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Sex differences in the estrogen-dependent regulation of temporomandibular joint remodeling in altered loading

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SUMMARY

Objective: Temporomandibular joint (TMJ) diseases predominantly afflict women, suggesting a role of estrogen in the disease etiology. Previously, we determined that decreased occlusal loading (DOL) inhibited collagen type II (Col2) expression in the mandibular condylar cartilage (MCC) of female wild-type (WT) mice whereas no change was observed in males. This decrease in chondrogenesis was abolished by estrogen receptor beta (ERβ) deficiency in females. Therefore, the goal of this study was to examine the role of estradiol − ERβ signaling in mediating DOL effects in male mice to further decipher sex differences.

Methods: Male 21 day-old WT and ERβKO male mice were treated with either placebo or estradiol and exposed to normal or DOL for 4 weeks. Cartilage thickness and cell proliferation, gene expression and immunohistochemistry of chondrogenic markers and estrogen receptor alpha (ERα), and analysis of bone histomorphometry via microCT were completed to ascertain the effect of estradiol on DOL effects to the TMJ.

Results: ERβKO male mice lack a MCC phenotype. In both genotypes, estradiol treatment increased Col2 gene expression and trabecular thickness. DOL in combination with estradiol treatment caused a significant increase in Col2 gene expression in both genotypes.

Conclusions: The sex differences in DOL-induced inhibition of Col2 expression do not appear to be mediated by differences in estradiol levels between male and female mice. Greater understanding on the role of estrogen and altered loading are critical in order to decipher the sex dimorphism of TMJ disorders.

Introduction

Temporomandibular joint (TMJ) pain afflicts approximately 10% of the United States population with roughly half of this pain associated with joint degenerative disease (TMJ-DD)[1,2]. It has been postulated that excessive TMJ remodeling in response to altered mechanical loading is a major factor in the development of TMJ-DD[3,4]. Further, women who engage in increased oral parafunctional behavior are the population most subject to developing TMJ-DD[5–8]. These clinical statistics suggest the role of estrogen and mechanical loading-induced TMJ remodeling in the disease process.

Unlike most hyaline articular cartilages of the appendicular joints, the mandibular condylar cartilage (MCC) is derived from periosteal tissue and comprised of fibrocartilage[9,10]. In response to altered loading, the MCC is known to regulate the size and properties of the tissue to adapt to the change in load[11–17]. This response is mediated by the osteochondral progenitor cells located within the proliferative zone of the cartilage[18–20]. These cells are typically quiescent and are activated to undergo proliferation. Afterwards, they either continue to proliferate or proceed through cell cycle arrest to then differentiate into chondrocytes which express

Abbreviations: TMJ, Temporomandibular joint; ERβKO, Estrogen receptor beta knock-out; ERα, Estrogen receptor alpha; ERβ, Estrogen receptor beta; MCC, Mandibular condylar cartilage; DOL, Decreased occlusal loading; NL, Normal Load; Plb, Placebo; Esd, Estradiol; Col2, Collagen type II; Col10, Collagen type X; Ihh, Indian hedgehog; PTHrP, Parathyroid hormone-related protein; Sox9, SRY-box containing gene 9; H&E, Hematoxylin and eosin.

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collagen type II and aggrecan[18,21]. This process is further regulated by estrogen signaling[22–24] which predominantly occurs through two receptor isoforms, estrogen receptor alpha (ERα) and beta (ERβ). Ligand binding of estrogen to either receptor induces a conformational change, receptor dimerization, nuclear translocation, and target gene transcription. Elucidating the combined role of altered loading and estradiol to the TMJ and deciphering sex differences that mediate these responses is vital for developing treatments to combat accelerated alterations to the joint.

In the TMJ, we have optimized a decreased occlusal loading (DOL) mouse model that consists of incisor trimming and soft diet administration[25]. Utilizing this model, we have observed a decrease in MCC thickness in both male and female mice in response to this underloading treatment[25–27]. Surprisingly, DOL reduced collagen type II (Col2) expression only in females, suggesting sex differences in the role estradiol plays in load altered load-induced MCC chondrogenesis[25,26]. Estrogen-mediated sex differences have been previously observed in periosteal-derived tissue. Male and female mice deficient in ERα express a characteristic skeletal phenotype[28,29]. In contrast, only female mice deficient in ERβ display a unique skeletal phenotype[29–32]. In bone, female mice deficient in ERβ experience an increase in mechanical loading-induced periosteal bone formation suggesting this receptor may inhibit altered load-induced remodeling[33]. However, the role of estradiol on the MCC of male mice in decreased loading conditions is unknown.

In the female MCC, we have previously determined that DOL inhibition of Col2 expression was abolished by ERβ deficiency suggesting that estradiol inhibition of TMJ chondrogenesis in this altered loading state is mediated by ERβ signaling[27]. Therefore, the major goal of this study was to examine whether the sex differences in DOL-induced inhibition of Col2 expression are controlled by an estradiol-ERβ signaling pathway utilizing male mice. In order to examine this, 21 day-old male WT and ERβKO mice were treated with placebo or estradiol and exposed to either normal or DOL for 4 weeks. We hypothesized that estradiol treatment would result in a DOL-induced inhibition of Col2 expression in male WT mice but not ERβKO mice. As such, cartilage thickness, cell proliferation, gene expression of chondrogenic markers and estrogen receptors, and microCT analysis of bone architecture were completed. Determining a mechanism by which estradiol mediates altered loading-induced TMJ remodeling is critical in order to decipher the sex dimorphism of the disease.

Materials and methods

Mice

All experiments were performed in accordance with animal welfare based on an approved Institutional Animal Care and Use Committee (IACUC) protocol (#AAAH9166) from Columbia University. Breeding pairs of C57BL/6 WT (Cat# 000664) and ERβKO mice (homozygous male, heterozygous female, Cat# 004745) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Twenty-one day-old WT and ERβKO male mice were divided into four groups ($n = 12$ for each group: $n = 6$ for histology/microCT and $n = 6$ for mRNA): placebo (Plb)/normal load (NL), placebo (Plb)/DOL, estradiol (Esd)/NL, and estradiol (Esd)/DOL (Table I). This age was chosen for two reasons: (1) eruption of molars and occlusion are complete at this age[34] (2) majority of MCC growth in mice is completed by 60 days of age[35]. Mice were administered placebo or 17β-estradiol (60 day release, Innovative Research of America, FL) for 28 days at a daily dose of 10 ng/g body weight. The dose of estradiol utilized in this study matches the dose found effective to restore MCC thickness in female, ovariectomized WT mice of the same age[36]. Half of the mice were subjected to DOL involving mandibular incisor trimming and a soft-dough diet (Transgenic Dough Diet; Bioserv, Frenchtown, NJ) as detailed previously[26]. The NL groups received a hard-dough diet. Following 4 weeks of treatment, mice were injected intraperitoneally with 0.1 mg bromodeoxyuridine (BrdU) per gram body weight at 3 and 19 h prior to euthanasia to track proliferating cells.

