Life Sciences 218 (2019) 308–313

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Primary cilia and autophagy interaction is involved in mechanical stress mediated cartilage development via ERK/mTOR axis

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ARTICLE INFO

Keywords:
Mechanical stress
Primary cilia
Autophagy
Cartilage development

ABSTRACT

Aims: Biomechanical reactivity is a special property of chondrocytes and mechanical stress can affect the development of cartilage. Primary cilia have been proved a cellular sensory which can detect physical and chemical stimuli extracellular and initiate multiple signaling transduction. Autophagy is an important environmental adaptive mechanism for cells maintenance of homeostasis. The aims of this study were to detect whether there is an interaction between primary cilia and autophagy in the regulation of mechanical stress-mediated cartilage development and to explore the underlying mechanism.

Main methods: In this study, chondrocytes were treated with cyclic tensile strain (CTS) by the four-point bending system. Chondrocytes viability, proliferation and differentiation capacities were analyzed by western blot and live/dead assays after CTS of different intensities. Meanwhile, primary cilia incidence and length changes, and autophagy expression were detected by immunofluorescence staining. The primary cilia and autophagy interaction regulation and the underlying mechanism were detected by immunofluorescence double staining and western blot.

Key findings: Mechanical stress could affect chondrocytes proliferation, phenotype and viability in an intensity dependent manner. The incidence and length of primary cilia as well as autophagy expression could be regulated by CTS. The integrity of primary cilia structure is vital for mechanical stress regulated ERK/mTOR signaling transduction and autophagy expression in chondrocyte.

Significance: These findings indicate that mechanical stress could affect the interaction between primary cilia and autophagy and help to reveal the underlying mechanism of stress regulated cartilage development.

1. Introduction

Mechanical stress is one of the most common physical stimuli [1]. Normally, articular cartilage can withstand all kinds of mechanical stress caused by body weight or joint motion [2]. Biomechanical reactivity is the most important functional property of chondrocytes, and it is also an important guarantee for the body to adapt to the external environment [1,2]. Currently, various studies have found that the effects of mechanical stress on chondrocytes proliferation, differentiation and apoptosis could be observed under different mechanical loading conditions [3,4]. However, mechanical stress also has been found a "double-edged sword" effect on chondrocytes. That is, proper mechanical stress is beneficial to cartilage development, whereas abnormal stress can accelerate cartilage degeneration and cause injury [5–8]. At present, the specific roles and mechanisms of different stress stimuli in the development and damage of cartilage are still unclear.

Primary cilia is microtubule-based organelle protruding from many mammalian cells membrane. As a kind of cellular sensory, it can detect physical and chemical stimuli extracellular and initiate multiple signaling transduction intracellular [9]. Previous studies have confirmed that primary cilia was involved in the transduction of extracellular mechanical signals [10–12]. Hydrodynamic stimulation could trigger primary cilia swing in the renal epidermis [13]. Disruption the structure of primary cilia could block the increase of calcium flux due to

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https://doi.org/10.1016/j.lfs.2019.01.001
Received 7 November 2018; Received in revised form 1 January 2019; Accepted 2 January 2019
Available online 03 January 2019

mechanical stimulation [14]. Recently, the role of primary cilia in cartilage development has been gradually recognized, which may be involved in important physiological activities like biomechanical reaction of chondrocytes. For example, primary cilia could promote the activation of Hedgehog signaling of growth plate chondrocytes when loaded with hydrostatic stimulation [11]. The effects of mechanical stress on cells are inseparable from the function of primary cilia.

Autophagy, as an important mechanism for cells maintenance of homeostasis, got more and more attention during the course of cartilage development. Previous study reported that autophagy was suppressed in the tissue of degenerated cartilage, while intra-articular injection of autophagy agonists could block the degeneration process of cartilage in rabbit [15]. The activation of autophagy could also inhibit the apoptosis of osteoarthritis chondrocytes and reduce the expression of MMP-13 and ADAMTS-5 [16]. Therefore, autophagy has been considered a potential target for protecting chondrocytes and blocking the progress of osteoarthritis.

