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Impact of miR-26b on cardiomyocyte differentiation in P19 cells through regulating canonical/non-canonical Wnt signalling

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Abstract

Background and objectives: The control of cardiomyocyte differentiation is tightly linked to microRNAs (miRNAs), which have been emerging as important players in heart development. However, the regulation mechanisms mediated by miRNAs in early heart development remains speculative. Here, we evaluated the impact of miR-26b during the progression of cardiomyocyte differentiation from the P19 cell line.

Materials and methods: The overexpression of miR-26b in P19 cells was performed by transduction with lentivirus vector. The levels of cardiac-related genes during P19 cell differentiation were detected using quantitative real-time PCR for mRNA abundance and Western blots for protein expression. ICG-001 was applied to elucidate the role of β-catenin on P19 cells differentiation. The Cell Counting kit-8 (CCK-8) was used to monitor the cell proliferation. The target genes of miR-26b were validated using the dual luciferase reporter system.

Results: Overexpression of miR-26b upregulates the expression level of cardiomyocyte-related genes such as Gata4, cTNT, α-MHC and α-Actinin that comprehensively represent cardiomyocyte differentiation by effecting Wnt5a signalling and Gsk3β activity. However, ICG-001 blocks the differentiation along with inhibition of the cell proliferation. In addition, miR-26b also regulates CyclinD1 to promote P19 cell proliferation, thereby, demonstrating the rapid aggregation and differentiation programming of these cells into cardiomyocytic types.

Conclusions: Our results indicated that miR-26b exerts a role on promoting cardiomyocyte differentiation of P19 cells by controlling the canonical and non-canonical Wnt signalling.

1 | INTRODUCTION

The heart is the first functional organ developed within embryo.[1] In mice, the formation of the heart goes through five stages, including the formation of the cardiac crescent following the formation of the linear heart tube then cardiac looping, chamber formation and to the mature heart.[2] At the cell level, cardiomyocyte differentiation from mouse embryonic stem cell (mESC) undergoes mesoderm phase, the cardiac progenitor cell phase and finally, the mature cardiomyocyte phase.[3] Five transcription factors such as Gata, Mef2, Nkx2.5, Tbx and Hand, all of which are involved with the cardiomyocyte commitment and have been identified to modulate the process of cardiomyocyte differentiation.[1] Growth factor signalling through the intracellular the wingless-type MMTV integration site for Wnt signalling controls the

cardiac lineage commitment.[4] Wnt proteins act as secreted ligands to regulate many essential cellular activities, including commitment of cell fates, cellular proliferation rates, cell survival as well as adhesion.[4,5] Wnt signalling consists of two pathways. The canonical pathway relies on β-catenin-mediated transcriptional activation, whereas, the non-canonical pathway utilizes Wnt signalling independent of β-catenin. For canonical pathway signalling, Wnt3a activates β-catenin to promote Wnt target gene transcription. While, non-canonical Wnt signalling pathway includes Wnt/Ca^{2+} and Wnt/planar cell polarity (PCP) pathways, in which the activation of Ca^{2+}-dependent signalling molecules, or the activation of c-Jun N-terminal Kinases (JNKs) through small Rho-GTPases is involved respectively.[6,7] Sequential activation and inhibition through both the canonical and the non-canonical Wnt signalling pathway is critical during the heart development. Inhibition of canonical Wnt signalling could lead to the reduction of the second heart field progenitor cells and affect the differentiation of ES cell into the cardiomyocyte.[8,9] The non-canonical Wnt signalling ligands, Wnt5a and Wnt11 express at the anterior pole to the heart tube and play roles during the second heart field migration. In addition, the non-canonical Wnt signalling can promote cardiogenesis in the non-cardiogenic posterior mesoderm of early embryos and the differentiation of stem cells into cardiomyocyte by inhibiting canonical Wnt signalling.[10] How to synergistically regulate the canonical and the non-canonical Wnt pathways in cardiomyocyte differentiation still maintains poorly understood.

As a group of endogenous non-coding and 19-23nt small RNA molecules,[11] miRNAs are crucial regulators in cardiomyocyte differentiation, cardiogenesis and heart diseases.[12-14] MiR-133 has been shown to be involved in the central role of transcript factor Mef2 in regulation of cardiac development.[15] MiR-143 is essential for cardiac chamber morphogenesis via directly repressing expression of Adducin3, which encodes an F-actin capping protein.[16] MiRNAs can also impact cardiomyocyte differentiation based on the Wnt signalling. For example, miR-19b knockdown increases the expression of Wnt and β-catenin, but Gsk3β can be increased with the overexpression of miR-19b in the P19 cell model for cardiomyocyte differentiation in vitro.;[17,18] In bone marrow-derived mesenchymal stem cells of rat,overexpression of miR-499 induces the expression of cardiac-specific genes, including Nkx2.5, Gata4, Mef2c and cTnI, and inhibits the ratio of phosphorylated/dephosphorylated β-catenin as well.[19] Moreover, the overexpression of miR-1 in human-induced pluripotent stem (iPS) cells enhances the expression of key cardiac transcriptional factors and sarcomeric genes, and facilitates cardiomyocyte commitment from human cardiovascular progenitors via targeting FZD7 to activate Wnt pathway.[20] MiR-26b is located in an intron of the Ctdsp2 gene and it can regulate neuro differentiation together with its host gene.[21] Expression levels of miR-26b have been showed a downregulation in the atrial fibrillation patients.[13] Forced-expression of miR-26b attenuates cardiac hypertrophy by targeting Gata4.[22] In addition, plasma miR-26b levels are downregulated in acute heart failure patients.[23] These results suggest that miR-26b is likely to play an important role in development and maintaining cardiac function. Previous studies have also shown that miR-26b could regulate β-catenin levels by inhibiting Gsk3β, then affected the Wnt/Gsk3β/β-catenin axis to elevate rheumatoid arthritis synovial fibroblasts (RAFLS) apoptosis and reduce the secretion of TNF-α, IL-1β and IL-6 to inhibit the inflammation.[24] Although miR-26b plays a potential role in the regulation of the Wnt signalling pathway by targeting Gsk3β in RAFLS, whether it can mediate Wnt signalling to regulate the cardiomyocyte differentiation remains unclear.

