miR-149 promotes the myocardial differentiation of mouse bone marrow stem cells by targeting Dab2

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Abstract. To investigate the role of microRNA (miR)-149 in the cardiac differentiation of mouse bone marrow mesenchymal stem cells (MSCs) *in vitro*, MSCs were infected with a lentivirus overexpressing miR-149 and the effect on cardiac differentiation was determined. The quantitative polymerase chain reaction results demonstrated that miR-149 promoted the expression of cardiac-specific markers in MSCs. Western blotting and a luciferase activity assay demonstrated that disabled homolog 2 (Dab2) was a direct target of miR-149. Dab2 ectopic expression and Wnt/β-catenin signaling pathway inhibition was able to reverse the increased expression of cardiac-specific markers induced by miR-149. In conclusion, miR-149 was able to target Dab2 and promote the cardiac differentiation of mouse MSCs *in vitro*, which depended upon the Wnt/β-catenin signaling pathway.

Introduction

Cardiovascular disease is one of the most harmful diseases to human health in the world, with a high incidence. Although surgery and medication reduce the mortality of patients with cardiovascular disease, numerous patients develop progressive myocardial failure, which may be fatal and is a threat to the quality of life of middle-aged and elderly patients following a heart attack (1,2). In the USA, a total of 20% of patients with heart failure succumb after 1 year, and 50% after 5 years (3). Since adult cardiomyocytes lose their regenerative ability, necrotic cardiomyocytes may only be replaced by fibroblasts to form scar tissue, eventually leading to heart failure (4). A feasible strategy to prevent heart failure is transplantation of exogenous cells into the injured myocardium to produce contractile cells.

A number of types of stem cells have been examined with respect to clinical applications, with the aim of replenishing necrotic cardiomyocytes or providing a more suitable environment for cardiac regeneration (5,6). Among them, mesenchymal stem cells (MSCs) have gained extensive attention due to their high proliferative ability, low immunogenicity and fewer ethical considerations. MSCs may be isolated from various tissues and may differentiate into numerous types of cells, including cardiocytes, bone cells, cartilage cells, adipocytes and neurons (7-9).

There are three methods for inducing bone marrow MSC differentiation into cardiomyocytes *in vitro*: The first is drug-induced differentiation, for example 5-azacytidine (5-aza) (10); the second is coculture with myocardial cells (11); and the third is genetic modification (12). Although the efficiency of differentiation induced by 5-aza requires further improvement, it remains a widely used model to differentiate MSCs into cardiomyocytes (13,14).

MicroRNAs are a type of non-coding RNA molecule of ~22 nucleotides in length. MicroRNAs are involved in a number of physiological processes by binding to the 3'untranslated region (3'UTR) of target genes, and thus promoting mRNA degradation or inhibiting the transcription of target genes (15). microRNAs additionally serve important roles in cardiovascular disease, affecting a number of facets of cardiac remodeling, including stem cell differentiation, apoptosis and cardiac contractility (8).

The present study investigated the role of microRNA-149 (miR-149) in the differentiation of mouse MSCs from bone marrow into cardiocytes, and examined the underlying mechanism and signaling pathway. The present study identified a microRNA, which was able to promote the differentiation of bone marrow MSCs, and lay the foundation for stem cell transplantation to repair myocardial injury.

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Materials and methods

Culture of cells. MSCs were purchased from Cyagen Biosciences, Inc. (Santa Clara, CA, USA; cat. no. MUCMX-01001). MSCs were cultured in DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 20% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.), in a humidified 5% CO_2 air incubator at 37˚C. The MSCs were passaged when they reached 80-90% confluence at 1:3 and used at passage (P)3. 5-aza was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and dissolved in dimethyl sulfoxide (Invitrogen; Thermo Fisher Scientific, Inc.). NIH/3T3 cells and 293T cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified 5% CO_2 air incubator at 37˚C. XAV-939 was purchased from Selleck Chemicals, Houston, TX, USA (cat. no. S1180), at working concentration of 10 nM.

5-aza induction of MSCs. MSCs at P3 were seeded into 6-well plates at a concentration of $5x10^5$ cells/well. At 24 h, MSCs were treated with 5-aza at a final concentration of 10 μM (day 0). The induction medium was changed following 24 h and cells were washed three times with PBS. Cells were cultured in DMEM/F12 containing 10% FBS for a further 6 (day 7) or 20 days (day 21).

Target prediction. Targetscan (http://www.targetscan.org/mamm_31/) and Pictar (http://www.pictar.org/) were used to determine potential target genes of miR-149.

