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Original Article

Targeted activation on Bnip3 enhances mitophagy to prevent the progression of osteoarthritis

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ABSTRACT

Background: The production of reactive oxygen species (ROS) and mitochondrial dysfunction in chondrocytes are closely related to cartilage degeneration in the procedure of osteoarthritis (OA). Mitophagy is responsible for the scavenging of ROS and dysfunctional mitochondria and is considered a key therapeutic target for the treatment of OA. Tiopronin, a classic thiol antioxidant, has been widely studied for the treatment of various oxidative stress-related diseases.

Methods: The expression of mitophagy (PINK1, PARKIN, and TOMM20) in intact and damaged cartilage of OA patients was analyzed by Western blot and histological analysis. RNA sequencing (RNA-seq) analysis was performed to explore the molecular mechanism of tiopronin in regulating mitophagy in chondrocytes, and then to find the specific target of tiopronin. The therapeutic effects of tiopronin were evaluated in the OA model induced by destabilisation of the medial meniscus (DMM), chondrocytes degenerative model with the primary chondrocytes from mouse and human cartilage explants experiment. The downstream molecular mechanisms of tiopronin were further investigated by si-RNA knockdown of mitophagy-related proteins.

Results: The level of mitophagy in cartilage was negatively correlated with the severity of OA. We revealed that tiopronin promoted the anabolism of the extracellular matrix (ECM) of hyaline chondrocytes and alleviates ROS *in vitro* and *in vivo* by strengthening mitophagy. Moreover, tiopronin strongly activated the expression of Bnip3, a protein anchored in the mitochondrial membrane, and subsequently enhanced the Pink1/Parkin signaling pathway.

Conclusion: These findings indicate that the Bnip3-Pink1-Parkin signaling pathway, targeted and activated by tiopronin, plays a key role in inhibiting the progression of OA.

The translational potential of this article: As a classical drug in clinic, tiopronin was developed a new therapeutic approach in the treatment in OA via this study. Based the significant and efficient effect of tiopronin in inhibiting the cartilage degermation and delay the progression of OA, it was believed that tiopronin may become an effective therapeutic candidate for OA treatment in clinical settings

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1. Introduction

Osteoarthritis (OA) is one of the most common degenerative joint diseases in the musculoskeletal system, clinically characterized by pain, limited mobility, and joint deformity [1]. However, the specific pathophysiological mechanisms and molecular signaling pathways involved in OA are not precisely understood, resulting in an inability to achieve satisfactory treatment outcomes for OA. Currently, conservative treatments for OA primarily focus on alleviating symptoms, such as pain and joint swelling. Cartilage degeneration represents the structural change and primary pathogenesis in the development of OA [2]. Therefore, it is crucial to explore strategies to inhibit OA progression and prevent further structural damage based on a precise understanding of OA mechanisms.

Chondrocytes are the sole cell type resident in articular cartilage, encased within a collagen-rich extracellular matrix (ECM) that serves as the primary target for cartilage degradation in OA [3,4]. Mitochondria, present in most cells including chondrocytes, are the site of aerobic respiration and energy production, and are also responsible for regulating oxidative reactions [5]. Notably, once mitochondria are damaged, they can induce the generation of a significant amount of reactive oxygen species (ROS) [6], which in turn leads to further mitochondrial injury, resulting in more intracellular ROS production [7]. To maintain normal mitochondrial function, damaged mitochondria are merged with lysosomes for autophagic degradation, a process known as mitophagy [8]. Mitophagy, which aids in the scavenging of damaged mitochondria within chondrocytes, is essential for regulating ROS levels and ensuring the normal function of the cell [9]. Notably, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (Bnip3), is considered an important protein which involved in mitochondrial quality control. Chondrocytes in OA cartilage exhibit mitochondrial dysfunction and oxidative stress, which are closely associated with the onset and progression of OA [10]. Therefore, mitophagy is suggested as an effective target for the treatment of OA.

Tiopronin, primarily composed of N-(2-mercaptopropionyl) glycine, has been utilized for treating oxidative stress-related conditions, liver diseases, and more. Although tiopronin is an important antioxidant and anti-inflammatory agent, its effect on preventing cartilage degeneration remains unknown. Furthermore, the role of tiopronin in regulating mitochondrial function and mitophagy has not yet been studied. Given the safety and efficacy of tiopronin in clinical applications, it is worthwhile to explore its therapeutic role and mechanism in alleviating OA progression and preventing cartilage degeneration.

In this study, we aimed to investigate the effect and mechanism of tiopronin in the development of OA. We identified a remarkable relationship between mitophagy and cartilage degeneration in human OA cartilage samples. In a mouse OA model and a chondrocyte degeneration model, it was revealed that tiopronin significantly enhanced hyaline cartilage ECM synthesis, repaired dysfunctional mitochondria, and suppressed ROS levels by upregulating mitophagy. Based on RNA transcriptional profiling and further identification, we unveiled that tiopronin strongly activated Bnip3 and subsequently promoted the Pink1/Parkin signalling pathway. These findings suggest that tiopronin is a potential therapeutic agent for inhibiting cartilage degeneration and alleviating OA by targeting Bnip3.

2. Results

1.The relationship between mitophagy and cartilage degeneration in OA

Firstly, to determine whether mitophagy can serve as a therapeutic target for ameliorating osteoarthritis (OA), we investigated the expression of mitochondrial-related proteins in intact and damaged human OA cartilage obtained from patients who underwent total knee replacement surgery. As shown in Fig. 1, the results of safranin O-fast green staining and histological staining for COL2 and MMPs revealed the presence of relatively healthy cartilage ECM and chondrocytes in intact cartilage (Fig. 1A and B). Compared to intact cartilage, the levels of TOMM20, PINK1, and PARKIN in damaged cartilage were significantly reduced (Fig. 1A and B), indicating a decrease in mitochondrial function and mitophagy in the chondrocytes of OA cartilage. To further demonstrate the relationship between cartilage degeneration and mitochondrial function, we induced a degeneration model in primary chondrocytes using tert-butyl hydroperoxide (TBHP) and further utilized the mitophagy inhibitor Mdivi-1 at 25 μM. The results showed that the expression of hyaline cartilage markers (Col2, Sox9) was downregulated, while the expression of cartilage degradation markers (Mmp1, Mmp3) increased in the tbhp group compared with the control group. Moreover, the addition of Mdivi-1 further suppressed the expression of hyaline cartilage markers and augmented the expression of degradation markers (Fig. 1C–F). We also observed that the addition of Mdivi-1 led to more detrimental effects than tbhp alone on the expression of antioxidant markers (Ho-1, Sod-1) and anti-apoptosis markers (Bcl2), while it increased the expression of the necrosis marker (Bax) (Fig. 1D–G). Additionally, we detected the expression of mitochondrial-specific proteins such as Pink1, Parkin, and Tomm20, as well as the autophagy-related protein Lc3 in the chondrocytes. The expression of these proteins was further reduced by the addition of Mdivi-1 in the presence of tbhp (Fig. 1E–H and Fig. 1K–M). Subsequently, we measured the mitochondrial membrane potential using the JC-1 fluorescence probe and ROS levels using the DCFH-DA fluorescence probe in chondrocytes. The results showed that the mitochondrial membrane potential (the ratio of red to green) decreased and ROS levels increased in chondrocytes with the addition of Mdivi-1 compared with tbhp group (Fig. 1I, J, L). In summary, the level of mitophagy is closely related to the progression of OA.

2.Tiopronin enhances mitochondrial function and inhibits chondrocyte degeneration *in vitro*

The CCK-8 assay was used to detect the influence of tiopronin on the viability of chondrocytes from mice, with or without tbhp treatment. The results showed that tiopronin promoted chondrocyte viability in both conditions when the dose ranged from 0.5 mM to 5 mM for 24 h (Fig. 2A). Consequently, we selected 5 mM as the dose of tiopronin for subsequent investigations. Western blot analysis and cellular immunofluorescent staining demonstrated that tiopronin treatment significantly increased anabolism and decreased catabolism in chondrocytes stimulated with tbhp (Fig. 2D–G, and O). Transmission electron microscopy (TEM) analysis revealed that, after tbhp treatment, mitochondria exhibited significant swelling and spine rupture. However, treatment with tiopronin increased the number of intact mitochondria with clear ridges (Fig. 2B and C). Anti-ROS factors, including Ho-1 and Sod-1 were upregulated following tiopronin treatment in degenerated chondrocytes.The intracellular ROS levels, indicated by 2′,7′-dichlorofluorescein diacetate (DCFH-DA) staining and mitochondrial oxidative stress levels indicated by Mito Sox staining, were lower in the tiopronin treatment group (Fig. 2J–L and O). Additionally, tiopronin treatment ameliorated the levels of apoptosis, as indicated by the expression of Bcl2 and Bax and TUNEL analysis, in chondrocytes stimulated with tbhp (Fig. 2E–H and Fig. S2A, B). Furthermore, mitophagy-related indicators (Pink1, Parkin, Lc3, Tomm20) were disrupted by tbhp compared with the control group, but were remarkably improved with the addition of tiopronin (Fig. 2F–I, N, and O). Meanwhile, mitochondrial-specific probe MitoTracker analysis and JC-1 analysis showed that the quantity and quality of mitochondria in chondrocytes were significantly enhanced after tiopronin treatment (Fig. 2K–M, and O). These results indicate that tiopronin prevents chondrocyte degeneration, inhibits oxidative stress, and reduces apoptosis by ameliorating mitochondrial function, likely through increasing the level of mitophagy.

