OSM is overexpressed in knee osteoarthritis and Notch signaling is involved in the effects of OSM on MC3T3-E1 cell proliferation and differentiation

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Abstract. Knee osteoarthritis (OA) is the most prevalent type of OA and the cytokine, oncostatin M (OSM), may contribute to the pathogenesis of OA. However, the exact role of OSM in the development of knee OA and the underlying mechanisms are not yet fully understood. This study was designed to detect the expression of OSM in the synovial tissue of patients with knee OA. Furthermore, we investigated whether Notch signaling is involved in the effects of OSM on MC3T3-E1 cell proliferation and differentiation. The synovial tissue of the knee joint was collected from 32 patients with knee OA. We detected OSM mRNA and protein expression (by RT-qPCR and western blot analysis, respectively) in the synovial tissue of the knee joint, and the expression level of OSM was higher in the patients with knee OA compared with the controls. MTT assay was used in the *in vitro* experiments to determine MC3T3-E1 cell proliferation, and cell differentiation was determined by measuring alkaline phosphatase (ALP) activity and osteocalcin (OCN) expression. The results from our *in vitro* experiments revealed that OSM induced bone formation by increasing osteoblast cell proliferation and differentiation. In addition, the expression levels of Notch ligand, receptor and target gene, including Delta-like 1 (Dll1), Notch homolog 1 (Notch1) and Hes family bHLH transcription factor 1 (Hes1) were decreased following treatment with OSM in a time-dependent manner in the MC3T3-E1 cells. A Dll1 overexpression vector was transfected into the cells to activate Notch signaling, and the results revealed that the activation of Notch signaling attenuated the effects of OSM on MC3T3-E1 cell proliferation and differentiation. In conclusion, our data demonstrate that elevated levels of OSM in synovial tissue induce bone formation by increasing osteoblast cell proliferation and differentiation. The Notch signaling pathway was found to be one of the signaling pathways that inhibit OSM-induced MC3T3-E1 cell proliferation and differentiation. The findings of this study may broaden our understanding of the mechanisms behing the role of OSM in the development of knee OA.

Introduction

Osteoarthritis (OA) is a musculoskeletal disorder (1) accounting for 3% of total number of years of living with a disability, particularly in the elderly (2,3). Knee OA is the most prevalent type of OA (4). It is characterized by the progressive degeneration and structural disorder of the articular cartilage, resulting in loss of joint space, accompanied by marginal and central new bone formation (5).

Over the past 30 years, a number of cytokines have been identified to be involved in OA, particularly interleukin (IL)-1 and IL-6 (6). Oncostatin M (OSM) is a glycoprotein which belongs to the IL-6 family of cytokines. It was firstly purified and biochemically characterized for its anti-proliferative effects on the A375 human melanoma cell line (7). An increasing amount of evidence has indicated that OSM participates in a variety of biological activities, such as differentiation, inflammation, development and the enhancement of metastatic capacity (8-11).

Osteoblasts and stromal cells isolated from the subchondral femoral heads of patients with OA express OSM (12). Mice transfected with the OSM gene have been shown to develop prominent characteristics of arthritis, such as joint inflammation, bone cell apoptosis, chondrophyte formation and the depletion of articular cartilage proteoglycans (13). However, the exact role of OSM in the development of OA and the underlying mechanism are not yet fully understood. To the best of our knowledge, this study is the first to detect the expression of OSM in the synovial tissue of patients with knee OA. Furthermore, a new signaling pathway was found to participate in the effects of OSM on MC3T3-E1 cell proliferation and differentiation. This study may broaden our understanding of the mechanisms behind the role of OSM in the development of knee OA.

Materials and methods

Sample collection. The present study was approved by the Ethics Committee of Beijing Shijitan Hospital, Capital Medical University and all the patients provided informed written consent prior to participation in the study. The OA synovial tissue of the knee joint was obtained from 32 patients with OA who underwent total knee replacement and arthroscopy. Normal control

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synovial tissue of the knee joint was obtained from 12 patients with a discoid meniscus. The tissues were immediately stored at -80°C until use in western blot analysis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Cell culture and transfection. The mouse osteoblast cell line, MC3T3-E1, was purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (both from Gibco-BRL Life Technologies, Grand Island, NY, USA) at 37°C in a humidified atmosphere with 5% CO_2. OSM was purchased from R&D Systems (Minneapolis, MN, USA) and was diluted with phosphate-buffered saline (PBS) into the indicated concentrations. The full-length cDNA fragment of the mouse Dll1 gene was amplified and cloned into the pEGFP-C1 vector (Clontech Laboratories, Inc., Mountain View, CA, USA) at the *Bgl*II and *Bam*HI sites to generate the Dll1 overexpression vector. The Dll1 overexpression vector was transfected into the MC3T3-E1 cells using Lipofectamine 2000 (Invitrogen Life Technologies, Grand Island, NY, USA) following the manufacturer's instructions.

