

TAZ accelerates osteogenesis differentiation of mesenchymal stem cells via targeting PI3K/Akt

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Abstract. – OBJECTIVE: To investigate the promoting effects of TAZ on the osteogenic differentiation of mesenchymal stem cells in a rat model of osteoporosis through the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signal.

MATERIALS AND METHODS: A total of 12 Sprague-Dawley (SD) rats were randomly divided into normal group (n=6) and model group (n=6). The rats in normal group were not given any treatment, while those in model group were used to establish a model of osteoporosis, and the bone mineral density (BMD) of the femur and lumbar in each group was detected. Then, the bone marrow mesenchymal stem cells were observed, of which those in normal group were divided into normal group and inhibitor group. The bone marrow mesenchymal stem cells in model group were enrolled as model group. Alkaline phosphatase (ALP) staining was adopted to observe the osteogenesis, cell counting kit-8 (CCK-8) was applied to determine the cell proliferation, and immunohistochemistry was performed to measure the expression of TAZ. Western blotting was utilized to detect the relative expressions of phosphorylated Akt (p-Akt) and bone morphogenetic protein 2 (BMP-2) proteins, and quantitative Polymerase Chain Reaction (qPCR) was used to measure the messenger ribonucleic acid (mRNA) expression of TAZ.

RESULTS: The average optical density of ALP-positive cells was decreased remarkably in model group and inhibitor group compared with that in normal group ($p<0.05$), while the cell proliferation rate was increased notably in model group and inhibitor group. In comparison with normal group, model group and inhibitor group had evidently declined positive expression and mRNA expression of TAZ ($p<0.05$). The relative expressions of p-Akt and BMP-2 proteins in model group and inhibitor group were significantly lower than those in normal group ($p<0.05$).

CONCLUSIONS: TAZ promotes the osteogenic differentiation of mesenchymal stem cells in the rat model of osteoporosis by repressing the PI3K/Akt signal.

Key Words:

Osteoporosis, TAZ, PI3K/Akt signaling pathway, Bone marrow mesenchymal stem cells.

Introduction

Osteoporosis is a systemic disease in which raised bone fragility due to osteopenia, as well as declined bone density and mass, increases the risk of fracture^{1,2}. As a common disease among the elderly, osteoporosis often leads to fracture and many secondary complications, thus threatening health and quality of life. According to statistics, the osteoporosis-induced disabling injuries have become the third most serious diseases following hypertension, cardiovascular, and cerebrovascular diseases that affect the life and health of the elderly³. It is currently believed that proliferation and differentiation of bone marrow mesenchymal stem cells, especially the osteogenic differentiation, have close correlations with the onset and recovery of osteoporosis, providing new ideas for the treatment and study of the disease.

As an important cell signal transduction pathway, the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway plays a vital role in mediating numerous processes, including cell proliferation, differentiation, apoptosis, and necrosis⁴. Currently, it is argued that the PI3K/Akt signaling pathway can accelerate the proliferation and differentiation of pre-osteoblastic cells. Regulation on this pathway is conducive to decreasing the proliferation of bone marrow mesenchymal stem cells and promoting the differentiation into osteoblasts.

TAZ, i.e., a transcriptional coactivator with PDZ-binding motif, has been proven to exert important effects in such processes as cell proliferation, differentiation, and self-renewal. It is not only a crucial transcription factor, but also

an important regulatory nuclear factor of the Hippo signaling pathway^{5,6}. This research aims to explore whether TAZ promotes the osteogenic differentiation of mesenchymal stem cells in the rat model of osteoporosis through the PI3K/AKT signal. Also, it aims to further clarify its mechanism of action.

Materials and Methods

Laboratory Animals

A total of 12 female SPF Sprague-Dawley (SD) experimental rats (aged 1-month-old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. [license number: SCXK (Shanghai, China) 2014-0003]. These rats were fed in the Laboratory Animal Center with normal diet and sterile filtered water every day under the conditions of a 12/12 h light/dark cycle, conventional room temperature, and humidity. This research was approved by the Animal Ethics Committee of the Heilongjiang University of Chinese Medicine.

