**Abstract**

**Background:** WNT and TGFβ signaling pathways play critical regulatory roles in cardiomyocyte fate determination and differentiation. MiRNAs are also known to regulate different biological processes and signaling pathways. Here, we intended to find candidate miRNAs that are involved in cardiac differentiation through regulation of WNT and TGFβ signaling pathways.

**Methods:** Bioinformatics analysis suggested hsa-miR-335-3p and hsa-miR-335-5p as regulators of cardiac differentiation. Then, RT-qPCR, dual luciferase, TOP/FOP flash, and western blot analyses were done to confirm the hypothesis.

**Results:** Human embryonic stem cells (hESCs) were differentiated into beating cardiomyocytes, and these miRNAs showed significant expression during the differentiation process. Gain and loss of function of miR-335-3p and miR-335-5p resulted in BRACHYURY, GATA4, and NKX2-5 (cardiac differentiation markers) expression alteration during the course of hESC cardiac differentiation. The overexpression of miR-335-3p and miR-335-5p also led to upregulation of CNX43 and TNNT2 expression, respectively. Our results suggest that this might be mediated through enhancement of WNT and TGFβ signaling pathways.

**Conclusion:** Overall, we show that miR-335-3p/5p upregulates cardiac mesoderm (BRACHYURY) and cardiac progenitor cell (GATA4 and NKX2-5) markers, which are potentially mediated through activation of WNT and TGFβ signaling pathways. Our findings suggest miR-335-3p/5p to be considered as a regulator of the cardiac differentiation process.

**Keywords:** miRNA, Cardiomyocyte differentiation, hESC, WNT, TGFβ

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**Introduction**

Human embryonic stem cells (hESCs) are pluripotent cells derived from the inner cell mass of blastocysts. These cells have self-renewal capacity and are able to differentiate into all derivatives of three germ layers [1]. The first lineage decision is chosen following gastrulation in which cells differentiate into the mesoderm, endoderm, and ectoderm [2]. Differentiation of hESCs is an ideal model for cardiogenesis research as these cells mimic the behavior of cells at early stages of embryonic development [3, 4]. Proper differentiation occurs by precise regulation of different transcription networks, signaling pathways, and epigenetic modifications [5, 6]. WNT/β catenin and TGFβ signaling pathways are known as two main and critical signaling pathways that regulate different stages of cardiac differentiation including mesendoderm cell commitment, cardiac progenitor differentiation, and final maturation [7–10]. Numerous factors tightly orchestrate these signaling pathways during differentiation [11]. MicroRNAs (miRNAs) are small (22 to 25 nucleotide in length) non-coding RNAs [12] that regulate gene expression and play important roles in proliferation, differentiation, cell fate decision, and various physiological processes [13–15]. Accumulating evidence has shown that miRNAs regulate signaling pathways related to cardiac differentiation at
posttranscriptional and posttranslational levels [16, 17]. For instance, miR-15, miR-16 [18], and miR-430 [19] are identified as regulators of cell fate acquisition through targeting the TGFβ signaling pathway. miR-1 and miR-133 promote mesoderm formation [20] and miR-499 promotes CPC differentiation into cardiomyocytes [21]. Here, we screen miRNAs that might be involved in cardiac differentiation through regulation of WNT/β catenin and TGFβ signaling pathways. Bioinformatics analyses indicate that miR-335-3p and miR-335-5p might regulate these two signaling pathways through targeting core members of the pathways. Gain- and loss-of-function studies were performed to verify the exact role of these two miRNAs in cardiac differentiation. Our findings demonstrate that these two miRNAs might regulate cardiac differentiation by activating WNT and TGFβ signaling pathways. This activation led to enhanced mesoderm cell commitment and promoted cardiac progenitor cell differentiation.

