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MiR-98-5p regulates myocardial differentiation of mesenchymal stem cells by targeting TBX5

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Abstract. – OBJECTIVE: This study aims to investigate whether miR-98-5p can participate in the myocardial differentiation of bone marrow mesenchymal stem cells (MSCs) by regulating TBX5.

MATERIALS AND METHODS: In this study, we first identified the MSCs that were isolated from rat bone marrow samples. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to detect mRNA expressions of cardiac-related genes, including brain natriuretic peptide (BNP), α-actinin, and Islet-1. The binding site of miR-98-5p and TBX5 was detected by dual-luciferase report gene assay. In addition, co-transfection of miR-98-5p mimics and TBX5 overexpression plasmids was conducted to assess whether miR-98-5p could regulate myocardial differentiation by targeting TBX5.

RESULTS: Overexpression of TBX5 or knockdown of miR-98-5p promoted myocardial differentiation of BMSCs. The mRNA expressions of α-actinin and Islet-1 were significantly increased after the miR-98-5p knockdown. Dual-luciferase report gene assay showed that miR-98-5p could bind to TBX5, which was further verified by qRT-PCR. Additionally, TBX5 overexpression reversed the inhibitory effect of miR-98-5p on regulating the mRNA expressions of α-actinin and Islet-1.

CONCLUSIONS: MiR-98-5p can inhibit the differentiation of rat MSCs into cardiomyocytes through targeting TBX5.

Key Words:
MiR-98-5p, Bone marrow mesenchymal stem cells, Myocardial differentiation, TBX5.

Introduction

Myocardial infarction can result in permanent loss of necrotic cardiomyocytes due to the lacked regenerative capacity, thus leading to decreased cardiac function and ultimately heart failure. Promotion of myocardial repair in ischemic necrosis area has become the key to prevent heart failure after myocardial infarction to reduce long-term mortality. In recent years, stem cell transplantation has become a research hotspot in infarct repair. Bone marrow mesenchymal stem cells (BMSCs) show advantages of easy extraction, no ethical constraints, and no immune response during transplantation. They have become one of the ideal seed cells for transplantation of infarcted areas. BMSCs can be differentiated into cardiomyocytes or cardiomyocyte-like cells *in vitro* and *in vivo*. After transplantation, BMSCs are more conducive to repairing infarct area and improving cardiac function[1-5]. Transplanted BMSCs can be differentiated into cardiomyocytes in the infarcted area and coupled electro-mechanically with the host's cardiomyocytes. They can be directly involved in synchronous contraction of the host myocardium and improve cardiac function[6]. However, transplantation of BMSCs has some limitations. Studies have shown that skeletal muscle-specific proteins such as actin, myosin heavy chain, phospholamban, and tropomyosin, can only express within the first 3-6 months after MSCs transplantation[7]. The long differentiation period, low differentiation efficacy and insufficient differentiation all restrict the clinical application of MSCs in the treatment of ischemic heart disease. Therefore, further studies should be carried out on exploring how to improve the differentiation efficacy of MSCs into cardiomyocytes or cardiomyocyte-like cells, so as to effectively treat ischemic heart disease.

MicroRNAs (miRNAs) are endogenous non-coding RNAs with 19-25 nucleotides in length that are found in a variety of biological ge-

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nomes and could regulate target gene expression. They inhibit the translation of mRNAs by completely or partially binding to the 3'-UTR of their target genes at post-transcriptional level[8,9].

MiR-98-5p was first cloned from Hela cells with 21 nt in length. It was expressed in various tissues with tissue-specific characteristics. Studies have shown that differentially expressed miR-98-5p is involved in the development, invasion, and metastasis of various tumors, such as lung cancer, thyroid cancer, uterine leiomyosarcoma, breast cancer, and colon cancer. The miR-98-5p family is highly conserved. It has been reported[1-12] that it can regulate target genes, such as RAS, MYC, HMGA2, CDC25A, CDK6, thus participating in various biological processes. It is closely related to physiological processes, especially in biological development, cell proliferation and differentiation, and tumor-related process. For example, abnormal expression of miR-98 plays a role in breast cancer, head and neck squamous cell carcinoma, and osteoarthritis[13-18]. However, whether miR-98-5p can participate in the regulation of cardiomyocyte differentiation of MSCs has not been reported yet.

