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Effect of lncRNA AK023948 on rats with postmenopausal osteoporosis *via* PI3K/AKT signaling pathway

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Abstract. – OBJECTIVE: To investigate the effect of long non-coding ribonucleic acid (lncRNA) AK023948 (AK0) on rats with postmenopausal osteoporosis *via* the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling pathway.

MATERIALS AND METHODS: Firstly, postmenopausal osteoporosis rat models were established to obtain osteoblasts. The phosphorylation level of AKT was analyzed by controlling the expression of AK0 gene in osteoblasts. Finally, XTT was used to analyze the proliferation of osteoblasts and the messenger ribonucleic acid (mRNA) expression level of caspase in AK0 gene knockout (KO) rat model.

RESULTS: In the bone tissue of postmenopausal osteoporosis rats, the levels of phospho-PI3K (p-PI3K), p-Akt, and p-phosphoinositide-dependent kinase-1(PDK1) were significantly decreased ($p<0.05$). In rat model osteoblasts, the overexpression of AK0 gene upregulated the phosphorylation level of AKT, while the interference with small interfering RNA (siRNA) in AK0 gene decreased that of AKT. Knocking out AK0 gene led to the down-regulation of phosphorylation level of AKT in cells. Moreover, if the AK0 gene was re-expressed in the KO rat model cells, the phosphorylation level of AKT was restored to a certain extent, but still lower than that after the overexpression of AK0 gene. Although the proliferation rate of osteoblasts in estrogen deficiency-related osteoporosis rats was low, the growth rate of osteoblasts with AK0 KO was remarkably lower than that in blank control group ($p<0.05$). It was also found that there was a certain correlation between AK0 gene and osteoblast apoptosis.

CONCLUSIONS: LncRNA AK0 can regulate the phosphorylation level of AKT in osteoblasts of rats with estrogen deficiency-related osteoporosis through the PI3K/AKT signaling pathway, thus regulating the proliferation of osteoblasts. It is speculated that lncRNA AK0 may be an important factor in regulating the PI3K/AKT signaling pathway.

Key Words:
LncRNA, AK023948 gene, PI3K/AKT signaling pathway, Postmenopausal osteoporosis.

Introduction

Osteoporosis is a systemic bone disease, mainly characterized by bone density reduction, bone mass decrease, microstructure degeneration of bone tissues, and fragile bone with a tendency to fracture[1,2]. Osteoporosis can be caused by many factors such as aging, heredity, and estrogen deficiency[3]. Menopausal women may suffer from estrogen deficiency due to ovarian hypofunction, thus inducing osteoporosis, which will lead to loss of bone mass and fragility of bone, and even cause the osteoporotic fracture in severe cases[4]. Therefore, the study on osteoporosis induced by estrogen deficiency has attracted much attention.

The phosphoinositide 3-kinase (PI3K)/ protein kinase B (AKT) is a vital signaling pathway which participates in regulating the proliferation, death, and survival of cells, and serves as a precondition for intervention treatment[5]. Some studies[6-8] on the PI3K signaling pathway have denoted that AKT and its related downstream signals are key factors for regulating endochondral ossification. AKT can affect bone formation and osteoblast survival by maintaining the class O forkhead box transcription factors (FOXOs) in the cytoplasm[9]. All these studies have indicated that the PI3K/AKT signaling pathway is closely related to the occurrence of osteoporosis.

Long non-coding ribonucleic acids (lncRNAs), discovered by Yamashita et al[10] in 2002, have been reported in studies since then to participate in various biological processes, including lncRNA-DNA, lncRNA-microRNA, and ln-

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cRNA-protein[11]. LncRNAs are equipped with multiple regulatory functions: they are closely related to various diseases of human beings, like tumor[12] and Alzheimer's disease[13]. By studying differential expressions of lncRNAs in patients with rheumatoid arthritis, researchers speculated that lncRNAs may play an important regulatory role in the occurrence and development of arthritis disease[14]. Hence, Jiang et al[15] determined the expression of lncRNAs in synovial tissues of osteoporosis rats, screened 260 kinds of lncRNAs with the common differential expression, and found that these lncRNAs participate in and regulate some important signal transduction pathways in cells. Therefore, lncRNAs are closely related to the occurrence and development of osteoporosis. However, it remains unclear through which pathway lncRNAs regulate osteoblasts and osteoclasts as they have a wide range of regulatory effects. In 2017, Koirala et al[16] denoted that AK023948 (AK0), a lncRNA, has positive regulation on AKT in the PI3K/AKT signaling pathway. The main purpose of this study is to investigate the effect of AK0 on estrogen deficiency-related osteoporosis in rats as the experimental model.

