

# Estrogen Deficiency Exacerbates Traumatic Heterotopic Ossification in Mice

Zheng Wang<sup>1,2,\*</sup>, Yifan Wu<sup>1,2,\*</sup>, Wanrong Yi<sup>1,2</sup>, Yifeng Yu<sup>1,2</sup>, Xue Fang<sup>1,2</sup>, Zonghuan Li<sup>1,2</sup>, Aixi Yu<sup>1,2</sup>

<sup>1</sup>Department of Orthopedic Trauma and Microsurgery, Zhongnan Hospital of Wuhan University, Wuhan, Hubei, People's Republic of China; <sup>2</sup>Hubei Clinical Medical Research Center of Trauma and Microsurgery, Wuhan, Hubei, People's Republic of China

\*These authors contributed equally to this work

Correspondence: Aixi Yu; Zonghuan Li, Email [yuaixi@whu.edu.cn](mailto:yuaixi@whu.edu.cn); [lizonghuan@whu.edu.cn](mailto:lizonghuan@whu.edu.cn)

**Background:** Traumatic heterotopic ossification (HO) is a devastating sequela of orthopedic surgeries and traumatic injuries; however, few studies have explored the effects of the estrogen-deficient state on HO formation. In the present study, we investigated the impact of estrogen deficiency on ectopic cartilage and bone formation in tendon after Achilles tenotomy in an ovariectomized mouse model.

**Methods:** A total of 45 female C57BL/6 mice were randomly divided into three groups: sham-operated (control), estrogen depletion by ovariectomy (OVX) and OVX with 17 $\beta$ -estradiol supplementation (OVX + E<sub>2</sub>), with 15 animals in each group. Three weeks after OVX, all mice were subjected to an Achilles tenotomy using a posterior midpoint approach to induce HO. At 1, 3 and 9 weeks after tenotomy, the left hind limbs were harvested for histology, immunohistochemistry and immunofluorescence evaluations. The volume of ectopic bone was assessed by micro-CT.

**Results:** Mice in the OVX group formed more ectopic cartilage 3 weeks after tenotomy, as well as ectopic bone 9 weeks after tenotomy, compared to the control group. Estrogen deficiency resulted in more severe inflammatory infiltration at the injury sites 1 week after tenotomy, involving the recruitment of more macrophages and mast cells, as well as increasing the expressions of pro-inflammatory mediators, including IL-1 $\beta$ , IL-6, and TNF- $\alpha$ . Moreover, the local TGF- $\beta$ /SMAD signaling pathway was dysregulated after OVX, which manifested as upregulated expressions of TGF- $\beta$  and pSMAD2/3. E<sub>2</sub> supplementation protected against OVX-induced HO deterioration, inhibited inflammatory infiltration, and downregulated the TGF- $\beta$ /SMAD signaling pathway.

**Conclusion:** Estrogen deficiency exacerbated HO formation in the Achilles tenotomy model. These findings might be attributable to the disturbance of the inflammatory response and the activation of TGF- $\beta$ /SMAD signaling at the injury sites during the early stages of HO development.

**Keywords:** heterotopic ossification, estrogen deficiency, inflammation, TGF- $\beta$ /SMAD signaling

## Introduction

Traumatic heterotopic ossification (HO) is characteristic as the pathological formation of bone and cartilage matrix in exoskeletal tissues including tendons, ligaments and muscles, which may occur in certain predisposing conditions, such as arthroplasty, arthroscopy, bone fracture or dislocation, traumatic brain and spinal cord injury, and severe burns.<sup>1</sup> Patients with HO would experience chronic pain, open wounds, restricted range of motion at joints, and nerve impingement, resulting in a poor quality of life. As such, considerable attention has been paid to the prevention and mitigation of HO formation.<sup>2</sup> However, the precise molecular mechanisms underlying HO formation remain unknown, and the management of HO is still challenging for physicians.<sup>3</sup> Therefore, understanding the factors that exacerbate its development is an important prerequisite.

Growing evidence supports the hypothesis that HO develops in part because of systemic factors, such as traumatic brain and spinal cord injury, diabetes, and burns, all of which have been shown to influence HO development.<sup>4,5</sup> Sex is

also a variable that appears to influence the probability of developing HO, with women having a lower incidence than men,<sup>6,7</sup> which could be partly attributable to differences in the osteogenic potential of mesenchymal stem cells (MSCs).<sup>6</sup> Previous studies have also shown that the abilities of chondrogenic and osteogenic differentiation of MSCs in male mice were higher than those in female mice.<sup>8,9</sup> For these distinctions, the effect of androgens on the osteogenic ability of MSCs may be a deeper cause.<sup>10</sup> However, the specific role of estrogen in HO formation in female animals has not been thoroughly investigated.

To date, only one epidemiological study has shown that HO is more prevalent among postmenopausal women in female patients after primary total hip arthroplasty (THA).<sup>11</sup> This seems paradoxical since postmenopausal estrogen deficiency is usually associated with reduced MSCs osteogenesis and decreased bone mass in osteoporosis.<sup>12</sup> Furthermore, the study was descriptive and did not provide a plausible mechanism. Thus, the association between estrogen deficiency and HO development remains controversial. Understanding whether estrogen deficiency affects HO formation could help elucidate new strategies for inhibiting or limiting this debilitating condition in postmenopausal women.

HO is an inflammatory disease and can be inhibited by non-steroidal anti-inflammatory drugs, such as indomethacin.<sup>13</sup> Estrogen deficiency could cause a chronic inflammatory state during tissue repair to traumatic insult, including increasing the inflammatory cell infiltration and levels of pro-inflammatory mediators in the injured muscles and tendons,<sup>14–17</sup> where predispose to HO formation.<sup>18</sup> Furthermore, estrogen replacement decreased the extent of local inflammation after muscle/tendon injury and accelerated their repair.<sup>14,15,17</sup> These relationships are important in examining the overlap between estrogen pathways and the inflammatory response during HO formation. Thus, the purpose of the current study was to clarify the role of estrogen in HO formation in ovariectomized (OVX) mice, and to identify the factors that explain why postmenopausal females are susceptible to HO.

