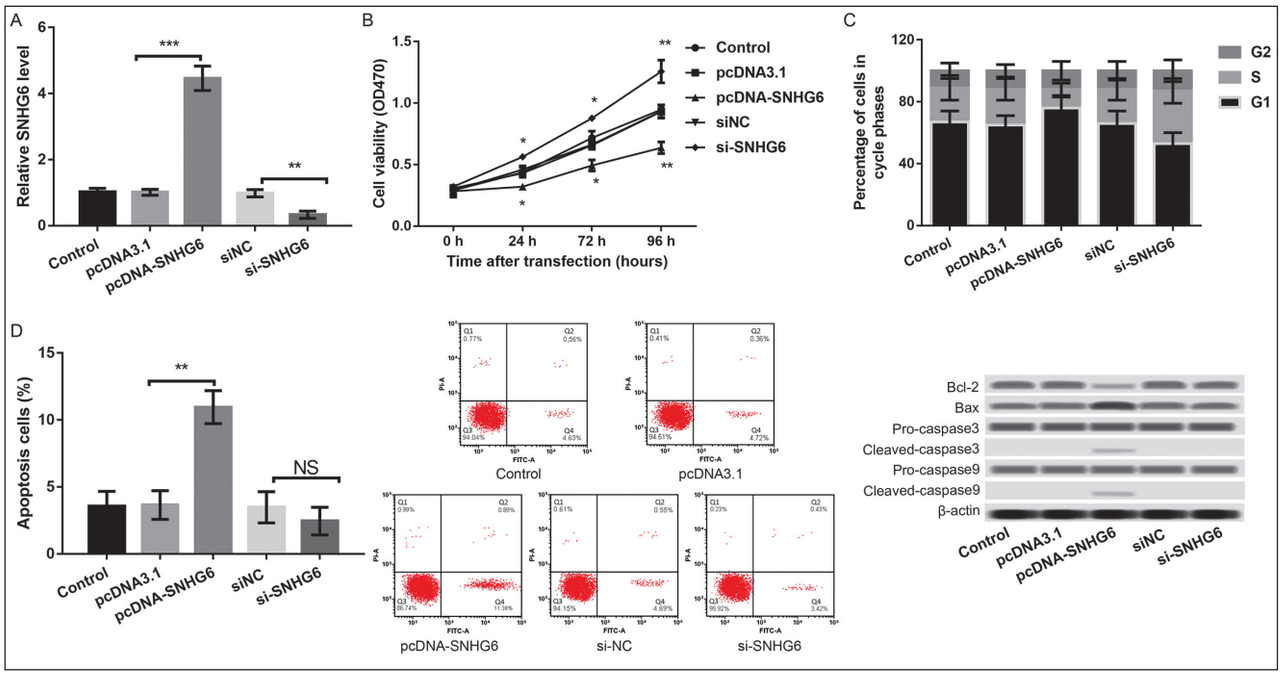

Fig. 2: The effects of aberrant expression of SNHG6 on P19 cell proliferation and induced apoptosis. A: The expression of SNHG6 in P19 cells after transfection with pcDNA-SNHG6 and si-SNHG6. B: MTT assay showed cell viability after transfection. C: Flow cytometry showed cell cycle after transfection. D: Flow cytometry showed cell apoptosis after transfection, and western blotting showed the expression of apoptosis-related proteins. All experiments were repeated three times. The data were presented as mean±SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with corresponding control.

2.3. Overexpression of SNHG6 induced P19 cell differentiation into cardiomyocytes

We further investigated the effects of overexpression of SNHG6 on P19 cell differentiation by determining the expression of key markers associated with cardiomyocyte differentiation (Fig. 3). The results

showed that there were no significant changes in the expression of these five markers (cTnT, MEF2C, Nkx2.5, GATA4, and TBX5) after overexpression of SNHG6 on day 1 and day 4 of cell differentiation. However the expression levels of the aforementioned five markers were significantly decreased after overexpression of SNHG6 on day 6 and day 10 of cell differentiation (all $P < 0.05$), indicating that SNHG6 could promote the differentiation of cardiomyocytes.

