Original Article

Activation of the extracellular-signal-regulated kinase (ERK)/c-Jun N-terminal kinase (JNK) signal pathway and osteogenic factors in subchondral bone of patients with knee osteoarthritis

Yaofeng Xu[1,2], Yuguo Gu[2], Wanbo Ji[2], Qirong Dong[1]

[1]Department of Orthopedics, the Second Affiliated Hospital of Soochow University, Suzhou, China; [2]Department of Orthopedics, Suzhou Hospital of Traditional Chinese Medicine Affiliated to Nanjing University of Traditional Chinese Medicine, Suzhou, China

Contributions: (I) Conception and design: Y Xu; (II) Administrative support: None; (III) Provision of study materials or patients: W Ji; (IV) Collection and assembly of data: Y Gu; (V) Data analysis and interpretation: Q Dong; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Qirong Dong. Department of Orthopedics, the Second Affiliated Hospital of Soochow University, 1055 Sanxiang Road, Suzhou 215004, China. Email: dongqirong@suda.edu.cn.

Background: The objectives of this study was to explore the activation of the extracellular-signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) signaling pathway and osteogenesis-related factors in the subchondral bone of patients with knee osteoarthritis (OA).

Methods: Ten patients with primary OA who underwent total knee arthroplasty in the Department of Arthritis Surgery of our hospital were enrolled, and subchondral bone tissue samples were obtained during the operation. He staining and saffron staining were used to observe the arrangement of chondrocytes in the patient tissues. The protein expression levels of JNK, p-JNK, ERK, p-ERK, Runx2 and OMD in subchondral bone were detected by Western Blot. Knee osteoarthritis mice were established. He staining was used to observe the arrangement of subchondral bone cells in the knee joint of mice. Cellular mineralized nodules were determined by alizarin red staining.

Results: Firstly, in general and staining, it was observed that the subchondral bone lesions of knee OA participants were obvious. Compared with normal knee joints, the levels of phosphorylation-c-Jun N-terminal kinase (P-JNK) and phosphorylation-extracellular-signal-regulated kinase (P-ERK) in the subchondral bone of knee arthritis participants were significantly increased ($P<0.05$). The level of osteomodulin (OMD) was significantly reduced ($P<0.05$). Secondly, compared with normal mice, the levels of JNK, P-JNK, OMD, ERK, and P-ERK in the model group were significantly different ($P<0.05$). At 2–8 weeks, the JNK and P-JNK levels in the mice model group increased significantly over time ($P<0.05$), and the OMD level decreased significantly over time ($P<0.05$). The levels of ERK and P-ERK fluctuated over time. Thirdly, osteoblasts were treated with different concentrations of anisomycin, and stained with alizarin red after continuous culture for 24 and 48 h, respectively. It was found that all the cells were stained with orange-red mineralized nodules. As the concentration of anisomycin was increased, the number of cell mineralization nodules was significantly larger, and the positive rate of chemical nodules increased. Different concentrations of anisomycin were given to interfere with the osteoblasts of mice. When anisomycin was administered at a dose of 25 ng, the OMD level reached the highest level. When the concentration of anisomycin was increased, the osteocalcin (OCN) level also showed an upward trend.

Conclusions: The process by which the JNK signaling pathway regulates OMD may be closely related to the pathological changes of subchondral bone in patients with knee OA, and is involved in the occurrence and development of knee arthritis.

Keywords: Arthritis; extracellular-signal-regulated kinase (ERK); c-Jun N-terminal kinase (JNK); osteogenesis-related factors; anisomycin

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Introduction

Osteoarthritis (OA) is a degenerative disease of joints, characterized by the gradual damage and erosion of articular cartilage, destruction of the articular surface, and exposure of subchondral bone. After long-term wear and tear on the exposed parts, the bone is prone to secondary hyperplasia, forming osteophytes, and subchondral bone sclerosis leading to joint deformities (1). Knee OA can cause chronic pain, restrict walking and movement, and reduce the quality of life, which will eventually lead to long-term pain and disability. At present, the pathophysiological and biochemical mechanisms of cartilage degeneration and pain induction have not been clarified. In 1972, Radin (2) first proposed the hypothesis that changes of subchondral bone is the initiating factor of OA. These changes have attracted the attention of many researchers. At present, the pathological changes are mainly considered to be vigorous bone metabolism, active but incomplete osteogenesis, and increased bone density but insufficient extracellular matrix mineralization. In the treatment of OA, the treatment of subchondral bone has also been paid increasing attention and has achieved remarkable curative effects, but the mechanism has not yet been fully elucidated.

Mitogen-activated protein kinases (MAPKs) are a type of protein kinases that are widely found in animal cells. They are important signal transduction pathways for cells to transmit information and regulate life activities. The signal pathways mainly include extracellular-signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) (3). The ERK and JNK signal transduction pathways are involved in cell growth, development, proliferation, differentiation, and other physiological and pathological processes. The ERK pathway is the most thoroughly studied MAPK pathway. It is highly expressed in OA and participates in and regulates the proliferation of OA chondrocytes and apoptosis (4). The JNK family is a conservative serine/threonine protein kinase, which is widely used in cell differentiation, apoptosis, stress response, and the occurrence and development of various diseases. It is an important regulatory target between normal state and disease state. Many diseases have been found to relate closely to activation of the JNK pathway, including diabetes, Parkinson's disease, and others, but currently there are few studies on OA and these pathways.

Through in-depth study of the pathological mechanism of arthritis, the abnormal expression and synthesis of type I collagen and low mineralization in human knee OA subchondral osteoblasts has been revealed as the main cause of abnormal bone metabolism. Osteomodulin/osteoadherin (OMD) is a monomeric protein in the extracellular matrix that interacts with type I collagen (5). Whether it is involved in the occurrence and development of OA, and its relationship with the ERK and JNK pathways is currently unknown. Therefore, this study mainly explored ERK and JNK pathway changes within pathologically changed knee OA subchondral bone. A mouse model of knee arthritis was established to explore changes in the levels of osteogenic factors, such as the ERK and JNK pathways and OMD, in the occurrence and development of arthritis. By culturing sclerotic bone osteoblasts in vitro, we explored the regulatory effect of the JNK-specific agonist, anisomycin, on the mineralization of sclerotic bone osteoblasts, and further determined the relationship between JNK and OMD.

We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/atm-21-1215).