Materials and Methods

Isolation and Culture of MSCs

Twenty healthy Sprague-Dawley rats aged 4 weeks were anesthetized using 2% sodium pentobarbital and soaked in a volume fraction of 75% ethanol for 10 min. The femur and tibia were taken under aseptic conditions, and washed 3 times with phosphate buffered saline (PBS). Metaphysis ends of the femur and tibia were cut off and the bone marrow cavity was exposed. The bone marrow was washed repeatedly with L-DMEM (Low-Dulbecco's Modified Eagle Medium) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with penicillin and streptomycin. The flushed bone marrow was prepared into a single cell suspension. After centrifugation at 1 000 r/min for 5 min, the cell supernatant was discarded. Cells were resuspended at a concentration of 1×10^9/L and seeded into a 25 cm^2 flask for cell culture at 37°C in a humidified CO_2 incubator. After 48 hours, the medium was completely replaced, and the medium was changed every 3 days. After cell adherence and cell density was up to 70% to 80%, they were digested with 0.25% trypsin and passaged at a ratio of 1:2.

Flow Identification of MSCs

Second-passage cells with good growth were digested with 0.25% trypsin and centrifuged at 1 000 r/min for 5 min, followed by cell count after washed 3 times with phosphate-buffered saline (PBS) containing 1% BSA (bovine serum albumin; Lianke, Hangzhou, China). Cells were incubated with CD73 and CD14. At the same time, an isotype negative control was set. Cells were incubated on ice for 45 min, and washed with PBS for 3 times to remove unbound antibody. Finally, cells were resuspended with 500 μL PBS and analyzed by flow cytometry.

Isolation and Culture of Neonatal Rat Cardiomyocytes

The heart of the newborn rat was aseptically removed and digested with trypsin overnight at 4°C. The next day, cells were incubated with collagenase and shaken at 37°C for 40 minutes. Dulbecco's modified MEM (DMEM) containing 20% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) was used to prepare for single cell suspension and the number of cells was adjusted to 1×10^5/mL. After pre-incubation for 90 min, the first adherent non-cardiomyocytes were removed using differential adherence separation technology. The cells were then cultured in DMEM containing 20% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Culture medium was replaced every other day.

Co-Culture of MSCs and CMs Induced Differentiation of MSCs Into Cardiomyocytes

CMs were seeded in the top layer of Transwell® (Corning Incorporated, Corning, NY, USA) nested Petri dishes separated by a 3 μm pore size polycarbonate membrane, while MSCs were seeded in the lower layer. Co-cultured MSCs and CM were transfected with TBX5 or not. The transfected and untransfected MSCs were respectively digested, seeded in 6-well plates at 1×10^5 cells/mL, and co-cultured with the CM cells at the ratio of 1:2 on the 3rd day. One week after co-cultivation, MSCs in the lower layer were collected for extracting total RNA and protein. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot analysis were performed to detect mRNA and protein expression of cardiac-specific proteins including brain natriuretic peptide (BNP), Islet-1 and α-actin.

RNA Extraction and qRT-PCR

RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA), chloroform, and isopropanol, and the extracted RNA was stored at -80°C until use. RNA concentration was measured with a micronucleimeter. Complementary Deoxyribose Nucleic Acid (cDNA) was obtained using reverse transcription and SYBR Green was used for PCR detection (TaKaRa, Otsu, Shiga, Japan). PCR amplification conditions were: pre-denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min and 30 s. Primer sequences were: BNP (F: 5'-TGGAAACGTCCGGGTTACAG-3', R: 5'-CTGATCCGGTCCATCTTCCT-3'), α-actinin (F: 5'-GTCATCTCAGGTGAACGCTTG-3', R: 5'-ACCACAGGAGTAACCCTTCTTT-3'), Islet-1 (F: 5'-TTTCCCTGTGTGTTGGTTGC-3', R: 5'-TGATTACACTCCGCACATTTCA-3'),GAPDH(F: 5'-ACCCACTCCTCCACCTTTGA-3', R: 5'-CTGTTGCTGTAGCCAAATTCGT-3'), TBX5 (F: 5'-CTGTGGCTAAAATTCCACGAAGT-3', R: 5'-GTGATCGTCGGCAGGTACAAT-3').