P19 cell line is derived from the CH3/He mice teratocarcinoma and can differentiate into three germ layers.[25] When treated with dimethyl sulphoxide (DMSO), P19 embryonic carcinoma cells aggregate and differentiate into cardiomyocytes, as widely used cell model of cardiomyocyte differentiation in vitro.[26-29] Here, we evaluated the ability of miR-26b to induce differentiation of pluripotent P19 cell into functional cardiomyocytes. Our data show that miR-26b can regulate Wnt signalling that give rise to early heart development. In the P19 cell model, we provides evidence that miR-26b contributes to the generation of functional cardiomyocytes. We further observe that overexpression of miR-26b actives canonical signalling and inhibits non-canonical Wnt to upregulate cardiac-related genes during differentiation. In addition, miR-26b can upregulate Cyclin D1 to promote P19 cell proliferation for further differentiation. These results suggest that miR-26b is potential regulator in promotion of cardiomyocyte differentiation from P19 cells by affecting both the canonical and non-canonical Wnt signalling.

2 | MATERIAL AND METHODS

2.1 | P19 cell culture and differentiation

P19 cells were cultured in accordance with previous description[28]: Cells were cultured in alpha-modified Eagle's minimal essential medium (Gibco, Carlsbad, CA, USA) with 10% foetal bovine serum (Gibco, Carlsbad, CA, USA) and 1% Penicillin/Streptomycin (Gibco, Carlsbad, CA, USA) at 37°C in a 5% CO_2 atmosphere.[28] To induce cardiac differentiation, the P19 cells were kept in the culture medium with 1% DMSO. The medium was daily changed in differentiation course process. The 25 μM of β-catenin inhibitor ICG-001 (SelleckChem, Houston, TX, USA) was added into P19 medium during differentiation.

2.2 | Construction of miR-26b overexpression P19 cell line

We used the lentivirus vector to construct the miR-26b overexpression vector and GFP-control vector. The mature sequence of mouse miR-26b (mmu-miR-26b) and GFP were amplified and cloned into the lentivirus vector using EcoRI and SalI to construct a miR-26b overexpression vector and GFP-control vector. Based on the mmu-miR-26b sequence of TTCAAGTAATTCAGGATAGGT, the sense strand of 5′-TGAATTCGACTTCATCATCCCGGTGGA-3′ and the anti-sense strand of 5′-ACCGGTTGCTGGCCAACAGGCTTAG-3′ were utilized.

For lentivirus preparation, 10 μg of DNA combined with 5 μg of VSVG and 7.5 μg of p-Pax2 was transfected into 293T cells in 10 cm dish. The medium was changed after transfection for10 hours and then the supernatant was collected after 60 hours. The supernatant containing the lentivirus of miR-26b and GFP was used to treat the

P19 for 48 hours. To establish the P19 cell line with miR-26b overexpressing or GFP expression, the puromycin was used to select positive cells, and the single positive cell was chose to obtain the stable-expression P19 cell lines.

2.3 | Transfection of miR-26b inhibitor into P19 cells

MiR-26b inhibitor and NC (Biotend, Shanghai, China) was transfected into P19 at a final concentration of 300 nM using X-treme GENE (Roche Diagnostics, Basel, Switzerland), the process of the transfection refers from the manufacturer protocol.

2.4 | Luciferase measurement

The wild-type or mutant mouse DNA sequences of the Gsk3β and Wnt5a 3′UTR with miR-26b target sites were inserted between the SpeI-Hind III restriction sites in the 3′UTR of the firefly luciferase gene in the pMIR reporter vector (Invitrogen, Carlsbad, CA, USA) respectively. The 293T cells were transfected with 150 ng of pMIR vector and 200 ng miR-26b mimic or negative control (Biotend, Shanghai, China) using X-treme GENE (Roche Diagnostics, Basel, Switzerland). The activities of the luciferases in cell lysates were detected with Firefly Luciferase Reporter Gene Assay Kit (Beyotime, Shanghai, China). The β-gal level was determined with β-galactosidase Assay Kit (Beyotime, Shanghai, China). Normalized data were calculated as the ratio of firefly/β-gal activities.

2.5 | Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted based on a standard TRIzol (Invitrogen, Carlsbad, CA, USA) protocol. After eliminating genomic DNA contamination, cDNA was reversely transcribed using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Kusatsu, Japan). SYBR® Premix Ex Taq™ II (TaKaRa, Kusatsu, Japan) was used in quantitative real-time PCR reaction, and the PCR reactions were run based on a Bio-Rad CFX Connect™ real-time system. The related primers are listed in, Table S1.

2.6 | Western blot

Total protein was extracted with RIPA buffer and supplemented with protease inhibitor cocktail (Roche). Protein concentrations were measured using the bicin choninic acid (BCA) assay. Then, the protein was separated with SDS/PAGE gel and transferred to PVDF membranes. Primary antibodies including β-catenin, WNT5a, GSK3β, Cyclin D1, cTNT, GATA4, αMHC, α-Actinin (Abcam, San Francisco, CA, USA) and GAPDH (Santa Cruz Biotechnology, Dallas, CA, USA). Detailed information about antibodies are listed in, Table S2.

2.7 | Immunostaining

For immunostaining assay, cells were washed by PBS, fixed with 4% paraformaldehyde for 20 minute and then permeabilized with 0.3% Triton X-100 for 15 minute. After washing the cells by PBS, we blocked it for 1 hour with 10% goat serum supplemented with 0.1% Triton X-100 (v/v in PBS) and incubated it overnight with primary antibodies at 4°C. Next, cells were stained with the fluorescent secondary antibodies for 1 hour and Hoechst was introduced for 10 minute. Primary antibodies were used in the immunostaining assay includes: cTNT and αMHC (Abcam, San Francisco, CA, USA). Detailed information regarding the antibodies used is listed in, Table S2.

2.8 | Proliferation activity assay

Cell Counting kit-8 (Beyotime, Shanghai, China) was used to monitor P19 cell proliferation. The Cell Counting kit-8 assay was carried out according to the manufacturer's instructions. The stable P19 cell lines of overexpressed miR-26b and GFP were cultured in 96-well plates. The cells were maintained in αMEM supplemented with 10% FBS, 100 mg/mL streptomycin and 100 U/mL penicillin. The CCK-8 solution was added to each well and incubated for 1 hour before analysis with a microplate reader with the absorbance measured at a wavelength of 450 nm.

2.9 | mESC differentiation

mESCs were induced to differentiation into the cardiomyocytes using the "hanging drop" method.[30] The D3 mESCs were dissociated by 0.25% trypsin-0.01% EDTA and re-suspended in the differentiation medium. The medium used to the embryoid body (EB) formation and differentiation was prepared as described previously.[30] The cell drops (10 000 cells/20 uL) hanged for 2 days for forming the EB. The EBs were then harvested and transferred onto 0.1% gelatin-coated six well plates. The medium was daily changed.