Table I
Groups and sample sizes investigated in this study

Group	Genotype	Estradiol	Loading condition	Sample size (n)
1	WT	Placebo	NL	6 (Histology); 6 (PCR and μCT)
2	WT	Estradiol	NL	6 (Histology); 6 (PCR and μCT)
3	WT	Placebo	DOL	6 (Histology); 6 (PCR and μCT)
4	WT	Estradiol	DOL	6 (Histology); 6 (PCR and μCT)
5	ERβKO	Placebo	NL	6 (Histology); 6 (PCR and μCT)
6	ERβKO	Estradiol	NL	6 (Histology); 6 (PCR and μCT)
7	ERβKO	Placebo	DOL	6 (Histology); 6 (PCR and μCT)
8	ERβKO	Estradiol	DOL	6 (Histology); 6 (PCR and μCT)

Histomorphometry and immunohistochemistry

Histomorphometry and immunohistochemistry techniques were employed to determine the effect of estradiol and DOL treatment on condylar cartilage cellular and matrix changes in male WT and ERβKO mice. Whole mouse heads were sectioned into halves, fixed in 10% formalin for 4 days at room temperature and decalcified in 14% ethylenediaminetetraacetic acid (EDTA) (pH 7.1) (Sigma, St. Louis, MO, USA) for 28 days. Subsequently, the samples were processed through progressive concentrations of ethanol, cleared in xylene, and embedded in paraffin. Sagittal serial sections of 5 μm thickness were made of the TMJ utilizing a Microm HM 355s microtome (Thermo Fisher Scientific, Waltham, MA, USA). Sections representing the mid-coronal portion of the mandibular head were stained with hematoxylin and eosin (H&E) and Safranin-O (SafO) and used as the representative central section for analysis.

Histomorphometry measurements were made in a blinded, nonbiased manner using the BioQuant computerized image analysis system (BioQuant, Nashville, TN, USA). MCC analysis was performed on H&E sagittal sections corresponding to the mid-coronal portion of the mandibular condylar head. Average thicknesses were determined for each region and summed for the total cartilage thickness. Condyles from six mice within each group were analyzed and the average of three-five sections was taken for each sample.

For immunohistochemistry, tissue sections were deparaffinized with xylene and rehydrated in progressive ethanol/water solutions with increasing concentrations of deionized water. Following rehydration, the sections were digested for 10 min with pepsin for unmasking (Lab Vision, Fremont, CA, USA), washed with PBS, and treated with a 3 vol% hydrogen peroxide in methanol solution to inhibit endogenous peroxidase activity. All sections were blocked with 10% normal goat serum (Life Technologies) to reduce nonspecific binding of the antigen with the primary antibody. Immunohistochemical staining was performed using the SuperPicture™ Polymer horseradish peroxidase (HRP) Broad Spectrum Detection Kit (Life Technologies) following the procedure recommended by the manufacturer. Collagen type II (Millipore; MAB8887, 1:100 dilution), ERα (Abcam; ab75635, 1:100 dilution) and androgen receptor (Abcam, ab74272, 1:100 dilution) primary antibodies were utilized in this study. After incubation with the primary antibodies (Col2: 60 min at RT; ERα and androgen receptor: overnight at 4°C), sections were washed twice in PBS. The secondary antibody – HRP conjugate (SuperPicture™, Life Technologies) was added and incubated for 10 min at RT. Following 2X PBS wash, sections were

stained with DAB chromogen (30 μL DAB in 1 mL diluent, ImmPACT DAB, Vector Laboratories) for 2 min. Sections were then counterstained with either hematoxylin (Col2 staining) or 0.2% Fast Green (ERα and androgen receptor staining) for 30 s, dehydrated, and mounted.

BrdU immunohistochemical analysis to determine proliferating cells was completed using a BrdU staining kit following the manufacturer's instructions (Zymed Laboratories-Invitrogen Corporation, Carlsbad, CA, USA). To quantify BrdU, the labeling index (number of BrdU positive cells divided by the total number of cells) was calculated. Three to six sections, corresponding to the same anatomical region utilized to determine total cell number (mid-coronal), were counted for each group and the average index of these sections was used for the labeling index.

mRNA Extraction and PCR amplification

After duration of treatment, mRNA from the condylar cartilage of all groups was extracted to analyze the effects of estradiol and DOL on the expression of chondrocyte markers and ERα. For each mouse, the MCC (left and right) was carefully isolated from all other soft tissue and dissected under a dissecting microscope. mRNA was extracted with TRIzol Reagent (Ambion by Life Technologies) following the manufacturer's protocol and treated with DNase treatment and removal kit (Ambion by Life Technologies). Reverse transcription was performed to convert mRNA to cDNA utilizing the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time polymerase chain reaction (RT-PCR) was conducted to assess the relative levels of genes of interest using the ViiA™ 7 Real-Time PCR System (Applied Biosystems, Life Technologies) following the protocol detailed in Chen *et al.*[37]. Expression of each gene of interest was determined relative to the Gapdh housekeeping gene (Gapdh – MM99999915_g1) utilizing the $\Delta\Delta C_T$ method. Gene expression was analyzed for the following chondrocyte markers: parathyroid hormone-related peptide (*PTHrP* – Mm00436057_m1), SRY-box containing gene 9 (*Sox9* – MM00448840_m1), collagen type II (*Col 2a1* – Mm00491889_m1), collagen type X (*Col 10a1* – Mm00487041_m1), sclerostin (*Sost* – Mm00470479_m1), indian hedgehog (*Ihh* – Mm00439613_m1), estrogen receptor α (*Esr1* – Mm00433149_m1), and estrogen receptor β (*Esr2* – *Mm00599819)*. All primers were purchased from Applied Biosystems.

Micro-CT analysis

The effect of estradiol and DOL on WT and ERβKO male mice subchondral bone architecture was accessed utilizing cone-beam micro-focus X-ray computed tomography (μCT40, Scanco Medical AG, Bassersdorf, Switzerland) as previously described[26]. Briefly, serial tomographic images from six mice per group were acquired at 55 kV and 145 μA and 2000 projections per rotation were collected at 300 ms integration time. Three-dimensional 16-bit grayscale images were reconstructed with standard convolution back-projection algorithms with Shepp and Logan filtering. Bone was segmented from marrow and surrounding soft tissue including cartilage using a constrained Gaussian filter to reduce noise at a threshold of 480 mg/cm^3. Volumetric regions were selected to include the mandibular condyle. Bone volume fraction, trabecular number, trabecular thickness, and trabecular spacing were determined.