Experimental Reagents and Instruments

L-glutamine (Gibco, Rockville, MD, USA), Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA), fetal bovine serum (FBS; Gibco, Rockville, MD, USA). Primary antibodies: anti-TAZ antibody (Abcam, Cambridge, MA, USA), anti-phosphorylated Akt (p-Akt) antibody (Abcam, Cambridge, MA, USA), anti-runt-related transcription factor 2 (RUNX2) antibody (Abcam, Cambridge, MA, USA), anti-bone morphogenetic protein 2 (BMP-2) antibody (Abcam, Cambridge, MA, USA), secondary antibodies (Abcam, Cambridge, MA, USA), alkaline phosphatase (ALP) staining kit, enzyme-linked immunosorbent assay (ELISA) kit, AceQ quantitative polymerase chain reaction (qPCR). SYBR Green Master Mix kit (Vazyme, Nanjing, China), HiScript II Q RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme, Nanjing, China), light microscope (Leica DMI 4000B/DF-C425C, München, Germany), fluorescence qPCR instrument (ABI 7500; Applied Biosystems, Foster City, CA, USA).

Animal Grouping and Treatment

All the 12 female SD rats were divided into normal group (n=6) and model group (n=6) using a random number table. The rats were fed adaptively in the Laboratory Animal Center for 7 d for subsequent experiments.

The rats in normal group were fed normally without any treatment. Their bone marrow mesenchymal stem cells were taken for *in vitro* culture. The rats in model group were utilized to establish an osteoporosis model and the bone marrow mesenchymal stem cells were taken for *in vitro* culture after successful modeling.

Establishment of Osteoporosis Model

The rats were anesthetized by intraperitoneal injection of 2% pentobarbital sodium (2 mL/kg) and then fixed on the operation table in supine position. Next, an abdominal midline incision was made, the junction of oviduct and uterus was ligated, and the ovary was excised. After that, the wound was washed and sutured layer by layer. At 12 weeks after the operation, the bone mineral density (BMD) was measured to determine whether the model was established successfully.

Detection of BMD

After successful anesthesia, the rats in each group were fixed on a dual-energy X-ray absorptiometer to detect, analyze, and compare the BMD of lumbar and femur to confirm the successful modeling.

In Vitro Culture and Grouped Treatment of Bone Marrow Mesenchymal Stem Cells

Once the rats in each group were anesthetized, the femur and tibia were exposed rapidly and the articular surfaces and metaphyses at both ends of the long bone were cut open to expose the medullary cavity. Then, the bone marrow in the medullary cavity was drawn to harvest the bone marrow mesenchymal stem cells, followed by pipetting and centrifugation. The harvested bone marrow mesenchymal stem cells were added into the prepared medium for resuspension, seeded into a culture dish at a density of 10^5 /mL, and placed in a cell incubator for culture. The medium was replaced every 3 d, and the culture was continued after passage. The cells in the third passage were used for experiments.

The bone marrow mesenchymal stem cells from the rats in normal group were divided into normal group and inhibitor group, while those from the rat model of osteoporosis were enrolled into model group. Osteogenic induction: the cells were seeded into a 6-well plate at a density of 10^5 cells/well for culture, and osteogenic induction was conducted after cell fusion. The osteogenic induction medium was directly added into the

cells in normal group and model group, and the cells were collected for detection after 3 days of induction. In inhibitor group, the cells were processed with 1 $\mu\text{mol/L}$ inhibitor LY294002 for 24 h first. Then, the osteogenic induction medium was added for induction for 3 d, after which the cells were collected for detection.