Transfection Experiment

Third-passage MSCs with good growth was used for the transfection experiment. MiR-98-5p mimics, miR-98-5p inhibitor, and control sequence were respectively transfected into MSCs. The sequences were as follows: miR-98-5p mimics sequence: (5'-GUGAAAUGUUUAGGACCACUAG-3', 5'-AGUGGUCCUAAACAUUUCACUU-3'); miR-98-5p inhibitor sequence: (5'-CUAGUGGUCCUAAACAUUUCAC-3'). The TBX5 overexpression plasmid was synthesized by Gene Pharma (Shanghai, China).

Luciferase Reporter Gene Assay

The 3'UTR sequence of TBX5 was downloaded from the NCBI website to construct the TBX5 wild-type sequence (TBX5 WT 3'UTR) and the mutant-type sequence (TBX5 MUT 3'UTR). The cells were then seeded in 96-well plates, and co-transfected with 50 pmol/L miR-98-5p mimics or negative controls and 80 ng TBX5 WT 3'UTR or TBX5 MUT 3'UTR. After 48 hours of transfection, cells were lysed for detecting fluorescence intensity using the dual luciferase reporter gene assay system.

Western Blot Analysis

The cells were collected after centrifugation and then sonicated at 0°C. After centrifugation at 5000 rpm/min for 5 min, the supernatant was collected. Then β-ME (0.5 mL to 9.5 mL) and bromophenol blue (0.5 mL to 9.5 mL) were added and boiled for 10 min. The protein samples were stored at -20°C until use. After the electrophoresis completed, the sample was transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and then blocked in 5% skim milk. The specific primary antibodies were used to incubate membrane overnight at 4°C. In the next day, the secondary antibody was incubated 1 h with the membrane. Finally, the exposure was performed.

Statistical Analysis

All experiments were repeated 3 times. Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM, Armonk, NY, USA) was used for analysis. Measured data were expressed as mean±standard deviation. Data were compared using the *t*-test. The difference was statistically significant at $p<0.05$.

Results

Isolation and Culture of Rat Bone Marrow Mesenchymal Stem Cells (BMSCs)

We selected bone marrow mesenchymal stem cells for constructing cell model. After the cells were seeded into culture flasks and cultured for 24 h, cells were adherent with a round, spindle or polygonal shape. The unattached contaminant cells were removed by changing the medium. MSCs grew to a spindle-shape on the 4th day and exhibited a high refractive index (Figure 1A). Flow cytometric identification of second-passage BMSCs showed positive CD73 (99.6%) and negative CD14 (3.4%), which were consistent with the immunophenotypic characteristics of BMSCs (Figure 1B).

Over-Expression of TBX5 or Low Expression of miR-98-5p Promoted Myocardial Differentiation of BMSCs

BMSCs were transfected with pcDNA-NC and pcDNA-TBX5 respectively, and then cardiomyocyte lysate was added to induce differentiation of BMSCs into cardiomyocytes. After overexpression of TBX5, mRNA expressions of BNP, α-actin, and Islet-1 were significantly increased (Figure 2A, 2B and 2C). Subsequently, miR-98-5p mimics and inhibitors were transfected into BMSCs and then cardiomyocyte lysates were added to induce differentiation of BMSCs into