## Materials and Methods

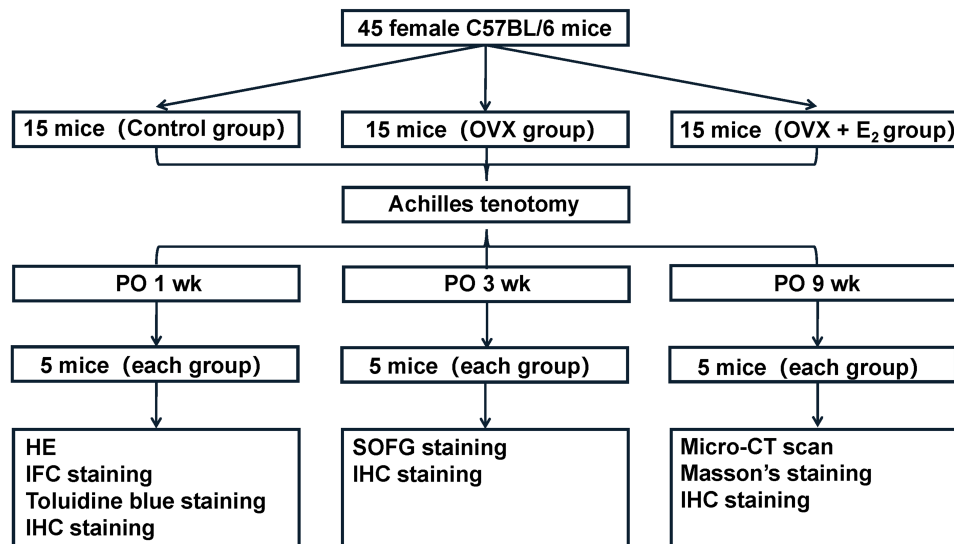
### Animal Experiment

All animal experiments were approved by the Experimental Animal Welfare Ethics Committee of the Zhongnan Hospital of Wuhan University (ZN2023212), and all operations were consistent with the guidelines of the National Institutes of Health Guide for Care and Use of Animals. Forty-five female C57BL/6 mice (12-week-old, body weight 18–20 g) were randomly divided into three groups of 15 animals each: sham-operated (control group), estrogen depletion by bilateral OVX (OVX group), and OVX with 17 $\beta$ -estradiol (E<sub>2</sub>) supplementation (OVX+E<sub>2</sub> group). After anesthetization with intraperitoneal pentobarbital sodium (1%, 5 mL/kg; Sigma-Aldrich, USA), bilateral ovariectomy was performed in the OVX and OVX+E<sub>2</sub> groups, as previously described.<sup>19</sup> Sham-operated mice underwent the same surgical procedure as OVX mice without ovary removal. To ensure that the back wounds of the animals were fully healed and to exclude the influence of the local inflammatory response on HO formation, we established a mouse HO model 3 weeks after the first surgery. All mice were subjected to Achilles tenotomy using the posterior midpoint approach to induce HO.<sup>20</sup> Briefly, a longitudinal incision was made along the medial aspect of the left Achilles tendon. The Achilles tendon was exposed and sharply severed at the midpoint and the incision was closed using absorbable sutures. When the animals recovered from anesthesia, they were injected subcutaneously with a meloxicam analgesic (0.2 mg/mL/kg; Boehringer-Ingelheim, Germany).

Mice in the OVX+E<sub>2</sub> group received estradiol supplementation by subcutaneous injection of E<sub>2</sub> (Beyotime Biotechnology, China) until sacrifice at a dose of 0.1 mg/kg every two days 19 immediately after Achilles tenotomy. The mice were sacrificed and the tendon injury sites were evaluated at 1, 3, and 9 weeks after tenotomy (Figure 1). Tissue histology, immunohistochemistry (IHC) staining, immunofluorescence (IFC), staining and micro-CT analyses were performed as described below.

### Histological Staining

At 1, 3, and 9 weeks after tenotomy, five animals in each group were sacrificed and the left hind limbs were harvested for histological evaluation. In brief, the samples were decalcified in 10% disodium ethylenediaminetetraacetate dihydrate for 4 weeks, embedded in paraffin, and sectioned at 5  $\mu$ m thickness. The sections were stained with hematoxylin and eosin



**Figure 1** Flow diagram of the study design indicating the group allocations and study procedures.

**Abbreviations:** OVX, ovariectomy; E<sub>2</sub>, 17 $\beta$ -estradiol; PO, post-operation; wk, week; HE, hematoxylin and eosin; IFC, Immunofluorescence; IHC, Immunohistochemistry; SOFG, Safranin O/Fast Green.

(HE; Baiqiandu, China) for routine histological examination. To reveal mast cells, the sections were stained with 1% toluidine blue (Baiqiandu) for 20 min, treated with alcohol for rapid color separation, permeabilized with xylene, and mounted. To evaluate ectopic cartilage formation at 3 weeks and ectopic bone formation at 9 weeks postoperatively, Safranin O/Fast Green (SOFG) staining and Masson's staining were performed using the corresponding kits (Baiqiandu), respectively. Briefly, for SOFG staining, sections were deparaffinized and stained with Fast Green for 5 min, washed, dehydrated, and stained with Safranin O for 2 min. For Masson's staining, the sections were placed in Bouin's solution for 40 min, Weigert's hematoxylin solution for 10 min, Biebrich scarlet solution for 10 min, phosphotungstic acid-phosphomolybdic acid solution for 15 min, and aniline blue solution for 2 min. The samples were washed under running water before changing the dye solution. Finally, they were rinsed in a 1% acetic acid bath, treated with ethanol for rapid color separation, and permeabilized with xylene. The sections were visualized under a light microscope (Olympus, Japan), and the positive areas were morphometrically analyzed using the ImageJ software (National Institutes of Health, USA).

## IHC Staining

IHC staining for IL-1 $\beta$ , IL-6, TNF- $\alpha$ , TGF- $\beta$ , Collagen-2, SOX9, OCN and RUNX2 at tendon injury sites was conducted using the respective primary antibodies. First, sections for histological staining were deparaffinized and rehydrated. After antigen retrieval, the sections were blocked with 3% bovine serum albumin for 30 min, incubated with corresponding primary antibodies overnight at 4°C, and incubated with an anti-rabbit immunoglobulin G (IgG) secondary antibody for 1 h at 25°C. Relevant information regarding all the primary and secondary antibodies is presented in [Supplementary Table S1](#). The sections were visualized under a light microscope (Olympus). To analyze the expression of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , TGF- $\beta$ , we calculated the average optical density of these indicators. To analyze the expression of Collagen-2, SOX9, OCN and RUNX2, the average immunoreactivity of the protein was calculated as the ratio of the positive antibody-stained area to the total area. The average optical density or positive area in five randomly selected fields was measured using ImageJ software.