Recently, various studies reported that there existed an interaction between primary cilia and autophagy during cells development, but whether mechanical stress affect cartilage development through regulating this interaction is still unclear. Therefore, this study is aimed to observe the effects of mechanical stress of different intensities on the development of chondrocytes, and investigate whether there is an interaction between primary cilia and autophagy under mechanical loading and the potential mechanism during cartilage development.

2. Materials and methods

This study was approved by the Ethics Committee on Animal Experimentation of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China).

2.1. Chondrocyte isolation and culture

Chondrocytes were isolated from the knee joint of 3 days old Sprague–Dawley rats. Dissected the cartilage tissue into 1 mm^3 pieces and digested with 0.25% trypsin solution for 30 min and 0.1% type II collagenase solution for 6 h successively. The cell suspension was centrifuged and discarded the supernatant. The cell pellet was washed with PBS for a time and then resuspended with Dulbecco's modified Eagle's medium/F-12 (DMEM/F12) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin). Cells were cultured in 37 °C incubator with 5% CO2.

2.2. Cyclic mechanical stress experiment

Cyclic tensile strain (CTS) was used to stimulate chondrocytes plated on culture plates by the four-point bending system according to our previous study [17]. Cells were exposed to CTS with different intensity (1000, 2500, 4000 μstrain) at a frequency of 1 Hz for 2 h when reached 80% confluence. Besides, 40 μM chloral hydrate was used to disturb primary cilia structure before 2500 μstrain CST stimulation. These experiments were conducted in 37 °C incubator with 5% CO2.

2.3. Immunofluorescence staining assay

The procedures of primary cilia staining refer to our previous study [18]. When conducting autophagy and primary cilia double staining, cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min. After being blocked with 5% BSA for 1 h at room temperature, cells were incubated with acetylated-α-tubulin antibody (1:400, Sigma, USA) at 4 °C overnight. Subsequently, cells were incubated with Cy3-conjugated goat anti-Mouse IgG secondary antibody (1:100, Boster, Wuhan, China) at room temperature for 1 h and 1 μg/μL DAPI stained for 10 min, and then washing with PBS for 3 times. According to the protocol of Cell Meter Autophagy Fluorescence Imaging Kit (AAT Bioquest, Sunnyvale, CA, USA), 250 μL of Autophagy Green working solution was added and incubated in 5% CO2 environment at 37 °C for 30 min protected from light. After washing with wash buffer for 3 times, the expression of autophagy was detected under a fluorescence microscope. Photos were taken by immunofluorescence microscope and the results were analyzed by Image J software.

2.4. LIVE/DEAD viability assay

According to the instruction of LIVE/DEAD Viability/Cytotoxicity Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), 20 μL 2 mM EthD-1 stock solution was added to 10 mL sterile PBS. After thorough mixing, 5 μL 4 mM calcein AM was added and vortexed the resulting solution. 0.5 mL result solution was added on the culture plate ensuring full coverage of cells and observed under fluorescence microscope after incubation at room temperature for 5 min protected from light. Green fluorescent labeled living cells and red fluorescent labeled dead cells, and the proportion of the two was calculated.

2.5. Western blot assay

Total protein was extracted from chondrocytes after stimulated under different conditions. 20 μg protein samples per lane was separated on SDS-PAGE polyacrylamide gels and transferred to PVDF membranes. After blocking with 5% BSA for 1 h at room temperature, membranes were respectively incubated at 4 °C overnight with primary cilia functional protein antibody IFT88 (1:400, Abgent, San Diego, CA, USA), autophagy related proteins antibody LC3-I/II (1:1000, CST, Boston, MA, USA) and Beclin1(1:1000, CST, Boston, MA, USA), Cartilage phenotypic protein antibody COL II (1:1000, Abcam, Cambridge, UK), proliferation related protein antibody Cyclin D1 (1:100, Boster, Wuhan, China), signaling pathway proteins antibodies ERK (1:1000, CST, Boston, MA, USA), p-ERK (1:1000, CST, Boston, MA, USA), mTOR (1:1000, CST, Boston, MA, USA), p-mTOR (1:1000, CST, Boston, MA, USA), and GAPDH (1:400, Boster, Wuhan, China). Then, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000, Boster, Wuhan, China) at room temperature for 1 h. ECL western blotting detection kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect the protein bands and visualized using an enhanced chemiluminescence system (Bio-Rad, Hercules, CA, USA).