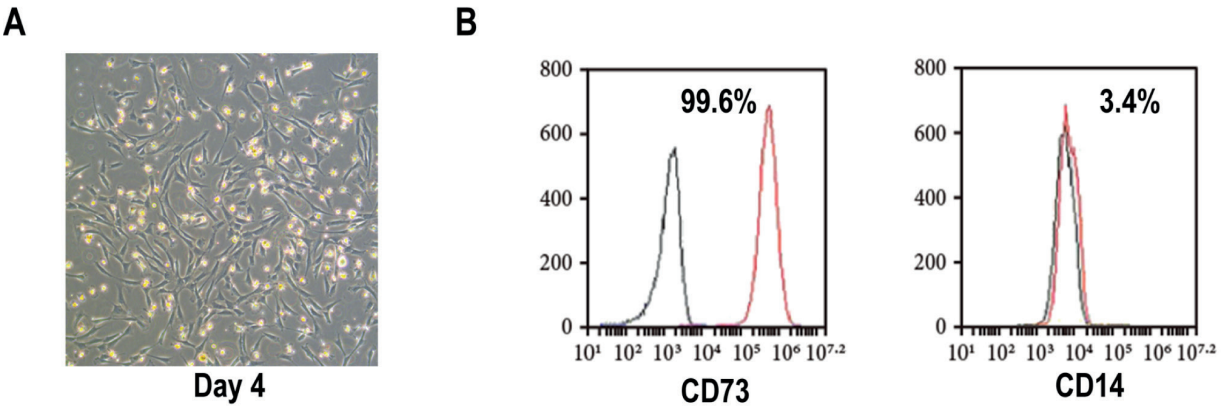

Figure 1. Isolation and culture of rat bone marrow mesenchymal stem cells (BMSCs). ***A,*** The morphology of BMSCs grown to the fourth day was long spindle. ***B,*** Flow cytometry identification of second-passage BMSCs showed a positive result of 99.6% for CD73 and negative result of 3.4% for CD14.

cardiomyocytes. After overexpression of miR-98-5p, the mRNA expressions of myocardial-related genes, including BNP, α-actin, and Islet-1 were found significantly reduced, whereas knockdown of miR-98-5p resulted in the opposite results (Figure 2D, 2E and 2F). The above results indicated that overexpression of TBX5 or knockdown of miR-9-5p could promote myocardial differentiation of BMSCs.

TBX5 Was Verified as the Target Gene of MiR-98-5p

The miRNAs can base pair with mRNA of their target genes to form a gene silencing complex and degrade the target gene mRNA to participate in the occurrence of the disease. The target genes of miR-98-5p were predicted by bioinformatics and functional analysis, and TBX5 was obtained. To verify the binding relationship between miR-98-5p and TBX5, miR-98-5p mimics and inhibitors were transfected into cells. After overexpression of miR-98-5p, TBX5 expression was significantly downregulated, whereas it was upregulated when miR-98-5p knockdown (Figure 3A). Subsequent detection in protein levels revealed that overexpression of miR-98-5p significantly reduced the protein expression of TBX5, whereas knockdown of miR-98-5p increased TBX5 expression (Figure 3B). To further confirm whether miR-98-5p could bind to TBX5, TBX5-WT 3'UTR and TBX5-MUT 3'UTR were constructed (Figure 3C). The result showed that after cell co-transfection, the luciferase activity of TBX5-WT 3'UTR group decreased, while TBX5-MUT 3'UTR group showed no significant difference (Figure 3D), indicating that TBX5 can bind to miR-98-5p.

MiR-98-5p Regulated Myocardial Differentiation of BMSCs by Targeting TBX5

To verify whether miR-98-5p could regulate myocardial differentiation of BMSCs by targeting TBX5, we transfected miR-98-5p mimics and induced differentiation of BMSCs into cardiomyocytes. It was found that overexpression of miR-98-5p remarkably decreased the mRNA expression of myocardial-associated genes including BNP, α-actin, and Islet-1, which were partially reversed by TBX5 overexpression (Figure 4A, 4B and 4C). Further protein levels detection showed the same result (Figure 4D). These results indicated that miR-98-5p could regulate myocardial differentiation of BMSCs by targeting TBX5.

Discussion

Acute myocardial infarction is one of the major diseases that seriously threaten human life and health. Since the 1980s, new anticoagulant and antiplatelet drugs, as well as the interventional catheter technology and coronary artery bypass grafting have been greatly advanced. The early survival rate of acute myocardial infarction has been significantly improved. However, the necrotic myocardial cells are lack of regenerative capacity, thus leading to the decline in cardiac function, and eventually develops to heart failure. Repair of myocardial infarction becomes the key to prevent heart failure after myocardial infarction and reduce the long-term mortality. In recent years, with the development of molecular biology and cell tissue engineering technology,