## IFC Staining

For IFC staining, rehydration, antigen retrieval, and blocking were performed as described for the IHC staining. Sections were incubated with anti-F4/80 antibody (Cell Signaling Technology, USA; Rabbit, 1:1000) and anti-pSMAD2/3 antibody (BIOSS, China; Rabbit, 1:200) at 4 °C overnight before being treated with CY3-conjugated anti-rabbit IgG secondary antibody (SeraCare, USA; Goat, 1:400) for 1 h at 37 °C. Finally, the nuclei were stained blue with

4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Solarbio, China) for 5 min and observed under a fluorescence microscope (Olympus). The relative expression of F4/80 or pSMAD2/3 was morphometrically analyzed using the ImageJ software.

## Micro-CT Scanning

Nine weeks after surgery, the left hind limbs ( $n=5$ ) of the mice in each group were scanned using a high-resolution micro-CT system (SkyScan 1276; Bruker, Belgium). Scans were conducted using the following settings: 90 kV polychromatic x-ray beam, 180  $\mu$ A current and 15  $\mu$ m resolution. Images were processed using NRecon Reconstruction software (Bruker) to align the scan images and generate the reconstructed 3D images. Total ectopic bone (differential new bone from native bone) volume in the left hind limbs was calculated using micro-CT volumetric software (Bruker).

## Statistical Analysis

All relevant data are presented as mean  $\pm$  standard deviation (SD), and GraphPad Prism 9 was used for data analysis. Comparisons among groups were performed using one-way ANOVA analysis of variance followed by Tukey's post-hoc test. Statistical significance was set at  $P < 0.05$ .

## Results

### Estrogen Deficiency Promoted Ectopic Cartilage Formation

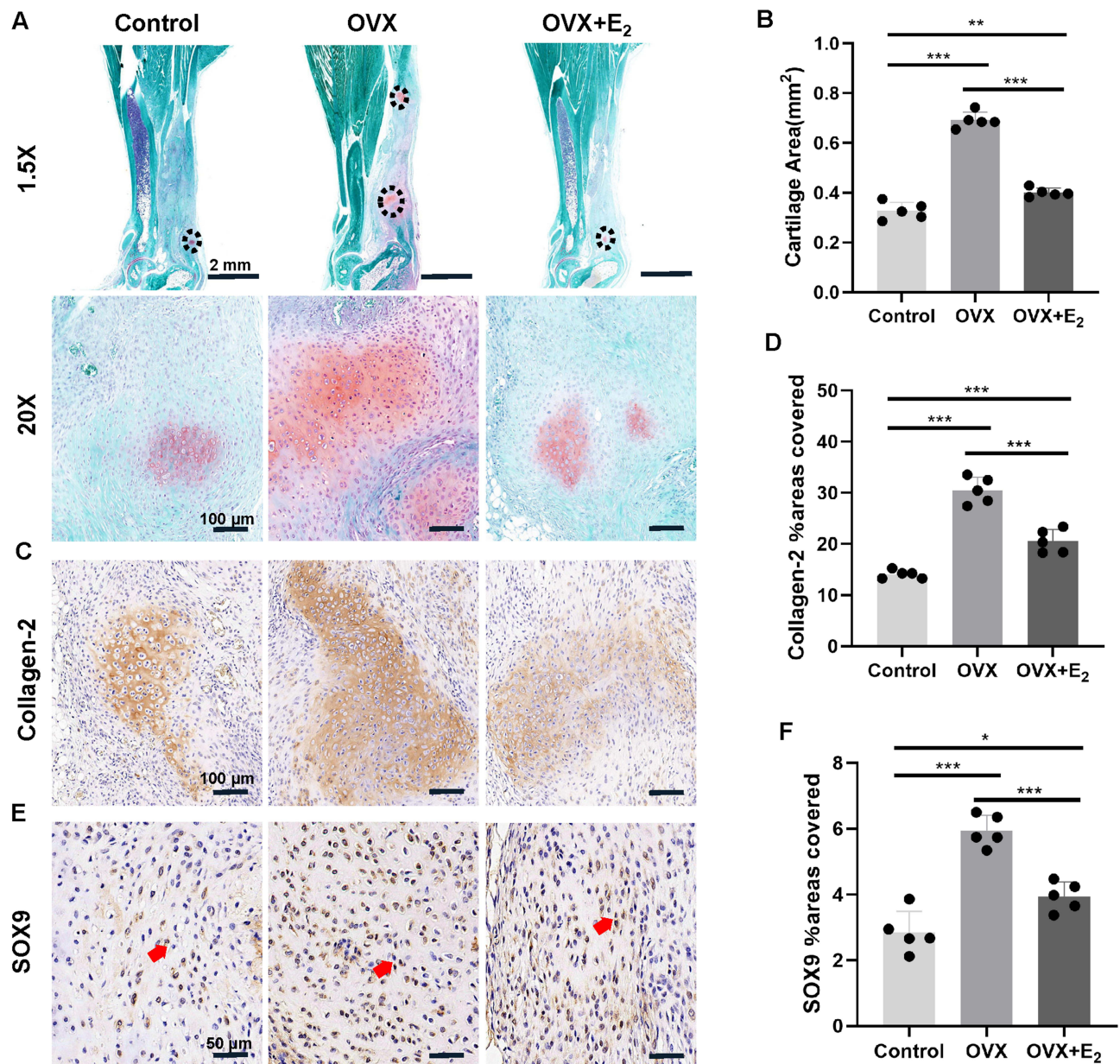
As HO is primarily formed in the context of endochondral ossification, we first performed histological analysis to determine the extent of cartilage deposition at the tenotomy sites. Three weeks after tenotomy, serum  $E_2$  levels decreased significantly after OVX ([Supplementary Figure S1](#)), and SOFG staining identified more ectopic cartilage deposition in OVX mice ( $0.69 \pm 0.03$  mm<sup>2</sup>) than that in the control mice ( $0.33 \pm 0.03$  mm<sup>2</sup>) ([Figure 2A and B](#)). Moreover, IHC staining showed that the relative expression of chondrogenic markers, Collagen-2 ( $30.45 \pm 2.60\%$ ) and SOX9 ( $5.93 \pm 0.48\%$ ), was increased at the tenotomy site in the OVX group relative to the control group ( $14.04 \pm 0.84\%$ ;  $2.85 \pm 0.64\%$ , respectively) ([Figure 2C–F](#)), demonstrating an acceleration of chondrogenesis in the estrogen-deficient state. After subcutaneous injection of  $E_2$ , serum estradiol levels in OVX+ $E_2$  mice significantly increased ([Figure S1](#)).  $E_2$  supplementation also inhibited chondrogenesis compared to the OVX group. Ectopic cartilage area ( $0.40 \pm 0.02$  mm<sup>2</sup>), as well as the percentages of the relative expressions of Collagen-2 ( $20.52 \pm 2.29\%$ ) and SOX9 ( $3.94 \pm 0.44\%$ ) were significantly downregulated at the tenotomy site in the OVX+ $E_2$  group compared with these in the OVX group.

