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FSH directly regulates chondrocyte dedifferentiation and cartilage development

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Abstract

Previous studies suggest that postmenopausal osteoarthritis is linked to a decrease in estrogen levels. However, whether follicle-stimulating hormone (FSH), the upstream hormone of estrogen, affects cartilage destruction and thus contributes to the onset of osteoarthritis has never been explored. To evaluate the potential involvement of FSH in joint degeneration and to identify the molecular mechanisms through which FSH influences chondrocytes, mouse cartilage chondrocytes and the ATDC5 chondrocyte cell line were treated with FSH and inhibitors of intracellular signaling pathways. We observed that FSH induces chondrocyte dedifferentiation by decreasing type II collagen (Coll-II) synthesis. Chondrocyte cytoskeleton reorganization was also observed after FSH treatment. The FSH-induced decrease in Coll-II was rescued by ERK-1/2 inhibition but aggravated by p38 inhibition. In addition, knocking down the FSH receptor (*Fshr*) by using *Fshr* siRNA abolished chondrocyte dedifferentiation, as indicated by the increased expression of Coll-II. Inhibition of the protein Gαi by pertussis toxin (PTX) also restored FSH-inhibited Coll-II, suggesting that Gαi is downstream of FSHR in chondrocyte dedifferentiation. FSHβ antibody blockade prevented cartilage destruction and cell loss in mice. Moreover, decreased Coll-II staining due to the progression of aging could be rescued by blocking FSH. Thus, we suggest that high circulating FSH, independent of estrogen, is an important regulator in chondrocyte dedifferentiation and cartilage destruction.

Key Words

- ▶ follicle-stimulating hormone
- ▶ chondrocyte
- ▶ dedifferentiation
- ▶ cartilage
- ▶ osteoarthritis

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Introduction

Osteoarthritis (OA) is a degenerative disease affecting more than 70% of the population ranging from 55 to 77 years of age (Johnson *et al.* 2012). Many risk factors, such as age, sex, obesity, injury, and mechanical factors, are involved in the progression of OA. Some studies have shown that the incidence of OA is higher in females than in males, especially after menopause (Zhang &

Jordan 2010, Hunter & Bierma-Zeinstra 2019). Until recently, the underlying mechanisms of OA have been linked to the decrease in estrogen that occurs with the onset of menopause (Martin-Millan & Castaneda 2013). Although FSH levels increase in parallel, the effect of FSH on cartilage and chondrocytes has never been explored.

FSH is a glycoprotein synthesized by the pituitary and is controlled by estrogenic feedback. FSH acts by binding its receptor FSH receptor (FSHR). Extragonadal FSHRs are expressed in a variety of cells, such as hepatocytes (Guo *et al.* 2019), adipocytes (Cui *et al.* 2012, Liu *et al.* 2015, 2017), and osteoclasts (Sun *et al.* 2006). Based on our previous study, FSHR is expressed on human and mouse chondrocytes (Kong *et al.* 2018). We speculate that FSH may exert effects on chondrocytes, consequently affecting osteoarthritis development.

Although OA is considered a disease that involves the whole joint, the articular cartilage is altered to some extent in affected joints with OA. Articular cartilage is an avascular organ consisting of distributed chondrocytes surrounded by an extracellular framework. Chondrocytes are the only cellular component of articular cartilage, and they can secrete extracellular matrix (ECM) components, such as type 2 collagen (Coll-II) and aggrecan (Poole 1997). Chondrocytes also produce catabolic molecules such as matrix metalloproteinases (MMPs) involved in the degradation of the ECM (van der Kraan & van den Berg 2012). In healthy cartilage, chondrocytes regulate cartilage homeostasis by maintaining a very low turnover ECM rate and balancing anabolism and catabolism. Chondrocytes acquire OA-associated phenotypes by changing their behavior. It is well documented that chondrocytes undergo mature hypertrophic differentiation and exhibited enhanced catabolism during the process of OA (Rim *et al.* 2020). However, recent studies have shown that chondrocytes can acquire other degenerative phenotypes (i.e. prehypertrophic, dedifferentiation) (Charlier *et al.* 2019, Li *et al.* 2019a, Speichert *et al.* 2019). It is therefore important to understand chondrocyte behavior in response to FSH in OA pathogenesis.

In the present study, we explored the effects and mechanisms of FSH on cartilage chondrocytes by using *in vitro* and *in vivo* studies. Mouse primary chondrocytes and the ATDC5 cell line were used to investigate whether FSH activation could disrupt ECM homeostasis and affect chondrocyte dedifferentiation. In addition, the possible signaling mechanisms underlying FSH-altered dedifferentiation were also evaluated. We also evaluated the effect of FSH absence on cartilage *in vivo* using FSH β antibody blockade. To our knowledge, this is the first study to demonstrate that FSH has a direct effect on chondrocyte dedifferentiation. Blocking FSH might be an option to treat joint disorders, such as postmenopausal osteoarthritis.

