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Biomechanically Induced Expression of Wnt5a and Ror2 Plays a Detrimental Role in Degenerative Remodeling of the Rat Temporomandibular Joint Induced by Abnormal Occlusion

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Abstract

The present study aimed to test whether abnormal mechanical loading could increase expression of Wnt5a and Ror2 and their effects on the degenerative changes in rat temporomandibular joints.

Primary chondrocytes and bone marrow stromal cells isolated from rat condylar cartilage and subchondral bone were stimulated using fluid flow shear stress, and pretreated with STAT3, NF-kappaB, and Notch pathway inhibitors. The gene and protein levels of Wnt5a and Ror2 were tested. The inhibitor of Wnt5a-Ror2 signaling was injected periarticularly into the temporomandibular joint of rats showing degenerative changes induced by abnormal molar occlusion. The effects of sRor2 on cartilage and subchondral bone pathology were evaluated.

In chondrocytes, Wnt5A and Ror2 expression increased and peaked at 120 min or 30 dynes/cm^2 of fluid flow shear stress, while those in bone marrow stromal cells peaked at 60 min or 20 dynes/cm^2 of FSS ($P<0.05$). Fluid flow shear stress-induced Wnt5a expression in chondrocytes and bone marrow stromal cells was regulated by the STAT3 pathway. The fluid flow shear stress induced expression of Ror2 was regulated by STAT3 and Notch in chondrocytes and by NF-kappaB and Notch in bone marrow stromal cells. Progressive cartilage degenerative changes and subchondral bone loss of rat temporomandibular joint were markedly reversed by 1 μg/week sRor2.

Keywords: Mechanical loading; Temporomandibular joint; Osteoarthritis; Wnt5A; Ror2

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Introduction

Temporomandibular Joint Osteoarthritis (TMJ-OA) is characterized by cartilage degradation and subchondral bone pathology, and is one of the most serious types of Temporomandibular Disorders (TMDs) [1]. Until now, no therapies have been developed that have an obvious impact on impeding or reversing the progression of TMJ-OA, mainly because of our limited understanding of its pathogenesis [2].

The subchondral bone and fibrocartilage form a functional entity termed the TMJ condyle to withstand the mechanical loading that occurs during jaw clenching and movement [3]. Normal biomechanical stress maintains the balance between the synthesis and degradation of the TMJ condyles, while abnormal mechanical loading can promote the onset of degenerative changes to the TMJ [4]. In previous studies, we have shown that abnormal loading of the TMJ induced by malocclusion could lead to OA-like changes in rat TMJ condyles [5]. During TMJ-OA progression, chondrocytes under shear stress express higher levels of pro-osteoclastic factors, which in turn induce loss of condylar subchondral bone via activation of osteoclastogenesis [6]. Conversely, other studies have shown that cells within the subchondral bone were more sensitive to the mechanical loading exerted on the joints [7,8]. Thus, it would be interesting to investigate the difference and common points between responses of chondrocyte and bone cells under the same mechanical environment.

Several Wnt proteins and their receptors are expressed in the cartilage, subchondral bone, and synovium, and have been shown to play important roles during OA progression [9-11]. Wnt5a

is a noncanonical Wnt-secreted ligand, and binds with its receptor, tyrosine kinase-like orphan receptor 2 (Ror2), to activate the signaling pathway [12]. It has been reported that interleukin-1 could upregulate the expression of Wnt5A in rabbit TMJ condylar chondrocytes, and Wnt5A signaling activation in human articular chondrocytes could induce the expression of several Matrix Metalloproteinases (MMPs) through JUN N-terminal Kinase (JNK) signaling [13,14]. In addition, Wnt5a-Ror2 signaling was activated in Bone Marrow Stromal Cells (BMSCs) of the condylar subchondral bone in TMJ-OA rats induced by abnormal mechanical loading [7]. These BMSCs promoted the migration and differentiation of osteoclast precursors, which in turn led to increased osteoclast activity and an overall TMJ subchondral trabecular bone loss in the TMJ-OA rats. These data suggested that Wnt5a-Ror2 signaling regulates critically the progression of cartilage degeneration and subchondral bone loss during TMJ-OA progression. However, whether the expression levels of Wnt5a and Ror2 in chondrocytes and BMSCs of TMJ condyles are regulated by mechanical loading and the signal pathway involved in this process remain unknown. Furthermore, it is also unknown whether blocking Wnt5a-Ror2 signaling could rescue the destruction of condylar cartilage and subchondral bone in TMJ-OA rats induced by abnormal mechanical loading.

Therefore, the present study aimed to investigate whether mechanical loading could induce increased expression of Wnt5a and Ror2 in primary chondrocytes from condylar cartilage and in BMSCs from subchondral bone, and if so, which signaling pathway(s) are involved in these processes. More importantly, whether blocking Wnt5a-Ror2 signaling could rescue the degenerative changes of TMJ condyles were tested. Fluid Flow Shear Stress (FSS) at different intensities and stimulation times was used to treat chondrocytes or BMSCs, and changes in the mRNA and protein levels of Wnt5a and Ror2 were examined [6]. The expression of Wnt5a and Ror2 is related to Signal Transducer and Activator of Transcription 3 (STAT3), Nuclear Factor kappa B (NF-κB), and Notch signaling pathways [15-17], inhibitors of these pathways were used to pretreat the cells before FSS to confirm the signaling pathway involved in FSS-induced expression of Wnt5a and Ror2. Furthermore, a soluble Ror2 fusion protein (sRor2) was prepared as previously reported, which could block Wnt5a-mediated signaling as a decoy receptor in several assay systems [17]. Whether injection of sRor2 into the local TMJ region could rescue TMJ degenerative remodeling was also investigated. The null hypotheses tested were as follows: (1) That FSS could not promote the expression of Wnt5a and Ror2 in chondrocytes and BMSCs; and (2) that periarticular injection of sRor2 could not rescue TMJ degenerative changes induced by abnormal loading.