Transfection. miR-149 mimics and mimics control were designed and synthesized by Shanghai Genepharma Co., Ltd. Transfection of NIH/3T3 and 293T cells was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. NIH/3T3 and 293T cells ($2x10^5$) were seeded in 3.5-mm dishes overnight and transfected with 50 pmol miR-149 or mimic control the following day. The medium was changed 6 h post-transfection. The sequence of miR-149 mimics was: UCUGGCUCCGUGUCUUCACUCCC (+) and GAGUGAAGACACGGAGCCAGAUU (-). The sequence of mimics control was: UUCUUCGAACGUGUCACGUTT (+) and ACGUGACACGUUCGGAGAATT (-).

Dual luciferase assay. 293T cells were seeded in 24-well plates at a density of $2x10^4$ cells/well. The 3'UTR of Dab2 containing the binding site of miR-149, was acquired by polymerase chain reaction (PCR) using MSCs and then cloned into pGL3 (Promega Corporation, Madison, WI, USA) with *Xba*I and *Xho*I restriction enzymes (New England BioLabs, Inc., Ipswich, MA, USA). PCR was performed with PrimeSTAR®HS DNA Polymerase (Takara Bio, Inc., Otsu, Japan) using the following thermocycling conditions: 30 cycles of 98˚C for 10 sec, 55˚C for 15 sec and 72˚C for 1 min. The primers used for PCR were as follows: Forward, 5'-GCCCTTTCGGAAATCCTTTTG-3' and reverse 5'-CTGGGAGAGATCACCAGAAT-3'. A plasmid with a mutant miR-149 binding site was acquired using a site-directed mutagenesis kit (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA). Plasmids were transfected into NIH cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The luciferase activity assay was performed using a Promega Dual-Glo® Luciferase Assay System (Promega Corporation), according to the manufacturer's protocol. The luminescence was measured using a Berthold LB9507 luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Relative luciferase activity is presented as the ratio of firefly to *Renilla* luminescence.

RNA isolation and reverse transcription-quantitative (RT-q)PCR. Total RNA from MSCs was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. For miRNA detection, cDNAs were synthesized at 37˚C for 60 min using the miRcute miRNA First-strand cDNA kit (Tiangen Biotech Co., Ltd., Beijing, China). For protein-coding genes, cDNAs were synthesized at 37˚C for 60 min using the Quant Reverse Transcriptase kit (Tiangen Biotech Co., Ltd.). The expression level of miR-149 was detected using miRNA qPCR detection kits (Tiangen Biotech Co., Ltd.) according to the manufacturer's protocol, and was normalized to the expression level of small nuclear RNA RNU6B (U6). For protein-coding genes, data were normalized to the expression level of GAPDH. The relative expression levels of mRNA were calculated using the $2^{-\Delta\Delta Cq}$ method (16). qPCR was performed using the ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBRGreen in a Super Real Pre Mix kit (Tiangen Biotech Co., Ltd.). The products were amplified using the following program: 94˚C for 10 min, followed by 40 cycles of 94˚C for 15 sec and 60˚C for 30 sec. The sequences of the PCR primers are listed in Table I.

Lentiviral infection. The lentiviruses overexpressing miR-149 and disabled homolog 2 (Dab2) were purchased from Shanghai Genepharma Co., Ltd. (Shanghai, China). Lentiviral infection was performed according to the manufacturer's protocol. MSCs ($5x10^5$ cells/well) were seeded into 6-well plates and then incubated at 37˚C with lentivirus overexpressing miR-149 or mock lentivirus (Shanghai Genepharma Co., Ltd.) at a multiplicity of infection (MOI) of 200 for 8 h. For MSCs overexpressing Dab2, the Dab2 lentivirus was added at an MOI of 100 1 day subsequent to the cells being infected with the lentivirus overexpressing miR-149, and the DMEM/F12 medium was changed at 24 h following incubation at 37˚C. Gene expression was separately analyzed using RT-qPCR as previously described on days 3, 7 and 14 post-infection.

Western blotting. Cells were collected in ice-cold PBS 3 days post-infection or transfection, and total proteins were extracted with ice-cold radioimmunoprecipitation assay buffer with protease inhibitors (Roche Applied Science, Penzberg, Germany) and quantified with a bicinchoninic acid kit (Thermo Fisher Scientific, Inc.). Equal amounts of proteins (40 μg) were separated by 10% SDS-PAGE and transferred to

Table I. List of primers for quantitative polymerase chain reaction analysis in the present study.