2.6. Statistical analysis

Software SPSS20.0 (IBM, Armonk, NY, USA) was used for statistical analysis. All data were described as means ± standard deviations. One-way analysis of variance was used to assess the significance of between-group differences. A p value < 0.05 was considered to reflect statistical significance.

3. Results

3.1. Cyclic tensile strain (CTS) regulated chondrocytes development

Chondrocytes were subjected to different intensities of CTS stimulation and phenotype related protein COL-II and proliferation related protein Cyclin D1 were detected. Mild (1000 μstrain) to moderate (2500 μstrain) intensity of CTS could up-regulate COL-II and Cyclin D1 expression, while high (4000 μstrain) intensity of CTS down-regulated both proteins (Fig. 1A–C). The results of live/dead viability assay showed that mild to moderate intensity of CTS had no significant effect on cells viability but high intensity of CTS could promote chondrocytes apoptosis (Fig. 1D–E).

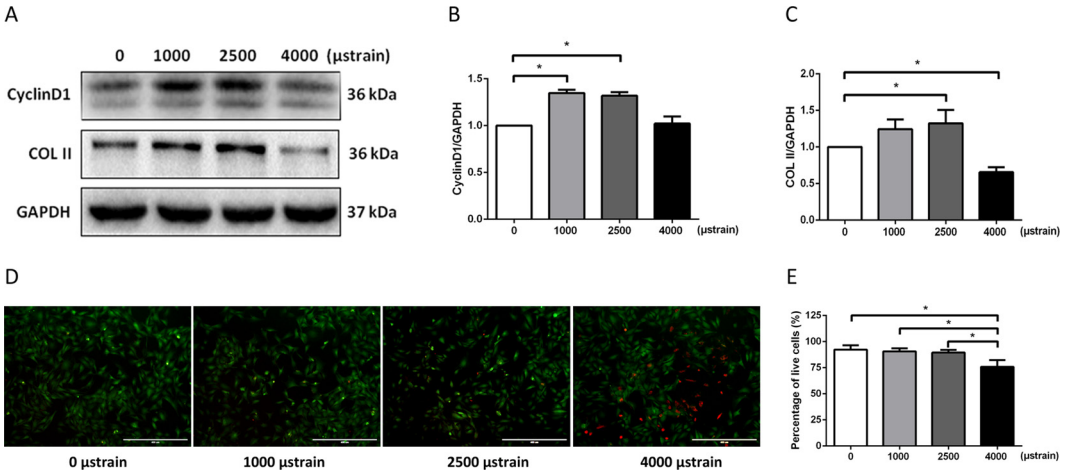

Fig. 1. CTS regulated chondrocytes development. Image A shows Cyclin D1 and COL II expression under different intensities of CTS. Image B and C show the quantitative analysis of proteins expression. Image D shows the live/dead staining under different intensities of CTS. Bars = 400 μm. Image E shows the percentage of live cells after stimulating by different intensities of CTS. (*$p < 0.05$).

3.2. CTS affected the expression of primary cilia and autophagy in chondrocyte

Chondrocytes were subjected with different intensities of CTS and primary cilia's incidence, length changes and autophagosome formation were detected by immunofluorescence staining assays respectively (Fig. 2A). The results showed that mild to moderate intensity (1000 and 2500 μstrain) stress had no obviously effect on primary cilia incidence and length (Fig. 2B–C). But high intensity (4000 μstrain) stress could suppress ciliogenesis and reduce cilia length, and the results showed statistical differences (Fig. 2B–C) ($p < 0.05$). Meanwhile, the number of autophagosome increased with the increase of CTS intensity to some extent (0–2500 μstrain), whereas autophagy was inhibited when loaded with a higher intensity of 4000 μstrain CTS (Fig. 2D) ($p < 0.05$).

