

Effect of estrogen on bone resorption and inflammation in the temporomandibular joint cellular elements

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Abstract. Several epidemiological studies have reported that temporomandibular disorder is more prevalent in women, which suggests the involvement of sex hormones, such as estrogen, in the pathogenesis of this disease. PCR amplification and Western blotting were employed to target the expression of estrogen receptors (ERs) in human fibroblast-like synovial and ATDC5 cells. The effect of estrogen was investigated through the expression of RANKL, osteoprotegerin (OPG), M-CSF/CSF-1 and *c-fms*. We showed expression of M-CSF/CSF-1 and *c-fms*, with time-dependent increase in both after the addition of estrogen. Based on previous studies reporting that M-CSF/CSF-1 regulates the proliferation and differentiation of hemopoietic progenitor cells into mature macrophages, we put forward a new hypothesis based on the increased inflammation and tendency of females to suffer more from temporomandibular disorder (TMD) in the presence of external exacerbating factors. Detection of RANKL and OPG in ATDC5 and expression of both in HFLS was confirmed with complete disappearance of the RANKL band, and marked increase in the expression of OPG after 1 h from the addition of estrogen.

Introduction

Estrogen, a representative steroid hormone, is known to regulate diverse physiological processes of target tissues, in both sexes (1). The biological activities of estrogen are initiated by binding to the specific receptor proteins, namely the estrogen receptors (ERs) (2). Two main isoforms of ER have been identified to date: ER α and β . Immunocytochemistry

using specific antibodies has revealed that ER α is widely distributed in various tissues, whereas the definitive distribution of ER β protein remains unclear (2).

The steroid nuclear receptors are a sub-family of the NR (nuclear receptor) super family, mediating the cellular effects of steroid hormones. NR genes are typically found on the nuclear membrane or less frequently, free in the cell cytoplasm (3).

The temporomandibular joint (TMJ) is a bilateral diarthrosis between the mandibular condyle and temporal bone. Temporomandibular disorder (TMD) is characterized by a triad of symptoms, including joint sounds, pain, and limited mandibular movement (4). These symptomatic variants indicate that TMD is caused by a combination of factors, such as occlusion, mental stress, strength, and hormones; however, the etiology of this disease is not fully understood. Several epidemiological studies have reported that TMD is more prevalent in women than in men (5-7), which suggests the involvement of sex hormones, such as estrogen, in the pathogenesis of this disease. Although estrogen is known to play important roles in the etiology of postmenopausal osteoarthritis or rheumatoid arthritis in systemic joints (8,9), little information has been available regarding the relationship between estrogen and the etiology of TMD (10).

The synovial membrane in the TMJ has an important role in joint movement because of the involvement of the synovial lining cells in the synovial fluid metabolism, which affects smooth jaw movement. Many ultra structural investigations have pointed out that the synovial membrane consists of two kinds of synovial lining cells: macrophage-like type A and fibroblastic type B cells (11). However, little previous information is available regarding the localization of ER α and β in the TMJ.

Macrophage colony-stimulating factor (M-CSF or CSF-1) is one of a family of acidic glycoproteins which regulate the survival, proliferation and differentiation of hemopoietic progenitor cells into mature macrophages and/or granulocytes (12). As its name implies, M-CSF/CSF-1 specifically promotes the growth and differentiation of cells in the monocyte-macrophage lineage and activates various functions of mature macrophages (13-15). The *c-fms* gene encodes the receptor for macrophage colony-stimulating factor-1 (16).

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Table I. The sequences of the used primers.

