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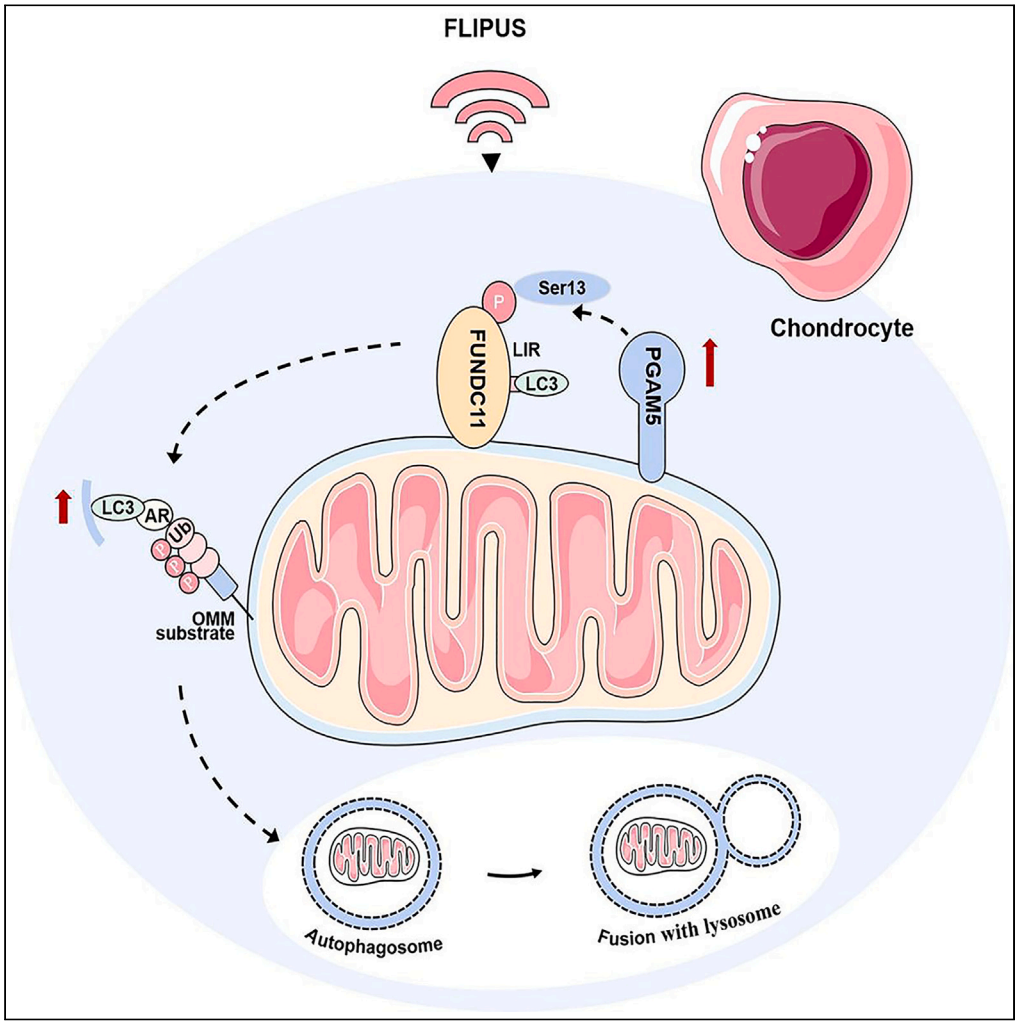

Haixia Ye, Dongqian Li, Xia Wei, Lehua Yu, Lang Jia

300895@cqmu.edu.cn (L.Y.)
jialang@hospital.cqmu.edu.cn (L.J.)

Highlights

Osteoarthritis (OA) is a degenerative disease of the joints and impaired mitophagy

FLIPUS has biological activities in various musculoskeletal disorders

FLIPUS alleviates OA by inhibiting apoptosis and restoring impaired mitophagy

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Focused low-intensity pulsed ultrasound alleviates osteoarthritis via restoring impaired FUNDC1-mediated mitophagy

Haixia Ye,[1,2,3] Dongqian Li,[1,3] Xia Wei,[1] Lehua Yu,[1,*] and Lang Jia[1,4,*]

SUMMARY

Mitophagy is critical for maintaining proper cellular functions, and it contributes to the onset and progression of osteoarthritis (OA). A recent study showed that focused low-intensity pulsed ultrasound (FLIPUS) could activate mitophagy, but the molecular mechanism remains unclear. This study aimed to elucidate the chondroprotective effects of FLIPUS in OA and the regulatory effects on FUN14-domain containing 1 (FUNDC1-mediated mitophagy. *In vitro*, FLIPUS improved inflammatory response, anabolism, and catabolism in interleukin (IL)-1β-induced OA chondrocytes. The chondroprotective effects of FLIPUS were attributed to promoting the expression of phosphoglycerate mutase 5 (PGAM5) and the dephosphorylation of FUNDC1 at serine 13 (Ser13), as well as promoting the mitophagy process. *In vivo*, FLIPUS reduced the cartilage degeneration and apoptosis and reversed the change of anabolic- and catabolic-related proteins in destabilized medial meniscus (DMM)-induced mouse model. Thus, the study indicates that FLIPUS exhibits a chondroprotective effect via activating impaired FUNDC1-mediated mitophagy.

INTRODUCTION

Osteoarthritis (OA) is a major chronic arthrosis and one of the leading causes of disability, pain, and impaired quality of life worldwide.[1] OA is a metabolically active repair process that involves localized loss of cartilage and remodeling of adjacent bone.[2] Cartilage degeneration is the main pathological manifestation of OA, resulting from an imbalance between the breakdown and repair of the cartilaginous tissue. The development of cartilage degeneration occurs as a result of multiple risk factors, such as weight, trauma, age, sex, genetic predisposition, overuse, and mechanical overload;[3] however, the pathogenesis of cartilage degeneration is complicated and requires further elucidation.

Chondrocytes play a vital role in maintaining the biological and mechanical functions of cartilage, including maintaining extracellular matrix (ECM) metabolism and the thickness of articular cartilage and distributing mechanical load across the subchondral bone.[4] Excessive intrinsic chondrocyte apoptosis was confirmed to be a major contributor to cartilage degeneration, while inhibiting apoptosis has been shown to be an effective and potential method for OA[5,6]; therefore, the inhibition of the excessive apoptosis pathway in OA deserves further study.

Autophagy plays an important part of cellular process, including chondrocyte fate. Autophagy-related proteins such as microtubule-associated protein light chain 3 (LC3) and Beclin-1 were markedly increased in chondrocytes and associated with increased apoptosis.[7] In an OA mouse model, cartilage damage is accompanied by an increase of apoptosis and a loss of key autophagy-related proteins.[8] Autophagy activation alleviated the severity of experimental OA while silencing of beclin-1 resulted in enhanced chondrocyte death.[9] These previous studies have strengthened knowledge of the protective role of autophagy against both OA development and chondrocyte apoptosis. Recently, mitochondrial dysfunction is observed in degenerative chondrocytes, and the relationship between mitochondrial dysfunction and apoptosis was investigated in a previous study.[10] Mitophagy is the selective degradation of mitochondria by autophagy, which is a pivotal mechanism for mitochondrial homeostasis and inhibits the initiation of excessive apoptosis.[11] Accumulating evidence also demonstrates the critical roles of mitophagy in the progression of OA. It has been found that mitophagy-related proteins including PINK1, LC3B, and SQSTM1 are highly expressed in cartilage from OA patients and monosodium iodoacetate (MIA)-induced rodent model of OA,[12] which shows a strong link between OA and mitophagy.

Ultrasound (US) treatment has been used as a noninvasive modality for the management of knee OA through thermal and nonthermal modalities (mechanical effects or cavitation effect). In recent years, the mechanical effects of focused low-intensity pulsed ultrasound (FLIPUS) have been indicated to be an effective and safe treatment modality for patients with knee OA.[13] Furthermore, in a prospective

[1]Department of Rehabilitation Medicine, The Second Affiliated Hospital of Chongqing Medical University, Chongqing 400010, China
[2]Department of Rehabilitation Teaching and Research, Chongqing City Management College, Chongqing 401331, China
[3]These authors contributed equally
[4]Lead contact
*Correspondence: 300895@cqmu.edu.cn (L.Y.), jialang@hospital.cqmu.edu.cn (L.J.)
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randomized trial, FLIPUS was more effective than pulsed shortwave diathermy in alleviating pain and in improving dysfunction and health status among subjects with knee OA in the short term.[14] In basic science studies, FLIPUS application promoted ECM production in a surgically induced OA rabbit model by downregulating prostaglandin E2 and nitric oxide, joint effusion volume, and chondrocyte apoptosis.[15] Meanwhile, electron microscopy analysis showed that FLIPUS induced typical autophagosomes that enclosed mitochondria in degenerative chondrocyte, which indicated that FLIPUS potentially inhibits the apoptosis via mitophagy activation.