3.3. There existed interaction between primary cilia and autophagy in chondrocyte

To explore the relationship between primary cilia and autophagy expression further, we respectively used serum free medium to trigger primary cilia and chloral hydrate to disturb cilia structure, and rapamycin to activate autophagy and 3MA to inhibit autophagosome formation. Primary cilia and autophagosome were double staining by immunofluorescence assays (Fig. 3A). The results showed that starvation by serum free medium could promote primary cilia expression and elongation but chloral hydrate could inhibit both simultaneously (Fig. 3B–C). Meanwhile, ciliary function protein IFT88 and the autophagy-related protein LC3-II showed the same tendency (Fig. 3D–E). Rapamycin had no obvious effect on ciliogenesis and length (Fig. 3B–C), but could up-regulated ciliary function protein IFT88 (Fig. 3D–E) ($p < 0.05$). Autophagy inhibitor 3MA could inhibit ciliogenesis and cilia elongation at the same time (Fig. 3B–C).

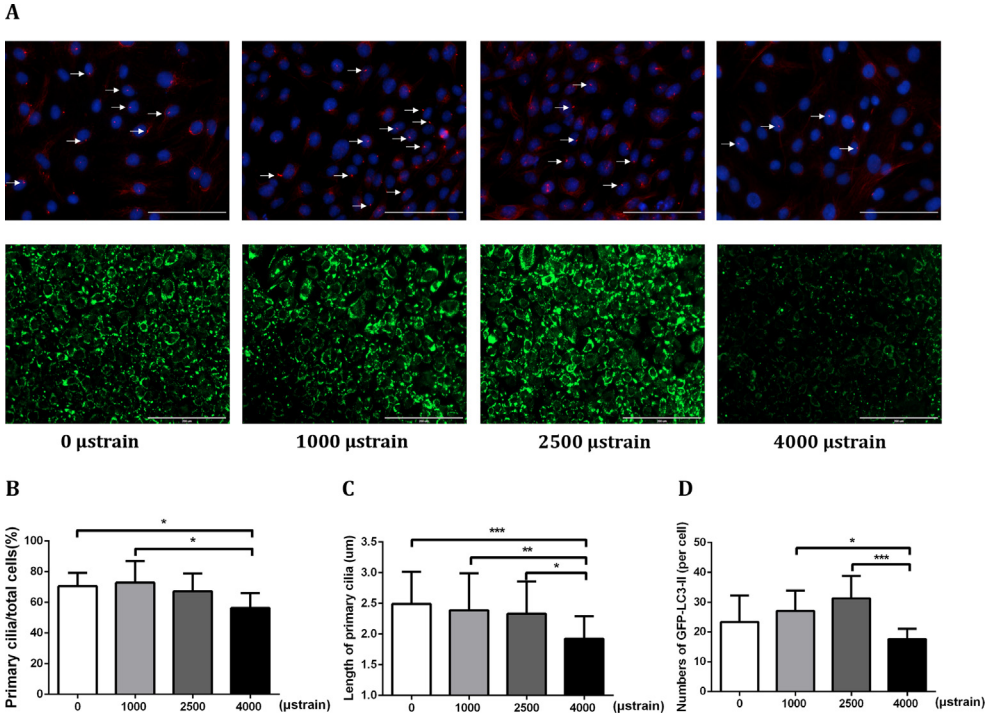

Fig. 2. CTS affected the expression of primary cilia and autophagy in chondrocyte. Image A shows the primary cilia and autophagy expression under different intensities of CTS (Bars = 200 μm; white arrow: primary cilia). Image B shows the incidence of primary cilia (*$p < 0.05$). Image C shows the average length of primary cilia (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Image D shows numbers of GFP-LC3-II per cell (*$p < 0.05$, ***$p < 0.001$).

