Impact of estrogen-related receptor α on the biological characteristics of rat mandibular condylar chondrocytes

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Abstract. It is well-known that estrogen-related receptor α (ERRα) affects numerous metabolic pathways and biological functions in the body, although the function of ERRα in the mandibular condylar chondrocytes (MCCs) of the temporomandibular joint remains unclear. The aim of the present study was to investigate the effect of ERRα on the biological characteristics of MCCs in female rats. Immunofluorescent staining was used to observe the expression level and distribution of ERRα in MCCs and tissues. Quantitative polymerase chain reaction (qPCR) was performed to detect the impact of estrogen intervention on the biological characteristics of female rat MCCs and ERRα expression levels. Liposome transfection and XCT-790 were used to overexpress and inhibit ERRα expression, respectively, and then qPCR was performed to detect changes in the biological characteristics of MCCs. ERRα expression was detected in the nucleus and cytoplasm of rat MCCs. 17-β estradiol (E2) (10^{-8} M) increased the mRNA and protein expression levels of ERRα, Sox9, GDF-5 and aromatase during *in vitro* MCC cultivation. In addition, E2 affected MCC proliferation through the regulation of ERRα expression levels. Overexpression of ERRα positively regulated the mRNA and protein expression levels of Sox9 and GDF-5, but did not exhibit a significant effect on the mRNA and protein expression levels of aromatase and Col2a1. In conclusion, ERRα exhibited an important regulatory role in the proliferation and differentiation of female Sprague-Dawley rat MCCs *in vitro* through regulating Sox9 and GDF-5.

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Introduction

The mandibular condylar chondrocyte (MCC) is the key functional cell of the temporomandibular joint (TMJ). A number of TMJ diseases are caused by damage to these cells. Epidemiological studies have identified a difference in TMJ morbidity between male and female patients; TMJ morbidity in female patients is higher than in male patients and data indicate that changes in estrogen levels affect the metabolism of MCCs (1), suggesting that abnormal levels of estrogen may be associated with TMJ disease (2). A previous study, which generated different estrogen-level experimental environments and specifically regulated the expression of estrogen receptors (ERs), found that ER expression did not explain all phenomena observed in clinical and animal studies (3). This suggests that in addition to the ER classical signaling pathway, there may be other signaling pathways, with and without ERα association, involved in estrogen-related diseases.

Estrogen-related receptor α (ERRα) is a type of orphan nuclear receptor that shares homology with ERs but without the binding of estrogen (4). *In vitro* studies have demonstrated that ERRα is expressed in the torso cartilage tissues of humans and animals, and is involved in a number of metabolic activities, including inflammation (5-10). The regulatory response of ERRα to estrogen may depend on the species, tissue and cell types (11,12), and the expression and functions of ERRα in temporomandibular tissues and cells remain unclear.

In the present study, the ERRα expression levels in the MCCs of female Sprague-Dawley (SD) rats were investigated, to analyze the impact of estrogen on the biological characteristics of MCCs through the mediative effects of ERRα. This may enable further understanding of MCC characteristics, as well as the mechanisms of physiological and pathological changes in MCCs.

Materials and methods

Primary cultivation and identification of MCCs. The present study was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional

Animal Care and Use Committee of the Fourth Military Medical University (Xi'an, China). Six female SD rats (age, eight weeks), were sacrificed by cervical dislocation. The rats were then soaked in 75% ethanol and MCCs were separated under sterile conditions. Serum-free RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) was used to remove and rinse blood and other impurities adhered to the articular cartilage. The articular cartilage was then repeatedly cut with ophthalmic scissors to form sections of ~1 mm^3. A total of 2-3 ml of 0.25% trypsin solution (Sigma-Aldrich) was added for digestion in the incubator at 37˚C for 20 min; 3 ml of 0.2% type II collagenase solution (Sigma) was then added and the mixture was further incubated for 2 h. The cell suspension was pipetted, filtered using 200 mesh and centrifuged at 300 x g for 5 min, and the supernatant was removed. RPMI-1640 medium supplemented with 10% fetal bovine serum was used to prepare the cell suspension. The cells were counted, then seeded and incubated at 37˚C, in 5% CO_2 and saturated humidity. The cells were then purified using the differential adhesion method (13). After 48 h, the medium was replaced to remove any nonadherent cells. The remaining cells were cultivated for 3-5 days, then the medium was replaced with the common medium. When cell fusion had reached 80-90%, passage was performed. The cell morphology was observed with an inverted microscope (Olympus, Tokyo, Japan),.