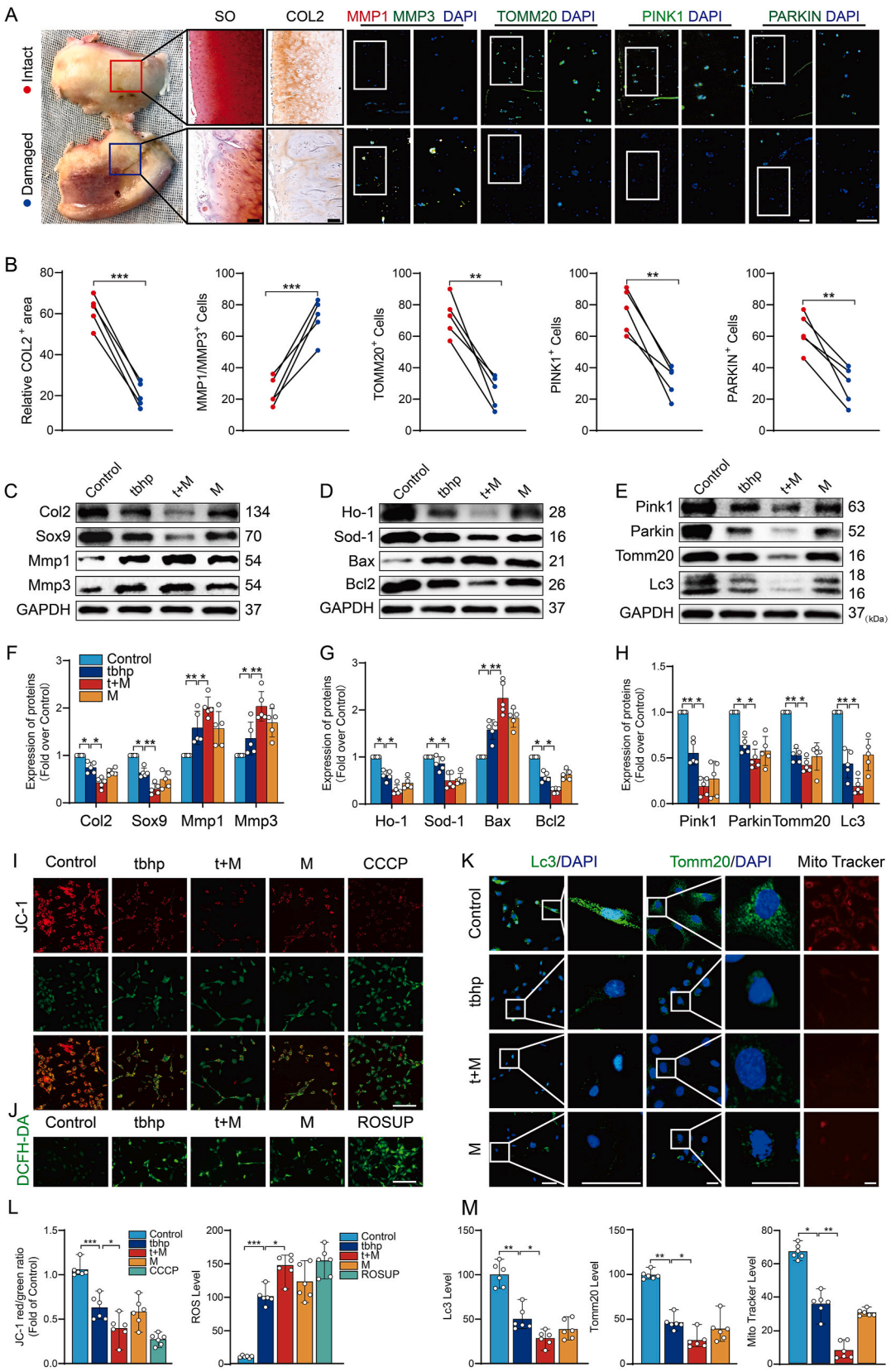

Fig. 1. The level of mitophagy is reduced in both human OA cartilage and mouse chondrocytes. (A,B) Macroscopic examination of knee joint specimens from patients undergoing total joint replacement surgery. Intact = The relatively normal part; Damaged = The damaged osteoarthritic part(n = 5).Scale bar, Safranin & Fast Green (S&O) staining,Col2 500 μm; The others250μm.(C–H) Western blot analysis Control, tbhp,tbhp + Mdivi-1(M),M Indicator protein in chondrocytes(n = 5).(I–L) Mitochondrial membrane potential levels and ROS levels in chondrocytes after Mdivi-1 treatment.Scale bar,100 μm(K,M) Immunofluorescence detection of LC3 and Tomm20 levels as well as Mito Tracker levels in chondrocytes(n = 6).Scale bar,50 μm *P < 0.05; **P < 0.01; ***P < 0.001.