### Estrogen Deficiency Promoted Ectopic Bone Formation

Next, ectopic bone volume at the injured sites was evaluated using micro-CT. At 9 weeks, there was more bone formation at the tenotomy site in the OVX mice ( $1.50 \pm 0.10$  mm<sup>3</sup>) compared to the control mice ( $0.57 \pm 0.03$  mm<sup>3</sup>) ([Figure 3A and B](#)). However, the ectopic bone volume in the OVX+ $E_2$  group ( $0.72 \pm 0.05$  mm<sup>3</sup>) was significantly smaller than that of the OVX group. Masson's trichrome staining of the same samples was used to highlight the ectopic bone matrix, which was consistent with the findings obtained from micro-CT analysis ([Figure 3C](#)). IHC staining also showed increased expression of OCN and RUNX2 at the tenotomy sites in OVX mice relative to control mice ( $9.24 \pm 1.34$  vs  $2.45 \pm 0.30\%$ ;  $6.56 \pm 0.87$  vs  $1.19 \pm 0.26\%$ , respectively) ([Figure 3D–G](#)). In contrast,  $E_2$  supplementation significantly inhibited osteogenesis compared to that in the OVX group. The percentages, the expression of OCN ( $3.96 \pm 0.40\%$ ) and RUNX2 ( $2.42 \pm 0.31\%$ ) was significantly downregulated at the tenotomy sites in the OVX+ $E_2$  group to these in the OVX group. These results demonstrated that estrogen deficiency after OVX increased endochondral bone formation, which could be alleviated by  $E_2$  replacement.

### Estrogen Deficiency Enhanced the Infiltration of Inflammatory Cells in the Early Stage of HO Development

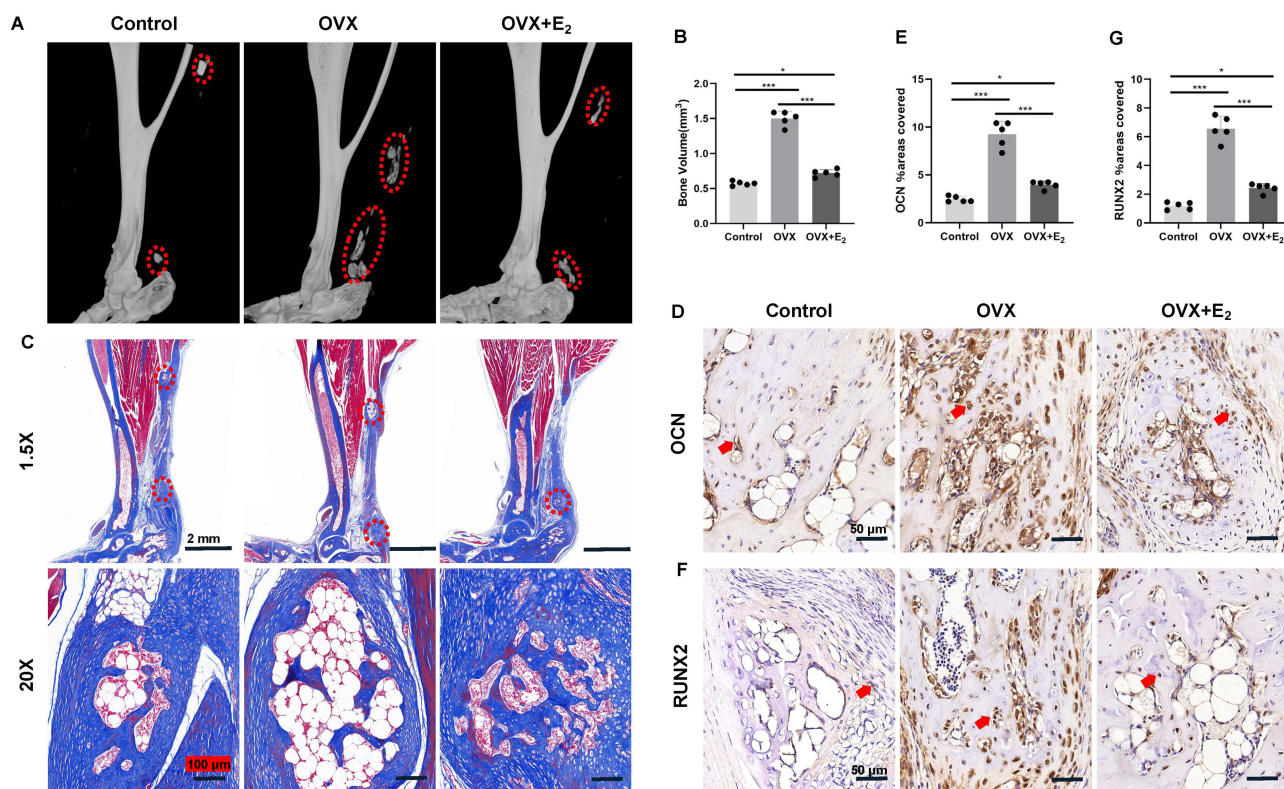
One week after injury, HE staining demonstrated numerous inflammatory cells at the tenotomy sites in the three groups. The results showed that inflammation within the soft tissues of OVX mice was more obvious than that in control mice



**Figure 2** Estrogen deficiency promoted ectopic cartilage formation at 3 weeks after tenotomy. **(A)** SOFG staining for ectopic cartilage (black circle) within the tenotomy sites from control, OVX and OVX+E<sub>2</sub> groups. **(B)** Histomorphometry quantifications of ectopic cartilage areas at the tenotomy sites. **(C)** IHC staining of Collagen-2 at the tenotomy sites. **(D)** Statistical analysis of the relative expression of Collagen-2. **(E)** IHC staining of SOX9 at the tenotomy sites. Arrows indicate positively staining cells for SOX9. **(F)** Statistical analysis of the relative expression of SOX9. \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001.

