NT-3 promotes osteogenic differentiation of mouse bone marrow mesenchymal stem cells by regulating the Akt pathway

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Introduction

Bone marrow mesenchymal stem cells (BMSCs) are multipotent stem cells found in the bone marrow. BMSCs have long-term self-renewal and multi-directional differentiation potential. Under the condition of in vitro differentiation, they can differentiate into osteoclasts, adipocytes, chondrocytes, nerve cells, muscle cells, epithelial cells, islet cells, and other cell types, and can be widely used in cardiovascular diseases, spinal joint diseases, liver diseases, skin diseases and other related diseasesⁱ². Under physiological conditions, osteogenic and adipogenic differentiation of BMSCs are in a dynamic equilibrium, and multiple transcription factors and different signaling pathways are involved in this process³. In some pathological conditions, such as osteoporosis, aging, osteoblasts are exhibit decreased proliferation ability and differentiation potential and the apoptosis of osteoblasts and bone cells is promoted. Also, adipogenic differentiation potential of BMSCs is increased while the osteogenic differentiation capacity is weakened, causing osteogenic defects, which are characterized by decreased bone mass, bone damage and accumulation of adipose tissue, etc⁴⁵. Therefore, finding a key regulatory factor in osteogenesis and adipogenic differentiation of BMSCs may be of great significance for the study of bone-related diseases such as osteoporosis.

Damaged bone is often difficult to heal on the ground of a lack of vascular nutrition or innervation in...
the injured area of bone\(^6\).\(^7\). Neurotrophin-3 (NT-3) is a multifunctional neurotrophic factor that maintains neuronal survival, promotes the differentiation of BMSCs into nerve cells, and repairs nerve development, differentiation, survival, and injury. It plays an essential role in promoting the proliferation, survival, and differentiation of neuronal progenitor cells\(^8\).\(^9\). NT-3 plays a specific role in the differentiation of BMSCs into neurons and vascular endothelial cells\(^10\). Also, it has been reported that NT-3 enhances the osteogenic capacity of human bone marrow mesenchymal stem cells stimulated by lipopolysaccharide\(^11\). It is suggested that NT-3 may be involved in the osteogenic differentiation of human bone marrow mesenchymal hepatocytes, but there are few studies concerning whether NT-3 can regulate bone marrow mesenchymal stem cell osteogenic, adipogenic differentiation, and the relationship between NT-3 and bone-related diseases such as osteoporosis. Therefore, this study investigates whether NT-3 is involved in osteogenic/adipogenic differentiation of bone marrow mesenchymal stem cells and whether it can play an essential role in bone formation, bone regeneration, and bone repair.

**Materials and Methods**

**Isolation and Culture of BMSCs**

All mice were purchased from Shanghai Weitong Lihua Co., Ltd., and the animal experiment was approved by the Ethics Committee of Qiqihar Medical College (SYKK (Black) 2016-001). Cell culture was carried out according to the literature report method\(^12\). 5-8 weeks old C57BL/6J male mice were sacrificed by cervical dislocation. The bilateral femur and tibia of the mice were isolated under sterile conditions. The bone marrow cavity was washed with F12 culture medium containing 10% fetal bovine serum, 100 U/mL penicillin, 100 U/mL streptomycin (Corning, USA). The flushing material was transferred into 25 cm\(^2\) cell bottle and cultured at 37°C 5% CO\(_2\). 24 hours later, the fresh culture medium was replaced. After the cell confluence reached about 90%, it was digested with 0.25-EDTA% trypsin (Biosharp, Cat. No. BL501A, China), passaged at a ratio of 1:2, and passed to the third generation for subsequent experiments.