Primer		Sequence
Mouse ER α	Forward	CAAGGAGGGAGTGCGTCTGG
	Reverse	CATCTAGGACCAGGTCCTCAGC
Mouse ER β	Forward	TCTGCAGTGATTATGCATCTGGG
	Reverse	CTTCGTGAGGGACATCATCATGG
Human ER α	Forward	AAGAGCTGCCAGGCCTGCC
	Reverse	TTGGCAGCTCTCATGTCTCC
Human ER β	Forward	GCTCAATTCCAGTATGTACC
	Reverse	GGACCACATTTTTGCACT
RANKL	Forward	ACGCAGATTTGCAGGACTCGAC
	Reverse	TTCGTGCTCCCTCCTTTCATC
OPG	Forward	TGGCACACGAGTGATGAATGCG
	Reverse	GCTGGAAAGTTTGCTCTTGCG
<i>c-fms</i>	Forward	GCG ATG TGT GAG CAA TGG CAG T
	Reverse	AGA CCG TTT TGC GTA AGA CCT G
CSF-1	Forward	ATGACAGACAGGTGGAAGTCCAGTGATAGAGG
	Reverse	TCACACAACCTTCAGTAGGTTTCAGGTGATGGGGC
G3PDH	Forward	GTCTACATGTTCCAGTATGAC TCC
	Reverse	AGCCTTCTCCATGGTGGTGAAGAC
β -actin	Forward	TGACGGGGTCACCCACACTGTGCCCATCTA
	Reverse	CTAGAAGCATTGCGGTGGACGATGGAGGG
L19	Forward	CTGAAGGTGAAGGGGAATGTG
	Reverse	GGATAAAGTCTTGATGATCTC

RANKL (receptor activator of NF κ B ligand) is a membrane-bound ligand expressed on osteoblasts. Binding of RANKL to its receptor RANK which is expressed on osteoclast precursors and mature osteoclasts, induces osteoclastogenesis and activation of mature osteoclasts (17-21). RANK is a member of TNF receptor super family, expressed as a transmembrane heterotrimer on the surface of haematopoietic osteoclasts progenitors, mature osteoclasts, chondrocytes, mammary gland epithelial cells (22-26). Estrogen has been shown to suppress RANKL-induced osteoclast differentiation *in vitro* (27).

Osteoprotegerin (OPG), a soluble tumor necrosis factor (TNF) receptor-like molecule termed TNF receptor super family 11B, is a naturally occurring RANKL inhibitor. OPG competitively binds RANKL to inhibit the action of the receptor activator of NF κ B (RANK) both *in vivo* and *in vitro*, which prevents osteoclastic bone resorption (28).

Our study targets the expression of ERs in normal human fibroblast-like synovial cells (HFLS) and ATDC5 (as a model for chondrocytes) and thus the effect of estrogen on synovial membrane fibroblastic type B cells represented by HFLS and chondrocytes represented by ATDC5 cells as regarding inflammation and bone resorption.

Materials and methods

Cell culture. HFLS are primary human fibroblast-like synovial cells, from synovial membrane of a 67-year old Caucasian

female, were purchased from (Cell Applications, Inc., San Diego, CA) and cultured in Synovocyte Growth Medium (Cell Applications). ATDC5 (differentiate into chondrocytes), were purchased from RIKEN Cell Bank and cultured in DMEM: HamF12 =1:1 (Sigma-Aldrich, St. Louis, MO), with 5% FBS. Cells were incubated in humidified 5% CO₂ atmosphere at 37°C.

Exogenous 17 β -estradiol treatment. At confluence (10⁻¹¹ M), 17 β -estradiol (in ethanol, Sigma-Aldrich) was added to the medium for periods of 1, 3, 6, 12 and 24 h, while controls were treated with an equivalent volume of ethanol (estrogen vehicle).

Reverse transcription (RT)-polymerase chain reaction (PCR). Total RNA was isolated using guanidinium thiocyanate-phenol-chloroform extraction (TRIzol, Invitrogen Corp., Carlsbad, CA). The RNA pellets were dissolved in distilled water and quantified using spectrophotometer (NanoDrop Tech., Inc., Wilmington, DE). Total RNA was reverse transcribed using oligo-(dT) primers and reverse transcriptase (Toyobo, Tokyo, Japan) according to the manufacturer's protocol.

PCR amplification: ER α and β were amplified with KOD-Dash (Toyobo). At first, gene-specific primers for human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were used to test cDNA quality. Amplification was performed as follows: 35 cycles at 94°C for 30 sec, annealing at 60°C for

