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MicroRNA-10-5p regulates differentiation of bone marrow mesenchymal stem cells into cardiomyocytes by targeting TBX5

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Abstract. – OBJECTIVE: The purpose of the study was to investigate the regulatory effect of microRNA-10-5p on TBX5 during myocardial differentiation of bone marrow mesenchymal stem cells (BMSCs) in rats.

MATERIALS AND METHODS: Rat BMSCs were first isolated, cultured and identified by flow cytometry. Expression levels of BNP (Brain Natriuretic Peptide), α-actinin, and Islet-1 in BMSCs co-cultured with rat cardiomyocytes were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Dual-luciferase reporter gene assay was conducted to verify the binding condition between microRNA-10-5p and TBX5. Subsequently, we detected TBX5 expression after overexpression or knockdown of microRNA-10-5p in BMSCs. Rescue experiments were conducted by overexpression of both microRNA-10-5p and TBX5 in BMSCs, and then, the expression levels of BNP, α-actinin, and Islet-1 were detected by qRT-PCR and Western blot.

RESULTS: Flow cytometry results showed positive-CD73 (99.3%), positive-CD90 (95.4%), and negative-CD34 (4.2%), which were consistent with immunophenotypic characteristics of BMSCs. TBX5 overexpression or microRNA-10-5p knockdown increased mRNA levels of BNP, α-actinin, and Islet-1 in BMSCs co-cultured with rat cardiomyocytes. Dual-luciferase reporter gene assay confirmed that microRNA-10-5p could bind to TBX5. Both mRNA and protein expressions of TBX5 were negatively regulated by microRNA-10-5p. The inhibited expression levels of BNP, α-actinin, and Islet-1 by microRNA-10-5p overexpression in BMSCs were partially reversed by TBX5 overexpression.

CONCLUSIONS: MicroRNA-10-5p regulates BMSCs differentiation into cardiomyocytes by binding to TBX5.

Key Words
MicroRNA-10-5p, BMSCs, Myocardial differentiation, TBX5.

Introduction

Acute myocardial infarction with the accompanying heart failure is one of the cardiovascular diseases that seriously threaten human health[1]. In recent years, stem cell transplantation has gradually been widely applied in the treatment of cardiovascular diseases[2]. Experimental researches and clinical trials have confirmed that stem cell transplantation can regenerate myocardium, reduce infarct size and improve cardiac function, thus providing a new alternative treatment for cell reconstruction of damaged heart and functional repair of debilitated heart[3].

Bone marrow mesenchymal stem cells (BMSCs) have multiple advantages, including a wide range of resources, easy collection and amplification, strong self-renewal ability, multi-directional differentiation potential, and low immunogenicity. BMSCs have become an ideal seed cell for cell transplantation in the treatment of cardiovascular diseases[4]. Relative studies have confirmed that BMSCs transplantation can improve heart failure after myocardial infarction[5]. However, the therapeutic efficacy of BMSCs transplantation is still far away from satisfactory. Low differentiation efficiency of BMSCs in the local infarct microenvironment greatly limits their ability to differentiate into cardiomyocytes[6]. Hence, it is of clinical

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significance to find an efficient way to promote BMSCs differentiation into cardiomyocytes.

MicroRNAs are a class of endogenous, non-coding RNA molecules of 19-25 nucleotides in length, which exert an important role in the regulation of gene expressions[7]. The developmental and differentiation processes of BMSCs involve the sequential activation of many genes, which are regulated by a variety of transcription factors or microRNAs. It is believed that the temporal activation of genes is determined by the epigenetic modification[8,9]. MicroRNAs can induce or inhibit the differentiation of BMSCs into the cardiomyocytes by silencing or overexpressing their target genes[10]. MicroRNA-10-5p belongs to the miR-10 family, which has been shown to be closely associated with a variety of tumor diseases[11,12]. However, the relationship between microRNA-10-5p and myocardial differentiation of BMSCs is rarely reported.

TBX5 belongs to the T-box transcription factor gene family. The human TBX5 gene is located at 12q24.1 and has a full-length complementary Deoxyribose Nucleic Acid (cDNA) of 2133 bp containing 8 exons. TBX5 binds to downstream target genes mainly through the T-box domain, which is capable of regulating normal heart development and upper limb formation in early embryonic stage[13]. Studies have reported that TBX5 blueprints for early atrial arrhythmogenic remodeling regulated by microRNAs[14]. Whether microRNA-10-5p can regulate myocardial differentiation of BMSCs through mediating TBX5 is rarely reported. Therefore, this study aims to reveal the regulatory effect of microRNA-10-5p on TBX5 gene during the differentiation of BMSCs into cardiomyocytes, which provides new ideas for clinical application.