Immunofluorescent staining. The third generation of logarithmically-growing MCCs was prepared as a single cell suspension, then inoculated into the Lab-Tek II chamber slide (NUNC, Roskilde, Denmark) with density of ~5x10^4 cells/chamber. The original culture medium was removed after 24 h conventional cultivation. Following washes with phosphate-buffered saline (PBS) solution for 3 times, 4% paraformaldehyde was added for fixation and 0.25% Triton X (Invitrogen Life Technologies, Carlsbad, CA, USA) was added for 15-min incubation at 37˚C. After 15 min soaking in 3% H_2O_2, goat serum (Boster, Wuhan, China) was added to cover the cell-attached coverslip for blocking. The slide was then incubated for 30 min at 37˚C, followed by washing with PBS three times. Type II collagen polyclonal antibody (titer 1:100; Sigma-Aldrich) was added and the mixture was incubated for 2 h at 37˚C. PBS was used to replace the primary antibody in the negative control group. Following three washes with PBS, the secondary FITC-conjugated goat anti-mouse IgG (Beyotime, Nantong, China) was added in the dark and the slide was incubated for 1 h at 37˚C then washed with PBS three times. The residue fluid was drained and DAPI was added for 15 min nuclear staining in the dark. Fluorescence microscopy (Olympus) was then used to observe and capture images of the staining situations.

Alcian blue staining. The MCCs were digested with 0.25% trypsin solution at 37˚C for 3 min, then seeded onto a 6-well plate and cultivated using the normal cultivation method. When the wall-adherent cells had grown to 80% confluence, the original culture medium was removed, the cells were washed with PBS buffer three times and 4% formaldehyde was used for fixation. The cells were again washed three times with PBS buffer, Alcian blue (Sigma) was added for 30 min staining and the cells were washed with water. PBS was used to replace the Alcian blue in the negative control group. A solution of 95% alcohol was used to dehydrate until the background was clear, then the cells were washed with water. The staining was then observed and recorded.

Table I. Primer sequences used in the reaction system.

Gene	Sequence
ERRα	F: 5'-GATGTGGCCTCTGGCTACCACTA-3'
	R: 5'-CGGACAGCTGTACTCGATGCTC-3'
Sox9	F: 5'-TGAAGGGCTACGACTGGACG-3'
	R: 5'-ACTTGTAATCGGGGTGGTCTTT-3'
Col2a1	F: 5'-TGGTGGAGCAGCAAGAGCA-3'
	R: 5'-CCCTCAGTGGACAGTAGACGGA-3'
GDF-5	F: 5'-AGGGAGGTAACAGCAGCGT-3'
	R: 5'-CTCCAAGGCACTGATGTCAAAC-3'
Aromatase	F: 5'-CTTTTGAGACGATTCCAGGTGA-3'
	R:5'-GGATAAGTAATGCCCCAGAGTAGAT-3'
β-actin	F: 5'-GGAGATTACTGCCCTGGCTCCTA-3'
	R: 5'-GACTCATCGTACTCCTGCTTGCTG-3'

ERRα, estrogen-related receptor α; F, forward; R, reverse.

Immunocytochemical staining. The MCCs were seeded on coverslips at a density of 5x10^3 cells/ml for 48 h and then fixed with cold acetone. Immunocytochemical analysis was performed using the streptavidin-biotin complex method according to the manufacturer's instructions (Zhongshan Golden Bridge Biotechnology Co. Ltd., Beijing, China). 3,3'-Diaminobenzidine (Boster) served as the chromogen. The primary antibody against ERRα was a monoclonal rabbit anti-human ERRα (ab41868; Abcam, Cambridge, UK) at a 1:100 dilution. For the negative control, the primary antibody was substituted with a commensurable volume of PBS. The samples were counterstained with hematoxylin and examined under an Olympus compound microscope (Olympus) equipped with a Nikon digital camera (Nikon, Tokyo, Japan).

qPCR. Third-generation MCCs were prepared as a cell suspension and inoculated into small culture flasks according to the grouping. To investigate the effects of E2 on ERRα and MCC-specific markers, MCCs were divided into an E2 group and control group. The MCCs in the E2 group were treated with 10^{-8} M 17-β estradiol (E2; Sigma-Aldrich) for 48-h stimulation, while the E2 was replaced by an equivalent solvent (anhydrous ethanol) in the control group. To investigate the effects of overexpression and inhibition of ERRα on MCCs, the cells were transfected with the plasmid pcDNA3.1(+)-cont and pcDNA pcDNA3.1(+)-ERRα, or treated with XCT790, respectively. After 48 h 10^{-8} M 17-β estradiol stimulation, the MCCs were collected. The total RNA from MCCs was isolated using TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. Complementary DNA was synthesized from mRNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). qPCR was conducted with a Mastercycler EP Realplex4 (Eppendorf

Table II. Sequences of the target fragmental primers.

Primer name	Primer sequence (5'-3')
ERRα-F	TTAGGTACCGCCACCATGTCCAGCCAGGTGGTGG
ERRα-R	CGGCGCTCGAGTCAGTCCATCATGGCCTCAAG

ERRα, estrogen-related receptor α.