Recently, specific pathways associated with mitophagy have been detected and identified, among which phosphoglycerate mutase 5 (PGAM5)/FUN14-domain containing 1 (FUNDC1) has become a hotspot and node for research on mitophagy.[16] PGAM5 is a mitochondria-localized serine/threonine protein phosphatase with the function of dephosphorylating protein serine/threonine.[17] PGAM5 is the key protein phosphatase that dephosphorylates serine at position 13 of FUNDCI under hypoxic signaling.[18] FUNDC1 is a mitochondrial autophagy protein, i.e., a tertiary transmembrane protein localized on the external mitochondrial membrane, that induces mitochondrial autophagy under hypoxic conditions.[19] FUNDC1 possesses a key autophagy molecule, the microtubule-associated protein 1 light chain 3 (LC3)-interacting region (LIR), and the phosphorylation state of serine at position 13 of the LIR affects the onset of mitochondrial autophagy. In particular, FUNDC1 dephosphorylation in response to hypoxia or reduced mitochondrial membrane potential allows the conversion of LC3I to LC3II and activation of mitochondrial autophagy.[19] TOM20 and TIM23 are mitochondrial membrane markers.[20] When mitochondrial autophagy occurs, LC3 expression increases, while TOM20 and TIM23 levels decrease, resulting in the degradation of damaged or aged mitochondria and thus reducing the release of proapoptotic factors, such as Bcl-2-associated X protein (BAX).[21,22] The cytoprotective effects of mitochondrial autophagy via the PGAM5/FUNDC1 pathway have been reported.[22] In this pathway, it is likely that PGAM5 senses mitochondrial stress and dephosphorylates FUNDC1 at serine 13 (Ser13). Dephosphorylated FUNDC1 then interacts with LC3 through its typical LC3-binding motif Y(18)xxL(21) and subsequently induces mitophagy.[19]

Based on our previous observation, we hypothesized that FLIPUS could activate mitophagy in degenerative chondrocytes. However, how mitophagy is regulated in pathophysiological conditions under FLIPUS application is still unclear. Therefore, in this study, we investigated whether FLIPUS promotes dephosphorylation of FUNDC1 at Ser13 through upregulation of PGAM5 expression in OA chondrocytes and activates mitophagy, thereby downregulating chondrocyte apoptosis. In general, this study allowed us to elucidate the role of FLIPUS in chondrocyte biology, particularly mitophagy, thereby providing an understanding of OA pathogenesis and potentially representing an essential molecular target for OA management.

RESULTS

FLIPUS decreased apoptosis and ECM loss in IL-1β-treated murine chondrocytes

First, we investigated the effect of FLIPUS on the ECM of primary chondrocytes. FLIPUS increased the expression of collagen II at the mRNA (Figure 1A) level in primary murine chondrocytes treated with interleukin (IL)-1β, thus suggesting the role of FLIPUS in increasing this cartilage marker. In addition, FLIPUS downregulated the levels of matrix metalloproteinase 13 (MMP13) at the mRNA (Figure 1B) and protein (Figures 1C and 1E) levels and increased type II collagen expression (Figures 1C and 1D). The present results suggest that FLIPUS attenuated IL-1β-induced ECM degradation in mouse chondrocytes.

Then, we determined the effects of FLIPUS on chondrocyte apoptosis. The western blot results showed that the protein expression of Bax (Figures 1F and 1G) and cleaved Caspase3 (Figures 1F and 1I) was increased, while the expression of Bcl2 (Figures 1F and 1H) was decreased after chondrocytes were treated with IL-1β. After FLIPUS treatment, the expression levels of Bax (Figures 1F and 1G) and cleaved Caspase3 (Figures 1F and 1I) were downregulated, and the level of Bcl2 (Figures 1F and 1H) was upregulated in chondrocytes that were treated with IL-1β. Chondrocyte apoptosis was further examined by flow cytometry, and the results showed that chondrocyte apoptosis in the IL-1β + FLIPUS (12.63 $\pm$ 1.168) group was lower than that in the IL-1β group (25.94 $\pm$ 3.042) (Figure 1J). These results indicate that FLIPUS decreases chondrocyte apoptosis *in vitro*.

FLIPUS ameliorated OA development in the destabilized medial meniscus (DMM) mouse model

To investigate the therapeutic role of FLIPUS in the development of OA *in vivo*, mice were treated with FLIPUS, and Safranin O–Fast Green staining, immunohistochemical staining, and TUNEL staining were performed to assess histomorphological differences in the knee joints. Articular cartilage erosion and proteoglycan loss were observed in DMM mice by Safranin O–Fast Green staining. In contrast, FLIPUS treatment effectively alleviated cartilage destruction and increased proteoglycan expression compared with the effect of DMM (Figure 2A). Consistent with the staining results, the Osteoarthritis Research Society International (OARSI) score was increased in both the medial tibial plateau (MTP) and medial femoral condyl (MFC) in the DMM group and was decreased in the FLIPUS treatment group (Figure 2B). Immunohistochemical staining showed that DMM surgery increased the expression of MMP13 and decreased the expression of Col2α1 and Aggrecan in articular cartilage. FLIPUS treatment relieved the loss of Col2α1 and Aggrecan and reduced the expression of MMP13 in mouse articular cartilage compared with that in the DMM group (Figures 2C–2F). TUNEL staining showed that the increased number of TUNEL-positive chondrocytes in the DMM group was reduced by FLIPUS treatment (Figures 2G and 2H). Collectively, these results suggest that FLIPUS alleviates the loss of cartilage matrix, decreases chondrocyte apoptosis, and ameliorates OA development in the DMM mouse model.

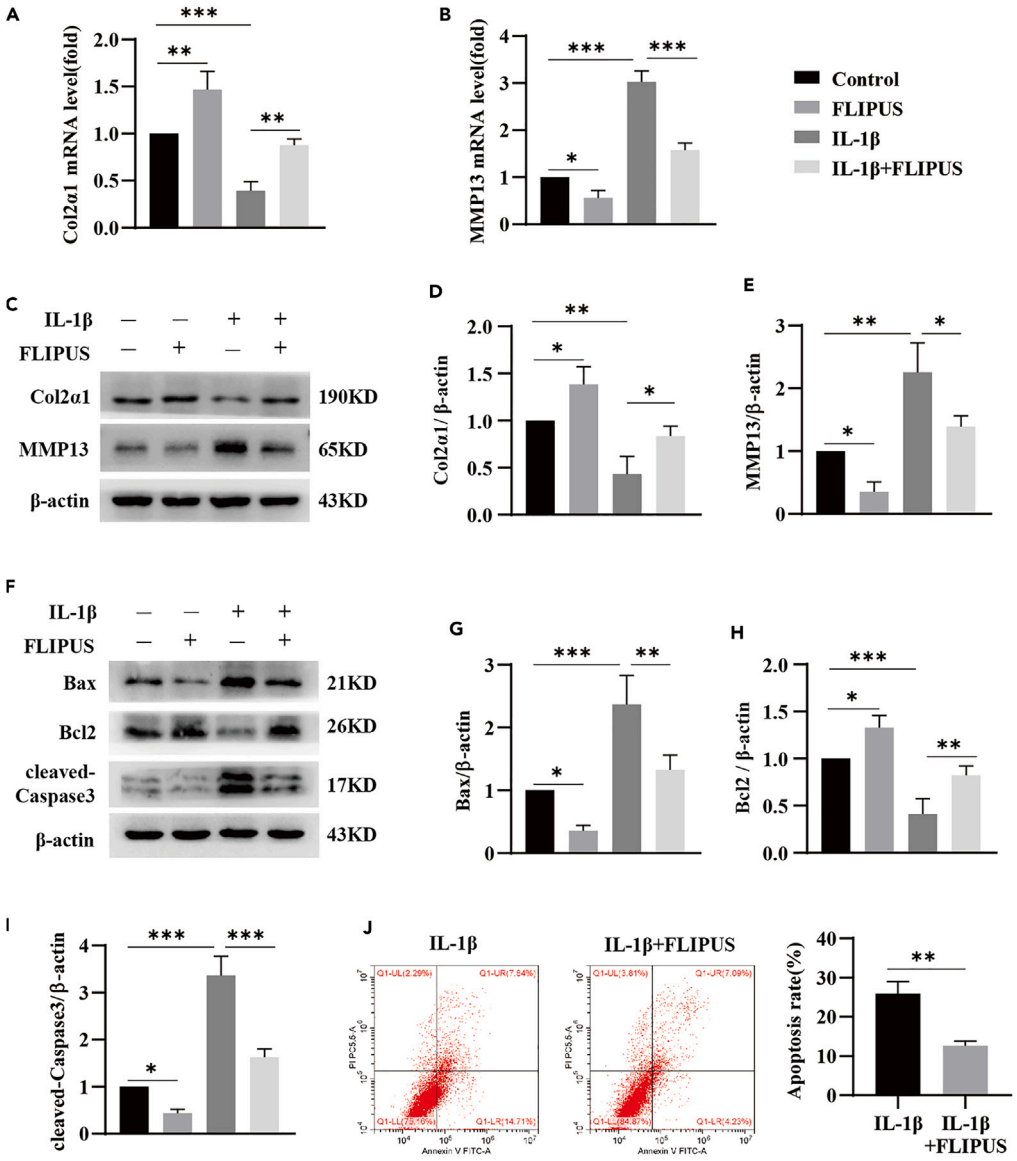

Figure 1. Effect of FLIPUS on apoptosis and the secretion of extracellular matrix by primary chondrocytes

(A and B) Relative mRNA levels of Col2α1 and MMP13 were examined by RT-PCR(n = 3).