Materials and methods

Cell culture and differentiation

HEK293 and SW480 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco), supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) (Gibco). Cells were grown at 37 °C in a humidified atmosphere with 5% CO2. The hESC line RH5 [22] was expanded under feeder-free conditions on Matrigel-coated plates. Cardiomyocyte differentiation occurred in a chemically defined medium, as previously described [23, 24] with minor modifications. Cells were stimulated with 20 ng/mL fibroblast growth factor 2 (FGF2), 20 ng/mL activin A, and 10 ng/mL BMP4 in the first 36 h for mesoderm induction; then, cells were treated with 20 ng/mL FGF2, 50 ng/mL BMP4, 0.5 mM retinoic acid, and 5 mM WNT inhibitor (IWP2) from day 1.5 to day 5. Finally, cells were treated with 5 ng/mL FGF2 and 10 ng/mL BMP4 which resulted in cardiomyocyte differentiation. Samples were collected at different time points (0, 0.5, 1, 1.5, 2, 5, and 12 days) of differentiation for expression analysis.

Transfection of hESCs

Gain- and loss-of-function studies were done in (day 0) D0 of differentiation. The miRCURY LNA™ microRNA mimic (Exiqon, Denmark) for miR-335-3p (MIMAT0004703), miR-335-5p (MIMAT000765), and mimic control as well as miRIDIAN microRNA miR-335-3p and miR-335-5p hairpin inhibitors and miRIDIAN microRNA hairpin inhibitor control (Dharmacon) were used for gain- and loss-of-function studies in which 8 x 10⁵ cells were plated in each 3.5-cm tissue culture dish, 24 h before transfection. When cells reached 80% confluence, they were transfected by 50 nM siRNA or 5 nM mimic structures using Lipofectamine® 3000 reagent, based on the manufacturer’s instructions. The efficiency of siRNAs and microRNA mimics transfection was evaluated using BLOCK-iT Alexa Fluor Red fluorescent oligo (Invitrogen).

RNA extraction and quantitative RT-PCR

Total RNA of harvested cells was extracted using TRIzol® reagent (Invitrogen, USA) according to the manufacturer’s protocol. The total RNA was used for cDNA synthesis after being treated with RNase-free DNase (Takara, Japan) in order to remove any DNA contamination. cDNAs were synthesized using RevertAid™ Reverse Transcriptase (Fermentase, Lithuania) according to the manufacturer’s instructions. For miRNA detection, polyA tail was added to 3’ end of RNAs before cDNA synthesis. RT-qPCR was performed using specific primers (Table S1) by StepOne Real-Time PCR system (Applied Biosystems). GAPDH and small nucleolar RNA, C/D box 48 (SNORD48) were used as internal controls for normalization of mRNAs and miRNA expression.

Immunocytochemistry (ICC)

The seeded cells were washed once with phosphate buffered saline (PBS) and fixed with 4% (w/v) paraformaldehyde (PFA) for 15 min at room temperature. The cells were permeabilized using PBS containing 0.2% Triton X-100 for 10 min followed by blocking in a solution containing PBS and 10% Donkey serum for 1 h at room temperature. Next, cells were treated with primary antibody overnight at 4 °C. After three washes in PBS, cells were incubated with secondary antibody for 1 h at room temperature in the dark. Finally, cells were incubated with DAPI for nucleic acid staining and imaged with a fluorescent microscope (IX71, Olympus, Japan). Image overlays and contrast enhancement were performed using ImageJ software.

Dual luciferase assay

Dual luciferase assay was utilized to validate the direct interaction between miR-335-3p and miR-335-5p and their target genes 3’-UTRs. To this aim, HEK293 cells were co-transfected with a psiCHECK-2 vectors including 3’-UTR of APC, AXIN-I, and SMAD7 and miR-335-3p, miR-335-5p mimics, and siRNA structures in 48-well plates. As negative controls, mimic and siRNA scramble were used in co-transfection. Luciferase activity was measured 48 h after transfection using the Dual Luciferase Reporter Assay System (Promega, USA) according to the manufacturer’s instructions.

TOP/FOP reporter assays

TOP/FOP reporter assays were carried out using the Dual-Glo luciferase assay kit (Promega), based on the
manufacturer’s instructions. SW480 cells were transfected with 1μg of constitutively active vector encoding Renilla luciferase, responsive firefly luciferase reporter plasmid Top Flash, each mimic and siRNA corresponding to the miR-335-3p, miR-335-5p and their related scrambled. Cells were harvested after 48 h, and both firefly and Renilla luciferase activity were measured in three biological replicates according to the manufacturer’s instructions. The firefly luciferase activity was normalized against Renilla luciferase activity.