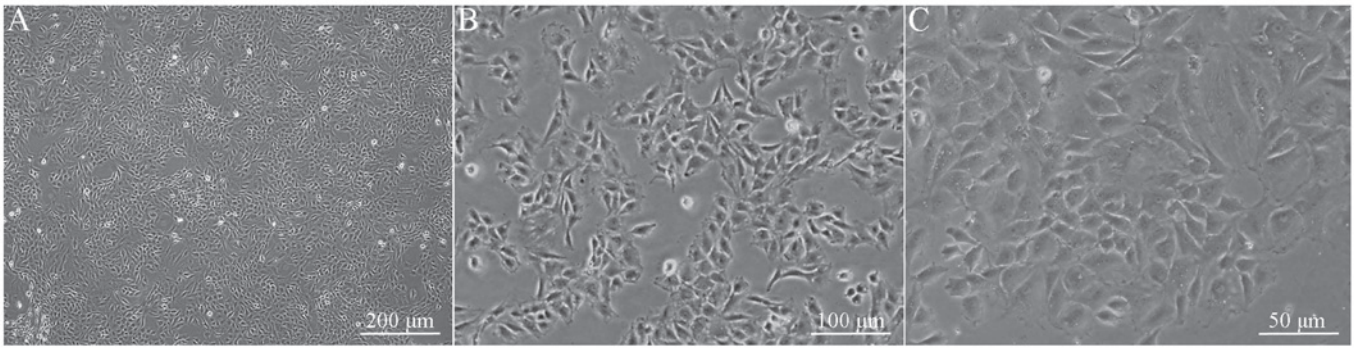


Figure 1. Cell morphology of the primary cultured rat mandibular condylar chondrocytes. Scale bar: (A) 200 μm; (B) 100 μm; and (C) 50 μm.

AG, Hamburg, Germany) and SYBR-Green (Invitrogen Life Technologies). The primer sequences used are shown in Table I. The reactions were performed under the following cycling conditions: 95˚C for 10 min, followed by 45 cycles of 95˚C for 15 sec and 60˚C for 1 min. The expression of the target genes was calculated using the formula $2^{-\Delta\Delta Ct}$. The expression data were normalized to the expression levels of the β-actin gene.

Western blotting. Briefly, cell extracts, containing 30 μg total protein, were subjected to SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked and then probed with primary antibodies against ERRα (ab41868; Abcam), Sox9 (sc-20095; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), GDF-5 (sc-6901; Santa Cruz Biotechnology, Inc.), aromatase (sc-14245; Santa Cruz Biotechnology, Inc.), Col2a1 (ab34712; Abcam) and β-actin (ab8227; Abcam). The secondary antibodies, which were selected according to the species of origin of the primary antibodies, included mouse anti-goat IgG-HRP (A0216; Beyotime), goat anti-rabbit IgG-HRP (A0208; Beyotime), and donkey anti-goat IgG-HRP (A0181; Beyotime). Following incubation, the luminescent signals were detected using an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA). The Quantity One software (Bio-Rad, Hercules, CA, USA) protein bands were used to measure the gray value of the target band corresponding to β-actin and the gray value of the ratio of the target protein in comparison with the strength of a sample index.

MCC proliferation detection. The experimental groupings were as follows: E2 (10^{-8} M) treatment group, XCT790 (5 μM; Abcam) treatment group, E2 (10^{-8} M)/XCT790 (5 μM) combined-treatment group and blank control group. The third generation of logarithmically-growing MCCs was prepared as a cell suspension, then inoculated into 96-well plates, with seeding density at $1x10^4$ cells/well. This was performed 5 times for each treatment group. The cells were observed 24 h after inoculation; if the cells were adherent to the wall, the original culture medium was discarded. According to the instructions of the WST-1 kit (Boster), WST-1 solution was added to the cells for 2 h-incubation at 37˚C and 5% CO_2. The plate was then agitated for 1 min to fully mix the system for detection. The WST-1 treatment was performed on the cells at 48 and 72 h, respectively, and then the relevant absorbance was determined using a spectrophotometer (BioPhotometer; Eppendorf). Absorbance was measured at 450 nm.

Construction of vector and transfection. The target fragments were amplified using the primers listed in Table II and the pcDNA3.1(+) vector (Invitrogen Life Technologies) was then digested with restriction enzymes: *Hin*dIII at the upstream digestion site and *Eco*RI at the downstream digestion site. Following identification, the endotoxin plasmid was extracted and the vector was precipitated with ethanol. According to the manufacturer's instructions, Lipofectamine 2000 (Invitrogen Life Technologies) was used to transfect the overexpressed ERRα; the transfection was performed when the third generation of logarithmically-growing MCCs had grown to 70-80% confluence.

After 48 h treatment, the cells were collected for the detection of changes in MCC-related biological-characteristic markers. All experiments were repeated three times.

