miR-30c regulates proliferation, apoptosis and differentiation via the Shh signaling pathway in P19 cells

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MicroRNAs (miRNAs) are small, non-coding single-stranded RNAs that suppress protein expression by binding to the 3′ untranslated regions of their target genes. Many studies have shown that miRNAs have important roles in congenital heart diseases (CHDs) by regulating gene expression and signaling pathways. We previously found that miR-30c was highly expressed in the heart tissues of aborted embryos with ventricular septal defects. Therefore, this study aimed to explore the effects of miR-30c in CHDs. miR-30c was overexpressed or knocked down in P19 cells, a myocardial cell model that is widely used to study cardiogenesis. We found that miR-30c overexpression not only increased cell proliferation by promoting cell entry into S phase but also suppressed cell apoptosis. In addition, we found that miR-30c inhibited dimethyl sulfoxide-induced differentiation of P19 cells. miR-30c knockdown, in contrast, inhibited cell proliferation and increased apoptosis and differentiation. The Sonic hedgehog (Shh) signaling pathway is essential for normal embryonic development. Western blotting and luciferase assays revealed that Gli2, a transcriptional factor that has essential roles in the Shh signaling pathway, was a potential target gene of miR-30c. Ptch1, another important player in the Shh signaling pathway and a transcriptional target of Gli2, was downregulated by miR-30c overexpression and upregulated by miR-30c knockdown. Collectively, our study revealed that miR-30c suppressed P19 cell differentiation by inhibiting the Shh signaling pathway and altered the balance between cell proliferation and apoptosis, which may result in embryonic cardiac malfunctions.

INTRODUCTION

MicroRNAs (miRNAs) are small, non-coding single-stranded RNAs that are ubiquitously expressed in plants, nematodes and human cells. miRNAs bind to the 3′ untranslated region (UTR) of their target genes, forming an RNA-induced silencing complex that mediates degradation of the target gene mRNA or inhibits translation of the target proteins.1,2 miRNAs are involved in development, apoptosis, differentiation, hormone secretion and various physiological processes.3–5 miRNAs have critical roles in cardiovascular development, and their expression profiles change with different pathological conditions. For example, miR-29 is involved in cardiac fibrosis; miR-145 and miR-92 regulate cardiac angiogenesis; miR-30 has a role in cardiac apoptosis; and miR-26 is affected by modified ionic channel function.6 Studies have indicated that miRNAs have important roles in cardiogenesis and congenital heart diseases (CHDs).7,8 The heart is known to be the earliest functional organ formed in the process of embryonic development. Heart development is spatiotemporally regulated, which includes accurate control of gene expression and signaling pathways, such as the Wnt signaling pathway, the Sonic hedgehog (Shh) signaling pathway, and a series of important morphological changes.9,10 Understanding the molecular mechanisms involved in CHDs is crucial for developing new therapeutic interventions.

Previously, our microarray data showed that miR-30c was highly expressed in the heart tissues of aborted embryos with ventricular septal defects, but the role of miR-30c in heart development is not known (data not shown). miR-30c belongs to the miR-30 family, which is evolutionarily conserved in different species (Table 1). Modulating the expression of miR-30b and miR-30c may affect vascular calcification.11

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miR-30c overexpression caused dilated cardiomyopathy. In addition, miR-30c was reported to be an independent predictor of a good response to tamoxifen therapy in advanced breast cancer patients, and the miR-30c/VIM/TWF1 signaling cascade has also been associated with clinical outcome in breast cancer patients.

Species Sequences

| Species | Sequences |
|---------|-----------|
| Dre     | UGUAAACAUCCUACUCUCAG |
| Ssc     | UGUAAACAUCCUACUCUCAG |
| Pmr     | UGUAAACAUCCUACUCUCAG |
| Mmu     | UGUAAACAUCCUACUCUCAG |
| Hsa     | UGUAAACAUCCUACUCUCAG |
| Rno     | UGUAAACAUCCUACUCUCAG |
| Gga     | UGUAAACAUCCUACUCUCAG |
| Lla     | UGUAAACAUCCUACUCUCAG |

Abbreviations: Dre, Danio rerio; Gga, Gallus gallus; Hsa, Homo sapiens; Lla, Lagothrix lagotricha; Mmu, Mus musculus; Pmr, Pan troglodytes; Rno, Rattus norvegicus; Ssc, Sus scrofa.