Materials and Methods

The isolation of chondrocyte and BMSCs

All the following experimental procedures and the operation administered to the animals were approved by the Ethics Committee of Lanzhou University (2016023), and were performed according to the institutional guidelines. Primary chondrocytes from TMJ condylar cartilage and BMSCs from condylar subchondral bone were isolated from female rats at 3 weeks old, as described previously [7,13]. Primary chondrocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, and harvested BMSCs (passage 3) were cultured in DMEM/F12 medium (Hyclone, Logan, UT, USA).

Fluid Flow Shear Stress (FSS) treatment

Chondrocytes and BMSCs were subjected separately to FSS treatment using a Flexcell Streamer™ system (FX4000, Flexcellint, Burlington, NC, USA), as described previously [6]. Cells were stimulated by FSS for 30, 60, or 120 min, at intensities of 10, 20, or 30 dynes/cm^2, based on our previous study and the preliminary data [6]. For the pathway inhibition assay, the primary chondrocytes and BMSCs were pre-treated for 1 h with 10 μM Stattic (S7024, a selective STAT3 inhibitor), 10 μM SN50 (S6671, a selective NF-κB inhibitor), or 10 μM FLI-06 (S7399, a selective Notch inhibitor) in Alpha Minimal Essential Medium (α-MEM) to block the corresponding pathways. All inhibitors were obtained from Selleck Chemicals (Houston, TX, USA). The inhibitor pre-treatment time and concentration were determined in accordance with the manufacturer's instruction.

MTT assay

5 mg/mL MTT (20 μL; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution were added to the culture medium of the chondrocytes or BMSCs which were stimulated by FSS for 2 h, at intensities of 10, 20, or 30 dynes/cm^2, and the supernatant was then discarded. Each well of the 96-well plate was supplemented with 150 μL DMSO. Absorbance was measured at 450 nm. The percentage of the viable cells was calculated using the following formula:

(%) = [100 × (sample absorbance)/(control absorbance)].

Total cell number was determined for each experiment and the fold change was determined for each experiment.

Quantitative Real-Time reverse transcription Polymerase Chain Reaction (RT-PCR)

Total RNA from the FSS-treated cells or the controls were extracted and the mRNA levels of Wnt5a and Ror2 were detected using qRT-PCR, as described previously [6]. Primers for targeted genes were designed as follow: Wnt5a: forward, TCACTGGTGCTGCTATGTCA, reverse, GAGTGGGACTGGATTATGGG; Ror2: forward, TTGGGAACCGAACTATTTATGTGGA, reverse, CAAACTGCGAGCAC TGGTCTG; GAPDH: forward, GGCACAGTCAAGGCTGAGAATG, reverse, ATG GTGGTGAAGACGCCAGTA. The $2^{-\Delta\Delta Ct}$ formula was used to calculate the amount of target cDNA relative to that of GAPDH (encoding Glyceraldehyde-3-Phosphate Dehydrogenase), and the relative quantification of the target genes were compared among different groups.

Western blotting

Total protein from FSS-treated cells or the control was extracted and the protein levels of Wnt5a, Ror2 and Proliferating Cell Nuclear Antigen (PCNA)were detected using western blotting, as described previously [6]. The primary antibodies were anti-Wnt5A (1:300, sc-376249, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Ror2 (1:300, 88639, Cell Signaling Technology, Danvers, MA, USA) and anti-PCNA (1:50, Clone PC10, Labvision Ltd., USA). The relative protein levels were calculated by normalization to the level of β-actin (1:1000, 3700, Cell Signaling Technology) and compared among different groups.

Animal experiments

Sixty female Sprague–Dawley rats at 8 weeks old were purchased from the Lanzhou University Animal Center (Gansu, China). As previously described, disordered molar occlusion was created in the