(C–E) The protein expression of Col2α1 and MMP13 was examined by western blotting (n = 3).

(F–I) The protein expression of Bax, Bcl2, and cleaved Caspase3 was examined by western blotting (n = 3).

(J) Chondrocyte apoptosis was examined by flow cytometry (n = 3). The data are presented as the mean ± SD of independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

FLIPUS enhanced mitophagic flux in chondrocytes

Previous studies have revealed that activating mitophagy in chondrocytes can reduce chondrocyte apoptosis, alleviate cartilage matrix degradation, and ameliorate OA development.[23,24] Thus, we examined whether the FLIPUS-mediated reduction in chondrocyte apoptosis was related to the regulation of mitophagy. First, we performed immunohistochemical staining to examine the autophagy-related marker LC3 and found that the protein level of LC3 was increased by FLIPUS in DMM mice (Figure 3A). Then, western blotting was performed to examine the expression of mitochondrial autophagy-related proteins in primary chondrocytes. The results showed that FLIPUS increased the protein level of LC3-II (Figures 3B and 3C) in IL-1β-treated chondrocytes, while the expression levels of the mitochondrial membrane proteins TOM20 (Figures 3B and 3D) and TIM23 (Figures 3B and 3E) were decreased, suggesting that FLIPUS increased the level of mitophagy and promoted mitochondrial autophagy. We further observed autophagic vacuoles in chondrocytes by TEM, and the results showed that there were few

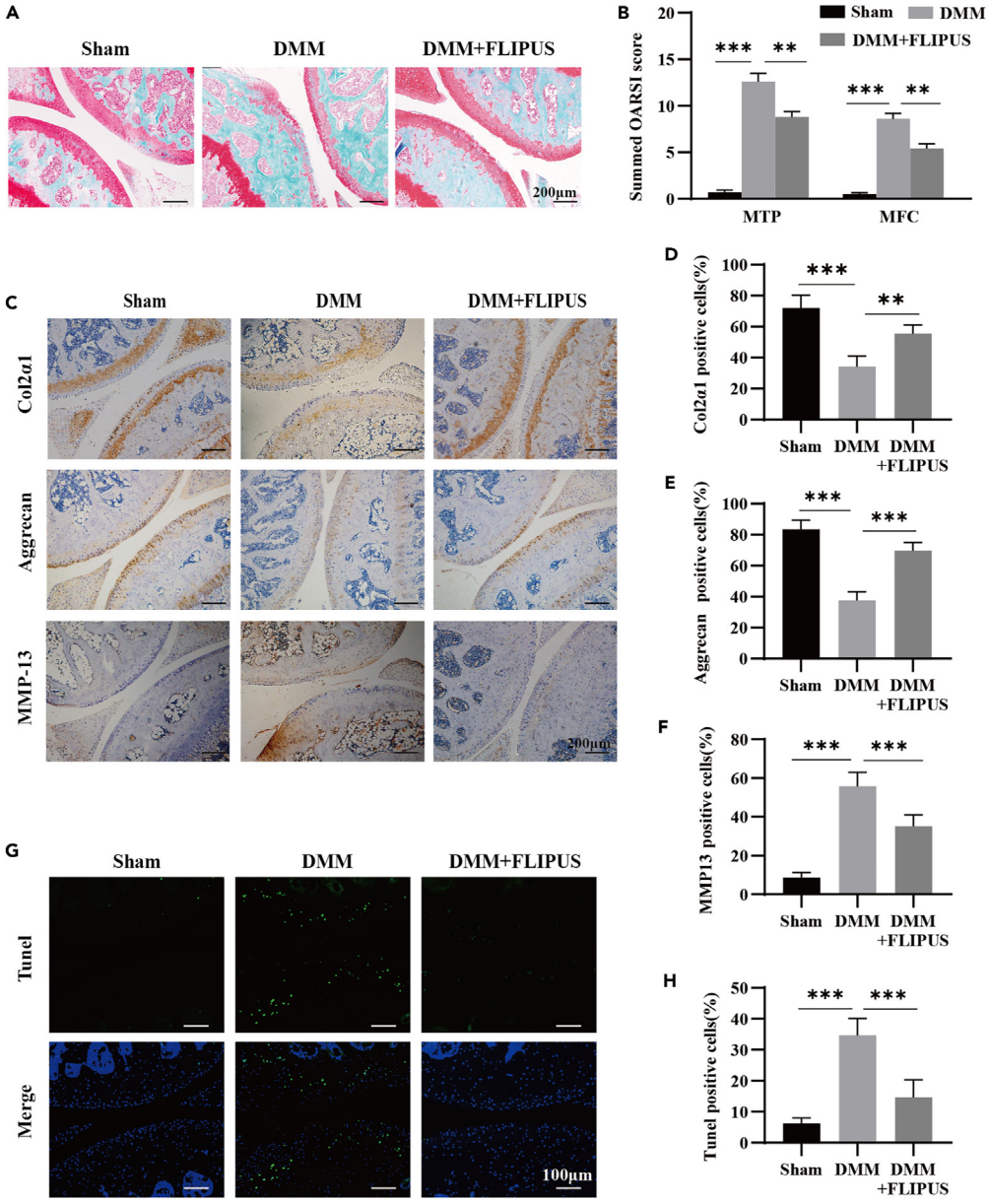

Figure 2. FLIPUS ameliorated OA development in the DMM mouse model

(A) Representative images of Safranin O–Fast Green staining in the Sham, DMM, and DMM + FLIPUS groups.

(B) The OARSI scores of the MTP and MFC in the three groups (n = 5/group).

(C) Immunohistochemical staining showed the expression of Col2α1, Aggrecan, and MMP13 in the three groups.

(D–F) Percentages of cells that were positive for Col2α1, Aggrecan, and MMP13 in the three groups (n = 5/group).

(G) Representative images of TUNEL staining.

(H) The TUNEL-positive rates in the three groups (n = 5/group). The data are presented as the mean ± SD of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

autophagic vacuoles in IL-1β-treated chondrocytes, while more autophagic vacuoles were observed after FLIPUS treatment (Figure 3F). As the increase in LC3-II and autophagic vacuoles may arise from either enhanced autophagic flux or decreased autophagic degradation,[25] we further transfected primary chondrocytes with a dual-tagged LC3 [red fluorescence protein (RFP)-green fluorescence protein (GFP)-LC3] adenovirus to determine the effect of FLIPUS on autophagic flux. The number of autolysosomes labeled with RFP in IL-1β-treated chondrocytes was increased by FLIPUS, and the number of RFP puncta exceeded that of GFP puncta (Figure 3G). These results suggest that FLIPUS enhances mitophagic flux in chondrocytes.

