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Increased mandibular condylar growth in mice with estrogen receptor beta deficiency

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Abstract

Temporomandibular joint (TMJ) disorders predominantly afflict women of childbearing age, suggesting a role for female hormones in the disease process. In long bones, estrogen acting via estrogen receptor beta (ER β) inhibits axial skeletal growth in female mice. However, the role of ER β in the mandibular condyle is largely unknown. We hypothesize that female ER β deficient mice will have increased mandibular condylar growth compared with wild type (WT) female mice. This study examined female 7-, 49- and 120-day-old WT and ER β knockout (KO) mice. There was a significant increase in mandibular condylar cartilage thickness, due to an increased number of cells, in the 49- and 120-day-old female ER β KO compared with WT controls. Analysis in 49-day-old female ER β KO mice revealed a significant increase in collagen type X, *Pthrp* and osteoprotegerin gene expression and a significant decrease in *Rankl* and *Ihh* gene expression, compared with WT controls. Subchondral bone analysis revealed a significant increase in total condylar volume and a decrease in the number of osteoclasts in the 49-day-old ER β KO compared with WT female mice. There was no difference in cell proliferation in condylar cartilage between the genotypes. However, there were differences in the expression of proteins that regulate the cell cycle; we found a decrease in the expression of *Tieg1* and *p57* in the mandibular condylar cartilage from ER β KO mice compared with WT mice. Taken together, our results suggest that ER β deficiency increases condylar growth in female mice by inhibiting the fibrocartilage turnover.

Introduction

The National Institute of Dental and Craniofacial Research, a division of the National Institutes of Health, reported that temporomandibular joint (TMJ) disease is the second most common musculoskeletal disease in the United States, with 10.8 million people suffering from TMJ problems at any given time. Eighty percent of individuals seeking treatment for TMJ disorders (TMDs) are females of childbearing age. Because of the high prevalence of TMDs in women of reproductive age, it has been postulated that estrogen may make an individual susceptible to TMD⁽¹⁻⁹⁾. Estrogen modulates multiple biological processes within the TMJ region including inflammation⁽¹⁰⁾, matrix metalloproteinase activity⁽¹¹⁾, and pain modulation⁽¹²⁾. However, none of these have been able to adequately explain the gender predilection for TMD, suggesting that other estrogen dependent mechanisms are involved.

Estrogen inhibits the growth of the mandibular condyle. Addition of estrogen to rat mandibular condylar organ cultures results in a decrease in condylar cartilage thickness and proliferation⁽¹³⁾. Numerous studies have shown that ovariectomy causes an increase in the thickness of the mandibular condylar cartilage in young rats^(14,15), newborn mice⁽¹⁶⁾ and young mice⁽¹⁷⁾; and this is reversed by the administration of exogenous estrogen⁽¹⁵⁾. Not only does lack of estrogen (ovariectomy) increase the condylar cartilage thickness but it also increases the width of the mandibular condylar head, which includes both the condylar cartilage and the subchondral bone, in young mice⁽¹⁷⁾. Furthermore, estrogen negatively regulates condylar cartilage differentiation in vivo. In 4-month-old mice, ovariectomy has

been shown to cause an increase in collagen type X expression after 1-8 weeks⁽¹⁸⁾. The ability of estrogen to decrease growth and differentiation of the mandibular condylar cartilage is consistent with the literature on growth plate cartilages; however, estrogen is believed to have a chondro-protective effect on articular cartilages. (see for review⁽¹⁹⁾). The distinct roles of estrogen in the different cartilages might help explain why the peak incidence of TMD occurs when estrogen levels are the highest; as opposed to the peak incidence of osteoarthritis in other joints, which occurs when estrogen levels are at the lowest (e.g., menopausal women).