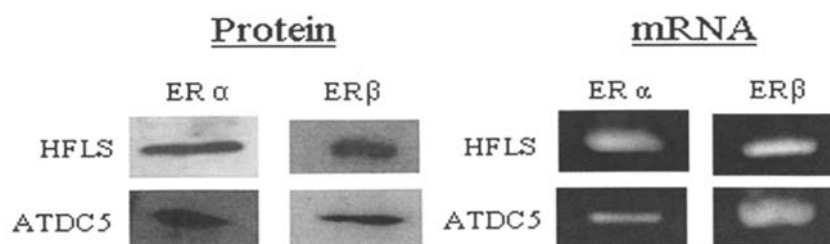


Figure 1. Right panels, estrogen receptors α and β RNA expression in HFLS and ATDC5 cells. Cells were analyzed by RT-PCR for the presence of the mRNA encoding ER α and β . Cells were cultured in standard growth conditions; RNA was extracted and retro-transcribed using the reverse transcriptase enzyme (RT). ER α and β were expressed as specific single bands. Left panels, Western blot analysis for ERs: the whole cell lysates from HFLS and ATDC5 were analyzed for the expression of ER α and β . The positive controls were MCF7 whole cell lysate for α and NIH/3T3 whole cell lysate for β . Cont, control.

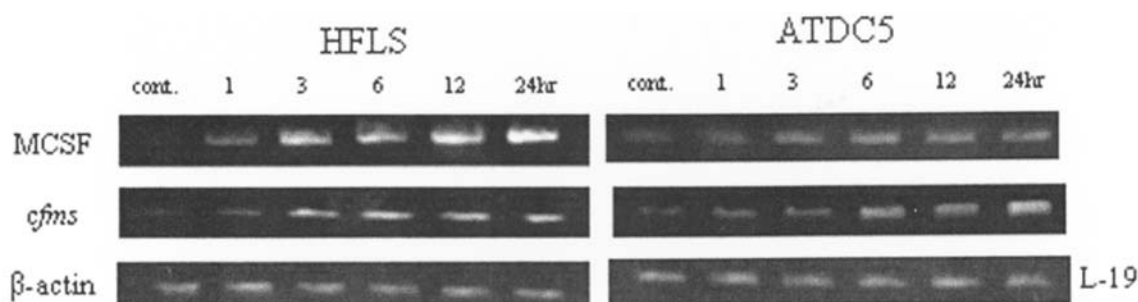


Figure 2. The effect of 17 β -estradiol on M-CSF and c-fms mRNA expression in HFLS and ATDC5. Cells were incubated with 10⁻¹¹ M 17 β -estradiol for periods of 1, 3, 6, 12 and 24 h. RNA was isolated and RT-PCR was performed. There was a gradual increase in expression of M-CSF/CSF-1 and c-fms after 1 h. Cont, control.

2 sec and 72°C for 30 sec. The first cycle was conducted at 94°C for 10 min and the final cycle at 72°C for 10 min.

For c-fms, M-CSF/CSF-1, RANKL and OPG, PCR was run for 27, 35, 28 and 25 cycles, respectively using Ampli-Tag Gold DNA polymerase (Perkin-Elmer, Boston, MA). Gene-specific primers for L19 and β -actin were used to test cDNA quality and equalization. Amplification was performed as follows: 95°C for 1 min, annealing at 58°C and 72°C for 1 min. The first cycle was conducted at 95°C for 11 min and the final cycle at 72°C for 10 min. The sequences of the used primers are as shown in Table I.

Following PCR, the reaction products were resolved on 2% agarose gel by electrophoresis. Gel was stained with ethidium bromide to visualize the PCR products. The levels of expression were analyzed with Image J 1.37 v (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, MD, USA) and normalized with G3PDH, L19 and β -actin mRNA expression (data not shown).

Western blotting. For protein extraction, at confluence, media were aspirated, cells were washed with Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium (1X PBS), and lysis buffer was added (10 mM HEPES-KOH at pH 7.5, 100 mM KCl, 0.1% NP-40). Cells were lysed thoroughly and dislodged from plate by repetitive pipetting. Protein concentrations were assessed by Bio-Rad protein assay kit. Controls for both α and β were used (MCF7 whole cell lysate for α and NIH/3T3 whole cell lysate for β , Santa Cruz Biotechnology, Inc., CA). Samples were separated on 10% polyacrylamide gels and transferred onto polyvinylidene

difluoride membranes. Membranes were blocked for 1 h in 5% skim milk followed by 3 washings with TBS containing 0.1% Tween buffer and were placed in a primary antibody [1:200 ER α (H-184) rabbit polyclonal IgG and 1:500 ER β rabbit polyclonal IgG, Santa Cruz Biotechnology] overnight at 4°C on shaker, followed by 3 washings with TBST and placed in secondary antibody (1:2500 HRP-goat anti-rabbit IgG, Zymed, San Francisco, CA) for 1 h at room temperature on shaker. After 3 washings for 15 min, the membranes were incubated in Western Lightning Chemiluminescence reagent Plus (Perkin-Elmer). Medical X-ray film was used to visualize the chemiluminescence.