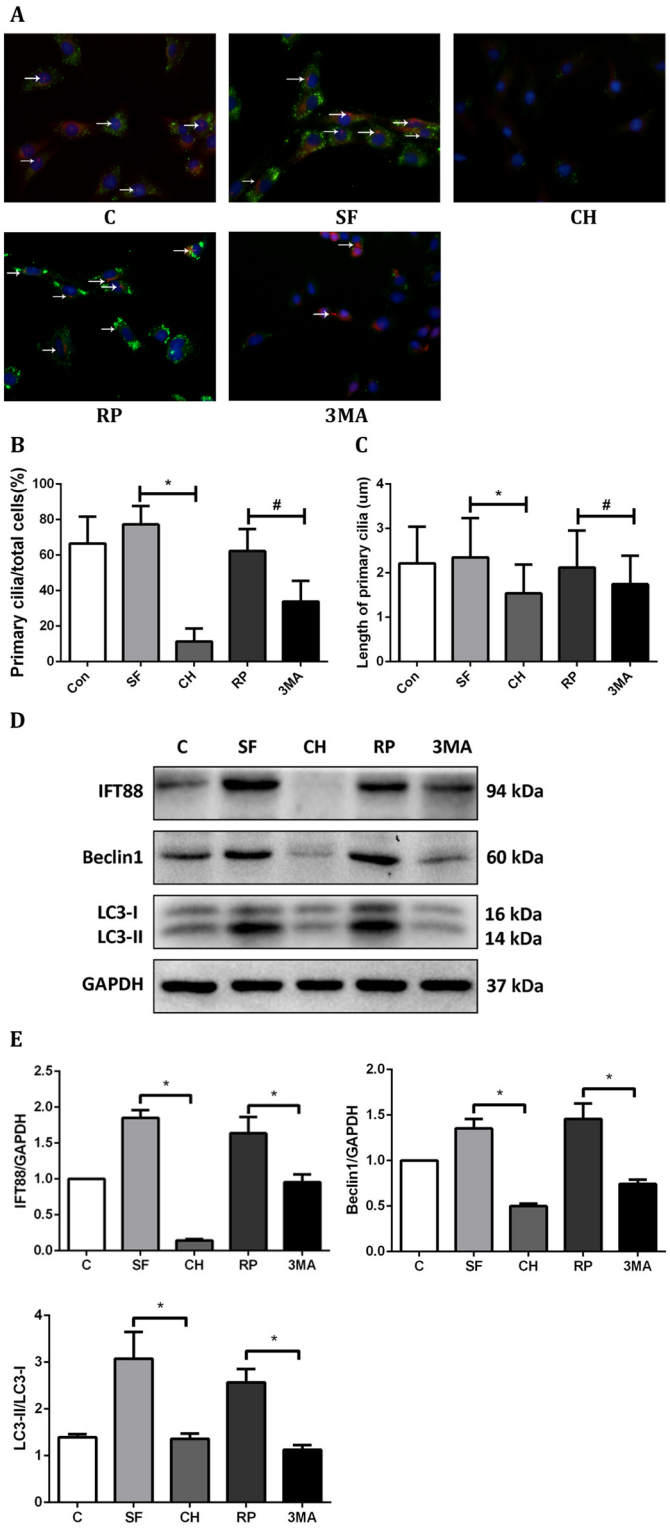

Fig. 3. The interaction between primary cilia and autophagy in chondrocyte. Image A shows the primary cilia and autophagy expression under different treatments (Bars = 100 μm, white arrow: primary cilia). Image B shows the percentage of primary cilia (*$p < 0.05$, #$p < 0.05$). Image C shows the average length of primary cilia (*$p < 0.05$, #$p < 0.05$). Image D shows the proteins expression of IFT88, Beclin1 and LC3-I/II. Image E shows the quantitative analysis of proteins expression (*$p < 0.05$).

3.4. ERK/mTOR signal axis is involved in the regulation of primary cilia and autophagy interaction

Chloral hydrate was used to disturb primary cilia structure and then CTS was loaded to detect the mechanism of interaction between primary cilia and autophagy. As is shown in Fig. 4A–C, the results demonstrated that 2500 μstrain CTS could up-regulate IFT88, Beclin1, LC3-II and p-ERK expressions, but downregulate p-mTOR expression. After disturbing primary cilia by chloral hydrate, IFT88, LC3-II and p-ERK expressions were suppressed but p-mTOR expression increased. What's more, the effects of CTS on promoting IFT88, Beclin1 and LC3-II proteins expression could be suppressed by chloral hydrate treatment. The promotion effect of p-mTOR by chloral hydrate could be inhibited by 2500 μstrain CTS loading to some degree, but still more than control group. The integrity of primary cilia is vital for mechanical stress regulated ERK/mTOR signaling transduction and autophagy expression in chondrocyte.