The classical estrogen receptors, alpha and beta, have distinct roles in estrogen's regulation of skeletal growth. ER β deficient mice have normal estrogen levels⁽²⁰⁾ and skeletal axial growth is affected only in adult female mice. Specifically, ER β deficient adult female mice (70-240 days old) have increased axial growth (crown-rump length) compared with the matched wild type mice^(20,21). On the other hand, estrogen receptor alpha (ER α) seems to promote skeletal linear growth in male mice but its effect on female mice is unclear⁽²⁰⁻²²⁾. Since ER β inhibits skeletal growth exclusively in females, the goal of this study was to examine whether a similar mechanism occurs in the TMJ. We hypothesized that female ER β deficient mice would have larger mandibular condyles than female WT mice. Greater understanding of estrogen signaling within the TMJ is critical to deciphering the gender predilection of the disease process.

Materials and Methods

Mice

Breeding pairs of C57Bl/6 wild type (Cat # 000664) and ER β KO mice (homozygous male, heterozygous female, Cat # 004745) were purchased from Jackson Labs. For the studies with ER β KO mice, only homozygous females were used. Seven- to 120-day-old female C57Bl/6 WT (total n=42) and ER β KO mice (total n=43) were used for the study (see Table 1 for details). At either three or sixteen hours prior to euthanasia, mice were injected intraperitoneally with 0.1 mg bromodeoxyuridine (BrdU) per gram body weight. All experiments were performed in accordance with animal welfare based on an approved IACUC protocol #AAAD0950 from Columbia University animal care committee.

RNA Extraction and PCR Amplification

For each mouse the mandibular condylar heads (left and right), containing both the mandibular condylar cartilage and subchondral bone, were carefully harvested and all the soft tissues were removed under a dissecting microscope. Total RNA was obtained from the condylar head and extracted with TRIzol Reagent (Invitrogen life technologies, Carlsbad, CA) following the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using the ABI High Capacity cDNA Archive Kit (Applied Biosystems, Foster city, CA) following the manufacturer's protocol. Real-Time PCR was performed, for expression of different genes, in separate wells (singleplex assay) of 96-well plates with reaction volume of 20 μ l. *Gapdh* was used as an endogenous control. Three replicates of each sample were amplified using Assays-on-Demand Gene Expression for the particular gene of interest, with predesigned unlabeled gene-specific PCR primers and TaqMan MGB FAM dye-labeled probes. The PCR reaction mixture (including 2X TaqMan Universal PCR Master Mix, 20X Assays-on-Demand Gene Expression Assay Mix, 50 ng of cDNA) was run in an Applied Biosystems ABI Prism 7300 Sequence Detection System instrument utilizing universal thermal cycling parameters. For the genes in which the efficiencies of target and endogenous control amplification were approximately equal, relative expression in a test sample was compared to a reference calibrator sample ($\Delta\Delta$ Ct Method) and used for data analysis. For the genes that were not amplified with the same efficiency as the endogenous control the

Relative Standard Curve method, in which target quantity is determined from the standard curve and divided by the target quantity of the calibrator, was used. Gene expression was performed for Proteoglycan 4 (*Prp4*), Parathyroid hormone related protein (*Pthrp*), SRY-box containing gene 9 (*Sox9*), collagen type II (*Col2a1*), indian hedgehog (*Ihh*), collagen type X (*Col10a1*), vascular endothelial growth factor (*Vegf*), insulin-like growth factor 1 (*Igf-1*), receptor activator for nuclear factor κ B ligand (*Rankl*), and osteoprotegerin (*Opg*).

Histology and immunohistochemistry

Whole mouse heads were sectioned into halves, fixed in 10% formalin for 4 days at room temperature and decalcified in 14% EDTA (pH 7.1) (Sigma, St Louis, MO) for 10 days and 14 days in 49-day and 120-day samples, respectively. Subsequently, the samples were processed through progressive concentrations of ethanol, cleared in xylene and embedded in paraffin. The TMJ was sagittally serially sectioned into 5 μ m sections by Microm HM 355s microtome (Thermo Fisher Scientific, Waltham, MA), and every 5th section was stained with H&E.