Experimental procedures

Animals

C57BL/6J mice were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Mice were housed at room temperature (25°C) under a 12 h light:12 h darkness cycle. Mice were intraperitoneal injected anti-FSH β antibody (Medix Mab, #6602, BiosPacific, Emeryville, CA) or anti-IgG antibody (Proteintech, Wuhan, China) at 100 μ g/day daily for 8 weeks (Guo *et al.* 2019). All animal protocols were conducted with the approval of the Animal Study Committee of Shandong Provincial Hospital.

Isolation and culture of primary chondrocytes

The direct effects of FSH on cartilage were assessed using primary chondrocytes. Primary chondrocytes were isolated from the distal femoral and proximal tibia of newborn mice (C57BL/6J) as described previously (Gosset *et al.* 2008). After careful dissection of adherent tissue, the cartilage was cut into 1 mm² pieces and digested in full culture medium containing 1 mg/mL collagenase P (Roche) and 0.145 mM L-cysteine. Then, chondrocytes were seeded and cultured in DMEM/F12 (HyClone, GE Healthcare) containing 10% FBS (Bio-Excel, New Zealand, Australia), 100 U/mL penicillin, 100 μ g/mL streptomycin (Gibco), 44 μ g/mL ascorbic acid (Solarbio, Beijing, China) and 130 μ g/mL ascorbic acid-2-phosphate (Sigma-Aldrich), at 37°C in a humid environment with 5% CO₂ (Xin *et al.* 2015). Primary chondrocytes were never passaged and were cultured for no more than 1 week. The cells isolated were identified by the expression of type II collagen (data not shown). Before treatment with FSH (R&D, #8576-FS), chondrocytes were starved 2 h before the experiment. Primary chondrocytes were treated with recombinant mouse FSH or vehicle. For MAPK signal analysis, PD98059 ERK inhibitor and SB203580 p38 inhibitor were purchased from Beyotime Biotechnology (Nanjing, China).

ATDC5 cell line culture

The mouse chondrogenic cell line ATDC5 was purchased from Wuhan Procell Life Science & Technology Co., Ltd. Cells were cultured in a 1:1 mixture of DMEM and DME/F12 medium containing 5% FBS at 37°C under 5% CO₂. Differentiation was induced by the addition of 10 μ g/mL insulin, 5.5 μ g/mL transferrin, and 5 μ g/mL sodium selenite (ITS; Sigma) with 250 μ M ascorbate for 2 weeks.

CCK-8 assay

The CCK-8 assay was used to assess whether FSH can affect chondrocyte apoptosis or proliferation. Briefly, mouse primary chondrocytes were seeded into a 96-well plate at a density of 4×10^3 /well. Then 10 μ L of the CCK-8 solution (Dojindo Laboratories, Kumanmoto, Japan) was added to each well. The cell viability was then detected at different time points (24, 48, and 72 h) at 450 nm using a microplate reader.

Immunoblotting

Cell culture supernatants were discarded and then washed with PBS. Then, the cell layer was scraped and lysed with RIPA and PMSF on ice. Total protein was loaded on 8–10% SDS-PAGE gels and transferred onto PVDF membranes (0.45 μ m, Millipore) and blocked with 5% non-fat milk. After incubated with primary antibodies against type II collagen (Abcam), SOX9 (Abcam, #ab185966), total ERK (CST, #4695), p-ERK (CST, #4370), total p38 (CST, #8690), p-p38 (CST, #4511), total CREB (CST, #9197), p-CREB (CST, #9198s), FSHR (Proteintech, #22665-1-AP) and GAPDH (Absin, #abs137959), the membranes were incubated with an HRP-conjugated secondary antibody. Protein bands were detected using an ECL detection kit (Millipore). GAPDH or ERK-1/2 was used as a control.

RNA extraction and qRT-PCR

Total RNA was extracted using TRIzol reagent (Takara) and reverse transcribed into cDNA using a Takara kit. The concentration of total RNA was measured by a NanoDrop1000 (NanoDrop, USA). Gene expression was quantified by real-time PCR in a Roche 480 Real-Time PCR system. The PCR primer sequences were as reported previously (Table 1).

Analysis of intracellular cAMP

For the analysis of adenosine 3',5'-cyclic monophosphate (cAMP) content, primary chondrocytes were incubated with forskolin (Forsk) as a positive control to stimulate cAMP production. Since G α i protein is sensitive to pertussis toxin (PTX), chondrocytes were pretreated with PTX at a concentration of 100 ng/mL for 12 h before FSH treatment. After treatment with FSH for 30 min, cell lysates were collected and measured using an ELISA kit (Cayman, #581001) according to the manufacturer's instructions.

Table 1 RNA primers.

