1. Introduction

Osteoarthritis (OA) is considered a bone and joint disease characterized by progressive loss of cartilage, osteophyte formation, and subchondral bone sclerosis and is affected by multiple factors, which causes joint pain, swelling, and other related symptoms [1,2]. The possible mechanism is that the elastic, blood-supplied hyaline cartilage covering the joint surface is defective, and the subchondral bone is exposed, which causes the surrounding cartilage tissue to be subjected to superphysiological compressive stress [3]. The primary pathological changes of OA are chondrocyte (CH) metabolism disorder and cartilage degeneration, which promotes the formation of new bones in abnormal parts and eventually joint stiffness and dysfunction [4]. Epidemiological studies have shown that in women, the loss of estrogen is often accompanied by an increase in the incidence of knee and hip joint OA [5]. In recent years, with the development of estrogen replacement therapy in bone and joint diseases, exploring the relationship between estrogen and OA at the molecular biology level provides a new option for the treatment of OA [6,7].

Most people believe that estrogen is only essential for the development of the reproductive system, but many studies have shown that estrogen also regulates the development of the nervous, cardiovascular, immune, and skeletal muscle systems [8,9]. Estrogen acts through estrogen receptors (ESR) and SOX6 in 17β-E2-mediated stimulation of ECM metabolism during chondrocyte (CH) degeneration. We compared the expression of anabolic genes (collagen II and aggrecan) and catabolic genes (MMPs and TIMPs) in IL-1β-induced CH degeneration in vitro, with and without 17β-E2 supplementation. We separately silenced the SOX6, ESR1, and ESR2 genes in CHs to determine their impact on 17β-E2 treatment. Additionally, we used Chromatin immunoprecipitation followed by DNA sequencing (ChIP-seq) and luciferase assays to investigate protein-DNA interactions within ESR2 and SOX6-promoter complexes. After three days of IL-1β treatment, ESR1/2, SOX6, collagen II, aggrecan, and TIMP1/3 were decreased, while MMP3/9/13 were increased. The addition of 17β-E2 partially reversed these effects, but silencing SOX6, ESR1, or ESR2 weakened the protective effects of 17β-E2. Silencing ESR2, but not ESR1, abolished the upregulation of SOX6 induced by 17β-E2. ESR2 was found to bind the SOX6 promoter and regulate SOX6 expression. 17β-E2 upregulates SOX6 through ESR2 mediation, and the synergistic effect of 17β-E2 and ESR2 on SOX6 balances ECM metabolism in CHs.

Keywords: Chondrocyte, 17β-estradiol, SOX6, Estrogen receptor 2, Extracellular matrix
have shown that Sox6 cooperates with Sox9 to activate the enhancer of the collagen II gene and maintain the extracellular matrix (ECM) of CHs. The Sox6 double-knockout mutation shows severe and general achondroplasia, characterized by the loss of the cartilage matrix. Sox6 and Sox9 are essential factors for chondrogenesis in mesenchymal cells [15]. Besides, 17-β estradiol (17-β-E2) [16], one of the most critical estrogenic hormones, is reported to up-regulate SOX9 rat mandibular condylar CHs via ESR1 [17]. Phytoestrogen (Daidzein) was also reported to promote the CH phenotype and ECM formation, including collagen II and Sox9 [18]. However, whether the estrogen would regulate SOX6 expression and affect CH metabolism remains unclear.

In this study, we used the human primary CHs to examine the role of 17-β-E2 and ERS in the ECM metabolism during CHs degeneration. Customary CHs degeneration was induced by IL-1β with suppression of anabolism and promotion of catabolism. 17-β-E2 supplement prevented the reduction of ERS and SOX6 expression and balanced the anabolism and catabolism in CHs, which was alleviated after SOX6 was silenced. Importantly, we found ESR2 binding sequences in the upstream regions of SOX6 promote. These results suggest that ESR2 is a critical regulator of 17-β-E2 to active SOX6 in CHs.

2. Materials and methods

2.1. Patient samples collection

This study got the support of the Ethics Committee of The Second Affiliated Hospital of Heilongjiang University of Traditional Chinese Medicine. We isolated the CHs from the cartilage of the OA patients undergoing total hip joint replacement surgery. A total of 10 female patients (every age: 52 years old, from 41 to 63 years) in our hospital from April to May 2020 participated in the study. The joint was conserved in cold culture medium immediately after cutting from patients for the following CHs isolation.