Statistical analysis

Data points, averages, and confidence intervals are presented in the scatter plots. For histomorphometry and BrdU analysis, each data point represents the average value for each mouse sample obtained from 3 to 6 histological sections. Confidence intervals were determined for the data sets using a t-distribution for sample sizes smaller than 30. *T* values were determined for a 95% confidence with degrees of freedom dependent on sample size (e.g., the value for 5 degrees of freedom for a sample size of six for a 95% confidence interval is 2.571). The standard deviation divided by the square root of the sample size was multiplied by the *t* value. This value was then added and subtracted from the sample means to determine the upper and lower bounds for the confidence interval. Normal distribution of the data was confirmed via the Shapiro-Wilk test using SPSS. Significant outliers were removed using Tukey's outlier method which was only necessary for the BrdU data. Specifically, the interquartile range was multiplied by 2.2 and subtracted from the lower quartile and added to the upper quartile. Observations were removed if they fell below or above these determined values. Statistical significance of differences among means was determined by a Student's *t*-test when comparing one factor (genotype, estradiol, or DOL alone) and two-way analysis of variance (ANOVA) with *post hoc* analysis by the Bonferonni method using SPSS when determining the combined effect of estradiol and DOL. The combined effects of estradiol and DOL were determined by significance between NL + Esd vs DOL + Esd and DOL + Plb vs DOL + Esd. Statistical significance was defined as $P < 0.05$ and the specific *P* values are denoted in the respective plots.

Results

Experimental groups and sample sizes utilized for this study are detailed in Table I. Estradiol treatment resulted in a significant decrease in body weight in both WT (WT Plb = 21.8 ± 1.3 vs WT Esd = 19.7 ± 1.6) and ERβKO (KO Plb = 21.7 ± 1.4 vs KO Esd = 19.2 ± 1.6) mice, an effect which has been shown previously[38]. However, DOL treatment had no significant effect on male mice body weight in either genotype.

ERβKO male mice do not exhibit a MCC or subchondral bone phenotype

There was no significant difference in cartilage morphology [Fig. 1(A) and (B)] or cartilage thicknesses as determined by histomorphometry [Fig. 1(C)] between male WT and ERβKO mice. Gene expression and immunohistochemical staining of Col2 and bone morphology as determined by μCT also revealed no statistically significant differences comparing the WT and ERβKO mice (Supplementary Fig. 1).

Estradiol treatment results in anabolic changes in the MCC and subchondral bone

Estradiol treatment did not result in any significant change in cartilage thickness in 49 day-old male WT and ERβKO mice [Fig. 2(A) and (C)]. However, estradiol treatment increased the production and localization of Col2 in both genotypes [Fig. 2(B) and (D)]. In the subchondral bone, estradiol treatment did not result in a significant change in bone volume fraction [Fig. 2(E)] but did result in an increase in trabecular number and trabecular thickness and a decrease in trabecular spacing in WT mice [Fig. 2(F)–(H)]. An increase in trabecular thickness was observed with estradiol treatment in ERβKO mice [Fig. 2(G)]. Overall, estradiol promotes remodeling of both the MCC and subchondral bone in WT and ERβKO male mice.

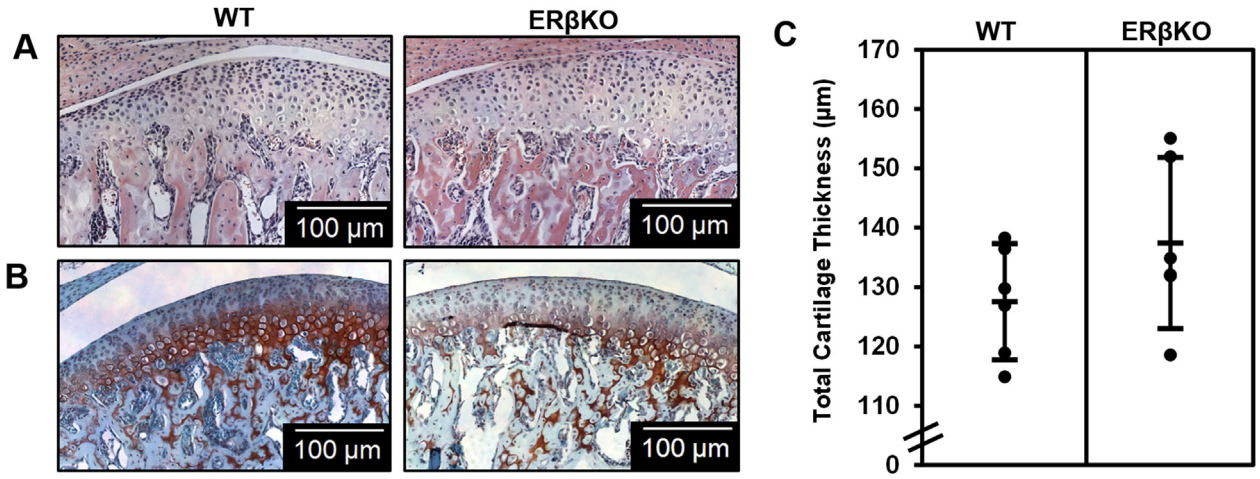

Fig. 1. ERβ MCC phenotype in male mice. Representative hematoxylin & eosin (H&E) images (A), Safranin-O images (B), and histomorphometric cartilage thicknesses (C) for 49-day WT and ERβKO male mice treated with placebo and subjected to NL. For histomorphometric analysis, $n = 6$ mice were utilized from each group and the average of 3–6 sections/mouse was analyzed. Statistical significance was determined by a Student's t-test and $P = 0.18$.

Estradiol promotes chondrogenesis in response to DOL independent of ERβ

While DOL resulted in a decrease in cartilage thickness for both genotypes, estradiol administered during this treatment partly inhibited the DOL-induced reduction in cartilage thickness [Fig. 3(A) and (B)]. Figure 3(C) and (D) illustrate the negligible effect of estradiol and DOL on cell proliferation for both genotypes. Figure 4(A) illustrates an increase in Col2 production and localization when estradiol is administered during DOL compared to DOL alone. A similar effect was observed with glycosaminoglycan staining with safranin O in Fig. 4(B). Gene expression analysis revealed that estradiol and DOL caused a significant increase in Col2 [Fig. 4(C)], Sox9 [Fig. 4(D)], and Sost [Fig. 4(E)] expression in both WT and ERβKO mice. In WT mice, Ihh expression [Fig. 4(F)] was significantly increased whereas Pthrp expression [Fig. 4(G)] was significantly increased in ERβKO. No significant change in Col10 expression was observed (data not shown). In the subchondral bone, estradiol and DOL resulted in an increase in bone volume fraction in both genotypes [Fig. 5(A)]. This effect is likely attributed to the increase in trabecular number [Fig. 5(B)] and/or trabecular thickness [Fig. 5(C)] with a resulting decrease in trabecular spacing [Fig. 5(D)].