Immunohistochemistry and ALP Staining

After the completion of the intervention, the culture medium was removed and the cells were fixed with 4% paraformaldehyde for 4 h. Next, endogenous peroxidase blocker was added dropwise onto the specimens and reacted for 10 min after rinsing. Subsequently, the specimens were rinsed and added with goat serum for sealing for 20 min. The anti-TAZ primary antibody (1:200) was added after the goat serum blocking buffer was shaken off, followed by culture in a refrigerator at 4°C overnight. On the next day, the specimens were rinsed and added with the secondary antibody solution for a reaction for 10 min. After rinsing adequately, the streptavidin-peroxidase solution was added for a reaction for 10 min, followed by color development with diaminobenzidine (DAB) added in drops, counterstaining of the nucleus with hematoxylin, mounting, and observation.

At 7 d after the osteogenic induction, the culture medium was removed, and the cells were fixed with fixative at room temperature for 1 min after rinsing. The staining solution was prepared in accordance with the kit instructions and added into the 6-well plate in drops, followed by reaction at room temperature for X h and observation. After rising, the cells were digested with trypsin and harvested, followed by centrifugation at 12,000 g for 5 min and lysis. Later, the ALP staining was quantified using p-nitrophenyl phosphate method and the average optical density at 450 nm in each well was measured.

Detection of Cell Proliferation Via Cell Counting Kit-8 (CCK-8) Assay

The cells in each group were seeded in a 96-well plate at a density of 10^5 cells/well. The cells in normal group and model group were cultured normally without any treatment, while those in inhibitor group were cultured in a medium containing 1 $\mu\text{mol/L}$ inhibitor LY294002. After culturing for 24 h, the CCK-8 reagent (Dojindo Laboratories, Kumamoto, Japan) was added to each group, and the cell proliferation rate in each group was detected according to the CCK-8 instructions.

Western Blotting

The lysis buffer was added into the cells for an ice bath for 1 h. Then, the cells were put into a centrifuge for centrifugation at 14,000 g for 10 min, and the protein was quantified using the BCA method. Next, the absorbance and standard curve of the protein were obtained through a microplate reader, which were applied to calculate the protein concentration in tissues. Subsequently, the proteins in tissue specimens were subjected to denaturation and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separation. The position of the Marker proteins was observed and the electrophoresis was stopped when the Marker proteins reached the bottom of the glass plate in a straight line. Then, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and reacted with blocking buffer for 1.5 h. After that, anti-p-Akt primary antibody (1:1000), anti-RUNX2 primary antibody (1:1000), anti-BMP-2 primary antibody, and secondary antibody (1:1000) were added in sequence. Later, the proteins were subjected to sufficient color development with the chemiluminescent reagent in the dark for 1 min after rinsing.

Detection Via qPCR

The knee joint tissue stored for standby use was added into the ribonucleic acid (RNA) extraction reagent to extract the total RNA in the specimens. Then, the extracted total RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the reverse transcription kit, with a reaction system of 20 μL . Reaction conditions are as follows: reaction at 53°C for 5 min, pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 s, and annealing at 62°C for 30 s for 35 cycles. ΔCt was calculated first, and then the expression difference of target genes was calculated. The detailed primer sequences are shown in Table I.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was adopted for statistical analysis. The enumeration data were expressed as mean \pm standard deviation. Comparison between multiple groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). *t*-test was performed for data meeting normal distribution and homogeneity of variance, corrected *t*-test for those meeting normal distribution and

Table I. Primer sequences.

Name	Primer sequence
TAZ	Forward primer: 5'ATGTTGACCTCCGGACTTTGG3' Reverse primer: 5'GAGGAAGGGCTCGCTTTTGT 3'
GAPDH	Forward primer: 5'ACGGCAAGTTCAACGGCACAG 3' Reverse primer: 5'GAAGACGCCAGTAGACTCCACGAC 3'

heterogeneity of variance, and non-parametric test for those not meeting normal distribution and homogeneity of variance. The rank-sum test was utilized for the ranked data and the chi-square test for the enumeration data. $p < 0.05$ was considered statistically significant.