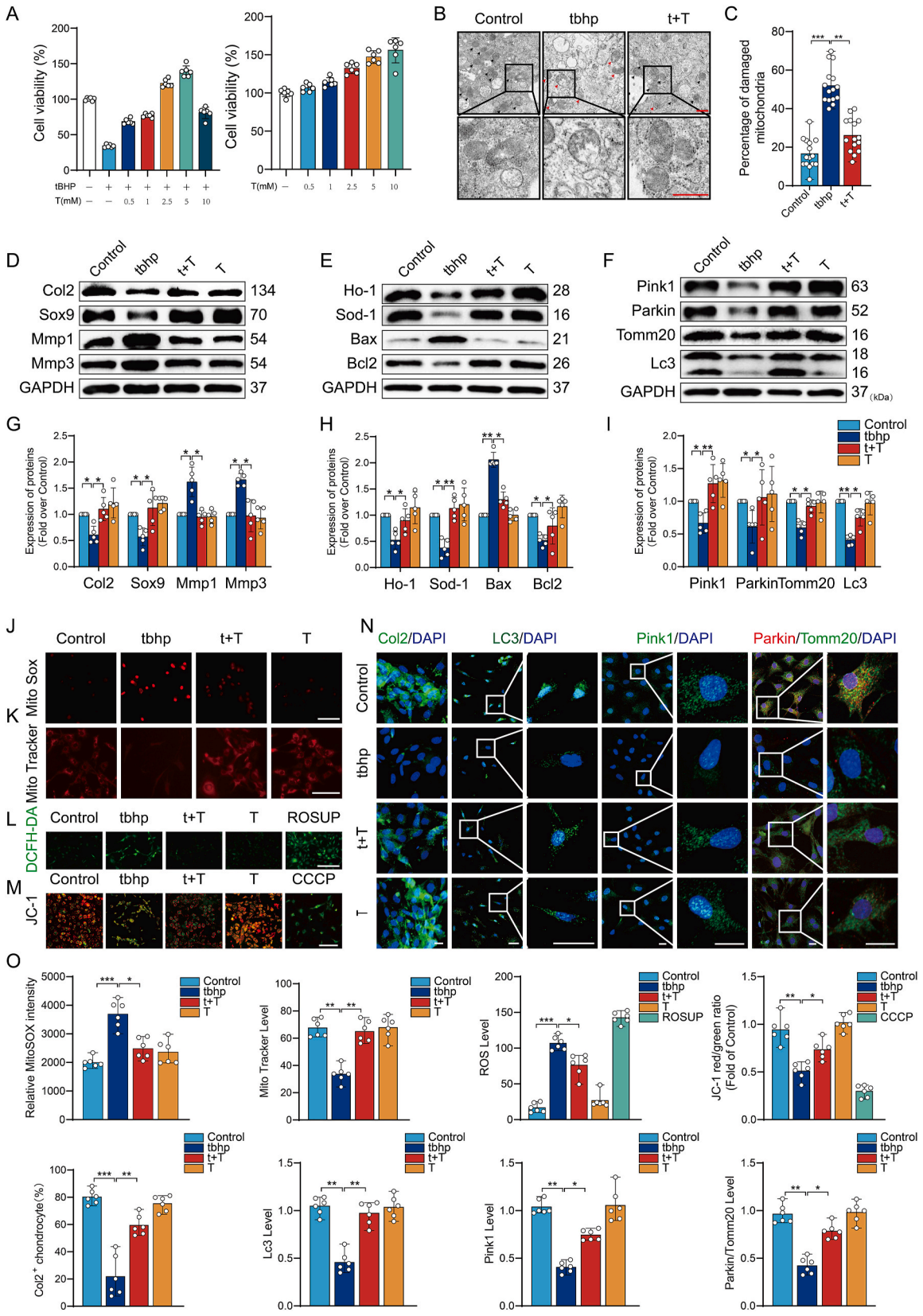

Fig. 2. Tiopronin improves mitochondrial function and inhibits chondrocyte degeneration. (A) CCK-8 assay to detect the cytotoxicity of sulforaphane after 24 h of treatment.(B,C) Detection of mitochondrial ultrastructure by TEM. Black arrow: normal mitochondria. White arrow: mitochondrial autophagosome. Red arrow: damaged mitochondria (mitochondria with swelling, broken cristae and vacuolation). The percent damaged mitochondria was quantified.(n = 15).Scale bar,5 μm. (D–I) Western blot analysis of the expression of related proteins in chondrocytes after treatment with sulforaphane (n = 5). (J–O) Immunofluorescence detection of the expression levels of Mito Sox, Mito Tracker, ROS, mitochondrial membrane potential(JC-1), Col2, LC3, Pink1, Parkin, and Tomm20 in chondrocytes after treatment with Tiopronin. (n = 6).Scale bar, J, L, and M 100 μm; K, N 50 μm*P < 0.05; **P < 0.01; ***P < 0.001.

3. The positive role of tiopronin in degenerated cartilage via mitophagy

To further verify the regulation of tiopronin on mitophagy, we utilized the mitophagy inhibitor Mdivi-1 to treat tiopronin-stimulated chondrocytes. The results of western blot analysis showed that after adding Mdivi-1 to the cells in the tbhp + tiopronin treatment group, the beneficial effects of tiopronin on chondrocyte metabolism, antioxidative capacity, anti-apoptosis, and mitochondrial function were diminished (Fig. 3A–F). Similarly, JC-1, ROS, TUNEL, and MitoTracker fluorescence staining showed consistent results (S3C, E). Furthermore, we performed *ex vivo* culture of human cartilage tissues to investigate the therapeutic effect of tiopronin on degenerated cartilage (Fig. 3G). Similar to the results in the animal OA model cartilage, tbhp treatment reduced the hyaline cartilage ECM and promoted cartilage degradation, as validated by safranin O staining and histological analysis (COL2, MMP1, and MMP3). However, after treatment with tiopronin, these harmful effects were significantly alleviated (Fig. 3G, H, Fig. S3F). Meanwhile, mitophagy-related proteins were also upregulated by the addition of tiopronin following tbhp-induced damage (Fig. 3H, I, Fig. S3F). In summary, these results indicate that tiopronin has the ability to promote mitophagy and thereby delay the progression of osteoarthritis.

4. Intra-articular injection of tiopronin prevents the progression of surgically induced OA *in vivo*

In this study, C57BL/6 mice were used to construct an OA model via the destabilisation of the medial meniscus (DMM) surgery on the right knee joint. One week after surgery, mice in the OA + tiopronin group received intra-articular injections of tiopronin (low, medium, high doses: 30 mg/kg, 60 mg/kg, 90 mg/kg) into the right knee joint twice a week (Fig. 4A). Gait analysis was performed and clearly demonstrated the limping of OA model mice due to pain during walking. Mice were allowed to freely walk on a 70 cm × 7 cm track with their front paws dipped in blue dye and their hind paws in red dye (Fig. 4A). The footprints of the mice were recorded, revealing that the front and hind paw prints of healthy mice overlapped relatively well, while those of the OA group were clearly separated. This separation was more pronounced in the OA group compared to the OA + tiopronin group. The average stride length (blue dashed line) and stride length (red dashed line) of the OA + high-dose tiopronin group were closer to those of the sham surgery group (Fig. 4B and C), indicating that tiopronin treatment effectively alleviated joint pain and relieved the symptoms of limping. Additionally, the maximum diameter of the affected knee joint (medial and lateral) was measured with vernier calipers before and 8 weeks after surgery to dynamically assess the degree of joint swelling. The results showed that tiopronin treatment significantly alleviated joint swelling during the progression of OA, with the high-dose OA + tiopronin group showing the most significant improvement (Fig. 4A–D).

Micro-CT analysis further confirmed that the formation of osteophytes (indicated by red arrows) was reduced in the tiopronin treatment group compared to the OA group. Additionally, as OA patients often experience subchondral bone sclerosis, it was observed that the tiopronin treatment group had lower subchondral bone sclerosis, closer to the sham group, with the high-dose group showing the most significant treatment effect (Fig. 4E–H, Fig. S4A). Histological analysis, including SO staining and grading of cartilage and osteophytes according to the OARSI scoring system, revealed that the OA + tiopronin group had less proteoglycan loss and fewer osteophytes compared to the OA group. H&E staining and synovitis scoring indicated that the OA group had synovial cell hypertrophy and thickening, while tiopronin treatment alleviated synovitis, with the OA + high-dose tiopronin group showing the most significant effect (Fig. 4F–H). Immunohistochemistry showed that the tiopronin treatment group had increased Col2 expression and decreased Col1 expression, as well as reduced expression of MMPs (Fig. 4G–I, and H). Consistent with the above analyses, the high-dose treatment group exhibited the best effect on cartilage protection. Subsequently, we analyzed the expression of mitophagy-related proteins in OA cartilage by immunofluorescent staining at the high dose of tiopronin that showed the best treatment effect (90 mg/kg). The results showed that the OA + tiopronin group significantly increased the levels of mitophagy-related proteins such as Pink1, Parkin, Tomm20, and Lc3 (Fig. 4I– Fig. S4B). The drug safety of tiopronin was confirmed with H&E staining of key organs in each group (Fig. S4C). In summary, intra-articular injection of tiopronin significantly alleviated the occurrence and progression of cartilage degeneration and OA in mice, identifying that this effect is achieved by increasing mitophagy levels. However, the specific target for this mechanism is yet to be determined.

4. Tiopronin act as an agonist of Bnip3 to enhance mitophagy through the Bnip3-Pink1/Parkin

According to our previous investigations, tiopronin enhances mitophagy levels to delay the progression of OA and cartilage degeneration. However, there is limited research on how tiopronin specifically increases mitophagy levels. Therefore, we conducted RNA-seq analysis on chondrocytes treated with tbhp with and without tiopronin (n = 3). Through this analysis, we identified 718 genes commonly involved in the normal, TBHP, and TBHP + tiopronin groups (S5A, C). To pinpoint genes showing significant differences before and after adding TBHP and tiopronin, we observed up-regulation of genes related to autophagy and mitochondria, and down-regulation of genes related to apoptosis (Fig. 5A). Importantly, we noted a substantial increase in Bnip3 expression by 10.55 times (log2 Fold of change = 3.406) in the tiopronin treatment group compared to the TBHP group, alongside significant changes in Pink1 and Parkin (Fig. 5A). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses based on the differentially expressed genes revealed biological changes following tiopronin treatment (Fig. 5B and C). The GO analysis indicated enrichment in processes such as positive regulation of autophagy, positive regulation of mitochondrion organization, negative regulation of ROS metabolic processes, and negative regulation of extrinsic apoptotic signaling in the tiopronin treatment group compared to the tbhp group (Fig. 5B). Additionally, KEGG analysis showed enhancement of pathways including mitophagy and autophagy in the tiopronin treatment group (Fig. 5C). Subsequently, we conducted immunofluorescent staining on human cartilage tissue to analyse BNIP3 expression in intact and damaged cartilage. Results showed significantly reduced BNIP3 expression in damaged cartilage tissue of OA patients (Fig. 5D and E). Conversely, *in vitro* and *ex vivo* experiments demonstrated that tiopronin treatment significantly upregulated Bnip3 expression in chondrocytes and human cartilage tissue compared to the TBHP group (Fig. 5F–I, S5D, E). Similarly, Bnip3 was notably up-regulated in OA-model mice treated with tiopronin (Fig. 5L– Fig. S5F). Furthermore, we examined LC3 expression in human cartilage tissue, linking autophagy with Bnip3 (Fig. 5J and K). Our research highlights Bnip3's crucial role in tiopronin's enhancement of mitophagy levels. Based on these findings and previous studies, we identified Bnip3, Pink1, and Parkin as targets for constructing siRNA to knock down their expression (S6A, B). Firstly, by using si-Bnip3 to knock down the expression of Bnip3, it was found that compared to the normal group, after the reduction of Bnip3, the level of mitophagy decreased, while mitochondrial oxidative stress and ROS levels increased, and this phenomenon could not be reversed by tiopronin (Fig. 5M– Fig. S6J). Secondly, Western blot results confirmed that Bnip3 siRNA significantly decreased Pink1 and Parkin expression in chondrocytes, whereas siRNAs targeting Pink1 and Parkin did not significantly affect Bnip3 expression (Fig. 5N–Q, Fig. S5I), suggesting that Bnip3 acts upstream of Pink1 and Parkin. Finally, after silencing these three proteins using siRNAs, we observed decreased levels of cartilage synthesis, antioxidants, and anti-apoptosis markers, alongside increased cartilage degradation. These changes were not reversed by tiopronin treatment (S5C-H). Therefore, we conclude that tiopronin acts