2.10 | Statistical Analysis

Student's *t* test (two-tailed) was used to detect the statistical significance, and the error bar represents the standard error of mean (SEM) of three independent experiments. Statistical differences were considered significant with a value of $P < .05$.

3 | RESULTS

3.1 | Impact of miR-26b in cardiomyocyte differentiation of P19 cells

To investigate potential role of miR-26b during the cardiomyocyte differentiation, we first compared the expression profile in the mouse embryonal carcinoma P19 cells with mESC. We found that miR-26b expression gradually increased with the cardiomyocyte differentiation and reached the highest level at day 12 in P19 cells or day 15 in mESC, respectively, when the functional cardiomyocytes formed (Figure 1A). We next forced expression of miR-26b in P19 cells using lentivirus vector, which mediates miR-26b stable expression at a higher level than that of the P19 cells transfected with GFP-control vector during

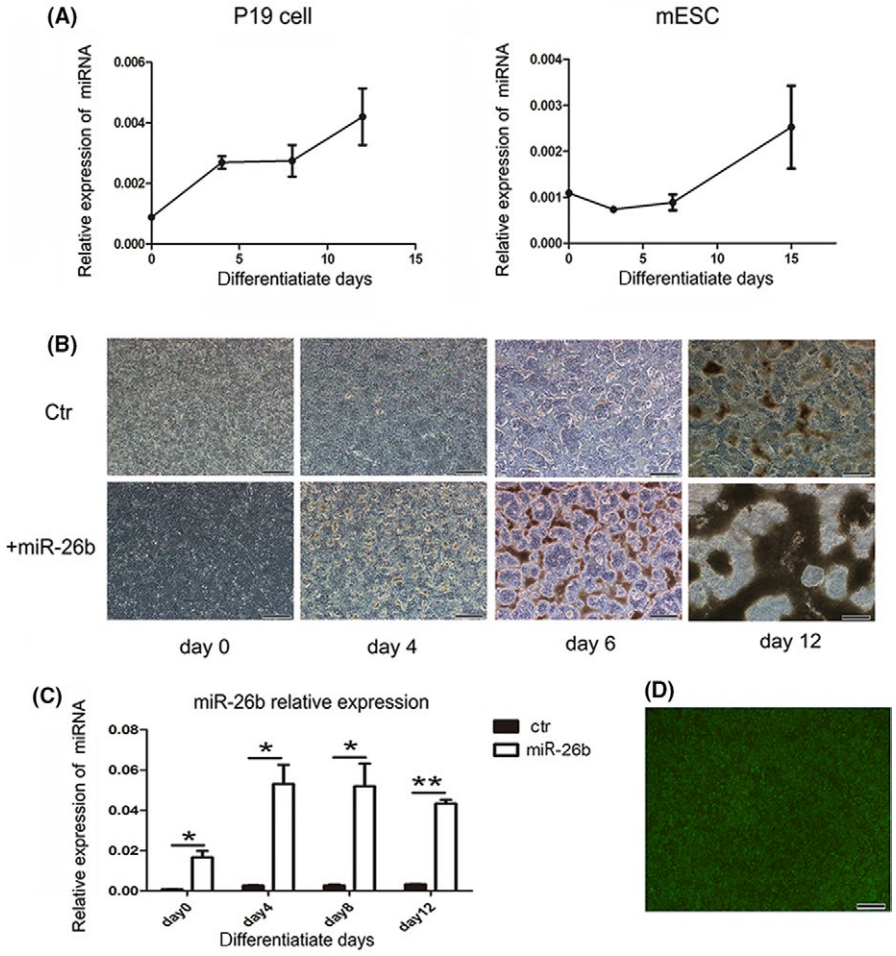


FIGURE 1 Construction of the miR-26b overexpression in P19 cell line. (A) The qPCR results of miR-26b expression tendency during the cardiomyocyte differentiation. (B) The cellular morphology during differentiation of the P19 into the cardiomyocyte (Scale bar: 500 μm). (C)The miR-26b mRNA expression of the overexpressed miR-26b P19 during differentiation. *P < .05, **P < .01, (D) The GFP fluorescence status of the control P19 cell line (Scale bar: 500 μm)

differentiation (Figure 1C,D). The overexpression of miR-26b obviously promoted the morphological changes of the differentiating P19 cells, exhibiting cellular aggregation at day 4 after differentiation (Figure 1B). The higher level of miR-26b expression also induced P19 cells to form more functional cardiomyocytes at day 12 (Figure 4A,B,D). However, the time point of synchronized twitching (beating cells) in differentiated cardiomyocytes was at day 10 (Video S1), which was not different in the condition of both overexpression and control levels indicated for miR-26b. The results suggest that miR-26b might mainly exert a role to induce more functional cardiomyocyte formation during P19 cell differentiation.

3.2 | MiR-26b targets both Gsk3β and Wnt5a

In order to identify the target gene regulated by miR-26b, the bioinformatics approach TargetScan (www.targetscan.org) was used to analysis. Among these candidate genes, we selected both Gsk3β and Wnt5a that involved in cell differentiation. The binding site of miR-26b with the 3′UTR of Gsk3β or Wnt5a was highly conserved in different species (Figure 2A). Next, we constructed the dual luciferase reporter vectors containing Gsk3β 3′UTR or Wnt5a 3′UTR, respectively, and then cotransfected reporter vectors with miR-26b mimic into 293T cells. The luciferase activity assay showed that miR-26b significantly repressed both Gsk3β and Wnt5a, and the inhibition was abolished when the corresponding seed sequences were mutated in 3′UTR of Gsk3β or Wnt5a (Figure 2B), suggesting both Gsk3β and Wnt5a could be directly controlled by miR-26b in 293T cells.