Results

Detection of BMD

As shown in Figure 1, the BMD of the femur and lumbar was (0.27 ± 0.03) and (0.31 ± 0.04) , respectively, in normal group, which was (0.18 ± 0.05) and (0.21 ± 0.04) , respectively, in model group. There were significant differences between the two groups ($p < 0.05$), suggesting that the rat model of osteoporosis was established successfully in this experiment.

ALP Staining for Osteogenesis

It was displayed in Figure 2 that the ALP-positive cells were in blue-violet, indicating the osteoblasts. ALP-positive cells were more in normal group but fewer in model group and inhibitor group. According to the statistical analysis of the

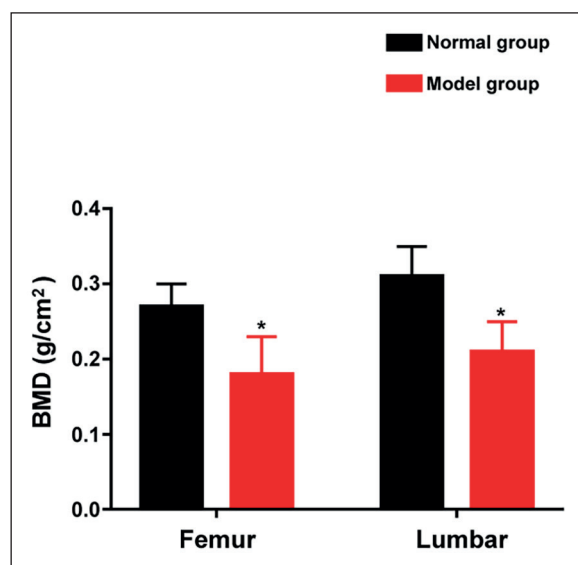


Figure 1. BMD in each group. Note: * $p < 0.05$ vs. normal group.

average optical density of ALP staining (Figure 3), the average optical density of ALP-positive cells was decreased markedly in model group and inhibitor group compared with that in normal

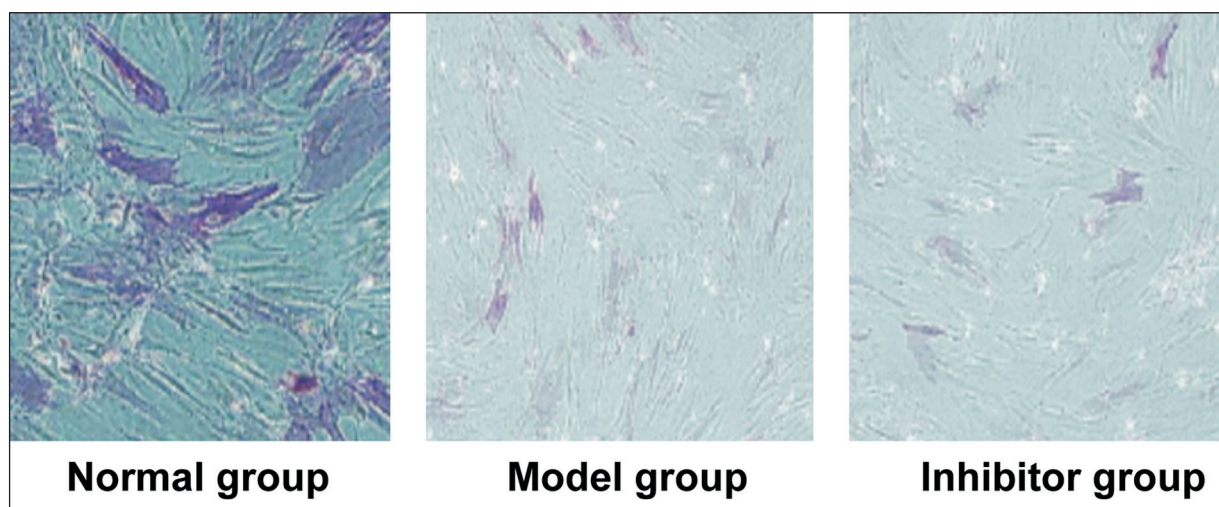


Figure 2. ALP staining in each group ($\times 200$).