Histomorphometry measurements were made in a blinded, nonbiased manner using the BioQuant computerized image analysis system (BioQuant, Nashville, TN). Mandibular condylar cartilage thickness measurements were performed on H&E sagittal sections corresponding to the mid-coronal portion of the mandibular condylar head. The entire mandibular condylar cartilage area was divided into 3 layers, superficial layer (layer S), flattened layer (layer F) and Hypertrophic layer (layer H) based on the cartilage cell size, shape and staining (Figure 2A). The entire anterior-posterior condylar cartilage area was measured for each section and the average of at least three sections was calculated as the cartilage thickness for that mouse. Measurements included cell number, thickness and area of each layer. Six to eight mice from each age and genotype group were analyzed.

For immunohistochemistry, tissue sections were deparaffinized with xylene and rehydrated with decreasing concentrations of ethanol. Following rehydration, the sections were treated with 3 % peroxide to block endogenous peroxidase activity and digested for 60 minutes with pepsin for unmasking (Lab Vision, Cat # AP-9007-006, Fremont, CA). All sections were blocked with Protein Block Serum-Free (DakoCytomation, code # X0909, Carpinteria, CA). Immunohistochemical staining was performed using the LSAB + System-HRP Kit (DakoCytomation, code # K0690) following the procedure recommended by the manufacturer. Primary antibodies used in this study were collagen type II antigen (Millipore, MAB8887, 1:200 dilution), Tieg1 (Santa Cruz, sc-23159, 1:100 dilution), p57 (Santa Cruz, sc-8298, 1:200 dilution) and IGF-1 (Abcam, ab40657, 1:100 dilution). BrdU immunohistochemical analysis was completed using a BrdU staining kit following the manufacturer's instructions (Zymed Laboratories-Invitrogen Corporation, Carlsbad, CA, USA). Negative controls were prepared by omitting the primary antibody step and incubating with blocking solution. To quantify BrdU, Tieg1 and p57 staining, the labeling index (number of BrdU, p57 or Tieg1 positive cells divided by the total number of cells) was calculated. Three to five sections, corresponding to the same anatomical area (mid sagittal), were counted for each animal and the average of these sections was used for the labeling index.

Osteoclastic cell measurement were performed on Tartrate-Resistant Acid Phosphatase (TRAP) stained sections by TRAP kit (Sigma Aldrich, St. Louis, MO). The measurements were made in the bone region beneath the mandibular condylar cartilage. The thickness of the measured region was designated to the approximate cartilage thickness of the correlated region. Osteoclast number, osteoclastic surface, and bone surface in this region were analyzed with BioQuant computerized image analysis system.

micro-CT Analysis

The subchondral bones of the mandibular condyles from C57Bl/6 WT and ER β KO female mice were analyzed. The three-dimensional morphology of the subchondral bone was evaluated by the micro-CT facility at University of Connecticut Health Center, Farmington CT. The analysis included bone volume, total volume, trabecular number, trabecular spacing, and trabecular thickness.

Serum IGF-1

Mouse serum was collected from 49-day-old WT and ER β KO female mice. Quantitative assay was performed with IGF1 Mouse ELISA Kit (Abcam, ab100695) following the manufacturer's protocol. The serum was diluted 1:250 in Assay Diluent A (from the Kit).

Statistical Analyses

Statistical significance of differences among means was determined by one way analysis of variance (ANOVA) with post-hoc comparison of more than two means by the Bonferroni method using GraphPad Prism (San Diego, Ca).

Results

Size measurements

At 49 days of age both WT and ER β KO female mice weighed the same, but by 120 days of age, ER β KO mice weighed significantly more than age-matched WT mice (Figure 1A). There was a significant increase in the female ER β KO mandibular condylar cartilage thickness at 49 and 120 days of age compared with age-matched female WT mice (Figure 1B).