Methods

General information

A total of 10 patients with primary OA who underwent artificial total knee arthroplasty were enrolled in our hospital. The average age of participants was 67.42±5.48 years, including 6 males and 4 females. The inclusion criteria were as follows: (I) diagnosed with knee OA according to the "Guidelines for the Diagnosis and Treatment of Osteoarthritis (2007 Edition)"; (II) severe knee joint pain, varying degrees of deformity, limited capacity for flexion and extension, and difficulty in daily life and walking; (III) conservative treatment had been ineffective or the effect was not significant; (IV) conventional X-ray examination of the knee joint at front and side view showed that the joint space was significantly narrowed or had even disappeared, the subchondral bone density increased, a large number of osteophytes formed on the joint edges, and the tibial

intercondylar crest became pointed and proliferated, and some showed bony loose body, in line with the 4th and 5th grades of the Ahlbäck classification system. The exclusion criteria were as follows: (I) recently suffered or is suffering from purulent knee joint infection; (II) There are uncured infections in the distance; (III) knee extension device rupture or severe loss of function; (IV) secondary to muscle weakness, reflex deformity, and painless, well-functioning knee joint fusion; (V) a history of arteriosclerosis in the affected limb, skin diseases such as psoriasis, neuropathic joint disease, morbid obesity, recurrent urinary tract infection, and osteomyelitis near the knee joint in the area of operation. In addition, the knee joint tissues of healthy people who needed amputation due to car accidents or other reasons were collected as normal controls. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The collection of all samples in this study was approved by the Second Affiliated Hospital of Soochow University (NO.:2021-021). Informed consent was taken from all individual participants.

Material and dyeing

The collected subchondral bone tissue was divided into 2 parts, and 1 part was immediately fixed in formalin solution, and then sent to the laboratory for staining microscopy. First, the soft tissues were rinsed with distilled water, and then soaked with different concentrations of alcohol to remove the water from the tissues. The alcohol concentration ranged from low to high, and the time for each level of alcohol soaking was 2 hours. Then, xylene was used to continue dehydration for 0.5–1 h until the specimen became transparent, indicating complete dehydration. Finally, the sample was embedded in paraffin, cut into 6 mm sections, and placed on a glass slide. The specimen was sliced for heat treatment and then stored at a low temperature. The other part of the cartilage tissue was rinsed 3 times with normal saline, dried with sterile gauze, and stored in liquid nitrogen in a cryotube.

Hematoxylin and eosin (HE) staining was performed following conventional dewaxing. After dewaxing, the sections were treated with 100% alcohol, 95% alcohol, 85% alcohol, 75% alcohol, and double distilled water for 3 minutes. Then, the slices that had been added to distilled water were stained in a hematoxylin aqueous solution for several minutes, and the ammonia water was separated. After coloring for a few seconds, they were rinsed with running water for 1 hour and added to distilled water for a while. Then, 70% and 90% alcohol were added for dehydration for 10 minutes, followed by alcohol eosin staining solution to dye for 2–3 min. Finally, the stained sections were dehydrated with pure alcohol, the sections were made transparent with xylene, and sealed with optical resin.

The samples were routinely deparaffinized to water prior to safranin staining. First, the samples were stained with freshly prepared Weigert dye solution for 3–5 min. They were then differentiated for 15 seconds in acid ethanol differentiation solution, and washed with distilled water for 1 min. Next, they were immersed in fast green dyeing solution for 5 min and washed with distilled water for 1 min. The slices were then stained in safranin O for 1–2 min, washed with distilled water for 1 min, washed with acetic acid solution for 1–2 min to remove residual solid green, and washed with distilled water for 1 min. Finally, the stained sections were dehydrated with pure alcohol, made transparent with xylene, and sealed with optical resin.

Detection of related proteins in subchondral bone by western blotting

After the subchondral bone was cut and homogenized, an appropriate amount of radioimmunoprecipitation assay (RIPA) lysis buffer was added. The samples were lysed on ice for 10 min, centrifuged at 15,000 r/min at –14 ℃ for 15 min, the sediment was discarded, supernatant was collected, and the total protein concentration was determined by bicinchoninic (BCA) method. Then, 30 g of protein was loaded on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After completion of the electrophoresis was, the membrane was transferred by the electro-membrane transfer machine, and sealed in 5% skimmed milk powder for 1 hour. The primary antibody (1:1,000) was added and incubated for 1 h. After washing with phosphate-buffered saline with Tween 20 (PBST), horseradish peroxidase (HRP) labeled rabbit anti-mouse IgG (1:2,000) secondary antibody was added and incubated for 1 h with B-actin as the internal control. Following enhanced chemiluminescence (ECL) reagent color development, Bio-Rad gel imaging system was used for calculation and statistical analysis of each group of bands, where each sample had 5 parallel holes.

Establish a rat model of knee OA

A total of 24 Sprague-Dawley rats, 10 weeks old, male or

female, weighing 225–250 g, were randomly divided into a model group (n=12) and control/sham operation group (n=12). The model group underwent anterior cruciate ligament (ACL) transaction and medial meniscus resection to construct a knee OA model. The rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal injection), and the right knee hair was shaved. In the model group of rats, an incision was made on the inside of the joint capsule (before the medial collateral ligament), the ACL was cut off and medial meniscus removed. After the operation, the joints were washed with saline and the skin was sutured. In the sham operation control group, the ACL was exposed through an incision on the inside of the joint capsule, but it was not cut and the medial meniscus was not removed. The joint capsule was opened, and the incision was closed layer by layer after flushing with saline.

Animal experiments were performed under a project license (NO.:2021-023) granted by Second Affiliated Hospital of Soochow University, in compliance with China guidelines for the care and use of animals.

Knee OA and tibial subchondral sclerotic bone osteoblast culture and anisomycin intervention