Gene	Forward (5'→3')	Reverse (5'→3')
Mmu-miR-149	TCTGGCTCCGTGTCTTCACTCCC	universal (provided by kit, sequence unavailable)
U6	CCTGCGCAAGGATGAC	GTGCAGGGTCCGAGGT
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Nkx2.5	CGA CGGAAGCCACGCGTGCT	CCGCTGTCGCTTGCACTTG
GATA4	CCCTACCCAGCCTACATGG	ACATATCGAGATTGGGGTGTCT
cTnI	GTCCTCCTTCTTCACCTGCTTG	CTCTGCCAACTACCGAGCCTAT
CX43	GTGCCGGCTTCACTTTCA	GGAGTAGGCTTGGACCTTGTC

Nkx2.5, homeobox protein Nkx2.5; GATA-4, transcription factor GATA-4; cTnI, cardiac troponin I; CX43, gap junction α-1 protein.

0.45-μm polyvinylidene fluoride membranes. The membranes were blocked with 5% fat-free milk in TBS containing 0.05% Tween-20 at room temperature for 1 h, and probed with the following primary antibodies overnight at 4˚C: Anti-Dab2 (cat. no. ab137866; 1:1,000; Abcam, Cambridge, UK) and anti-β-actin (cat. no. ab227387; 1:3,000; Abcam). Membranes were subsequently washed three times with TBST and incubated with peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (cat. no. 32460; 1:1,000; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Signals were visualized using enhanced chemiluminescence reagents (Roche Applied Science). Dab2 antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Densitometric quantification was performed using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All experimental data are presented as the mean ± standard deviation. Statistical significance was determined by Student's t-test for two groups, or single factor analysis of variance followed by the Tukey's multiple comparisons test. Statistical analysis was performed using GraphPad Prism software (version 6.01; GraphPad Software Inc., La Jolla, CA, USA). All experiments were performed in triplicate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-149 promotes the expression of cardiac differentiation markers. The cardiac differentiation of mouse MSCs from bone marrow was induced with 5-aza, and the expression of miR-149 was detected with qPCR at different time points. It was identified that the expression level of miR-149 was upregulated with increasing treatment time of 5-aza (Fig. 1A). Therefore, it was hypothesized that miR-149 may serve a role in the cardiac differentiation of MSCs. To test this hypothesis, miR-149 was overexpressed in MSCs using a lentivirus and the expression of cardiac differentiation markers was detected using qPCR. Compared with the control group which was infected with mock lentivirus, miR-149 was overexpressed (Fig. 1B). The early markers of cardiac differentiation, Nkx2.5 and GATA-4, were upregulated when miR-149 was expressed for 3 days and the high level was maintained between 7 and 14 days (Fig. 1C and D). The expression of the late markers of cardiac differentiation, cTnI and CX43, continued to increase over time (Fig. 1E and F).

Dab2 is the direct target of miR-149. The present study aimed to elucidate the mechanism through which miR-149 may affect the cardiac differentiation of MSCs (Fig. 2). Targetscan and Pictar were used to analyze the potential target genes of miR-149, and it was identified that Dab2 may be regulated by miR-149 (17-19). First, the expression level of Dab2 was detected in 5-aza-induced MSCs, and it was observed that the expression of Dab2 was downregulated at the mRNA and protein level (Fig. 2A, C and F). Similarly, overexpressing miR-149 decreased the mRNA expression level of Dab2 at different time points (Fig. 2B). The protein expression level of Dab2 was detected following overexpression of miR-149 for 3 days in MSCs, and it was demonstrated that Dab2 was downregulated (Fig. 2D and G). Since Dab2 expression decreased during the process of cardiac differentiation, to rule out the influence of cardiac differentiation on Dab2, NIH 3T3 cells were transfected with miR-149 mimics. It was demonstrated that miR-149 was able to downregulate the protein expression level of Dab2 in NIH 3T3 cells (Fig. 2E and H). A dual luciferase assay was performed to detect whether Dab2 was the direct target of miR-149. miR-149 was able to decrease the relative luciferase activity of the wild type 3'UTR of Dab2, while it had no effects on the mutant 3'UTR of Dab2 (Fig. 2I and J).

miR-149-induced cardiac differentiation is mediated by Dab2. To detect whether Dab2 mediates the cardiac differentiation of MSCs induced by miR-149, Dab2 was overexpression using a lentivirus in miR-149-overexpressing MSCs. Western blotting was performed to test the expression of Dab2 on the 7th day. As hypothesized, miR-149 decreased the expression of Dab2, while exogenous expression of Dab2 recovered the protein expression level (Fig. 3A and B). Cardiac differentiation markers were subsequently detected. Since CX43 was undetectable at baseline in MSCs (Fig. 1F), and on the 7th day all markers exhibited a detectable level, Nkx2.5, GATA4 and cTnI were detected on the 7th day. It was observed that miR-149 increased the expression of these genes, while overexpression of Dab2 was able to reverse the expression level of these genes almost to basal levels (Fig. 3C-E).