The Shh signaling pathway is known to regulate specification, patterning and growth of multiple embryonic organs, including cardiac development. Shh initiates signaling by binding to its receptor Ptc (Patched), resulting in Smo (Smoothened) dissociation from Gli proteins and activation of the Gli proteins. Gli proteins are transcriptional factors that control expression of Shh target genes. The Shh pathway has a critical role during development in a time- and position-dependent manner by regulating patterning and maintenance of proliferative niches. In animals, loss of Shh leads to several cardiac abnormalities, including ventricular hypoplasia, septation defects and outflow tract shortening. It is a key signal in determining left–right asymmetry, including positioning the heart on the left side of the body.

We confirmed that Gli2, one of the primary transcriptional factors in the Shh signaling pathway, is a potential target gene of miR-30c. Shh signaling via Gli1/2 was considered to be sufficient to initiate the expression of cardiac muscle transcription factors, leading to cardiomyogenesis in aggregated P19 cells.

In this study, we examined the underlying mechanisms of heart development by analyzing proliferation, apoptosis and differentiation in response to miR-30c overexpression or knockdown in P19 cells. Our findings suggest that miR-30c has an important role in the regulation of cardiac cell proliferation and/or cell survival and provide further insight into heart development.

**MATERIALS AND METHODS**

**Plasmid constructs**

The mature sequence of mouse mmu-miR-30c (5′-TGTTAACATCC TACACTTCGAC-3′) was annealed and inserted into the EcoRI/AgeI sites of the pPG/miR/eGFP/blasticidin vector (GenePharma, Shanghai, China) using the following primers: sense, 5′-ATTCTCGTAACAT CCTACACTTCGACGTTTGGCCACTGACTGAGTAGTA GGATGTTCAGAC-3′ and antisense, 5′-CCGGTTGTAACATCC TACCTCGTGTCGTGCGC-3′. To knock down mmu-miR-30c, a miRNA sponge was used to suppress endogenous expression of mmu-miR-30c. The complementary binding sites (5′-GCTGAGAGTGGATGT-3′) were inserted into the same vector.

The 3′ UTR or mutant 3′ UTR regions of Gli2 were PCR-amplified and inserted into the Xhol/AacI-digested vector psiCHECK-2 (GenePharma) containing firefly and Renilla luciferase reporter genes. The mutant sites are shown in bold in the following sequences: wild type 3′ UTR region of Gli2: 5′-GGGTCTCTCCTGGCCTGTTTACA-3′; mutant 3′ UTR region of Gli2: 5′-GGGTCTCTCCTGCCAAACAACA-3′. To validate the efficiency of the miRNA sponge, the miR-30c sponge sequence was inserted into the psiCHECK-2 vector. To confirm that miR-30c overexpression or knockdown by the miRNA sponge was functional, the wild type or mutant 3′ UTR of twinflim1 (Twf1), a previously identified target gene of miR-30c, was inserted into the psiCHECK-2 vector as described.

**Cell culture, miRNA transfection and establishment of stable cell lines**

P19 cells obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in modified Eagle’s medium (α-MEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 mg ml⁻¹ streptomycin, and 100 U ml⁻¹ penicillin in a 5% CO₂ incubator at 37 °C. A miR-30c mimic or inhibitor (Ambion, Foster City, CA, USA) was transiently transfected into P19 cells using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. To establish stable cell lines, the plasmids were transfected into P19 cells using Lipofectamine 2000. The cells were then selected with 60 μg ml⁻¹ of blasticidin for 2 weeks (Invitrogen). The establishment of miR-30c-overexpressing cells was confirmed by quantitative real-time RT-PCR (qRT-PCR) (Table 2).