Gene name	Forward and reverse primer (5'-3')
<i>Col2a1</i>	F: CACACTGGTAAGTGGGGCAAGACCG R: GGATTGTGTTGTTTCAGGGTTCGGG
<i>Acan</i>	F: CTGTCTTTGTACCCACACACA TG R: GAAGACGACATCACCA TCCAG
<i>Sox9</i>	F: ATCTCCACCCAGAACCTCT R: CAGGGCAGGAGAGTTGAGAC
<i>Sox5</i>	F: TGGCAATGGGATCAGGGAAC R: ATCATACCCATGAGCTGCCG
<i>Sox6</i>	F: ATCTCCACCCAGAACCTCT R: CAGGGCAGGAGAGTTGAGAC
<i>Mmp3</i>	F: AGGGATGATGATGCTGGTATGG R: CCA TGTCTCCAAGTCAAAGG
<i>Mmp13</i>	F: GGAGTTTACAGGAAGCAGACA R: CATCCACA TGGTTGGGAAGTTCT
<i>Col10a1</i>	F: CTTTGTGTGCCTTTCAATCG R: GTGAGGTACAGCCTACCAGTTT
<i>Prg4</i>	F: GACGCCCAATGTAAGAAG R: GCAAGGGTGAGACAAGAA
<i>Egr1</i>	F: TCGGCTCCTTCTCTCACTCA R: CTCATAGGGTTGTTGCTCGG
<i>Pthlh</i>	F: CATCAGCTACTGCATGACAAGG R: CTGTGTGGATCTCCGCGAT
<i>Ihh</i>	F: GCGCTACGAAGGCAAGAT R: CTTGAAGATGATGTCGGGATTGT
<i>Fshr</i>	F: CCTTGCTCCTGGTCTCCTTG R: CTCGGTCACCTTGCTATCTTG
<i>Gapdh</i>	F: TCACTGCCACCCAGAAGAC R: TGTAGGCCATGAGGTCCAC

Immunofluorescence

Chondrocytes were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 30 min. In total, 5% BSA was used as a blocking solution. Rabbit polyclonal anti-FSHR antibody (Proteintech, #22665-1-AP) was added to the cells overnight at 4°C followed by staining with Cy3-conjugated anti-rabbit antibody in the dark. For actin cytoskeleton analysis, ATDC5 cells were stained with Rhodamine phalloidin (Cytoskeleton, #PHDR1) for 30 min at 37°C. For Egr1 staining, rabbit polyclonal anti-Egr1 antibody (Proteintech, #22008-1-AP) was added and incubated overnight at 4°C. Alexa Fluor Plus 488 anti-rabbit antibody (Invitrogen, #A32731) was used as the secondary antibody. Nuclei were counterstained with DAPI (Abcam) for 5 min. The cells were visualized with darkfield microscopy (LSM 780, Zeiss).

Transfection of siRNA

Three pairs of siRNA (siRNA) (#1, #2, #3) (Table 2) specific for FSHR and a scrambled control were synthesized by Genomeditech Co., Ltd. (Shanghai, China). The siRNA sequences specific for FSHR were selected based on their potency to inhibit more than 80% *fshr* expression.

Table 2 Sequences of *Fshr* siRNA.

Fshr siRNA	Forward and reverse primer (5'–3')
#1	F: UAGAAAGAUGGCAUACCAU tt R: AUGGUAUGCCAUCUUUCUA tt
#2	F: GUCUCCGAUAUCUGUAAU tt R: AUUAAACAGAUACGGAGAC tt
#3	F: GAGUCUUGAUUGGUUUUAU tt R: AUAACCAUAUCAAGACUC tt

The transfection of 100 nM *fshr*-siRNA was performed using Lipo3000 transfection reagent (Invitrogen) according to the manufacturer's instructions.

Histology

Knee joint tissue samples were fixed in 4% paraformaldehyde overnight and decalcified at 0.5 M EDTA (Servicebio, China) for 3 weeks. After decalcification, the mouse knee joints were dehydrated and embedded in paraffin. Five- μ m serial sagittal sections were cut from the lateral tibiofemoral joint (~100 sections were cut). Then sections corresponding to 1/4 (sections ~25), 2/4 (sections ~50), 3/4 (sections ~75) and 4/4 (sections ~100) of the joint were selected to evaluate the whole cartilage. For comparison, the sections were selected from the same location. Safranin O/Fast Green was stained to observe the cartilage. Histological data were analyzed by assessing the Mankin score (Mankin *et al.* 1971, Aigner *et al.* 2010) and OARSI score (Pritzker *et al.* 2006). The scoring of the sections was performed by two experienced observers in a blinded manner.

Immunohistochemistry and Alcian blue staining

Mouse cartilage sections were stained to assess FSHR location. Briefly, sections were deparaffinized, rehydrated, and immunostained according to the manufacturer's instructions. The sections were incubated with FSHR (Proteintech, #22665-1-AP), type II collagen, Ki67 (Abcam, #ab15580), and Egr1 (Proteintech, #22008-1-AP) antibody at 37°C for 1 h. The sections were counterstained with toluidine blue to verify cartilage. The slides were visualized under a light microscope (Leica).

ATDC5 cells were fixed in 4% paraformaldehyde for 15 min at room temperature and then stained with Alcian blue (Sigma-Aldrich) overnight. Then, the cells were washed with PBS and 0.1 M HCl to remove the background.

Statistical analysis

All experiments were performed at least three times. Data are expressed as the mean $\pm$ S.E.M. and were

analyzed using GraphPad Prism software version 6.0 (GraphPad Software, Inc.). Non-parametric test followed by Mann–Whitney test was used for the comparison of two groups and non-parametric test followed by Kruskal–Wallis test was used for the comparison of multiple groups. *P*-values less than 0.05 were considered statistically significant.