**Abbreviations:** OVX, ovariectomy; E<sub>2</sub>, 17β-estradiol.

(Figure 4A). However, following E<sub>2</sub> supplementation, the inflammatory response in OVX+E<sub>2</sub> mice was significantly inhibited compared to that in the OVX group. To further characterize the local inflammatory cells, we conducted IFC staining on similar serial sections with F4/80 as a marker of macrophages and toluidine blue staining to reveal mast cells. There was a dramatic increase in F4/80 positive cells within the soft tissues at the tenotomy site of OVX mice (59.74 ± 6.57%) compared to control mice (25.81 ± 2.38%) (Figure 4B and C). Toluidine blue staining also suggested that mast cell number in the OVX mice (112.90 ± 8.50 cells/mm<sup>2</sup>) was significantly upregulated relative to that in the control mice (68.02 ± 7.88 cells/mm<sup>2</sup>) (Figure 4D and E). However, the number of macrophages and mast cells was markedly reduced in samples from the OVX+E<sub>2</sub> group (38.74 ± 4.58%; 72.26 ± 7.61 cells/mm<sup>2</sup>, respectively) compared to samples from the OVX group.



**Figure 3** Estrogen deficiency promoted ectopic bone formation at 9 weeks after tenotomy. **(A)** Micro-CT reconstruction images of ectopic bone formation (red circle) at the tenotomy sites from different groups. **(B)** Micro-CT quantifications of the total ectopic bone (differential new bone from native bone) volume at the tenotomy sites. **(C)** Masson's trichrome staining for ectopic bone (red circle) within the tenotomy sites from control, OVX, and OVX+E<sub>2</sub> groups. **(D)** IHC staining of OCN at the tenotomy sites. Arrows indicate positively staining cells for OCN. **(E)** Statistical analysis of the relative expression of OCN. **(F)** IHC staining of RUNX2 at the tenotomy sites. Arrows indicate positively staining cells for RUNX2. **(G)** Statistical analysis of the relative expression of RUNX2. \* $p < 0.05$ , \*\*\* $p < 0.001$ .

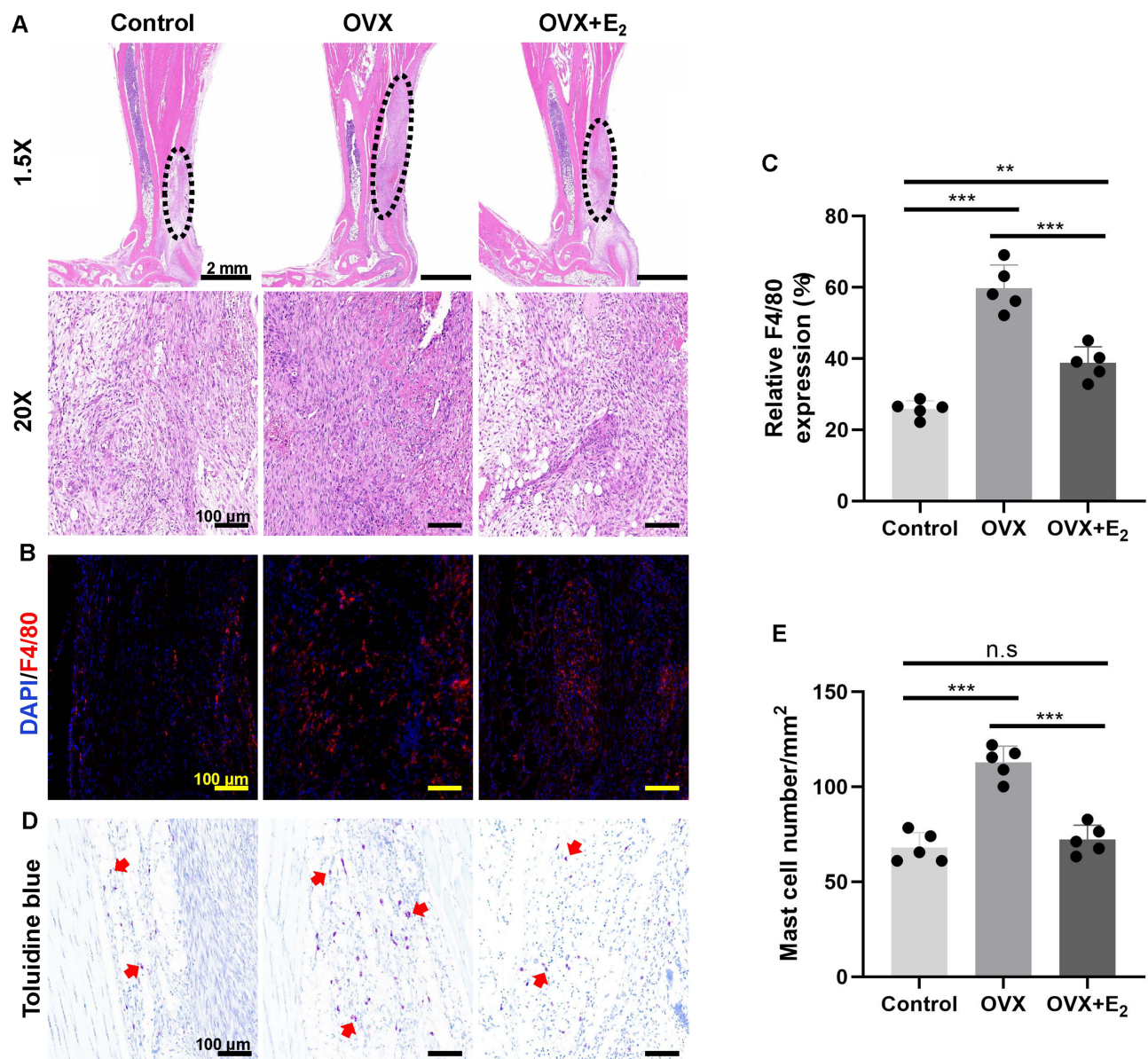
**Abbreviations:** OVX, ovariectomy; E<sub>2</sub>, 17 $\beta$ -estradiol.