Materials and Methods

Isolation and Culture of BMSCs

Twenty 4-week-old Sprague Dawley rats were executed with dislocation of the cervical vertebra. Rat femur and tibia were collected under aseptic condition. The marrow cavity was washed with DMEM (Dulbecco's Modified Eagle Medium) (Gibco, Rockville, MD, USA) containing 15% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). After centrifugation at 1000 r/min for 7 min, BMSCs were resuspended in DMEM containing 10% FBS and maintained in a 5% CO_2 incubator at 37°C. Culture medium was replaced for the first time until cell adherence, and then replaced every three days. Cell passage was performed with 0.25% trypsin when the confluence was up to 90%.

BMSCs Identification

Second-passage BMSCs were digested with 0.25% trypsin, centrifuged at 1000 r/min for 7 min, and resuspended in 5 mL of phosphate buffered saline (PBS). Resuspension was centrifuged again at 1000 r/min for 7 min. Subsequently, BMSCs were incubated with anti-CD73, anti-CD90, and anti-CD34 at 4°C for 30 min. After another centrifugation at 1000 r/min for 7 min, BMSCs were resuspended in 500 μL of PBS for flow cytometry detection.

Isolation of Cardiomyocytes Extracted From Neonatal Rats and Cell Culture

Myocardial tissues of neonatal rats were harvested and cut into small pieces with 1 mm^3 in size and digested with 0.25% trypsin. After a gentle blow, the mixture was maintained 2 minutes and the supernatant was discarded. The remaining precipitate was gently blown again for discarding the supernatant. Subsequently, pre-cooled DMEM containing 10% FBS was added for terminating digestion, followed by centrifugation at 4°C, 1500 r/min for 10 min, and precipitate preservation. The centrifugation was performed three times, so as to clean out the remaining trypsin. Finally, rat cardiomyocytes were cultured in a culture bottle and maintained in a 5% CO_2 incubator at 37°C.

Co-Culture of BMSCs and Rat Cardiomyocytes

The extracted rat cardiomyocytes were cultured three days and then incubated with 1.5 mL DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich, St. Louis, MO, USA) at a final dose of 50 μg/mL. After 2 h incubation at 37°C, DAPI dyeing solution was cleaned 6 times by 0.01 mol/L PBS wash (pH 7.2). BMSCs were seeded in the plates where rat cardiomyocytes were already dyed with DAPI at a density of 3.5×10^4 cells per well. Culture medium was replaced 24 hours later for the first time and then replaced every 2-3 days.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The TRIzol kit (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA, which was then reversely transcribed into cDNA. After the cDNA was amplified, qRT-PCR was performed to detect the expressions of related genes. Primers used in this

study were as follows: BNP (Brain Natriuretic Peptide) (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').

Cell Transfection

BMSCs in good growth condition were seeded in the 96-well plates and collected for cell transfection according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Culture medium was replaced 6 h later. Primers used in the study were as follows: MicroRNA-10-5p (F: 5'-CGCTACCCTGTAGATCCGAA-3', R: 5'-GTGCAGGGTCCGAGGT-3'); TBX5 (F: 5'-GAGATAGTCGCTATCGCCTGG-3', R: 5'-AGGTTCTGCTCTCCAACTATCC-3').

Dual-Luciferase Reporter Gene Assay

The binding site of microRNA-10-5p and TBX5 was predicted to construct wild-type (TBX5-WT 3'UTR) and mutant-type TBX5 (TBX5-MUT 3'UTR). Cells were seeded in 12-well plates and co-transfected with 50 pmol/L microRNA-10-5p mimics or negative control and 80 ng TBX5-WT 3'UTR or TBX5-MUT 3'UTR for 48 h, respectively. Luciferase activity was finally detected according to the relative commercial kit instructions.