Results

Expression of ERs. ERs are central to the study, so we examined the expression of ER α and β in HFLS and ATDC5 cells. Using PCR (35 cycle amplification) both ER α and β were expressed as a specific single band in HFLS and ATDC5 cells (Fig. 1). Further more, Western blotting confirmed the expression of both ER α and β in both cells (Fig. 1).

Expression of M-CSF/CSF-1 and c-fms and effect of estrogen in HFLS and ATDC5. PCR amplification for 35, 27 cycles respectively, showed novel expression of M-CSF/CSF-1 and c-fms in HFLS and ATDC5 cells (Fig. 2). At confluence, following addition of 10⁻¹¹ M 17 β -estradiol (in ethanol) to the medium for periods of 1, 3, 6, 12 and 24 h, the effect of estrogen on M-CSF/CSF-1 and c-fms in HFLS and ATDC5 was investigated. PCR amplification for 35, 27 cycles

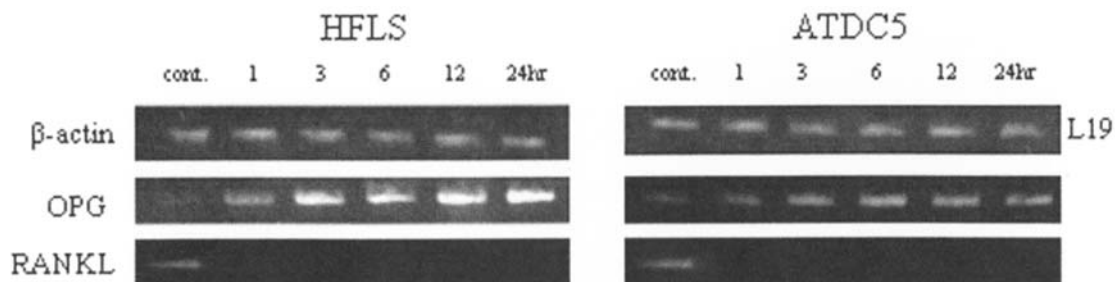


Figure 3. The effect of 17 β -estradiol on RANKL and OPG mRNA expression in HFLS and ATDC5. Cells were incubated with 10^{-11} M 17 β -estradiol for periods of 1, 3, 6, 12 and 24 h. RNA was isolated and RT-PCR was performed. There was marked increase in the expression of OPG and complete disappearance of the RANKL band, after 1 h. Cont, control.