Materials and Methods

Reagents

Pentobarbital sodium (purity >99%) was purchased from Sinopharm Group Co., Ltd. (Beijing, China). P-PI3K, p-AKT, and AKT antibodies, Tris, NaCl, ethylene diamine tetraacetic acid (EDTA), nonyl phenoxypolyethoxylethanol (NP-40), and glycerol were bought from Sigma-Aldrich (St. Louis, MO, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin were purchased from Shanghai Yuyou Biology Company (Shanghai, China). Small interfering RNA (siRNA) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and AK0 LNA probe from Eton Bioscience (San Diego, CA, USA).

Animal Experiments

Thirty female SD rats (180-210 g) purchased from Shanghai Kaixue Biotechnology Co., Ltd. (Shanghai, China) were divided into three groups: blank control group (Control group), sham operation group (Sham group), and model group [Ovariectomy (OVX) group], with 10 rats in each one. After all rats were adaptively fed for one week, the rats in Sham group and OVX group were injected with pentobarbital sodium for anesthesia. Then, bilateral OVX was performed with abdominal fixation, and those in Sham group were removed with fat instead of ovaries. They were cultured for 2 months after the operation. The serum of rats was isolated and the concentration levels of calcium ion, phosphorus ion, and alkaline phosphatase (ALP) were determined. The bilateral femur and spinal column of rats were made into pathological sections to observe the histological structure of femur. Bone mineral density of spine was measured with the help of the bone densitometer.

The operation procedure of animal *in vivo* experiment strictly followed the methods described in Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. This investigation was approved by the Animal Ethics Committee of Jinan Traditional Chinese Medicine Hospital Animal Center. All animals received humanitarian care as presented in the Guide for the Care and Use of Experimental Animals (Animal Care Association, 2002).

Cell Culture

Osteoblasts were isolated from the postmenopausal osteoporosis rat model and cultured in a matrix containing 10% fetal bovine serum (FBS; Hyclone, South Logan, UT, USA), 1% penicillin-streptomycin and 5% CO_2 at 37°C. Cells were collected on the 5th, 10th, and 14th day of cell culture, and the cell concentration was tested with the cell proliferation kit (XTT).

Calcium Concentration Analysis

The calcium concentration in serum was analyzed based on instructions of Sigma-Aldrich (St. Louis, MO, USA) Diagnostic Kit 587.

Phosphorus Concentration Analysis

The phosphorus concentration in serum was analyzed by molybdic acid method according to the instructions of the kit.

ALP Activity Analysis

The activity of ALP in cells was analyzed as described in reference[17]. The cells needed for the experiment were collected, the matrix was removed, the cells were washed with phosphate-buffered saline (PBS), and then placed in lysis buffer (20 nM Tris, pH =8.0, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% NP-40, 10% glycerol). A total of 20 μL of cell lysate was mixed with 100 μL of Tris-glycine buffer, 2 mM of $MgCl_2$, and 100 μL of p-nitro-

phenyl phosphate. After incubating the mixture at 37°C for 30 min, 50 μL of NaOH was added to stop the reaction. The absorbance value of various samples was tested at 405 nm using the microplate reader.

XTT Analysis

The cell concentration was tested using the Sigma-Aldrich (St. Louis, MO, USA) XTT cell visibility kit. Cell samples cultured for 5, 10, and 14 days were taken to remove the matrix, and 100 μL of fresh matrix and 25 μL of XTT solvent were added to each sample. After incubation for 5 h, the cell density of each sample was determined with the microplate reader at 450 nm of absorbance.

Plasmid Construction

AK0 expression vector was constructed on pCDH-MSCV-EF1-GFP-T2A-Pu. Koirala et al[16] showed that an expression vector of siRNA interfering AK0 gene was constructed, and the AK0 gene in osteoblasts was knocked out *via* the CRISPR/Cas9 method. High fidelity deoxyribonucleic acid (DNA) ligase was adopted for polymerase chain reaction (PCR) amplification. All PCR products were verified by DNA sequencing.