Results

FSHR activation leads to decreased Coll-II synthesis

FSH is the pituitary hormone responsible for the stimulation of estrogen, and it is increased after menopause. Although the receptor of FSH, FSHR, is expressed on chondrocytes (Kong *et al.* 2018), whether FSH plays role in cartilage homeostasis has never been investigated. To examine FSH affected cell viability, the CCK-8 assay was performed. No significant changes in cell viability were observed after FSH treatment. The *in vitro* results indicate that FSH has no cytotoxicity and does not affect cell apoptosis (Fig. 1A).

Given that ECM shifts during changes in chondrocyte behavior and during the progression of OA, we performed real-time quantitative RT PCR (qRT-PCR) and immunoblotting to evaluate the effects of FSH on primary chondrocytes. mRNA analyses demonstrated that the expression of genes related to ECM synthesis (*Sox9*, *Sox5*, *Sox6*, *Col2a1*, *Acan*) and chondrocyte hypertrophic differentiation (*Col10a1*, *Mmp3*, *Mmp13*) were decreased after FSH treatment (Fig. 1B). This is consistent with former studies that the general DNA methylation level is increased during dedifferentiation, leading to a decrease of gene expression (Ma *et al.* 2013, Duan *et al.* 2017). Chondrocyte differentiation is regulated by the PTHrP-Indian hedgehog (*Ihh*) negative feedback loop. PTHrP (also known as *Pthlh*) acts downstream of *Ihh* to inhibit chondrocyte hypertrophic differentiation (Kobayashi *et al.* 2002). In our present study, *Pthlh* was sharply increased and *Ihh* was decreased (Fig. 1A), suggesting that the hypertrophic differentiation is inhibited.

Moreover, immunoblotting analysis showed that Coll-II, the main collagen in cartilage and a differentiation marker, was reduced in a dose-dependent manner after FSH treatment (Fig. 1C), indicating a trend toward chondrocyte dedifferentiation. The chondrogenic transcription factor SRY-type high-mobility-group box (SOX9) was also reduced in a dose-dependent manner (Fig. 1C). A previous study suggested that the

Figure 1

FSH reduces ECM synthesis and promotes cell dedifferentiation in primary chondrocytes. (A) FSH did not affect chondrocyte viability at 24, 48 and 72 h by the CCK-8 assay. (B) The effect of FSH (10 ng/mL) on genes related to ECM synthesis, chondrocyte hypertrophy and differentiation, * $P < 0.05$ vs control. (C) Effect of different doses of FSH on Coll-II, SOX9, total-CREB (t-CREB) and phospho-CREB (p-CREB). GAPDH was used as a control.

cAMP-response element-binding protein (CREB) CBP/p300 interacts with SOX9 to enhance Coll-II transcription (Tsuda *et al.* 2003). Therefore, we also analyzed the phosphorylation level of CREB and observed a dose-dependent decrease after FSH treatment (Fig. 1C).

The decreases in Coll-II was in parallel with decreases in SOX9 and p-CREB (Fig. 1C).

FSH activation induces actin cytoskeleton reorganization

Chondrocytes change to a round and large phenotype during hypertrophic differentiation, while they change to a fibroblast phenotype during dedifferentiation (Charlier *et al.* 2019). Therefore, we performed an immunofluorescence assay to analyze the effect of FSH on the cell cytoskeleton.

The ATDC5 cell line is a well-validated chondrogenic cell line in chondrocyte differentiation. We stained ATDC5 cells with a FSHR antibody and confirmed that the FSH receptor is located on the surface of ATDC5 cells (Fig. 2A). Then, we treated ATDC5 cells with FSH, showing that FSH markedly alters the actin cytoskeletal architecture by changing ATDC5 cells to a fibroblast-like cell phenotype, as evidenced by phalloidin staining (Fig. 2B). These data support that FSH induces chondrocyte cytoskeleton reorganization, which is a sign of chondrocyte dedifferentiation.

FSH modulates Coll-II by binding to FSHR

To examine whether FSH-modulated chondrocyte dedifferentiation was mediated by FSHR, we knocked down *Fshr* in primary chondrocytes by using *Fshr* siRNA. The efficiency of *Fshr* siRNA to knockdown FSHR was detected by qRT-PCR (Fig. 3A) and western blot (Fig. 3B). And the results showed that only the second pair of siRNA (#2) has the highest efficiency to knockdown *Fshr*, so we use pair #2 in the following study. Interfering with *Fshr* siRNA upregulates Coll-II (Fig. 3C), suggesting that FSH regulates Coll-II by binding its receptor FSHR. In the present study, even though we interfered with *Fshr* by using *Fshr* siRNA, we still observed a nonsignificant decrease in Coll-II after FSH treatment in the *Fshr* siRNA group (Fig. 3C). A plausible explanation for this may be that FSH stimulation upregulates the expression of FSHR (Cui *et al.* 2012). To explore FSHR-mediated signaling pathways, we measured the levels of the second messenger cyclic AMP (cAMP) by using an ELISA kit after FSH treatment. Forskolin is known to increase cAMP levels, so we used it as a positive control. The results showed that FSH inhibits cAMP production in primary chondrocytes. The reduced cAMP can be restored by pertussis toxin (PTX), a $G_{\alpha i}$ inhibitor