**Western blotting**

The protein was extracted from the samples. Samples containing 40 μg purified protein were separated by 12% SDS/PAGE, transferred to PVDF membranes (Santa Cruz), and run at 100 V for 1.5 h at room temperature. The PVDF membrane was subsequently blocked by 5% BSA in phosphate-buffered saline (PBS) containing 0.1% Tween for 1 h at room temperature, followed by overnight incubation at 4°C with phospho BRACHYURY primary antibodies (1:500, Cell Signaling). The blot membrane was washed and incubated with anti-rabbit secondary antibody (1:1000, Santa Cruz) for 1 h at room temperature. The protein bands were visualized by an enhanced chemiluminescence (ECL) detection system (Amersham, Piscataway, NJ). The membranes were stripped and re-probed with β-actin for verification of protein loading. The bands were quantified using an image analyzer program (ImageJ).

**Results**

**Bioinformatics analyses introduced miR-335 as a potential regulator of cardiac differentiation**

WNT and TGFβ signaling pathways are known as main players in cardiac differentiation. In this study, we aimed to find out miRNAs that are crucially important for driving cardiac differentiation through regulation of WNT and TGFβ signaling pathways. To this aim, a four-step filtering approach was performed to nominate some miRNAs (Fig. 2a). At the first step, 973 miRNAs were predicted to target WNT and TGFβ signaling pathways using miRWalk, a target prediction resource that uses several miRNA target prediction tools. Then, considering the number of genes which are targeted by a miRNA in each pathway, the numbers of MREs (miRNA recognition elements) in 3′UTR sequences of each target gene, and the annealing and conservation status of each MRE, seven candidate miRNAs were chosen for further analyses (Additional file 1: Table S2). Among candidate miRNAs, miR-335-3p/5p hosted by mesoderm-specific transcript (MEST) gene were chosen for further investigations concerning its role in cardiogenesis.

**Differentiation of hESCs into cardiomyocytes**

In vitro differentiation of hESC, RH5, to cardiomyocyte-like cells was successfully performed (Fig. 1a). Different time points representing different cell fates during cardiac differentiation were considered (i.e., pluripotent stem cells (day 0), mesendoderm (day 1.5), cardiac progenitor (day 5), and cardiomyocyte cells (day 12)). NANOG stem cell marker expression was substantially decreased while the BRACHYURY (mesendodermal marker) expression level reached its highest level in mesendoderm stage (day 1.5) (Fig. 1b). In addition, NKX2-5 and ISL1 (early cardiac differentiation markers) expression levels elevated from day 2 and substantially increased during the process. As expected, cardiac-specific markers (TNNT and MYH6) were significantly increased at the final stage (day 12) (Fig. 1b), followed by spontaneous beating of the cardiac cells (Additional file 2: Movie S1). The flow cytometry analysis indicated that ~51% of the cells were BRACHYURY positive on day 1.5, ~87% of the cells were NKX2-5 positive on day 5 (cardiac progenitor cells) and ~96% of the cells were MYH6 positive on day 12 (the final step of cardiomyocyte differentiation) (Fig. 1c). Furthermore, immunostaining indicated that hESCs were positive for OCT4, whereas hESC-CMs stained positive for cardiac-specific markers MYH6 (Fig. 1d), approving that these cells were successfully differentiated into cardiomyocytes.

**MiR-335 expression pattern during the human cardiac differentiation process**

MiR-335-3p and miR-335-5p expression status was measured at seven time points of human cardiac differentiation process, using RT-qPCR. Both miR-335-3p and miR-335-5p had relatively high levels of expression during this process (data not shown); however, their expression pattern was different. While miR-335-3p was transiently upregulated at day 1 and substantially downregulated to the end of differentiation process, miR-335-5p showed no significant alteration during the differentiation (Fig. 2b).