4. Discussion

Mechanical stress, as a micro-manifestation of exercise training can directly function on chondrocyte and affect its growth and development process [1,17]. In this study, we found that within a certain range, proper intensities of mechanical stress could promote the proliferation and phenotype maintenance of chondrocytes, while high intensity stimulation could induce apoptosis. Primary cilia and autophagy could play an important role in the development of cartilage under mechanical stress. The expression of primary cilia and autophagy could be regulated in an intensity-dependent manner by mechanical stress. Especially, high intensity stress could reduce the incidence and length of primary cilia and the expression of autophagy.

Autophagy is a key mechanism for cells to maintain intracellular metabolic stability when confronted with extracellular environment changes [18]. The level of autophagy is involved in the regulation of cartilage development and the occurrence of cartilage-related diseases. For example, the level of autophagy was found decreased in chondrocytes with age increasing, and this decline always occurred before cartilage damage [19]. Aggressive accumulation of damaged macromolecules induced by autophagy decreasing could ultimately lead to cellular dysfunction and death, and this is a common feature of aging lesions, including osteoarthritis [20]. Knockdown of autophagy related gene ATG5 in normal cartilage could trigger chondrocyte senescence prematurely and cartilage degeneration, indicating that reduced autophagy could result in a change in cartilage homeostasis and extracellular matrix destruction and promote the progression of osteoarthritis [21]. Besides, activation of autophagy could inhibit inflammation-mediated apoptosis which could delay the process of cartilage degeneration [20].

Primary cilia, as a mechanical sensory, also plays an important role in the regulation of cartilage development. Primary cilia had a close relationship with the Golgi and involved in the regulation of endocytosis and extracellular matrix secretion to affect the phenotype maintenance of chondrocytes [22]. Primary cilia helped to maintain the polarity and columnar growth state of chondrocytes, and IFT mutations could induce abnormal metaphyseal growth and result in long bone dysplasia eventually [23]. Mechanical stimulation could down-regulate the expression of MMP-1 and MMP-13 genes in chondrocytes by acting on primary cilia, and these two genes often mediate the degradation of extracellular matrix [24]. Our study also revealed that mechanical stress could regulate ciliary functional protein IFT88 expression in promoting the proliferation and differentiation of chondrocytes, and the integrity of cilia structure was indispensable.

Recently, the interaction of primary cilia and autophagy has attracted more and more attention [25]. Autophagy could strongly induce the elongation of primary cilia by blocking the disassembly process, and inhibition of autophagy could suppress ciliogenesis [26,27]. Primary