2.2. CHs isolation and treatments

We separated the smooth part of the cartilage in the joint and washed sterile phosphate-buffered saline solution (PBS) to eliminate the hemocytes. Cartilage was then cut into small pieces and digested with a mixture of 0.25% trypsin and type I collagenase (Sigma, St. Louis, MO, USA) for 12 h. The NP cell pellets were re-suspended in trypsin and type I collagenase (Sigma, St. Louis, MO, USA) for 12 h. The NP cell pellets were re-suspended in cold culture medium immediately after cutting from patients for the following CHs isolation.

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2.3. Protein Preparation and Western Blot

After treatments, CHs were washed with PBS, and the total protein was harvested with the RIPA lysis buffer (Beyotime, Shanghai, China). The concentration was measured with a Bicinchoninic Acid Kit (Beyotime, Shanghai, China) according to the manufacturer’s instructions. The equal sample was separated by SDS gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes. After being blocked with 5% milk, the membranes were incubated with primary antibodies: ESR1, ESR2, SOX6, and GAPDH (as a loading control) overnight at 4°C. Membranes were washed with Tris-buffered saline containing Tween 20 (TBST) and incubated with anti-species secondary antibody for 1 h at room temperature. All the antibodies were purchased from Abcam (Cambridge, UK). Finally, the protein was visualized with the BeyoECL substrate (Beyotime, Shanghai, China) and analyzed by ImageJ software.

2.4. Real-time polymerase chain reaction (RT-PCR)

To determine the expression levels of indicated genes, total RNA was extracted from the CHs using TRIzol reagent (Life Technologies, USA) according to the manufacturer’s instructions. Then, mRNA was reverse-transcribed into cDNA with a reverse transcription kit (Roche, Basel, Switzerland). The RT-PCR was performed using SYBR Green Master (TOYOBO, Japan) with the reactions: 94°C for 45 s followed by 40 cycles of 95°C for 5 s followed by 60°C for 30 s. Data were calculated based on the GAPDH mRNA expression according to the method of 2-∆∆Ct. The primers used for real-time PCR are listed in Table 1.

2.5. Chromatin immunoprecipitation (ChIP)

We got the 2000-bp promoter sequences upstream of SOX6 from the Genome Browser Gateway database (http://genome.ucsc.edu/cgi-bin/hgGateway). Following, the DNA-binding sites for ESR2 in the promoter region of SOX6 were predicted from the JASPAR core database. We confirm the facticity of the binding sites using the ChIP

Table 1. Primer sequences for RT-PCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward (5’&gt;3’)</th>
<th>Reverse (5’&gt;3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen II</td>
<td>TGGACGATCAGGCACAAACC</td>
<td>GCTGCGGATGCTCTCAATCT</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>ACTCTCTGTTTCTCAGCTCT</td>
<td>ACACTCAGCGATGTGCATGG</td>
</tr>
<tr>
<td>MMP3</td>
<td>AGTCTCCATCTACTGTTGCT</td>
<td>TCCCCGTACCTCAAATCC</td>
</tr>
<tr>
<td>MMP9</td>
<td>ACTGAGAGGCTCCGAGAAATG</td>
<td>GAAACCGCAGCATTTGGCTT</td>
</tr>
<tr>
<td>MMP13</td>
<td>ACTGAGAGGCTCCGAGAAATG</td>
<td>GAAACCGCAGCATTTGGCTT</td>
</tr>
<tr>
<td>TIMP1</td>
<td>CTTCTGCAATTCGGACGCTCT</td>
<td>AGGCTGTATAAGGTTTGCTG</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>CATGTGCAGTACATCCATCGG</td>
<td>CATATACAGCGACCGATCT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACAACTTGGATCATGTTGAAGG</td>
<td>GCCATCACGCCACAGTTC</td>
</tr>
</tbody>
</table>

RT-PCR: Reverse Transcription-Polymerase Chain Reaction.
assay (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The empty beads or crosslinked with ESR2 antibody (Abcam, Cambridge, UK) and goat IgG were used for immunoprecipitation with the ultrasonic lysed CHs. The whole chromatin of the CHs was used as Input DNA templates. After the solution of crosslinking, PCR was used to amplify the DNA fragment in AGE (agarose gel electrophoresis). The primers were designed using the Premier 5.0 software (Premier Biosoft International, USA) and listed in Table 2.

### 2.6. Luciferase assays

The dual-luciferase assay was used to verify that ESR2 activates the SOX6 promoter expression. We used the plasmid with empty (as control) or ESR2 coding to overexpress the ESR2 protein. The elements of verified SOX6 promotes binding sequences for ESR2 were subcloned into a pGL3 (pGL3-WT) based firefly luciferase reporter vector (Promega, Madison, USA), and the pGL3-WT was mutated by using Directed Mutagenesis System (Invitrogen, Carlsbad, CA, USA). Meantime, pRL containing the Renilla luciferase reporter vector was used as an internal control. Subsequently, CHs were co-transfected with the plasmid, pGL3, and pRL using lipofectamine 2000 reagent (Beyotime, Shanghai, China). After 24 h transfection, the firefly luciferase activity was normalized by Renilla with a Dual-Luciferase Reporter Assay System (Thermo Fisher Scientific, Waltham, MA, USA).