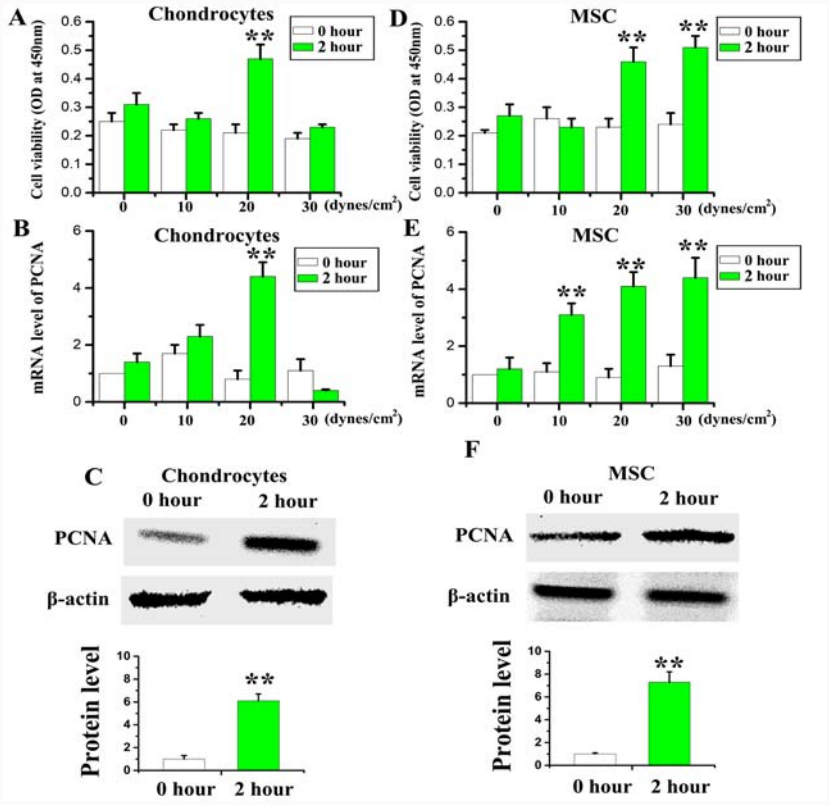

Figure 1: The viability and gene and protein levels PCNA of chondrocyte and BMSC in response to FSS. MTT assay of cell viability of chondrocytes (**A**) and BMSCs (**D**) stimulated by FSS for 2 h at 10, 20, or 30 dynes/cm^2. Gene (**B** and **E**) and protein (**C** and **F**) expression of PCNA by chondrocytes or BMSCs under FSS treatment for 2 h at intensities of 10, 20, or 30 dynes/cm^2. **P<0.01: *vs*. 0 h control group.

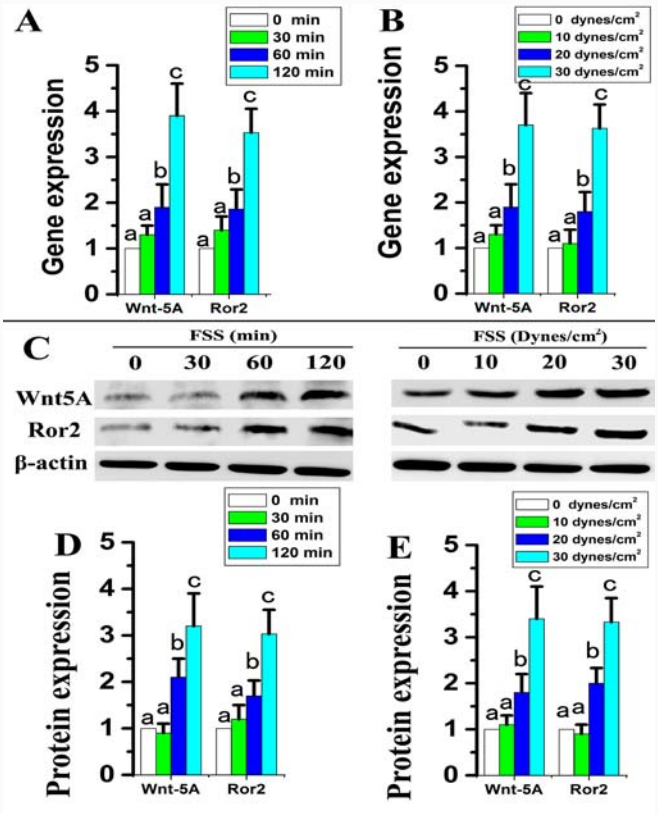

Figure 2: Chondrocyte gene expression in response to FSS. Gene (**A** and **B**) and protein (**C-E**) expression of Wnt 5A and Ror2 by chondrocytes under FSS treatment for 30, 60, and 120 min at intensities of 10, 20, or 30 dynes/cm^2.

experimental groups (Exp). In the sham Control groups (Con), rats received the same procedure, but their dental occlusions were not disturbed [5,6]. The Ror2 fusion protein (sRor2), composed of the extracellular region of Ror2, was prepared as described previously [17], and was confirmed to block Wnt5a-mediated signaling as a decoy receptor; however, it did not affect Wnt3a-mediated signaling. We administered Vehicle (Veh) and Ror2 fusion protein (sRor2) to the Exp and Con rats by TMJ local area injection as described previously. Fifty microliters containing 0.2 μg or 1 μg of sRor2, or the same volume of vehicle were respectively injected into the local TMJ regions of the Exp or Con rats once every week, from the first day of the experiment [17]. After 8 weeks, all experimental and control rats were sacrificed humanely.

Histochemical and immunohistochemical staining

TMJ tissue was paraffin-embedded and serial mid-sagittal sections at 5-μm thick were prepared from the paraffin-embedded tissue. The sections were stained with Hematoxylin and Eosin (H&E), toluidine blue, and Safranin O for histological evaluation (N=6) [6]. In addition, immunohistochemical staining was carried out as described previously. The primary antibodies were anti-collagen II (sc-7763; 1:75 dilution) and anti-MMP13 (sc-30073; 1:75 dilution), both purchased from Santa Cruz Biotechnology. Histomorphometric evaluations and comparisons were performed as reported previously (N=6) [6].

Micro-computed tomography

Bone Mineral Density (BMD) and microstructural trabecular parameters of the condylar subchondral bone were analyzed using micro-Computed Tomography (μCT) as described previously [6]. The microstructural trabecular parameters included bone volume fraction (BV/TV), Trabecular Thickness (Tb.Th), Trabecular Number (Tb.N), Trabecular Space (Tb.Sp), and Bone Surface to Volume ratio (BS/BV), which were determined using the MicroView Advanced Bone Analysis 2.1.2 software (GE Healthcare, Pittsburgh, PA, USA).