**Luciferase assay**

Reporter plasmids were transfected into the P19 cells stably overexpressing miR-30c or the P19 miR-30c knockdown cells. The Dual Luciferase Reporter Assay Kit (Promega, Madison, WI, USA) was used to analyze the reporter plasmid to determine the firefly and Renilla luciferase activities according to the manufacturer’s protocols.

**Induction of cell differentiation**

Cell differentiation was carried out as previously described. Briefly, cells were cultured in α-MEM medium supplemented with 10% FBS, 100 U ml⁻¹ penicillin, 100 mg ml⁻¹ streptomycin and 1% DMSO (Sigma, St Louis, MO, USA) for 4 days. The cells formed embryoid bodies, which were then transferred and cultured in 6 cm dishes with
harvested using trypsin/EDTA, washed twice with PBS, and
medium for the indicated times (0, 8, 16, 24 and 32 h). The cells were
serum deprivation for 24 h, followed by replacement with complete
medium. Cells were then dehydrated with an ethanol gradient for 10 min.

The cells were fixed in 2.5% glutaraldehyde for 2 h. Then, the cells were stained with
4% osmium tetroxide for 2 h and dehydrated with a graded ethanol series.

For electron microscopy analysis, cells were harvested using trypsin/
EDTA, washed with PBS, and

mg-actin. Western blotting showed that GFP was stably
expressed after DMSO treatment for 10 days, indicating that
expression (Figure 1a). The stable cell lines were selected by
GFP fluorescence analysis.

For analysis of apoptosis by
flow cytometry, cells were harvested on
indicated time point according to the manufacturer’s instructions.

Stable cell lines were seeded in 96-well plates for 5 days. Cell proliferation was determined using a BrdU Cell Proliferation ELISA Kit (Abcam, Cambridge, MA, USA) according to the manufacturer’s instructions.

To analyze the cell cycle, stable cell lines were synchronized by
serum deprivation for 24 h, followed by replacement with complete
medium for the indicated times (0, 8, 16, 24 and 32 h). The cells were
harvested using trypsin/EDTA, washed twice with PBS, and fixed in
70% ethanol at −20 °C overnight. Then, the cells were stained with
propidium iodide (PI) solution and analyzed using a BD FACS
Canto cytometer and Cell Quest software (BD Biosciences, San Jose,
CA, USA).

Apoptosis assays: Hoechst 33258 staining, electron
microscopy, measurement of caspase-3 activity and flow
cytometry

Cells were cultured in serum-deprived α-MEM for 24 h to induce
apoptosis. For Hoechst staining, cells were cultured with PBS for
3 min and then stained with Hoechst 33258 (Beyotime, Nantong,
China) for 5 min. Digital images were taken using a SPOT camera
system (Nikon, E4500, Japan).

For electron microscopy analysis, cells were harvested using trypsin/
EDTA, washed with PBS, and fixed with 2.5% glutaraldehyde for 2 h. The
particles were then dehydrated with an ethanol gradient for 10 min
each time and coated with gold (15 min). Images were acquired using
a scanning electron microscope system (SEM; Supra 55, Carl Zeiss).

A caspase-3 colorimetric assay kit (KeyGen, Nanjing, China) was
used to determine apoptosis activity. Cells were collected, washed with
PBS, lyzed on ice for 1 h, vortexed for 10 s every 20 min and then
centrifuged for 1 min at 10 000 g at 4 °C. Aliquots of the supernatant
were mixed with 5 μl of substrate and incubated at 37 °C for 4 h in
dark. The absorbance values were measured at 400–405 nm.

For analysis of apoptosis by flow cytometry, cells were harvested,
washed and resuspended in 1 ml binding buffer, followed by staining
with annexin V-fluorescein isothiocyanate (V-FITC) and PI at room
temperature for 15 min (BioVision, CA). Flow cytometry was used to
analyze the FITC and PI fluorescence signals.