**Cell Transfection**

The full-length pcDNA plasmid of NT-3 was designed and constructed by Beijing Saibaisheng Bioengineering Co., Ltd. (China). The plasmid was extracted with reference to the plasmid extraction kit (Invitrogen, USA), and the plasmid concentration was adjusted to 1 μg/μL, stored at -20°C. The siRNA used to knock down NT-3 was designed and synthesized by Invitrogen. The third generation mouse BMSCs were transfected with Lipo2000 transfection reagent (Invitrogen, USA), transfected with NT-3 overexpression plasmid (OE NT-3), and transfected with siRNA knockdown NT-3 (KO NT-3). Empty vector negative control (ie NC) and siRNA negative control (ie siRNA-NC) were transfected separately. The KO NT-3 sequence is 5’-GGUCAG AGUCCAGCCAATT-3’ and 5’-AUUGCUGAGACUGACCTT-3’. The KO NT-3 sequence is forward, 5’-GCCAGAUCGCUAGATT-3’ and reverse, 5’-ACUUAGGCAUCGUUGCT-3’. The expression of NT-3 was detected after 48 hr of transfection, and osteogenic differentiation or adipogenic differentiation was induced.

**Osteogenic Differentiation of BMSCs**

The third generation mouse BMSCs were inoculated into a 24-well plate or a six-well plate, and after the cell, confluence reached about 70%.

Osteogenic induction was carried out with 10% fetal bovine serum containing 10% FBS, 100 U/mL penicillin, 100 U/mL streptomycin, 0.1 μmol/L dexamethasone, 50 μmol/L ascorbic acid, and 10 mmol/L β-glycerophosphate. The induction solution was changed every 3 days, and alkaline phosphatase (ALP) staining, alizarin red (ARS) staining, or real-time qPCR experiments were performed 14 days after induction.

**Adipogenic Differentiation of BMSCs**

The third generation mouse BMSCs were inoculated into a 24-well plate or a six-well plate, and after the cell, confluence reached about 80%. The lipid-inducing liquid A (MUD-90031, Sailing, China) was induced by BMSCs containing 10% fetal bovine serum, 100 U/mL penicillin, 100 U/mL streptomycin, 1 μmol/L of dexamethasone, 10 μg/mL of insulin, 0.5 mmol/L of 3-isobutyl-1-methylxanthopterin, and 100 μmol/L indomethacin. After 3 days of adipogenic induction, the adipogenic induction solution B containing 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin was used for 1 day, 4 days for one cycle for 16 days.

**Alkaline Phosphatase (ALP) Staining**

Mouse BMSCs inoculated in 24-well plates (Corning, USA) were osteogenic induced for 14 days, washed with 0.01 mmol/L PBS three times for 2 minutes each time, and added 4% paraformaldehyde solution (Solebao, China), fixed at room temperature for 30 min. ALP staining was carried out according to the methods in previous reports\(^13\). The cell culture plate was placed under an inverted light microscope (XDS200, Olympus, Japan) to observe the photograph.

**Alizarin Red (ARS) Staining**

After 14 days of osteogenic induction of BMSCs inoculated in 24-well plates, the net induction solution was discarded and washed three times with 0.01 mmol/L PBS. After fixed with 4% paraformaldehyde, PBS (PBS-10001, Saiye, China) with pH of 7.4 was washed 3 times. The ARS staining solution (Saiye, China) was added and incubated at room temperature for 20 min under an inverted optical microscope, and 10 visual fields were collected and photographed.
Oil Red O (ORO) Staining

After 16 days of adipogenic induction of mouse BMSCs inoculated in 24-well plates, ORO staining was performed. First, the net induction solution was discarded, washed 3 times with PBS, 4% paraformaldehyde was added and fixed at room temperature for 30 min. The cells were washed 3 times with PBS, added with ORO staining solution (Saiye, China), and incubated at room temperature for 30 min, observed under an inverted optical microscope, and took a picture.

Real-time Quantitative Polymerase Chain Reaction (Real-Time qPCR)

After 14 days of osteogenic induction of mouse BMSCs, the results of ALP staining showed that compared with the control group, the black particles in the induction group increased significantly (Figure 1A). ARS staining results showed that compared with the control group, calcium nodules and calcium salt significantly increased in the induced group (Figure 1B). The results of the real-time qPCR experiment showed that the expression of osteoblast-related genes Runx2, Osterix, OCN, and ALP increased to about 5.5-fold, 3.2-fold, 9-fold, and 4.5-fold, respectively, after 14 days of osteogenic induction, and the difference was statistically significant (P is 0.0095, 0.0002, 0.0003, 0.0017, respectively (Figure 1C).