Sex steroid levels in response to DOL and estradiol treatment

Estradiol treatment did not significantly affect ERα expression for either genotype. DOL resulted in a decrease in ERα expression in WT mice which was not changed when estradiol was administered as seen by immunohistochemistry [Fig. 6(A)] and gene expression analysis [Fig. 6(B)]. Neither DOL nor estradiol treatment significantly affected the gene expression of ERβ in the WT groups as seen in Fig. 6(C). However, estradiol treatment did increase the localization of androgen receptor in the MCC in the WT mice but not the ERβKO mice as shown in Supplementary Fig. 2.

Discussion

The major goal of this work was to examine whether the sex differences in DOL-induced inhibition of chondrocyte maturation were mediated by an estradiol-ERβ signaling pathway. Overall, two major findings resulted from this study. First, male mice lack an ERβ mandibular condylar phenotype indicating estrogen-induced TMJ growth and remodeling are not directly mediated by ERβ in male mice. Second, the sex differences in DOL-inhibition of chondrocyte maturation, specifically decreased Col2 gene expression and protein production seen in females but not males, are not mitigated by supraphysiological estradiol levels.

ERβ has no significant effect on mandibular condylar phenotype or remodeling in male mice

In 49 day-old male mice, no statistically significant differences were observed in any cartilage or subchondral bone measurements of the WT mandibular condyle compared to ERβKO mice. In addition, we found no significant change in the response to DOL in placebo treated WT mice compared to placebo treated ERβKO mice. This is in contrast to 49 day-old female mice, in which we found that ERβKO mice exhibited increased cartilage thickness, increased chondrocyte maturation gene expression, and increased total volume in the subchondral bone compared to age-matched WT controls[36]. We also found that DOL-induced inhibition of Col2 expression that occurs in WT female mice did not occur in female ERβKO mice[27]. These combined results are consistent with previous findings that illustrate an ERβ phenotype solely in the cortical bone, trabecular bone, and growth plate cartilage of female mice[29,32,39]. These results highlight the fundamental differences in the role of ERβ in mediating TMJ growth and remodeling in males vs females.

Effects of estradiol on the mandibular condyle in male mice

Multiple studies have illustrated the effect of estradiol on MCC metabolism in females[22,23,40–42]. However, the role of estradiol supplementation in male mice is unclear. Estradiol treatment resulted in differential effects to the MCC thickness and proliferation in male mice compared to effects previously observed in female mice. In females, it has been shown that estrogen treatment causes a decrease in MCC thickness in ovariectomized WT mice but not in ERβKO mice[23,37]. Further, ovariectomy results in increased proliferation in female WT but not ERβKO mice[37,42]. In this study, we found that estrogen treatment did not significantly affect the MCC thickness or proliferation in male WT or ERβKO mice. One reason for the observed sex differences may be that estradiol treatment was conducted in gonad-intact mice in this study,