3.3 | MiR-26b inhibits expression of Wnt5a and Gsk3β during cardiomyocyte differentiation of P19 cells

The early phase of heart development is commitment to the cardiomyocyte differentiation, in which Wnt signalling pathway involved in.[31] Next, we examined the effects of miR-26b on expression of Wnt5a and Gsk3β along with P19 differentiation. At day 0 and at day 4, after induced differentiation using DMSO, mRNA level of Wnt5a or Gsk3β was reduced by the overexpression of miR-26b in P19 cells (Figure 3A). Consistently, the protein levels of both Wnt5a and Gsk3β were also downregulated in presence of high level of miR-26b (Figure 3C,D). These results reveal that miR-26b is directly involved in cardiomyocyte differentiation from P19 cells via regulating Wnt signalling in a coordinated manner, whereby, miR-26b simultaneously blocks Gsk3β to activate the canonical Wnt pathway and inhibit Wnt5a for controlling the non-canonical Wnt signalling. Moreover, miR-26b inhibitor could reverse the β-catenin upregulation induced

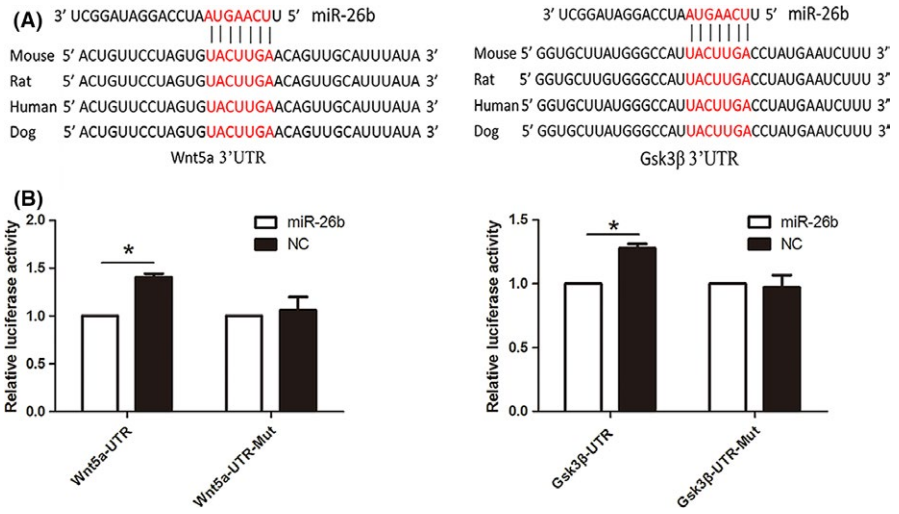


FIGURE 2 MiR-26b targets the Wnt5a and the Gsk3β. (A) The comparison between Gsk3β 3′UTR and Wnt5a 3′UTR containing the miR-26b binding site in different species. (B) Dual luciferase reporter results. MiR-26b can target the Gsk3β 3′UTR and Wnt5a 3′UTR respectively.*$P < .05$

by miR-26b-forced expression (Figure 3B), confirming again that miR-26b can indeed balance Wnt signalling to promote cardiomyocyte differentiation from P19 cells.

3.4 | MiR-26b promotes the expression of cardiac-related genes in differentiation

Cardiomyocyte differentiation was mainly derived by the expression of mesoderm and cardiac-related genes. The overexpression of miR-26b was found to upregulate the expression of both Braychyury and Mesp1 at day 4 after starting differentiation as early stage (Figure 4C), and then the expression of α-MHC and a-Actinin, the markers of mature cardiomyocyte, obviously increased at day 12 with the cell beating (Figure 4A,B). α-MHC could be up-regulated at earlier stages such as at day 8 and maintain normal level in functional cardiomyocytes in the presence of high level of miR-26b (Figure 4B). Also, more positive cells with the markers α-MHC or cTNT were detected at day 12 after differentiation using immunofluorescence assay (Figure 4D). We also detected the cardiomyocyte progenitor cells with the marker of Nkx2.5 or Isl1 at day 8, and observed that the progenitor cell proportion increased along with miR-26b overexpression in P19 (Figure 4E). Interestingly, cardiac transcription factor Gata4 was shown to be upregulated at day 4 in miR-26b-overexpression P19 cells, whereas this increase was absent in WT P19 cells until day 12 (Figure 4A,B), suggesting the launch of cardiomyocyte differentiation is considerably earlier. As a potential target of miR-26b, Kcnj2, which codes a potassium channel, was inhibited by overexpression of miR-26b with the differentiation, being consistent with a previous study[13] even though Plcβ1 levels[22] were not obviously affected in the study (Figure 4C). In order to confirm the effects of miR-26b overexpression on cardiomyocyte differentiation, we applied a miR-26b antagonist in the miR-26b-overexpression cells during differentiation. Indeed, the acceleration of the growth and aggregation of P19 cells was disappeared with same cell morphology like the control cells in presence of miR-26b inhibitor (Figure S1A). Moreover, we further detected the mature cardiomyocyte makers of cTNT, α-MHC and α-Actinin at day12, where no significant change was observed (Figure S1B,C). Similarly, the mRNA expression level of Tnnt2 and Gata4 at day12 also showed no obvious change (Figure S1D).

3.5 | Inhibition of β-catenin curbs the cardiomyocyte differentiation from miR-26b overexpressed P19 cells

Canonical Wnt signalling has been showed to decide the cardiomyocyte commitment mainly involving both initial and mesoderm phases in differentiation.[31-34] Our results suggest that miR-26b upregulates β-catenin level to promote P19 cell differentiation into cardiomyocyte. To further test the possibility, the miR-26b overexpressed P19 cells were treated during differentiation with β-catenin inhibitor ICG-001. Interestingly, the cardiomyocyte differentiation from the P19 cells is really inhibited in presence of ICG-001, in which the cells show an undifferentiated status in morphology without obvious aggregation (Figure 5A). Meanwhile, Western blot shows both expression levels of GATA4 and cTNT are downregulated via ICG-001during the differentiation (Figure 5B,C). These results indicate that miR-26b could play a role on promoting cardiomyocyte differentiation from P19 cells through upregulating β-catenin level.

3.6 | miR-26b promotes P19 cell proliferation during differentiation

The aggregation of proliferating P19 cells with proliferation is an early event to exert differentiation. To understand if miR-26b plays a role in early stage of P19 cell differentiation, we observed the proliferation activity using CCK-8 assay. Starting from day 1 to day 4 in early differentiation of P19 cells, CCK-8 was significantly increased by the presence of miR-26b overexpression (Figure 6B). β-catenin has been showed to affect cell proliferation and growth via modulating Cyclin D1.[35] Given that an increase in cell proliferation can be mediated by increased speed of the cell cycle, we next detected the expression level of Cyclin D1 which can induce cell growth and transformation by shortening cell cycle G1 phase. WT and P19 cells overexpressing