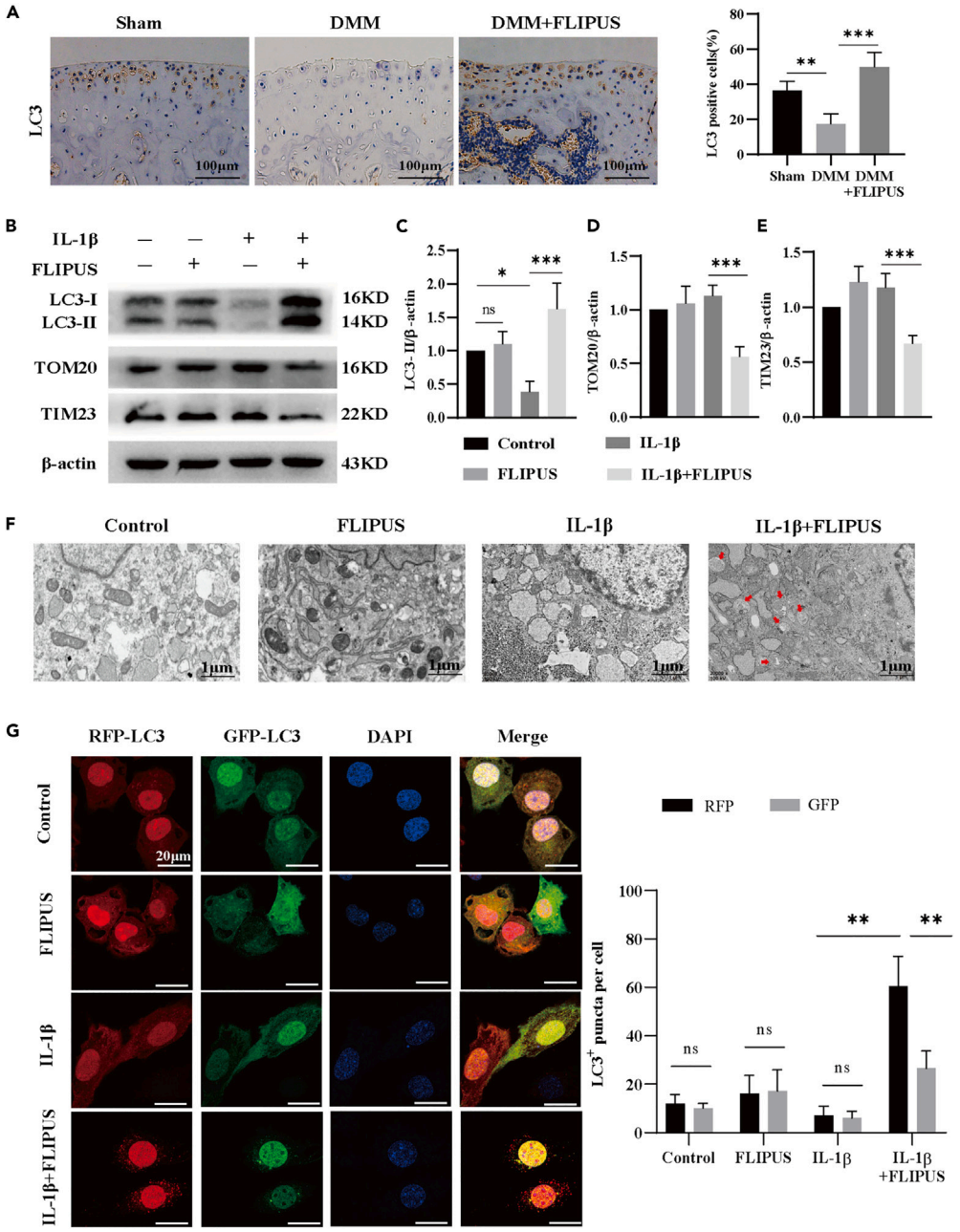

Figure 3. Effect of FLIPUS on mitophagic flux in chondrocytes

(A) Immunohistochemical staining showed the expression of LC3 in the articular chondrocytes of mice (n = 5/group).

(B–E) The protein expression of LC3, TOM20, and TIM23 was examined by western blotting (n = 3).

(F) TEM was used to examine autophagic vacuole (AV) formation in primary chondrocytes treated with Control, FLIPUS,IL-1β or IL-1β + FLIPUS. A red arrowhead indicates an AV.

(G) Confocal images of primary chondrocytes stably expressing Ad-RFP-GFP-LC3. Five different arbitrary areas were counted (right panel, n = 5). Statistical analysis was performed using two-way ANOVA. The data are presented as the mean ± SD of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

FLIPUS activated mitophagic flux to regulate apoptosis-related protein

We further used bafilomycin A1 (BafA1) and Liensinine to inhibit autophagic flux and mitophagy to explore the effect of FLIPUS-enhanced mitophagic flux on chondrocyte apoptosis. Western blot analysis showed that the protein level of Col2α1 was decreased in the IL-1β + BafA1 + FLIPUS group and and IL-1β + Liensinine + FLIPUS group, while the level of MMP13 was increased (Figures 4A and 4B). The results also showed that the

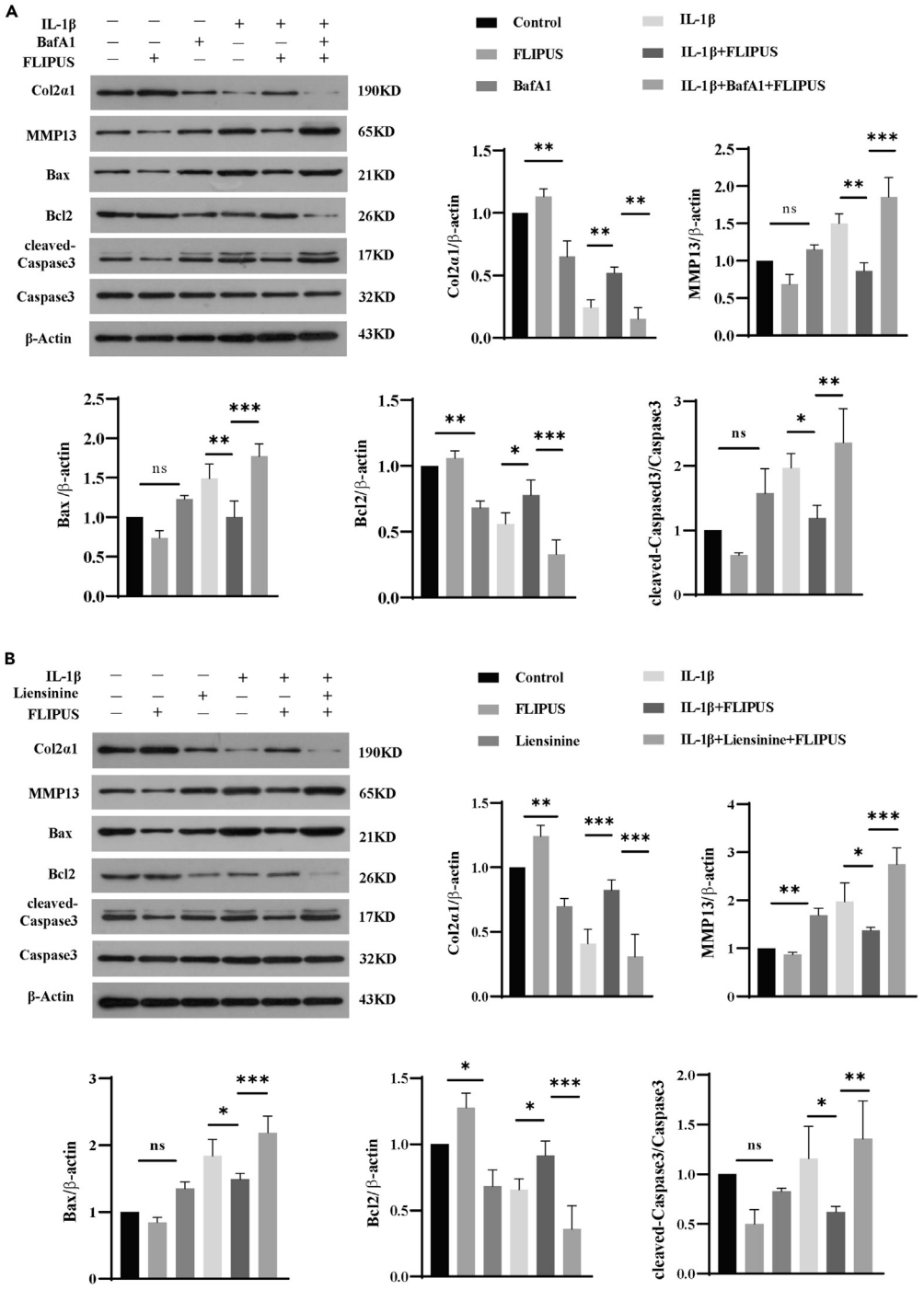

Figure 4. FLIPUS reduced chondrocyte apoptosis by enhancing autophagic flux

(A and B) Primary chondrocytes were treated with IL-1β with or without 20 nM BafA1, 20 μM Liensinine and then treated with or without FLIPUS. Then, the protein levels of Col2α1, MMP13, Bax, Bcl2, and cleaved Caspase-3 were analyzed by western blotting (n = 3). The data are presented as the mean ± SD of independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

expressions of Bax and cleaved Caspase3 in chondrocytes in the IL-1β+ BafA1 + FLIPUS group and IL-1β+ Liensinine + FLIPUS group were higher than those in the IL-1β + FLIPUS group, and the expression of Bcl2 was significantly lower than that in the IL-1β + FLIPUS group (Figures 4A and 4B). These results reveal that FLIPUS-enhanced mitophagic flux decreases chondrocyte apoptosis and alleviates ECM degradation.