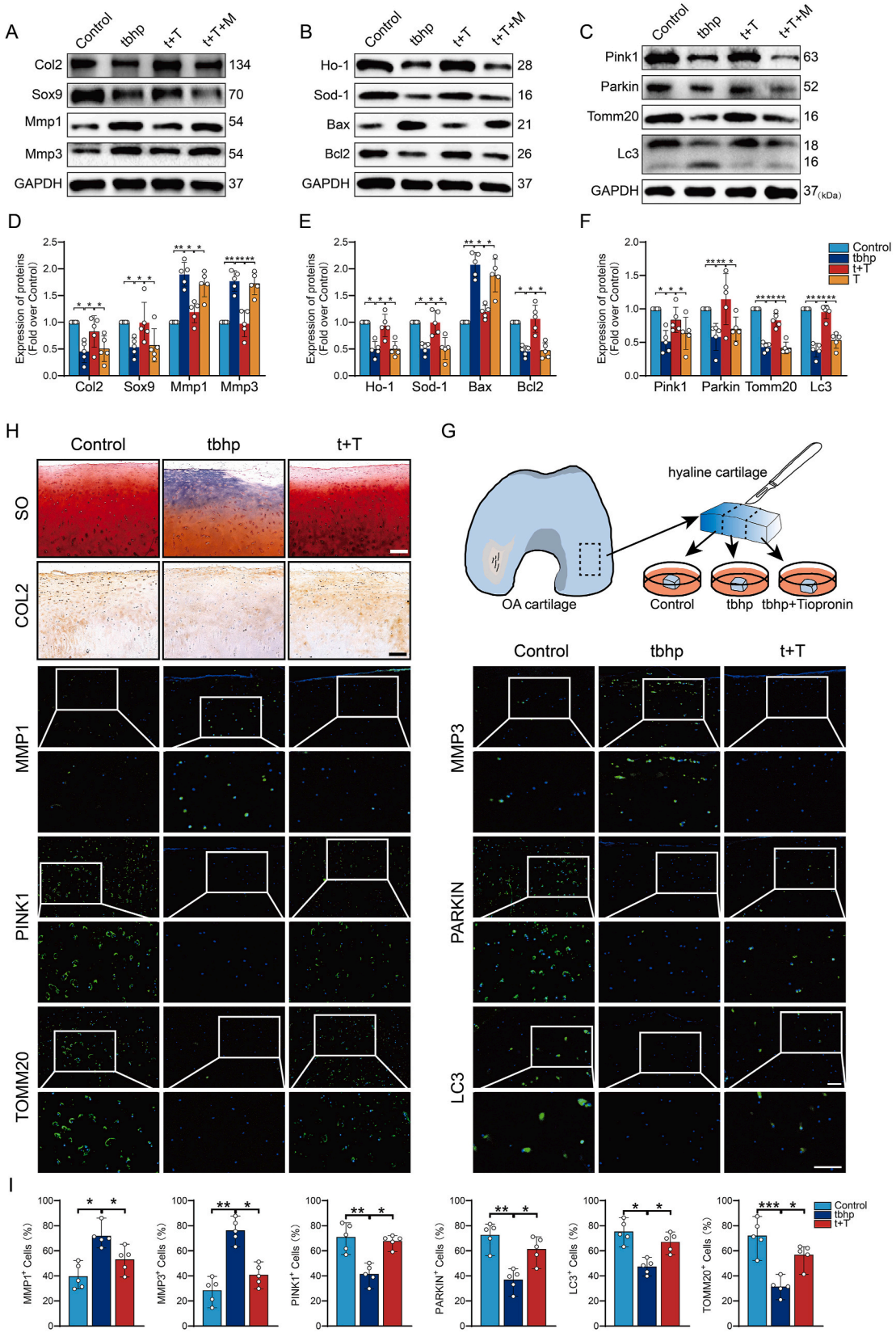

Fig. 3. Tiopronin inhibits chondrocyte degeneration by enhancing mitophagy levels. (A–F) Western blot analysis of the expression of related proteins in chondrocytes treated with sulforaphane and/or Mdivi-1 (n = 5). (G) Illustration of Steps for Explant Culture Experiment. (H,I) Detection of the expression levels of related indicators in tissue explants after sulforaphane treatment using Safranin & Fast Green (S&O) staining, immunohistochemistry, and immunofluorescence (n = 5). Scale bar,100 μm *P < 0.05; **P < 0.01; ***P < 0.001.