Transfection

Koirala et al[16] found that DNA plasmids or siRNAs were transfected into osteoblasts of postmenopausal osteoporosis rats with DNAfectin or RNAfection, respectively.

Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)

The levels of PI3K, AKT, phosphoinositide-dependent kinase-1 (PDK1), caspase 3, caspase 7, and caspase 9 in cells were detected *via* qRT-PCR. The reaction conditions are as follows: 50°C for 2 min for 1 cycle, 95°C for 15 min, 95°C for 15 s for 40 cycles, 60°C for 30 s, and 72°C for 30 s. The primers used in this study were shown in Table I.

Western Blotting

After the lysis buffer was prepared, the cultured cells were placed in it. Cell proteins were collected after some time, treated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and detected with its corresponding antibody.

Statistical Analysis

The data collected in this experiment were analyzed by one-way variance (ANOVA) followed by Post-Hoc Test (Least Significant Difference) using SPSS 19.0 software (IBM, Armonk, NY, USA). $p<0.05$ suggested that the difference was statistically significant.

Results

Establishment of Rat Models of Menopausal Osteoporosis

At first, 30 rats were divided into three groups: Control group, Sham group, and OVX group. Two months after OVX, indicators in the serum of rats were measured (Table I). According to the observation of sections and bone mineral density analysis (Figure 1), there was no false positive osteoporosis sample in the operation, and the postmenopausal osteoporosis rat models were successfully established.

Table I. The primers used in this study.

Gene		Primer sequences
PI3K	Forward	5'-CACTCAGCCCATCTATTTCCAG-3'
	Reverse	5'-TCTTGGATCTTCACCTTCAGC-3'
Akt	Forward	5'-GACTGACACCAGGTATTTCGATGA-3'
	Reverse	5'-CTCCGCTCACTGTCCACACA-3'
PDK1	Forward	5'-CTATGCTGTGTTACTTCTTGGAGCACAG-3'
	Reverse	5'-TGCCGAATATCATGGTGGAAAATGGCCG-3'
Caspase 3	Forward	5'-CTCGCTCTGGTACGGATGTG-3'
	Reverse	5'-TCCCATAAATGACCCCTTCATCA-3'
Caspase 7	Forward	5'-GAAGAGGCTCCTGGT TTGTG-3'
	Reverse	5'-TCATGGAAG TGTGGGTCATC-3'
Caspase 9	Forward	5'-GGCTGTTAAACCCCTAGACCA-3'
	Reverse	5'-TGACGGGTCCAGCTTCACTA-3'
GAPDH	Forward	5'-ACTGTGCCGACTTGACGTTT-3'
	Reverse	5'-ATCGTAGTGAACGGTCGATTGT-3'

Expression Level of PI3K/AKT Signaling Pathway in Rat Model

To investigate the effect of the PI3K/AKT signaling pathway on estrogen deficiency-related osteoporosis rat models, Western blotting and RT-PCR were employed to detect the activity of the PI3K/AKT signaling pathway in three groups of rat models. The results showed that the levels of p-PI3K, p-AKT, and p-PDK1 were significantly decreased in postmenopausal osteoporotic rats ($p<0.05$, Figure 2A), but there were no significant differences in mRNA expressions of PI3K, AKT, and PDK1 ($p>0.05$, Figure 2B). The above results confirm that the PI3K/AKT signaling pathway plays a key role in the regulation of osteoporosis.

Effect of AK0 on AKT Activity in Rat Model Osteoblasts

After the rat osteoblasts were obtained, the effects of AK0 overexpression, RNA interference (AK0 siRNA), AK0 KO, and re-expression of AK0 in AK0 KO cells on AKT activity were explored respectively. Molecular experiments in rat osteoblasts were conducted by transfecting the constructed plasmid into rat cells. The results showed that the phosphorylation level of AKT was still low in the rat model (Figure 3). The overexpression of AK0 enhanced the phosphorylation of AKT, while the interference with RNA in AK0 gene reduced the phosphorylation of AKT (Figure 3A). After the AK0 gene was knocked out, the phosphorylation level of AKT