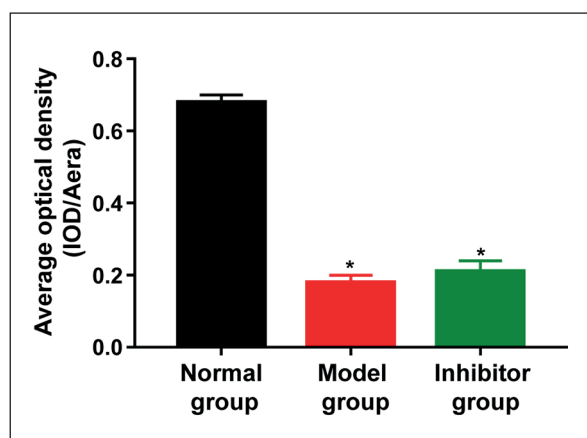


Figure 3. Average optical density of ALP-positive cells in each group. Note: * $p < 0.05$ vs. normal group.

group, with statistically significant differences ($p < 0.05$). However, no significant difference in the average optical density of ALP-positive cells was discovered between model group and inhibitor group ($p > 0.05$).

Expression of TAZ Detected Via Immunohistochemistry

The positive expression of TAZ was in sepia. There was a more positive expression of TAZ in normal group and inhibitor group and less posi-

tive expression of TAZ in model group (Figure 4). The statistical results (Figure 5) manifested that the average optical density of cells with TAZ positive expression declined significantly in model group in comparison with that in normal group, displaying a statistically significant difference ($p < 0.05$). The difference in the average optical density of cells with TAZ positive expression between normal group and inhibitor group was not significant ($p > 0.05$).

Relative Expressions of Related Proteins Detected Via Western Blotting

As shown in Figure 6, normal group had higher expressions of p-Akt and BMP-2 proteins than model group and inhibitor group. According to the statistical results (Figure 7), the relative expressions of p-Akt and BMP-2 proteins were reduced remarkably in model group and inhibitor group compared with those in normal group, and the differences were statistically significant ($p < 0.05$). There were no significant differences in the relative expressions of p-Akt and BMP-2 proteins between model group and inhibitor group ($p > 0.05$).

The expression of TAZ mRNA was decreased evidently in model group in comparison with that in normal group, displaying a statistically significant difference ($p < 0.05$), while no significant difference was detected between normal group and inhibitor group ($p > 0.05$) (Figure 8).