Measurement of alkaline phosphatase (ALP) activity. ALP activity was determined in the MC3T3-E1 cells in U/ml sample using an Alkaline Phosphatase Activity Colorimetric Assay kit (BioVision Inc., Mountain View Milpitas, CA, USA) according to the manufacturer's instructions. Briefly, the cells were homogenized in the assay buffer and centrifuged at 13,000 x g for 3 min. Different volume of samples were added into a 96-well plate and assay buffer was used to increase the total volume to 80 μl. A total of 50 μl of the 5 mM pNPP solution was added to each well followed by incubation for 60 min at room temperature. A standard curve was created by the addition of 1 mM pNPP 0, 4, 8, 12, 16 and 20 μl into a 96-well plate in duplicate to generate 0, 4, 8, 12, 16 and 20 nmol/well pNPP standard followed by the addition of 10 μl of ALP enzyme solution to each well containing the pNPP standard. Subsequenlty, the reactions were terminated by the addition of 20 μl Stop Solution and the optical density (OD) was measured at 405 nm using a microplate reader (Ascent 354; Thermo Labsystems, Waltham, MA, USA).

MTT assay. The MC3T3-E1 cells were seeded into a 96-well plate and allowed to grow for 24 h. Following treatment with various concentrations of OSM (5-100 ng/ml) for the appropriate periods of time, 50 μl MTT solution (Beyotime, Shanghai, China) were added to the well followed by incubation at 37°C for 4 h. Formazin granules were dissolved with 150 μl DMSO (Sigma, St. Louis, MO, USA) and the OD at 570 nm was measured using a microplate reader (Ascent 354; Thermo Labsystems).

RT-qPCR. Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). A total of 2 μg of RNA was reverse transcribed using a cDNA synthesis kit (RevertAid™ First Strand cDNA Synthesis kit; Fermentas, Vilnius, Lithuania). Quantitative (real-time) PCR was performed using the SYBR PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on the Applied Biosystems

7900HT Fast Real-Time PCR system. The relative expression level was calculated using the comparative Ct method.

Western blot analysis. Total protein was separated using ice-cold lysis buffer [1 mM EDTA, 20 mM Tris-HCl (pH 7.5), 10 mg/ml soybean trypsin inhibitor, 15 mM CHAPS, 0.05% Tween-20 and 10 mM PMSF] and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred onto a PVDF membrane (EMD Millipore Corp., Billerica, MA, USA). The membrane was then blocked with 5% BSA at 4°C overnight and incubated with antibodies against OSM (Cat. no. sc-50296), Notch homolog 1 (Notch1; Cat. no. sc-9170), Hes family bHLH transcription factor 1 (Hes1; Cat. no. sc-25392) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), osteocalcin (OCN; Cat. no. ab93876), Delta-like 1 (Dll1; Cat. no. ab10554) (Abcam, Cambridge, MA, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cat. no. 10494-1-AP) (Proteintech Group Inc., Chicago, IL, USA) at 37°C for 2 h. After washing with PBS, the membrane was incubated with horseradish peroxidase (HRP)-labeled antibody (Cat. no. sc-2004) at 37°C for 1 h. Immunoreactive bands were visualized using a chemiluminescence-based detection system (ECL Western Blotting Detection kit; Pierce Biotechnology, Inc., USA).

Statistical analysis. Data were shown as the means ± SD of at least 3 independent studies. The Student's t-test was used to assess differences between 2 groups. P-values <0.05 were considered to indicate statistically significant differences.

Results

Expression of OSM in the synovial tissue of the knee joint. OSM mRNA and protein expression in the synovial tissue of the knee joint was detected by RTqPCR and western blot analysis. The synovial tissue of the knee joint obtained from patients with discoid menisci was used as a control (Fig. 1). The relative mRNA expression level of OSM was significantly increased in the patients with OA compared with the controls (P<0.01; Fig. 1A). The results from western blot analysis revealed that the OSM protein expression level was also higher in the synovial tissue of patients with knee OA compared to that of the controls (P<0.01; Fig. 1B).