Statistical analyses

Histomorphometric measurements were performed blindly by two independent observers (JW and QM) using Photoshop CS 14.0 software (Adobe, San Jose, CA, USA). A high level of agreement between the two observers' data was observed (all r>0.9), and thus the mean of the two measurements was used for the statistical analysis. The Shapiro–Wilk test and Levene's test showed that the data had a normal distribution and homogeneity of variance. The statistical comparison among groups was performed using Analysis of Variance (ANOVA), and the post hoc comparison between groups were performed using Tukey's test. P-values less than 0.05 were considered statistically significant.

Results

FSS increased the expression of Wnt5a and Ror2 by chondrocytes and BMSCs in time- and intensity-dependent manners

Comparing to the un-stimulated controls, the viability of chondrocytes and their gene and protein expression of PCNA significantly increased when they stimulated by 20 dynes/cm² FSS for 2 h (Figures 1A-1C, all P<0.05), while those parameters of BMSCs increased significantly when they were stimulated by 20 and 30 dynes/cm² FSS comparing to the un-treated control (Figures 1D-1F, all P<0.05). The viability and levels of PCNA of chondrocytes or BMSCs didn't exhibit significant difference when they stimulated by 10 dynes/cm² FSS for 2 h, comparing to the un-stimulated controls (Figure 1, all P>0.05). When the chondrocytes were stimulated with 20 dynes/cm² FSS for different times, the mRNA and protein levels of

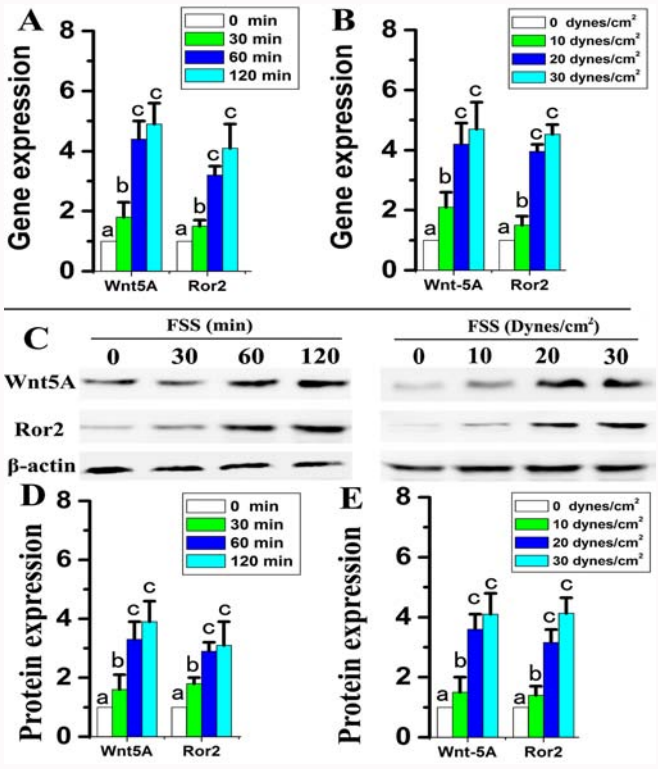

Figure 3: BMSC gene expression in response to FSS. Gene (**A** and **B**) and protein (**C-E**) expression of Wnt 5A and Ror2 by BMSCs under FSS treatment for 30, 60, and 120 min at intensities of 10, 20, or 30 dynes/cm².

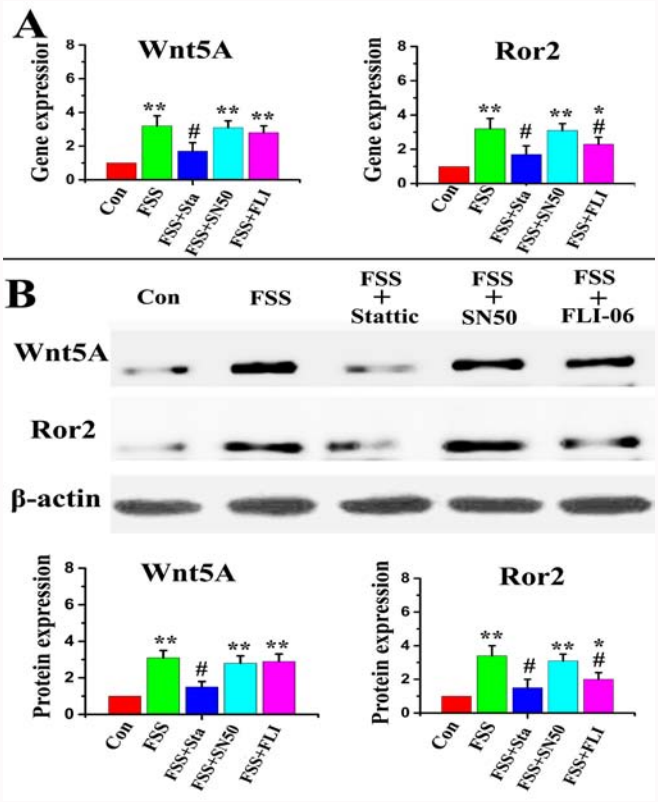

Figure 4: Chondrocyte gene expression in response to FSS and STAT3, NF-κB, and North inhibitors. Gene (**A**) and protein (**B**) expression of Wnt 5A and Ror2 by FSS-treated chondrocytes in combination with 10 μM Stattic (a selective STAT3 inhibitor), 10 μM SN50 (a selective NF-κB inhibitor), or 10 μM FLI-06 (a selective Notch inhibitor).