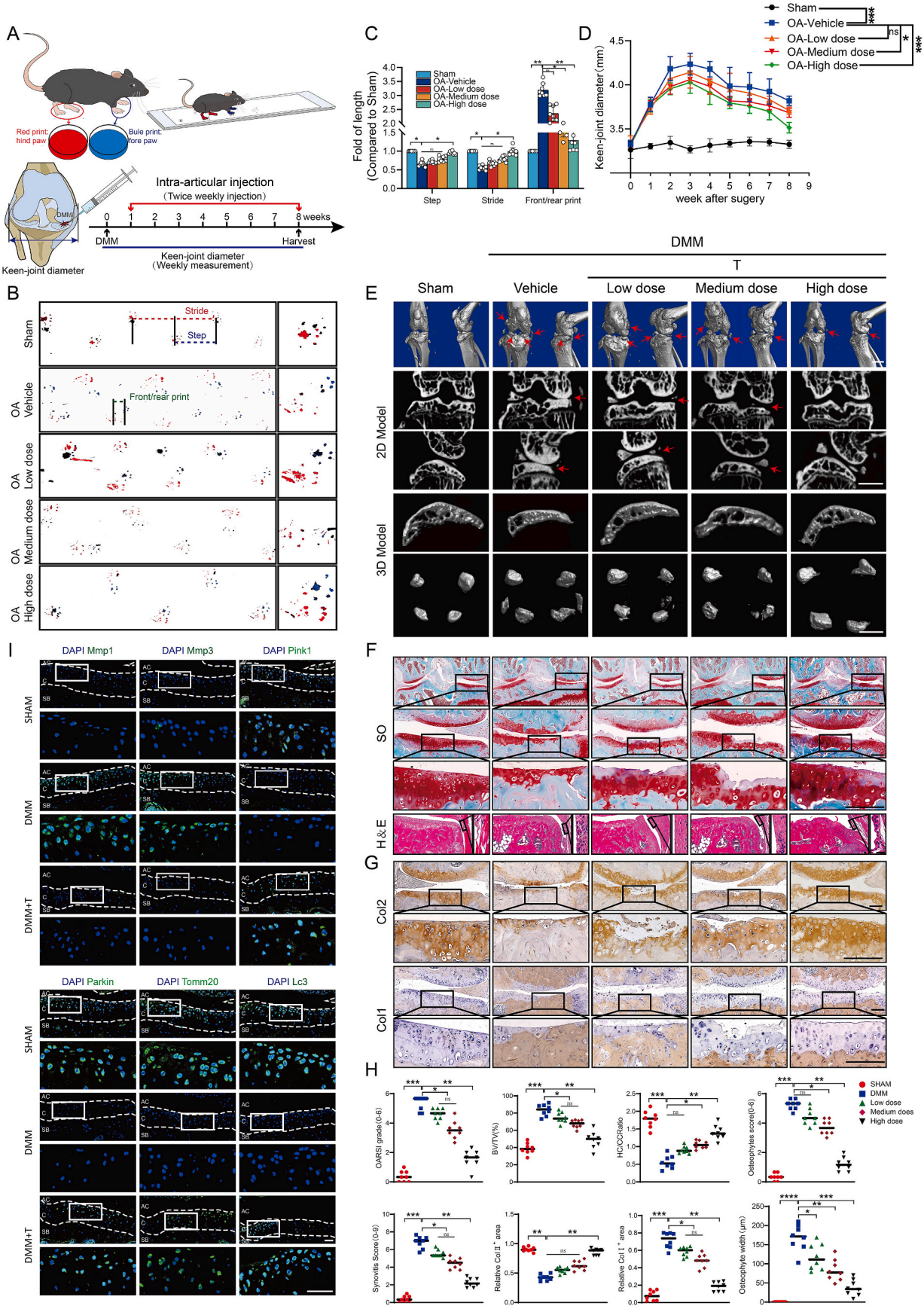

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Fig. 4. Intra-articular injection of Tiopronin can improve the progression of surgically induced osteoarthritis. (A) Schematic of mouse gait and administration.(B,C) Gait analysis results for different groups. The blue dotted line represents stride length, the red dotted line represents step length, and the green dotted line represents the length of front/rear paw prints: blue print, fore paw; red print, hind paw. (n = 8).(D) Knee joint diameter of the affected limb in mice 8 weeks post-surgery (n = 10).(E) Representative images of subchondral bone using micro-computed tomography (μCT) reconstruction (n = 8). Scale bar, 2.0 mm(F) After 8 weeks of modeling, the joints from the control group and the treatment group were stained with S&O and HE, respectively, to evaluate cartilage formation and synovitis.(n = 8).Scale bar, S&O 500 μm; HE 250 μm. (G) After 8 weeks of modeling, the joints from the control group and the treatment group were stained with immunohistochemistry for Col1 and Col2.(n = 8) Scale bar, 500 μm. (H) Based on the aforementioned μCT, S&O, and HE results, quantitative analyses were performed for OARSI, BV/TV, HC/CC, Osteophytes score, Synovitis Score, and Osteophyte width. (I) Immunofluorescence analysis of the levels of related proteins in the control group, the model group, and the high-concentration group after 8 weeks of modeling.(n = 8)Scale bar,100 μm*P < 0.05; **P < 0.01; ***P < 0.001. "ns" means not significant (P > 0.05).

through these three targets to treat OA, indicating strong activation of Bnip3 expression and regulation of the Bnip3-Pink1-Parkin signalling axis to enhance mitophagy.

6. The effect of Oral tiopronin on preventing the progression of surgically induced OA

In this study, we aimed to simulate oral administration of tiopronin in mice, with celecoxib used as a positive control according to osteoarthritis treatment guidelines. Behavioral and histological assessments were conducted to evaluate the therapeutic effects of oral tiopronin, laying the groundwork for potential clinical translation in OA treatment. Weekly measurements of knee joint diameter demonstrated that the high-dose tiopronin group exhibited significant reduction in joint swelling, comparable to the effects observed with celecoxib treatment and sham surgery groups (Fig. 6A). Gait analysis further indicated that compared to the OA group, the OA + high-dose tiopronin group showed step lengths, stride lengths, and front/rear paw distances closer to those of the celecoxib treatment and sham surgery groups (Fig. 6B and C), underscoring tiopronin's efficacy in alleviating pain and improving mobility. Micro-CT, safranin O staining (SO), and hematoxylin and eosin (HE) staining revealed that oral administration of high-dose tiopronin reduced osteophyte formation (indicated by red arrows), subchondral bone sclerosis, degradation of cartilage extracellular matrix (ECM), and synovitis (Fig. 6D–F, Fig. S7A, B). Analysis of collagen levels in the OA model cartilage showed that oral tiopronin, especially in the high-dose group, promoted the recruitment of hyaline cartilage ECM (high expression of Col2 and low expression of Col1), compared to the OA group (Fig. 6F). Additionally, toxicity testing of key organs indicated no adverse effects from oral tiopronin administration (S7C). In summary, oral administration of tiopronin demonstrated significant therapeutic effects in an OA animal model, effectively preventing cartilage degeneration and suggesting its potential for clinical application in OA treatment.

5. Discussion

Cartilage degeneration stands as a pivotal characteristic of OA [11,12]. The erosion and degeneration of cartilage not only contribute to the demise of articular chondrocytes but also significantly influence the pathology of OA. Several critical molecular signals regulate the homeostasis, survival, and death of chondrocytes [13]. Consequently, symptoms such as chronic knee pain, damage to articular cartilage, synovitis, subchondral bone remodeling, and osteophyte formation are exacerbated by cartilage degeneration, ultimately leading to joint deformity and functional decline [14,15]. Increasingly, excessive oxidative stress and disturbances in mitochondrial homeostasis are linked to the initiation and progression of OA and cartilage degeneration. Abnormal oxidative stress disrupts the cartilage microenvironment's homeostasis, leading to elevated levels of reactive oxygen species (ROS), accumulation of matrix metalloproteinases (MMPs), and impaired cell function [16]. At the cellular level, excessive oxidative stress causes severe damage to mitochondrial DNA (mtDNA) and nuclear DNA, affecting cell signaling pathways and protein transcription [17]. Moreover, excessive oxidative stress and mitochondrial imbalance further contribute to abnormal bone and cartilage metabolism [18], structural degeneration, and stimulate synovial cells, resulting in synovitis and joint swelling [19]. During the onset of osteoarthritis, elevated oxidative stress levels can increase ROS levels and inhibit mitophagy, disrupting cellular function [20].

Mitophagy plays a critical role in maintaining mitochondrial function by eliminating damaged mitochondria, thereby reducing ROS levels and restoring the cartilage microenvironment's homeostasis [21]. The progression of OA is closely linked to the regulation of mitophagy. Our studies have demonstrated that mitochondrial function is compromised in human OA cartilage, accompanied by reduced mitophagy in chondrocytes. This imbalance results in decreased expression of anabolic genes involved in cartilage matrix synthesis and increased expression of catabolic genes responsible for ECM degradation. Thus, mitophagy emerges as a pivotal factor contributing to metabolic dysregulation in chondrocytes during OA progression [22]. Enhancing mitophagy to alleviate oxidative stress has therefore been identified as a critical strategy for preventing the progression and treating OA. Impaired mitophagy leads to the gradual accumulation of dysfunctional mitochondria, triggering chondrocyte apoptosis, ECM degradation [9], and intracellular ROS accumulation, ultimately causing cell damage and subsequent cartilage degeneration [23,24]. Mitochondrial dysfunction is implicated in cellular injury and various age-related diseases, including degenerative joint diseases. Previous research underscores the importance of maintaining mitochondrial stability and function [25,26]. In this study, we observed a close association between mitophagy and OA progression in human cartilage, tbhp-treated chondrocytes, and a mouse model of DMM surgery. We found a negative correlation between mitophagy levels and the severity of OA. In summary, mitophagy represents a critical pathological mechanism underlying OA development and warrants consideration as a potential therapeutic target for OA treatment.

Tiopronin, a thiol antioxidant, has been extensively investigated for its therapeutic potential in various oxidative stress-related conditions, including chemotherapy-induced renal and hepatic toxicity, radiation poisoning, and ischemia-reperfusion injury of cardiac and pulmonary tissues [27,28].Previous studies have shown that tiopronin has a good therapeutic effect in chronic active or chronic persistent hepatitis, with good tolerance and minimal side effects [28]. Its metabolite, 2-mercaptopropionic acid, is known for its robust free radical scavenging capabilities [29]. Given these antioxidant properties, tiopronin has shown promise in clinical settings for mitigating oxidative damage. Despite its established role in other conditions, the specific mechanisms through which tiopronin operates in cartilage degeneration and oxidative stress in the context of OA remain largely unexplored. In recent studies, tiopronin has emerged as a potential therapeutic agent for promoting mitophagy, a process crucial for maintaining mitochondrial health and reducing oxidative stress, thereby potentially slowing down OA progression. In investigations using human and mouse OA cartilage models, tiopronin treatment was found to upregulate mitophagy levels. This was evidenced by transmission electron microscopy revealing a reduction in damaged mitochondria and an increase in mitochondrial autophagosomes. Notably, when mitophagy was inhibited using Mdivi-1 in cellular experiments, tiopronin's beneficial effects on mitochondrial health were reversed, further supporting its role in enhancing mitophagy to combat OA-related mitochondrial dysfunction. Clinical administration of tiopronin typically involves oral delivery rather than local application