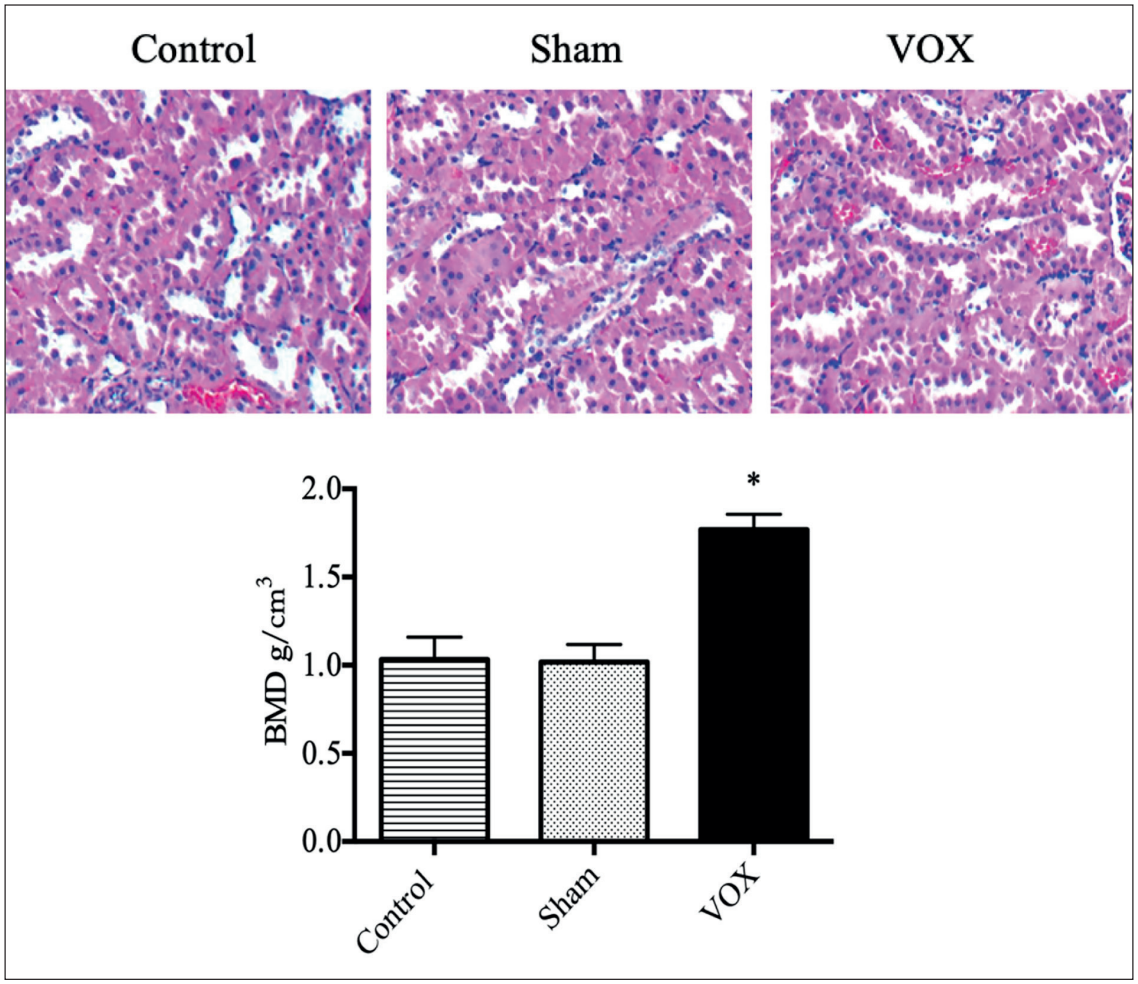

Figure 1. Observation of pathological sections and bone mineral density comparison of femur of rats in three groups (magnification 200×) [* indicates there is a significant difference ($p<0.05$)].