## Estrogen Deficiency Increased the Expression of Pro-Inflammatory Mediators in the Early Stage of HO Development

Next, we explored the effect of estrogen deficiency on the expression of pro-inflammatory mediators in injured Achilles tendon tissues. The results of IHC staining indicated that the relative expression levels of IL-1 $\beta$  ( $0.33 \pm 0.07$ ), IL-6 ( $0.13 \pm 0.01$ ) and TNF- $\alpha$  ( $0.30 \pm 0.02$ ) were significantly higher in the OVX group than these in the control group ( $0.16 \pm 0.01$ ;  $0.05 \pm 0.01$ ;  $0.16 \pm 0.01$ , respectively) (Figure 5A–F). However, E<sub>2</sub> supplementation in the OVX+E<sub>2</sub> group decreased the relative expression levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  by 48.0%, 61.3%, and 47.4%, respectively, compared with those in the OVX group.

## Estrogen Deficiency Upregulated the TGF- $\beta$ /SMAD Signaling Pathway in the Early Stage of HO Development

To further elucidate the mechanism by which estrogen deficiency aggravates HO development, changes in the TGF- $\beta$  signaling pathways 1 week after surgery were evaluated. IFC analysis revealed that the expression of TGF- $\beta$  at tenotomy sites increased significantly in the OVX group ( $0.15 \pm 0.01$ ) compared with the control mice ( $0.07 \pm 0.01$ ) (Figure 6A and B). We further examined the expression of downstream pSMAD2/3 molecules in this canonical signaling pathway in the HO anlagen 1 week after injury. There was a dramatic increase in p-SMAD2/3 positive cells within the tenotomy site of OVX mice ( $75.69 \pm 4.93\%$ ) compared with control mice ( $34.97 \pm 1.45\%$ ) (Figure 6C and D). In contrast, the relative expression levels of TGF- $\beta$  ( $0.07 \pm 0.01$ ) and p-SMAD2/3 positive cells ( $36.07 \pm 1.45\%$ ) were significantly downregulated at the tenotomy site in the OVX+E<sub>2</sub> group than these in the OVX group. Taken together, these results indicate that estrogen



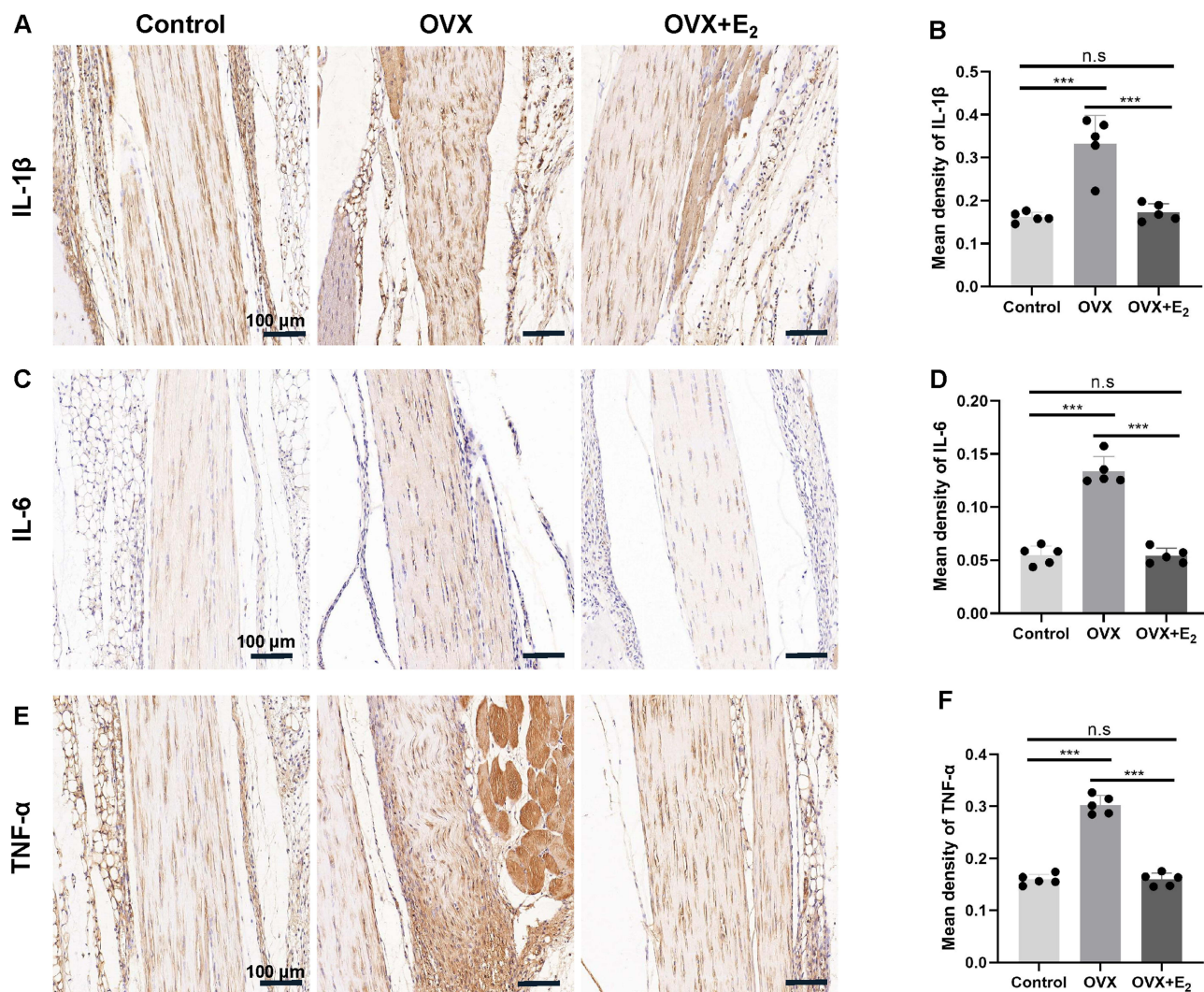
**Figure 4** Estrogen deficiency enhanced the infiltration of inflammatory cells at 1 week after tenotomy. **(A)** HE staining for observation of gross inflammatory response (black circle) at the tenotomy sites. **(B)** IFC staining of F4/80 for observing macrophages at the tenotomy sites. **(C)** Quantification of F4/80 positive cells in IFC staining images. **(D)** Toluidine blue staining for observation of mast cells (red arrows) at the tenotomy sites. **(E)** Quantification of the mast cells in toluidine blue staining images. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

**Abbreviations:** OVX, ovariectomy; E<sub>2</sub>, 17 $\beta$ -estradiol; n.s, non-significant.

deficiency can upregulate the TGF- $\beta$ /SMAD signaling pathway during the early phases of endochondral ossification, thereby accelerating HO.