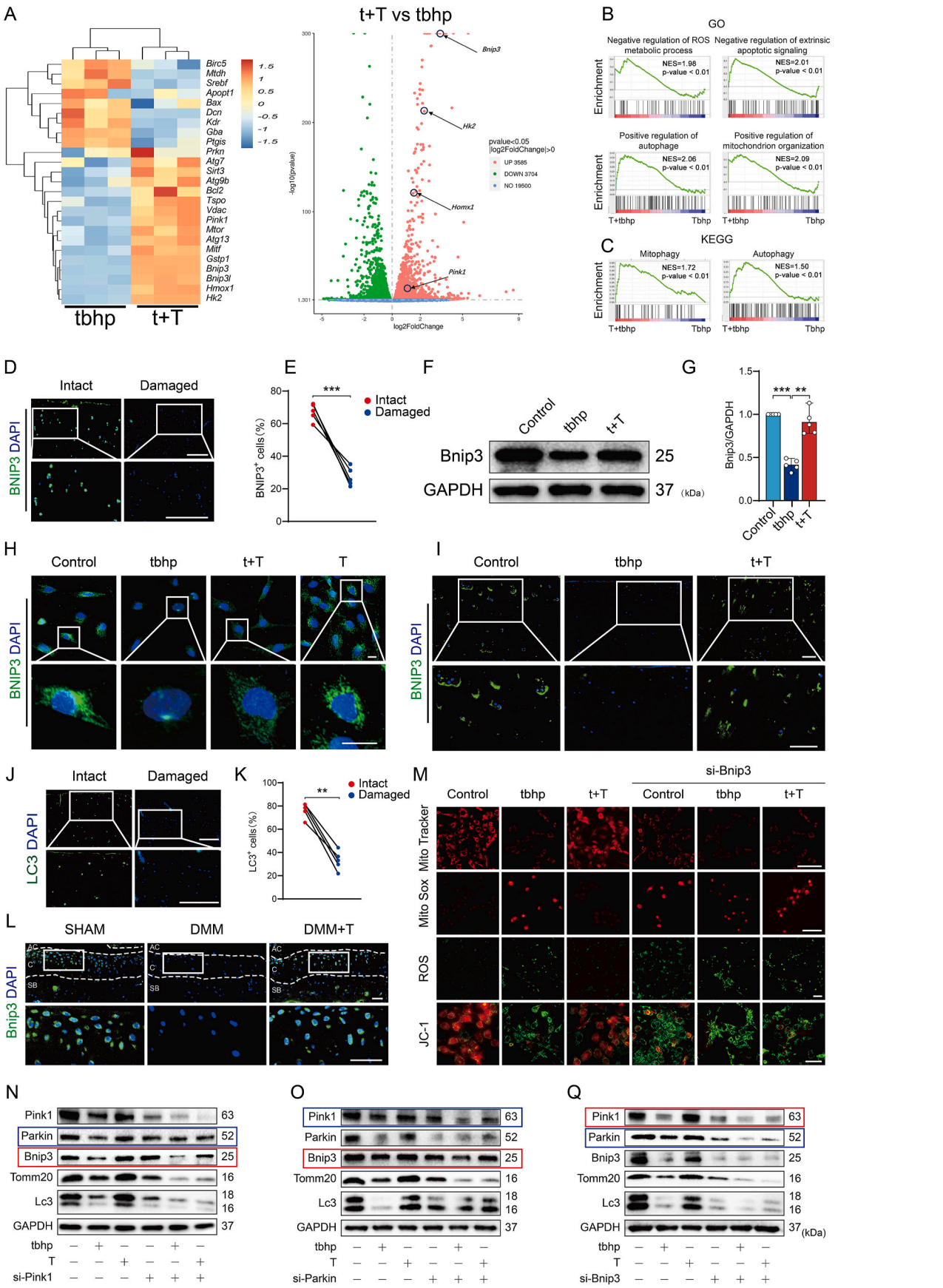

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Fig. 5. Tiopronin enhances mitochondrial autophagy levels through the Bnip3-Pink1/Parkin axis. (A) Heatmap shows the differentially expressed proteins between tbhp-stimulated chondrocytes or tbhp + Tiopronin-stimulated chondrocytes, and the volcano map for differentially expressed genes. (B,C) Gene set enrichment analysis (GSEA) revealing the enrichment of Gene Ontology (GO) terms and KEGG in t + T. (D, E) Immunofluorescence analysis of Bnip3 expression in human cartilage. (n = 5). Scale bar, 100 μm. (F, G) Western blot analysis of Bnip3 levels in mouse chondrocytes after Tiopronin treatment.(n = 5). (H) Immunofluorescence detection of Bnip3 expression levels in mouse chondrocytes after sulforaphane treatment. (n = 6). Scale bar, 50 μm. (I) Immunofluorescence detection of Bnip3 expression levels in human explant tissue after sulforaphane treatment. (n = 5). Scale bar, 100 μm. (J,K) Immunofluorescence detection of LC3 expression levels in human cartilage tissue.(n = 5) Scale bar, 250 μm.(L) Immunofluorescence detection of Bnip3 expression levels in mice after high-concentration Tiopronin administration.(n = 8). Scale bar, 100 μm. (M) Immunofluorescence analysis of mitophagy (Mito Tacker, Mito Sox, and JC-1) and oxidative stress (ROS) after si-Bnip3-mediated Bnip3 knockout.(n = 6). Scale bar, Mito Tacker,JC-1 50 μm. Mito Sox, Ros 100 μm. (N–Q) Western blot analysis of mitochondrial autophagy-related proteins in chondrocytes transfected with Pink1-siRNA, Parkin-siRNA, and Bnip3-siRNA, after TBHP modeling with or without sulforaphane treatment.(n = 3).** $P < 0.01$; ***$P < 0.001$.

[30]. In mouse models of OA, oral administration of tiopronin was compared with celecoxib, a conventional treatment for OA, and demonstrated significant efficacy in alleviating OA progression. This underscores the therapeutic potential of tiopronin through its ability to enhance mitophagy. Meanwhile, the drug instructions for tiopronin indicate that few patients may experience allergic reactions or proteinuria after using the drug. Therefore, patients with severe allergic reactions or renal dysfunction should use the drug with caution. However, the specific side-effect of tiopronin in treating OA with intra-articular injection or long-term oral administration needs to be validated in clinical trials in the future. In conclusion, tiopronin holds promise as a therapeutic agent for OA treatment, potentially through its antioxidative properties and enhancement of mitophagy. Further research into its precise mechanisms and clinical efficacy in human trials is warranted to validate its role in managing OA and improving patient outcomes.

Tiopronin demonstrated a significant effect on up-regulating mitophagy and preventing cartilage degeneration in osteoarthritis (OA). However, the specific target through which tiopronin exerts its effects on mitophagy remains unknown. Previous studies have shown that mutations in Pink1 (PTEN-induced putative kinase 1) are associated with early-onset familial Parkinson's disease (PD) [10]. Regarding mitochondria, Pink1 accumulates on the outer membrane of damaged mitochondria and subsequently recruits Parkin to promote mitophagy [31]. To elucidate the specific mechanism by which tiopronin enhances mitophagy, we performed RNA sequencing on chondrocytes from mice treated with tiopronin. The results indicated that the expression of Bnip3 differential genes was significantly prominent following tiopronin treatment. We also confirmed that the expression of Bnip3 increased both *in vitro* and *in vivo* with tiopronin treatment. Several reports have indicated that BCL2-interacting protein 3 (Bnip3) is a hypoxia-inducible mitochondrial adapter protein that directly interacts with mitochondria and targets sub-entities [12]. Under hypoxic stimulation, Bnip3 is upregulated and anchored to the outer membrane of the mitochondria (MOM) [17]. Notably, both Bnip3 and Pink1 act on the MOM and can interact to promote the accumulation of full-length Pink1 on the MOM, thereby facilitating Parkin recruitment and Pink1/Parkin-mediated mitophagy [32]. Our research revealed that tiopronin primarily promoted mitophagy levels through the marked activation of Bnip3 expression, which subsequently increased the expression of Pink1 and Parkin. Previous studies have identified Bnip3 and Pink1/Parkin as two independent pathways in the process of mitophagy [33,34]. However, recent studies suggested that Bnip3 could affect the Pink1/Parkin induced mitophagy via suppressing PINK1 kinase proteolytic cleavage or regulating the Sirt1/Foxo3 signaling [32,35]. In this study, we used siRNAs to knock down their expression to investigate their relationships and found that Bnip3 is a mitochondrial BH3-only protein that interacts with Pink1. This interaction promotes the accumulation of full-length Pink1 on the outer mitochondrial membrane, thereby enhancing Parkin recruitment and Pink1/Parkin-mediated mitophagy. It was ultimately confirmed that tiopronin promotes the expression of Bnip3 in chondrocytes, activates the downstream Pink1/Parkin signaling pathway, promotes mitophagy, and delays the progression of OA.