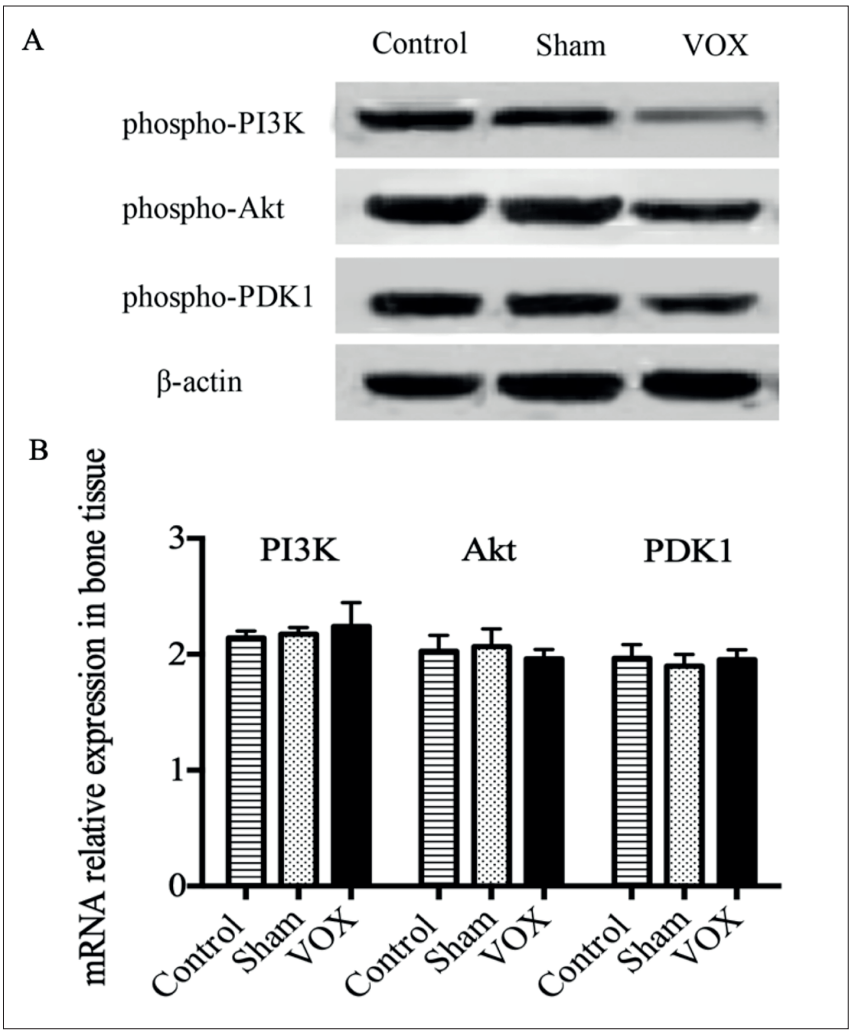

Figure 2. Changes of PI3K/AKT signaling pathway in postmenopausal osteoporosis rats.