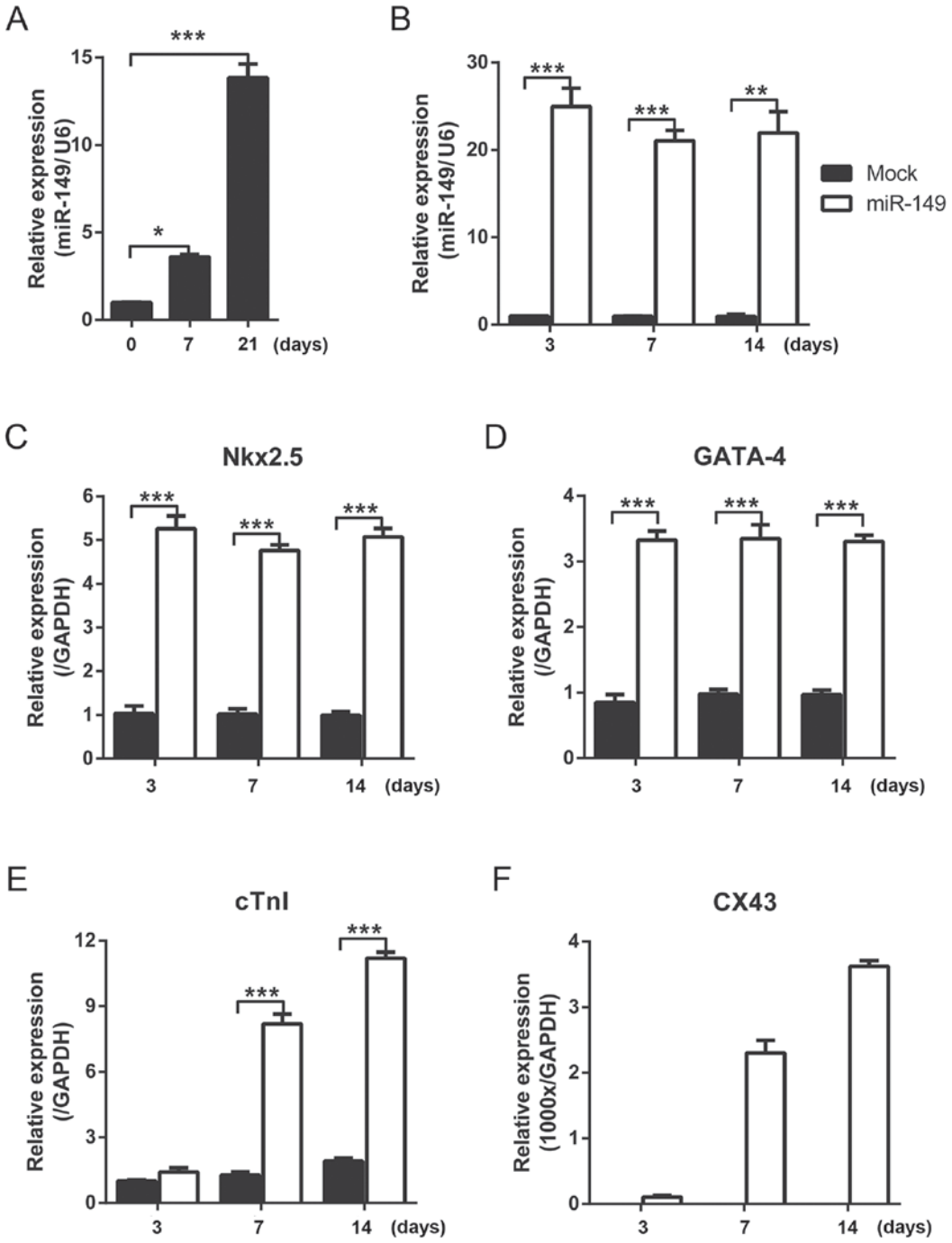

Figure 1. miR-149 promotes the expression of cardiac markers of MSCs. (A) The expression of miR-149 in MSCs treated with 5-azacytidine at different time points. (B) The expression of miR-149 in MSCs following infection with the miR-149-overexpressing lentivirus at different time points. The expression of cardiac markers following infection with miR-149-overexpressing lentivirus was assessed at different time points: (C) Nkx2.5; (D) GATA-4; (E) cTnI; and (F) CX43. *P<0.05, **P<0.01, ***P<0.001. MSCs, mesenchymal stem cells; miR-149, microRNA-149; Nkx2.5, homeobox protein Nkx2.5; GATA-4, transcription factor GATA-4; cTnI, cardiac troponin I; CX43, gap junction α-1 protein; d, days.