In summary, we have confirmed that tiopronin delays the progression of OA by enhancing mitophagy levels through the Bnip3-Pink1-Parkin signalling pathway. Based on studies of animal and human cartilage tissue, we identified that tiopronin has a promising effect in inhibiting cartilage degeneration. However, further research is necessary, including investigations into the specific interactions between the Bnip3 and Pink1/Parkin pathways, as well as the mechanism by which tiopronin promotes the expression of Bnip3. Our findings on the remarkable activation of Bnip3 by tiopronin not only advance the understanding of tiopronin's antioxidant effects but also facilitate the clinical translation of tiopronin in the treatment of OA.

6. Materials and methods

6.1. Ethics statement

Human articular cartilage tissue was obtained from patients with knee osteoarthritis (OA) who underwent total knee arthroplasty, a procedure approved by the Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University (MR-33-24-042514). All experiments involving mice were authorized by the Wenzhou Medical University Animal Experiment Center and were conducted in strict accordance with its regulations (xmsq2023-0672).

6.2. Clinical specimen collection

Cartilage specimens from patients with knee osteoarthritis (OA) (aged 64–82 years, Kellgren–Lawrence grade 4) were obtained from the Second Affiliated Hospital of Wenzhou Medical University. These specimens were divided into intact areas (lateral condyle) and damaged areas (medial). In the cartilage specimens, five pairs were used for the measurement of mitochondrial autophagy-related proteins and cartilage synthesis and degradation markers. The information of these patients was showed in Table S2. Additionally, five patients with knee osteoarthritis (aged 64–71 years, Kellgren–Lawrence grade 4) had specimens taken from relatively intact areas (lateral condyle), stored aseptically at low temperature, cultured for articular cartilage, and then measured for mitochondrial autophagy-related proteins and cartilage synthesis and degradation markers. The information of these patients was showed in Table S3.

6.3. Animal study

Mice of the C57BL/6 strain were procured from the Experimental Animal Research Center of Wenzhou Medical University. All mice were maintained in a sterile environment under a 12-h light/dark cycle and had unrestricted access to food and water. To investigate the therapeutic efficacy of tiopronin during the progression of osteoarthritis, 10-week-old male C57BL/6 mice underwent right knee joint DMM surgery and were euthanized 8 weeks post-operation. The intra-articular injection groups were as follows: (1) Sham surgery; (2) Tiopronin treatment (intra-articular injection, 30/60/90 mg/ml/d, 10 μL per mouse, twice a week); (3) DMM (destabilisation of the medial meniscus); (4) DMM + NS (saline treatment). The gavage groups were as follows: (1) Sham surgery; (2) Tiopronin treatment (gavage, 10/20/40 mg/ml/d, 0.2 ml per mouse, every other day); (3) DMM (destabilisation of the medial

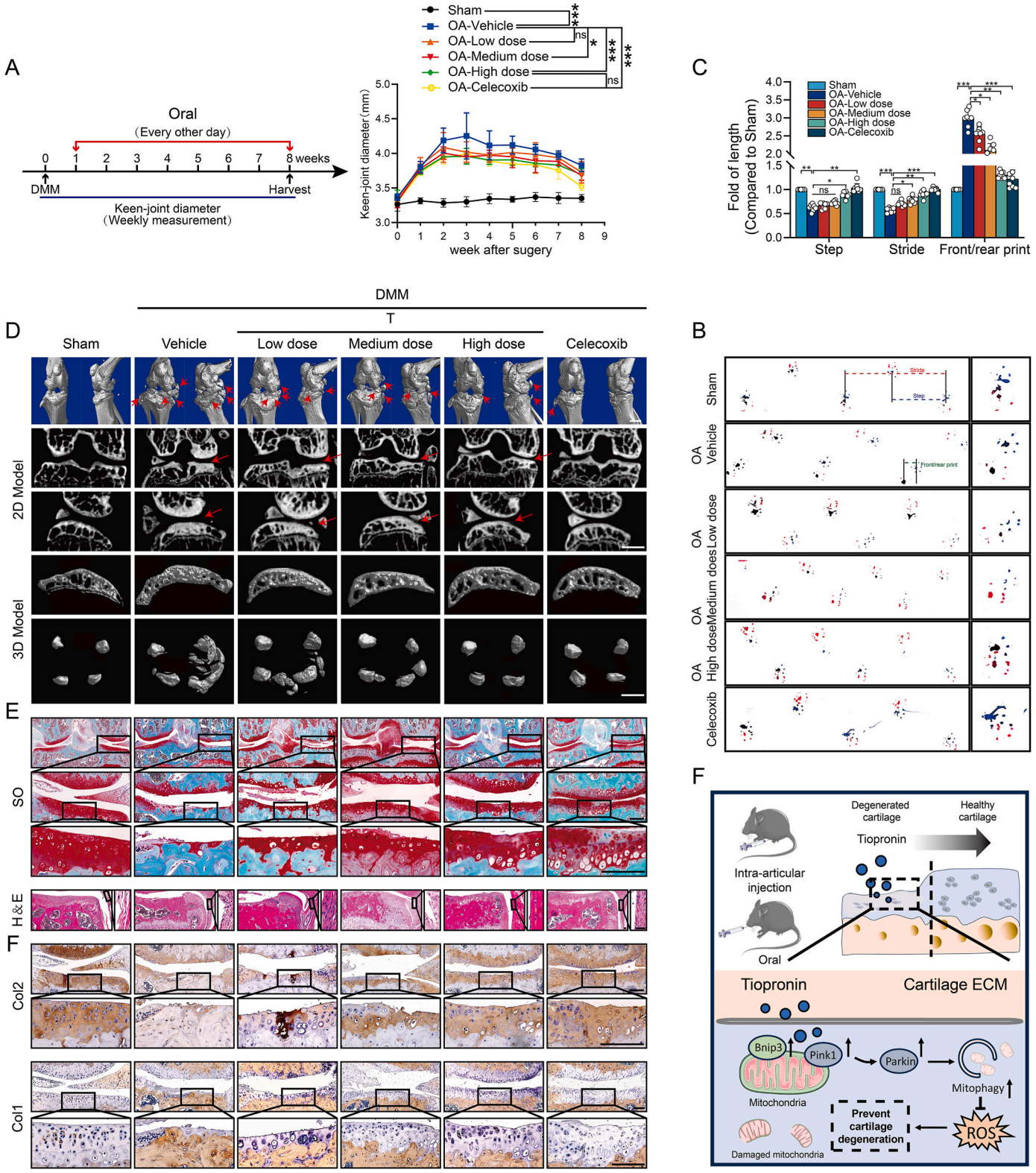

Fig. 6. In comparison to gavage of celecoxib, gavage of Tiopronin can also ameliorate the progression of surgery-induced osteoarthritis. (A) illustrates the schematic of gavage, and the diameter of the knee joint in the affected limb of mice 8 weeks post-surgery (n = 8).(B,C)Gait analysis results for different groups. The blue dotted line represents stride length, the red dotted line represents step length, and the green dotted line represents the length of front/rear paw prints: blue print, fore paw; red print, hind paw. (n = 8)。 (D) Representative images of subchondral bone using micro-computed tomography (μCT) reconstruction (n = 8). Scale bar, 2.0 mm. (E) After 8 weeks of modeling, the joints from the control group and the treatment group were stained with S&O and HE, respectively, to evaluate cartilage formation and synovitis.(n = 8). Scale bar, S&O 500 μm; HE 250 μm.(F) After 8 weeks of modeling, the joints from the control group and the treatment group were stained with immunohistochemistry for Col1 and Col2.(n = 8)Scale bar,500 μm*P < 0.05; **P < 0.01; ***P < 0.001. "ns" means not significant (P > 0.05).

meniscus); (4) DMM + NS (saline treatment); (5) DMM + celecoxib (20 mg/kg/d). Mice in the Sham and DMM groups received 0.2 ml of saline by gavage. The number of mice in each group was calculated according to the formula reported by Charan and Kanthari [15]. All animal studies were conducted in accordance with ethical guidelines.

6.4. DMM surgery-induced OA mouse model

Surgically induced osteoarthritis was induced using the destabilisation of the medial meniscus (DMM) method, as previously described [36]. Following anaesthesia with sodium pentobarbital, mice underwent DMM surgery on their right knee joint, involving transection of the medial meniscus-tibial medial ligament and securing of the medial meniscus to the tibial plateau. Control mice underwent sham surgery involving the same procedure but without ligament transection. The mice were euthanized 8 weeks after surgery.