The model mice were euthanized by spinal bleaching, and the leg skin and subcutaneous muscle tissue were incised layer by layer under aseptic conditions. The femur, tibia, and fibula were then separated with a scalpel. A sterilized scalpel was used to carefully cut along the closing line of the bone growth plate, then the epiphyseal plate (rich in chondrocytes, fibroblasts, and bone marrow stem cells) was peeled off at the growth end of the femur and tibia, and discarded. The muscle tissue attached to the bone was thoroughly removed, and the bone was soaked in a sterile PBS solution containing 5% bi-antibody for 30 min, and then transferred to a biological safety cabinet. The long bone was placed in a 10 cm cell culture dish, and a 2% sterile PBS solution of the double antibody was repeatedly pipetted to rinse the long bone 3 times. The periosteum attached to the surface of the bone was scraped with a scalpel, and the irregular bone segments about 3 mm at the ends of the long bones were cut and discarded (it is difficult to completely remove the muscle fiber tissue at the ends, which allows foreign cells being introduced easily). Sterile rongeurs and hemostatic forceps were used to remove the bones and clip them into pieces about 2 mm × 2 mm. The bone fragments were washed twice with a sterile PBS solution containing 1% bi-antibody, bone fragments were gathered in a 15 mL centrifuge tube, and an appropriate amount (to submerge all bone fragments) of sterile PBS solution containing 1% bi-antibody was added to the tube. The centrifuge tube was placed on a vortex shaker for 10 seconds, and allowed to stand for another 10 seconds. This process was repeated 5 times, to remove any red bone marrow and foreign cells remaining in the bone fragments, until the bone fragments appeared milky white and semi-permeable. After the liquid was agitated in the tube, the bone pieces were carefully removed with sterile forceps and placed in a 10 cm petri dish. A scalpel was used to scrape away potential residual bone marrow on the side of the bone marrow cavity. The bone pieces were rinsed again with a sterile PBS solution containing 1% double antibody. Around 6–10 pieces of bone per well were inoculated into a 6-well plate, with the addition of 5 mL high-sugar Dulbecco's modified eagle medium (DMEM) containing 1% L-ascorbic acid, 1% double antibody, and 20% fetal bovine serum (FBS) to each hole. All bone pieces were soaked, and without letting the bone fragments float, they were put in a 37 ℃, 5% CO_2, saturated humidity incubator to culture.

Alizarin red staining to determine mineralized nodules

A 1% alizarin red aqueous solution was created by dissolving1 g Alizarin Red powder (Shanghai Yuanye Biological Co., Ltd, China) in 100 mL distilled water. This was filtered to remove impurities, the pH was adjusted to 4.2 with 10% ammonia water, and the solution was then stored at 4 ℃ until use. The Osteoblasts were cultured in a 12-well plate, then the medium was discarded, PBS was used to rinse the osteoblasts, 0.5 mL of 95% ethanol was added, and they were fixed for 30 min, or overnight, at 4 ℃. After fixation, the ethanol was discarded and a little air-drying was allowed. Next, 0.3 mL of the prepared Alizarin Red S staining solution was added to each well for 15 min, the time of which can also be extended appropriately according to the staining situation. The staining solution was vacuumed off (recyclable), followed with rinsing in distilled water 3 times. The cells were then routinely dehydrated, mounted, observed, and photographed under a light microscope (Guangzhou Minghui Technology Co., Ltd., China).

Statistical methods

The software SPSS version 18.0 (IBM Corp., Armonk, NY, USA) was used for statistical analyses, measurement

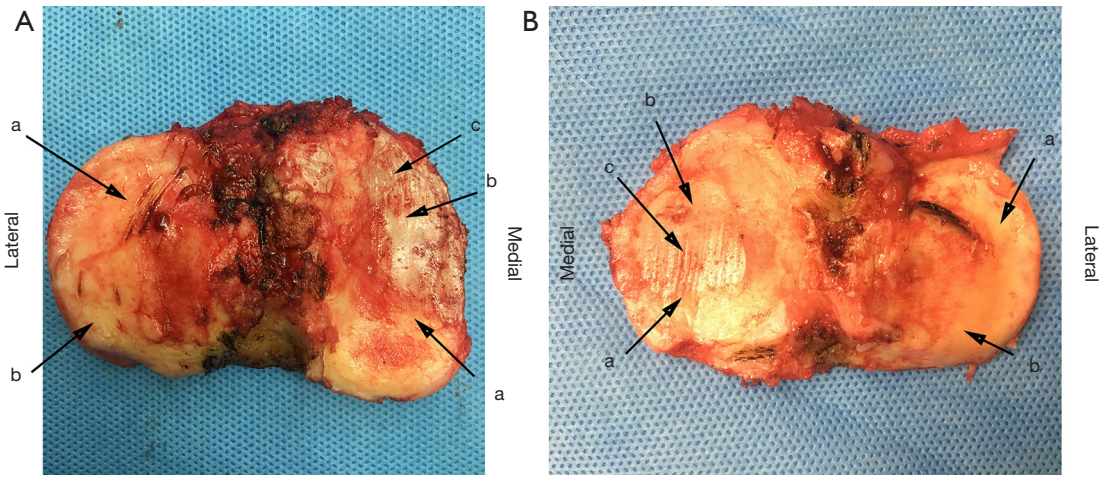

Figure 1 Observation of subchondral sclerotic matrix in patients with knee OA. (A) The medial tibial plateau of the knee in a patient with OA. a, the medial plateau cartilage gradually thinned from the margin to the center and disappeared; b, the subchondral bone is exposed and sclerotic; c, the surface of the hardened subchondral bone shows signs of wear. (B) The lateral tibial plateau of the knee in a patient with OA. a, cartilage morphology intact, no obvious wear degeneration (the surface scratches were electrosurgical cutting injuries); b, cartilage thickness normal. OA, osteoarthritis.

data were expressed as mean ± standard deviation, and *t*-test analysis was applied. A P value <0.05 was considered statistically significant.

Results

Expression of the ERK and JNK signaling pathways and osteogenesis-related factors in the subchondral bone of patients with knee OA

General observation

Cartilage degeneration of the medial platform of all specimens was more serious than that of the lateral. The cartilage surface of the medial platform was rough, dull, grayish-yellow, with extensive softening foci, huge fissures, exposure of subchondral bone, ivory and pannus-like tissue coverage, and multiple osteophytes at the center and edges of the platform. The cartilage of the lateral plateau was relatively normal, and the cartilage surface was still flat and partially shiny. Scattered superficial ulcers and softened foci were more common, and fissure formation was rare. There was no full-thickness cartilage loss, no pannus-like tissue formation, and marginal osteophytes were seen in some specimens (*Figure 1*).

HE staining

Normal chondrocytes were arranged loosely, in regular order, normal in number, evenly distributed, matrix staining was basically uniform, thickness and density were moderate, the nucleus and cytoplasm staining were deeper than the cartilage layer, and the tide line was complete. There were many fissures in the subchondral trabecular bone of knee OA, hypertrophy of chondrocytes, disordered cell arrangement and increased dispersion, evidence of cell clusters, uneven tide lines, irregular distortions, and uneven staining or loss of subchondral bone. The thickness of bone was reduced, color was difficult to distinguish from the cartilage layer, and there was a tendency to hardening (*Figure 2*).

Safranin staining

In the normal controls, the subchondral bone cells were evenly arranged, and the safranin stain was lightly stained (*Figure 3A*). In knee OA participants, the layers of subchondral bone were unevenly arranged, number of cartilage cells was significantly reduced, size was different, outline was not clear, and some cell displayed necrosis (*Figure 3B*).