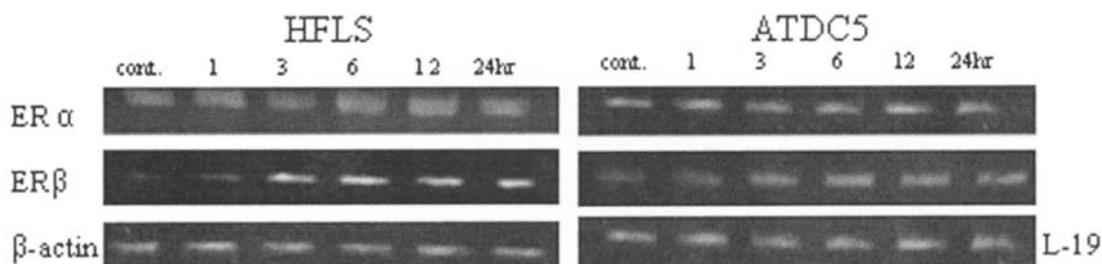
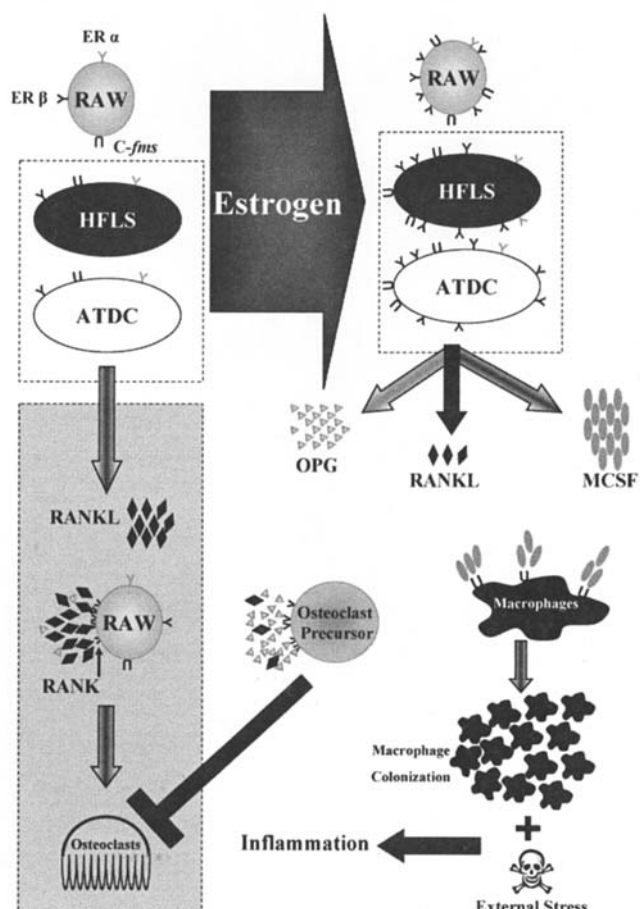


Figure 4. The effect of 17 β -estradiol on ER α and β mRNA expression in HFLS and ATDC5 cells. Cells were incubated with 10^{-11} M 17 β -estradiol for periods of 1, 3, 6, 12 and 24 h. RNA was isolated, and RT-PCR was performed. A gradual increase in expression of ER β with time was observed, with no change occurring to the expression of ER α . Cont, control.



respectively, showed time-dependent increase in expression of M-CSF/CSF-1 and *c-fms* 1 h after the addition of estrogen (Fig. 2). Same result was obtained in both cell types (Fig. 2).

Expression of RANKL and OPG and the effect of estrogen. Novel detection of RANKL and OPG in ATDC5 and expression of both in HFLS was confirmed by PCR amplification for 28 and 25 cycles respectively. At confluence, following addition of 10^{-11} M 17 β -estradiol (in ethanol) to the medium, PCR amplification revealed complete disappearance of the RANKL band, with marked increase in the expression of OPG after 1 h from the addition of estrogen, with a continuous time-dependent increase of expression (Fig. 3). Same result was obtained in both cell types.

Effect of estrogen on ER α and β receptors. The capacity of estrogen to increase expression of M-CSF/CSF-1, *c-fms*, and OPG, and diminishing the expression of RANKL, together with the detection of ERs in both cells suggested mediation

Figure 5. Estrogen increases inflammation and decreases bone resorption. The HFLS, ATDC5 and RAW 264.7 cells show increase in expression of ER β after the addition of estrogen. HFLS and ATDC5 express RANKL that helps the differentiation of osteoclast precursors into osteoclasts. However with the addition of estrogen, there is diminished expression of RANKL and up-regulated expression of OPG that binds to RANKL, blocking the interaction between RANKL and RANK. Estrogen increases MCSF expression, causing proliferation and differentiation of macrophages. When chronically or erroneously stimulated, macrophages exert damaging effects on the surrounding tissue (inflammation).



ER α or β) so we studied the effect of estrogen on expression of both ER α and β after addition of 10^{-11} M 17 β -estradiol for periods of 1, 3, 6, 12 and 24 h was studied. PCR amplification of 35 cycles was conducted for both ERs. For HFLS and ATDC5, there was a gradual increase in expression of ER β with time, with no change occurring to the expression of ER α (Fig. 4).