2.4. miR-101 was related with human VSD, the embryonic development of mice and differentiation of P19 cells into cardiomyocytes

It has been reported that SNHG6 regulates gastric cancer cell proliferation and invasion *via* sponging miR-101-3p (Kai et al. 2017), we

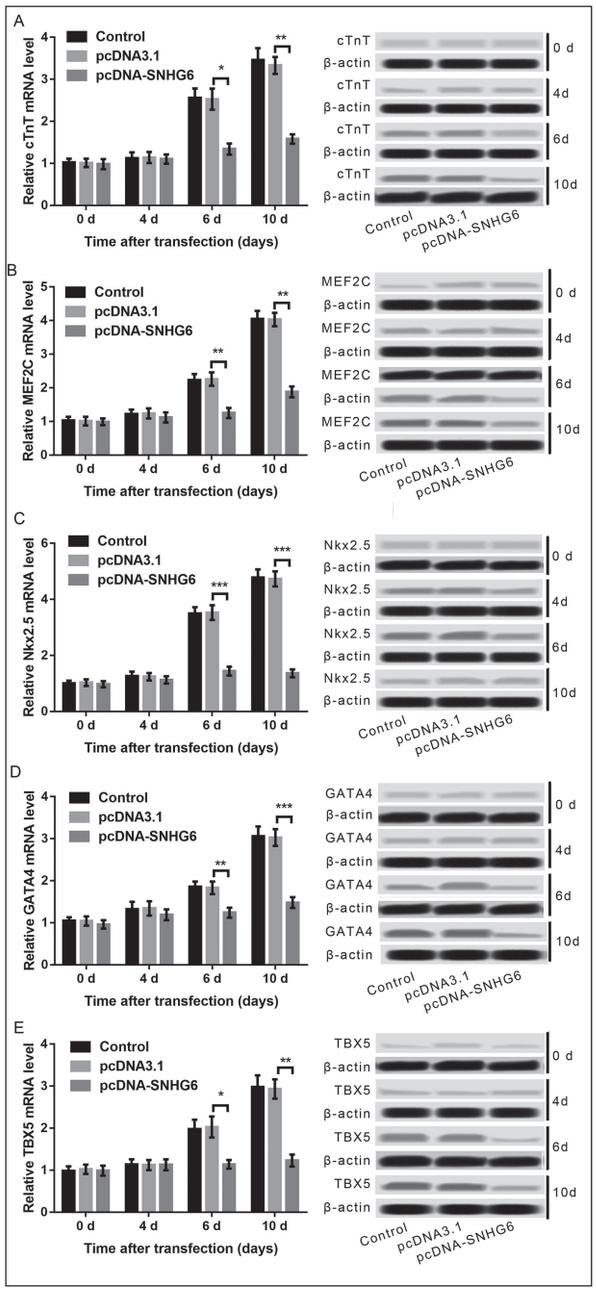

Fig. 3: Overexpression of SNHG6 induced P19 cell differentiation into cardiomyocytes. qRT-PCR and western blotting showed the expression changes of cardiomyocyte differentiation associated markers, including cTnT (A), MEF2C (B), Nkx2.5 (C), GATA4 (D), and TBX5 (E) after overexpression of SNHG6 on days 1, 4, 6 and 10 of cell differentiation. All experiments were repeated three times. The data were presented as mean±SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with corresponding control.

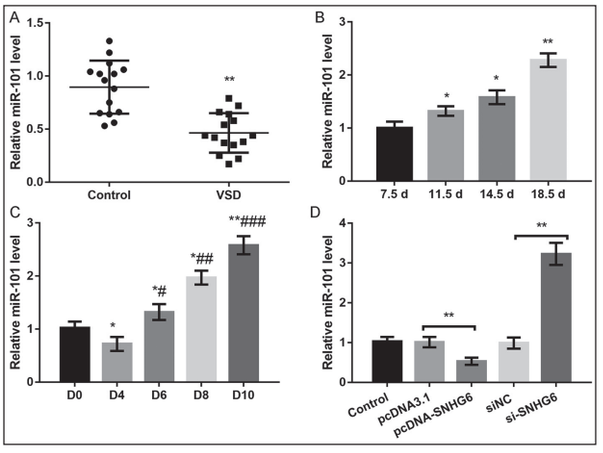

Fig. 4: miR-101 was downregulated in fetal cardiac tissues with VSD (A), increased in the embryonic development of mice (B) and differentiation of P19 cells into cardiomyocytes (C), and inverse expressed with SHNG6 (D). All experiments were repeated three times. The data were presented as mean ± standard deviation (SD). * $P < 0.05$, AND ** $P < 0.01$compared with corresponding control. In figure 4C, # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ compared with D4.