Figure 2

FSH activation induces actin cytoskeleton reorganization in ATDC5 cells. (A) FSHR localization was visualized by immunofluorescence in ATDC5 cells. The cell nucleus was defined by DAPI (blue). Darkfield microscope was obtained from Zeiss (Axio Image A2, Germany). Scale bars = 100 μm. (B) The cytoskeleton of ATDC5 cells was stained with Rhodamine phalloidin (red) after FSH treatment for 30 min, and the nucleus was stained with DAPI (blue). Scale bars = 200 μm.

(Fig. 3D), suggesting that FSH may function by binding to its Gαi coupled FSHR.

ERK-1/2 and p38 kinase pathways are involved in FSH-mediated dedifferentiation

ERK and p38 kinase pathways have been confirmed to be involved in chondrogenesis and chondrocyte dedifferentiation (Kim *et al.* 2002, Yu & Kim 2015, Yu *et al.* 2016a,b). Thus, we measured whether the coupling of FSHR could alter ERK-1/2 and p38 activities. Western blot analysis results showed that FSH stimulates ERK-1/2 phosphorylation but inhibits p38 phosphorylation within 30 min in a dose-dependent manner (Fig. 4A). We next examined the roles of ERK-1/2 and p38 signaling in FSH-mediated pathways by using signaling inhibitors. Inhibition of the ERK-1/2 and p38 pathways was achieved with the ERK inhibitor PD98059 (PD) and p38 inhibitor

SB203580 (SB). As revealed by Western blotting results, the FSH-mediated inhibition of Coll-II was rescued by PD but aggravated by SB (Fig. 4B). Proteoglycans are indicators of chondrocyte differentiation, which can be detected by Alcian blue staining. The results showed that the proteoglycan accumulation was slightly inhibited after FSH treatment. However, the decrease in proteoglycan could be restored by PD but aggravated by SB (Fig. 4C). These data suggest that ERK-1/2 and p38 play critical roles in FSH-mediated chondrocyte dedifferentiation.

It is well established that Gαi is one of the most potent G proteins that regulates the MAPK signaling pathway (Leroy *et al.* 2007, Li *et al.* 2019b). The pretreatment with PTX restores FSH increased p-ERK-1/2 (Fig. 4D), indicating that the change in ERK-1/2 in response to FSH is sensitive to Gαi. In line with this, PTX restores FSH downregulated Coll-II (Fig. 4D), suggesting that Gαi involves in FSH

Figure 3

FSH inhibits Coll-II by binding to FSHR in primary chondrocytes. (A) The efficiency of three pairs *Fshr* siRNAs (#1, #2, #3) were detected by qRT-PCR. *Gapdh* was used as a control. * $P < 0.05$ vs control. (B) The expression of FSHR after *Fshr* siRNAs (#2) interfering was detected by Western blot. (C) Western blot analysis of FSHR in the FSH-mediated Coll-II. The effect of FSHR knockdown on the activating effects induced by FSH (5 ng/mL) was assessed with *Fshr*-siRNA transfection (100 nM). Western blotting analysis was quantified by ImageJ software, * $P < 0.05$ vs control. (D) Cyclin AMP (cAMP) analysis of primary chondrocytes in the presence of FSH, PTX (100 ng/mL), Forsk was used as a positive control. PTX, pertussis toxin; Forsk, forskolin. (* $P < 0.05$ vs control).

downregulated Coll-II. The same mechanism was found in a previous study showing that FSHR interacts with $G_{\alpha i}$ and induces osteoclast differentiation (Sun *et al.* 2006). We also observed a slightly inhibited p-p38 phosphorylation after PTX treatment (Fig. 4D). The same phenomenon was found in previous studies showing that PTX inhibits p38 and increases ERK-1/2 in chondrocytes (Kim *et al.* 2005, Xie *et al.* 2018).

Blocking FSH prevents chondrocyte loss and superficial zone destruction

Chondrocytes exhibit ranged phenotypic plasticity and have different functions in the different zones of articular cartilage (Stokes *et al.* 2002). For example, chondrocytes in the superficial zone (SZ) have a flattened, fibroblast-type appearance and have the capability to secrete lubricin to

Figure 4

Effects of FSH on the ERK-1/2 and p38 pathways. (A) FSH dose-dependently induces ERK-1/2 phosphorylation but reduces p38 phosphorylation in primary chondrocytes. Total ERK-1/2 and total p38 were used as controls. (B) MAPK signaling was inhibited by the ERK-specific inhibitor PD98059 (10 nM) or the p38 inhibitor SB203580 (10 nM) for 30 min in primary chondrocytes. The cells were then treated with FSH (30 ng/mL) for 30 min in the presence of these inhibitors. Western blotting analysis was quantified by ImageJ software, * $P < 0.05$ vs control. (C) ATDC5 cells were cultured in differentiation medium. MAPK signaling was inhibited by the ERK-specific inhibitor PD98059 (10 nM) or the p38 inhibitor SB203580 (10 nM) for 30 min. Then, the cells were treated with 100 ng/mL FSH in the presence of the inhibitors. Alcian blue staining was performed after 3 days. (D) Immunoblotting analysis of FSH (30 ng/mL) with and without PTX (100 ng/mL) on Coll-II, ERK-1/2 and p38 signaling in ATDC5 cells (* $P < 0.05$ vs controls).