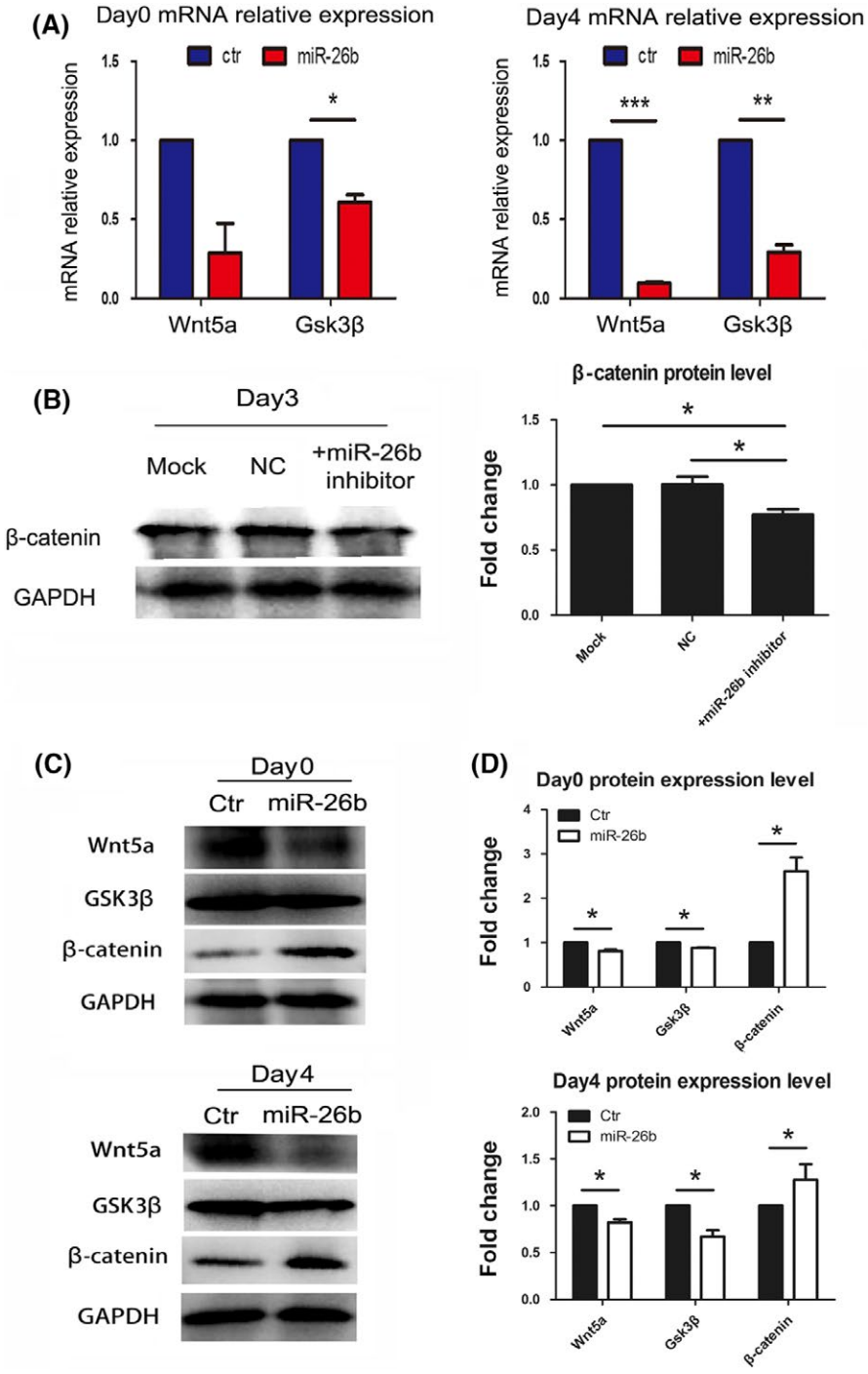
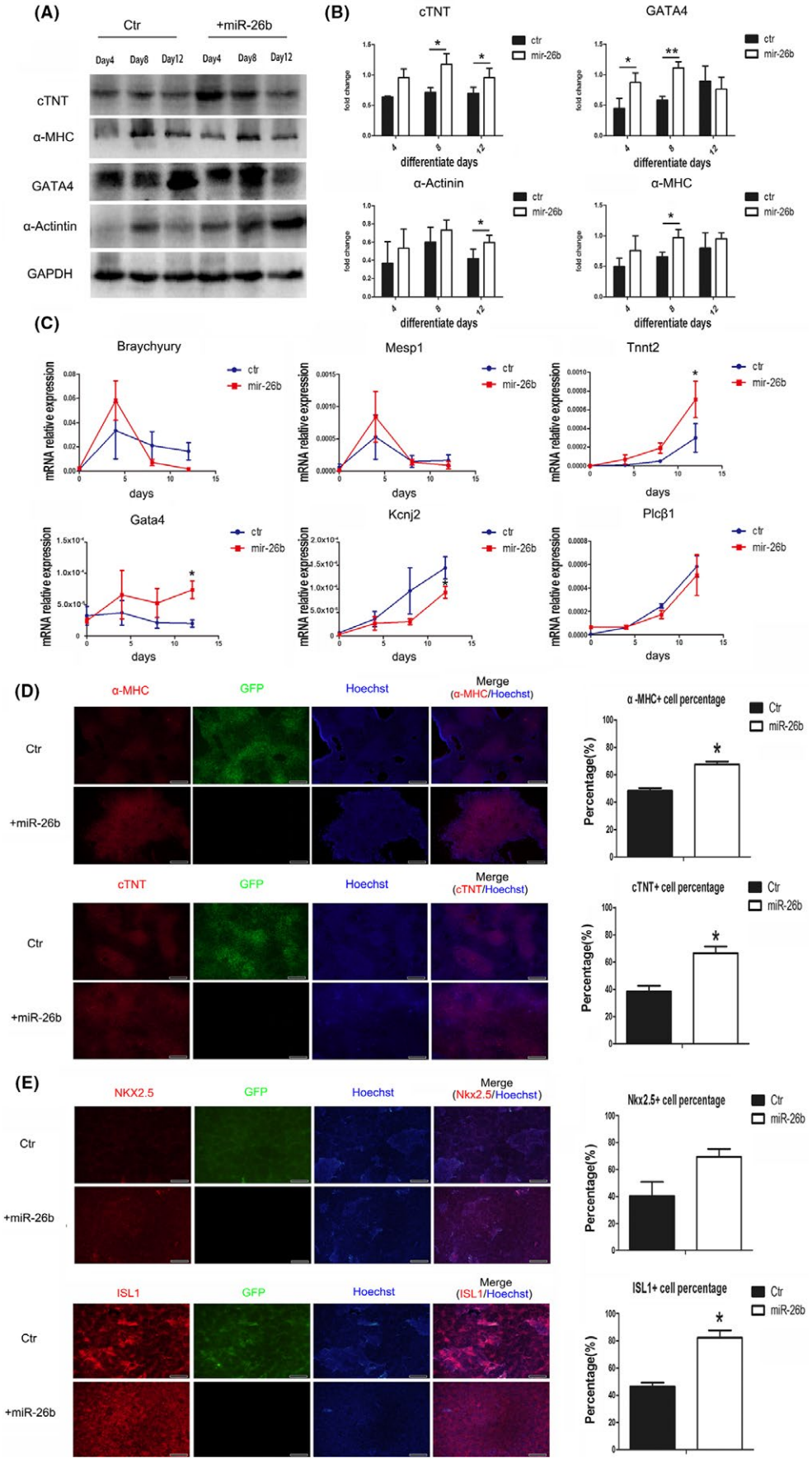