thus explored whether miR-101 was dysregulated in cardiomyocytes. The results showed that miR-101 expression in fetal cardiac tissues with VSD was significantly decreased compared with normal control tissues ($P < 0.01$, Fig. 4A). Moreover, from embryonic day 7.5 to 18.5, the expression levels of SNHG6 were gradually increased with the increase of embryo development time ($P < 0.05$, Fig. 4B). Furthermore, during the induction of P19 cell differentiation into cardiomyocytes, the expression of SNHG6 was significantly decreased at day 4 of differentiation compared with day 0, but then significantly increased with the increase of differentiation time compared with day 4 ($P < 0.05$, Fig. 4C). These data indicated that miR-101 expression is also related to human VSD, the embryonic development of mice and differentiation of P19 cells; moreover, miR-101 and SNHG6 were inversely expressed in these processes. In addition, we further tested the expression of miR-101 after aberrant expression of SNHG6. The results showed that the miR-101 expression was markedly downregulated in pcDNA-SNHG6 group compared to that in pcDNA3.1 group, but obviously upregulated in si-SNHG6 group related to that in si-NC group ($P < 0.01$, Fig. 4D), confirming that SNHG6 could negative regulate the expression of miR-101.

2.5. SNHG6/miR-101 axis was involved in the regulation of P19 cell function

To further explore the effects of SNHG6/ miR-101 axis in heart development, miR-101 mimic and miR-101 inhibitor were transfected into P19 cells to overexpress and suppress the expression of miR-101 in P19 cells. As displayed in Fig. 5A, the expression of miR-101 was significantly increased in miR-101 mimic group compared to that in mimic NC group, and markedly decreased in miR-101 inhibitor group related to that in inhibitor NC group ($P < 0.01$, Fig. 5A), indicating that the high transfection efficiency could be used for following experiments. Subsequently, the combined effects of overexpression of SNHG6 and miR-101 synchronously were investigated. The results showed that overexpression of SNHG6 and miR-101 synchronously significantly reversed the effects of overexpression of SNHG6 alone on cell viability ($P < 0.05$, Fig. 5B), apoptosis ($P < 0.05$, Fig. 5C), the expression changes of apoptosis-related proteins (Fig. 5D), and the expression changes of cardiomyocyte differentiation-markers ($P < 0.05$, Fig. 5E). These data indicated that SNHG6 might be involved in the modulation of P19 cell function *via* negative regulation of miR-101.

2.6. Effects of SNHG6 on P19 cells were through Wnt/β-catenin pathway

Activation of Wnt/β-catenin signaling is shown to play key roles in cardiac development and repair (Buikema et al. 2014; Pahnke