Western Blot

Cells were lysed for protein extraction. The concentration of each protein sample was determined by a BCA (bicinchoninic acid) kit (Abcam, Cambridge, MA, USA). The protein sample was separated by gel electrophoresis and transferred to PVDF (polyvinylidene difluoride) membranes (Millipore, Billerica, MA, USA). After incubation with primary and secondary antibody, immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

Statistical Analysis

We used Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) for the statistical analysis. The quantitative data were represented as ($\bar{x}\pm s$).The *t*-test was used for comparing differences between the two groups. The $p<0.05$ was considered statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Results

Isolation and Culture of BMSCs in Rats

BMSCs were elongated with a strong refractivity on the third day of cell culture (Figure

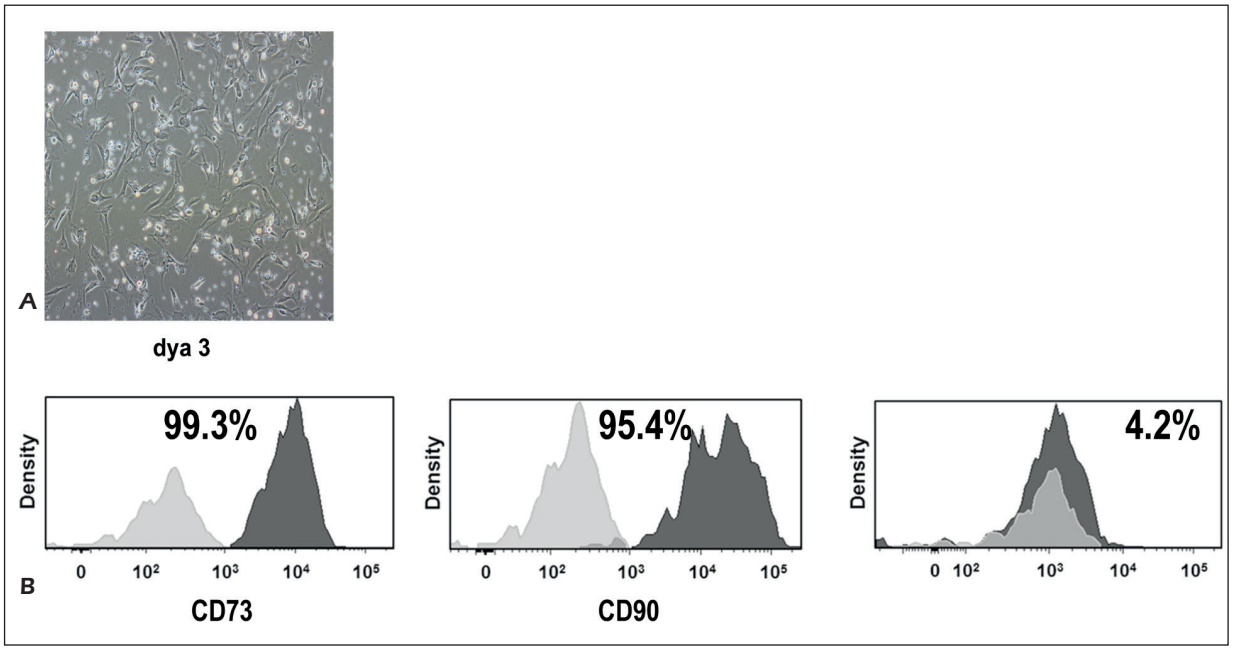

Figure 1. Isolation and culture of BMSCs in rats. **A**, Morphology of BMSCs at the third day of isolation (magnification 200). **B**, Flow cytometry identification of second-passage BMSCs.

1A). Second-passage BMSCs were collected for phenotype identification using flow cytometry. The data showed positive-CD73 (99.3%), positive-CD90 (95.4%), and negative-CD34 (4.2%), which were consistent with immunophenotypic characteristics of BMSCs, rather than hematopoietic stem cells (Figure 1B).

TBX5 Overexpression or MicroRNA-10-5p Knockdown Promoted Myocardial Differentiation of BMSCs

BMSCs overexpressing TBX5 were co-cultured with cardiomyocytes extracted from neonatal rats. QRT-PCR data showed that mRNA levels of BNP, α-actinin, and Islet-1 are remarkably upregulated (Figure 2A). Similarly, mRNA levels of BNP, α-actinin, and Islet-1 remarkably increased as well after BMSCs with microRNA-10-5p knockdown were co-cultured with rat cardiomyocytes (Figure 2B). The opposite results were obtained in BMSCs with overexpression of microRNA-10-5p (Figure 2B). It is indicated that TBX5 overexpression or microRNA-10-5p knockdown promotes myocardial differentiation of BMSCs.