Statistical analysis. The collected experimental data were processed with SPSS 18.0 statistical software (SPSS, Inc., Chicago, IL, USA) and Student's t-test was used for intergroup comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

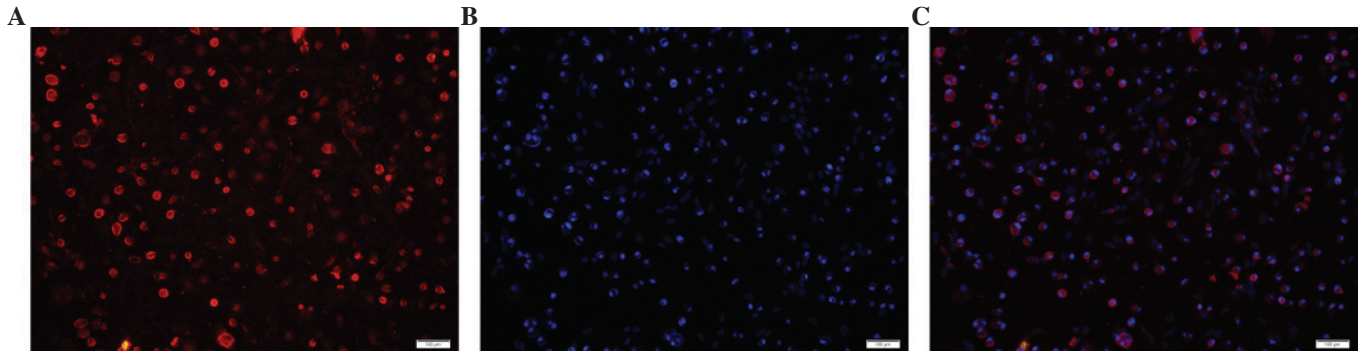


Figure 2. Immunofluorescent staining of collagen type II in rat mandibular condylar chondrocytes using phycoerythrin (PE) and DAPI fluorescence. (A) Type II collagen was widely expressed only in the cytoplasm demonstrated by PE fluorescence; (B) nuclei stained with DAPI; (C) merged PE and DAPI fluorescence. Scale bar, 100 μm.

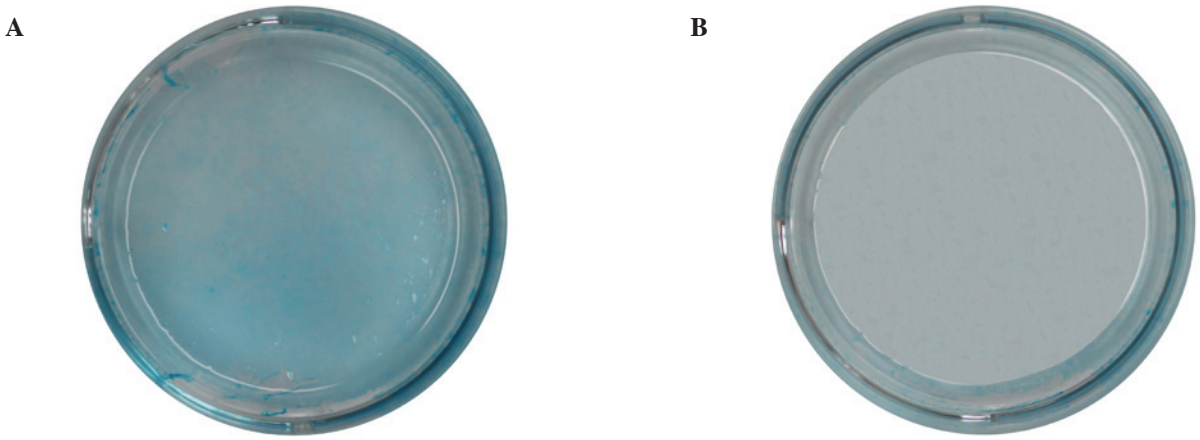


Figure 3. Alcian blue staining in rat mandibular condylar chondrocytes. (A) Positive Alcian blue staining of the third-passage mandibular condylar chondrocytes in normal culture medium. (B) Negative staining in the phosphate-buffered saline control group.