Effect of the Wnt signaling pathway on miR-149-induced cardiac differentiation. It has been reported that Dab2 may negatively regulate the canonical Wnt-β-catenin signaling pathway (20). The results of the present study demonstrated that overexpressing miR-149 increased the expression of Dab2-target Wnt pathway genes, including Axin2 and pterin-4 alpha-carbinolamine dehydratase 1 (Fig. 4A and B). In order to assess whether the effect of miR-149 depended on the Wnt/β-catenin signaling pathway, overexpressing miR-149 MSCs were treated with XAV-939, a Wnt/β-catenin signaling pathway inhibitor. It was identified that XAV-939 reversed the upregulation of cardiac differentiation markers induced by miR-149 (Fig. 4C-F), which suggested that miR-149 may promote the cardiac differentiation of MSCs via the Wnt/β-catenin signaling pathway.

Discussion

The present study demonstrated that expression of miR-149 in mouse MSCs from bone marrow promoted the expression

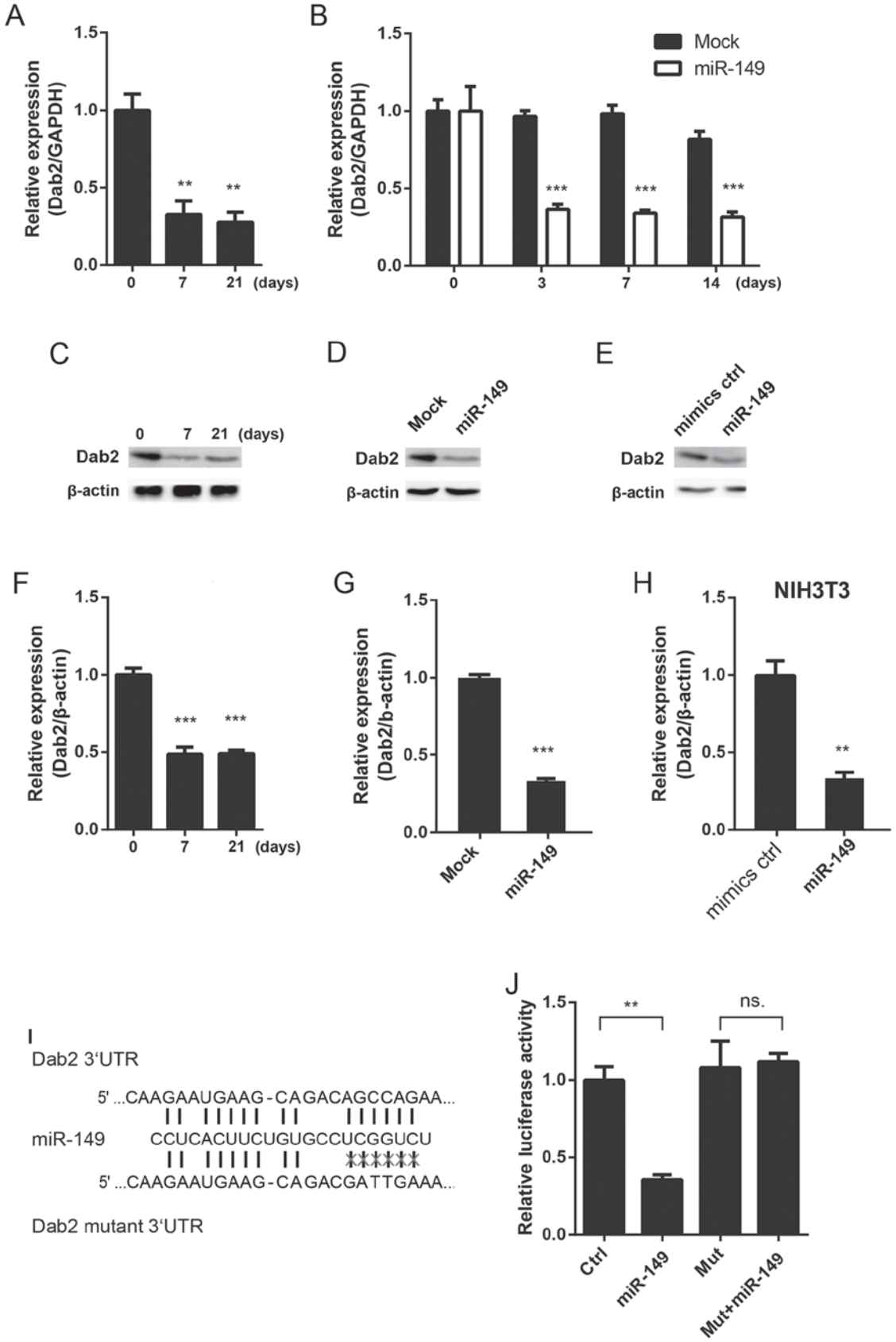

Figure 2. Dab2 is a direct target of miR-149. (A) The mRNA expression of Dab2 in MSCs treated with 5-aza at different time points. (B) The mRNA expression of Dab2 in MSCs following infection with miR-149-overexpressing lentivirus at different time points. (C) The protein expression of Dab2 in MSCs treated with 5-aza at different time points. (D) miR-149 decreased the protein expression of Dab2 following infection with miR-149-overexpresseing lentivirus on the 3rd day. (E) miR-149 mimics decreased the protein expression of Dab2 in 3T3 cells following 3 days' transfection. (F) Quantification of the protein expression of Dab2 in MSCs at different time points. (G) Quantification of the protein expression of Dab2 post-lentiviral infection. (H) Quantification of Dab2 expression in 3T3 cells. (I) Schematic diagram of the miR-149 binding site in the 3'UTR of Dab2 and the mutant site. (J) Identification of miR-149 in Dab2 with the luciferase assay. *P<0.05, **P<0.01, ***P<0.001 vs. respective control. MSCs, mesenchymal stem cells; 5-aza, 5-azacytidine; Dab2, disabled homolog 2; miR-149, microRNA-149; d, days; UTR, untranslated region; ns, not significant; ctrl, control; mut, mutant.