Effect of OSM on MC3T3-E1 cell proliferation. Following 2 and 4 days of treatment with various concentrations of OSM, MC3T3-E1 cell proliferation was measured by MTT assay. We found that treatment with 5 ng/ml OSM did not affect cell viability following 2 and 4 days of treatment (P>0.05). However, treatment with OSM at 10-100 ng/ml significantly increased MC3T3-E1 cell viability following 2 and 4 days of incubation (Fig. 2).

Effect of OSM on MC3T3-E1 cell differentiation. The MC3T3-E1 cells were incubated for 3 days with various concentrations of OSM, and the early- and middle-stage osteogenic markers, ALP activity and OCN expression, respectively, were then examined. Treatment with OSM did not induce ALP activity and OCN at the concentration of 5 ng/ml. Treatment with OSM at 10-100 ng/ml induced ALP activity with the
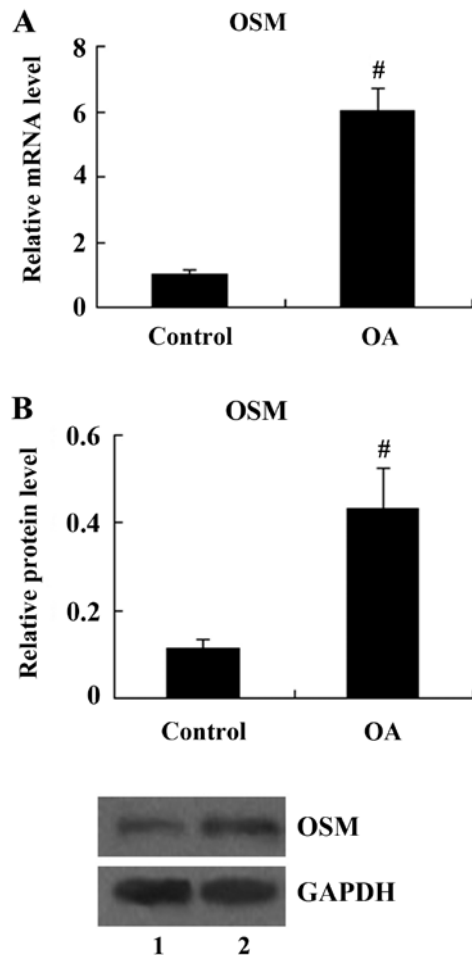
Figure 1. Expression of OSM. (A) mRNA and (B) protein expression in the synovial tissue of the knee joint in patients with OA. #P<0.01 vs. control. Lane 1, control; lane 2, OA. OSM, oncostatin M; OA, osteoarthritis.
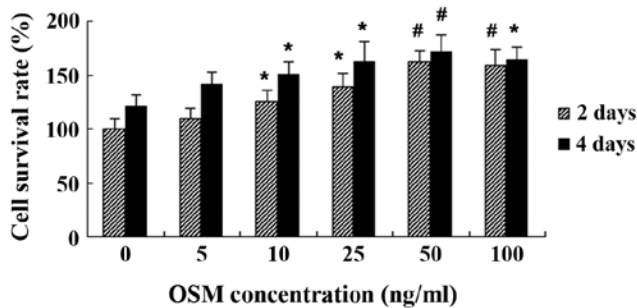

Figure 2. Effect of OSM on MC3T3-E1 cell proliferation. *P<0.05 and #P<0.01 vs. 0 ng/ml. OSM, oncostatin M.

Figure 3. Effect of OSM on ALP activity. *P<0.05 and #P<0.01 vs. 0 ng/ml. OSM, oncostatin M; ALP, alkaline phosphatase.

Figure 4. Effect of OSM on OCN (A) mRNA and (B) protein expression. *P<0.05 and #P<0.01 vs. 0 ng/ml. Lane 1, 0 ng/ml OSM; lane 2, 5 ng/ml OSM; lane 3, 10 ng/ml OSM; lane 4, 25 ng/ml OSM; lane 5, 50 ng/ml OSM; lane 6, 100 ng/ml OSM. OSM, oncostatin M; OCN, osteocalcin.

maximum effect (peak value) being observed at the dose of 50 ng/ml (Fig. 3). OCN mRNA and protein expressin were also induced following treatment with increasing concentrations of OSM. The increase in its expression was observed with OSM at the dose of 10-100 ng/ml (Fig. 4).