alleviate the friction of joints (Neu *et al.* 2007). Spherical chondrocytes in the deep zone can express abundant type X collagen (Coll-X) and are involved in osteogenesis (Sophia Fox *et al.* 2009). To better illustrate the roles of FSH in cartilage, it is necessary to identify the location of FSHR on cartilage. Our previous study demonstrated that FSHR is located on human and mouse primary chondrocytes using immunoblotting and immunofluorescence (Kong *et al.* 2018). Here, we found that FSHR is mainly located on the superficial layer of articular cartilage and infrequently in calcified chondrocytes (Fig. 5A). The expression of FSHR

strongly implicates a potential role of FSHR signaling in the SZ of healthy and diseased articular cartilage.

Then, we used a polyclonal antibody against FSH β to block FSH in mice to study the effects of FSH absence. Previous studies demonstrated that blocking FSH efficiently decreases cholesterol (Guo *et al.* 2019) and bone loss (Zhu *et al.* 2012) in mice. Thus, blocking FSH with a FSH antibody in mice is an efficient approach to study the effects of FSH absence. There were no differences in bone length between the two groups (Fig. 5B). Compared with IgG mice, FSHAb mice had significantly increased

Figure 5

Blocking FSH prevents chondrocyte loss and superficial zone destruction. Mice were injected with FSH antibody or mouse IgG (100 μg/day) for 8 weeks. (A) Immunostaining of FSHR in mouse knee cartilage. Sections were counterstained with toluidine blue to verify proteoglycans. A light microscope was obtained from Leica (DMI 400B, Germany). Scale bars = 50 μm. (B) Imaging and quantification of femur and tibia length were performed for two groups. (C) Histological images of articular cartilage stained with Safranin O (red) and Fast Green. (D) The thickness of different cartilage zones in the medial tibial plateau of knee cartilage was quantified (* $P < 0.05$ vs IgG mice). Uncal.Th, uncalcified thickness. Cal.Th, Calcified thickness. Total.Th, Total thickness. (E) The number of chondrocytes in the uncalcified zone was quantified (** $P < 0.01$ vs IgG). (F) Mankin score shows that mice in the FSHAb group had an improved cartilage phenotype compared with those in the IgG group (* $P < 0.05$ vs IgG mice). (G) OARSI score shows that mice in the FSHAb group had an improved cartilage phenotype compared with those in the IgG group (** $P < 0.01$ vs IgG mice).

total cartilage thickness (Fig. 5C and D). Besides, the increased cartilage thickness was also observed in both the uncalcified and calcified zones. Decreased chondrocyte cell numbers (Fig. 5E) and destroyed cartilage (Fig. 5C, see black arrow) were noticeable in the cartilage in IgG mice. While blocking FSH can prevent chondrocyte loss and maintain an intact cartilage surface in FSHAb mice. Moreover, FSHAb mice had enhanced Safranin O staining, decreased Mankin score (Fig. 5F) and OARSI score (Fig. 5G) compared with IgG mice. Thus, blocking FSH in knee cartilage may provide therapeutic strategies for postmenopausal osteoarthritis.

Blocking FSH increases Ki67 and decreases Egr1 staining in cartilage

Then, we stained Coll-II to explore whether blocking FSH can affect cartilage ECM composition. Results showed

that the staining of Coll-II was markedly enhanced in FSHAb mice compared with IgG mice. Moreover, compared with IgG mice, FSHAb mice had increased Ki67 staining, suggesting that FSH signaling may be harmful in chondrocyte survival and proliferation.

To explore whether FSH plays roles in the onset or progression of OA, we analyzed two OA markers, proteoglycan 4 (Prg4, also known as lubricin) and early growth response 1 (Egr1). Prg4 is secreted by SZ chondrocytes and is crucial for reducing the friction of cartilage, while Egr1 has been shown to promote cartilage degeneration and OA onset. By using qRT-PCR, we showed that FSH inhibits *Prg4* but increases *Egr1* expression in chondrocytes (Fig. 6B). *Egr1* expression was further analyzed by using immunofluorescence in ATDC5 cells; results showed an increased staining after FSH treatment (Fig. 6C). However, blocking FSH decreased *Egr1* staining in FSHAb mice (Fig. 6D).