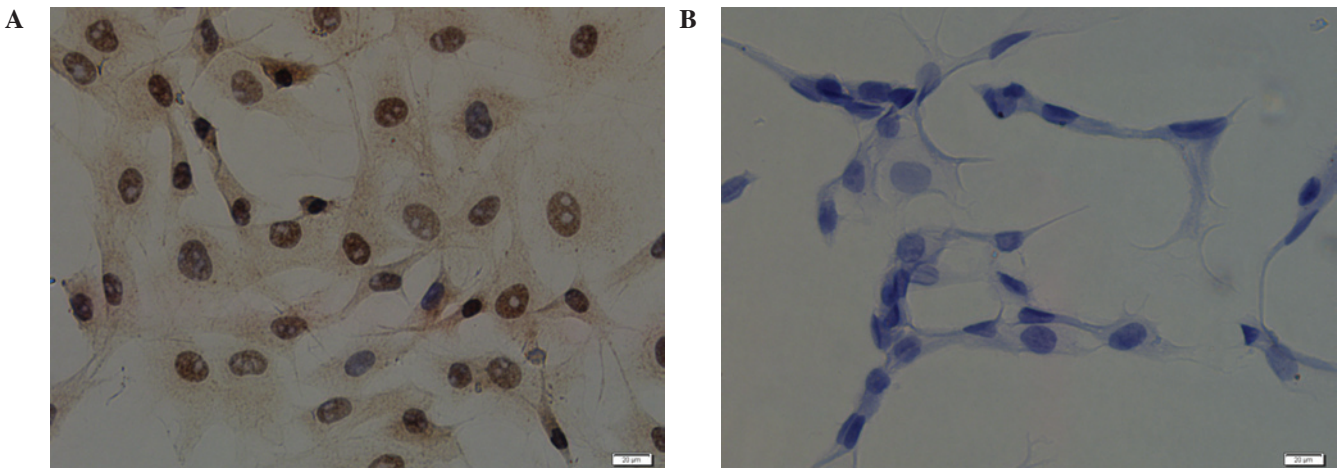


Figure 4. Immunocytochemical staining of estrogen-related receptor α (ERRα) in rat mandibular condylar chondrocytes (MCC)s. (A) Positive staining of ERRα in the cytoplasm and nuclei of MCCs, as detected using anti-human ERRα. (B) Negative immunoreactivity for ERRα in MCCs in the negative control group treated with phosphate-buffered saline. Scale bar, 20 μm.

Results

Characterization of MCCs. Observed under an inverted microscope, the primary cultured rat MCCs appeared spherical with a refractive extracellular matrix within 12 h and the cells were adherent to the wall within 12 to 48 h. Following adherence, the cells stretched and exhibited polygon- or spindle-shaped morphology, with blunt cell bodies (Fig. 1). The cells aggregated in ~1 week, confluenced in ~10 days and formed a monolayer in ~14 days, and secreted large quan-

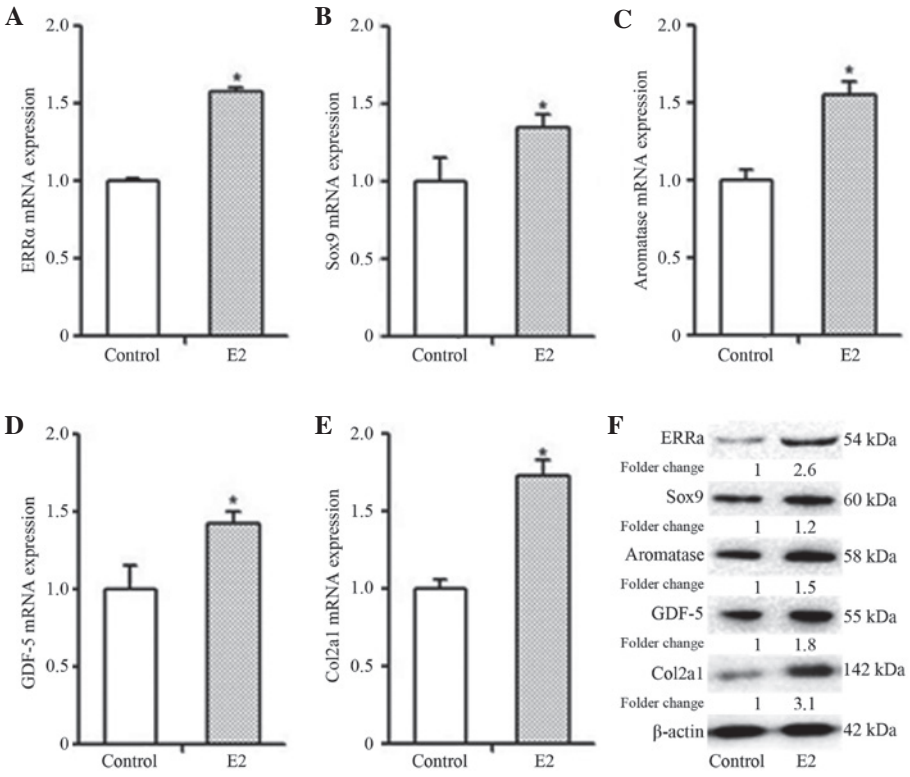


Figure 5. Changes in estrogen-related receptor α (ERRα) and mandibular condylar condrocyte (MCC)-specific marker expression levels after 48 h 17-β estradiol (E2) (10^{-8} M)-simulated cultivation of MCCs. (A-E) Quantitative polymerase chain reaction detection results; (F) western blotting results. *P<0.05, vs. the control group.

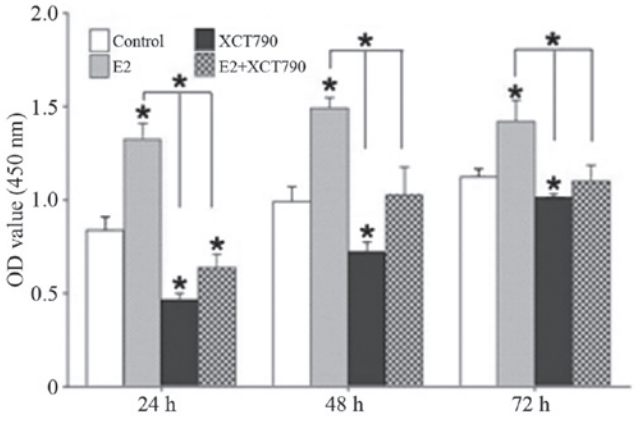


Figure 6. Impact of 17-β estradiol (E2) and XCT790 treatment on mandibular condylar condrocyte proliferation, measured using WST-1 *P<0.05.