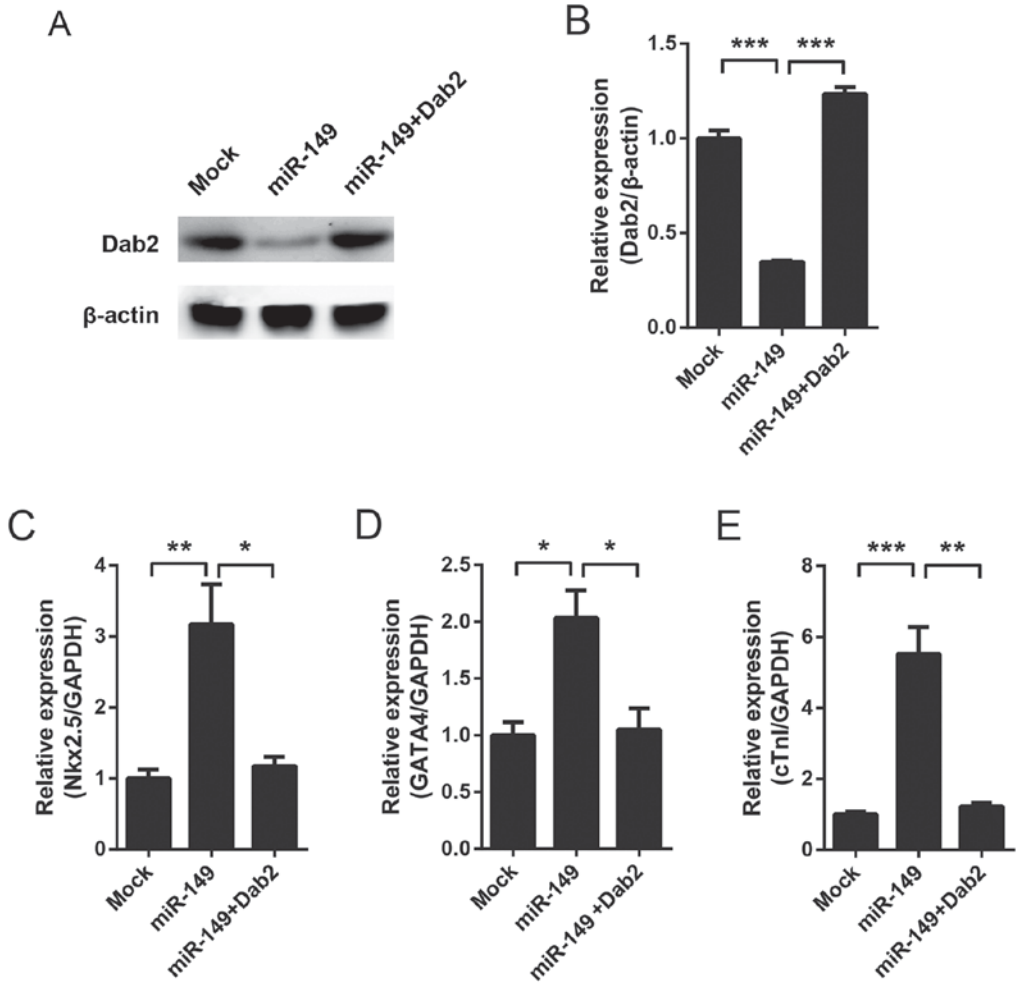

Figure 3. miR-149-induced cardiac differentiation is mediated by Dab2. (A) The protein expression of Dab2 in mesenchymal stem cells following 3 days' lentiviral infection. (B) Quantification of western blotting results. The mRNA expression of the cardiac markers (C) Nkx2.5, (D) GATA-4 and (E) cTnI in MSCs following 7 days' lentiviral infection. *P<0.05, **P<0.01, ***P<0.001. miR-149, microRNA-149; Dab2, disabled homolog 2; Nkx2.5, homeobox protein Nkx2.5; GATA-4, transcription factor GATA-4; cTnI, cardiac troponin I.

of cardiac phenotypic markers, suggesting that miR-149 may serve a role in the induction of differentiation from MSCs into cardiocytes. Furthermore, it was observed that Dab2 was a direct target gene of miR-149, and that exogenous expression of Dab2 was able to reverse the upregulation of cardiac markers induced by miR-149. Further mechanistic analysis demonstrated that miR-149 likely regulated cardiac differentiation through the Wnt/β-catenin signaling pathway.

Increasing microRNAs have been reported to serve important roles in cardiac differentiation and myocardial repair following a heart attack (8,15). MSCs are able to secrete exosomes and microvessels rich in microRNAs, in order to shape the microenvironment to promote myocardial regeneration following a myocardial infarction (21). Therefore, it is important to elucidate the potential function of microRNAs to comprehensively understand the process of cardiac differentiation.