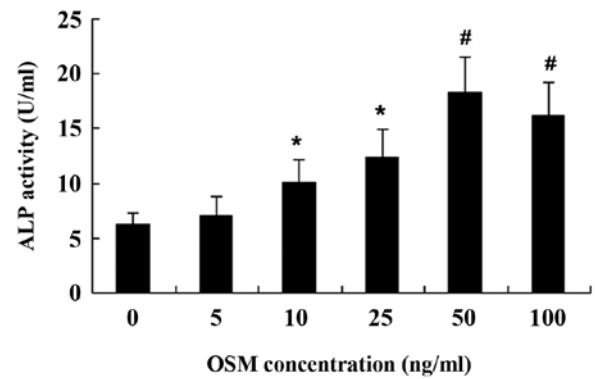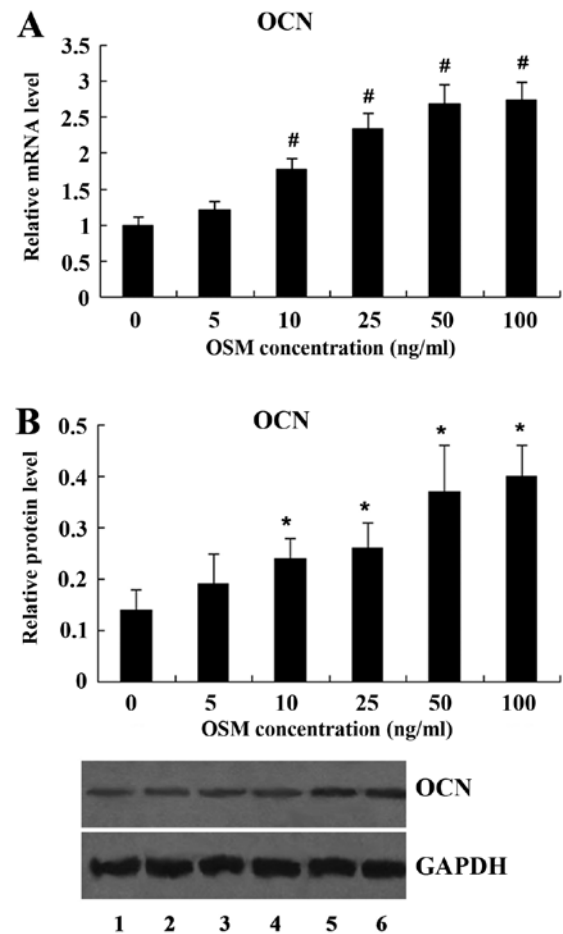*Effect of OSM on the expression of Notch signaling molecules.* The MC3T3-E1 cells were incubated with 50 ng/ml OSM for 1-4 days, and the expression of Dll1, Notch1 and Hes1 was determined by western blot analysis. OSM at the concentration of 50 ng/ml inhibited the expression of Dll1, Notch1 and Hes1 following treatment for 1-4 days compared with the untreated cells (Fig. 5).