Quantitative real-time RT-PCR

To determine miRNA levels, miRNAs were exacted using a mirVana
PARIS kit (Ambion, Foster City, CA, USA) according to the
manufacturer’s protocols. RNA concentration was determined with
a NanoDrop 2000 spectrophotometer (NanoDrop Tech., Rockland,
DE, USA). A TaqMan MicroRNA Reverse Transcription Kit and
miRNA-specific stem-loop primers (Life Technologies, Carlsbad, CA,
USA) were then used to carry out reverse transcription of the total
RNA into cDNA. U6 levels were used as an internal control. Real-time
PCR (TaqMan) was conducted in 96-well plates on an ABI 7500
real-time PCR system (Applied Biosystems, Foster City, CA, USA).
The TaqMan probe IDs of miR-30c and U6 are shown in Table 2.

To determine gene expression levels, total RNA was extracted from
P19 cells using TRIzol reagent (Invitrogen). cDNA was reverse
transcribed using a High Capacity cDNA Reverse Transcription Kit
(Life Technologies). qPCR (TaqMan) was initiated at 95 °C for
10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.
β-actin was used as an internal control. The TaqMan probe IDs of all
genes are listed in Table 2.

Western blotting

Western blotting was performed using antibodies against Gli2, cTnT or Gata4 (Abcam) overnight. Cells were then stained with
FITC-conjugated secondary antibody. Nuclei were stained with DAPI
(4',6-diamidino-2-phenylindole, Sigma). Images were collected using a
Zeiss Axiosplan-2 epifluorescence microscope.

Statistical analysis

Experiments were performed three times. All data are shown as the
mean ± s.d. Statistical analysis was performed using an unpaired
Student’s t-test with SPSS software version 13.0 (SPSS, Inc., Chicago,
IL, USA). P-values < 0.05 were considered statistically significant.

RESULTS

Establishment of stable cell lines

Plasmids, including the overexpression vector, the knockdown
vector of mmu-miR-30c and the empty vector, were transiently
transfected into P19 cells. The transfection efficiencies were similar between different groups as indicated by GFP
expression (Figure 1a). The stable cell lines were selected by
blasticidin. Western blotting showed that GFP was stably
expressed after DMSO treatment for 10 days, indicating that
the stable cell lines were successfully established
(Supplementary Figure 1). The stable miR-30c-overexpressing
P19 cell line was confirmed by qPCR (Figure 1b). To validate

Table 2 TaqMan assay IDs of genes used in the TaqMan qPCR

| Gene         | TaqMan assay ID |
|--------------|----------------|
| mmu-miR-30c  | 000104         |
| U6           | 001973         |
| cTnT         | Mm01290256_m1  |
| Nkx2.5       | Mm01398913_s1  |
| Gata4        | Mm00484689_m1  |
| Gli2         | Mm01293111_m1  |
| Ptch         | Mm00436026_m1  |
| β-actin      | Mm00607939_s1  |
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**Figure 1** Establishment of stable miR-30c-overexpressing or silenced P19 cell lines. (a) GFP expression was observed by fluorescence microscopy after transfection of the miR-30c overexpression or knockdown vector or a control vector into the P19 cells at the indicated time points. Scale bar, 50 μm. (b) qRT-PCR analysis of miR-30c expression in P19 cells after blasticidin selection. The data are presented as the mean±s.d. of three experiments. (c) Luciferase assay of the miR-30c sponge efficiency. The data are presented as the mean±s.d. of three experiments. (d) Luciferase assay of miR-30c with a Twf1 3′ UTR. Luciferase reporters containing a wild type (WT) or mutant (mut) 3′ UTR of Twf1 were transfected into P19 stable cell lines. The data are presented as the mean±s.d. of three experiments. (*P<0.05 comparing miR-30c overexpression with control cells; #P<0.05 comparing miR-30c knockdown with control cells).
the efficiency of the miR-30c sponge, we co-transfected a luciferase reporter containing the miR-30c sponge sequence and a miR-30c-overexpressing plasmid into P19 cells. A luciferase assay showed that the luciferase activity was significantly suppressed by miR-30c, indicating that the miR-30c sponge was effective (Figure 1c). To confirm that the miR-30c overexpression or knockdown in P19 cells was functional, we transfected a luciferase reporter containing a wild type or mutant 3′ UTR of Twf1, a previously identified target gene of miR-30c, 27 into stable miR-30c-overexpressing or miR-30c knockdown P19 cells. Luciferase assays showed that miR-30c overexpression significantly reduced the luciferase activity, while miR-30c knockdown increased it (Figure 1d).