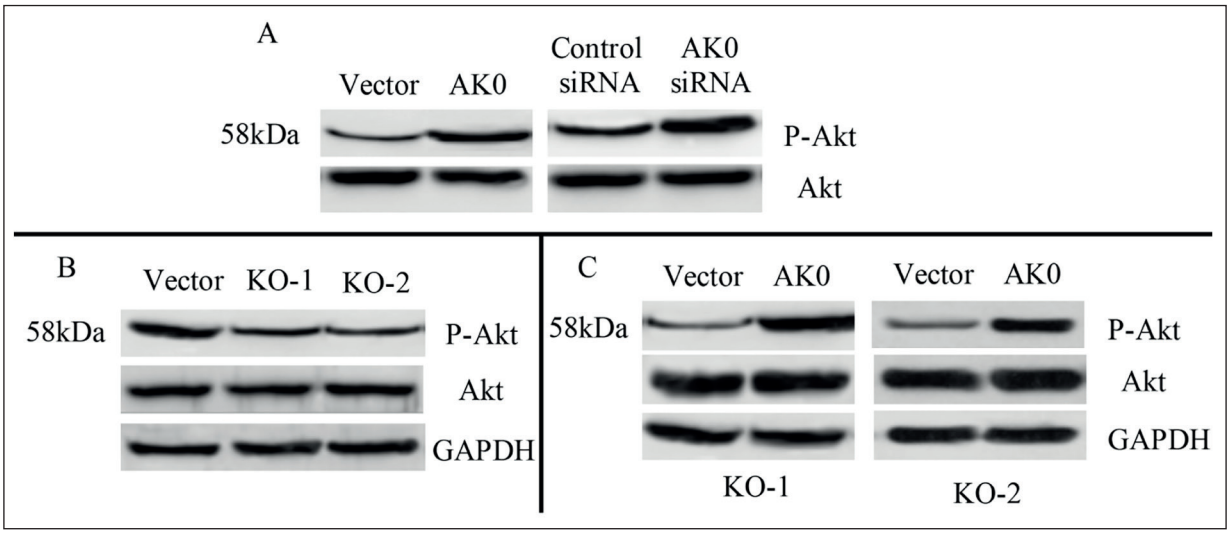

Figure 3. Effect of AK0 on AKT activity in rat models: **A,** overexpression of AK0 gene and interference with siRNA in AK0 gene, **B,** AK0 gene KO, **C,** re-expression of Ak0 gene in KO cells.

was lowered (Figure 3B). However, if the AK0 gene was re-expressed in the KO rat model cells, the phosphorylation level of AKT was restored to a certain extent, but lower than that after the overexpression of AK0 gene (Figure 3C). The results suggest that AK0 gene plays a key role in regulating the phosphorylation level of AKT in osteoblasts of rat models with estrogen deficiency-related osteoporosis.

Effects of AK0 KO on PI3K/AKT Signaling Pathway

To further clarify the effect of AK0 gene, an experiment was designed to investigate the influence of AK0 gene KO on the proliferation of rat osteoblasts. The osteoblasts with AK0 KO were cultured for 14 days, and cell samples were taken on the 5th, 10th, and 14th d for analysis and compared with the blank control without KO. The results indicated that although the proliferation rate of osteoblasts in estrogen deficiency-related osteoporosis rats was low, the growth rate of osteoblasts in rats with AK0 KO was significantly lower than that in the control samples (*p<0.05; Figure 4), suggesting that AK0 gene affects the proliferation of osteoblasts by regulating the PI3K/AKT signaling pathway.

Moreover, on the basis of the exploration of cell proliferation, this experiment selected rat osteoblasts on the 14th day, and further compared the mRNA expression levels of caspase-3, caspase-7, and caspase-9 of rat osteoblasts after AK0 gene KO after 14 days of culture. The effect of AK0 on osteoblast apoptosis was deduced. The results presented that the mRNA expression levels of caspase-3, caspase-7, and caspase-9 were notably higher than those of the non-KO group (*p<0.05; Figure 5), which point out that AK0 gene has a certain correlation with osteoblast apoptosis.

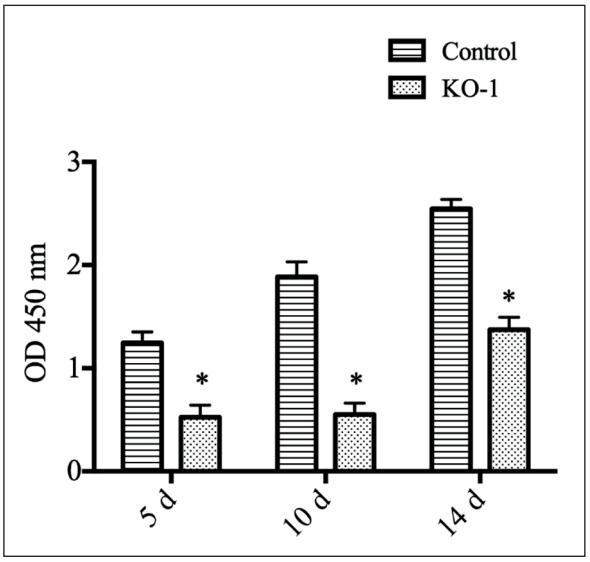

Figure 4. Effect of AK0 gene KO on the proliferation of rat osteoblasts [* suggests there is a significant difference (p<0.05)].