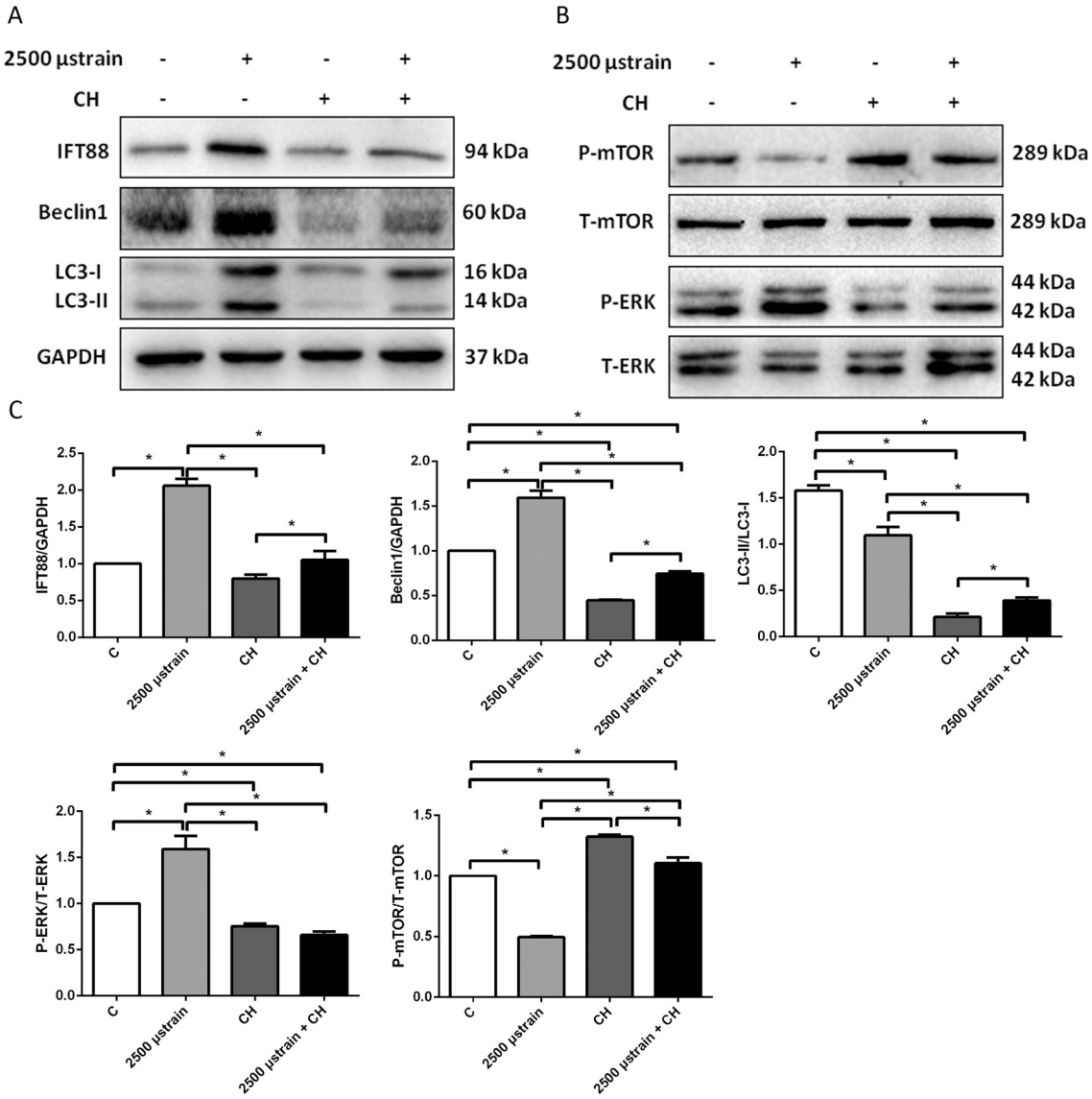

Fig. 4. ERK/mTOR signal axis is involved in the regulation of primary cilia and autophagy interaction. Image A shows the expression of IFT88, Beclin1 and LC3-I/II proteins under different treatment. Image B shows the expression of p-mTOR, T-mTOR, p-ERK and T-ERK. Image C shows the quantitative analysis of proteins expression (*$p < 0.05$).

cilia assembly could recruit autophagy related protein ATG16L to localize in the basal body of cilia, and knocking out of cilia functional protein IFT could downregulate basal autophagy level induced by nutritional deficiency [28,29]. Tang et al. reported that autophagy could promote primary cilia assembly by degrading OFD1 protein at the centrosome microsatellite [30]. The elongation of cilia could recruit autophagy specific protein ATGs to the basal body by activating Hedgehog pathway [29]. Furthermore, both autophagy and ciliogenesis could be regulated by the status of mTOR signaling pathway. It has been reported that autophagy induction with mTOR inhibitor could trigger cilia elongation while mTOR activation would shorten the length [31]. Our study found that mechanical stress could activate ERK pathway but inhibit mTOR signaling, and upregulate IFT88 and LC3-II to promote cilia and autophagy function. After disturbing cilia structure, autophagy was inhibited under stress stimulation, indicating that mechanical stress promoting autophagy depended on the integrity structure of primary cilia. It is suggested that there may exist an interaction between primary cilia and autophagy to maintain the balance of cilium function and autophagy level under mechanical stress conditions.

5. Conclusion

This study preliminarily illustrated that mechanical stress could affect the development of chondrocytes by regulating the interaction between primary cilia and autophagy. It will help us understand the mechanism of mechanical stress in affecting chondrocyte development and cartilage related disease from a new perspective. In the future, exploring how to regulate the crosstalk between primary cilia and autophagy will help to promote cartilage repair and block cartilage damage.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (Grant No. 81772440).

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