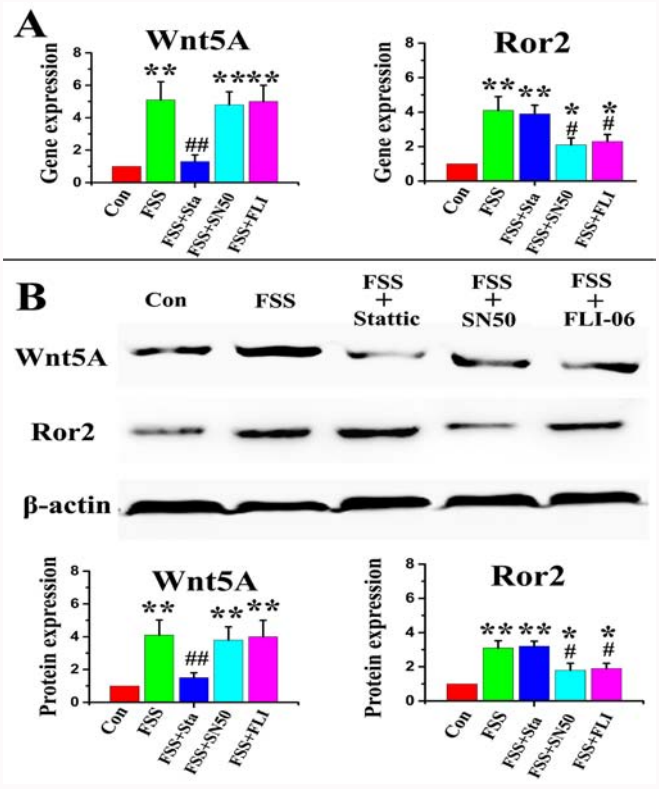

Figure 5: BMSC gene expression in response to FSS and STAT3, NF-κB, and North inhibitors. Gene (**A**) and protein (**B**) expression of Wnt 5A and Ror2 by FSS-treated BMSCs in combination with 10 μM Stattic (a selective STAT3 inhibitor), 10 μM SN50 (a selective NF-κB inhibitor) or 10 μM FLI-06 (a selective Notch inhibitor).

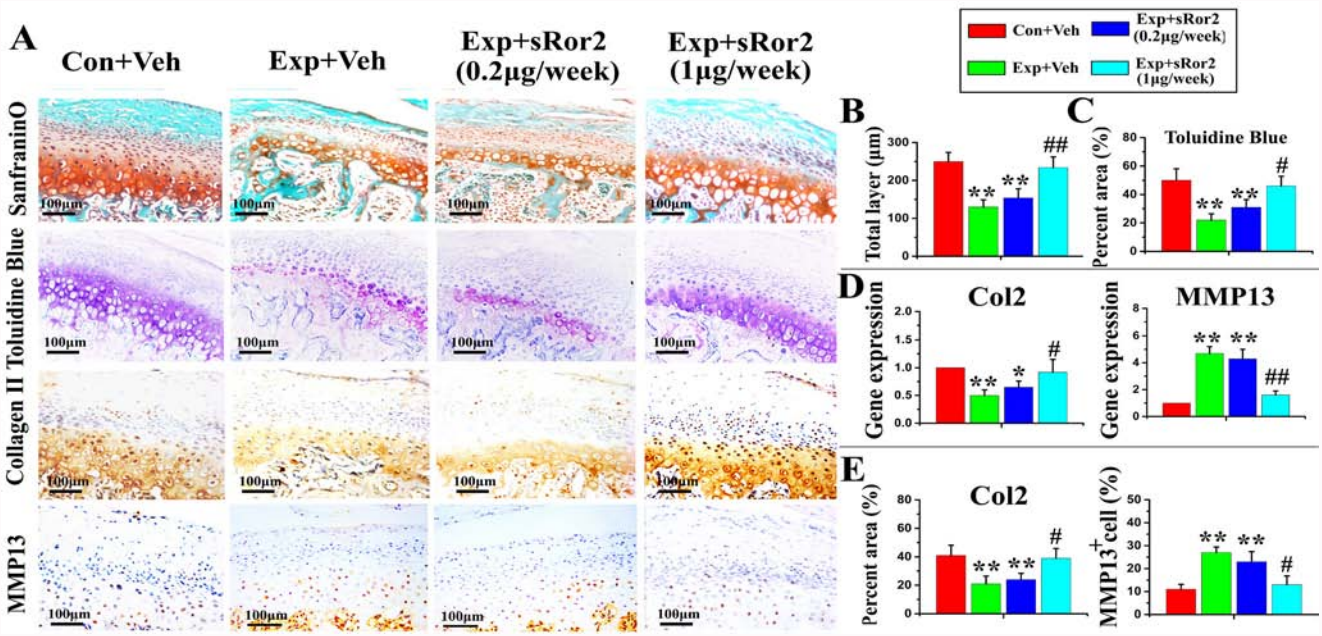

Figure 6: Condylar cartilage changes of control rats (Con) and experimental rats (Exp) treated with physiological saline (Veh), sRor2 (0.2 μg/per week) or sRor2 (1 μg/per week). (**A**) Representative images of safranin O-fast green (first row) and toluidine blue (second row) staining, collagen II (third row), and MMP13 (forth row) immunohistochemical staining of the mandibular condyle. (**B**) Comparison of the values of the thickness of the total cartilage layer. (**C**) Comparison of the values of the percent area of toluidine blue staining. (**D**) qRT-PCR analysis of the expression of Col2 (collagen II) and Mmp13 in the above condylar cartilage samples. (**E**) Quantification of the immunohistochemical staining of t collagen II (Col2) and MMP-13 in the above condylar cartilage samples. *P<0.05: *vs*. Con+Veh group; **P < 0.01: *vs*. Con+Veh group; #P < 0.05: *vs*. Exp++Veh group, ##P<0.01: *vs*. +Veh group.