Table 1. Primer sequences required for real-time qPCR experiments.

| Gene name | Sequence (5' to 3') |
|-----------|---------------------|
| Runx2 | F: AATGCTCTCGGTGTTATG  
R: TCTGCTGACTCGGTCTTTG |
| Osterix | F: TCCTGCTGGGAGCGAAGTC  
R: AGGAACTCTGACGTATGGT |
| OCN | F: AAGCAGGAGGGCAATAAGG  
R: AGCTGCTGACATACCTAC |
| ALP | F: TTGATTTATGTTAAGGTTAAAGAG  
R: ACTACAAACAAAATAAACCC |
| FABP4 | F: GGCCAGGAATTGGACGAGTC  
R: AGCGTAACCTTGAGATGTTGTAAGACA |
| PPARγ | F: ACAGGAAAGGACACGACAAAAATC  
R: 5'-GGATCATGACGACACCTT |
| CEBPα | F: AAGAACTCGGTCAGACAAGACAG  
R: TGGCCACCGACGATG |
| LPL | F: CCCAGTGCCTTTCCCTGAT  
R: CTTCTGCTGCACCTTGAGT |
| GAPDH | F: TCAATGACACTGTTGACGCTCA  
R: GTGGTGGGTCAAGGTTCTTACT |

Western Blot Experiment

The cells of each group were treated with protein lysate (P0013, Biyuntian, China), and the total protein was extracted. The protein concentration was determined by BCA protein concentration assay kit (P0012S, Biyuntian, China). Under a condition of 110 V, 10% polyacrylamide gel electrophoresis (SDS-PAGE) was performed, and 30 μg of each protein was loaded. After electrophoresis, the membrane was transfected, and rabbit anti-p-Akt (3165, 1:2000, CST, USA) or rabbit anti-Akt (1462, 1:1000, CST, USA) or rabbit anti-β- was incubated overnight at 4°C (1477, CST, USA). The next day, rabbit anti-secondary antibodies (3690, 1:1000, CST, USA) were incubated for 2 h at room temperature. The gray value of each band was analyzed using a gel imaging system (Bio Rad Chemi Doc XRS imaging system, USA). The relative content of each protein of interest was calculated.

Statistical Method

All data were analyzed using the statistical software GraphPad Prism 5 (GraphPad, USA). All data were expressed as mean ± standard deviation. All data were repeatedly processed at least 3 times. The difference between the two groups was analyzed by paired t-test. p<0.05 was considered to be significant.

Results

Identification of Mouse BMSCs Osteogenic and Adipogenic differentiation

After 14 days of osteogenic induction of mouse BMSCs, the results of ALP staining showed that compared with the control group, the black particles in the induction group increased significantly (Figure 1A). ARS staining results showed that compared with the control group, calcium nodules and calcium salt significantly increased in the induced group (Figure 1B). The results of the real-time qPCR experiment showed that the expression of osteoblast-related genes Runx2, Osterix, OCN, and ALP increased to about 5.5-fold, 3.2-fold, 9-fold, and 4.5-fold, respectively, after 14 days of osteogenic induction, and the difference was statistically significant (P is 0.0095, 0.0002, 0.0003, 0.0017, respectively (Figure 1C).

After 16 days of adipogenic induction of mouse BMSCs, the results of ORO staining showed that compared with the control group, red lipid droplets in the induction group increased significantly (Figure 1D). The results of real-time qPCR experiment showed that the expression of osteoblast-related genes Runx2, Osterix, OCN, and ALP increased to about 5.5-fold, 3.2-fold, 9-fold, and 4.5-fold, respectively, after 16 days of adipogenic induction, and the difference was statistically significant (P is 0.0002, 0.0005, 0.0054 and 0.0004) (Figure 1E).
Figure 1. Osteogenic and adipogenic differentiation of mouse BMSCs. (A) Identification of the osteogenic differentiation of mouse BMSCs by ALP stain; (B) Identification of bone formation and differentiation of BMSCs in mouse by ARS stain; (C) real-time qPCR was used to detect the expression of osteogenic genes in mouse BMSCs; (D) Identification of adipogenic differentiation of mouse BMSCs by ORO staining; (E) Detection of BMSCs Lipid related Gene expression in mice by real-time qPCR Test. n=3, **p<0.01, ***p<0.001.