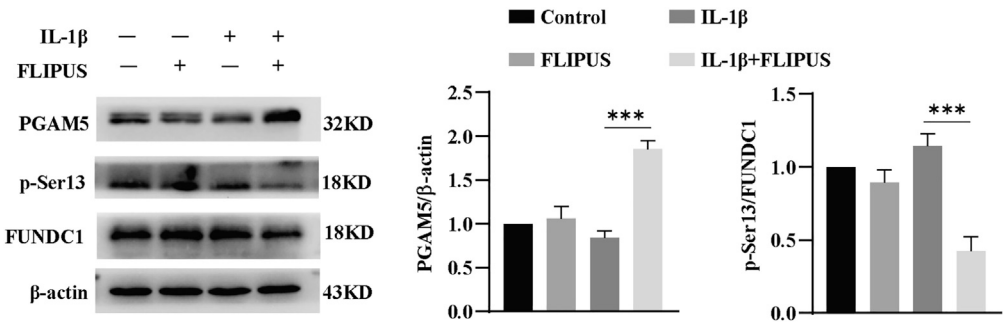

Figure 5. Effect of FLIPUS on mitophagy via the PGAM5/FUNDC1 signaling pathway

The protein expression levels of PGAM5, FUNDC1, and *p*-Ser13 were examined by western blotting (n = 3). The data are presented as the mean ± SD of independent experiments. ***p < 0.001.

FLIPUS promoted chondrocyte mitophagy through the PGAM5/FUNDC1 signaling pathway

To investigate whether FLIPUS activates mitophagy through the PGAM5/FUNDC1 signaling pathway, we examined protein expression and found that FLIPUS induced significant FUNDC1 dephosphorylation at Ser13 in IL-1β-treated chondrocytes, accompanied by the degradation of mitochondrial proteins and an increase in LC3-II (Figure 5). We also found that FLIPUS increased the protein level of PGAM5 in IL-1β-treated chondrocytes (Figure 5). As a result, we suggest that FLIPUS enhances chondrocyte mitophagy through the PGAM5/FUNDC1 signaling pathway.

FUNDC1 dephosphorylation at Ser13 was associated with mitophagy activation in response to FLIPUS

To further examine whether FLIPUS dephosphorylated FUNDC1 at Ser13, we transfected chondrocytes with Ad-FUNDC1 to knock down the expression of FUNDC1 at both the RNA (Figure 6A) and protein levels (Figure 6B). The results indicated that FLIPUS induced significant FUNDC1 dephosphorylation at Ser13 in both the control group (IL-1β group) and the control adenovirus group (IL-1β+ Ad-GFP group), thereby activating mitophagy in chondrocytes (Figure 6C). However, FLIPUS could not activate mitophagy in FUNDC1-knockdown chondrocytes (Figure 6C). These results suggest that FLIPUS enhances mitophagy by dephosphorylating FUNDC1 at Ser13.

FLIPUS upregulated PGAM5 expression to dephosphorylate FUNDC1 at Ser13

PGAM5 is the major phosphatase that is responsible for FUNDC1 dephosphorylation at Ser13, as previously described. To verify whether FLIPUS dephosphorylates FUNDC1 at Ser13 by upregulating the expression of PGAM5, we knocked down the PGAM5 gene in chondrocytes with Ad-PGAM5 (Figures 7A and 7B). The data showed that FLIPUS upregulated the protein level of PGAM5 in both the IL-1β and IL-1β + Ad-GFP groups, which reduced the phosphorylation of FUNDC1 at Ser13 and thus activated mitophagy (Figure 7C). After knocking down PGAM5 expression, FLIPUS could not dephosphorylate FUNDC1 at Ser13 and subsequently activate mitophagy (Figure 7C). These results demonstrate that FLIPUS dephosphorylates FUNDC1 at Ser13 by upregulating the expression of the phosphatase PGAM5 and ultimately activates mitophagy in chondrocytes.

DISCUSSION

In the current study, we discovered that FLIPUS activated FUNDC1-mediated mitophagy. Under hypoxic conditions, we showed that FLIPUS led to PGAM5 overexpression, which catalyzed the dephosphorylation of FUNDC1 at Ser13. Subsequently, the dephosphorylated form of FUNDC1 interacts with LC3 to activate mitophagy.[26] Our data demonstrate that FLIPUS finely tunes mitophagy and broaden our view of the effect of FLIPUS on selective mitophagy through the PGAM5/FUNDC1 signaling pathway in chondrocytes. To the best of our knowledge, this is the first study to describe the effects of FLIPUS on the mitophagy signaling pathway for the management of OA.

FLIPUS has been proven to be an effective and safe modality for relieving pain and improving the joint functions and quality of life of patients with knee OA.[13] In an *in vivo* study, FLIPUS relieved the severity of cartilage degeneration and inhibited apoptosis in a surgically induced osteoarthritic rabbit model.[27] Present US parameters were selected according to our previous study.[15] Compared with sham stimulation, FLIPUS (0.6 MHz, 120 mW/cm^2, 300 Hz, 20%, 20 min) attenuated the release of type II collagen and proteoglycan, reduced chondrocyte apoptosis and total joint effusion volume, and decreased the levels of prostaglandin E2 and nitric oxide in the synovial fluid. The potential biological effects of US arise from thermal effects, mechanical effects, and cavitation effects, which are closely related to physical parameters and biological characteristics of body tissue, including pulsing modes, intensity, frequency, duration of sonification, and the attenuation coefficient of tissue. A previous study reported that only 0.21°C increases in tissue-mimicking materials were detected after 10 min of exposure to Doppler US with an intensity of 290 mW/cm^2 and a frequency of 3 MHz.[28] In the present study, FLIPUS had an intensity of 120 mW/cm^2 and a frequency of 0.6 MHz, which were lower than those of Doppler US. In theory, an increase in temperature induced by FLIPUS should be smaller than 0.21°C. Therefore, the thermal effect was negligible after FLIPUS application. Moreover, cavitation generated through

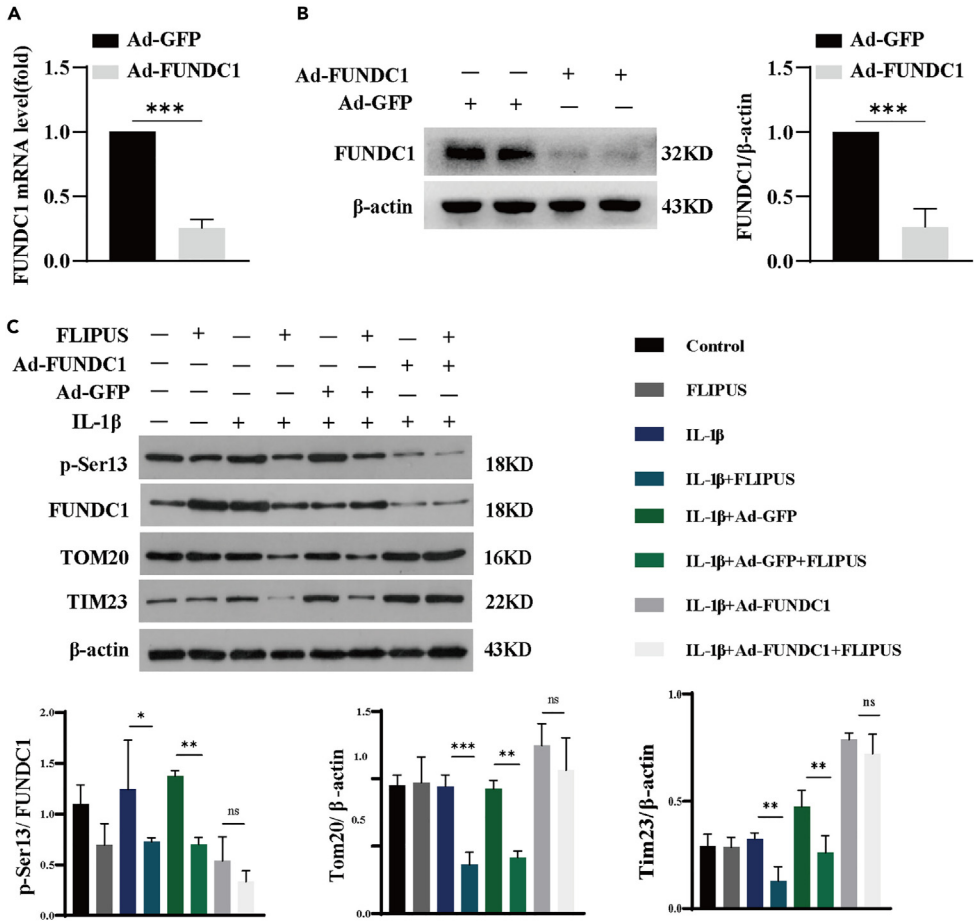

Figure 6. Effects of FLIPUS on mitophagy in chondrocytes after FUNDC1 knockdown

(A) After IL-1β-treated chondrocytes were transfected with Ad-GFP or Ad-FUNDC1, the relative mRNA level of FUNDC1 was examined by RT-PCR (n = 3).

(B) The protein expression of FUNDC1 was examined by western blotting in the Ad-GFP and Ad-FUNDC1 groups (n = 3).