Histological analysis

Sagittal sections of the mandibular condylar cartilage from 49- and 120-day-old female ER β KO and WT mice were stained with H&E (Figure 2A). The mandibular condylar cartilage can be divided into 4 zones: articular, polymorphic, flattened and hypertrophic. In the mandibular condylar cartilage from the 49-day-old ER β KO mice cells in the polymorphic and flattened zones were vertically stacked, reminiscent of the proliferating zone of the growth plate cartilage. The vertically stacked cells were not apparent in the mandibular condylar cartilage from WT mice of the same age. Due to the difficulty in delineating articular from polymorphic zones in 49- and 120-day-old mice, cell counts were done for the layer S, which includes both the articular and polymorphic zones; the layer F, which includes cells in the flattened zone; and the layer H, which includes hypertrophic chondrocytes not embedded in the subchondral bone. We found significant increases in the total number of cells and in the number of cells in the flattened layer in 49- and 120-day-old ER β KO mice compared with the WT mice (Figure 2B and 2C). Thickness of the F layer was also significantly increased in 49- and 120-day-old KO mice (Figure 2B and 2C).

Gene expression

Gene expression analysis revealed significant increases in *Prg4*, *Pthrp*, *Col10a1* and *Opg* mRNA expression and significant decreases in *Sox9*, *Rankl*, *Vegf*, and *Ihh* mRNA expression in the 49-day-old ER β KO compared with age-matched WT mice (Figure 3B). Immunohistochemistry of collagen type 2 expression revealed similar levels of expression between ER β KO and WT mice (Figure 3A), which was consistent with the gene expression analysis results. However, when gene expression assay was performed with 120-day-old WT and ER β KO mice, there were no significant differences in any of the genes examined (Figure 3C).

Cell cycle exit

Proliferation was measured by BrdU immunohistochemistry in the mandibular condylar cartilage from female WT and ER β KO mice injected with BrdU three hours prior to sacrifice. We found no significant difference in the number of BrdU labeled cells/ total number of cells in the mandibular condylar cartilage from 49 – (Figure 3B) and 120 day-old (data not shown) female ER β KO compared to aged matched female WT mice. In order to determine if the increase in cell numbers in the ER β KO mice was due to a delay in the number of cells exiting the proliferative pool, we examined markers of cell cycle exit. Immunohistochemistry for BrdU was performed on the mandibular condylar cartilage of mice that received BrdU either 3 hours or 16 hours prior to sacrifice. There was a significant decrease in the percentage of BrdU labeled cells after 16 hours compared with 3 hours in WT mice; but, no significant difference was evident in the ER β KO mice (Figure 4). This decrease in BrdU labeling represents cells that have exited the proliferative pool of the mandibular condylar cartilage⁽²³⁾. Immunohistochemistry was also performed for two regulators of cell cycle exit, Tieg1 and p57. Both were predominantly localized to the area of transition between flattened and hypertrophic zones in the mandibular condylar cartilage. We also found that there was a significant decrease in p57 and Tieg1 in the female ER β KO compared with age-matched WT mice (Figure 5).

Local and serum IGF-1

ELISA assay showed a tendency toward increased serum IGF-1 levels in 49-day-old ER β KO mice compared with WT. However, the difference is not statistically significant (Figure 1C, $p=0.052$). Real-time PCR detected no significant difference in condylar head mRNA levels (Figure 3C) between wild type and ER β KO mice, in either the 49- or 120-day-old groups. Similarly, immunohistochemistry showed no difference in the IGF-1 protein distribution pattern in the condylar cartilage of wild type and ER β KO female mice (data not shown).

Subchondral bone

Micro-Ct analysis revealed a significant increase in the total volume and a significant decrease in the bone density (BV/TV) and trabecular thickness in 49-day-old ER β KO compared with age-matched WT mice (Figure 6). Micro-CT of 120-day-old WT and ER β KO mice revealed no significant differences in any of the measurements. TRAP staining was performed to quantitate the number of osteoclasts and the ratio of osteoclast surface/ bone surface (OcS/BS) in 49- and 120-day-old groups. We found that in 49-day-old mice, there were significant decreases in both the number of osteoclasts and OcS/BS in the subchondral bone of ER β KO mice compared with age-matched WT mice (Figure 7).