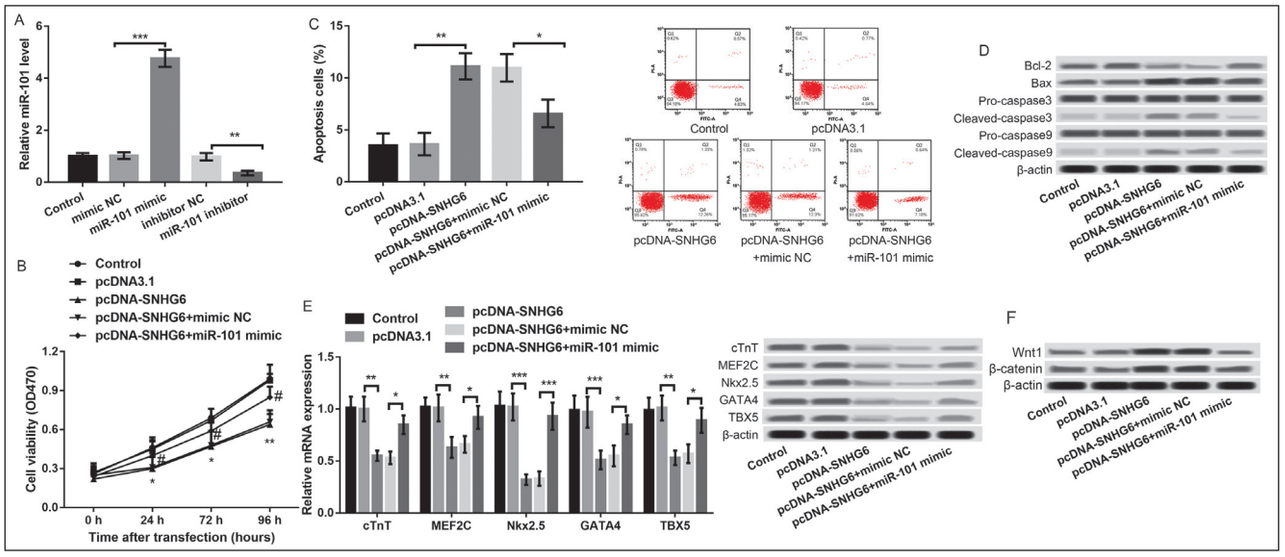

Fig. 5: SNHG6 was involved in the modulation of P19 cell function via negative regulation of miR-101. A: The expression of miR-101 in P19 cells after transfection with miR-101 mimic and miR-101 inhibitor. B: MTT assay showed cell viability after transfection. C: Flow cytometry showed cell apoptosis after transfection. D: Western blotting showed the expression of apoptosis-related proteins. E: qRT-PCR and western blotting showed the expression changes of cTnT, MEF2C, Nkx2.5, GATA4, and TBX5 after transfection. F: The protein expression of Wnt1 and β-catenin after overexpression of SNHG6 and/or miR-101.All experiments were repeated three times. The data were presented as mean±SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with corresponding control. In Fig. 5B, # $P < 0.05$ compared with pcDNA-SNHG6+mimic NC group.

et al. 2016). Therefore, we further investigated whether SNHG6/miR-101 axis could regulate the activation of Wnt/β-catenin pathway to modulate heart development. As shown in Fig. 5F, overexpression of SNHG6 promoted the protein expression of Wnt1 and β-catenin, which was significantly reversed after overexpression of SNHG6 and miR-101 synchronously. These data indicated that the effects of SNHG6 on P19 cells might be achieved through regulating the activation of Wnt/β-catenin pathway.

3. Discussion

This study explored the potential role of SNHG6 in heart development. We found that SNHG6 was upregulated in fetal cardiac tissues with VSD, and decreased in the embryonic development of mice and differentiation of P19 cells into cardiomyocytes. Overexpression of SNHG6 inhibited P19 cell proliferation and induced apoptosis, as well as promoted cell differentiation into cardiomyocytes. Further experiments confirmed that SNHG6 could negative regulate the expression of miR-101, and the effects of SNHG6 on the modulation of P19 cell function were through negative regulation of miR-101. In addition, overexpression of SNHG6 activated the Wnt/β-catenin pathway, which was reversed after overexpression of SNHG6 and miR-101 synchronously. These important findings merit further discussed.