(C) IL-1β-treated chondrocytes were transfected with Ad-GFP or Ad-FUNDC1 and then treated with or without FLIPUS. Then, the protein expression of FUNDC1, *p*-Ser13, TOM20, and TIM23 was examined by western blotting (n = 3). The data are presented as the mean ± SD of independent experiments. *p < 0.05, **p < 0.01.***p < 0.001.

ultrasonic waves of 0.6 MHz is as high as 100–400 W/cm^2,[15] and the cavitation effect of FLIPUS (120 mW/cm^2) was negligible in the present study as well. The advantage of FLIPUS over low-intensity ultrasound (LIPUS) is that focused transducers have the capability to concentrate US energy in the "focus"; as a result, higher intensities can be reached with respect to unfocused transducers driven with the same electric field. In summary, the main biological effect of FLIPUS in our experiment is a mechanical effect.

Mechanical load is usually a double-edged sword in the physiology and pathology of chondrocytes. Overloading promotes OA-like degradation of cartilage and chondrocyte apoptosis, which causes OA development;[27] however, in the current study, moderate mechanical load induced by FLIPUS efficiently decreased apoptosis and attenuated the release of ECM and the structural progression of OA *in vitro* and *in vivo* (Figures 1 and 2). Previously, we showed that FLIPUS induced typical autophagosomes that enclosed mitochondria in chondrocytes. We concluded that FLIPUS protected chondrocytes by activating mitophagy.

Mitophagy is considered to be protective against the pathological conditions through degrading useless proteins and dysfunctional mitochondria, including OA.[29] Different mitochondrial responses are triggered by different cellular stresses, including hypoxia, phenylhydrazones carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), and carbonyl cyanide-p-(trifluoromethoxy) phenylhydrazone (FCCP).[30] The main mitophagic pathways involve Parkin-dependent pathways and non-Parkin-dependent pathway. In Parkin-dependent pathways, Parkin follows mitochondrial accumulation of PINK1 and promotes mitochondrial engulfment by autophagosomes, indicating mitophagy. The non-Parkin-dependent pathway involves the direct interaction of LC3 and the mitochondrial receptors that activate mitophagy through the activation of NIX/BNIP3[31,32] L, BNIP3,[33] and FUNDC1.[34]

FUNDC1 was reported as a unique receptor that participated in the occurrence of mitophagy.[19] FUNDC1 possesses a classical LIR that interacts with LC3 to recruit isolation membranes for subsequent mitophagy. The PGAM5 is a serine/threonine (Ser/Thr) phosphatase that is located in mitochondria and exists in equilibrium between dimeric and oligomeric states that can sense oxidative load to alter the

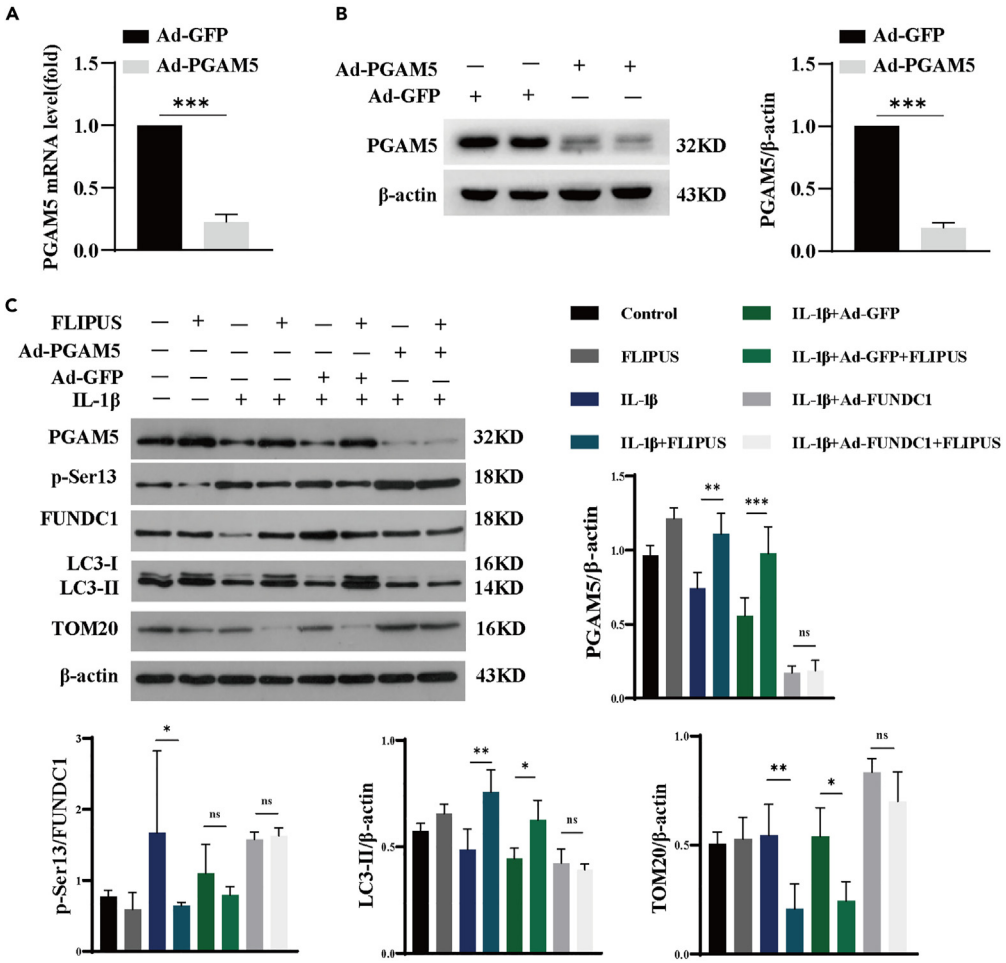

Figure 7. Effects of FLIPUS on mitophagy in chondrocytes after PGAM5 knockdown

(A) After chondrocytes were transfected with Ad-GFP or Ad-PGAM5, the relative mRNA level of PGAM5 was examined by RT-PCR (n = 3).

(B) The protein expression of PGAM5 was examined by Western blotting in the Ad-GFP and Ad-PGAM5 groups (n = 3).

(C) IL-1β-treated chondrocytes were transfected with Ad-GFP or Ad-PGAM5 and then treated with or without FLIPUS. Then, the protein expression levels of PGAM5, FUNDC1, *p*-Ser13, LC3, and TOM20 were examined by western blotting (n = 3). The data are presented as the mean ± SD of independent experiments. **p < 0.01, ***p < 0.001.

multimeric status of mitochondrial PGAM5, thus freeing PGAM5 from BCL-xL.[32] The dephosphorylation of FUNDC1 at Ser13 by the PGAM5 enhances its binding affinity for LC3 and facilitates the anchoring of mitochondria into autophagosomes.[30]

In our current study, the IL-1β induces cell senescence, ECM metabolic unbalance, and apoptosis, downregulating FUNDC1 dephosphorylation at Ser13 and PGAM5, and decreasing mitophagy. Interestingly, FLIPUS shows significant therapeutic effects against cell senescence and apoptosis. To investigate the protective effect of FLIPUS via mitophagy, we performed Liensinine[35,36] to block mitophagy in chondrocytes. Our results suggested that FLIPUS activated mitophagy flux to decrease chondrocyte apoptosis, and the protective effects of FLIPUS were suppressed by the mitophagy inhibitor Liensinine, which confirmed our previous hypothesis.

In agreement with previous study,[37] mitophagy played an important role in preventing apoptosis and aging of chondrocytes to postpone OA. As a physical therapy, FLIPUS could elevate type II collagen and proteoglycans activity via mechanical effect.[15] Among them, COL2A1 and proteoglycans protect chondrocyte function and maintain ECM anabolism. Besides, FLIPUS reduced apoptosis, cellular reactive oxygen species (ROS), and MMP13 and alleviated articular cartilage damage *in vivo* and *in vitro*.[38] Therefore, these substantial findings suggested that FLIPUS played a vital role in maintaining the ECM integrity in articular cartilage. Furthermore, we found that FLIPUS not only enhanced the ECM synthesis at the transcriptional level but also inhibited the IL-1β-induced ECM degradation through enhancing mitophagy. To explore the mechanism of FLIPUS on mitophagy activation, the expression PGAM5/FUNDC1 signaling pathway was investigated in chondrocytes. FLIPUS induced significant FUNDC1 dephosphorylation at Ser13 and the protein level of PGAM5 in IL-1β-treated chondrocytes, accompanied by the degradation of mitochondrial proteins and an increase in LC3-II and ratio of LC3-II/LC3-I. The ratio of LC3-II/LC3-I had been increased slightly after FLIPUS application since the LC3-II/LC3-I ratio is an indicator of mitophagy. Though there was barely statistically

significant difference between FLIPUS+IL-1β group and IL-1β group, which may be relevant to relative small sample size, an increasing trend was observed in the present study. Moreover, we performed the FUNDC1 small interfering RNA (siRNA) and PGAM5 siRNA to knock down the FUNDC1 and PGAM5 expression in chondrocytes, respectively. The siRNA PGAM5 and siRNA FUNDC1 could significantly reduce the LC3 expression and mitophagy under FLIPUS application, indicating that PGAM5/FUNDC1 was a significant mechanism involved in FLIPUS protective effect.