Related protein levels

Compared with normal knee joints, the levels of phosphorylation-c-Jun N-terminal kinase (P-JNK) and phosphorylation-extracellular-signal-regulated kinase (P-ERK) in knee OA subchondral bone were significantly up-regulated (P<0.05), and the OMD level was significantly

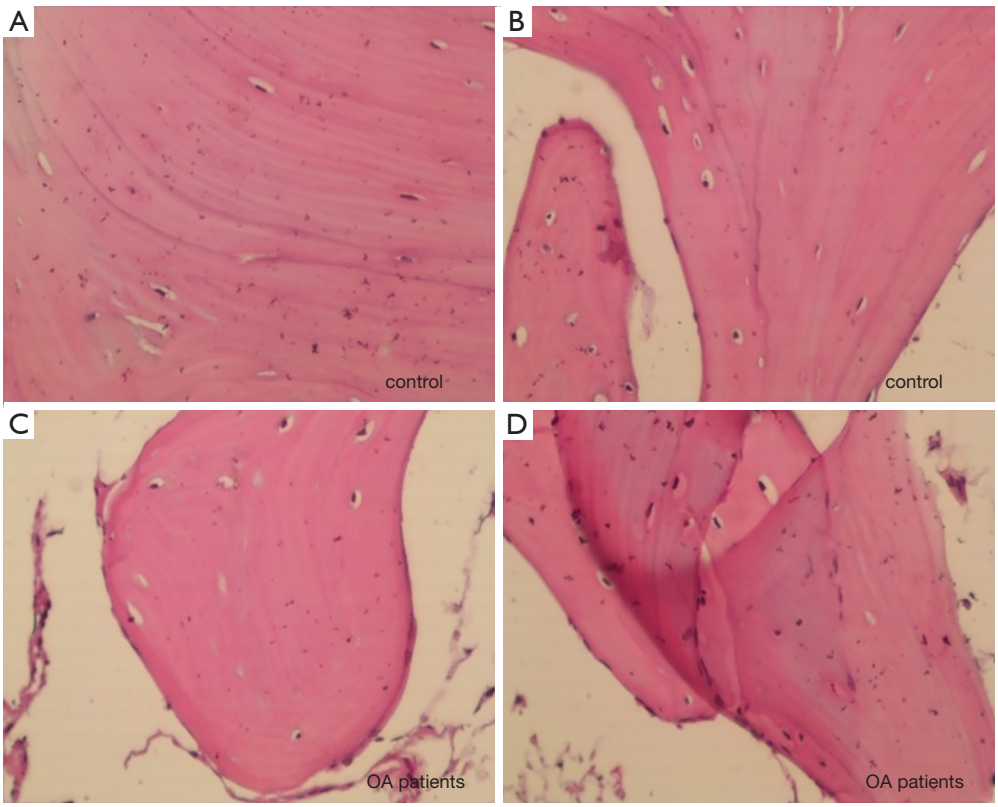

Figure 2 HE staining results of human knee joint subchondral bone cells. (A,B) Normal knee joint subchondral bone (200×); (C,D) knee arthritis patient subchondral bone (200×). HE, hematoxylin and eosin.

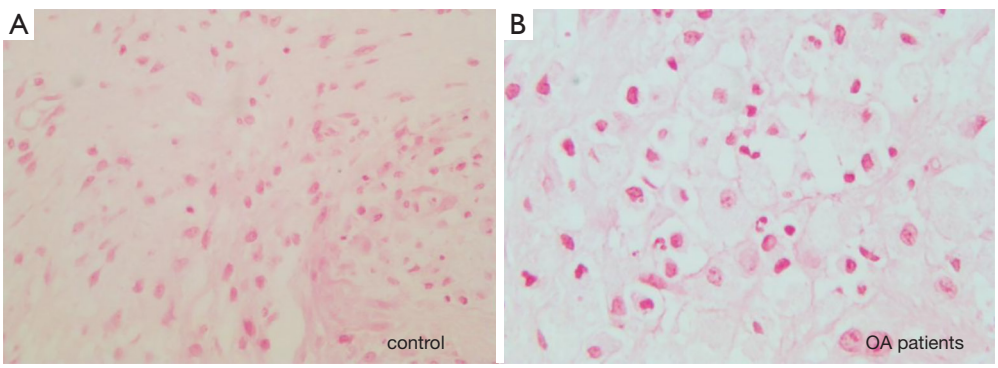

Figure 3 Human knee joint subchondral bone safranin staining results. (A) Normal knee joint subchondral bone (200×); (B) knee OA participant subchondral bone (200×). OA, osteoarthritis.

down-regulated (P<0.05).In the electrophoretic map, compared with normal subjects, the protein bands of p-JNK and p-ERK in OA patients were darker in color, while the protein bands of OMD were lighter in color. (*Table 1*, *Figure 4*).

Observation of serum and subchondral bone immunity in rat OA model

HE staining observation

In normal mice, the subchondral bone cells of the knee joint were arranged in an orderly manner, number was

Table 1 Gray values of related proteins

Project	Normal group	OA group
P-ERK	10,002.69±841.91	10,531.24±818.68*
ERK	19,141.78±945.15	19,930.24±873.22
P-JNK	13,909.93±965.89	22,069.43±901.54*
JNK	19,162.44±237.98	19,116.27±271.82
RUNX2	10,902.47±798.82	10,851.58±710.32
OMD	14,192.23±1481.76	9,027.75±1080.29*
GAPDH	23,755.49±897.02	23,587.86±873.26

*P<0.05, compared with the normal group. OA, osteoarthritis; P-ERK, phosphorylation-extracellular-signal-regulated kinase (P-ERK); ERK, extracellular-signal-regulated kinase; P-JNK, phosphorylation- c-Jun N-terminal kinase; JNK, c-Jun N-terminal kinase; RUNX2, runt-related transcription factor 2; OMD, osteomodulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