Figure 6

Blocking FSH increases Ki67 but decreases Egr1 staining. (A) Histological images of articular cartilage stained with Coll-II and Ki67. (B) qRT-PCR analysis of *Prg4* and *Egr1* in primary chondrocytes. *Gapdh* was used as a control (* $P < 0.05$ vs control). (C) *Egr1* expression after FSH (30 ng/mL) treatment for 30 min was analyzed by immunofluorescence in ATDC5 cells. The cell nucleus was labeled with DAPI (blue). Darkfield microscope was obtained from Zeiss (Axio Image A2). Scale bars = 100 μ m. (D) Histological images of articular cartilage stained with Egr1, scale bars = 50 μ m.

These data suggest that FSH may be a negative regulator in the onset or progression of OA.

Discussion

The effects of FSH on postmenopausal osteoporosis have been extensively studied by several groups, including our own (Sun *et al.* 2006, Wang *et al.* 2015). Whether FSH contributes to cartilage destruction, thus promoting the onset of osteoarthritis, remains unclear. Our previous study has shown that FSHR is located on human and mouse chondrocytes (Kong *et al.* 2018). However, the underlying mechanisms of FSHR on chondrocytes remain to be elucidated.

During the progression of OA, chondrocytes exhibit multiple pathologic behaviors, such as altered proliferation and cell death; unbalanced synthesis and degradation; changed phenotype and formation of osteophytes (Sandell & Aigner 2001). Traditionally, the terminal hypertrophic differentiation is considered as one of the key pathologic phenotypes in OA (Dreier 2010, Ripmeester *et al.* 2018). During which period, chondrocytes start to express Coll-X. Meanwhile, chondrocytes express enhanced degradation enzymes, such as MMP13 and MMP3, stimulating Coll-II degradation and subsequently cartilage catabolism (van der Kraan & van den Berg 2012). However, recent study suggests that inflammatory factors, such as IL-1 and TNF- α can promote chondrocytes into a fibroblast phenotype, a phenotype of dedifferentiation (Murakami *et al.* 2000, Speichert *et al.* 2019). And it is increasingly believed that the chondrocyte 'dedifferentiated-like' phenotype also contributes to OA progression (Stokes *et al.* 2002, Charlier *et al.* 2019, Ji *et al.* 2019). Compared to differentiated conditions, dedifferentiated OA chondrocytes can secrete vascular endothelial growth factor (VEGF) in response to proinflammatory factors (Honorati *et al.* 2004), which may subsequently involve in the vasculogenesis in the progression of OA.

Chondrocyte hypertrophic differentiation and dedifferentiation share some characteristics, such as decreased Coll-II and aggrecan. However, the behavior of the two chondrocyte functions occur via two different mechanisms. Recent study suggests that dedifferentiated chondrocytes start to express some mesenchymal stem cell (MSC) markers (CD49, CD90) (Vinod *et al.* 2020). Besides, chondrocytes reorganize their actin cytoskeleton and change their morphology to a fibroblast phenotype (Yu *et al.* 2016b). Much of this was related to the increase

of F-actin, which then increased cell stiffness upon dedifferentiation (Sliogeryte *et al.* 2016). Our results showed that FSH activation shifted the phenotype of ATDC5 cells from a round morphology to a fibroblastic morphology (Fig. 2B), suggesting a trend toward dedifferentiation. Meanwhile, Coll-II was decreased by FSH treatment (Fig. 1C). Therefore, our results suggest that FSH induces dedifferentiation in chondrocytes, which has been suggested to be an early sign of OA onset (Charlier *et al.* 2019).

Next, we investigated the relationship between Coll-II and its transcription factor SOX9 in FSH-induced dedifferentiation. We detected a decrease in phospho-CREB in our study (Fig. 1C), which was consistent with the decreases in SOX9 and Coll-II. These data suggest that the FSH-mediated downregulation of Coll-II is likely to be regulated by the SOX9/CREB-dependent pathway, which is consistent with a former study (Tsuda *et al.* 2003).

Previous reports suggest that MAPK signaling is involved in chondrocyte dedifferentiation (Kim *et al.* 2002, Yu *et al.* 2016a). In this study, opposite changes in ERK-1/2 (increase) and p38 kinase (decrease) were observed after FSH treatment, the mechanisms of which are similar to the effect of the proinflammatory cytokine IL-1b (Kim *et al.* 2002). In addition, the FSH-mediated inhibition Coll-II could be rescued by the ERK inhibitor PD98059, suggesting that ERK-1/2 activation is involved in FSH-induced dedifferentiation. However, the FSH-mediated inhibition of Coll-II was aggravated by the p38 inhibitor SB203580, suggesting that FSH may exert its effect by inhibiting p38 kinase. In our study, we observed that SOX9 and phospho-p38 were both decreased after FSH treatment. The interactions of P38 MAPK and SOX9 in chondrocyte differentiation have been investigated by Zhang *et al.*, who showed that SOX9 is downstream of p38 MAPK (Zhang *et al.* 2006). However, further studies need to be employed to discover the interactions of ERK-1/2 and SOX9 in FSH-induced dedifferentiation.