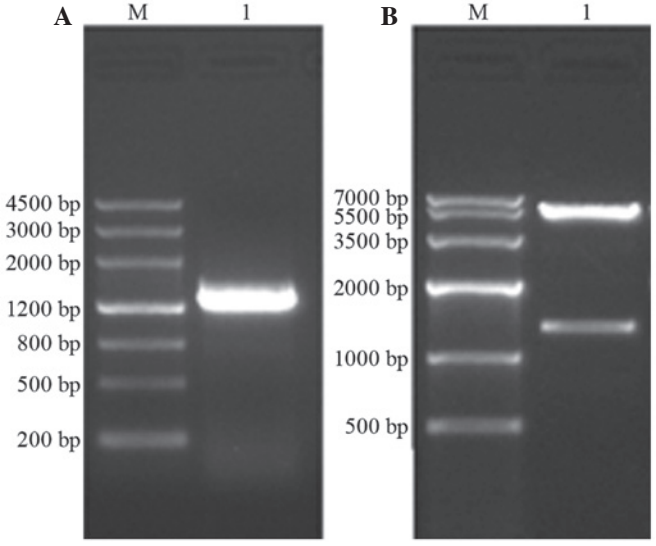


Figure 7. Agarose gel electrophoresis results. (A) Polymerase chain reaction products; lane 1, a band at 1,300 bp was observed, corresponding to estrogen-related receptor α, ERRα. (B) Recombinant plasmids following double-enzyme digestion; lane.1, pcDNA3.1 (+)-ERRα. M, marker lane.

tities of refractive extracellular matrix. After three weeks, overlapping growth was observed in certain cells, with multilayer-clump-like growth zones in certain areas. The cells that exhibited positive immunofluorescent staining for cytoplasmic type II collagen accounted for >90% cells in the random view field, while DAPI blue staining was observed, only in the nucleus (Fig. 2).

Alcian blue staining. Alcian blue staining revealed that the cultured cells were positively blue stained (Fig. 3A), exhibiting characteristics of cartilage-specific extracellular matrix, while the PBS control group exhibited no positive staining (Fig. 3B).

Expression of ERRα and MCC-related biological characteristic markers. As shown in Fig. 4A, ERRα was expressed in the MCCs; the nuclei and cytoplasm were brown-stained, although the expression in the nucleus was markedly greater. Fig. 4B shows the PBS negative control staining.

In order to investigate the impact of E2 on the biological characteristics of MCCs, different markers of MCC prolifera-

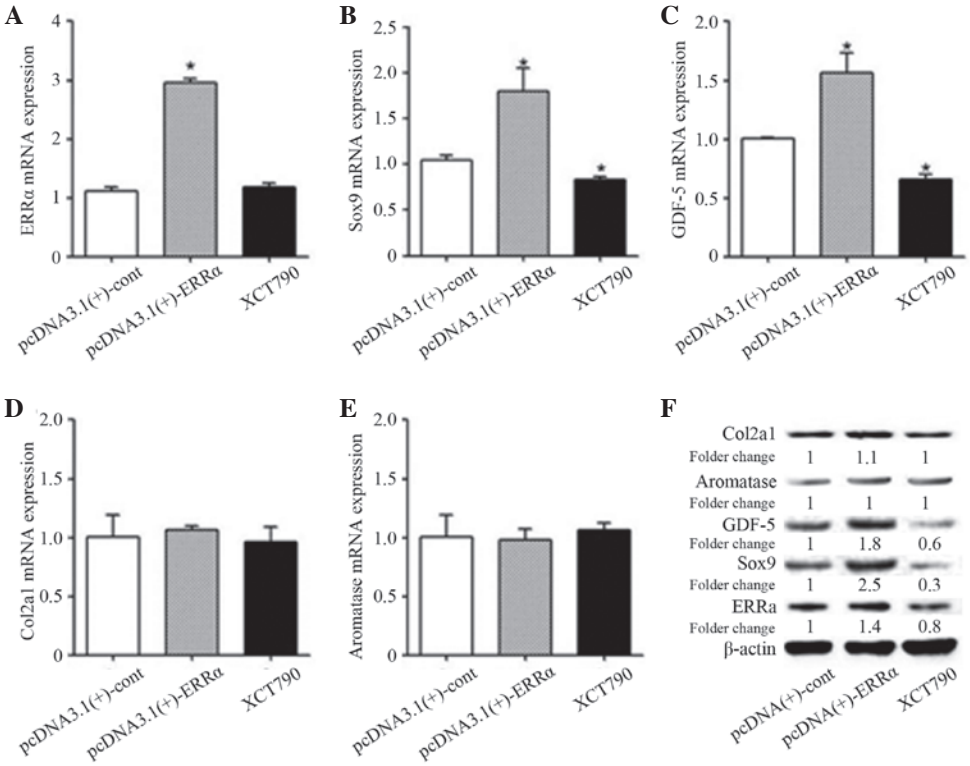


Figure 8. Transfected plasmid overexpressed estrogen-related receptor α (ERRα); the expression levels of markers 48 h after XCT790 treatment of mandibular condylar chondrocytes (MCC)s. (A-E) mRNA expression changes. (F) Protein changes. *P<0.05, vs. the control group.

tion and differentiation were analyzed. Following E2 (10^{-8} M) stimulation for 48 h, the mRNA and protein expression levels of ERRα, Sox9, GDF-5, aromatase and Col2a1 in the MCC cells were significantly higher than those in the cells of the control group (Fig. 5; P<0.05). Therefore, E2 promoted the gene and protein expression of ERRα, Sox9, GDF-5, aromatase and Col2a1 in the MCCs.