The growth and development of the fetal heart lie on the balance of cardiomyocyte proliferation and apoptosis (Fiorina et al. 2004). It has been suggested that the imbalance of proliferation and apoptosis may result in heart deformity or congenital heart disease (Lévy et al. 2007), which may be caused by altered proliferation and/or apoptosis in the septum or myocardium(Gittenberger-de Groot et al. 2005). In this study, we found that overexpression of SNHG6 inhibited P19 cell proliferation and induced apoptosis. Overexpression of SNHG6 arrested cell cycle at the G1 phase and contributed to an S phase reduction. We thus conclude that overexpression of SNHG6 may exert key functions on growth and development of the fetal heart. In addition, overexpression of SNHG6 induced P19 cell differentiation into cardiomyocytes by decreasing the expression of five markers (cTnT, MEF2C, Nkx2.5, GATA4, and TBX5) associated with cardiomyocyte differentiation. Nkx2.5, GATA4 and CTnT are considered as key indicators of cardiomyocyte lineage commitment and maturation during induction (Qin et al. 2013; van der Heyden and Defize 2003). Paffett-Lugassy et al. (2013) showed that NKX2.5 was identified to be associated with early heart development. GATA4, a transcription factor active in cardiac development, has been shown to regulate cardiogenesis in embryonic stem cells (Grépin et al. 1997). Garg et al. (2003) revealed that GATA4 mutations might cause human congenital heart defects including VSD *via* interaction with TBX5 (Garg et al. 2003). LncRNA uc.167 is revealed to regulate P19 cell proliferation, apoptosis and differentiation *via* regulating MEF2C (Song et al. 2016). These findings confirm the key role of SNHG6 in differentiation of P19 cells into cardiomyocytes. In addition to the upregulation of SNHG6 in fetal cardiac tissues with VSD, we speculate that SNHG6 may play a role in VSD formation.

Furthermore, the potential regulatory mechanisms of SNHG6 in VSD were investigated. The results showed that SNHG6 could negatively regulate the expression of miR-101. In previous studies, downregulation of miR-101 has been shown to be involved in rheumatic heart disease via regulating TLR2 (Dong et al. 2015); miR-101 is also found to repress post-infarct cardiac fibrosis and the deterioration of cardiac performance via the FOS/TGFβ1 pathway (Pan et al. 2012). These data reveal the potential role of miR-101 in heart diseases. Our results demonstrated that the effects of SNHG6 on the modulation of P19 cell function were through negative regulation of miR-101, implying that SNHG6 may contribute to VSD *via* negative regulation of miR-101. Furthermore, the association between SNHG6 and the Wnt/β-catenin pathway was explored. Activation of Wnt/β-catenin signaling is shown to play key roles in various aspects of cardiac development and repair, such as progenitor expansion, proper cardiac specification, and myocardial growth (Buikema et al. 2014; Pahnke et al. 2016). Moreover, Cantu et al. (2018) revealed that mutations in Bcl9 and Pygo genes might cause congenital heart defects by regulating Wnt/β-catenin signaling. Based on our results, we hypothesize that SNHG6/miR-101 might play a role in VSD formation by regulation of the Wnt/β-catenin pathway activation.

In conclusion, our study strengthened the idea that SNHG6 might play a role in VSD formation *via* negative regulation of miR-101 and activation of Wnt/β-catenin pathway. SNHG6 may constitute a potential therapeutic target in this disease. Further studies using various cell types and animal models should be conducted to validate our findings and speculation.

4. Experimental

4.1. Collection of fetal heart tissues

Fetal heart tissues were collected from pregnant women who were diagnosed with VSD and underwent artificial abortion in our hospital. The gestational age of recruited

individual was between 17 and 20 weeks. Moreover, the fetuses with other developmental defects were excluded. In addition, the pregnant women with the same gestational age who underwent voluntary abortion because of private reasons were enrolled, and normal fetal heart tissues were collected as controls. Therefore, 15 fetal cardiac tissues with VSD and 15 normal control tissues were obtained. The study was approved by the ethics committee of our hospital, and all patients signed written informed consent for research.

4.2. Isolation of heart tissues from mice embryo

Heart tissues of mice with a C57BL/6 genetic background were collected at four time points: embryonic day 7.5, 11.5, 14.5 and 18.5. Subsequently, embryonic tissues were carefully dissected by micro-instruments under a microscope and then rinsed with DEPC-treated PBS buffer. All procedures were performed with the approval of the Institutional Animal Care and Use Committee (IACUC).

4.3. Cell culture and the induction of cardiomyocyte differentiation

The P19 mouse embryonic carcinoma cell line (American Type Culture Collection, USA) was obtained and then cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Paisley, Scotland, UK) containing 10% fetal bovine serum (FBS; Gibco BRL, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at a 37 °C incubator with 5% CO_2. To induce cardiomyocyte differentiation, P19 cells were cultivated as aggregates from day 0 to 4 in bacteriologic dishes filling with DMEM containing 10 % FBS and 1 % DMSO (Sigma, USA) at a 37 °C incubator with 5% CO_2, followed by transferring to cell culture flasks on day 5. Cells were harvested at dayS 0, 4, 6, 8 and 10 of differentiation.