**The effect of MiR-335 expression alteration on cardiac differentiation**

In order to examine the effect of miR-335-3p/5p expression alteration on cardiac differentiation, gain- and loss-of-function study was performed according to miR-335-3p and miR-335-5p expression profiles (Fig. 2c). In this part, miR-335-3p and miR-335-5p mimics or their corresponding siRNAs were transfected into differentiating hESCs on D0 to induce specific overexpression and downregulation, respectively. Red fluorescent oligo transfection indicated ~45% transfection efficiency (Fig. 2d), and specific up- and downregulation of each miRNA was further confirmed using RT-qPCR, 24 and
Fig. 1 (See legend on next page.)
Fig. 1 Characterization of hESC-derived cardiomyocyte.

a Schematic description of cardiomyocyte differentiation protocol. Activin A and BMP4 compounds were used to induce differentiation of hESCs into mesoderm. Then, WNT inhibitor reagent (IWP2) was used to induce cardiac progenitor cell development followed by cardiomyocyte formation. 

b Time-dependent expression of NANO <sub>G</sub> (pluripotency marker), BRACHYURY (mesoderm marker), NKX2-5, ISL1 (cardiac progenitor cell markers), TNNT2, and MYH6 (cardiomyocyte markers) during the cardiac differentiation process. RT-qPCR data are presented as mean ± SEM normalized against day 0 data, for n = 3 independent experiments. GAPDH was used as a housekeeping gene. 

c Flow cytometry results confirmed the expression of BRACHYURY (day 1.5), NKX2-5 (day 5), and MYH6 (day 12) during the differentiation process. 

d Immunocytochemistry analysis of OCT4 (pluripotent hESCs) and MYH6 (cardiomyocytes) revealed the stemness potency of hESCs and successful differentiation of cardiomyocyte. Differentiated cardiomyocytes are shown in two magnitudes.

Fig. 2 MiR-335 expression alteration during cardiac differentiation.

a Schematic presentation of different stages of miRNA screening. 

b miR-335-3p (top) and miR-335-5p (bottom) expression pattern during hESC cardiac differentiation. 

c Gain- and loss-of-function strategy in which miR-335-specific mimics and siRNAs were transfected on day 0 of differentiation process. 

d Flow cytometry results showed that hESCs were efficiently transfected by miR-335 specific mimics or siRNAs along with 50 nM BLOCK-IT Alexa Fluor Red Fluorescent oligonucleotide. 

e The RT-qPCR confirmed significant (P value < 0.05) overexpression of miR-335-3p (top-left) and miR-335-5p (bottom-left) but significant downregulation of miR-335-3p (top-right) and miR-335-5p (bottom-right), 24 and 48 h after transfection. RT-qPCR data are presented as mean ± SEM normalized against day 0 data. GAPDH was used as a housekeeping gene.
48 h post transfection (Fig. 2e). Immunostaining of the cells transfected by mimic-scr, mimic-3p, or mimic-5p indicated successful cardiac differentiation (Fig. 3a). To determine the differentiation stage which has been most affected by miR-335 expression alteration, the expression level of stage-specific markers was measured by RT-qPCR. Here, the expressions of BRACHYURY and MESP1 as endodermal markers, NKK2-5 and GATA4 as cardiac progenitor markers, HCN4 and ISL1 as progenitor markers of the first and second heart field, and TNNT2 and CNX43 as cardiomyocyte markers were analyzed. RT-qPCR data indicated that 48 h post-transfection of mimic-scr, mimic-3p, or mimic-5p, the expression level of BRACHYURY was significantly increased (Fig. 3b). Consistently, downregulation of miR-335-3p was followed by significant reduction of BRACHYURY expression (Fig. 3b). The results of western blotting also confirmed the increased protein level of BRACHYURY following mimic-3p and mimic-5p treatments compared to the mimic-scr control (Fig. 3b, right). Increased or decreased level of miR-335 did not show a significant effect on MESP1 expression level (Fig. 3c). Interestingly, GATA4 and NKK2-5 expression levels were significantly increased following miR-335-3p overexpression while they decreased after using their related siRNA (Fig. 3d, e). Similar significant results were obtained for miR-335-5p against GATA4, but results were non-significant against NKK2-5 expression (Fig. 3d, e).