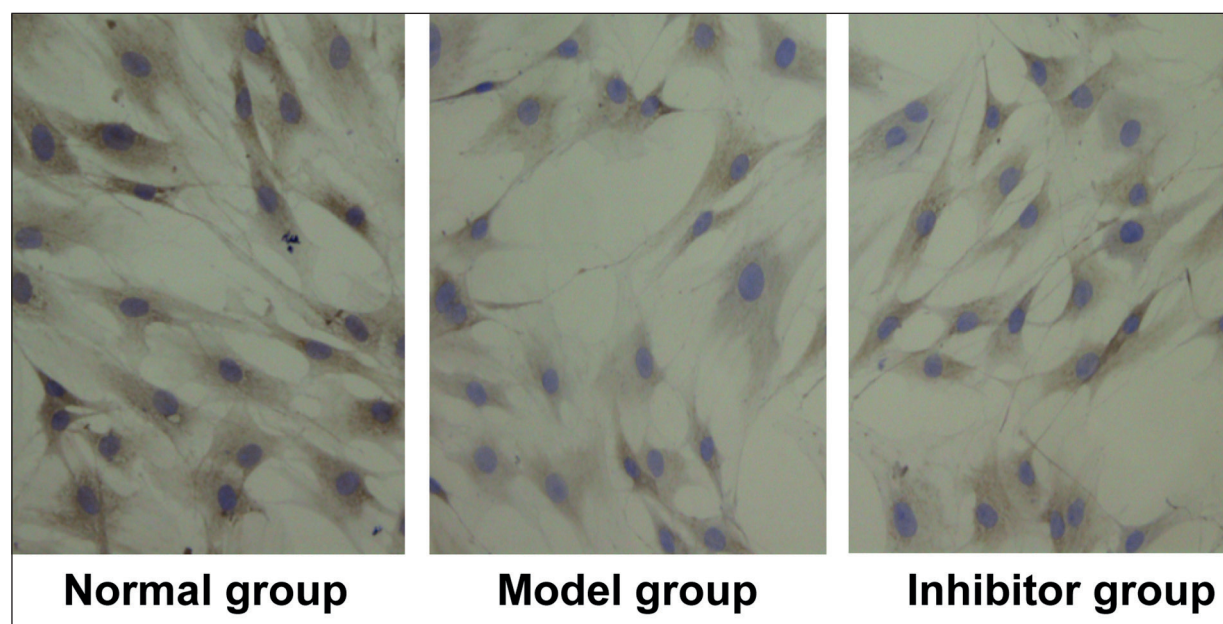


Figure 4. Positive expression of TAZ in each group ($\times 200$).

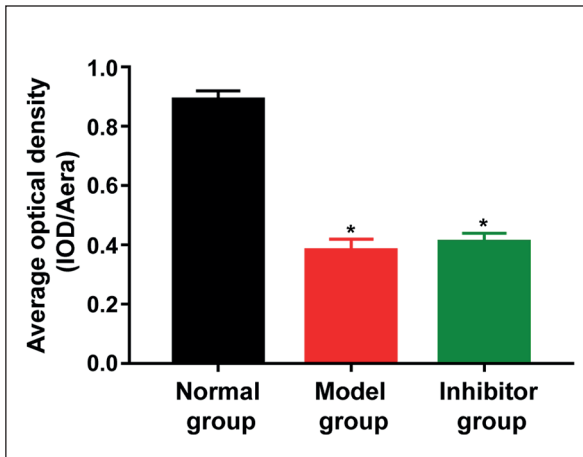


Figure 5. Average optical density of TAZ positive expression. Note: * $p < 0.05$ vs. normal group.

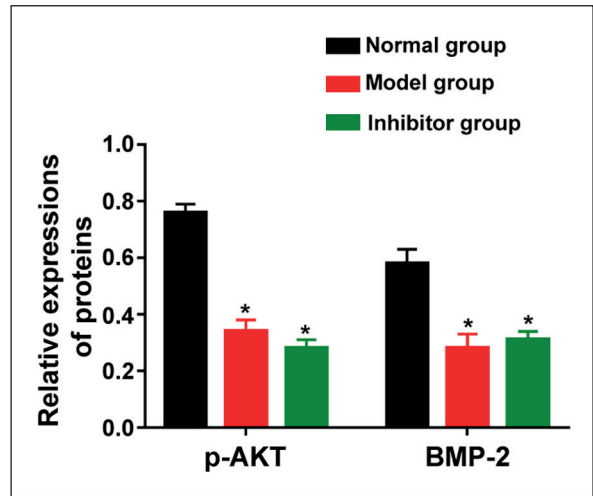


Figure 7. Relative expressions of proteins in each group. Note: * $p < 0.05$ vs. normal group.

Cell Proliferation Detected Via CCK-8

Model group and inhibitor group had prominently higher cell proliferation rates than normal group, with statistically significant differences ($p < 0.05$). Compared with that in model group, the cell proliferation rate was lowered in inhibitor group, and the difference was statistically significant ($p < 0.05$) (Figure 9).