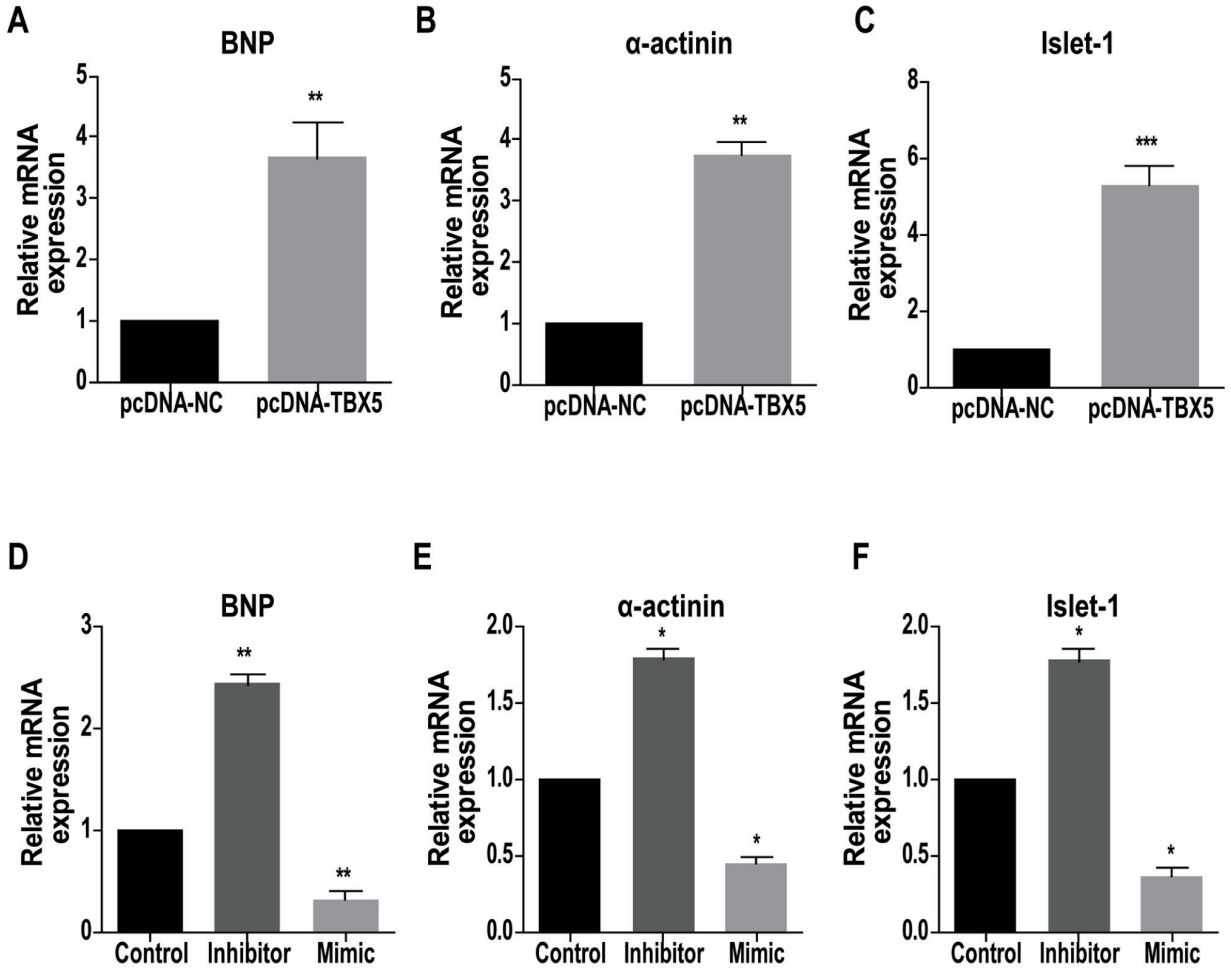

Figure 2. Overexpression of TBX5 or knockdown of miR-98-5p promoted myocardial differentiation of BMSCs. ***A-C,*** After TBX5 was overexpressed, BMSCs were co-cultured with CM cells. The mRNA levels of BNP, α-actin and Islet-1 were significantly increased in BMSCs. ***D-E,*** After knockdown of miR-98-5p in BMSCs, they were co-cultured with cardiomyocytes. It was found that mRNA levels of BNP, α-actin, and Islet-1 were significantly upregulated, and the results were reversed after overexpression of miR-98-5p.

stem cell transplantation has become a new hot spot in the treatment of myocardial infarction.

Bone marrow-derived mesenchymal stem cells (BMSCs) are fibroblast-like stem cells existing in the bone marrow and are able to self-renew in the body in an undifferentiated state. They have multiple differentiation potentials of cells including osteoblasts, chondrocytes, fat cells, endothelial cells, nerve cells, smooth muscle cells and cardiomyocytes[19,20]. Some studies have demonstrated that BMSCs can be differentiated into cardiomyocytes or cardiomyocyte-like cells *in vitro* and *in vivo*. Transplantation of induced BMSCs to the infarct zone is more conducive to improve the cardiac function[21]. In this study, BMSCs were induced to differentiate into cardiomyocytes by extracting cardiomyocyte lysates.