Following transfection of mimic-3p and mimic-5p, HCN4 (the first heart field marker) was significantly upregulated as well; however, miR-335-3p and miR-335-5p downregulation results were not consistent with it (Fig. 3f). MiR-335 expression alteration also has effect on ISL1 (the second heart field marker) expression. Transfection of both mimic-3p and mimic-5p resulted in downregulation of ISL1 expression while, miR-335 downregulation resulted in increased ISL1 expression (Fig. 3g). Overexpression of miR-335-3p significantly upregulated CNX43 expression but suppressed the expression of TNNT2 at the final stage of the differentiation process (Fig. 3h, i). Interestingly, miR-335-5p overexpression could significantly upregulate TNNT2 with no significant effects on CNX43 expression.

**MiR-335 as an activator of WNT signaling pathway**

Target prediction tools predicted 5 and 1 potential target sites for miR-335-3p within the 3′ UTR sequences of APC and AXIN-I genes, respectively. For miR-335-5p, a single recognition site was predicted within 3′ UTR sequences of both APC and AXIN-I genes (Fig. 4a). Alterations in the expression of APC and AXIN-I target genes were investigated 48 h post-transfection of miR-335 mimics or siRNAs on hESC differentiation. Also, direct interaction of each miRNA mimic and 3′ UTR sequences of APC and AXIN-I predicted target genes was investigated in HEK293 cells, using dual luciferase reporter assay.

RT-qPCR indicated that up- or downregulation of both miR-335-3p (Fig. 4b, top) and miR-335-5p (Fig. 4c, top) did not significantly affect APC gene expression in differentiating cells. Co-transfection of reporter construct containing APC-3′ UTR and miR-335-3p mimic in HEK293 cells resulted in a significant reduction of luciferase activity, and consistently, luciferase activity was increased after the application of siRNA against miR-335-3p, compared to scrambled controls (Fig. 4b, bottom). This effect was abrogated using off target UTR (the same length of UTR with no available target site for miRNA), thereby approving the direct interaction between miR-335-3p and APC 3′ UTR. Similar dual luciferase assay results indicated that miR-335-5p was not interacting with APC 3′ UTR sequence (Fig. 4c, bottom).

Transfection of differentiating hESCs with miR-335-3p mimic resulted in significant downregulation of AXIN-I expression, compared to scrambled control transfection (Fig. 4d, top). Furthermore, dual luciferase assay confirmed the direct interaction between miR-335-3p mimic and 3′ UTR sequence of AXIN-I (Fig. 4d, bottom). Transfection with miR-335-5p mimic also caused a significant reduction in AXIN-I transcripts as examined by RT-qPCR (Fig. 4e, top). However, dual luciferase assay did not show a direct interaction between miR-335-3p and 3′ UTR sequence of AXIN-I gene (Fig. 4e, bottom).

TOP/FOP flash assay indicated that overexpression of miR-335-3p or miR-335-5p led to significant upregulation of WNT signaling pathway in SW480 cells, compared to mimic-scr controls (Fig. 4f). Similar results to those of Top/Fop controls were observed when miR-335 was downregulated by specific siRNA (Fig. 4f). The RT-qPCR analysis against CYCLIND1 and c-MYC (downstream targets of WNT signaling pathway) also confirmed the upregulation of WNT signaling, following miR-335-5p overexpression (Additional file 1: Figure S1). Alterations in miR-335-3p expression did not significantly affect CYCLIND1 and c-MYC expression (Additional file 1: Figure S1). Altogether, these data suggested that miR-335-3p might enhance the WNT signaling through targeting the inhibitory components of WNT signaling pathway (i.e., APC and AXIN-I) while miR-335-5p could activate this pathway indirectly without any direct effect on these two target genes.