Effects of miR-30c on P19 cell proliferation

The CCK-8 assay was used to determine the effect of miR-30c overexpression or knockdown on the growth of P19 cells. As shown in Figure 2a, miR-30c-overexpressing P19 cells grew much faster than control cells, while miR-30c knockdown cells grew much more slowly (P<0.05). BrdU ELISA analysis showed that miR-30c overexpression increased P19 cell proliferation, while miR-30c knockdown decreased it (Figure 2b). In addition, miR-30c had similar effects on the cell cycle. Flow cytometry of the cell cycle distribution showed a significantly higher percentage of miR-30c-overexpressing cells in S phase (Figures 2c, *P<0.05; **P<0.001 comparing miR-30c overexpression with control cells; #P<0.05 comparing miR-30c knockdown with control cells).

Effects of miR-30c on P19 cell apoptosis

Hoechst staining is a classic, quick and easy method used to detect apoptosis. The chromatin in apoptotic cells is condensed, and the nuclei from apoptotic cells are dense or fragmented white spots under a fluorescence microscope after Hoechst staining, while the nuclei of normal cells appear blue. As shown in Figure 3a, miR-30c overexpression significantly suppressed serum starvation-induced apoptosis, while miR-30c knockdown increased cell apoptosis. Electron microscopy, which is a powerful method for morphologically observing differentiating nuclei and organelles, was used to confirm apoptosis. As shown in Figure 3b, there were few typical morphological changes in the miR-30c overexpression group, but obvious apoptotic morphological changes were observed in the miR-30c knockdown group after serum starvation for 24 h. Annexin V-FITC, which binds to phosphatidylserine, was used to detect apoptosis. Cells were induced to undergo apoptosis by

Figure 2 Effects of miR-30c on cell proliferation. (a) Cell growth was measured for seven consecutive days using a CCK-8 assay kit. (b) BrdU ELISA analysis for measuring cell proliferation after cells were cultured for 5 days. (c, d) Flow cytometry analysis of the cell cycle of miR-30c-overexpressing (c) or miR-30c knockdown (d) cells. The data are presented as the mean±s.d. of three experiments (*P<0.05; **P<0.01 comparing miR-30c overexpression with control cells; #P<0.05 comparing miR-30c knockdown with control cells).
serum starvation for 24 h. Overexpression of miR-30c reduced the percentage of apoptotic cells, while knockdown of miR-30c increased it in response to serum deprivation (Figures 3c, *$P<0.05$). The same results were observed in P19 cells transiently expressing either a miR-30c mimic or inhibitor (Supplementary Figure 2C, *$P<0.05$). In addition, caspase-3 activity assays showed that miR-30c overexpression suppressed cell apoptosis, while miR-30c knockdown increased the number of apoptotic cells in response to serum deprivation (Figure 3d, *$P<0.05$). Collectively, these results indicated that miR-30c protected P19 cells from serum deprivation-induced apoptosis.