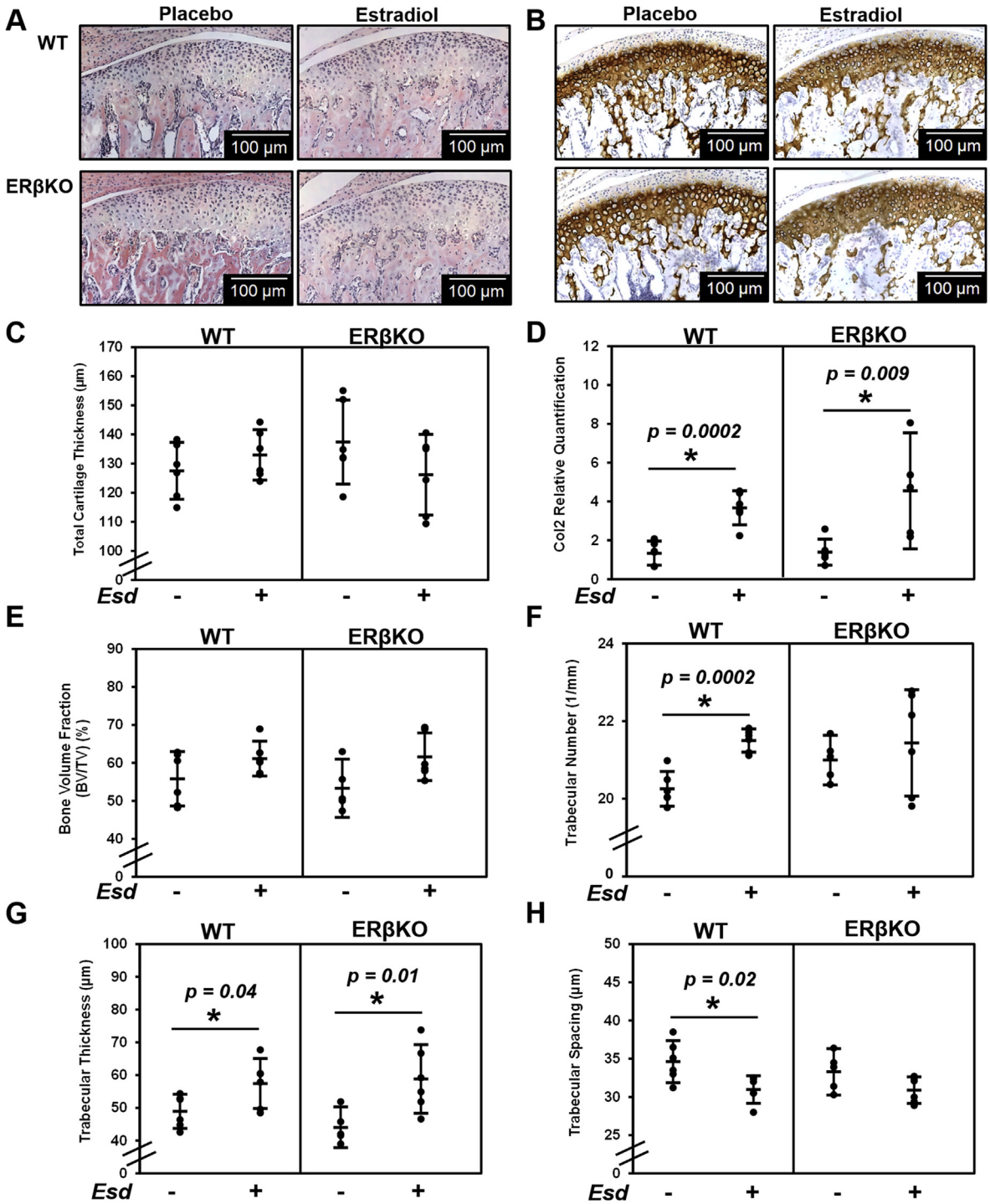

Fig. 2. Effect of estradiol on cartilage thickness, Col2 expression and production, and bone architecture. The data represent WT and ERβKO mice under NL with either placebo or estradiol treatment. Representative hematoxylin & eosin (H&E) images (A), Col2 immunohistochemical staining (B), cartilage thickness as determined by histomorphometry (C), Col2 gene expression (D), bone volume fraction (E), trabecular number (F), trabecular thickness (G), and trabecular spacing (H) are shown. For histomorphometric analysis, $n = 6$ mice were utilized for all groups and the average of 3–6 sections/mouse was analyzed. For gene expression, $n = 6$ mice were utilized for all groups and MCC from left and right were pooled together. Statistical significance was determined by a Student's *t*-test and $P < 0.05$.

whereas estradiol treatment was performed in ovariectomized female mice in our previous studies. However, Figueroba *et al.* showed that estradiol treatment in gonadectomized male rats did not drastically effect MCC thickness compared to the changes observed in female rats supporting the findings from this study[43]. Therefore taken together, the results suggest that estrogen via ERβ inhibits proliferation and subsequent thinning of the MCC in female but not in male WT mice.

On the other hand, estradiol treatment promoted chondrogenesis in male mice. In this study, we found that similar to female mice, estradiol caused a significant increase in Col2 gene expression and protein production in both genotypes[37]. Estradiol is known to

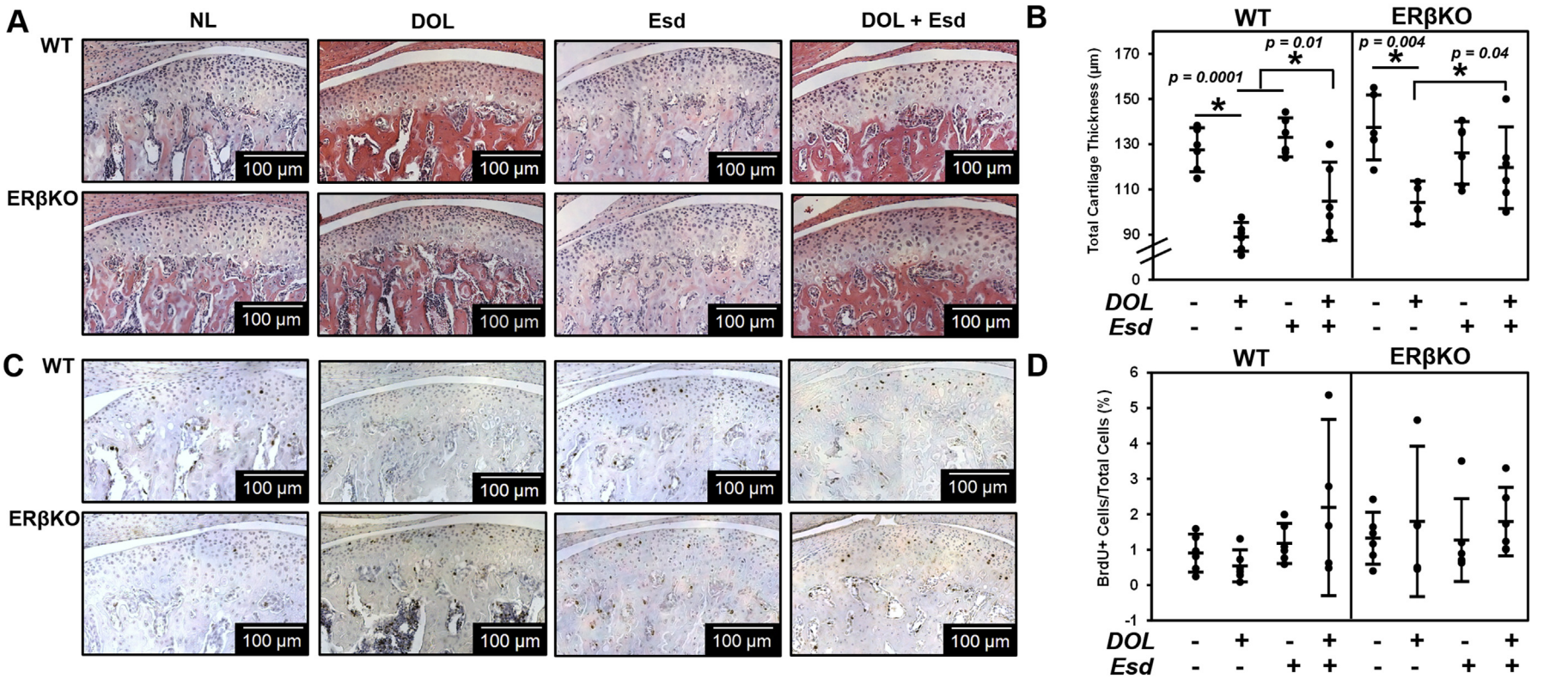

Fig. 3. Effect of estradiol and DOL on cartilage thickness and cell proliferation. The data represent WT and ERβKO mice under NL or DOL with either placebo (Plb) or estradiol (Esd) treatment. Specifically, the labels indicate the following: NL = normal load and placebo; DOL = decreased occlusal loading and placebo; Esd = normal load and estradiol; DOL + Esd = decreased occlusal loading and estradiol. Representative hematoxylin & eosin (H&E) images (A), cartilage thickness as determined by histomorphometry (B), BrdU proliferative immunohistochemical staining (C), and quantified percentage of BrdU + cells (D) are shown. For histomorphometric and BrdU analysis, $n = 6$ mice were utilized for all groups and the average of 3–6 sections/mouse was analyzed. BrdU + cells were normalized to the total number of cells in the cartilage region as determined from the adjacent H&E section. Statistical significance was determined by a two-way ANOVA followed by posthoc analysis with the Bonferonni method with $P < 0.05$. Exact P values are listed above the bars that denote significance.