miR-149 has been extensively studied in tumor biology, and was demonstrated to be involved in different processes of tumorigenesis and development, including proliferation, migration, invasion and epithelial-mesenchymal transition (22,23). In addition, miR-149 serves important roles in a number of other diseases, including stroke, type II diabetes and non-alcoholic fatty liver disease (24-28). A recent study reported that the circulating level of miR-149 decreased in mouse models with severe heart failure (29). An earlier study in 2013 demonstrated that the alteration of the binding site of miR-149 located in the 3'UTR of methylenetetrahydrofolate reductase due to a single-nucleotide polymorphism was associated with coronary heart disease susceptibility (30). These reports suggested that miR-149 may have important roles in heart disease. However, the function and mechanism of miR-149 in heart disease remained unclear. The results of the present study revealed a novel function of miR-149 in cardiac differentiation from bone marrow MSCs.

The present study further identified the target gene of miR-149, Dab2, which mediated the cardiac differentiation induced by miR-149. Dab2 is a scaffold protein with multiple modules. It has important roles in signaling transduction and affects numerous biological processes, including cell growth, vesicle trafficking, cell interaction, macrophages polarization and platelet activation (31-33). Certain previous studies demonstrated that Dab2 is important for the expression of cardiac markers, and decreased expression of Dab2 was able to promote the transforming growth factor-β-stimulated