Effects of miR-30c on the differentiation of P19 cells
We observed morphological changes during DMSO-induced differentiation of the P19 cells into myocardial cells and quantified specific myocardial markers (cTnT, Gata4 and Nkx2.5) in the miR-30c overexpression or knockdown cell lines. Figure 4a shows the cell morphology changes from day 0 to day 10. Compared with the control groups, there were fewer beating cell clusters in the miR-30c overexpression group, while there were many more beating cell clusters in the miR-30c knockdown group. In addition, we used qRT-PCR to analyze the relative RNA expression levels of the myocardial markers cTnT, Gata4 and Nkx2.5 on days 0, 2, 4, 6, 8 and 10 after DMSO treatment. The expression of these markers is known to be upregulated during differentiation of mouse P19 cells into myocardial cells (Figure 4b, *$P<0.05$). Expression of all the marker genes gradually increased during the process of differentiation. Compared to the controls, the expression levels of these marker genes were globally much lower in the miR-30c-overexpressing cells, and they were much higher in the miR-30c knockdown cells. Furthermore, an immunofluorescence assay was performed to show the expression level of cTnT and Gata4 in response to DMSO treatment. As shown in Figure 4c, the expressions of both cTnT and Gata4 were induced by DMSO treatment, and their levels were much lower in the miR-30c-overexpressing cells and much higher in the miR-30c knockdown cells compared with the control group.

miR-30c targets Gli2 and regulates the Shh signaling pathway
Gli2, a transcriptional factor of the Shh signaling pathway, was predicted to be a potential target gene of miR-30c by a variety
of online gene analysis programs, including TargetScan 5.1. The binding of miR-30c to the target site in the Gli2 3′ UTR was detected using the Dual Luciferase Reporter system. As shown in Figure 5a, miR-30c overexpression significantly reduced the luciferase activity of the pGL3-Gli2-3′ UTR wild type (WT) reporter but not the mutated construct (mut), while miR-30c knockdown significantly rescued the luciferase activity of the pGL3-Gli2-3′ UTR reporter but not the mutated construct (**P<0.05). Furthermore, western blotting showed that miR-30c reduced, while miR-30c knockdown increased, the protein levels of Gli2 in P19 cells (Figure 5b). These results suggest that Gli2 is a potential target gene of miR-30c. We also conducted immunofluorescence staining of Gli2 in the cells and found that Gli2 expression was much weaker in miR-30c-overexpressing P19 cells, while it was slightly stronger in miR-30c knockdown cells compared with the control group (Figure 5c). Ptch1 is an important receptor of the Shh signaling pathway and is transactivated by Gli2. Because miR-30c targets Gli2, we therefore tested whether miR-30c regulates Ptch1 expression when the cells were induced to differentiate by DMSO. As shown in Figures 5d and e, DMSO treatment globally induced both protein and mRNA levels of Gli2 and Ptch1. miR-30c overexpression substantially inhibited Gli2 and Ptch1 expression before DMSO treatment and maintained a lower expression of the genes compared with the controls after DMSO treatment. miR-30c knockdown had the opposite effect. Furthermore, we transiently transfected a miR-30c mimic or inhibitor into P19 cells. Consistent with the results in the stable

Figure 4 Effects of miR-30c on the differentiation of P19 cells. (a) miR-30c overexpressing or knockdown P19 cells were stimulated to differentiate by DMSO for 10 days. Images are shown from days 0, 2, 4, 6, 8 and 10. Scale bar, 50 μm. (b) qRT-PCR analysis of expression levels of myocardial cell markers at the indicated time points during the induction of differentiation. The data are presented as the mean±s.d. of three experiments (*P<0.05 comparing miR-30c overexpression with control cells; #P<0.05 comparing miR-30c knockdown with control cells). (c) Immunostaining of myocardial cell markers cTnT and Gata4 at the indicated time points. DAPI was used to stain the nuclei. Scale bar, 50 μm.
cell lines, expression levels of Gli2 and Ptch1 were reduced by the miR-30c mimic and increased by the miR-30c inhibitor in P19 cells (Supplementary Figure 3A). The Shh signaling pathway is involved in various developmental pathways. To determine if miR-30c targets the Shh signaling pathway, we employed the MC3T3-E1 cell line, a widely used murine osteoblast development model. Transient transfection of a miR-30c mimic or inhibitor in the MC3T3-E1 cells suppressed or increased Gli2 and Ptch1 expression, respectively (Supplementary Figure 3B), similar to the P19 cardiac development model.