6.5. Cell culture

Primary mouse chondrocytes were harvested and cultured following previously described methods [37]. Briefly, costal cartilage from 3-day--old C57BL/6 mice was isolated and digested at 37 °C for 4 h using 0.2 % Collagenase 2 (Gibco, USA). After removal of soft tissue, the chondrocytes were cultured in Dulbecco's Modified Eagle's Medium with 1 g/L glucose (Gibco, Carlsbad, CA), supplemented with 10 % fetal bovine serum (Gibco) and 1 % penicillin-streptomycin (Gibco) at 37 °C in a 5 % CO_2 atmosphere. The cell culture medium was refreshed every 72 h. Chondrocytes were treated as specified with 50 μM tbhp (#MKCH9944, Sigma, USA), 5 mM Tiopronin (#HY-B0373, MCE), and 25 mM Mdivi-1 (#HY-15886, MCE).

6.6. Protein extraction and western blot analysis

Chondrocytes were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with protease inhibitors and/or a phosphatase inhibitor cocktail. Total cell lysates were prepared in lysis buffer containing 150 mM NaCl, 1 % Nonidet P-40, 50 mM Tris, and 5 mM NaF. These lysates were then separated by SDS polyacrylamide gel electrophoresis (PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. Following blocking with 5 % nonfat dry milk in 0.1 % Tween 20 Tris-buffered saline (TBST), membranes were incubated overnight at 4 °C with specific primary antibodies. After washing thrice with TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature and visualized using the BIO-RAD ChemiDoc XRS + system. Details of the primary antibodies used are provided in Table S1.

6.7. Immunofluorescence and immunohistochemical staining

The animal and human OA cartilage sections were incubated overnight at 4 °C with the primary antibodies, details of which are provided in Table S1. For immunofluorescent staining, FITC- or tetramethyl rhodamine isothiocyanate (TRITC)–conjugated secondary antibodies were applied to the sections and left in darkness for 1 h. Alternatively, for immunohistochemical staining, the sections were exposed to HRP-conjugated secondary antibodies. Stained sections were imaged using a fluorescence microscope (Zeiss, Heidelberg, Germany).

6.8. Histological analysis

Following decalcification with a 10 % EDTA solution (#1340, BioFroxx, Germany), human cartilage and mouse knee joints were embedded in paraffin blocks and sectioned into 5 μm thick coronal sections using a microtome (Thermo, Germany). As per a previously documented procedure, 15 sections from each tissue sample were stained with Safranin-O/Fast Green (#G1371, Solarbio, Beijing, China) and Hematoxylin-Eosin (H&E) (#C0105S, Beyotime) to assess cartilage damage and synovitis, respectively. Articular cartilage destruction was blindly scored using the Osteoarthritis Research Society International (OARSI) scoring system (0–6), while synovitis was scored on a scale of 0–9 [38]. Scores for OARSI and synovitis were recorded for each segment, and average scores were computed.

6.9. Short interfering RNA (siRNA) transfection of mouse primary chondrocytes

The transfection of siRNAs (Hippobio,Nan Jing, China)was conducted following methods described previously [39]. The siRNAs used were designed and synthesized with the following sequences:

mouse Bnip3: 5′-GCUUUGCAGGAUGAGGAUUTT-3'; mouse Pink1:5′-GCGGUAAUUGACUACAGCAAA-3'; mouse Parkin:5′-CUUGCUGGGACGAUGUCUUAA-3'

6.10. Micro-computed tomography (Mirco-CT) analysis

Following soft tissue dissection, the harvested knee joints were fixed in 4 % paraformaldehyde overnight. Subsequently, the samples were scanned and reconstructed using high-resolution microcomputed tomography (CT) equipment (SkyScan 1172) and CT reconstruction software (NRecon v1.6). For three-dimensional model visualization and further data analysis, CTAn v1.9 and μCTVol v2.0 software tools were employed. The scanning parameters were set at 100 kVp voltage, 200 μA current, and a resolution of 9.066683 μm per pixel. The entire subchondral bone of the specimens was defined as the region of interest, and bone volume fraction (BV/TV) was quantified.

6.11. The culture and treatment of human OA cartilage explants

The culture of human articular cartilage from osteoarthritis (OA) patients who underwent total knee arthroplasty was conducted following previously described methods [40]. Briefly, full-thickness cartilage samples were obtained and rinsed with sterile saline solution. The cartilage was then cut into small pieces measuring 0.5 cm on each side and cultured overnight in low-glucose Dulbecco's Modified Eagle's Medium at 37 °C. Subsequently, the cartilage pieces were randomly assigned to three groups: (1) Control; (2) 50 μM tert-butyl hydroperoxide (tbhp); (3) 50 μM tbhp + 5 mM Tiopronin. The culture medium containing the respective drugs was refreshed every 72 h. After 7 days of treatment, the cartilage pieces were fixed with 4 % paraformaldehyde, embedded in paraffin, and sectioned into 5 μm thick slices for histological analysis.

6.12. OA severity assessment

Assessment of osteoarthritis (OA) severity, including measurement of the affected knee joint diameter and gait analysis, was conducted before surgery and 8 weeks post-surgery. The knee joint diameter was defined as the maximum length in the coronal plane of the mice knee joint, measured using a Vernier caliper (from the medial femoral condyle to the lateral condyle plus the thickness of the swollen joint synovium). Gait analysis was performed to evaluate claudication of the affected limb induced by OA. Animals were placed in a 100 × 10 cm open gait arena and allowed to freely walk from one side to the other without external stimuli or food enticement. Footprints of each mouse were recorded by dyeing the hind paws with red dye and forepaws with blue dye. Outcome measures were assessed by three independent examiners who were blinded to the experimental conditions.

6.13. RNA sequencing (RNA-seq) for the chondrocyte transcriptome

We utilized the NovelBrain Cloud analysis platform to conduct RNA-seq analysis on mouse chondrocytes harvested from C57BL/6 mice rib

samples. Chondrocytes were treated with tbhp and tiopronin for 24 h, after which total RNA was extracted using TRIzol reagent and categorised into Control (Ctrl), tbhp-treated, and tbhp + tiopronin-treated groups (n = 3). RNA-seq was employed to generate cDNA libraries for each mouse RNA sample. Differential gene and transcript expression analysis were performed using TOPHAT and Cufflinks. Gene and long non-coding RNA (LncRNA) counting were conducted using HTSeq software. Flow cytometry was employed for gene expression detection. The DESeq algorithm was applied to identify differentially expressed genes, with significance assessed using P-values and false discovery rate (FDR). Differentially expressed genes were defined by fold changes greater than 2 or less than 0.5, with an FDR threshold of less than 0.05. GO analysis was employed to elucidate the biological significance of differentially expressed genes across biological processes (BP), cellular components (CC), and molecular functions (MF). GO annotations were sourced from NCBI (http://www.ncbi.nlm.nih.gov/), UniProt (http://www.uniprot.org/), and Gene Ontology (http://www.geneontology.org/). Pathway analysis utilising the KEGG database identified pathways significantly influenced by differentially expressed genes. Fisher's exact test was applied to determine the statistical significance of GO categories and pathways, with the significance threshold defined by the P-value.

6.14. Cell viability assay

To evaluate the cytotoxic effects of tbhp on chondrocytes treated with or without Tiopronin, and tbhp on chondrocytes treated with or without the mitochondrial autophagy inhibitor (Mdivi-1), we conducted the Cell Counting Kit-8 (CCK-8) assay (Dojindo Co, Kumamoto, Japan) following the manufacturer's instructions. Chondrocytes were seeded in equal numbers into 96-well plates and treated with tbhp and/or Tiopronin and/or Mdivi-1 for 24 h. After washing with PBS, the cells in each well were incubated with DMEM/F12 containing 10 % (v/v) CCK-8 solution at 37 °C for 2 h. Absorbance was measured at 450 nm using a microplate reader (Thermo Scientific, Logan, UT, USA).

6.15. Statistical analysis

Statistical analysis was performed using SPSS software (version 25.0) and GraphPad Prism software (version 10.1.2). Quantitative data represent results from at least three independent experiments. No samples or animals were excluded during the analysis. The Shapiro–Wilk test was used to assess data normality, and the Levene method was employed to evaluate homogeneity of variance. For comparisons between two groups, paired or unpaired two-tailed Student's t-tests were used as appropriate. One-way or two-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests were conducted to determine statistical significance among mean values of more than two groups. Data are presented as mean values ± standard deviation (SD), and statistical significance was set at $P < 0.05$.

Contribution

YG and JWL conducted the conception and design. YG and JWL performed the drafting of the main part of article. CGW, KJF, SKS, HJZ, CKB, HN, XZ, YYX, QC, XCG, BTC, LZ, CLX, and MZ participated the experiment of this study. JWL and EXX conceived the project and designed the study. JWL, YG and EXX performed the edition and final approval of the article.

Data statement

All data relevant to the study are included in the article.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2025.01.012.

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