Discussion

Osteoporosis is a severe disease threatening the health and quality of life of the elderly, whose incidence rate is rising year by year with the progression of population aging in the society. In particular, the incidence rate of osteoporosis is extremely high among the postmenopausal elderly women, and complications induced thereby, such as pain and osteoporotic fracture, seriously affect

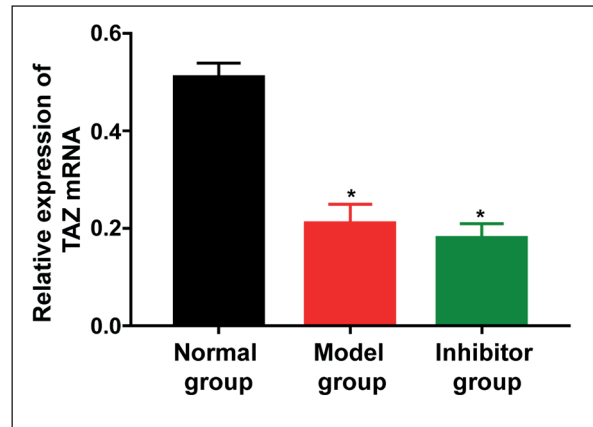


Figure 8. Relative expression of TAZ mRNA in each group. Note: * $p < 0.05$ vs. normal group.

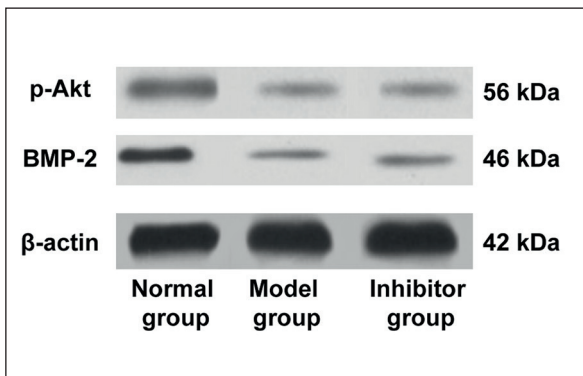


Figure 6. Protein expressions in each group.

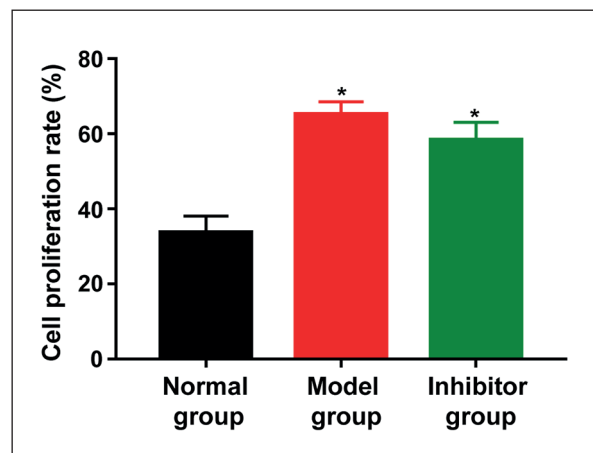


Figure 9. Cell proliferation rate in each group. Note: * $p < 0.05$ vs. normal group.

the patient's physical and mental health, as well as quality of life. Besides, substantial human, material, and financial resources have been put into the prevention and treatment of the disease by the whole society⁷. Therefore, it is of significant importance for the clinical treatment of osteoporosis to deeply study its pathogenesis. Currently, it is argued that the occurrence of osteoporosis is closely correlated with bone metabolism disorder caused by the dysfunctions and disproportions of osteoclasts and osteoblasts in the body. Reports^{8,9} have revealed that the content of estrogen in postmenopausal women declined dramatically, which results in osteoclast hyperfunction and osteoblast inhibition in the body, thus enabling the osteoclast-induced bone destruction to significantly exceed the osteoblast-induced bone formation and leading to osteopenia. Meanwhile, the bone marrow mesenchymal stem cells are the major source of osteoblast and osteoclast differentiation in the body, while the proportion of massive bone marrow mesenchymal stem cells differentiating into the osteoblasts is decreased under the stimulation of reduced estrogen, further influencing the bone formation and leading to osteopenia. In this experiment, castration of male and female rats was adopted to remove their ovaries to cause an abrupt decrease in the estrogen. At 12 weeks after modeling, the BMD of femur and lumbar of the rats was detected and it was shown that the BMD of the castrated rats declined prominently, illustrating that the method for preparing the rat model of osteoporosis is feasible, and the model was successfully established in this experiment.