Figure 2. Effect of overexpression of NT-3 on osteogenic differentiation of mouse BMSCs. (A) Real-time qPCR assay for NT-3 transfection efficiency; (B) Identification of the osteogenic differentiation of mouse BMSCs by ALP stain; (C) Identification of bone formation and differentiation of BMSCs in mouse by ARS stain; (D-G) Real-time qPCR assay The expression of osteogenic related genes in mouse BMSCs was detected. n=3, *p<0.05, **p<0.01, ***p<0.001.
Effect of Overexpression of NT-3 on Osteogenic Differentiation of Mouse BMSCs

As shown in Figure 2A, compared with NC, the expression of NT-3 in the cells transfected with NT-3 overexpressing plasmid increased by about 11 times ($p<0.0001$) (Figure 2A). After 14 days of osteogenic induction, ALP staining results showed that compared with NC, the number of black particles in NT-3 overexpression group increased significantly (Figure 2B). The results of ARS staining showed that compared with NC, calcium nodules, and calcium salt increased significantly in NT-3 overexpression group. (Figure 2C). The real-time qPCR experiment results showed that compared with NC, the expression of Runx2, Osterix, OCN, and ALP in NT-3 overexpression group increased to about 2.8 times, 1.6 times, 5 times, and 2 times, respectively. The difference was statistically significant. (P is 0.0152, 0.0047, 0.0004 and 0.0064, respectively) (Figure 2D-2G).

Effect of Knockdown of NT-3 on Osteogenic Differentiation of Mouse BMSCs

As shown in Figure 4A, the expression of NT-3 in cells transfected with siRNA was reduced to about 30% ($p=0.0189$) compared with NC (Figure 4A). After 14 days of osteogenic induction, the results of ALP staining showed that compared with NC, black particles decreased significantly in the NT-3 knockdown group (Figure 4B). The results of ARS staining showed that compared with NC, calcium nodules, and calcium salts decreased significantly in the NT-3 knockdown group (Figure 4C). The results of the real-time qPCR experiment showed that the expression of Runx2, Osterix, OCN, and ALP in the NT-3 knockdown group was reduced to about 45%, 50%, 25%, and 30% compared with NC. The difference was statistically significant ($p$ is 0.0014, 0.0007, 0.0004 and 0.0005, respectively) (Figure 4D-4G).

Effect of Overexpression of NT-3 on Adipogenic Differentiation of Mouse BMSCs

The results of ORO staining showed that compared with NC, red lipid droplets decreased significantly in NT-3 overexpression group (Figure 3A). The real-time qPCR experiment results showed that compared with NC, the expression of NT-3 overexpressing lipid-related genes FABP4, PPARγ, CEBPα, and LPL decreased to about 40%, 60%, 45%, and 55%, and the difference was statistically significant. ($p=0.0049, 0.0229, 0.0025$ and 0.0171, respectively) (Figure 3B-3E).

Effect of Knockdown of NT-3 on Adipogenic Differentiation of Mouse BMSCs

The results of ORO staining results showed that compared with NC, red lipid droplets increased significantly in NT-3 knockout group (Figure 5A). The real-time qPCR experiment results showed that compared with NC, the expression of FA-3, PPARγ, CEBPα, and LPL in NT-3 knockout group increased to about 4, 1.7, 1.5, and 3 times, and the difference was statistically significant. ($p$ is 0.0004, 0.002, 0.0009 and 0.014, respectively) (Figure 5B-5E).
Figure 4. Effect of knockdown of NT-3 on osteogenic differentiation of mouse BMSCs. (A) NT-3 transfection efficiency by real-time qPCR assay; (B) Identification of the osteogenic differentiation of mouse BMSCs by ALP staining; (C) Identification of bone formation and differentiation of BMSCs in mouse by ARS stain; (D-G) Detection of BMSCs osteogenic related gene expression in mice by real-time qPCR assay. n=3, *p<0.05, **p<0.01, ***p<0.001.