Wnt5A and Ror2 peaked at 120 min of stimulation (Figure 2A, 2C, 2D; all P<0.01). The levels of Wnt5A and Ror2 increased significantly in the 60 min FSS-treated groups compared with those in the controls (all P<0.05), while those in the 30 min FSS-treated group did not exhibit a significant difference compared with those in the controls (P>0.05). When the chondrocytes were stimulated for 60 min at different intensities, the mRNA and protein levels of Wnt5A and Ror2 peaked at 30 dynes/cm^2 in the FSS-treated groups (Figure 2B, 2C, 2E; P<0.01). The levels of Wnt5A and Ror2 increased significantly in the 20 dynes/cm^2 FSS-treated groups compared with those in the controls (P<0.05), while those in the 10 dynes/cm^2 FSS-treated group did not exhibit a significant difference compared with those in the controls (Figure 2, P>0.05). When the BMSCs were stimulated with 20 dynes/cm^2 FSS for different times, the mRNA and protein levels of Wnt5A and Ror2 peaked in the 60 min FSS-treated group, and their levels were maintained in the 120 min FSS-treated groups (Figure 3A, 3C, 3D; P<0.05), while those in the 30 min FSS-treated group did not exhibit a significant difference compared with those in the controls (P>0.05). When the BMSCs were stimulated for 60 min at different intensities, the mRNA and protein levels of Wnt5A and Ror2 peaked in the 20 dynes/cm^2 FSS-treated groups and their levels were maintained in the 30 dynes/cm^2 FSS-treated groups (Figure 3B, 3C, 3E; P<0.05), while those in the 10 dynes/cm^2 FSS-treated group did not exhibit a significant difference compared with those in the controls (P>0.05). Therefore, the peak increases of Wnt5A and Ror2 expression in chondrocyte were at 120 min or 30 dynes/cm^2, while those of BMSCs were at 60 min or 20 dynes/cm^2, indicating that BMSCs are more sensitive to FSS than chondrocytes.

Signaling pathways involved in FSS-induced increased expression of Wnt5a and Ror2 by BMSCs and chondrocytes

In both chondrocytes and BMSCs, only the STAT3 inhibitor Stattic could significantly suppressed the FSS-induced increase in the gene and protein levels of Wnt5a (Figure 4, 5, all P<0.05); neither the NF-kappaB inhibitor SN50 nor the Notch inhibitor FLI-06 had any blocking effect on the FSS-induced increased in Wnt5a levels (all P>0.05). The gene and protein levels of Wnt5a did not exhibit a significant difference between the FSS+Stattic group and the control group (Figures 4, 5, all P>0.05). For the FSS-induced increased levels of Ror2, in chondrocytes, the STAT3 inhibitor Stattic and the Notch inhibitor FLI-06 both suppressed the FSS-induced increase in the gene and protein levels of Ror2 significantly (Figure 4, all p<0.05); however, the NF-kappaB inhibitor SN50 did not have any blocking effect (P>0.05). The gene and protein levels of Ror2 did not exhibit a significant difference between the FSS+Stattic group and the control group (Figure 4, P>0.05), while those in the FSS+FLI 06 group were still significantly higher than those of the control group (Figure 4A, P<0.05). In BMSCs, the NF-kappaB inhibitor SN50 or the Notch inhibitor FLI-06 both suppressed FSS-induced increased levels of Ror2 significantly (Figure 5, all P<0.05); however, the STAT3 inhibitor Stattic did not have any blocking effect (P>0.05). Both the gene and protein level of Ror2 in the FSS+SN50 and FSS+FLI-06 groups were significantly higher than those in the control group (Figure 5, P<0.05). Taken together, these results indicated that the FSS-induced increases in the levels of Wnt5a in both chondrocytes and BMSCs were regulated by the STAT3 pathway. However, the FSS induced increases in the levels of Ror2 in chondrocytes and BMSCs were regulated by different pathways: The former was regulated by the STAT3 and Notch pathways, whereas the latter was regulated by the NF-B and Notch pathways.

Periarticular injection GST-sRor2 largely rescued condylar cartilage degradation induced by abnormal occlusion