### 2.7. siRNA transfection

The siRNA was purchased from Thermo Fisher Scientific: SOX6 (catalog # s30970), ESR1 (catalog #145538), ESR2 (catalog #145909), and transfected in CHs by Lipofectamine 2000 reagent (Beyotime, Shanghai, China) according to the manufacturer’s instruction.

### 2.8. Statistical Analysis

Data were presented as mean ± standard deviation (SD). We used unpaired one-way ANOVA to compare the differences between the groups. The statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 15.0 (SPSS Inc., Chicago, IL, USA). P<0.05 was considered statistically significant.

### 3. Results

#### 3.1. 17β-E2 promotes ESR1/2 expression and balances the ECM metabolism of CH via SOX6

To induce the CHs degeneration, we cultured CHs with 10 ng/ml IL-1β for three days, which suppressed the ESR1, ESR2, and SOX6 protein expressions. After supplying the 17β-E2 in the IL-1β-containing medium, the ESR1, ESR2, and SOX6 levels were maintained (Figure 1A). Moreover, the 17β-E2 supplement protected the anabolism of CHs by maintaining the synthesis of collagen II aggrecan and prevented the catabolism by maintaining the TIMP1 and TIMP3 and suppressing the MMP3, MMP9, and MMP13 expression (Figure 1B, C, D). Thus, 17β-E2 is sufficient to resist the IL-1β caused ESR1/2 and SOX6 reduction and the metabolism disruption. To further determine the role of SOX6 in the protection of 17β-E2 in CHs, we silenced the SOX6 gene in CHs and cultured the CHs with IL-1β and 17β-E2 as well. The data of the western blot showed that the silencing of SOX6 did not affect the ESR1 and ESR2 protein expression. However, blocking SOX6 abolished the protection of 17β-E2 on collagen II and aggravated the IL-1β-induced reduction of aggrecan (Figure 1B). Meanwhile, SOX6 silencing affected the effects of 17β-E2 on TIMP3, MMP3, and MMP9 and promoted the IL-1β caused MMP3/13 upregulation (Figure 1C, D). Therefore, we concluded that the SOX6 silencing aggravated the IL-1β induced ECM metabolism disorder of CHs and weakened the underlying 17β-E2 protection.

#### 3.2. ESR2 deficiency abolishes 17β-E2 induced SOX6 upregulation

To further determine whether the 17β-E2 prevented the CHs degeneration and SOX6 reduction via the ESR, we silenced the ESR1 and ESR2 of CHs separately, and the CHs with or without silencing were treated with IL-1β for three days to induce degeneration. The supplement of 17β-E2 was also used to alleviate the effects of IL-1β. Interes-
tingly, under IL-1β treatment, silencing of ESR1 did not affect the ESR2 and SOX6 expression, but the ESR2 and SOX6 protein levels were compensatory increased after adding the 17β-E2. In contrast, silencing of ESR2 did not affect the ESR1 and SOX6 expression, and the 17β-E2 also compensatory increased the ESR1 expression. However, ESR2 deficiency abolished the 17β-E2 triggered SOX6 upregulation (Figure 2A). Additionally, to a certain extent, silencing ESR1 or ESR2 affected the role of 17β-E2 in protecting collagen II, aggrecan, and TIMP1/3, and the prevention of MMP3/9/13 in CHs degeneration (Figure 2B, C, D). Therefore, the dysfunction of one ESR will compensatory increase the expression of another, but it eventually affects the effect of 17β-E2 on CHs.

3.3. ESR2 upregulates SOX6 expression via promoter activation

Due to the ESR2 silencing affecting the upregulation of SOX6 and the compensatory increase of ESR2 promoting the SOX6 expression, we speculated that ESR2 regulated the SOX6 expression. Since ESR2 is a transcription factor, we first verified whether ESR2 could promote SOX6 expression via activating its promoter. Thus, we used the JASPAR core database to forecast the putative elements for ESR2 to bind to the promoter region of SOX6. As shown in Figure 3A, we found two putative binding sites in the 2000 bp upstream of the SOX6 promoter. One (site 1, red color) starts at 284 bp, and another one (site 2, green color) begins at 939 bp. We determined the binding efficiency of the two sites using ChIP. We used the bead-linked ESR2 antibody or IgG to pull down the indicated protein in the ultrasonic cell lysis solution. The empty bead was used as a negative control. All the DNA chromatin in the cell lysis was used as Input to test the PCR primers. The immuno-precipitated DNA was eluted from the bead and amplified by PCR. The result from agarose gel electrophoresis (Fi-