TBX5 Was the Target Gene of MicroRNA-10-5p

The potential target genes of microRNA-10-5p were predicted through bioinformatics, followed by function analyses. TBX5 was finally screened out for the following experiments. To further verify the binding condition between microRNA-10-5p and TBX5, TBX5-WT 3'UTR, and TBX5-MUT 3'UTR were first constructed (Figure 3A). BMSCs showed decreased luciferase activity after co-transfection with microRNA-10-5p mimic and TBX5-WT 3'UTR. However, no significant change in luciferase activity was found in BMSCs transfected with TBX5-MUT 3'UTR (Figure 3B). Subsequently, we detected the TBX5 expression after overexpression or knockdown of microRNA-10-5p. The TBX5 expression was downregulated after overexpression of microRNA-10-5p, whereas it increased by microRNA-10-5p knockdown (Figure 3C and 3D).

MicroRNA-10-5p Regulated BMSCs Differentiation into Cardiomyocytes Through Targeting TBX5

After overexpression of microRNA-10-5p, mRNA and protein expression of BNP, α-actinin, and Islet-1 significantly decreased, which were reversed by TBX5 overexpression (Figure 4A and 4B). These results indicated that microRNA-10-5p regulates myocardial differentiation of BMSCs by targeting TBX5.

Discussion

Cardiovascular diseases severely threaten human health and lives. So far, there are 22.5 million patients with heart failure globally, which still increases at a speed of 2 million per year. The 5-year survival rate of heart failure is similar to that of malignant tumors[15].

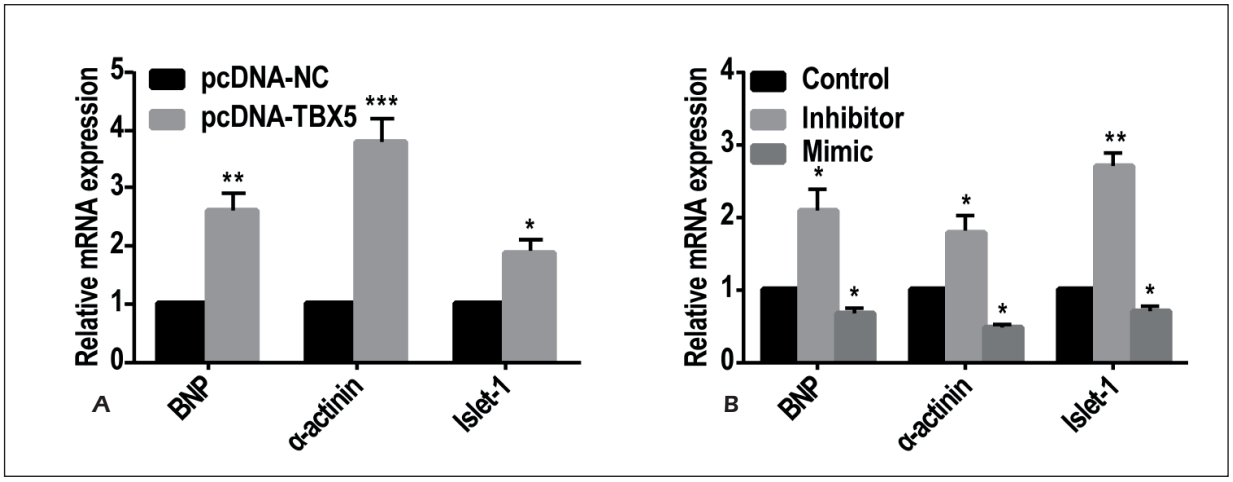

Figure 2. TBX5 overexpression or microRNA-10-5p knockdown promoted myocardial differentiation of BMSCs. **A**, The mRNA levels of BNP, α-actinin, and Islet-1 remarkably increased in BMSCs overexpressing TBX5 co-cultured with cardiomyocytes. **B**, The mRNA levels of BNP, α-actinin, and Islet-1 remarkably increased after BMSCs with microRNA-10-5p knockdown were co-cultured with rat cardiomyocytes.