FIGURE 3 Expression of Wnt5a, GSK3β and β-catenin. (A)The mRNA expression of Wnt5a and GSK3β at differentiation day 0 and day 4. (B) β-catenin protein levels treated with miR-26b inhibitor. The right panel is quantification of the western blot results. (C)The protein expression levels of Wnt5a, GSK3β and β-catenin at differentiation day 0 and day 4. (D)The quantitative of the Figure C. **P* < .05, ***P* < .01, ****P* < .001

miR-26b were used to analyse Cyclin D1 levels at varying periods. As shown in Figure 6A,C, Cyclin D1 expression was markedly increased approximately 1/3 fold compared to controls at both starting point and day 4 after differentiation. To confirm whether β-catenin regulates Cyclin D1 in the process of cardiomyocyte differentiation, ICG-001 was applied in the miR-26b overexpressed P19 cells. We found the number of P19 cells was decreased with downregulation of CCK-8 at differentiation day4 (Figure 6D,E). Importantly, the upregulation of Cyclin D1 induced by miR-26b overexpression in P19 cells could be inhibited by ICG-001, even showing a downward tendency

FIGURE 4 The expression status of cardiomyocyte differentiation-related genes. (A) The protein levels of cardiomyocyte-related genes during cardiomyocyte differentiation. (B) The quantitative summary of the Figure A. (C) The mRNA levels of cardiomyocyte-related genes during cardiomyocyte differentiation. (D) Immunofluorescence results of cTNT and α-MHC in the cardiomyocyte derived from P19 at day 12 (Scale bar: 500 μm). The right panel shows quantification of the immunofluorescence results. (E) Immunofluorescence results of Nkx2.5 and Isl1 in the cardiomyocyte derived from P19 at day 8 (Scale bar: 500 μm). The right panel is quantification of the Immunofluorescence results. **P* < .05, ***P* < .01


(A)
Ctr
+miR-26b
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Day8
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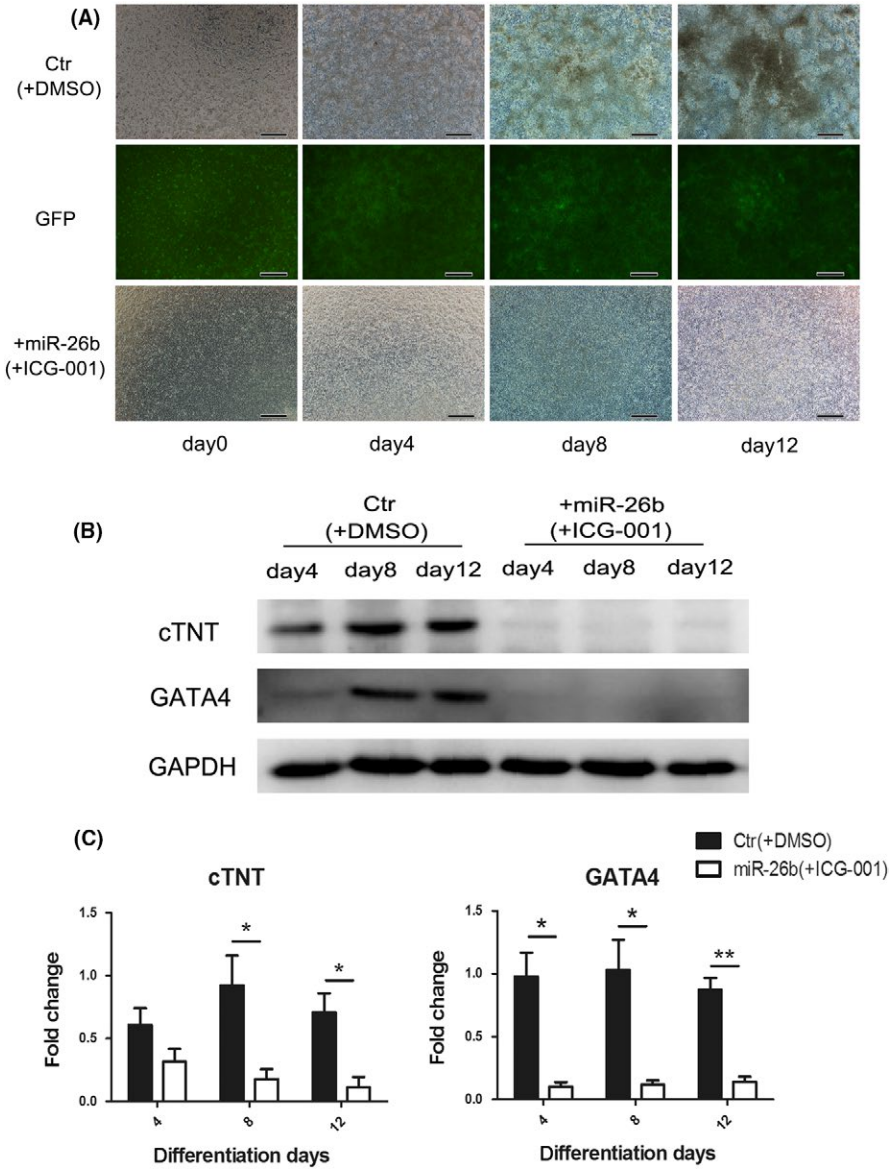

FIGURE 5 Effect of β-catenin Inhibition on the cardiomyocyte differentiation from miR-26b overexpressed P19 cells. (A) The cellular morphology of miR-26b overexpressed P19 cells treated with ICG-001 (Scale bar: 500 μm). (B) The expression levels of cTNT and GATA4 during the cardiomyocyte differentiation. (C) The quantification of the (B). $^{*}P < .05$, $^{**}P < .01$

(Figure 6F,G). These results confirm that the rapid proliferation of P19 cells with the overexpression of miR-26b is associated with early differentiation of cardiomyocytes due to the regulation of Cyclin D1 which is modulated directly by GSK3β.[36]

3.7 | Inhibition of miR-26b suppresses Wnt signalling during differentiation

To elucidate the mediation of Wnt pathway in miR-26b effect on regulation of cardiomyocyte differentiation, P19 cells with miR-26b inhibitor were performed to differentiation. This result shows an opposing trend at the levels of β-catenin, a Wnt signalling regulator, and GATA4, a Wnt-regulated transcription factor, compared with miR-26b-overexpression P19 cells (Figure 4A,B). The expression of β-catenin and GATA4 was overtly downregulated at the mesoderm stage (Figure 7A,C,D). These results further confirm that miR-26b can mediate Wnt signalling during the cardiomyocyte differentiation. Although β-catenin and GATA4 was suppressed when miR-26b was inhibited, the mature cardiomyocyte marker cTNT was not obviously affected (Figure 7A,B), suggesting the involved mechanism is complicated course, and needs to be cleared in future.