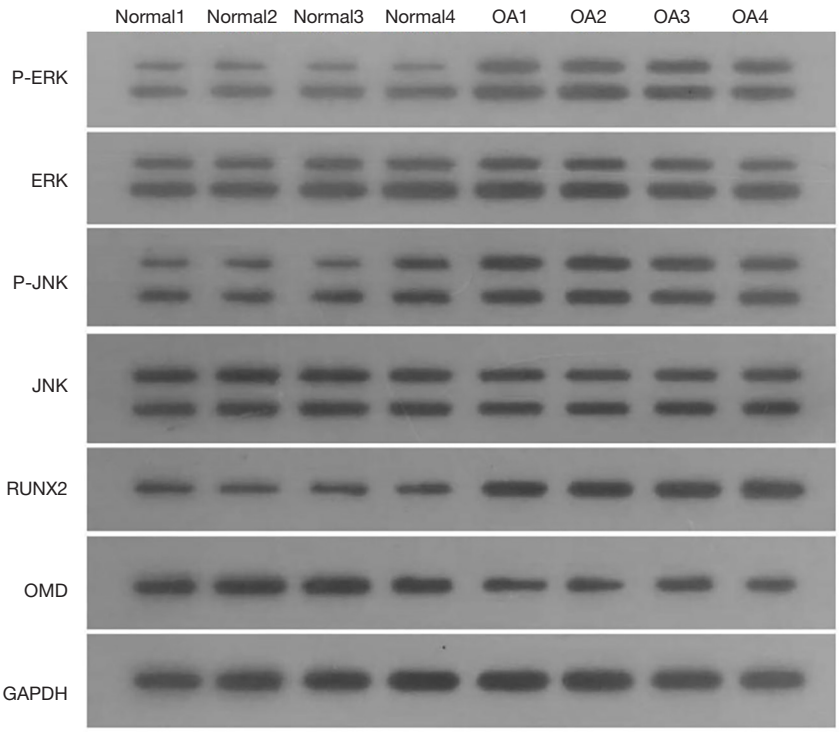

Figure 4 Related protein electropherograms of some OA participants and normal controls. OA, osteoarthritis; P-ERK, phosphorylation-extracellular-signal-regulated kinase; ERK, extracellular-signal-regulated kinase; P-JNK, phosphorylation- c-Jun N-terminal kinase; JNK, c-Jun N-terminal kinase; RUNX2, runt-related transcription factor 2; OMD, osteomodulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

normal, there was no fibrous tissue proliferation, and none or occasional infiltration of lymphocytes and monocytes (*Figure 5A*,*B*). In the 2-week model mice, the arrangement of knee joint subchondral osteocytes was slightly disordered, and a small amount of fibrous tissue was occasionally infiltrated with lymphocytes and monocytes (*Figure 5C*). In the 4-week model mice, knee joint subchondral osteocytes were reduced, cell arrangement was disordered, fibrous tissue formation could be seen, and some lymphocytes and monocyte infiltration was apparent (*Figure 5D*). In the

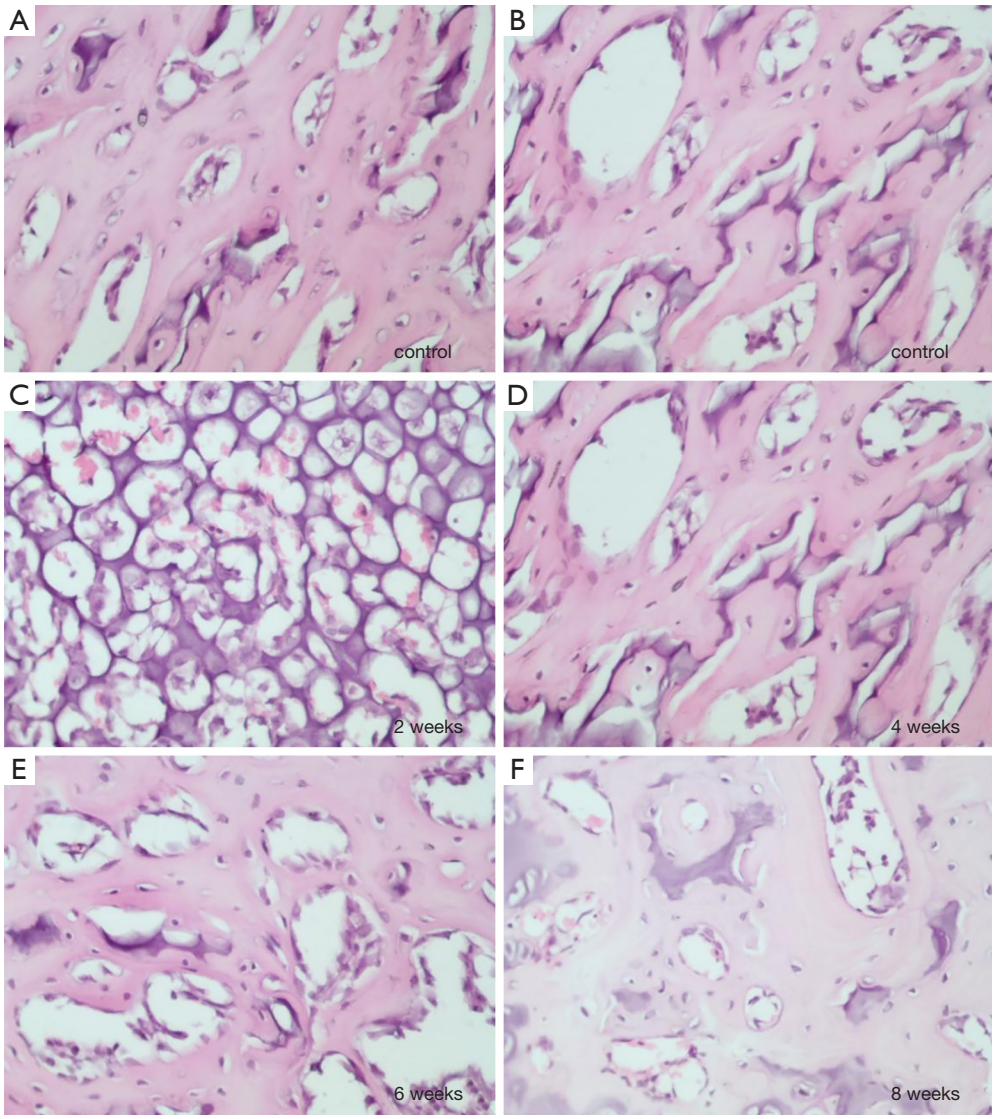

Figure 5 HE staining results of subchondral bone cells in mouse knee joints (200×). (A,B) knee subchondral bone cells of normal mice; (C) 2-week knee OA mouse model knee joint subchondral bone cells; (D) 4-week knee OA mouse model knee joint subchondral bone cells; (E) 6-week knee OA mouse model knee joint subchondral bone cells; (F) 8-week knee OA mouse model knee joint subchondral bone cells. HE, hematoxylin and eosin; OA, osteoarthritis.

6-week model mice, knee joint subchondral bone cells were significantly reduced, cell arrangement was disordered, fibrous tissue was more than that of 4-week model mice, lymphocyte and monocyte infiltration was visibly greater than in the 4-week model mice (*Figure 5E*). In the 8-week model mice, the knee joint subchondral bone cells were significantly reduced, the cell arrangement was disordered, there was more fibrous tissue proliferation, and more visible infiltration of lymphocytes and monocytes (*Figure 5F*).