## Discussion

A clinical study demonstrated an increased incidence of HO formation after primary THA among postmenopausal women in female patients.<sup>11</sup> However, the study was retrospective, which complicates the determination of whether the higher occurrence could be attributed to intrinsic differences in estrogen levels or to the inclusion of a predominantly postmenopausal female cohort that underwent THA surgery. Furthermore, no animal studies have investigated the impact of estrogen deficiency on the production of pathological heterotopic bone after trauma. In this study, we used OVX mice as a model of estrogen deficiency to determine the effect of estrogen on HO following trauma. We demonstrated for the



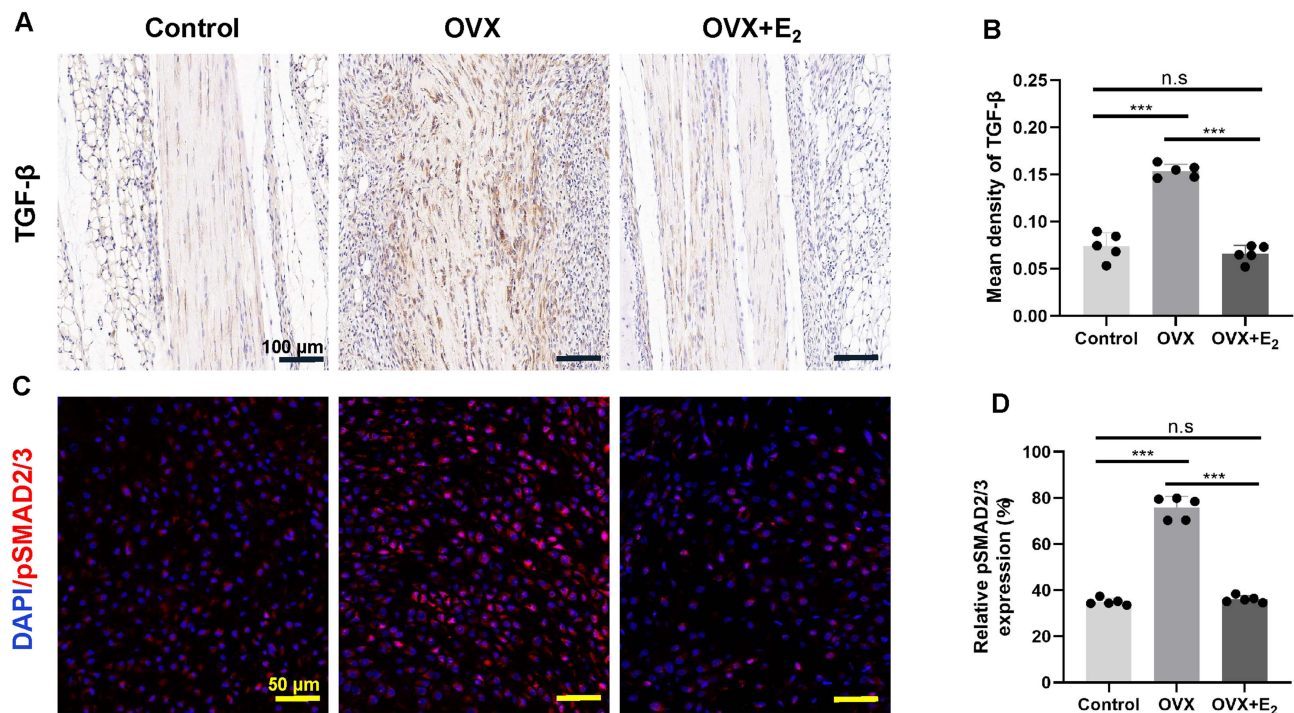
**Figure 5** Estrogen deficiency increased the expression of pro-inflammatory mediators at 1 week after tenotomy. (A, C and E) IHC staining of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  expressions at tenotomy sites. (B, D and F) Statistical analysis of the average optical density of IL-1 $\beta$ , IL-6 and TNF- $\alpha$ . \*\*\* $p$ <0.001. **Abbreviations:** OVX, ovariectomy; E<sub>2</sub>, 17 $\beta$ -estradiol; n.s., non-significant.

first time that estrogen deficiency aggravated HO formation. In addition, we further enriched the issue regarding its role in the tissue inflammation response and TGF- $\beta$ /SMAD signaling during HO progression after tendon injury.

HO is often attributed to abnormal proliferation and osteochondral differentiation after acute inflammatory insult with tissue-resident mesenchymal progenitor cells (TMPCs) located at the injury site.<sup>3,21</sup> In the current study, cartilage deposition and expression levels of SOX9 and Collagen-2 increased in the state of estrogen deficiency. Moreover, we observed greater ectopic bone formation and expression levels of RUNX2 and OCN in OVX mice after tenotomy than in control mice. However, it is well known that postmenopausal estrogen deficiency could reduce MSCs osteogenesis and decrease bone mass, often resulting in osteoporosis.<sup>12</sup> Therefore, we may not be able to explain the interesting phenomenon that estrogen deficiency promotes HO formation from the perspective of the direct effect of estrogen on TMPCs.

Histologically, traumatic HO is believed to develop through a process of endochondral ossification involving four stages: inflammation, chondrogenesis, osteogenesis, and maturation stages.<sup>22</sup> Among them, inflammation as a trigger plays an important role in the formation of HO. The inflammatory response induced by tissue damage is characterized by a series of cellular events, including changes in the inflammatory cells and their secreted cytokines. The increase in inflammatory cytokines recruits TMPCs to local injury sites and induces abnormal chondrogenic and osteogenic





**Figure 6** Estrogen deficiency upregulated the TGF- $\beta$  signaling pathway at 1 week after tenotomy. (A) IHC staining at tenotomy sites. (B) Statistical analysis of the average optical density of TGF- $\beta$ . (C) IFC staining of pSMAD2/3 at tenotomy sites. (D) Quantification of pSMAD2/3 positive cells in IFC staining images. \*\*\* $p < 0.001$ .