4 | DISCUSSION

MiRNAs have been shown as critical regulators in cardiomyocytes for differentiation, proliferation, beating rhythm, hypertrophy and apoptosis.[37,38] Using expression profiling strategies, studies have identified that the pattern of miR-1, miR-133, miR-208 and miR-499 strongly correspond with cardiac differentiation from stem cells.[39] Importantly, the combination of these miRNAs introduced with a lentivirus into the infarcted myocardium in mice really leads to direct reprogramming of non-cardiac myocytes to cardiac myocytes and improve cardiac function.[40] Whereas, deletion of miR-133

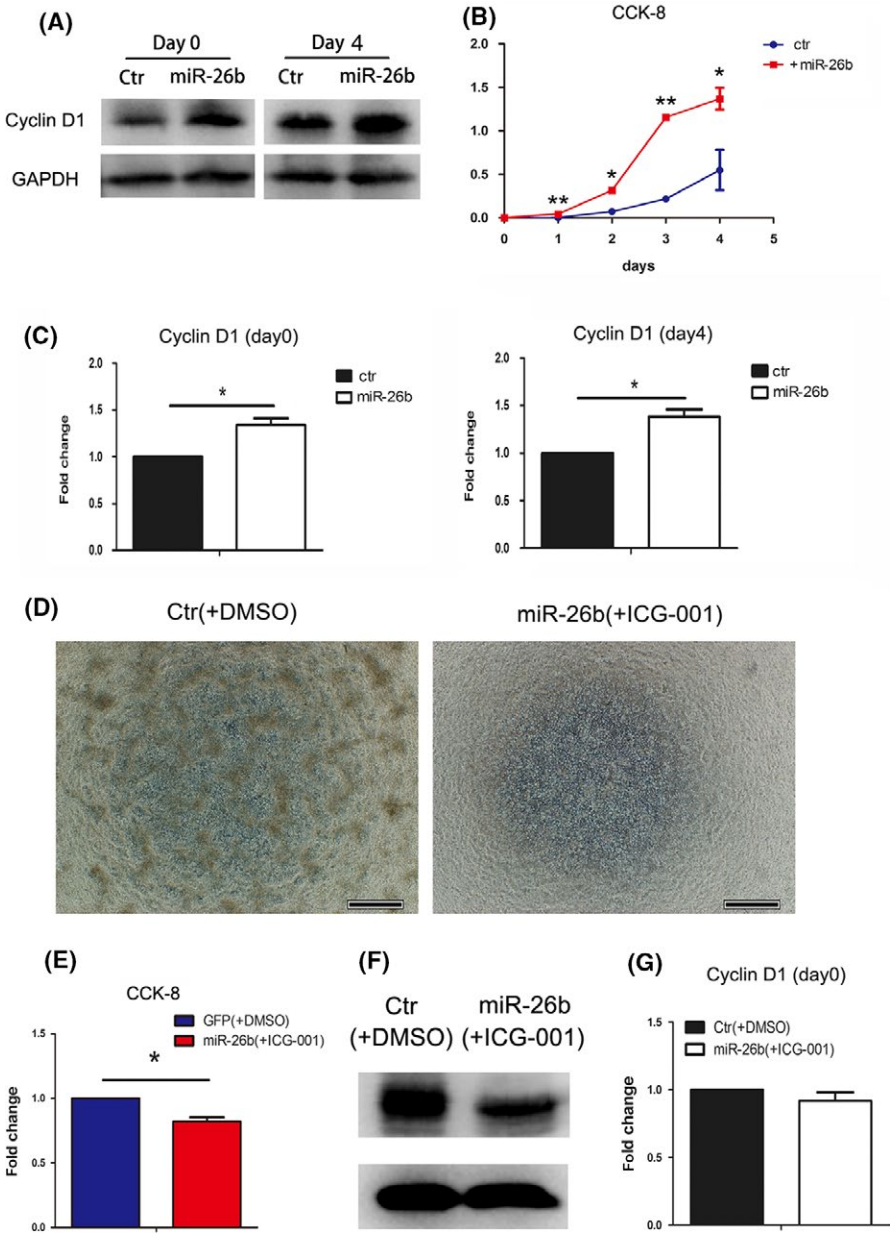

FIGURE 6 Effects of miR-26b on p19 proliferation. (A) Cyclin D1 protein level changes at day 0 and day 4. (B) The CCK-8 assay results for cell proliferation. (C) The quantification of the (A). (D) The cellular morphology treated with ICG-001 at differentiation day4 (Scale bar: 500 μm). (E) The CCK-8 assay results for cell proliferation in presence of ICG-001 at differentiation day4. (F) Cyclin D1 protein levels at day 4. (G) The quantification of the (E).*P < .05, **P < .01

causes ventricular-septal defects in mice with dilated cardiomyopathy and heart failure.[41] Recently, the potential of miRNAs has been investigated as developmental cues to promote the differentiation of stem cell derivatives.[42-44] For example, expression of miRNAs, including miR-1, miR-133a/b, miR-208a/b, miR-143, miR-145 and let-7 miRNA family, dramatically increase during a period of cardiomyocyte differentiation from hiPSC. The underlying mechanism of miR-1-mediated differentiation might implicate suppression of Wnt proteins and fibroblast growth factor (FGF) signalling pathways.[20] Here, we found that miR-26b was robustly elevated corresponding with the cardiomyocyte differentiation of both mES cells and P19 cells, suggesting miR-26b might modulate cardiomyocyte commitment from pluripotent cells. Herein, we generated the P19 stable cell line to express high levels of miR-26b to begin to explore how miR-26b can induce cardiomyocyte differentiation using the in vitro cell differentiation model.