**MiR-335 as an inducer of TGFβ signaling pathway**

The in silico analyses suggested that miR-335 could regulate TGFβ signaling through targeting SMAD7 transcripts. It was predicted that both miR-335-3p and miR-335-5p target inhibitory SMAD7 gene (Additional file 1: Figure S2A). RT-qPCR indicated that SMAD7 expression was
MiR-335 regulates earlier stages of hESC cardiac differentiation. a Immunocytochemistry analysis showed that overexpression of hsa-miR335-3p or hsa-miR335-5p on day 0 did not affect the final outcome of cardiac differentiation on day 12 (cardiomyocytes), as detected by MYH6 expression in all groups. b Overexpression of both hsa-miR335-3p (top) and hsa-miR335-5p (bottom) resulted in significant elevation of BRACHYURY (mesoderm marker) expression. Consistently, hsa-miR335-3p downregulation resulted in reduced expression of BRACHYURY. Western blot results indicate an increased level of BRACHYURY following the mimic-3p and mimic-5p treatment, compared to mimic-scr control (right). c No significant expression alteration was detected for MESP1 following alterations in hsa-miR335 expression. d, e Expressions of GATA4 and NKX2-5 (main cardiac progenitor markers) were both increased following miR-335-3p (top) and miR-335-5p (bottom) overexpression. Downregulation of miR-335 had reverse effect on both of these cardiac progenitor markers. f Expression of HCN4 (the first heart field marker) was significantly increased following miR-335-3p (top) and miR-335-5p (bottom) overexpression. g Alterations in the expression of miR-335-3p (top) and miR-335-5p (bottom) had a significant reverse effect on ISL1 (the second heart field marker) expression. h, i Has-miR-335-3p (top) overexpression increased CNX43 expression but decreased TNNT2 expression. However, has-miR-335-5p decreased CNX43 but enhanced TNNT2 expressions. RT-qPCR data are presented as mean ± SEM normalized against mimic-scr and siRNA-scr. GAPDH was used as a housekeeping gene.
significantly reduced following miR-335-3p and miR-335-5p overexpression, 48 h after transfection in differentiating hESCs. Consistently, downregulation of these miRNAs had reverse effects (though non-significant) on SMAD7 expression (Fig. 5a, top).

Luciferase activity was decreased following miR-335-3p overexpression, and this suppression was relieved by downregulation of miR-335-3p as detected by dual luciferase assay (Fig. 5b, top). Significant reductions in luciferase activity were also detected after miR-335-5p overexpression (Fig. 5a, bottom), while miR-335-5p downregulation could not rescue luciferase activity, significantly (Fig. 5b, bottom). Overall, these data suggested that both miR-335-3p and miR-335-5p were capable of targeting SMAD7 transcript. The effect of MiR-335 overexpression on other TGFβ signaling pathway components
was also investigated using RT-qPCR. Results indicated that both miR-335-3p and miR-335-5p overexpression led to TGFβR-I upregulation (Fig. 5c, d). Alterations in the expression of miR-335 had no significant effect on SMAD2 and SMAD3 expression (Additional file 1: Figure S2B-C). Altogether, these data suggest that both miR-335-3p and miR-335-5p might activate TGFβ signaling pathway through targeting SMAD7 transcript.

Discussion

TGFβ and WNT signaling pathways have some pleiotropic and multifunctional effects and regulate several bioprocesses including embryonic development, as well as cell differentiation, proliferation, and survival [25–27]. These two pathways play critical roles in orchestration of cardiac development and differentiation [28–30]. It is known that regulation of TGFβ and WNT signaling pathway is essential for mesoderm cell fate commitment, as well as cardiomyocyte progenitor cell formation and cardiomyocyte maturation [31]. These cardiogenesis steps are closely regulated by different mechanisms at posttranscriptional and posttranslational levels. miRNAs are known to regulate different bioprocesses through targeting related signaling pathways.
Following miR-335-3p or miR-335-5p gain- and loss-of-functions, cardiac progenitor markers (GATA4 and NKK2-5) were also up- and down-regulated, respectively (Fig. 3d, e). Upregulation of GATA4 and NKK2-5 progenitor markers could enhance the first heart field progenitor cell commitment through upregulating HCN4 (Fig. 3f) and downregulating ISL1 expression (Fig. 3g). RT-qPCR results against late cardiac differentiation markers (TNNT2 and CNX43) suggest that miR-335-3p and miR-335-5p might have complementary effects on the process.

While miR-335-3p downregulated TNNT2 expression, miR-335-5p significantly upregulated it within the cells (Fig. 3i). Similar results were also obtained for CNX43 expression after alterations in miR-335-3p and miR-335-5p expression (Fig. 3h). Altogether, these data suggest that miR-335 enhances cardiac differentiation through upregulation of the expression of mesoderm (BRACHYURY) and cardiac progenitor marker (GATA4 and NKK2-5) genes. Nevertheless, molecular mechanism(s) via which these miRNAs regulate the expression of these markers remained undiscovered.