In conclusion, our results reveal a potential mechanism for mitophagy that is a crucial pathway in OA chondrocytes treated with FLIPUS. Specifically, FUNDC1 was related to the OA progression and regulated mitochondrial dysfunctions via mitophagy in the damaged chondrocytes. FLIPUS could significantly inhibit apoptosis and chondrocytes senescence and recover ECM metabolic unbalance in the OA-like chondrocytes via FUNDC1-mediated mitophagy.

Limitations of the study

This study has limitations, and there are directions for future research. First, we conducted an *in vitro* study only on isolated chondrocytes grown in monolayers and did not recapitulate the characteristics of OA cartilage well. Similarly, this surgically induced OA model could not replicate or simulate actual OA conditions. Therefore, a three-dimensional chondrocyte culture system and specific gene knockout animal models may be more suitable to investigate the OA process in future studies. Second, there are significant differences between cell and mouse treatments during FLIPUS application. It is important to ensure that the accurate ultrasonic energy actually reaches the different targets (chondrocytes *in vitro* and mouse tissues *in vivo*). The present US parameters and protocol were selected based on our previous *in vivo* study, and an important direction for future research is elucidating the optimum acoustic exposure parameters of FLIPUS *in vitro*. Third, mitophagy and apoptosis are dynamic processes, often accompanied by the increase and decrease of proteins (e.g., LC3, TOM20, TIM23, and caspase-3). The time course experiments are important to show the progress of apoptosis and mitophagy after treatment with IL-1β or IL-1β+FLIPUS in future studies. Additionally, it is not clear whether there are other pathways that link FUNDC1 or other receptors (Parkin/pink1, BNIP3/NIX) to mitophagy. Despite these limitations, this study is the first to report that FLIPUS induces FUNDC1-mediated mitophagy in osteoarthritic chondrocytes.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107772.

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AUTHOR CONTRIBUTIONS

Conceptualization, L.J.; methodology, L.J. and L.Y.; validation, L.J. and L.Y.; formal analysis, H.Y. and D.L.; investigation, L.J. and L.Y.; resources, L.J.; data curation, X.W.; writing original draft preparation, L.J. and H.Y.; writing review and editing, L.J. and H.Y.; supervision, L.J. and L.Y.; project administration, L.J.; funding acquisition, L.J. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-Col2α1	Proteintech	Cat#28459-1-AP; RRID: AB_2881147
Rabbit polyclonal anti-MMP13	Proteintech	Cat#18165-1-AP; RRID: AB_2144858
Rabbit polyclonal anti-Aggrecan	Proteintech	Cat#13880-1-AP; RRID: AB_2722780
Rabbit polyclonal anti-Bax	Proteintech	Cat#50599-2-Ig; RRID: AB_2061561
Rabbit polyclonal anti-Bcl2	Proteintech	Cat#26593-1-AP; RRID: AB_2818996
Rabbit monoclonal anti-cleaved Caspase3	Abcam	Cat#ab214430
Rabbit monoclonal anti-LC3I/II	Cell Signaling Technology	Cat#12741
Rabbit polyclonal anti-TOM20	Proteintech	Cat#11802-1-AP; RRID: AB_2207530
Rabbit polyclonal anti-Tim23	Proteintech	Cat#11123-1-AP; RRID: AB_615045
Rabbit polyclonal anti-PGAM5	Abcam	Cat#ab131552
Rabbit polyclonal anti-FUNDC1	Abcam	Cat#ab224722
Rabbit phospho-FUNDC1	Affinity Biotechnology	N/A
Mouse monoclonal anti-β-actin	Proteintech	Cat#66009-1-Ig; RRID: AB_2687938
HRP Conjugated AffiniPure Goat Anti-mouse IgG (H+L)	BOSTER	Cat#BA1050
HRP Conjugated AffiniPure Goat Anti-rabbit IgG (H+L)	BOSTER	Cat#BA1054
Bacterial and virus strains		
PGAM5-knockdown adenovirus	Shanghai GeneChem Co., Ltd.	N/A
FUNDC1-knockdown adenovirus	Shanghai GeneChem Co., Ltd.	N/A
GFP control adenovirus	Shanghai GeneChem Co., Ltd.	N/A
mRFP-GFP-LC3 adenovirus	Hanbio Biotechnology Co., Ltd.	N/A
Chemicals, peptides, and recombinant proteins		
IL-1β	Pepro Tech	Cat#211-11B
DMEM/F12	Gibco	Cat#C11330500BT
10% fetal bovine serum	Gibco	Cat#10099141
Annexin V-FITC apoptosis detection kit	Beyotime	Cat#C1062S
TRIzol	Takara Bio	N/A
SYBR Premix Ex Taq kit	Takara Bio	Cat#RR820A
DAB kit	ZSGB-BIO	N/A
Collagenase II	Sigma Aldrich	N/A
Fluorescein(FITC) Tunel Cell Apoptosis Detection Kit	Servicebio	Cat#G1501-100T
Critical commercial assays		
PrimeScript™ RT reagent Kit	Takara Bio	Cat#RR047A
Experimental models: Cell lines		
Mouse: C57BL/6 Primary chondrocytes	Chongqing Medical University	N/A
Experimental models: Organisms/strains		
Mouse: C57BL/6	Chongqing Medical University	N/A
Oligonucleotides		
Primer: Col2α1 Forward: CATCCAGGGCTCCAATGATGTA	This paper	N/A
Primer: Col2α1 Reverse: ATGTCCATGGGTGCGATGTC	This paper	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Primer: Matrix metalloproteinase (MMP) 13 Forward: CTTCTTCTTGTTGAGCTGGACTC	This paper	N/A
Primer: Matrix metalloproteinase (MMP) 13 Reverse: CTGTGGAGGTCACTGTAGACT	This paper	N/A
Primer: PGAM5 Forward: ATCTGGAGAAGACGAGTTGACA	This paper	N/A
Primer: PGAM5 Reverse: CCTGTTCCCGACCTAATGGT	This paper	N/A
Primer: FUNDC1 Forward: CCCCCTCCCCAAGACTATGAA	This paper	N/A
Primer: FUNDC1 Reverse: CCACCCATTACAATCTGAGTAGC	This paper	N/A
Primer: β-actin Forward: GTGCTATGTTGCTCTAGACTTCG	This paper	N/A
Primer: β-actin Reverse: ATGCCACAGGATTCCATACC	This paper	N/A
Software and algorithms		
GraphPad Prism 8.0	Graphpad Software	https://www.graphpad.com/
Quantity One	Bio-Rad	N/A
Other		
FLIPUS	Chongqing Haifu Medical Technology Co., Ltd.	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Lang Jia (jialang@hospital.cqmu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Construction of mouse models

Twenty 10-week-old C57BL/6 male mice were obtained from the Animal Center of Chongqing Medical University. All animals were housed in individual cages with a 12:12 h light–dark cycle at 20°C–25°C and were given a standard laboratory diet and drinking water *ad libitum*. For establishment of the OA model, the medial meniscotibial ligament of the right knee was removed using aseptic surgical procedures.[39] As a control, sham operation without transaction of the medial meniscotibial ligament was performed on the left knee joint. Activity, weight, food consumption, rectal temperature, and wound healing were monitored daily during postoperative week 1. After postoperative week 1, the mice were forced to move for 30 min daily, 5 d per week for 3 weeks to induce OA progression. The experimental and animal care protocols (No. 2018011) were approved by the Committee on the Ethics of Animal Experiments at the Second Affiliated Hospital of Chongqing Medical University (Chongqing, China). In addition, this study was performed in accordance with the recommendations described in the ''Guide for the Care and Use of Laboratory Animals'' from the Ministry of Science and Technology of the People's Republic of China. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize animal suffering during the course of this study.

Isolation and culture of primary chondrocytes

Chondrocytes were isolated from the knee articular cartilage of 5-day-old C57BL/6 mice. Briefly, cartilage tissue was minced and digested with 0.25% trypsin (Beyotime, Shanghai, China) for 30 min and 0.25% collagenase II (Sigma Aldrich, St. Louis, MO, USA) at 37°C for 6 h. Chondrocytes were collected and cultured in an incubator containing 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco) and antibiotics (100 U/ml penicillin G sodium, 100 μg/ml

streptomycin sulfate) (Gibco). Primary chondrocytes were passaged when the density reached 80% or more fusion and treated with IL-1β for 24 h prior to subsequent Western blot, RT–PCR, flow cytometry, confocal photography, and transmission electron microscopy experiments.