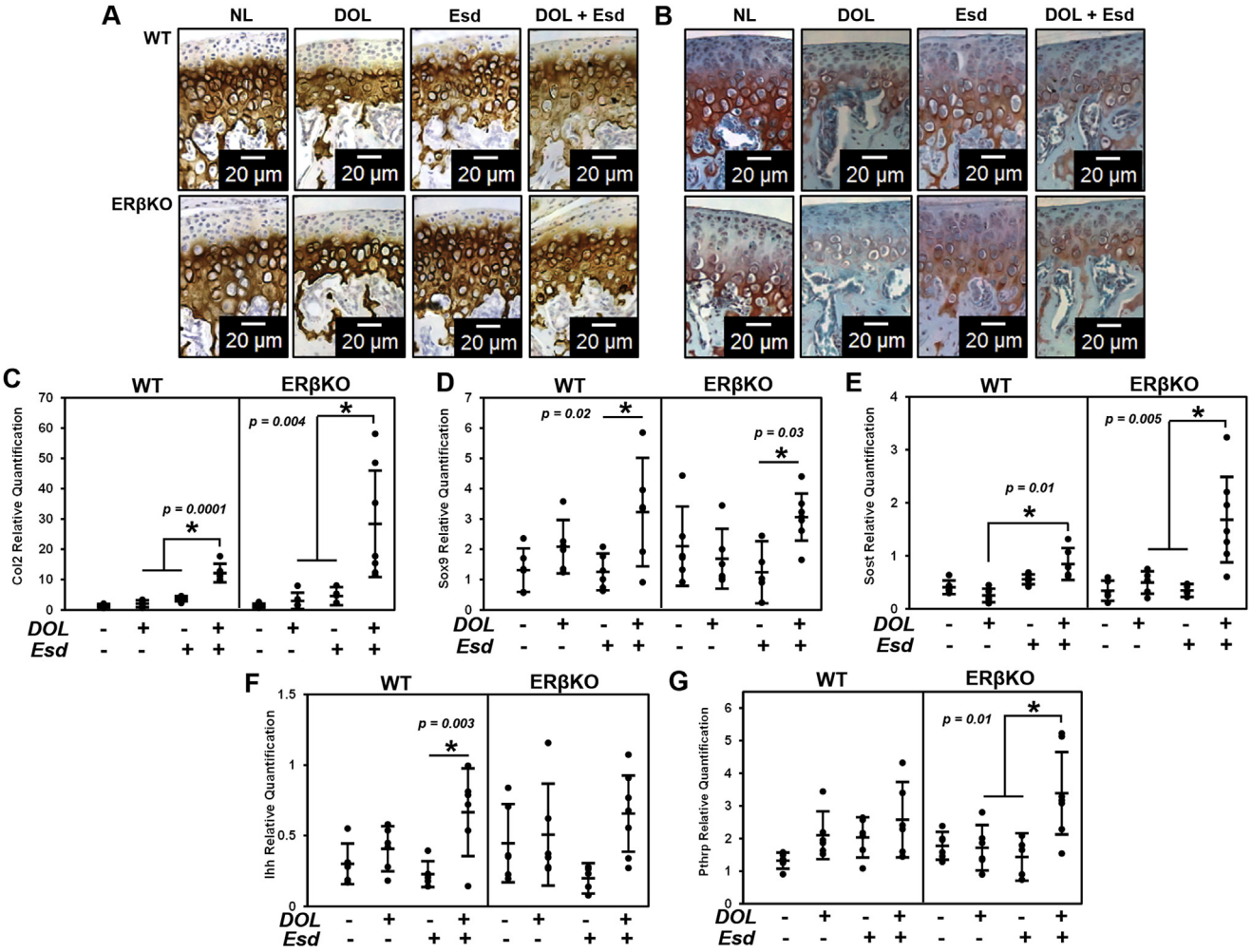

Fig. 4. Effect of estradiol and DOL on chondrogenic markers. The data represent WT and ERβKO mice under NL or DOL with either placebo or estradiol treatment. Specifically, the labels indicate the following: NL = normal load and placebo; DOL = decreased occlusal loading and placebo; Esd = normal load and estradiol; DOL + Esd = decreased occlusal loading and estradiol. Representative Col2 immunohistochemical images (A), representative safranin O images (B) and gene expression of Col2 (C), Sox9 (D), Pthrp (E) and Ihh (F) and Sost (G) are shown. For gene expression, $n = 6$ mice were utilized for all groups and MCC from left and right were pooled together. Statistical significance was determined by a two-way ANOVA followed by posthoc analysis with the Bonferonni method with $P < 0.05$. Exact P values are listed above the bars that denote significance.

promote chondrogenesis in hyaline cartilage and delay experimental arthritis[44], emphasizing the anabolic effects of estrogen observed in this study[45]. Specifically in males, estradiol has been shown to increase bone mineral density and exhibit a bone sparing effect[46–49] and promote epiphyseal closure[50]. These results strongly suggest that estrogen promotes TMJ chondrogenesis independent of ERβ in both sexes.

While a few studies have looked at the role of estrogen in mediating the microarchitecture of the mandibular condylar subchondral bone in females, not much is known in male mice. In this study, we found that estrogen caused an increase in trabecular thickness independent of ERβ. In females, estrogen deficiency induced bone loss as measured by trabecular thickness and bone volume fraction in the mandibular subchondral bone of female rats[51]. Further, studies investigating the role of genistein, a phytoestrogen that structurally resembles 17β-estradiol, illustrated an increase in trabecular thickness and bone volume fraction dependent on ERβ in female rats[52]. Thus, estradiol promotes anabolic changes in the mandibular condylar subchondral bone of both sexes; however, the increase in trabecular thickness may only depend on ERβ in females.

Sex differences in the effects of estradiol and DOL on the TMJ

We previously found that DOL caused inhibition of Sox9 and Col2 expression in female mice only[26] and these effects were abolished by ERβ deficiency[27]. Thus, we hypothesized that estradiol treatment to DOL male mice would cause inhibition of Col2 expression in WT but not in ERβKO mice. However, we observed the opposite effect. Estradiol treatment with DOL resulted in an increase in Col2 and Sox9 expression and a decrease in Sost expression in both genotypes. These results suggest that the sex differences in response to DOL are not mediated by differences in estradiol levels between male and female mice.