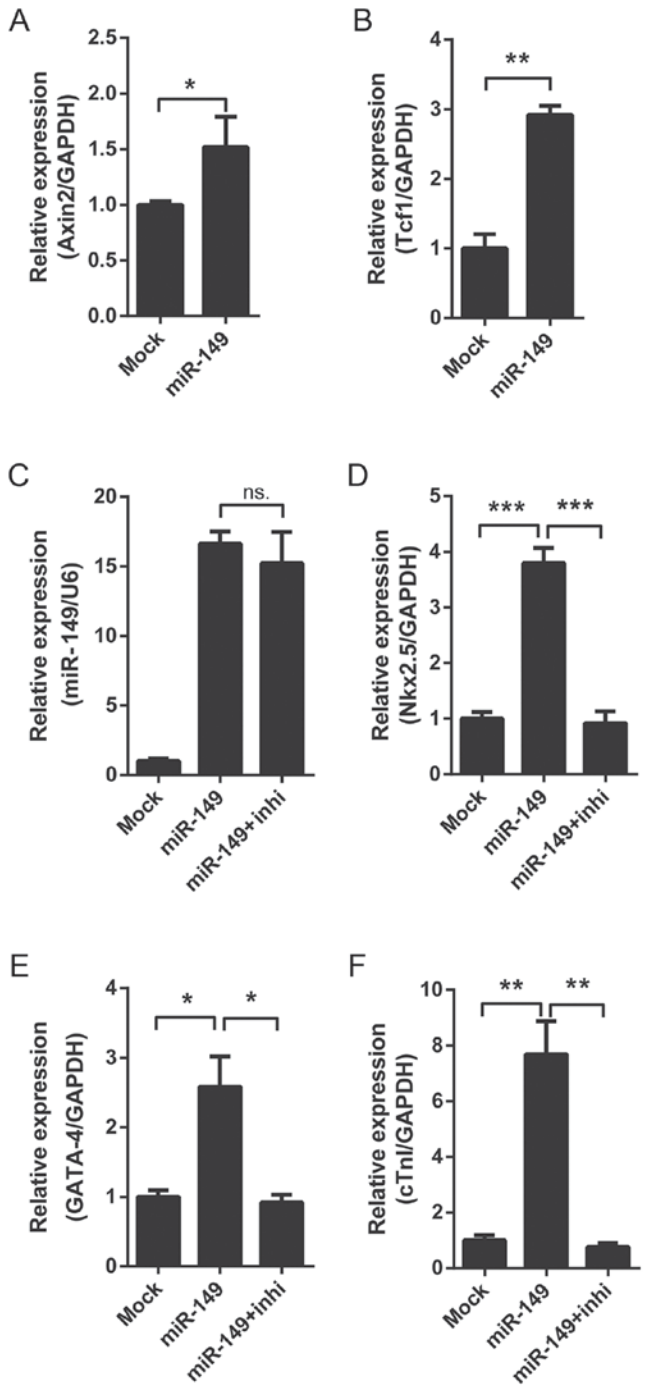

Figure 4. Effect of the Wnt signaling pathway on miR-149-induced cardiac differentiation. (A) The mRNA expression of Axin2 in MSCs following 7 days' lentiviral infection. (B) The mRNA expression of Tcf1 in MSCs following 7 days' lentiviral infection. (C) The expression of miR-149 in MSCs following 7 days' lentiviral infection, with or without treatment with 10 nM XAV-939. The mRNA expression of the cardiac markers (D) Nkx2.5, (E) GATA-4 and (F) cTnI in MSCs following 7 days' lentiviral infection, with or without treatment with 10 nM XAV-939. *P<0.05, **P<0.01, ***P<0.001. miR-149, microRNA-149; MSCs, mesenchymal stem cells; Tcf1, pterin-4 alpha-carbinolamine dehydratase 1; Nkx2.5, homeobox protein Nkx2.5; GATA-4, transcription factor GATA-4; cTnI, cardiac troponin I; ns, not significant; inhi, inhibitor XAV-939.

cardiac differentiation of MSCs and improve cardiac function following MSC transplantation (20,34). These results support the present findings that downregulation of Dab2 by miR-149 promoted cardiac differentiation. However, whether these cells may differentiate to mature cardiocytes *in vivo* and promote the impaired cardiocyte repair requires further study.

The Wnt/β-catenin signaling pathway serves important roles during the process of heart development and regeneration. In the present study, it was observed that the reduction in Dab2 increased the expression of β-catenin target genes, which suggested that Dab2 may be a negative regulator of the Wnt/β-catenin signaling pathway. Through treatment with a Wnt/β-catenin inhibitor, it was illustrated that the regulatory effect of miR-149 on the expression of cardiac markers is dependent upon the Wnt/β-catenin signaling pathway. This finding is supported by a previous study that demonstrated that the deletion of Dab2 in zebrafish led to an abnormal cardiomyocyte number and increased Wnt/β-catenin signaling (20). These results demonstrated that miR-149 may regulate cardiac differentiation through the Dab2/Wnt/β-catenin signaling pathway. Whether other signaling pathways are involved in this process requires investigation in the future.

In conclusion, the results of the present study demonstrated that miR-149 promoted the cardiac differentiation of mouse MSCs from bone marrow *in vitro*, which depended on Dab2 and the Wnt/β-catenin signaling pathway. The present study provides a potential molecular target for the cardiac differentiation of MSCs, and a foundation for further study with animals and in the clinic.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ML and SY designed the experiments. ML, LX, MW, TG, FL and NS performed the experiments. ML, LX and TC analyzed the data and organized the figures. ML, SY and TC wrote and revised the manuscript. SY and TC supervised the work.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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