Changes in the level of related proteins

There was significant difference in the JNK, P-JNK, OMD level between the model mice and the normal mice at all time points (P<0.05) (*Table 2*, *Figure 6*). At 2–8 weeks, the JNK and P-JNK levels of the model group mice increased significantly over time (P<0.05) (*Figure 6A,B*), and the OMD level decreased significantly over time (P<0.05) (*Figure 6C*). The levels of ERK and P-ERK fluctuated steadily over time (*Figure 6D,E*).

Table 2 Gray values of related proteins at different time points in two groups of mice

Group	Time (w)	JNK	OMD	ERK	P-ERK	P-JNK
Normal group	2	7,478.98±812.42#	10,458.11±1,057.35	13,635.74±1,279.63	13,489.78±1,882.34	6,860.03±721.86
	4	8,413.30±855.76#	11,457.34±1,386.84	13,928.98±1,428.76	13,965.98±1,583.56	6,960.59±754.39
	6	6,017.29±724.95#	12,808.47±1,799.31	15,661.88±2,219.48	15,061.88±1,748.75	7,413.640±891.46
	8	6,117.10±683.16#	11,680.35±1,435.26	16,077.17±1,877.12	14,900.17±1,622.13	7,242.81±805.17
Model group	2	10,079.76±1,578.53*#	12,786.93±929.54*#	14,678.50±1,856.53	14,338.50±1,758.96	9,777.57±1,033.58*#
	4	10,854.64±1,086.49*#	8,772.47±917.47*#	14,271.93±1,704.67	14,198.93±1,632.87	10,458.10±1,156.65*#
	6	13,900.59±1,803.92*#	5,454.05±652.69*#	15,575.76±1,582.95	15,275.76±1,701.79	12,033.64±1,178.32*#
	8	14,720.76±2,031.86*#	5,293.69±633.45*#	15,384.11±1,698.33	15,184.12±1,964.54	12,787.93±1,276.25*#

*P<0.05, compared with the normal group; #P<0.05, compared with the four points in time. P-ERK, phosphorylation-extracellular-signal-regulated kinase (P-ERK); ERK, extracellular-signal-regulated kinase; P-JNK, phosphorylation-c-Jun N-terminal kinase; JNK, c-Jun N-terminal kinase; OMD, osteomodulin.

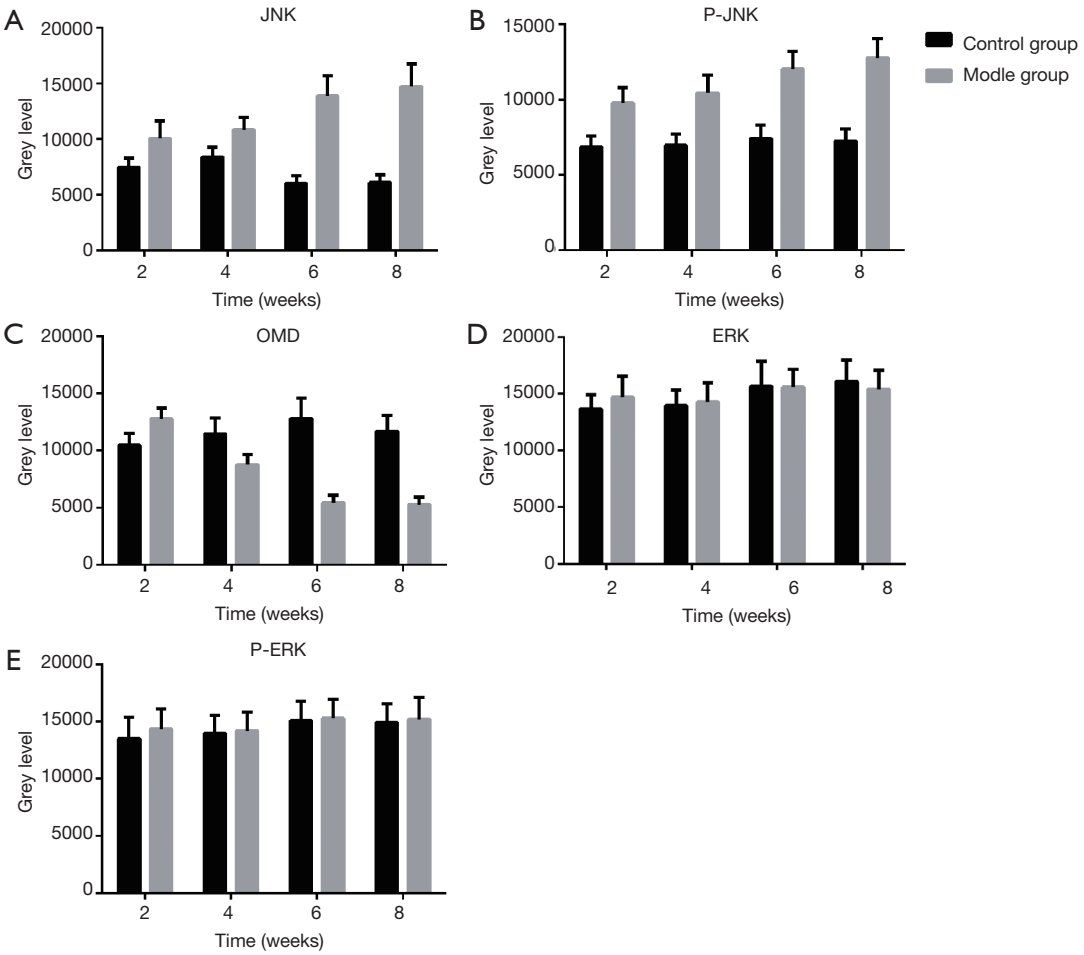

Figure 6 Correlated protein gray values at different time points. (A) JNK expression at 2–8 weeks; (B) P-JNK expression at 2–8 weeks; (C) OMD expression at 2–8 weeks; (D) ERK expression at 2–8 weeks; (E) P-ERK expression at 2–8 weeks. JNK, c-Jun N-terminal kinase; P-JNK, phosphorylation-c-Jun N-terminal kinase; OMD, osteomodulin; ERK, extracellular-signal-regulated kinase; P-ERK, phosphorylation-extracellular-signal-regulated kinase.

Table 3 Effects of different concentrations of alizarin red s posiyivr

Concentration (ng)	24 h	48 h
0	18.46±1.48	26.48±2.15
10	24.18±2.56a	34.56±3.94[a]
25	31.01±3.47[ab]	43.03±3.29[ab]
40	35.46±3.89[abc]	44.79±3.55[abc]

[a]P<0.05, compared with the 0 ng; [b]P<0.05, compared with the 10 ng; [c]P<0.05, compared with the 25 ng.