![Fig. 2. ESR2 deficiency abolishes 17β-E2 induced SOX6 upregulation. The normal CHs, ESR1 or ESR2-silenced CHs were cultured in IL-1β (10 ng/ml) with or without 17β-E2 (10 nM) for three days. (A) Western bolt analysis for ESR1, ESR2, SOX6, and GAPDH protein expression after treatments, and the quantitative analysis. (B-D) RT-PCR analysis for collagen II, aggrecan, TIMP1, TIMP3, MMP3, MMP9, and MMP13 mRNA expression by normalization to GAPDH expression. Results are expressed as mean ± SD. (n=4, *P < 0.05, **P < 0.01, ***P < 0.001).](image)

![Fig. 3. ESR2 upregulates SOX6 expression via promoter activation. (A) Two predicted binding elements (site 1: red, site 2: green) in the SOX6 promoter region. (B) site 1 and site 2 sequences were recovered by PCR from ESR2 immunoprecipitation but not from IgG or empty bead immunoprecipitation. (C) The cartoon of the plasmid or vector-transfected in CHs. (D) Luciferase activity was driven by the site 1 WT sequences, which was more dramatic following ESR2 protein overexpression and no significant difference in luciferase activity was observed when the sequences were mutated. Results are expressed as mean ± SD. (n=6, ***P < 0.001).](image)

![The supplement of 17β-E2 helps CHs tolerate the injury from IL-1β dependent on the increased ESR2 and SOX6. We wondered whether only upregulation of ESR2 could also protect the anabolism and suppress catabolism of CHs. Thus, we compared the normal CHs or ESR2-plasmid transfected CHs in the IL-1β and 17β-E2 containing cultures. All the cells were cultured for three days. Under the IL-1β treatment, both plasmid transfection and 17β-E2 stimulation upregulated the ESR2 and SOX6 protein expression. Meanwhile, adding 17β-E2 in the ESR2](image)
Estradiol balances chondrocyte metabolism

Our study argues for the protection of 17β-E2 in the ECM metabolism of CH based on the mediation of ESR2 and SOX6. The ECM of articular cartilage is mainly composed of collagen II and aggrecan. The destruction of cartilage in the pathogenesis of OA is not only related to the dysfunction of CHs but also the loss of ECM. Estrogen is an essential female hormonal hormone that maintains normal sexual and reproductive functions in women. It plays a vital role in bone metabolism, which promotes bone matrix metabolism, helps the reabsorption of calcium, and the deposition of phosphate salts to maintain standard bone quality [19]. Epidemiological studies have found that the incidence of OA in men under 50 years old is higher than that in women, while the incidence of OA in women over 50 years old is much higher than that of men, and the clinical symptoms are more severe [20]. The reason for this phenomenon may be related to the decline in estrogen levels in postmenopausal women. Nowadays, estrogen replacement therapy and selective estrogen receptor modulators (SERMs) have been effective in delaying and reducing the risk of OA in women.

A previous study from Maneix et al. [21] reported that 17β-E2 upregulates the collagen II via the ESR1 and SOX9. SOX9 is one of the key transcription factors to maintain CH phenotype and ECM metabolism of cartilage. It regulates collagen II/IX, proteoglycans, MMPs, and TIMPs. Like SOX9, SOX6, another member belonging to the SOX family, has also been confirmed to have the function of maintaining CH phenotype [22,23]. However, the regulation of SOX6 during the ECM metabolism of CHs remains unclear. In this study, we particularly investigated the effects of 17β-E2 and its receptor ESR1/2 with the interaction of SOX6. In the IL-1β caused CHs degeneration model [24,25], we found the supplement of the 17β-E2 reversed the reduction of collagen II, aggrecan, and TIMP1/3 and suppressed the MMP3/9/13 expression, which was accompanied by up-regulation of ESR1/2 and SOX6. As we all know, estrogen mediates its effects by binding to its receptors. ESR1 and ESR2 can be activated by the estrogen and then translocate into the nucleus and bind to DNA to regulate the activity of different genes [26,27]. To further determine whether the SOX6 gene is also mediated by ESR, we silenced the ESR1 and ESR2 in the CHs, respectively. When one of the ESR and ESR2 was silenced, the other one was compensatory increased after 17β-E2 stimulation, which is consistent with the previous study [28,29]. By contrast, ESR2 deficiency abolished the 17β-E2-induced SOX6 upregulation, whereas ESR1 deficiency did not significantly affect the SOX6 expression.