Discussion

The mandibular condylar cartilage is unique compared with other cartilages in the body in a number of ways. For example, other articular cartilages are composed of hyaline cartilage, whereas the mandibular condylar cartilage is composed of fibrocartilage (reviewed in⁽²⁴⁾) displaying distinct layers of cells at various stages of differentiation. Furthermore, the mandibular condylar cartilage undergoes endochondral ossification and is derived from periosteum⁽²⁵⁾. Interestingly, estrogen acting via the ER β pathway has been shown to inhibit endochondral ossification of the femur⁽²⁶⁾, mechanical load-induced periosteal bone formation⁽²⁷⁾ and fibrocartilage maturation of the fracture callus⁽²⁸⁾.

In our study we found that the female ER β KO mice had increased mandibular condylar cartilage thickness and an increased number of cells in the flattened zone at 49 and 120 days of age compared with age-matched female WT mice. We believe that this increase may be

due to a delay in cell cycle exit in the cells of the flattened zone. In support of this claim, we found a significant decrease in the number of BrdU labeled cells in female WT mice that were injected with BrdU 16 hours prior to sacrifice when compared with those injected with BrdU 3 hours prior to sacrifice. This reduction in BrdU labeling signifies cells have exited the flattened zone and undergone hypertrophic maturation. In contrast, ER β KO female mice exhibited no significant differences in BrdU labeling when BrdU was injected 16 hours prior to sacrifice as compared to 3 hours prior to sacrifice. A significant decrease in the expression of the cell cycle regulators, p57 and Tieg1, was found in the mandibular condyle of female ER β KO compared with female WT mice. In growth plate cartilage, p57 has been shown to inhibit cyclin-dependent kinases, and its activation is associated with cells exiting the proliferative pool and undergoing hypertrophic maturation^(29,30). Tieg1 has been shown to act downstream of estrogen receptor beta signaling⁽³¹⁾. Furthermore, in tenocytes⁽³²⁾ and myoblasts⁽³³⁾ Tieg1 deficiency caused a delay in cell cycle exit.

Another possible explanation for the increased mandibular condylar cartilage thickness is that ER β KO mice have been shown to have increased serum IGF-1 levels⁽²⁰⁾, and exogenous IGF-1 treatment causes increased mandibular condylar cartilage thickness⁽³⁴⁾. However, we do not believe this is the major mechanism involved as we did not find any significant changes in serum IGF-1 levels or in the local expression of IGF-1 mRNA within the mandibular condylar cartilage of female ER β KO compared with wild-type mice. Additionally, IGF-1 treatment was recently shown to cause increased mandibular condylar cartilage proliferation⁽³⁵⁾, which was not part of our ER β KO mandibular condylar cartilage phenotype.

A more pronounced phenotype was present in the mandibular condylar cartilage of ER β KO female mice at 49 days of age than at 120 days of age. For example, 49-day-old female ER β KO mice presented with decreased osteoclast numbers, increased chondrocyte maturation markers and a larger mandibular condylar total volume compared with the age-matched WT mice. At 2-4 months of age, the long bones of ER β KO female mice exhibit an increased femur length compared with age-matched WT mice; this difference is not evident in 8-month-old mice^(21,26). Unlike the femur, the majority of growth of the mandibular condyle occurs before puberty⁽³⁶⁻³⁸⁾, which may explain the more pronounced female ER β KO mandibular condylar phenotype observed at 49 days of age.

There were decreased osteoclast numbers in the subchondral bone of the mandibular condylar cartilage in female ER β KO mice, which is consistent to what has been reported in ER β KO long bones⁽³⁹⁾. The decrease in the number of osteoclasts could be due to either decreased RANKL expression in the hypertrophic chondrocytes of the mandibular condylar cartilage⁽⁴⁰⁾ or decreased Tieg1 expression, which has been shown to repress OPG promoter activity in female ER β KO mice⁽⁴¹⁾. We speculate that the increase in Col X expression in the mandibular condylar cartilage of ER β KO mice is due to a decrease in osteoclasts (as seen in Figure 7). In support, accumulation of hypertrophic chondrocytes in the mandibular condyle has been reported in models of defective osteoclasts⁽⁴²⁾.