**Abbreviations:** OVX, ovariectomy; E<sub>2</sub>, 17 $\beta$ -estradiol; n.s, non-significant.

differentiation, thereby contributing to the pathological formation of the bone and cartilage matrix.<sup>23</sup> It has been reported that estrogen exerts anti-inflammatory effects in various disease models. Reduced estrogen levels exacerbate A $\beta$ -induced memory impairment by enhancing neuroinflammation and amyloidogenesis in ovariectomized mice.<sup>24</sup> Moreover, estrogen is also important in tissue recovery, including muscles and tendons, after traumatic injuries by directly modifying the local inflammatory response.<sup>14–17</sup> As a complication of inflammatory insult after soft tissue injury, HO might also be exacerbated by increased inflammation due to estrogen deficiency.

Abnormal persistent inflammatory activation of macrophages in the post-traumatic immune microenvironment is the most important stage in the pathological process of HO, which promotes abnormal osteogenic differentiation of local stem cells.<sup>25</sup> Macrophages appeared early at 3 days and peaked at 7 days after Achilles tenotomy, which are believed to be particularly relevant to the initiation of the HO process.<sup>26</sup> In this study, we used F4/80 to mark macrophages and found that more macrophages invaded the injured tendon in mice after OVX than these in mice without OVX in the early stage of HO. In contrast, the number of these cells was markedly reduced in specimens from E<sub>2</sub> treated mice versus OVX mice. Previous studies have shown that estrogen reduces inflammatory damage in the skeletal muscle by controlling intramuscular infiltration of monocytes/macrophages,<sup>14</sup> which is in agreement with the anti-inflammatory properties of estrogen during HO development, as revealed by our work. In addition, mast cells can act as primary producers of inflammation and contribute to soft tissue healing and remodeling. Several studies have proposed an emerging notion that neuroinflammation induces mast cell degranulation as a central mechanism in HO formation<sup>27</sup> and inhibition of mast cell degranulation has in fact been found to reduce HO formation in animal models.<sup>28,29</sup> Mast cells are increased in numbers in the bone marrow of postmenopausal osteoporotic patients, but they compromise the healing of bone fractures under estrogen-deficient conditions by releasing inflammatory mediators.<sup>30</sup> Our data first demonstrated that OVX also initiated a more severe mast cell infiltration at the tenotomy site. However, under osteoporosis and HO conditions, OVX-induced mast cell proliferation plays an opposite role, and the specific mechanism needs to be further studied.

Additionally, the excessive paracrine of pro-inflammatory cytokines by macrophages or mast cells after inflammatory destabilization are indispensable in promoting HO formation.<sup>23,26</sup> Estrogens regulate the expression of cytokines

involved in bone cell biology and, in estrogen deficiency, the increased cytokine levels, such as IL-1 $\beta$ , IL-6 and TNF $\alpha$  could affect TMPCs function,<sup>31–33</sup> thereby contributing to the development of HO.<sup>23</sup> Our results showed that the above inflammatory cytokines were elevated in the OVX group while E<sub>2</sub> treatment suppressed their expression levels. These findings suggest that E<sub>2</sub> treatment inhibits the inflammatory response during HO development. In this regard, the observed increase in ectopic cartilage and bone lesions was associated with an elevated inflammatory response in injured tendons of OVX mice. Although estrogen deficiency has been shown to reduce the osteogenic differentiation ability of MSCs,<sup>12</sup> the increased inflammatory response caused by estrogen deficiency may counteract or even reverse this effect and ultimately enhance osteogenic behavior during HO formation.

TGF- $\beta$ , specifically secreted by myeloid cells, was highly expressed in the early stages of HO.<sup>34</sup> In the mouse model for traumatic HO, deletion of macrophage TGF- $\beta$  resulted in decreased HO formation.<sup>35</sup> TGF- $\beta$  could activate SMAD2 and SMAD3 and increase the expression of SOX-9, resulting in the differentiation of MSCs into the cartilage stage.<sup>36</sup> In addition, TGF- $\beta$  also plays an important role in inducing endothelial cells to differentiate into TMPCs before the formation of HO, enhancing the differentiation potential of TMPCs, and initiating their differentiation into osteoblasts.<sup>37</sup> Therefore, we explored the potential mechanism by which estrogen deficiency influences HO formation from the perspective of TGF- $\beta$  signaling pathway. Previous studies have also demonstrated that the binding of estrogen and estrogen receptors regulates TGF- $\beta$ /SMAD signaling by forming a protein complex with SMAD2 and SMAD3 and promoting their degradation.<sup>38</sup> Here we showed that the expression levels of TGF- $\beta$  and its downstream pSMAD2/3 molecules in the tenotomy sites were all increased after OVX. In contrast, elevated TGF- $\beta$  signaling can be reversed by estrogen replacement therapy. Thus, estrogen deficiency could also disturb protein expression in the TGF- $\beta$ /SMAD signaling pathway, resulting in enhanced chondrogenesis and osteogenesis, thereby accelerating HO.

Our study has some limitations. First, OVX not only causes estrogen deficiency, but also leads to changes in testosterone levels.<sup>6</sup> Therefore, it is necessary to use female estrogen receptor knockout mice in the future to further clarify the role of estrogen deficiency in HO development. Second, estrogen deficiency may affect not only one signaling pathway, but also other related pathways involved in HO formation, such as mTOR signaling,<sup>39</sup> which has not been investigated. Thirdly, there are still many physiological differences between mice and humans. At present, we have only revealed that estrogen deficiency promotes the formation of heterotopic ossification in mice by aggravating local inflammatory response, and this conclusion needs to be verified in large animals and human clinical specimens in the future. Finally, it is unclear whether estrogen replacement therapy has an inhibitory effect on HO formation in healthy females. Therefore, future research is warranted to investigate these problems and develop precise strategies to inhibit or limit this debilitating condition in postmenopausal women.

## Conclusion

In this study, we demonstrated that an estrogen-deficient state in a female mouse model exacerbated the development of HO. These findings could be attributed to the disturbance of inflammatory response mechanisms and the activation of TGF- $\beta$ /SMAD signaling. In addition, estrogen replacement therapy can protect against OVX-induced HO deterioration. By revealing the role of estrogen deficiency in the development of HO, we can better understand the pathophysiology of HO and develop targeted pharmacological interventions to improve HO prophylaxis and treatment for the growing population of menopausal patients.

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## Disclosure

The authors report no conflicts of interest in this work.

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