MiR-26b has been identified as an important regulator in carcinogenesis and in other biological processes, including proliferation, angiogenesis, apoptosis and inflammation.[45,46] Interestingly, the apoptosis of RAFLS induced by miR-26b is attributed to the upregulation of β-catenin through targeting Gsk3β, which activates the Wnt signalling pathway.[24] In cardiac hypertrophy, Gata4 has been showed to be upregulated with a decrease of miR-26b level, while with the pathogenesis of pulmonary arterial hypertension, increased levels of miR-26b are involved in the activation of Wnt/β-catenin.[22] Collectively, these results suggest that miR-26b has a demonstrated role in maintenance of heart function by regulating Wnt pathway. Wnt signalling has been identified to be critical in cardiovascular differentiation, cell proliferation and carcinogenesis.[47] The inhibition of GSK3β by Wnts 1, 3A and 8 promotes nuclear localization of β-catenin, which appears to enhance cardiac differentiation at early stage, whereas the non-canonical Wnt appears to enhance cardiac

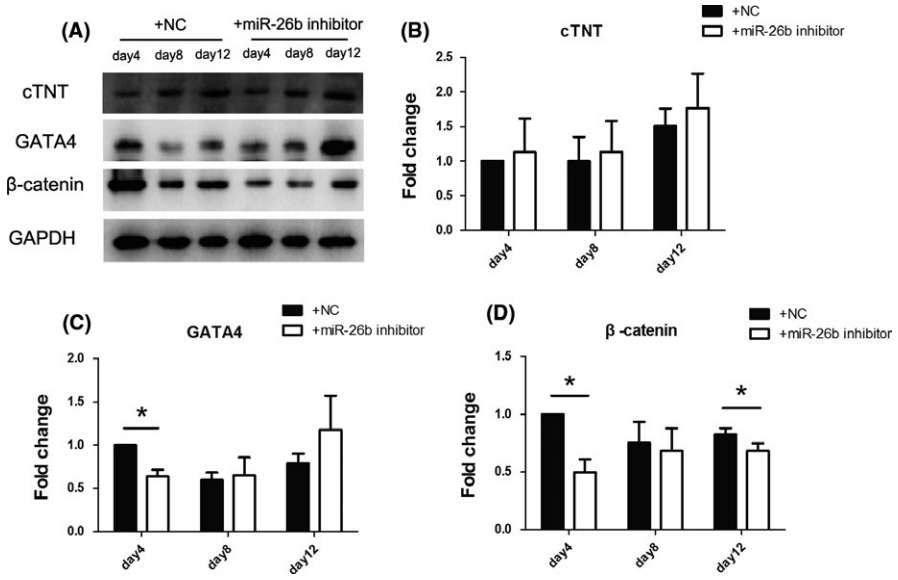

FIGURE 7 Inhibition of miR-26b on cardiomyocyte differentiation. (A) The protein levels of cardiomyocyte related genes during cardiomyocyte differentiation when miR-26b was inhibited. Panels (B), (C) and (D) are quantification of (A), respectively, $^{*}P < .05$

differentiation following.[48,49] In the present study, luciferase assays confirm that miR-26b directly regulates both Gsk3β and Wnt5a. Given the features of miRNAs, generally, as negative regulators for their targets, overexpression of miR-26b indeed inhibits Gsk3β and Wnt5a, which results in Gsk3β-mediated upregulation of β-catenin with activation of the canonical Wnt signalling and blocks the non-canonical Wnt pathway. The effects of canonical and non-canonical Wnt signalling in development exhibit temporal and spatial specificity, however, how the canonical and non-canonical Wnt signalling are regulated in coordination at different stages of differentiation is unclear. Our results predict a potential mechanism whereby microRNAs fine-tune the canonical/non-canonical Wnt signalling pathway during cardiomyocyte differentiation. We demonstrate that the effect of miR-26b on the regulation of canonical and non-canonical Wnt activities cooperatively contributes to the activation of Gata4 and in turn induces P19 cells differentiate into cardiomyocyte. On the other hand, cardiac development is also regarded as a period of cardiomyocyte proliferation.[50] MiRNAs have been shown to participate in cardiomyocyte proliferation in various aspects of cell cycle. MiR-26b was characterized as a negative regulator of proliferation, angiogenesis, and apoptosis in hepatocellular carcinoma.[45] Importantly, miR-26b can also regulate human umbilical cord-derived mesenchymal stem cell proliferation by targeting oestrogen receptor.[51] The present result uncovered that miR-26b could allow P19 cell proliferation via targeting Cyclin D1 mediated by Gsk3β, appearing to promote differentiation. Similarly, miR-26b was also demonstrated to promote differentiation partly by targeting Cyclin D2 which involves cell proliferation by arresting the G1/S transition in human preadipocytes.[52] Taken together, these results indicate that miR-26b participates in cardiomyocyte differentiation of P19 cells by modulating Wnt pathways signalling through targeting both Gsk3β and Wnt5a.

Through the differentiation of P19 cells, the cardiac-like cells exhibit features of synchronized cell twitching, a functional marker of cardiomyocytes in vitro. Previous studies have indicated that miR-26b can target Kcnj2, encoding KIR2.1 potassium channel that has an important role in maintaining action potential in cardiomyocytes through negative control. Likewise, our research also indicated that Kcnj2 was downregulated in the cardiomyocyte derived from the overexpressed miR-26b P19 cells. Moreover, the beating cells were accordingly induced to increase in presence of high-level miR-26b.

In summary, we find that miR-26b expression could be increased with the differentiation of pluripotent cells. The high level of miR-26b in the embryonal carcinoma P19 cell model plays a role in the promotion of cardiomyocyte differentiation, involving the mechanism in which miR-26b activates canonical pathway by inhibiting Gsk3β and also inhibits non-canonical Wnt signalling via blocking Wnt5a. The expression of cardiac-related genes, includingGata4, cTNT, α-MHC and α-Actinin, could be accelerated with the expression status of miR-26b during cardiomyocyte differentiation of P19 cells. Moreover, upregulation of miR-26b and Cyclin D1 promotes P19 cell proliferation, which in turn facilitates the differentiation process. These results show miR-26b as a potential player to balance the canonical and non-canonical Wnt pathways in cardiomyocyte differentiation.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

L.Peng and L.Li conceived the research; L.Peng and D.Wang designed the experiment; D.Wang performed major of the experiments. C.Liu constructed the lentivirus vector and established the miR-26b-overexpression P19 cell line; D.Wang, Y.Wang, W.Wang K.Wang analysed the data; X.Wu, Z.Li and C.Zhao contributed reagents; all authors discussed the manuscript; D.Wang wrote the manuscript; L.Peng and L.Li revised the manuscript, and supervised the project.

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SUPPORTING INFORMATION

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