**DISCUSSION**

CHDs, which account for ~40% of perinatal deaths, are the most common group of major birth defects. They are a serious medical problem that causes heavy social and economic burdens worldwide. CHDs have been intensively studied by the international community and much progress has been made in recent years. CHDs comprise numerous abnormalities in the structure and function of the heart and great vessels. Most CHDs are correlated with gene deletions and mutations. Thus, we explored the genetic and molecular mechanisms of CHDs. miRNAs have critical roles in cardiovascular development, and their expression profile changes correspond to different pathological conditions. miRNAs can be used as novel biomarkers and therapeutic targets of CHDs.

In this study, we investigated the function of miR-30c in CHDs. We used P19 cells, which are commonly used to study cardiogenesis, as a myocardial cell model. We established stable P19 cell lines overexpressing miR-30c or knocked down miR-30c using a ‘miRNA sponge’ that contains multiple binding sites in its 3′ UTR for a miRNA or miRNA seed family. The stable cell lines were induced to differentiate into cardiomyocytes using DMSO. Significant changes were observed in their morphology and the levels of cardiac markers, such as cTnT, Gata4 and Nkx2.5. cTnT is a myocardial-specific protein that indicates late myocardial differentiation. Gata4, which belongs to the cardiac Gata subfamily, is an important transcription factor in heart development. Gata4 interacts with Nkx2.5 to
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regulate myocardial cell migration and differentiation. We found that miR-30c overexpression inhibited, while miR-30c knockdown increased, the differentiation of P19 cells into cardiomyocytes, as indicated by the morphological changes and the expression of myocardial markers.

Embryonic fetal heart growth depends on the balance between cardiomyocyte proliferation and apoptosis.47 Inadequate proliferation or excess apoptosis will directly or indirectly lead to CHDs,48 which are often caused by altered proliferation and/or apoptosis in the septum, neighboring tissues or myocardium.49 miR-30c overexpression/knockdown may affect cell proliferation or apoptosis, resulting in inhibition of the differentiation of P19 cells into cardiomyocytes. miR-30c overexpression promoted cell proliferation, and miR-30c knockdown increased cell proliferation. Using Hoechst staining, electron microscopy, annexin V-FITC staining and caspase-3 activity assays, we found that miR-30c overexpression suppressed P19 cell apoptosis, while miR-30c knockdown increased cell apoptosis. Thus, by promoting cell growth and protecting cells from apoptosis, miR-30c inhibited the differentiation of P19 cells to cardiomyocytes.

Furthermore, we demonstrated that miR-30c regulates the Shh signaling pathway via a newly identified miR-30c target gene, Gli2. The three Gli proteins, Gli1, 2 and 3, are highly similar in amino-acid sequences.50 Gli1 knockout mice did not display organ malformations, but Gli2 knockout mice showed heart malformations, which were similar to Shh knockout mice. The phenotype of Gli3 knockout mice was different from the Shh knockout mice.51 Silencing of Gli2 inhibited Shh signaling.52 It has been reported that Shh signaling promoted the expressions of the cardiac progenitor markers Nkx2.5 and Gata4 in P19 cells,53 which were blocked by Shh inhibition.54 Ptc1, which is the receptor of Shh and a transmembrane target of Gli2, transduces the Shh signal. Our data showed that Ptc1 expression was suppressed by miR-30c and increased by miR-30c knockdown. Given that miR-30c inhibited expression of its target gene gli2, miR-30c may suppress differentiation of P19 cells by inhibiting the Shh signaling pathway.

In summary, we demonstrated that miR-30c overexpression suppressed, while miR-30c knockdown increased, cardiomyocyte differentiation in P19 cells by regulating the Shh signaling pathway. In addition, miR-30c altered the balance between cell proliferation and apoptosis, which might result in embryonic cardiac malformations. These findings contribute to a better understanding of the mechanisms underlying cardiac abnormalities.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Author contributions: Conceived and designed the experiments: XHL, ZBY and SPH. Performed the experiments: XHL. Analyzed the data: XHL and XSH. Contributed reagents/materials/analysis tools: ZBY, SSZ, JX and MML. Wrote the paper: XHL. Study supervision: SPH and YZP.

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