As a vital category of stem cells in the body, characterized by proliferation and differentiation, the bone marrow mesenchymal stem cells can differentiate into cell types with specific actions, such as osteoblasts and osteoclasts¹⁰. It is currently believed that many important cells signaling pathways participate in controlling the fate of bone marrow mesenchymal stem cells, in which they can regulate the cells to maintain in the proliferative status or to differentiate toward a certain type of cells. The PI3K/Akt signaling pathway, a crucial cell signal transduction pathway, has been verified to play important regulatory roles in such physiological and pathological processes as cell proliferation, differentiation, apoptosis, and necrosis. Researches^{11,12} have discovered that the PI3K/Akt cell signaling pathway can activate β -catenin and its phosphorylation. Also, it can promote the transcription of target genes and translation of proteins in downstream signaling

pathways. In the meantime, the p-Akt produced by the phosphorylation of Akt in the PI3K/Akt signaling pathway is capable of promoting the phosphorylation of β -catenin and triggering cell proliferation^{13,14}. In a word, the PI3K and P85 in the cells can be activated by various stimulations to recruit and activate P110 around the cells, thereby exerting effects on and activating phosphatidylinositol-bisphosphate on the surface of the cell membrane, forming a large number of phosphatidylinositol-triphosphate¹⁵. As a second messenger, phosphatidylinositol-triphosphate can further activate the phosphorylation of Akt to generate p-Akt, thus playing a role in signal transduction, exerting synergistic effects *via* the downstream GSK3 β and Wnt pathways and regulating the occurrence of cell differentiation^{16,17}. Therefore, the PI3K/Akt signaling pathway is capable of controlling the fate of bone marrow mesenchymal stem cells, whose activation can accelerate the differentiation of bone marrow mesenchymal stem cells, and its repression can regulate the proliferation of those cells. According to the results of this experiment, in the bone marrow mesenchymal stem cells of osteoporosis rats, the PI3K/Akt signaling pathway was inhibited, the expression level of p-Akt protein was decreased notably, and the cell proliferation rate was remarkably higher than that in normal bone marrow mesenchymal stem cells. As a result, it is essential to effectively regulate the PI3K/Akt signaling pathway to facilitate the differentiation of bone marrow mesenchymal stem cells into osteoblasts.

TAZ, a kind of transcriptional coactivator and a vital effector molecule, can affect multiple downstream cell signaling pathways, thereby exerting crucial regulatory effects in cell proliferation, differentiation, and other physiological processes^{18,19}. In this research, the expressions of TAZ and TAZ mRNA, as well as the proliferation rate of bone marrow mesenchymal stem cells in osteoporosis rats, were markedly lower than those in normal bone marrow mesenchymal stem cells, indicating that the expression of TAZ is abnormal in the bone marrow mesenchymal stem cells of osteoporosis rats, thus leading to persistent cell proliferation and weakened osteogenic capacity. Taken together, these are all involved in the pathogenesis of osteoporosis. It was further discovered that after applying the inhibitor of the PI3K/Akt signaling pathway, the effect of TAZ on the bone marrow mesenchymal stem cells was attenuated prominently, the

osteogenic differentiation capacity of normal bone marrow mesenchymal stem cells was decreased, and their proliferation capacity was enhanced. Therefore, TAZ promotes the osteogenic differentiation of mesenchymal stem cells in the rat model of osteoporosis by repressing the PI3K/Akt signal.

Conclusions

We demonstrated that TAZ promotes the osteogenic differentiation of mesenchymal stem cells in the rat model of osteoporosis by repressing the PI3K/Akt signal.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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