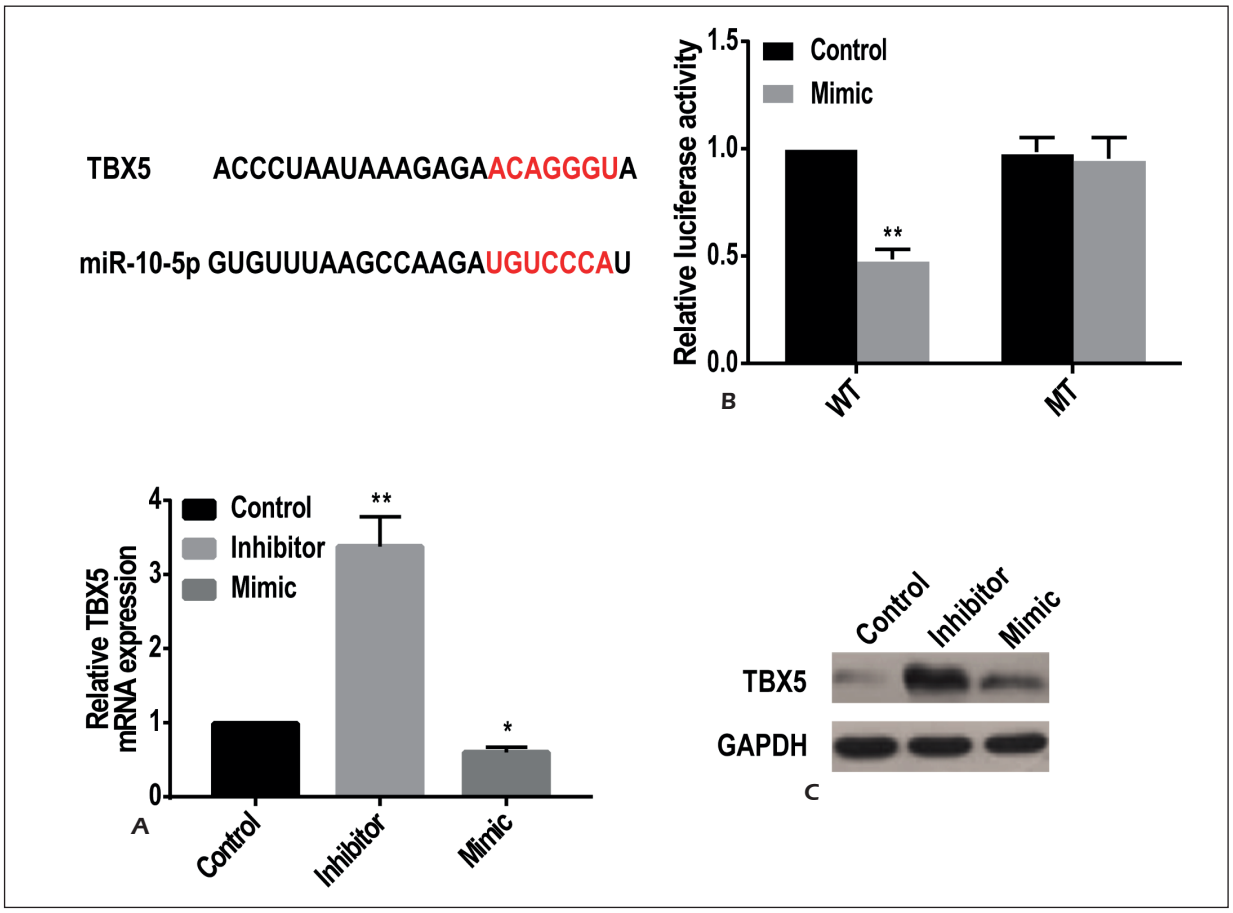

Figure 3. TBX5 was the target gene of microRNA-10-5p. **A**, Binding site between TBX5 and microRNA-10-5p. **B**, BMSCs showed decreased luciferase activity after co-transfection with microRNA-10-5p mimic and TBX5-WT 3'UTR. However, no significant change in luciferase activity was found in BMSCs transfected with TBX5-MUT 3'UTR. **C**, Overexpression of microRNA-10-5p decreased mRNA level of TBX5, whereas knockdown of microRNA-10-5p increased mRNA level of TBX5. **D**, Overexpression of microRNA-10-5p decreased protein level of TBX5, whereas knockdown of microRNA-10-5p increased protein level of TBX5.