Effects of XCT790 on MCC proliferation. In order to reveal the impact of E2 on MCC proliferation and the possible regulatory effects of ERRα, E2 (10^{-8} M) and an inverse agonist of ERRα, XCT790 (5 μM), were used to treat the MCCs. WST-1 was performed at different time points to detect cell proliferation. Statistical analysis (Fig. 6) demonstrated that MCC proliferative capacity in the E2 (10^{-8} M) treatment group was higher than that in the control group and the E2 (10^{-8} M)/XCT790 (5 μM) treatment group. In addition, the MCC proliferative capability of the XCT790 (5 μM) treatment group was lower than that of the control group and the E2 (10^{-8} M) treatment group; and the MCC proliferative capability in the E2 (10^{-8} M)/XCT790 (5 μM) treatment group was lower than that in the E2 (10^{-8} M) but higher than that in the XCT790 (5 μM) treatment groups.

Construction of the ERRα eukaryotic expression vector. For electrophoretic identification of the PCR products, agarose gel electrophoresis was used to separate the samples and the 1,300 bp band of ERRα was observed.

For the electrophoretic identification of recombinant plasmids, the recombinant plasmid pcDNA3.1 (+)-ERRαby was obtained following enzyme digestion and ligation reactions. The minipreparation-extracted plasmids were double-enzyme digested and agarose gel electrophoresis was performed, which revealed two DNA bands (Fig. 7), one at ~5.4 kb, matching the size of the pcDNA3.1 (+) vector, and the other at ~1,300 bp, matching the base pair number of ERRα.

DNA sequence analysis of the recombinant plasmids. The sequencing results of pcDNA3.1-ERRα were performed using BLAST analysis following the correction. The nucleic acid sequences were consistent with the coding sequences of ESRRA in the GenBank database.

Expression levels of MCC-associated biological characteristic markers. Once ERRα overexpression in the transfected MCCs was confirmed, statistical analysis of the ERRα group compared with the control group revealed that the expression levels of the ERRα mRNA and protein were significantly increased (P<0.05) and the expression levels of Sox9 and GDF-5 mRNA and protein were also significantly increased (P<0.05). However, no significant difference was identified in the change in mRNA and protein expression levels of Col2a1 and aromatase (Fig. 8). The results showed that the transfection was successful, resulting in overexpression of ERRα mRNA and protein, and that ERRα overexpression exibited a positive regulatory role on the early proliferation and differentiation markers of MCCs, namely Sox9 and GDF-5, although showed no significant correlation with Col2a1 and aromatase, which were expressed by the mature MCCs.

In order to further clarify the role of ERRα, XCT790 (5 μM) was used to inhibit ERRα activity in MCCs. The changes in gene and protein expression levels of ERRα, Sox9,

GDF-5, Col2a1 and aromatase after 48 h were detected by qPCR and western blotting. The results revealed that compared with the control group, no significant difference was detected in the expression levels of ERRα mRNA in the XCT790 (5 μM) treatment group, although the protein expression level was reduced. In addition, the mRNA and protein expression levels of Sox9 and GDF-5 were reduced, but no significant changes were identified in the expression levels of Col2a1 and aromatase mRNA and protein. The results indicated that the inhibition of ERRα in MCCs with XCT790 (5 μM) occurred mainly at the protein level, not at the gene level. In conclusion, these results suggest that XCT790 (5 μM) inhibited the mRNA and protein expression of Sox9 and GDF-5 through downregulation of ERRα protein expression.

Discussion

In the present study, the bodies of the primary MCCs were obtuse, appearing polygonal or spindle-like. Type II collagen immunofluorescent staining revealed that the positive staining cells covered >90% of the area in the view field, indicating that the cultivated cells maintained the ability to synthesize type II collagen. The positive Alcian blue staining revealed that the cultured cells possessed the ability to secrete proteoglycans, thus, exhibiting the phenotype of MCCs (14,15).

A study demonstrated that the expression of ERRα in tissues and cells is important with regard to the metabolic activities and biological functions of the body (16). ERRα is expressed in the bone and cartilage of the limbs and trunk, and may mediate the regulation of bone metabolism balance (17,18). The TMJ is involved in speech, chewing and other functions (19). Due to its special structures, the characteristics of the TMJ are different from that of other joints in the trunk and limbs. MCCs are an important component of TMJ, which undergoes constant change to respond to the external stress stimuli and internal hormone levels. To the best of our knowledge, the present study was the first study to reveal positive ERRα expression in the MCCs. ERRα expression in the nuclei and cytoplasm of MCCs further suggests that ERRα may act as a transcription factor, shuttling between the nucleus and cytoplasm, and actively regulating signal transduction in cells.