4.4. Cell transfection

The P19 cells were seeded into a 96-well plate and grown to 70–90% confluence before transfection. Cells were then transfected with appropriate concentrations of pcDNA3.1-SNHG6 (overexpression vector), pcDNA3.1 blank control, si-SNHG6, si-negative control (NC), miR-101 mimic, mimic NC, miR-101 inhibitor, and inhibitor NC using Lipofectamine™ 2000 reagent (Invitrogen, USA). The medium was replaced at 4 h after transfection. The transfected cells were harvested at 48 h of post-transfection.

4.5. MTT assay

Cell viability was detected using MTT assay. Briefly, after different treatments, 10 μl of 5 mg/ml MTT (in PBS) was added to each well of a 96-well plate to incubate P19 cells for 3 h at 37 °C. The formazan granules were then dissolved in dimethyl sulfoxide for 15 min. The absorbance of each well at 570 nm wavelength was detected using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

4.6. Cell cycle assay

After different treatments, P19 cells were cultivated in serum-free α -MEM for 24 h and then incubated with complete medium for 0 and 24 h. Following incubation, cells (10^4 cells) were harvested using trypsin/EDTA and fixed in 70 % ethanol at 4 °C overnight. After further stained with 500 μL propidium iodide (PI) (BD Bioscience, San Diego, CA, USA), the DNA content of P12 cells in different groups was analyzed by a FACSCalibur flow cytometer (BD Bioscience), followed by analysis of the population in each cell cycle phase using FlowJo software (FlowJo, Ashland, OR, USA).

4.7. Apoptosis assay

To measure apoptosis, P19 cells were collected after 24 h of different transfections. After rinsing with PBS, cells were stained with fluorescein isothiocyanate (FITC)-labelled annexin V and propidium iodide (PI) using the Annexin V-FITC kit (Biosea Biotechnology Co., Beijing, China). Apoptotic cells were immediately assayed by flow cytometric analysis on a flow cytometry (FACSCalibur, Becton-Dickinson, San Jose, CA).

4.8. Real-time quantitative PCR (qPCR)

Total RNAs from different transfected P19 cells were extracted using TRIzol reagent (Invitrogen), and then reverse transcribed into cDNA using a RevertAid First Strand cDNA SynthesisKit (Thermo Scientific, Pittsburgh, PA, USA). Real-time qPCR (SYBR Green method) was performed using a GoTaq 2-Step RT-qPCR System (Promega, Madison, WI, USA) on the 7500 Fast System Real-Time PCR cycler (Applied Biosystems). The gene expression was normalized against a reference gene, GAPDH, and the relative gene expression levels were quantified based on the Ct.

4.9. Western blotting

Total proteins from different transfected P19 cells were extracted using the Total Protein Extraction Kit (KeyGen, inc., China) and then quantified by a bicinchoninic acid (BCA) assay kit (KeyGen, Nanjing, China). The proteins were then separated by 10 % SDS-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Primary antibodies (1:1000) were then added to incubate the membranes overnight at 4°C. Anti-Bcl-2, anti-Bax, anti-pro-caspase3, anti-cleaved-caspase3, anti-pro-caspase9, anti-cleaved-caspase9, anti-GATA4, anti-TBX5, anti-cTnT, anti-Nkx 2.5, anti-Wnt, anti-β-catenin and anti-β-actin antibodies were obtained from Abcam (Abcam plc, Cambridge, UK). β-actin was used as the internal control. Followed by further incubation with appropriate horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h, the protein signals were detected using ECL plus reagents (Beyotime, Shanghai, China).

4.10. Statistical analysis

All experiments were repeated three times. The obtained data were presented as mean±standard deviation (SD). Statistical significance between groups was analyzed by one-way analysis of variance (ANOVA) in SPSS 13.0 software (SPSS, USA). A value of $P < 0.05$ indicated a significant difference.

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Conflict of interest: None declared.

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