Discussion

Although many epidemiological studies have reported a higher frequency of TMD in females than in males (5-7), there has been controversy concerning the presence of ERs in the TMJ. Abubaker *et al* (29) found ER α immunoreactivity in the articular disc of human TMJs; however, Campbell *et al* (5) found no such immunoreactivity in the articular disc of a TMJ obtained from a TMD patient. In our previous study (30), with the use of Western blotting and PCR amplification, we were able to demonstrate the novel detection of ER β and confirmed presence of ER α in RAW 264.7 cells (representing macrophage-like type A synovial lining cells). In this study we showed expression of ER α and β in HFLS (synovial lining cells type B), thus exploring both kinds of synovial lining cells.

The effects of estrogen on bone metabolism are well established. In contrast, there have been few studies regarding the effect of estrogen on cartilage in the TMJ, or even in other systematic joints (31). To have an overview of all joint cellular elements, we investigated and confirmed the presence of ER α and β in ATDC5 cells (as a model for chondrocytes), denoting possible estrogen effects on joint cells through these receptors.

In our previous study (30) we showed that estrogen increased expression of *c-fms* in macrophage-like cells. In the present study, we detected the expression of M-CSF/CSF-1 and *c-fms* in ATDC5 and HFLS cells and we further demonstrated increased expression of M-CSF/CSF-1 and *c-fms* via estrogen. Based on previous studies reporting that M-CSF/CSF-1 regulates the survival, proliferation and differentiation of hemopoietic progenitor cells into mature macrophages and/or granulocytes (12), and as M-CSF/CSF-1 increased via estrogen, we hypothesize a resultant increase in macrophage colonization (Fig. 5). Macrophages are patrolling cells of the innate immunity, involved in the recognition of foreign pathogens, in the elimination of toxic molecules and in the reconstitution of tissue integrity. Macrophage cells orchestrate these diverse pathways by producing several different mediators, such as nitric oxide (NO), cytokines, including interleukin 1 β (IL-1 β) and tumor necrosis factor- α (TNF α), and matrix degrading enzymes, such as metallo-protease-9 (MMP-9). The production of these molecules, although beneficial for killing bacteria and further activating the immune system, exerts damaging effects on the surrounding tissue when chronically or erroneously stimulated (32). Thus, in the presence of external exacerbating factors such as stress, the increase in macrophage colonization could be a contributing factor in the increased inflammation and tendency of females to suffer more from TMD.

Until recently, inflammatory cytokines, such as interleukin (IL)-1, IL-6, IL-11, and TNF- α were implicated as important mediators of bone lysis (33-35). Recent findings,

however, indicate that RANKL might be the central mediator of osteoclast development in bone loss pathologies. RANKL expressed on synovial fibroblasts and activated T-lymphocytes enhance differentiation of synovial macrophages and osteoclast precursors in bone marrow into bone resorbing osteoclasts (20). Using PCR we showed expression of RANKL and OPG in HFLS and their novel detection in ATDC5. In contrast to other studies stating that estrogen does not have any effect on RANKL, we can show that after 1 h from the addition of 17 β -estradiol there is completely diminished expression of RANKL with an enhanced expression of OPG.

We hereby show that estrogen prevents bone resorption by up-regulating OPG production to increase OPG binding of RANKL, which subsequently blocks the interaction between RANKL and RANK in osteoclasts (Fig. 5).

Estrogens have been demonstrated to act via ER β in tissues of the central nervous system, cardiovascular system, immune system, urogenital tract, gastrointestinal tract, kidney and lungs (36). The capacity of estrogen to increase expression of MCSF, *c-fms*, and OPG, and diminishing the expression of RANKL, together with the detection of ERs in HFLS and ATDC5 cells suggested mediation by ERs (ER α or β). So we studied the effect of estrogen on ERs. For HFLS and ATDC5, there was a gradual increase in expression of ER β with time, with no change occurring to the expression of ER α . This coincides with our previous study showing increase in expression of ER β in RAW 264.7 cells following application of 17 β -estradiol and the shown relation between receptor expression and different estrogen concentrations.

Exploring various cellular elements, showing presence of estrogen receptors α and β , and presenting the coexisting relation between estrogen and ER β denotes possible estrogen effects on joint cells through these receptors. Referring to the increased expression of M-CSF/CSF-1 and *c-fms* we hypothesize increased macrophage colonization to be a contributing factor in the increased inflammation and tendency of females to suffer more from TMD in the presence of external exacerbating factors. An attempt to scientifically explain female tendency to TMD, may provide novel targets of both prevention and therapy.

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