Therefore, we speculated that, unlike the SOX9 regulated by ESR1 [21,30], SOX6 is more likely controlled by ESR2. Interestingly, we found two putative elements for ESR2 to bind with the SOX6 promoter, and one of them was confirmed to be linked to the ESR2 protein. What’s more, the ESR2 protein also effectively determined the activation of SOX6 through the promoter regions. Thus, the 17β-E2 upregulated SOX6 via the mediator of ESR2, and the silencing of ESR2 would reduce the SOX6 upregulation caused by 17β-E2. Further, we tested, without the supplement of 17β-E2, whether the upregulated ESR2 would balance the anabolism and catabolism of CHs under the IL-1β stimulation. However, we only found the overexpressed ESR2 and the resulting SOX6 suppressed the catabolism process by decreasing the MMP3 and MMP9, but it did not significantly change the anabolism process. After superimposing 17β-E2 to the overexpressed ESR2, the efficiency was enhanced compared to applying 17β-E2 alone in the IL-1β stimulation. Therefore, it is the synergy of 17β-E2 and ESR2 and the resulting upregulated SOX6 that protect the ECM metabolism, not any of them alone.

Collectively, our in vitro study provides a shred of evidence to support that 17β-E2 protects the anabolism and prevents the catabolism of CHs via the SOX6. Further, the data suggest that the ESR2, not ESR1 regulates the SOX6 expression via the promoter activation. Without

e transfectected CHs enhanced the ESR2 and SOX6 protein expression (Figure 4A). Without 17β-E2, the collagen II and aggrecan content was not increased even though the ESR2 and SOX6 protein levels were elevated. However, the ESR2 overexpression amplified the effect of 17β-E2 on the promotion of collagen II and aggrecan (Figure 4B, C). Moreover, only up-regulation of ESR2 and SOX9 did not significantly affect the expression of TIMP1 and TIMP3, which was markedly increased after adding 17β-E2 (Figure 4D, E). However, the MMP3 and MMP9 levels were decreased when the CHs were transfected with ESR2 plasmid, and adding 17β-E2 enhanced the suppression of MMP9 and MMP13 compared to the ESR2-transfected CHs. Therefore, ESR2 overexpression might contribute to suppressing the catabolism under IL-1β treatment, but it could not protect the anabolism of CHs. The existence of 17β-E2 is necessary for maintaining the balance of ECM metabolism.

4. Discussion

Our study argues for the protection of 17β-E2 in the ECM metabolism of CH based on the mediation of ESR2 and SOX6. The ECM of articular cartilage is mainly composed of collagen II and aggrecan. The destruction of cartilage in the pathogenesis of OA is not only related to the dysfunction of CHs but also the loss of ECM. Estrogen is an essential female hormonal hormone that maintains normal sexual and reproductive functions in women. It plays a vital role in bone metabolism, which promotes bone matrix metabolism, helps the reabsorption of calcium, and the deposition of phosphate salts to maintain standard

**Fig. 4.** ESR2 relies on 17β-E2 to balance the ECM metabolism of CH. The normal CHs or ESR2-overexpressed CHs were cultured in IL-1β (10 ng/ml) with or without 17β-E2 (10 nM) for three days. (A) Western blot analysis for ESR1, ESR2, SOX6, and GAPDH protein expression after treatments, and the quantitative analysis. (B-E) RT-PCR analysis for collagen II, aggrecan, TIMP1, TIMP3, MMP3, MMP9, and MMP13 mRNA expression by normalization to GAPDH expression. Results are expressed as mean ± SD. (n=4, *P < 0.05, **P < 0.01, ***P < 0.001).
the 17β-E2, the protection of ESR2 and SOX6 in the CHs is limited. Such insights not only enrich our fundamental understanding of the 17β-E2 in ECM metabolism of CHs but also help develop novel and improved strategies associated with SOX6 in the OA of female patients.

Conflict of Interests
The authors declared no conflict of interest.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
This study was approved by the ethics committee of The Second Affiliated Hospital of Heilongjiang University of Traditional Chinese Medicine.

Informed Consent
Signed written informed consent were obtained from the patients and/or guardians.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
Ying Cao and Hongpeng Liu designed the study and performed the experiments, Yongji Li and Xiangjun Yang and Hongxu Wang analyzed the data, Ying Cao and Hongpeng Liu prepared the manuscript. All authors read and approved the final manuscript.

Funding
None.

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