Sex differences in the estrogen receptor expression levels and/or the role of these receptors in mediating mechanical loading-induced TMJ remodeling may exist. For example, in the rat condylar cartilage, the number of cells expressing ERα and ERβ were significantly larger in males compared to females from through 12 months[53]. It has previously been shown that estrogen deficiency alone or in combination with decreased loading increases ERα expression in female rats[40]. While we did observe a decrease in ERα expression with DOL in WT male mice in this study,

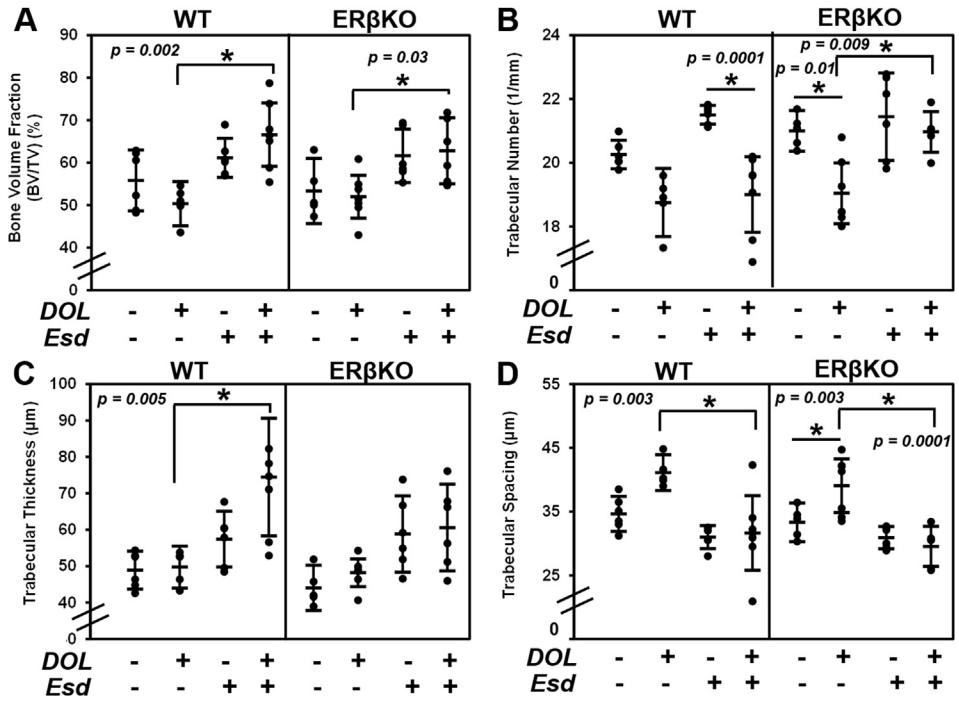

Fig. 5. Effect of estradiol and DOL on bone architecture. The data represent WT and ERβKO mice under NL or DOL with either placebo or estradiol treatment. Bone volume fraction (A), trabecular number (B), trabecular thickness (C), and trabecular spacing (D) as determined by microCT are shown. Statistical significance was determined by a two-way ANOVA followed by posthoc analysis with the Bonferonni method with $P < 0.05$. Exact P values are listed above the bars that denote significance.

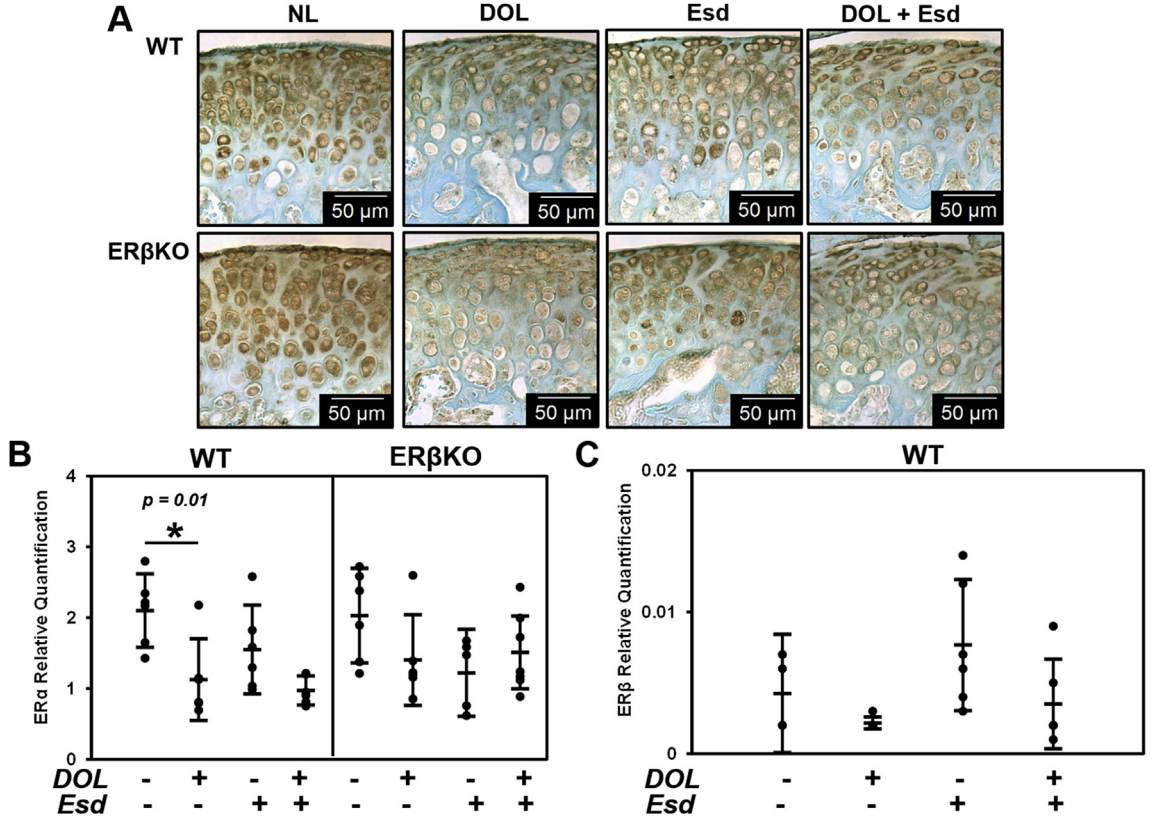

Fig. 6. Effect of estradiol and DOL on ER-gene expression and immunostaining. The data represent WT and ERβKO mice under NL or DOL with either placebo or estradiol treatment. Specifically, the labels indicate the following: NL = normal load and placebo; DOL = decreased occlusal loading and placebo; Esd = normal load and estradiol; DOL + Esd = decreased occlusal loading and estradiol. Representative ERα immunohistochemical images (A), and gene expression of ERα (B) and ERβ (C) are shown. For gene expression, $n = 6$ mice were utilized for all groups and MCC from left and right were pooled together. Statistical significance was determined by a two-way ANOVA followed by posthoc analysis with the Bonferonni method with $P < 0.05$. Exact P values are listed above the bars that denote significance.

no significant change was observed with estradiol or the combined treatments. Thus, the significant increase in Col2 expression in response to estradiol treatment and DOL is not directly dependent on ERα expression levels in male mice.