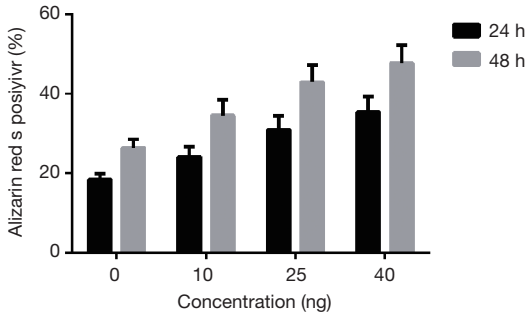

Figure 8 Effect of different concentrations of anisomycin on the positive rate of mineralized nodules.

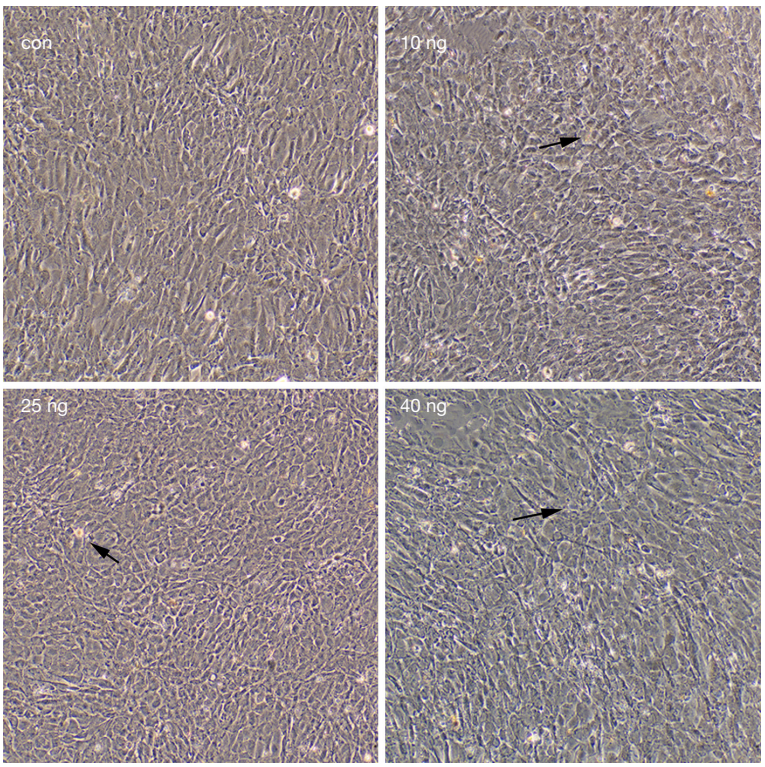

Figure 7 Effect of different concentrations of anisomycin on osteoblasts (100×).

Knee OA tibial subchondral sclerotic bone osteoblast culture and anisomycin intervention

The effect of different concentrations of anisomycin on the mineralized nodules of mouse knee osteoblasts

The osteoblasts that had not been treated with anisomycin were mainly irregular, long fusiform, and triangular, with many protrusions, mononuclear oval, 1–3 nucleoli, rich in cytoplasm, and clearly adjacent. Different concentrations of anisomycin were given to treat osteoblasts, and after continuous culture for 24 and 48 h, Alizarin Red staining was performed, revealing that all cells were stained with orange-red mineralized nodules. As the concentration of anisomycin increased, the number of cell mineralization nodules was significantly higher. Regardless of the effect of giving anisomycin to culture for 24 or 48 h, as the concentration of anisomycin increased, the positive rate of mineralized nodules increased (*Table 3*, *Figures 7*,*8*).

Table 4 Effects of different concentrations of anisomycin on osteoblast protein in mouse knee joint

Concentration (ng)	24 h		48 h	
	OCN	OMD	OCN	OMD
0	18.54±1.50	22.26±2.36	18.22±1.65	14.69±1.34
10	21.64±1.76[a]	24.27±3.28[a]	20.01±2.14a	13.86±1.47
25	29.98±3.39[ab]	44.67±3.04[ab]	26.56±2.82[ab]	16.62±1.98[a]
40	34.99±3.88[abc]	53.98±5.46[abc]	24.72±2.09[ab]	14.57±1.53

[a]P<0.05, compared with the 0 ng; [b]P<0.05, compared with the 10 ng; [c]P<0.05, compared with the 25 ng.

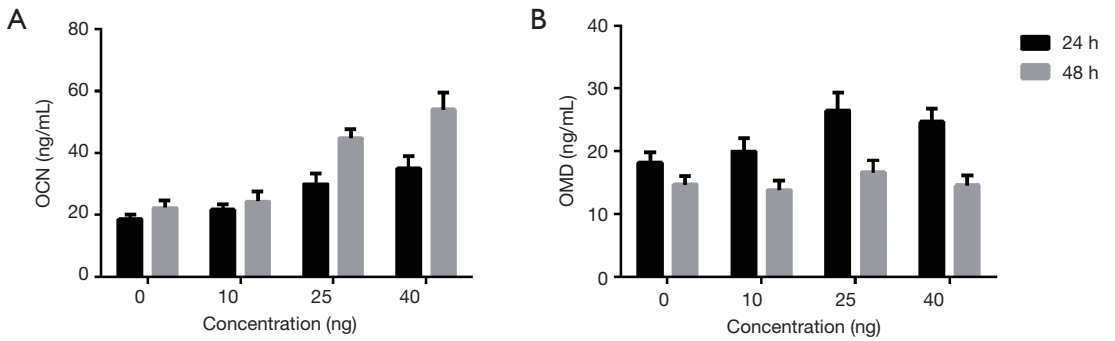

Figure 9 The effects of anisomycin on the levels of OCN and OMD in osteoblasts. (A) The effect of anisomycin on the level of OCN in osteoblasts; (B) the effect of anisomycin on the level of OMD in osteoblasts. OCN, osteocalcin; OMD, osteomodulin.

The effect of different concentrations of anisomycin on subchondral bone cell protein of mouse knee joints

After administering different concentrations of anisomycin to dry mouse knee joint subchondral bone pre-cells, the OMD level did not change significantly after 24 and 48 h of treatment. When anisomycin was administered at 25 ng, the OMD level reached the highest. Whether it was 24 or 48 h, OCN levels increased with the increase of anisomycin concentration (*Table 4*, *Figure 9*).