Condylar cartilage from rats in the Con+Veh groups was well

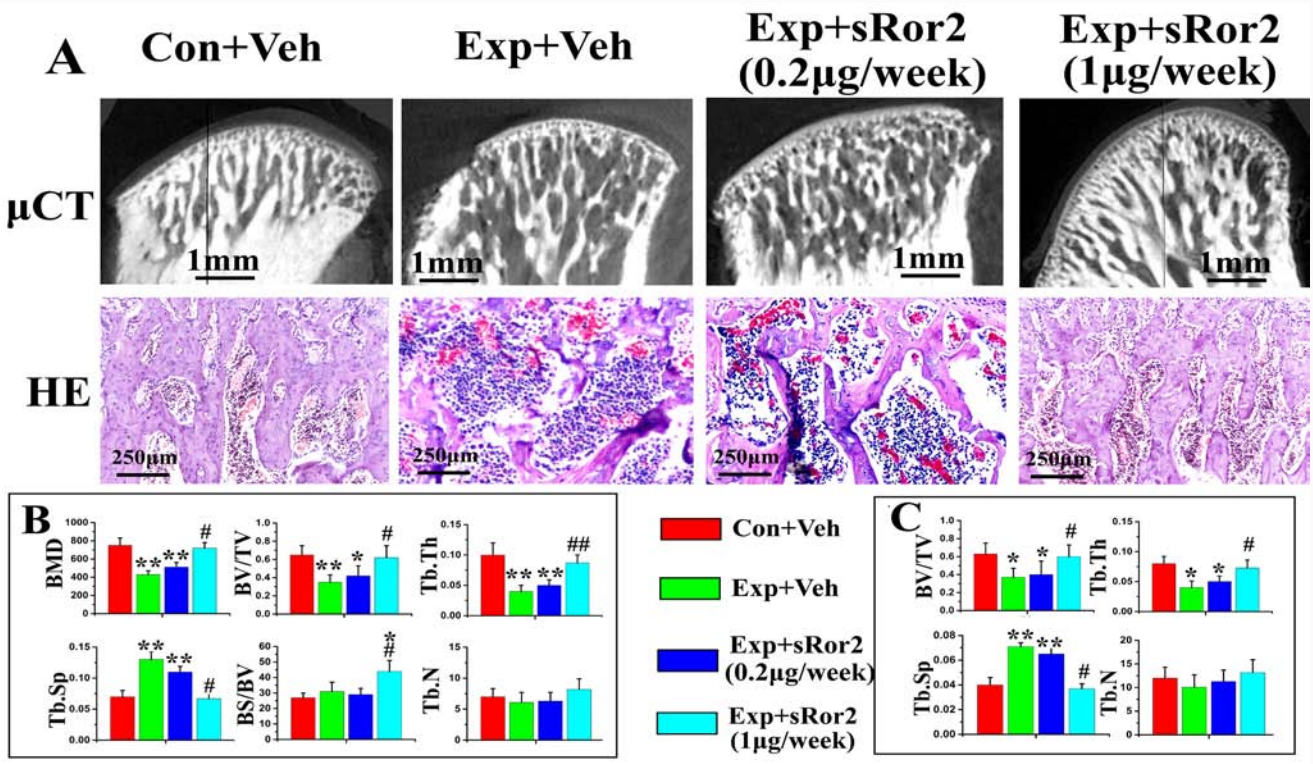

Figure 7: Condylar subchondral bone changes of Control rats (Con) and Experimental rats (Exp) treated with physiological saline (Veh), sRor2 (0.2 μg/per week) or sRor2 (1 μg/per week). (**A**) Representative images of micro-computed tomography (μCT, first row) and H&E staining (second row) of the mandibular condyle. (**B**) Analysis of Bone Mineral Density (BMD) and different parameters representing trabecular microstructures of the condylar subchondral bone based on the reconstructed μCT images. (**C**) Histomorphometric analysis of trabecular microstructure parameters of the condylar subchondral bone based on the images of H&E staining.

organized, and exhibited even and rich distribution of proteoglycans (Figure 6A). Degenerative cartilage changes were observed in both the Exp+Veh and the Exp+ sRor2 (0.2 μg/week) groups, in the form of cartilage thinning and loss of proteoglycans (Figure 6A). Data analyses revealed significant decreases in cartilage thickness and proteoglycan area in both the Exp+Veh and Exp+ sRor2 (0.2 μg/week) groups compared with those in the Con+Veh group (Figure 6B, 6C, all P<0.05). In addition, decreased mRNA and protein levels of collagen II and MMP13 were also observed in both the Exp+Veh and Exp+sRor2 (0.2 μg/week) groups compared with those in the Con+Veh group (Figure 6D, 6E, all P<0.05). Blocking of Wnt5a-mediated signaling by GST-sRor2 at 1 μg/week markedly reversed the cartilage degeneration in the experimental rats, as evidenced by the increased cartilage thickness, and proteoglycans and collagen II expression, and decreased expression of MMP13 levels in the condylar cartilage (Figure 6, all P<0.05). Cartilage thickness, and proteoglycans and collagen II distribution and expression were not significantly different between the Exp+sRor2 (1 μg/week) group and Con+Veh group (Figure 6, all P>0.05).

Periarticular injection GST-sRor2 largely rescued subchondral bone loss induced by abnormal occlusion

The condylar subchondral bone of rats in the Con+Veh groups was well aligned, and the bone marrow cavities were evenly distributed throughout the whole joint (Figure 7A). Local bone loss and enlarged bone marrow cavities were evident in both the Exp+Veh and Exp+sRor2 (0.2 μg/week) groups (Figure 7A). Data analyses revealed significant decreases in BMD, BV/TV, and Tb. Th (Figure 7B, 7C), but significant increases in Tb.Sp (Figure 6B, 6C), in both the Exp+Veh and Exp+sRor2 (0.2 μg/week) groups compared with those in the Con+Veh groups (all P<0.05). Similarly, blocking of Wnt5a mediated signaling by sRor2 at 1 μg/week markedly reversed the condylar subchondral bone loss in the experimental rats, as evidenced by the increased BMD, BV/TV, BS/BV, and Tb.Th, and decreased Tb.Sp, compared with those in the Exp+Veh and Exp+sRor2 (0.2 μg/week) groups (Figure 7, all P<0.05). The BMD and microstructural parameters of the condylar subchondral bone did show no significant differences between the Exp+sRor2 (1 μg/week) group and the Con+Veh group (Figure 7, all P>0.05).

Our results showed that both chondrocytes and BMSCs under shear tress expressed higher levels of Wnt5a and its receptor Ror2 via activation of the STAT3, NF-kappaB, and Notch pathways. More importantly, inhibition of Wnt5a-Ror2 signaling by sRor2 markedly reversed the cartilage degeneration and subchondral bone loss in TMJ-OA rats induced by abnormal mechanical loading.