Figure 5. Effect of overexpression of NT-3 on adipogenic differentiation of mouse BMSCs. (A) Identification of the osteogenic differentiation of mouse BMSCs by ORO staining; (B-E) Detection of BMSCs Lipid related Gene expression in mice by real-time qPCR test. n=3, *p<0.05, **p<0.01, ***p<0.001.
**Discussion**

Because BMSCs are easy to obtain, easy to expand in vitro, and multi-directional differentiation potential, they are often used as seed cells for bone tissue engineering\(^4,15\). When bone tissue is damaged, bone regeneration mainly comes from the progressive differentiation of BMSCs into osteogenic progenitor cells, pre-osteoblasts, and osteoblasts, and finally forms matrix and mineralizes into bone tissue\(^6,17\). In addition, it differentiates into osteoblasts and also differentiates into adipogenic direction\(^18\). Therefore, in order to improve the ability of BMSCs to differentiate into osteoblasts, it is essential to explore how to enhance their osteogenic differentiation and inhibit adipogenic differentiation\(^19,20\).

In clinical work, bones without innervation or weakened innervation are prone to fractures or bone-related diseases such as osteoporosis. Therefore, neurological factors play an important role in bone formation and repair. NT-3, a member of the neurotrophin family, can participate in the survival of spinal neurons and its axon regeneration, embryonic blood vessels, limbs, and vascular endothelial cell migration, limb bone formation and other processes\(^21,23\). However, studies on bone formation and bone repair by NT-3 have rarely been reported. Therefore, this study investigated whether NT-3 is involved in bone formation and adipogenic differentiation of bone marrow mesenchymal stem cells, whether it can be formed in bone formation and bone regeneration. It plays an important role in bone repair.

In the present study, the results of ALP staining and ARS staining showed that overexpression of NT-3 could promote the differentiation of mouse BMSCs into osteoblasts, while knockdown of NT-3 could inhibit the differentiation of mouse BMSCs into osteoblasts. Runx2, Osterix, OCN, and ALP serve as indicators of osteogenic differentiation of BMSCs, reflecting their ability to differentiate into osteoblasts. The results of real-time qPCR results showed that overexpression of NT-3 could increase the expression of osteoblast genes Runx2, Osterix, OCN, and ALP in mouse BMSCs, while knockdown of NT-3 could inhibit osteogenic gene expression in mouse BMSCs.

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**Figure 6.** Effect of overexpression and knockdown of NT-3 on p-Akt/Akt expression. (A) Statistics of p-Akt/Akt protein expression. n=3, **p<0.01, ***p<0.001.
The results of ORO staining showed that overexpression of NT-3 could inhibit the differentiation of mouse BMSCs into adipogenesis, and knockdown of NT-3 could promote the differentiation of mouse BMSCs into adipogenesis. Real-time qPCR results showed that overexpression of NT-3 could decrease the expression of FABP4, PPARy, CEBPα, and LPL in mouse BMSCs, and knockdown of NT-3 could increase the expression of adipogenic genes in mouse BMSCs.

Moradian H. et al. have found that NT-3 can promote the differentiation of BMSCs into neurons, and the application of BMSCs and PLGA microcarriers expressing NT-3 can promote the intracellular pathway of dopaminergic neuron differentiation, which may be used in the treatment of brain tissue engineering such as Parkinson’s disease24. Zhu T et al. found that the glial cell-derived neurotrophic factor (GDNF) and NT-3 induce BMSCs to differentiate into neurons in the fetal intestinal medium, and the combination of GDNF and NT-3 with BMSCs may be a potential method for the treatment of neurological diseases25. This study indicates that NT-3 can promote the differentiation of mouse BMSCs into osteoblasts and inhibit their differentiation into adipogenesis, which may be used to treat bone tissue engineering such as bone loss. This experiment’s results indicate that overexpression of NT-3 can significantly increase p-Akt/Akt levels, while knockdown of NT-3 can significantly reduce p-Akt/Akt levels. We analyzed that NT-3 may restart the osteogenic differentiation mechanism by acting on p-Akt/Akt, thereby exerting a regulatory effect on osteogenic differentiation of BMSCs. Still, the specific mechanism needs to be further explored.

In conclusion, by regulating the Akt signaling pathway, NT-3 promotes the differentiation of BMSCs into osteoblasts and inhibits their differentiation into adipocytes.

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Authors’ contributions
SZ and LS: conceived and designed the study, drafted the manuscript. SZ, SS, and JH: collected, analyzed, and interpreted the experimental data. SZ: revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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