Up-regulated or down-regulated miRNAs act on target genes to activate corresponding molecular signaling pathways and regulate various biological and pathological processes. For example, miRNAs play important role in myocardial remodeling and ventricular remodeling after myocardial ischemia and ischemia[22]. Studies have demonstrated that miR-1 plays an important role in cardiomyocyte apoptosis and arrhythmia[23,24], which can regulate the apoptosis of ischemic myocytes by targeting HSP60, HSP70 and Bcl-2[25,26]. The expression of miR-21 in a normal heart is very low, but it is significantly upregulated un-

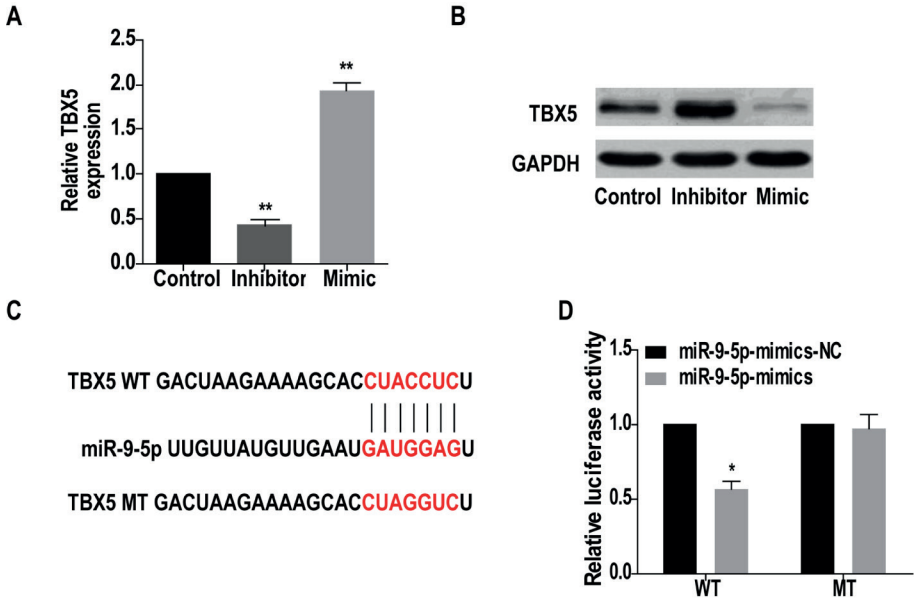

Figure 3. TBX5 was confirmed as the target gene of miR-98-5p. ***A,*** After overexpression of miR-9-5p, the mRNA level of TBX5 significantly decreased, while miR-98-5p knockdown significantly increased the mRNA level of TBX5. ***B,*** After over-expression of miR-98-5p, the protein level of TBX5 significantly decreased. After knockdown of miR-9-5p, the protein level of TBX5 significantly increased. ***C,*** Binding sites of TBX5 and miR-98-5p. ***D,*** Luciferase activity in BMSCs.

der the conditions of heart failure, cardiac hypertrophy, ischemia, and hypoxia. Studies[27,28] have indicated that miR-21 is involved in cardiomyocyte apoptosis and ischemia-reperfusion injury. In this study, low expression of miR-98-5p was verified to promote myocardial differentiation in BMSCs.

The T-box gene family is highly conserved during evolution and serves as a transcription factor that regulates embryonic growth and development. The TBXS gene is an important member of the T-box gene family and controls heart development and upper extremity formation during the early embryonic stage[29]. The human TBXS gene was cloned and identified in Holt-Oram syndrome in 1997. TBXS is located in 12q24.1, which is 2133 bp in length and contains 8 exons encoding 518 amino acids. As a transcription factor, TBXS binds to downstream target genes through its unique T-BOX domain. The TBXS gene is involved in the regulation of the whole process of heart development. The precise expression of TBXS in time and space is critical to the initial differentiation of the atrioventricular cavity, correct formation of the ventricular and interventricular septum. Differentially expressed TBXS can cause cardiac malformations[30]. In the present study, TBX5 is confirmed to be the target gene of miR-98-5p, which can inhibit cardiomyocyte differentiation of BMSCs by selectively combining with TBX5.