Bioinformatics analyses indicated that miR-335 regulates WNT and TGFβ signaling pathways. Moreover, RT-qPCR (Fig. 4b–e, top) and dual luciferase assay (Fig. 4b–e, bottom) showed that miR-335-3p specifically target 3′UTR sequences of APC and AXIN-I, which are two main members of WNT inhibitor complex. These data suggested that miR-335-3p but not miR-335-5p could regulate APC expression at the post-transcriptional level.

Although miR-335-5p had no direct interaction with 3′UTR of these two target genes, but RT-qPCR results indicated a significant downregulation of AXIN-I transcript (Fig. 4e, top). Thus, it could be concluded that miR-335-5p indirectly downregulated AXIN-I expression without interacting with its transcripts. Consistently, both of these miRNAs were capable of activating WNT signaling pathway as detected by Top/Fop flash assay (Fig. 4f). This is also consistent with a previous report which showed that miR-335-5p activates WNT signaling pathway through DKK1 downregulation [40].

Interaction between both miR-335-3p and miR-335-5p and SMAD7 3′UTR was also confirmed by RT-qPCR (Fig. 5a) as well as dual luciferase assay (Fig. 5b). Results indicated that these two miRNAs could enhance TGFβR-I expression (Fig. 5c, d). There are two SMAD-binding elements within TGFβR-I promoter recognized by SMAD7, which inhibit TGFβR-I expression [41]. Downregulation of SMAD7 by these two miRNAs could lessen the inhibitory effect of SMAD7 and enhance TGFβR-I expression. Altogether, the results proposed that miR-335-3p and miR-335-5p could activate WNT and TGFβ signaling pathways.
LEF-1/β-catenin complex is reported to bind to the TCF binding site at the BRACHYURY promoter sequence and enhance its expression [42]. TGFβ1 also induces the expression of BRACHYURY in human carcinoma cells [43]. In other words, activation of WNT and TGFβ signaling pathways could enhance BRACHURY expression, as also observed in the current experiment. Furthermore, WNT signaling pathway activation was shown to enhance GATA4 [44] as well as NMX2-5 expression through hindering HDAC1 inhibitory effect [45]. There is also evidence showing that TGFβ activation enhances GATA4 and NKX2-5 expression [46–48].

**Conclusion**

Based on the bioinformatics and experimental validations, we propose a regulatory network for miR-335-3p and miR-335-5p during cardiomyocyte differentiation (Fig. 6). Accordingly, both miR-335-3p and miR-335-5p might activate WNT and TGFβ signaling pathways that, in turn, induce mesoderm (BRACHYURY) and progenitor (GATA4 and NKX2-5) marker expression and cardiac differentiation.

**Additional files**

**Additional file 1:** Table S1. Primer sequences used in this research. Table S2. Final list of candidate miRNAs. Table S3. List of potential miR-335-3p target genes related to WNT and TGFβ signaling pathways. Table S4. List of potential miR-335-5p target genes related to WNT and TGFβ signaling pathways. Figure S1. RT-qPCR results of C-MYC and CCND1 after mimics and siRNA treatment for miR-335-3p (A) and miR-335-5p (B). All experiments were done in three biological replicates and presented as mean ± SEM. Figure S2. RT-qPCR results of SMAD2 and SMAD3 expression. A) Pairing status of miR-335-3p (left) and miR-335-5p (right), with 3′ UTR of SMAD7 gene. B) SMAD2 expression was not significantly changed followed by miR-335-3p (top) and miR-335-5p (bottom) overexpression. C) RT-qPCR data also showed no significant alterations in SMAD3 expression following miR-335-3p (top) and miR-335-5p (bottom) overexpression. All data are presented as mean ± SEM normalized against mimic-scr and siRNA-scr. GAPDH was used as a housekeeping gene. Figure S3. Genomic location of miR-335 presented in UCSC genome browser. miR-335 is...
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Availability of data and materials. All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Authors' contributions. MK contributed to do experiment design and carried out the experiment, and also contributed in the manuscript preparation. HA and FHA did the experiment. BMS and HB did the experiment design, result interpretation, and manuscript preparation. All authors read and approved the final manuscript.

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