Estrogen exhibits extensive effects on body metabolism, and is closely associated with cell proliferation, differentiation and maturation (20). An increasing number of studies have demonstrated that estrogen is important in the metabolism of bone/cartilage tissue, and that its biological effects are associated with the estrogen dose and response pathways (21-25). The imbalance in estrogen regulation depends largely on the changes in its response pathways. In addition to the traditional ER signaling pathway, there are a number of associated by-pathways. Thus, to investigate the impact of E2 on the biological characteristics of MCCs and ERRα expression, the physiological concentration of E2 (10^{-8} M) (26) was used in the present study to process *in vitro* cultured MCCs. The mRNA and protein expression levels of ERRα, Sox9, GDF-5, aromatase and Col2a1, were elevated following stimulation of MCCs by E2 (10^{-8} M) for 48 h, indicating that the physiological concentration of E2 exhibited the capacity to promote early proliferation and differentiation, and late maturation of *in vitro*-cultured MCCs.

Considering that the ERRα expression levels increased in the MCCs following E2 (10^{-8} M) treatment and that ERRα has been reported to promote cell proliferation (27), the physiological concentration of E2 and the specific ERRα inverse agonist, XCT790, were used in the present study to treat the MCCs, and WST-1 was used to observe the changes in cell proliferation. The WST-1 results revealed that E2 promoted the proliferation of MCCs and that when XCT790 (5 μM) was used to inhibit ERRα expression, the proliferative capacity of the MCCs was reduced. Therefore, ERRα promoted MCC proliferation and mediated the E2-induced promotion of cell proliferation. The results suggest that the ERRα expression in MCCs was directly or indirectly influenced by E2, and that ERRα mediated the signal transduction through which E2 affected MCC proliferation, differentiation and maturation, although the specific signaling pathway for these effects remains unclear.

ERRα has been confirmed to regulate different metabolic activities of humans and multiple animals under different conditions, and is crucial in controlling metabolic balance (28). In the present study, following plasmid transfection to overexpress ERRα, the gene and protein expression levels of Sox9 and GDF-5 were found to significantly increase. The gene and protein expression of Col2a1 and aromatase increased marginally, although not significantly. In the XCT790 treatment group, ERRα protein expression, but not mRNA expression, was significantly inhibited, consistent with the results of a study of the mechanism of XCT790 inhibition of ERRα (29). Following XCT790 treatment, the gene and protein expression levels of Sox9 and GDF-5 declined along with the reduction in ERRα protein expression levels, although no significant changes were identified in the gene and protein expression levels of Col2a1 and aromatase.

In MCCs, the observation that changes in ERRα protein expression levels may increase or reduce mRNA and protein expression levels of Sox9 and GDF-5 suggested that Sox9 and GDF-5 were regulated by ERRα, and that these genes were downstream responsive genes. Activation of the Sox9 gene may promote proliferation and aggregation of cartilage precursor cells, and further differentiation towards chondrocytes (30). The changes in Sox9 expression levels were consistent with those of ERRα, indicating that ERRα may be a direct regulator of Sox9 in MCCs. In addition, ERRα affected the signal response of cell proliferation and accumulation in the early stages of MCC metabolic change. GDF-5 is the predominant regulator of cartilage growth and differentiation, and it was also positively regulated by ERRα. In the early stages of bone/cartilage metabolism, changes in the expression levels of Sox9 may induce changes in the subsequent bone/cartilage metabolism (17). Whether the effects of ERRα on GDF-5 were mediated through Sox9 remains to be confirmed by further studies.

Col2a1 and aromatase are expressed by mature cartilage, and are important in secreting type II collagen and topically synthesizing estrogen to maintain the stable chondrocytes function. Col2a1 and aromatase expression levels were not sensitive to ERRα regulation, further suggesting that the effect of ERRα on MCCs is mainly reflected in upper-end regulation, namely proliferation, differentiation and maturation. This does not exclude the possibility that ERRα may exert indirect effects

on Col2a1 and aromatase. As the course of cell proliferation, differentiation, maturation and apoptosis is a continuous metabolic process, all stages influence each other; the associations are close rather than isolated. In order to maintain normal biological functions, the balance of cell proliferation, differentiation and maturation are precisely regulated by hormone levels, transcription factors and numerous other factors in the body (31). The interactions among ERRα and other transcription factors remain to be elucidated.

In conclusion, ERRα was identified as an important regulator in the early proliferation and differentiation of MCCs, positively regulating Sox9. Overexpressed ERRα promoted proliferation of MCCs, although the impact on cell maturation is unclear. ERRα expression may be affected by multiple factors, including estrogen levels. Further studies regarding the ERRα-mediated biological characteristic changes of MCCs may aid further understanding of the impact of changes in the body estrogen levels on the physiological and pathological changes in the TMJ.

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