Discussion

The metabolism and the functional properties of chondrocytes and BMSCs are influenced substantially by mechanical forces [18,19]. Abnormal mechanical loading could promote the degeneration of cartilage and subchondral bone by increasing the levels of catabolic factors in chondrocytes and BMSCs, such as prostaglandins 2, IL-6, and nitric oxide [20]. Here, we showed that FSS could increase the levels of Wnt5 and its receptor Ror2 in both chondrocytes and BMSCs, in a time and intensity-dependent manner. Thus, the first null hypothesis, that flow shear stress could not promote the expression of Wnt5a and Ror2 in chondrocytes and BMSCs, has to be rejected.

We have shown that abnormal mechanical loading on the TMJ induced by disturbed molar occlusion in rats could cause TMJ condyle degradation [5,6]. In addition, changes in mechanical loading, such as that caused by extended periods of bed rest or microgravity in space, are associated with altered bone remodeling and formation *in vivo* [21-23]. In the present study, our *in vitro* data showed that both chondrocytes and BMSCs are sensitive to FSS, which increased the expression of Wnt5a and its receptor Ror2 in a time and intensity-dependent manner. Interestingly, in response to FSS treatment, the peaks of the increases in Wnt5A and Ror2 expression by chondrocytes were at 120 min or 30 dynes/cm^2, while those of BMSCs were at 60 min or 20 dynes/cm^2, indicating that BMSCs are more sensitive to the FSS than chondrocytes. These data support those of previous studies, which showed that subchondral bone changes precede cartilage degeneration during OA initiation and thus play important roles in cartilage degeneration [23]. Further studies are needed to clarify the mechanism involved in the different sensitivity to mechanical loading between chondrocytes and BMSCs.

Soluble Ror2 fusion protein (sRor2), comprising the extracellular region of Ror2, has been used successfully to block Wnt5a-mediated signaling *in vitro* and *in vivo* [17]; however, it is unknown whether blocking Wnt5a-Ror2 signaling by sRor2 could rescue the TMJ degradation induced by abnormal loading. In the present study, although sRor2 at 0.2 μg/week had no obvious rescue effects on the degradation of TMJ condyle in the experimental rats, sRor2 at 1 μg/week markedly reversed condyle degradation, as evidenced by the increased cartilage thickness and percentage area of proteoglycans and type II collagen; the decreased expression of MMP13 in condylar cartilage specimens; and the increased BMD, BV/TV, Tb.Th, and decreased Tb.Sp in the subchondral bone, when compared with those in the Exp+Veh groups. Therefore, the second null hypothesis, that periarticular injection of sRor2 could not rescue TMJ degenerative remodeling induced by abnormal loading, has also to be rejected.

Wnt5a-Ror2 signaling, by promoting chondrocyte differentiation and inhibiting chondrocyte maturation, regulates cartilage development and metabolism. Wnt5a is the most upregulated Wnt family member in the knee cartilage of OA mice induced by destabilization of the medial meniscus [24-26]. In addition, chondrocyte expression and release of Wnt5a was stimulated by Fibronectin fragments (FN-f), a matrix fragment found in OA cartilage and synovial fluid, which has been shown to stimulate catabolic signaling [27,28]. Furthermore, Wnt5a treatment reduced aggrecan expression, but promoted MMP1 and MMP13 protein production, in human chondrocytes [13]. In the present study, we added to the current knowledge by showing that inhibiting of Wnt5a-Ror2 signaling by sRor2 at 1 μg/week greatly attenuated cartilage degenerative changes and subchondral bone loss in TMJ-OA rats. These data were agreed with the results of a previous study, which showed that intraperitoneal injection of sRor2 at 2 μg per day for 19 days suppressed subchondral bone destruction in a type II collagen-induced arthritis and collagen-specific antibody-induced arthritis mouse model [17]. Taken together, these data suggested that the Wnt5a-Ror2 pathway is crucial for cartilage degradation and subchondral bone loss in OA, and represents a therapeutic target in OA.

The aim of the present in vitro study is to investigated the mechanism of FSS-induced increased expression of Wnt5a and Ror2. In terms of the mechanism regulating the expression of Wnt5a, it has been reported that the STAT3 pathway could activate WNT5A expression via binding to its promoter, while transfection of chronic lymphocytic leukemia cells with a STAT3-targeted short hairpin RNA downregulated Wnt5a mRNA and protein levels [15,29]. In addition, it has been reported that WNT5a and ROR2 transcription is influenced by other mechanisms, such as NF-B and Notch signaling cascades [15,17,30,31]. Our inhibition experiments showed that the FSS induced increases in Wnt5a levels in both chondrocytes and BMSCs were regulated by the STAT3 pathway. However, the FSS-induced increase in the expression of Ror2 in chondrocytes and BMSCs was regulated by different pathways: In chondrocytes, it was regulated by STAT3 and Notch pathways, whereas in BMSCs, it was regulated by the NF-B and Notch pathways. Further studies are needed to investigate whether inhibition of the STAT3 pathway could reverse TMJ-OA induced by abnormal mechanical loading.

In conclusion, our results showed that both chondrocytes and BMSCs under shear tress expressed higher levels of Wnt5a and its receptor Ror2 *via* activation of the STAT3, NF-kappaB, and Notch pathways. More importantly, inhibition of Wnt5a-Ror2 signaling by sRor2 markedly reversed the cartilage degeneration and subchondral bone loss in TMJ-OA rats induced by abnormal mechanical loading.

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