METHOD DETAILS

Total experimental Plan

In this study, we used destabilization of the medial meniscus (DMM) to mimic osteoarthritic injury and evaluated the effect of FLIPUS on chondrocyte mitochondrial autophagy and apoptosis in an *in vivo* experiment. We then successfully isolated primary mouse chondrocytes to evaluate the effect of FLIPUS on autophagy- and apoptosis-related proteins and to investigate whether the expression of PGAM5 in chondrocytes is upregulated and leads to dephosphorylation of FUNDC1 at Ser13 under FLIPUS-induced mechanical loading. In parallel, we assessed the link between FLIPUS in mitochondrial autophagic flow and apoptosis. Additionally, we silenced the expression of PGAM5 and FUNDC1 by adenoviral transfection to assess whether FLIPUS enhances mitochondrial autophagic flux in chondrocytes via the PGAM5/FUNDC1 signaling pathway.

Transfection

A PGAM5-knockdown adenovirus (Ad-PGAM5), a FUNDC1-knockdown adenovirus (Ad-FUNDC1), and a GFP control adenovirus were designed by Shanghai GeneChem Company as adenoviruses encoding shRNA. In brief, chondrocytes were transfected with Ad-PGAM5 or Ad-FUNDC1 for 24 h, treated with IL-1β for 24 h, and cultured in a hypoxic incubator containing a gas mixture of 1% O_2, 5% CO_2, and 94% N_2 for 12 h. To determine the role of FLIPUS in autophagic flux, chondrocytes were infected with dual-tagged LC3 (mRFP-GFP-LC3) adenovirus (Hanbio Biotechnology, Shanghai, China) for 24 h and treated with IL-1β for 24 h.

FLIPUS treatment

FLIPUS was applied to chondrocytes by using the rehabilitation mode (Chongqing Haifu Medical Technology Co., Ltd., Chongqing, China). The FLIPUS probes were fixed in the bottom of 3.5 cm dishes covered by the coupling agent (Figure S1A). Chondrocytes were cultured in dishes and treated with FLIPUS. FLIPUS had an ultrasonic transducer diameter of 25 mm, a radius-of-curvature of 28 mm, a frequency of 0.6 MHz, a pulse repetition frequency of 300 Hz, a spatial and temporal average intensity (Ista) of 120 mW/cm^2, and a duty cycle of 20% for 20 min.[15,21] After FLIPUS treatment, the cells were lysed, and mRNA and protein were extracted for further analysis. Furthermore, the cells were harvested and stained with DAPI for 10 min when fusion reached 60%. The numbers of RFP and GFP LC3B+ puncta/cell were counted after confocal microscopy and photography.

In animal experiments, after 4 weeks of OA induction, 20 mice were randomized into the DMM+ FLIPUS group and the DMM group (n = 10, each group). Mice in the DMM + FLIPUS group were treated with FLIPUS for 20 min/d, 6 d/week per cycle for two cycles in total. Mice in the DMM group received a sham treatment without energy output as a control (Figures S1B and S1C). A Model CZG200 Ultrasound Therapeutic Device for Arthritis was used. The detailed FLIPUS therapeutic methods and parameters have been described above.[15]

Real-Time Polymerase Chain Reaction (RT–PCR)

Total RNA was extracted from chondrocytes with TRIzol reagent (Takara Bio, Otsu, Shiga, Japan) according to the manufacturer's instructions, and 1 μg of total RNA per sample was converted to cDNA using a cDNA Synthesis Kit (Takara Bio). Quantitative RT–PCR was performed with a SYBR Green PCR kit (Takara Bio). All samples were measured in triplicate, and fold changes in mRNA expression were calculated according to the $2^{-\Delta\Delta CT}$ method. The oligonucleotide pairs used were as follows: Col2α1 (5′-CATCCAGGGCTCCAATGATGTA-3′ and 5′-ATGTCC ATGGGTGCGATGTC-3′), matrix metalloproteinase (MMP) 13 (5′-CTTCTTCTTGTTGAGCTGGACTC-3′ and 5′-CTGTGGAGGTCACTGTA GACT-3′), PGAM5 (5′-ATCTGGAGAAGACGAGTTGACA-3′ and 5′-CCTGTTCCCGACCTAATGGT-3′), FUNDC1 (5′-CCCCCTCCCCAAGAC TATGAA-3′ and 5′-CCACCCATTACAATCTGAGTAGC-3′), and β-actin (5′-GTGCTATGTTGCTCTAGACTTCG-3′ and 5′-ATGCCACAG GATTCCATACC-3′).

Protein extraction and Western blot analysis

Cells were collected when the cell density reached 90% or more. Protein was extracted from chondrocytes using RIPA lysis buffer containing protease inhibitors (Beyotime). Protein concentrations were quantified with a BCA protein quantification kit (Beyotime). Equal amounts of protein were loaded on a 10% or 12% SDS–PAGE gel and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk for 1.5–2 h at room temperature and probed with primary antibodies specific for Col2α1 (Proteintech, 28459-1-AP, Wuhan, China), MMP13 (Proteintech,18165-1-AP), Aggrecan (Proteintech,13880-1-AP), Bax (Proteintech, 50599-2-Ig), Bcl2 (Proteintech 26593-1-AP), cleaved Caspase3 (Abcam, ab214430, Cambridge, UK), LC3 (Cell Signaling Technology, #12741, Danvers, USA), TOM20 (Proteintech, 11802-1-AP), TIM23 (Proteintech, 11123-1-AP), PGAM5 (Abcam, ab131552), FUNDC1 (Abcam, ab224722), phosphorylated FUNDC1 (p-Ser13) (Customized by Affinity Biotechnology, Jiangsu, China), and β-actin (Proteintech, 66009-1-Ig) overnight at 4°C. The following day, the membranes were incubated with the appropriate secondary antibodies for 1 h at 37°C. Next, the blots were visualized with an ECL chemiluminescence system (Bio-Rad, Hercules, CA, USA), and antigen–antibody complexes were quantified with Quantity One analysis software (Bio-Rad).

Flow cytometric analysis

After being treated, chondrocytes in six-well plates (1×10^6) were harvested and incubated with FITC-annexin V and PI (Beyotime) for 10 min in the dark at room temperature according to the manufacturer's instructions. The percentage of apoptotic cells was analyzed by flow cytometry (BD, San Jose, CA, USA).

Transmission electron microscopy (TEM)

Chondrocytes were harvested (1×10^6) and fixed in 2% glutaraldehyde overnight at 4°C. Ultrathin sections (60–80 nm) were prepared and stained with uranyl acetate and lead citrate. Autophagic vacuoles were observed using an electron microscope (Hitachi, Tokyo, Japan).

Histological analysis

The joint tissues were fixed in 4% paraformaldehyde for 24 h and decalcified in 15% EDTA for approximately 10 d. The tissues were dehydrated, embedded in paraffin, and cut into 5 μm sagittal sections. Sections of the mouse knee joints were stained with Safranin-O Fast Green. Histological changes in the medial femoral condyle (MFC) and the medial tibial plateau (MTP) of the knee joints were evaluated by the Osteoarthritis Research Society International (OARSI) scoring system (score 0 to 24).[40] Scoring was carried out by three independent researchers in a blinded manner.

Immunohistochemistry

Knee joint sections were deparaffinized and rehydrated, and endogenous peroxidase was blocked with 3% H_2O_2. After being blocked with goat serum for 1 h, the sections were incubated with primary antibodies against Col2α1 (Proteintech), MMP13 (Proteintech), Aggrecan (Proteintech), and LC3 (Cell Signaling Technology) at 4°C overnight. The sections were then incubated with HRP-conjugated secondary antibodies for 1 h at 37°C, and the immunohistochemical signals were visualized by using a DAB kit (ZSGB-BIO, Beijing, China). Images were analyzed by ImageJ software (Bethesda, MD, USA). Five mice from each group were used for quantitative analysis.

TUNEL assays

Chondrocyte apoptosis was examined by TUNEL staining. Knee joint sections were deparaffinized, rehydrated, and incubated with 0.1% Triton X-100 for 20 min. The sections were then incubated with the TUNEL reaction mixture (Servicebio, Wuhan, China) according to the manufacturer's instructions for 2 h at 37°C, and nuclei were stained with DAPI for 10 min. The images were observed and captured under a fluorescence microscope (Nikon, Tokyo, Japan), and TUNEL-positive cells were counted using ImageJ software (Bethesda).

QUANTIFICATION AND STATISTICAL ANALYSIS

The results were analyzed using GraphPad Prism version 8.0 (GraphPad Inc., La Jolla, CA, USA). All data are presented as the means ± SEMs of at least three independent experiments. Comparisons between two groups were performed using Student's t test. Statistical analyses for multiple comparisons were performed using one-way analysis of variance (ANOVA), followed by Tukey's test for pairwise comparisons. n represents the number of animals/samples in each group. p values less than 0.05 were considered to indicate statistical significance. Significance was defined based on exceeding different p value thresholds: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.