FSH exerts its roles by binding to its receptor FSHR, a member of GPCR family. FSHR can couple to multiple G proteins in a tissue-specific manner. For example, FSH couples with G α s and elevates cAMP in ovarian cells (Conti 2002), while it couples with G α i and inhibits cAMP in osteoclasts (Sun *et al.* 2006). In the present study, we found that FSH inhibits cAMP production. To identify the underlying mechanism, we introduced a G α i inhibitor (PTX) and found that the inhibition of G α i restored the FSH-mediated decrease in cAMP. Moreover, the FSH-induced decrease in Coll-II was also rescued by PTX,

implicating a role of the G α i pathway in FSH-induced dedifferentiation. Thus, our results suggest that FSHR may regulate chondrocyte dedifferentiation by interacting with G α i.

The FSH antibody approach has been adopted for studying osteoporosis and adipogenesis after menopause (Zhu *et al.* 2012, Liu *et al.* 2017, Guo *et al.* 2019) and is regarded as a well-characterized method to study FSH absence. Interestingly, blocking FSH increases Coll-II staining in the cartilage, suggesting an inhibition of dedifferentiation. And the *in vivo* results indicate that blocking FSH prevents chondrocyte loss and increases Ki67 staining in the cartilage, suggesting blocking FSH may be helpful for cell proliferation. Since we did not observe obvious effect of FSH on chondrocyte proliferation in CCK-8 assay. The possible explanation may be that the 72h in our *in vitro* CCK-8 assay is not sufficient to observe the effect of FSH on cell proliferation since primary chondrocytes have a very slow proliferation rate (Farnum & Wilsman 1993). Thus, the *in vivo* results suggest that blocking FSH is a useful tool to promote chondrocyte survival and to resolve the serious problem of dedifferentiation.

Notably, we found that FSH affects OA markers, such as Prg4 and Egr1 (Fig. 6B). The SZ is responsible for producing superficial zone protein (also called Prg4 or lubricin) to reduce the friction of articular cartilage (Poole 1997). Downregulated expression of Prg4 has been observed in early OA in animal osteoarthritis models (Flannery *et al.* 2009, Coles *et al.* 2010), while overexpression of Prg4 protects against OA (Ruan *et al.* 2013). Thus, Prg4 acts as a marker of early OA. Moreover, a previous study suggested that Prg4 is a CREB-dependent gene in articular cartilage (Ogawa *et al.* 2014). As shown in our results, decreases in Prg4 (Fig. 6B) and phospho-CREB (Fig. 1C) were observed after FSH treatment. Thus, FSH may have roles in the onset of OA by reducing lubricin secretion and increasing the friction of cartilage. Previous study showed that Egr1 promotes cartilage degeneration, chondrocyte hypertrophy, and inhibits extracellular matrix gene expression in chondrocytes (Sun *et al.* 2019). In addition, Egr1 suppresses *col2a1* promoter activity (Tan *et al.* 2003) and is increased in OA cartilage (Feng & Lian 2015, Zhang *et al.* 2016). In our present study, *Egr1* transcription was sharply increased after FSH treatment (Fig. 6B). However, Egr1 staining was decreased after blocking FSH (Fig. 6D). Based on published studies, FSH-enhanced *Egr1* might be regulated by upregulated ERK-1/2 since ERK-1/2 acts upstream of *Egr1* (Rockel *et al.* 2009, Shan *et al.* 2019).

Figure 7

Schematic diagram depicting mechanisms of FSH on chondrocyte dedifferentiation and osteoarthritis. FSH exerts roles by binding to its G α i coupled FSHR, which then inhibits p38 and activates ERK-1/2 phosphorylation in chondrocytes. The activated ERK-1/2 signaling increases Egr1 accumulation, which then reduces the Col2a1 synthesis. The inhibited p38 signaling decreases SOX9 transcription, the transcription factor of Col2a1. Besides, FSH also decreases the phosphorylation of CREB. As a result, FSH activation leads to decreased Col2a1 transcription and chondrocyte dedifferentiation. Upon dedifferentiation, chondrocytes reorganize their actin cytoskeleton and change their morphology to a fibroblast phenotype. In summary, FSH activation leads to decreased extracellular matrix (ECM) synthesis and chondrocyte dedifferentiation which may exert roles on osteoarthritis (OA).

There are some limitations in our present study. We did not investigate the direct interactions of G α i and MAPK signaling since many studies have shown that MAPK signaling is downstream of G α i (Sun *et al.* 2006, Goldsmith & Dhanasekaran 2007, Leroy *et al.* 2007). Another limitation is that we cannot exclude whether FSH has roles in other cells, such as osteoclasts, that affect the impact of FSH on cartilage. Future considerations of a cartilage-specific knockout animal model could be used to assess the direct relationship between FSH and knee OA.

In conclusion, our present study (summarized in Fig. 7) suggests that FSH acts as a negative regulator in chondrocyte differentiation through the suppression of Coll-II. Plausible mechanisms may be that FSH oppositely regulates ERK-1/2 and p38 kinase. In summary, our study may provide insights into therapeutic options in cartilage disorders, such as postmenopausal osteoarthritis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Y W and J X designed the research. M Q Z and Z K H conducted the animal experiments. Y W, X J Z and S S S performed WB, qRT-PCR and IHC. Y W and D H K analyzed the data. Y W and J X wrote the paper.

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