BMSCs have the characteristics of self-renewal and multi-directional differentiation. Based on different inductions, they can differentiate into osteoblasts, chondrocytes, myogenic cells, adipocytes, etc. BMSCs can also be directly differentiated into nerve cells and pericytes under certain environmental and stimulating factors[16,17]. Studies have shown that 5-azacytidine (5'-aza) can induce the differentiation of BMSCs into myocardium by downregulating the expression of the methyltransferase DNMT1 and inhibiting DNA methylation[18].

MicroRNAs are widely involved in the regulation of gene expressions in biological activities[19]. Some microRNAs exert important regulatory roles in cardiac development and cardiomyocyte differentiation[20]. For example, miRNA-1, miRNA-133, and miRNA-499 are abundantly expressed in muscle tissues, which exert biological regulations in cardiac development, cardiac hypertrophy, and arrhythmia[21]. MiRNA-1 and miRNA-133 may serve as key regulators in stem cell differentiation to myocardium[22]. MiR-1 is capable of promoting stem cell differentiation into myocardial precursor cells, while miR-133 inhibits this differentiation[23,24]. In this investigation, we found that low expression of microRNA-10-5p promotes BMSCs differentiation into cardiomyocytes.

As a member of the T-box transcription factor family, TBX5 is necessary for the formation and maturation of the cardiac conduction system. Experimental studies have shown that TBX5 is widely expressed in the atrioventricular and conduction bundles of mice[25]. Torrado et al[14] found that microRNAs may be astonishingly altered even in a short-term duration of atrial arrhythmia. Currently, it is believed that TBX5 regulated by microRNA-10-5p could mediate the joint

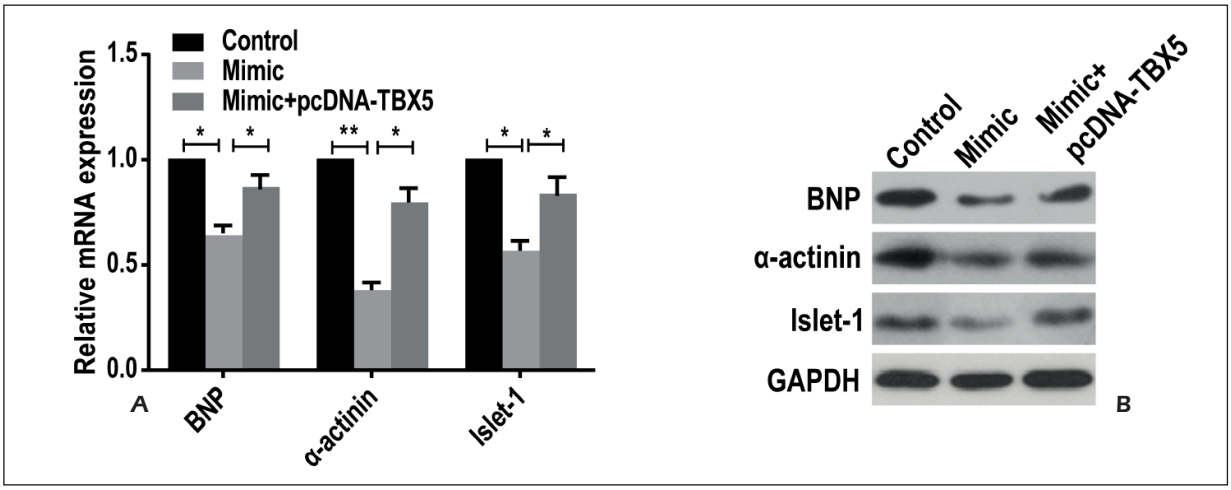

Figure 4. MicroRNA-10-5p regulated BMSCs differentiation into cardiomyocytes through targeting TBX5. Overexpression of microRNA-10-5p decreased mRNA and protein expression of BNP, α-actinin, and Islet-1, which were reversed by TBX5 overexpression.

inflammation[26]. In this research, we found that TBX5 is the target gene of microRNA-10-5p. MicroRNA-10-5p regulated BMSCs differentiation into cardiomyocytes through targeting TBX5.

Conclusions

We showed that microRNA-10-5p regulates BMSCs differentiation into cardiomyocytes by binding to TBX5.

Conflict of Interests

The authors declare that they have no conflict of interest.

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