Discussion

Recent studies have indicated that OA is an organ-level disease, which is characterized by cartilage degeneration and subchondral bone changes (6). Although cartilage degeneration is regarded as the main symptom of OA, a large number of studies have shown that subchondral bone changes also play an important role in the occurrence and development of OA, and are the main signs of OA progress. Regardless of whether the subchondral bone changes occur before cartilage injury or the development of the disease, subchondral bone is currently recognized as a potential target for the treatment of OA (7,8), so monitoring the pathological changes of subchondral bone in OA patients is very important. In the process of OA, the subchondral bone and cartilage plate often show structural changes such as thickening of the calcified cartilage layer, double tidal lines, subchondral bone sclerosis, and bone marrow lesions (9). This study found that the medial plateau cartilage degeneration of all participants with knee OA was more severe than that of the lateral. There were obvious lesions in the cartilage of the medial plateau, such as extensive softening, huge fissures, pannus-like tissue coverage, and multiple osteophytes at the center and edges. The cartilage of the lateral plateau was relatively normal. Scattered superficial ulcers and softened foci were more common, and fissure formation was rare. The lesions were less obvious than those of the medial side. The results of HE and safranin staining showed that the cytomorphology of the subchondral bone of participants with knee OA was significantly different from that of normal people. There were more fissures in the trabecular bone cartilage of knee OA, hypertrophy of chondrocytes, and disordered and diffuse cell arrangement. Cells were clustered, stained

unevenly or out of stain, subchondral bone was stained unevenly or out of stain, thickness was reduced, the color was indistinguishable from the cartilage layer, and there was a tendency to harden. In participants with knee OA, the layers of subchondral bone were unevenly arranged, number of cartilage cells was significantly reduced, size was different, outline was not clear, and some cell necrosis had appeared. All of the above pathological changes are similar to the results of the above studies (10), which re-emphasizes the importance of the pathological changes of subchondral bone in the pathogenesis of knee OA.

As an important part of the joint, subchondral bone has a substantial relationship with the occurrence and development of OA (10), but the specific mechanism of action has not been elucidated. The ERK and JNK signal transduction pathways may be involved in the regulation of chondrocytes, and are currently the research hotspot of OA (11). Hayashi *et al.* (12) and other studies have shown that activation of the ERK1/2 signaling pathway promotes the proliferation of human OA chondrocytes, and ERK1/2 specific inhibitors can inhibit cell proliferation. Starkman *et al.* (13) and other studies have shown that activation of the ERK12 signaling pathway does not promote the enhancement of human chondrocyte protein secretion. Yin *et al.* (14) found that the ERK1/2 signaling pathway inhibited the secretion of human chondrocyte protein. The above studies indicate that the ERKI2 signal is involved in the dynamic balance regulation of chondrocyte proliferation and apoptosis and that of chondrocyte anabolism and catabolism. At present, JNK has also been shown to be activated (phosphorylated) in OA and play a key role in cartilage destruction (15). The activation of the JNK and ERK pathways activates c-Jun, which is a key component of AP-1. It regulates the expression of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin 1 (IL-1), resulting in a decrease in the synthesis of proteoglycan and increased production of chondrodegrading enzyme MMP-13 (16-18). Yang *et al.* (19) and other studies have suggested that CXCL8 and CXCL11 promote the apoptosis of chondrocytes and inhibit the proliferation of chondrocytes by activating the JNK signaling pathway. The results of this study showed that, compared with normal people, the levels of P-JNK and P-ERK in the subchondral bone of participants with knee OA were significantly up-regulated ($P<0.05$), confirming that JNK and ERK pathways are very active in patients with knee OA. By constructing a mouse model of knee OA, we could see the changes of the JNK and ERK pathways at different stages of knee OA. At all time points, the levels of JNK, P-JNK, ERK, and P-ERK in the model group were significantly different from that of the normal mice ($P<0.05$). At 2–8 weeks, the levels of JNK and P-JNK in the model group increased significantly over time ($P<0.05$), and the levels of ERK and P-ERK fluctuated over time. It was further confirmed that the JNK and ERK pathways are involved in the occurrence and development of knee OA in the subchondral bone.

At present, studies have shown that the abnormal expression and synthesis of type I collagen and low mineralization in knee OA subchondral osteoblasts are the main causes of abnormal bone metabolism. The study of Tashima *et al.* (5) found that OMD regulates the shape and diameter of type I collagen by reducing the charge between collagen molecules, thereby making it fully mineralized to form a mature bone structure; Luo *et al.* (20) and other authors have found that osteoblasts do not have JNK 1. Under these circumstances, the ability to perform later functions such as the secretion of extracellular matrix and mineralization is severely impaired, and the expression of advanced osteoblast markers such as OCN is significantly reduced. From this we infer that the JNK pathway is closely related to OMD and other osteogenesis-related factors. Anisomycin is a JNK-specific agonist, which can interfere with the activity of the p38 gene of the MAPK family and the c-Jun amino-terminal kinase JNK pathway (21). In this study, the results confirmed that the level of OMD in the subchondral bone of the knee joint of participants with knee OA was significantly down-regulated ($P<0.05$), and in a mouse model of 2-8 weeks, its level was significantly decreased over time ($P<0.05$). The above results indicate that OMD may be related to the pathogenesis of arthritis and participate in the occurrence and development of arthritis. In addition, this study explored the relationship between the JNK pathway and other osteogenic factors such as OMD by administering different concentrations of anisomycin to dry mouse knee joint subchondral bone pre-cells. After the cells were cultured continuously for 24 and 48 h, they were stained with Alizarin Red. It was found that all the cells used were stained with orange-red mineralized nodules. As the concentration of anisomycin increased, the number of cell mineralized nodules was significantly increased. The positive rate of metamorphic nodules increased, indicating that the mineralization ability of osteoblasts is enhanced by anisomycin. Different concentrations of anisomycin were administered to interfere with the osteoblasts of mice. When anisomycin

was delivered at 25 ng, the OMD level reached its highest. When the concentration of anisomycin increased, the OCN level also showed an upward trend. The above results indicated that the JNK pathway may regulate the levels of OMD and other osteogenic factors to guide the mineralization of extracellular matrix. Anisomycin may be a potential drug for the JNK pathway to treat arthritis, which needs to be confirmed by further experiments.

In summary, the process of the JNK signaling pathway regulating OMD may be closely related to the pathological changes of subchondral bone in patients with knee OA, and it is involved in the occurrence and development of knee arthritis.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study conformed to the provisions of the Declaration of Helsinki (as revised in 2013). Informed consent was taken from all individual participants. The study was approved by Second Affiliated Hospital of Soochow University (NO.:2021-021). Animal experiments were performed under a project license (NO.:2021-023) granted by Second Affiliated Hospital of Soochow University, in compliance with China guidelines for the care and use of animals.

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