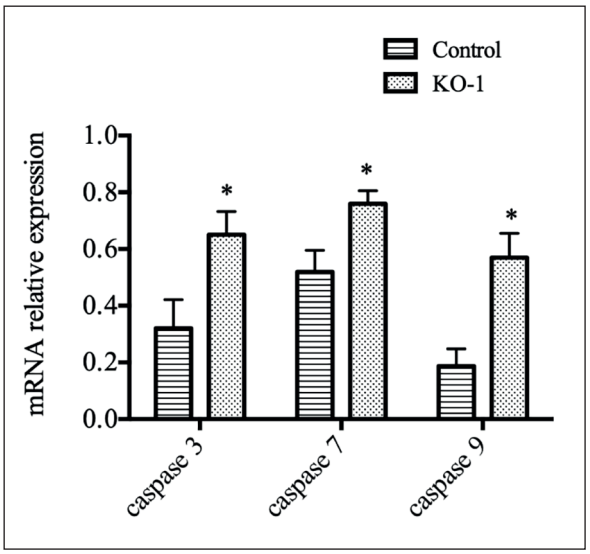

Figure 5. Effect of AK0 gene KO on the proliferation of rat osteoblasts [* suggests there is a significant difference (p<0.05)].

Discussion

Epidemiological statistics demonstrate that the number of osteoporosis patients worldwide increases distinctly every year, and at this rate, it is estimated that the medical expenses on osteoporosis-induced fractures will exceed 25.3 billion USD by 2025[18]. In particular, the lack of estrogen secretion in postmenopausal women will lead to an overt increase in their susceptibility to osteoporosis[19]. Osteoporosis has seriously disturbed people's lives, so it has attracted the attention of researchers.

The PI3K/AKT signaling pathway can regulate the activity of osteoblasts and osteoclasts, thus controlling the balance of bone density in bone cells. Although the PI3K/AKT signaling pathway has been determined to be an important factor in regulating osteoporosis, which upstream factors can regulate the PI3K/AKT signaling pathway remains unclear. In 2017, Koirala et al[16] found

that AK0 gene, a lncRNA, has a positive regulatory effect on AKT in cells. Therefore, with the finding mentioned above as a reference, this study explored the effect of AK0 on postmenopausal osteoporosis rats through the PI3K/AKT signaling pathway.

Firstly, osteoporosis rat models were established. Compared with other types of animals, rats boast advantages of low cost, easy nurture, and the like, and the characteristics of bone metabolism of rats after castration are very close to those of postmenopausal women with osteoporosis, which can well simulate estrogen deficiency-related osteoporosis. Hence, the ovariectomized rats were selected as research models. In this study, whether the estrogen deficiency-related osteoporosis rat models were successfully established was judged by the levels of calcium, phosphorus, and ALP in rat serum as well as the observation of pathological sections and bone mineral density.

After the establishment of rat models, the influence of the PI3K/AKT signaling pathway on rat models was explored by Western blotting and RT-PCR. The results revealed that the levels of p-PI3K, p-AKT, and p-PDK1 in osteoporosis rats were markedly lower than those in blank control group, which verified that the PI3K/AKT signaling pathway is an important factor in regulating the occurrence and development of osteoporosis. Next, taking the results of the p-AKT as a reference, the regulation of lncRNA AK0 on AKT activity was discussed.

LncRNA AK0 is an exon gene, 2807 bp long. In this experiment, AK0 gene was transfected into rat model osteoblasts, and the expression level of p-AKT was assayed by Western blotting. It was discovered that the overexpression of AK0 gene and its re-expression in cells with AK0 KO can up-regulate the phosphorylation level of AKT. Additionally, in the case of interference or knockout of the expression of AK0 gene, the phosphorylation level of AKT was markedly reduced, which presents that AK0 gene plays a key role in regulating the phosphorylation level of AKT in osteoblasts of estrogen deficiency-related osteoporosis rat models.

To further reveal the influence of AK0 gene on rat model osteoblasts, this study investigated the effect of AK0 gene KO on the proliferation of rat osteoblasts. The results showed that the growth rate of rat osteoblasts with AK0 KO was remarkably lower than that of the control sample ($p<0.05$), suggesting that AK0 gene can affect the proliferation of osteoblasts by regulating the PI3K/AKT signaling pathway. Moreover, the mRNA expression of caspase in osteoblasts after 14 days of cell culture was determined. The results denoted that AK0 gene can regulate the level of caspase to affect the apoptosis of osteoblasts.

Conclusions

To sum up, lncRNA AK0 can regulate the phosphorylation level of AKT in osteoblasts of estrogen deficiency-related osteoporosis rats through the PI3K/AKT signaling pathway, thus affecting the proliferation of rat osteoblasts. As a result, it is speculated that lncRNA AK0 may be an important factor in regulating the PI3K/AKT signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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