Conclusions

We showed that miR-98-5p inhibits myocardial differentiation of mesenchymal stem cells by targeting TBX5.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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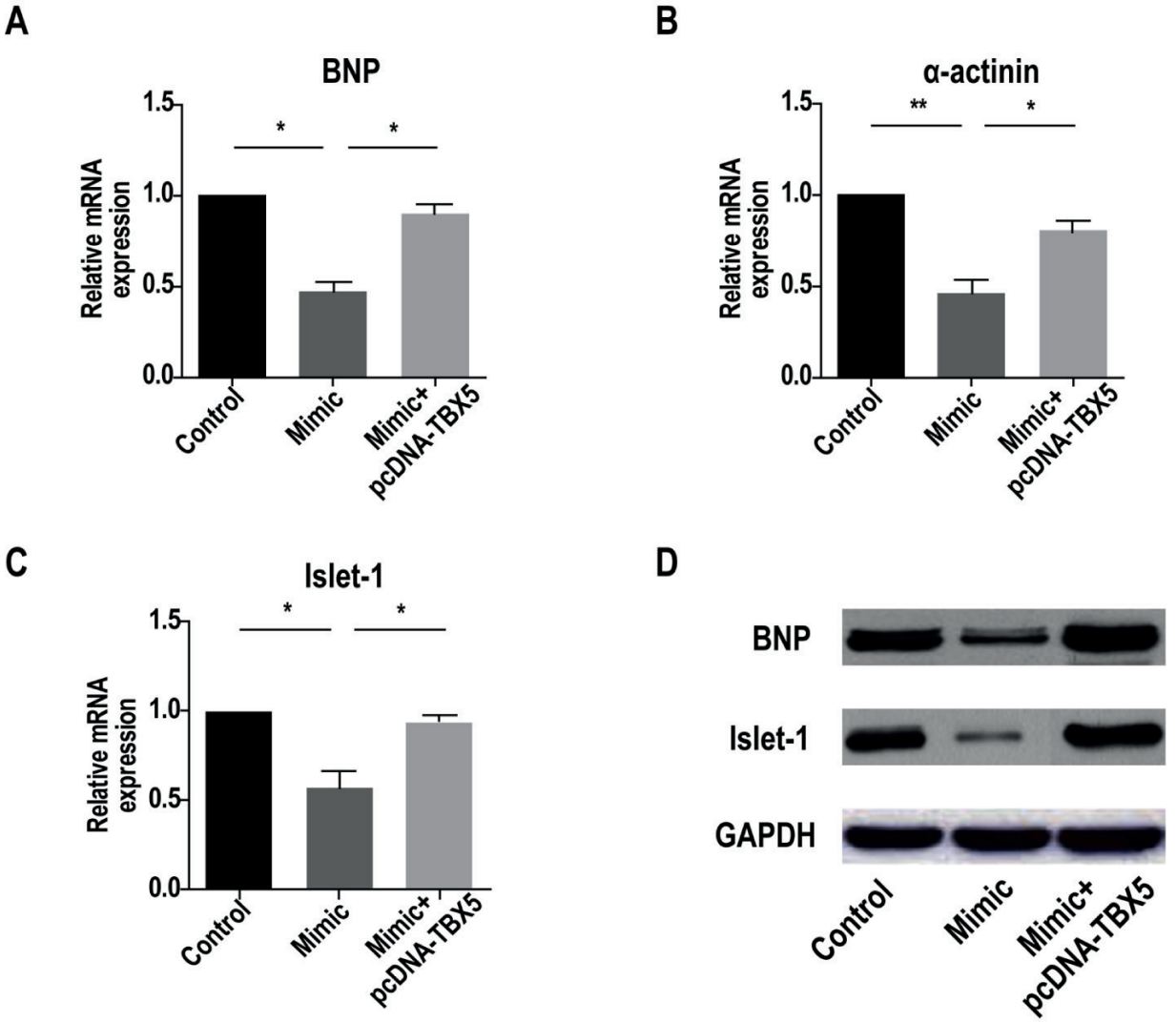

Figure 4. MiR-98-5p regulated myocardial differentiation of BMSCs by targeting TBX5. ***A-C,*** Simultaneous overexpression of miR-98-5p and TBX5 reversed the inhibitory effect of overexpressed miR-98-5p on myocardial differentiation-related mRNA expression (BNP, α-actin, Islet-1). ***D,*** Simultaneous overexpression of miR-98-5p and TBX5 reversed the inhibitory effect of overexpressed miR-98-5p on myocardial differentiation-related proteins (BNP, Islet-1).

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