Decreased levels of estrogen by ovariectomy resulted in increased mandibular condylar cartilage thickness and increased osteoclast numbers compared with female sham operated controls^(16,43). The fact that we see decreased osteoclast numbers in the mandibular condyles of the ER β KO mice, suggests that other estrogen mediated pathways are involved. For example, estrogen receptor alpha may be also involved; estrogen through the estrogen receptor alpha has been shown to cause increased osteoclast apoptosis⁽⁴⁴⁾.

In this study we show that deletion of estrogen receptor beta reduces osteoclast surface/ bone surface and delays the number of cells exiting the flattened zone, resulting in a larger

mandibular condyle at 49 days of age. In the growth plate cartilage, there is believed to be a finite number of cells capable of proliferation⁽⁴⁵⁾. We have previously shown that mechanical loading also causes increased mandibular condylar cartilage proliferation⁽⁴⁶⁾. Therefore, it is possible that high estrogen levels during puberty may deplete the number of proliferative cells in the mandibular condylar cartilage, making post-pubertal females less responsive to mechanical load-induced TMJ remodeling. A similar phenomenon occurs in periosteal bones, where pre-pubertal females exhibit a greater increase in load-induced periosteal bone formation than post-pubertal females⁽⁴⁷⁾. In our future studies we plan to investigate the effects of estrogen receptor beta deficiency in decreased versus increased TMJ loading models.

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Figure 1. Body weight (A) and mandibular condylar cartilage thickness (B) of female WT and ERβ KO mice at 7, 49 and 120 days of age. n= 6-8 for each age and genotype. * = significant difference p<0.05. C. Serum IGF-1 level of 49-day-old female WT and ERβ KO mice. p=0.052.

Figure 2.

H & E staining of the mandibular condylar cartilage (A) from female WT and ERβ KO mice at 49 days and 120 days of age. B, C-Cell counts and thickness measurements were performed for the Superficial layer (S, Articular and Polymorphic zones), Flattened Zone (F) and Upper Hypertrophic zone (H). n=6-8 for each age and genotype, * = significant difference p<0.05. Bars = 100μm.

Figure 3.

Col2 Immunohistochemistry (A) and Quantification of Gene Expression (B, C) by Q-PCR was performed on the mandibular condyles of female 49- (B) and 120 day-old (C) WT and ER β KO mice. n=6-8 for each age and genotype, * = significant difference $p < 0.05$. Bars = 100 μ m.

Figure 4.

Immunohistochemistry for BrdU in the mandibular condyle was performed on female 49-day-old WT and ER β KO mice (A). Mice were injected with BrdU at either 3 hours or 16 hours prior to sacrifice. B. Quantification of BrdU labeling. n=6-8 for each age and genotype, * = significant difference p<0.05. Bars = 100 μ m.

Figure 5.

A. Immunohistochemistry for Tieg1 and p57 was performed on the mandibular condyles of female 49-day-old WT and ERβ KO mice. B. Quantification of Tieg1 and p57 labeling. n=6-8 for each age and genotype, * = significant difference $p < 0.05$. Bars = 100μm.

Figure 6. micro-Ct analysis of the subchondral bone from female WT and ER β KO at 49 and 120 days of age on: Total Volume, Bone Volume, Bone Volume Fraction (BV/TV), Trabecular Thickness, Trabecular Number, and Trabecular Spacing. n=6-8 for each age and genotype, * = significant difference p<0.05.

Figure 7.

A. TRAP staining of the mandibular condyles of female 49 day- and 120-day-old WT and ERβ KO mice. B. Quantification of osteoclast surface/bone surface (OcS/BS). C. Quantification of osteoclast number, n=6-8 for each age and genotype, * = significant difference $p < 0.05$. Bars = 200μm.

Table 1

The numbers of mice.

Mouse Genotype	Total Number	Age (days)	Real Time PCR	Histology/micro-CT	
				3 hr BrdU ^a	16 hr BrdU
Wild Type	42	7		8	
		49	6	8	7
		120	6	7	
ERβ KO	43	7		8	
		49	7	8	8
		120	6	6	

^athese mice were also used for measuring body weight, serum IGF-1 level in 49 day-old mice and condylar cartilage thickness.