Further, it is probable that estradiol treatment may affect TMJ growth and remodeling differently in males and females resulting in differential responses to altered mechanical loading. Estradiol inhibits proliferation solely in female mice which may result in fewer cells available for chondrogenesis compared to male mice that are exposed to DOL. In support, it has previously been shown that ovariectomized female rats exposed to a soft diet exhibited an increased Col2 area compared to non-ovariectomized female rats exposed to a soft diet suggesting that estradiol and decreased loading reduce the number of cells undergoing chondrogenesis[41]. Also, sex differences in cell proliferation in response to strain have been shown in murine osteoblasts[54]. Collectively, these results suggest the male MCC exhibits an increased estrogen-mediated adaptive capacity to resist altered mechanical loading.

A working model illustrating the effects of estradiol and DOL in the MCC is provided in Fig. 7. We posit that DOL causes cell cycle arrest in both sexes. However, we propose that DOL and/or estradiol via ERβ inhibits TMJ progenitor cell activation solely in female mice. Therefore, when estradiol and DOL treatment are combined in the male mice, we propose that DOL results in an increase in the number of cells undergoing cycle arrest and primed for chondrogenic differentiation, which is then promoted by estradiol. This results in the increase in Col2 expression and one possible mechanism for the partial increase in cartilage thickness compared to DOL alone. In contrast, ERβ causes a decrease in the number of activated progenitor cells in female mice, thereby causing a decrease in the number of cells available for chondrogenesis. These sex differences related to MCC chondrogenesis provide one mechanism to explain the discrepancies in TMJ disorders between males and females.

We recognize that our study has many limitations. First, the mice utilized in this study were young, growing 49-day old mice. Thus, any changes observed cannot be directly translated to effects that may occur in older mice. Also, the aim of this study was to determine if supraphysiological estradiol levels in male mice resulted in similar results compared to the female studies. Naturally, circulating estradiol levels in male mice are negligible[55]. However, aromatization of testosterone is a common way in which males produce estrogen suggesting that estradiol levels may have been fluctuating throughout the experiment[39,56]. Also, the supraphysiological levels of estrogen administered to male mice in this study may be suppressing androgen levels and causing the observed effects[57]. Supplementary Fig. 2 illustrates an increase in androgen receptor staining solely in WT mice after estradiol treatment possibly indicating a change in androgen levels in these mice. Lastly, this study was conducted using global ERβKO mice that may express ERβ splice variants. Thus, future studies using conditional ERβKO mice with complete deletions of ERβ in specific cell populations will be advantageous in ascertaining the local effects of estradiol and DOL.

In conclusion, this study illustrates that estradiol treatment independent of ERβ results in an increase in chondrogenesis in the male MCC. Further, estradiol treatment administered with DOL causes enhanced chondrogenesis in male mice independent of ERβ. Taken together, these results suggest that there are sex differences in response to DOL and estradiol in the TMJ. Thus, additional investigation of the role of estradiol signaling in both males and females is necessary to gain greater understanding of the sexual dimorphism in TMJ disorders and develop sex-specific regeneration strategies.

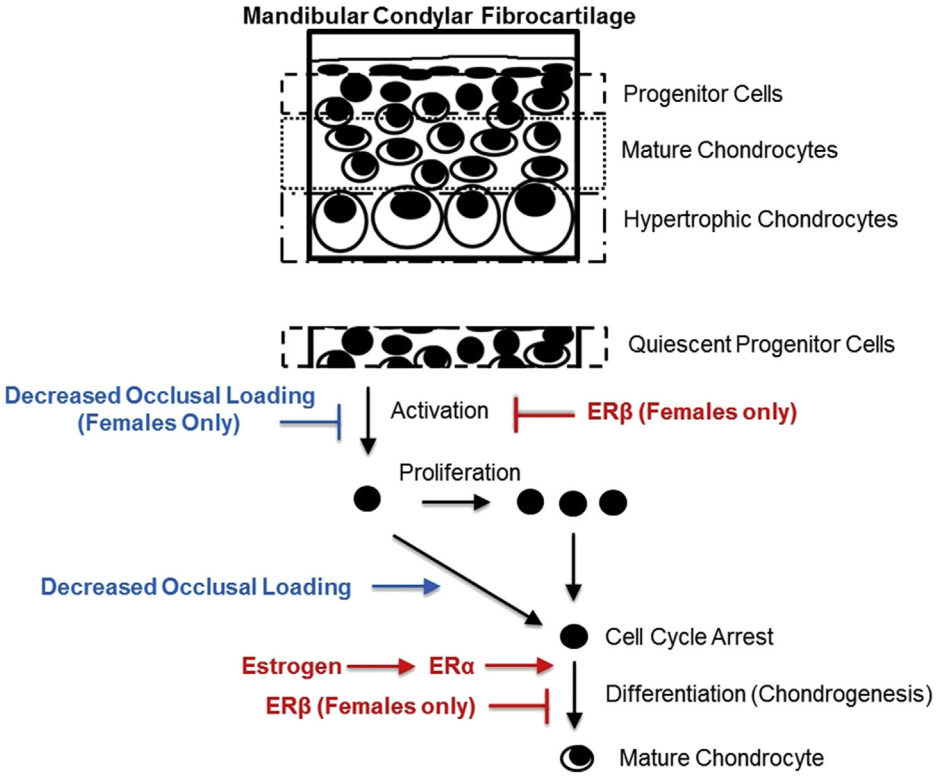

Fig. 7. Working model illustrating the sex-dependent role of estradiol via ERβ and DOL on the MCC growth and remodeling.

Author contributions

J. Robinson was involved in study design, data collection, data analysis, drafting and approval of the manuscript.
K. Cass was involved in data collection, drafting and approval of the manuscript.
R. Aronson was involved in data collection, drafting and approval of the manuscript.
T. Choi was involved in data collection, drafting and approval of the manuscript.
M. Xu was involved in data collection, drafting and approval of the manuscript.
R. Buttenbaum was involved in data collection, drafting and approval of the manuscript.
H. Drissi was involved in study design, drafting and approval of the manuscript.
H. H. Lu was involved in data analysis, drafting, and approval of the manuscript.
J. Chen was involved in study design, data analysis, drafting and approval of the manuscript.
S. Wadhwa was involved in study design, data analysis, drafting and approval of the manuscript.
J. Robinson and S. Wadhwa take responsibility for the integrity of the work as a whole.

Conflict of interest

No competing financial interests exist for any of the authors.

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Supplementary data

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