Activation of Notch signaling attenuates the effects of OSM on MC3T3-E1 cell proliferation. Following transfection with
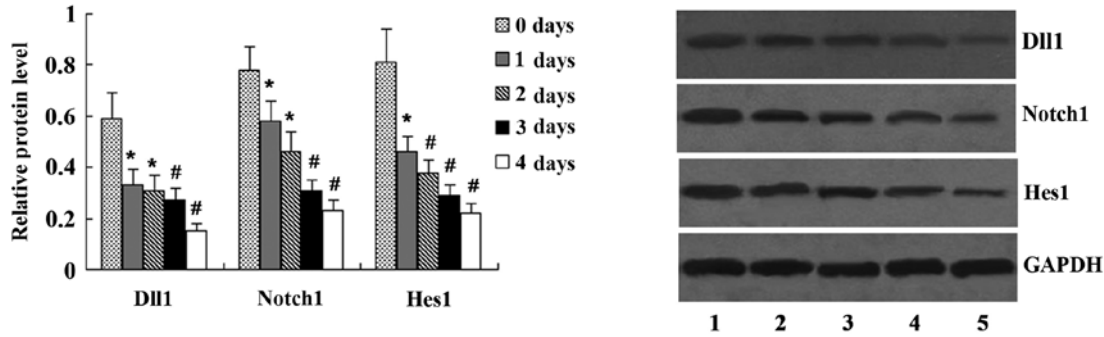
Figure 5. Effect of OSM on the protein expression of Notch signaling molecules. *P<0.05 and #P<0.01 vs. 0 day. Lane 1, 0 day; lane 2, 1 day; lane 3, 2 days; lane 4, 3 days; lane 5, 4 days. Dll1, Delta-like 1; Notch1, Notch homolog 1; Hes1, Hes family bHLH transcription factor 1.
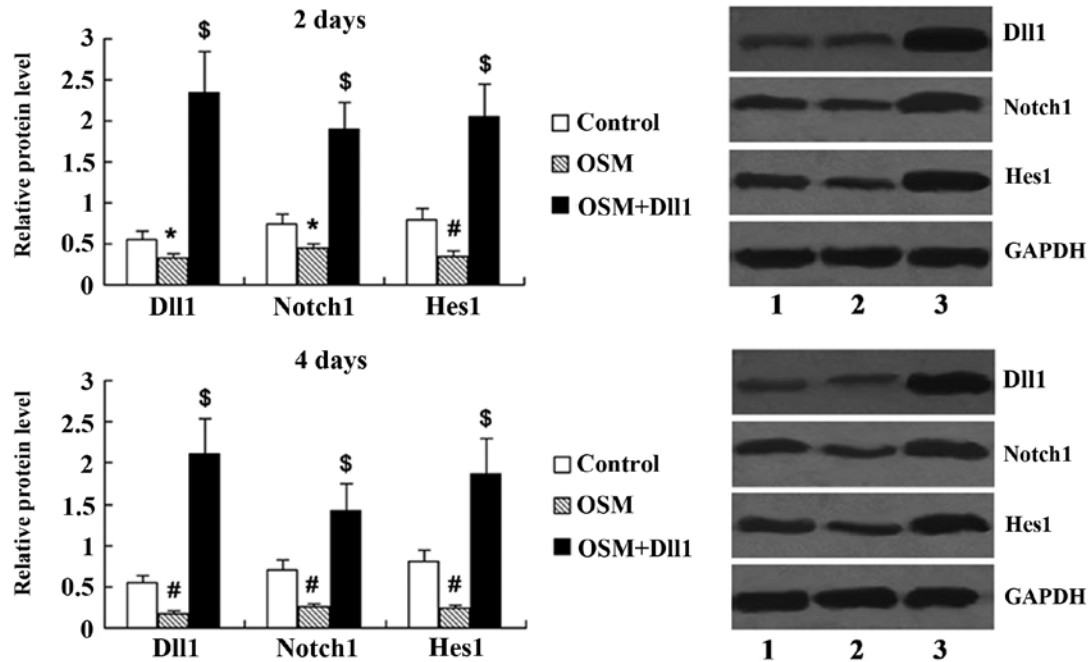

Figure 6. Expression of Notch signaling molecules in MC3T3-E1 cells transfected with the Dll1 overexpression vector followed by incubation with OSM for 2 and 4 days. *P<0.05 and #P<0.01 vs. control group; $P<0.01 vs. OSM group. Lane 1, control; lane 2, OSM; lane 3, OSM + Dll1. OSM, oncostatin M; Dll1, Delta-like 1; Notch1, Notch homolog 1; Hes1, Hes family bHLH transcription factor 1.
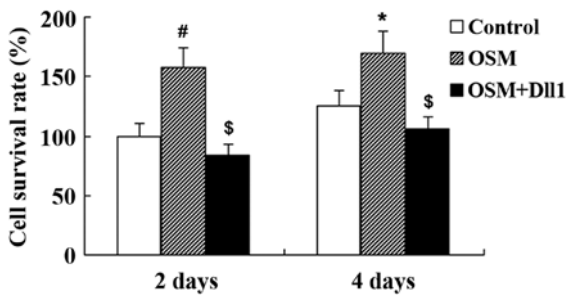

Figure 7. Activation of Notch signaling attenuates the effects of OSM on MC3T3-E1 cell proliferation. *P<0.05 and #P<0.01 vs. control group; $P<0.01 vs. OSM group. OSM, oncostatin M; Dll1, Delta-like 1.

with OSM for 2 and 4 days were determined by western blot analysis. The protein expression of Dll1 was significantly upregulated in the OSM + Dll1 group compared with the OSM group (P<0.01; Fig. 6). Notch1 and Hes1 expression was also significantly increased in the MC3T3-E1 cells transfected with the Dll1 overexpression vector (P<0.01).

We then investigated whether the activation of Notch signaling affects the OSM-induced MC3T3-E1 cell proliferation. Cell viability was assessed by MTT assay following treatment with 50 ng/ml OSM. We found that cell viability was significantly increased in the OSM-treated group compared with the control group (P<0.01). However, the activation of Notch signaling led to a decrease in OSM-induced cell proliferation; cell viability was significantly decreased in the OSM + Dll1 group compared with the OSM group (P<0.01; Fig. 7).

Activation of Notch signaling attenuates the effects of OSM on MC3T3-E1 cell differentiation. The MC3T3-E1 cells were
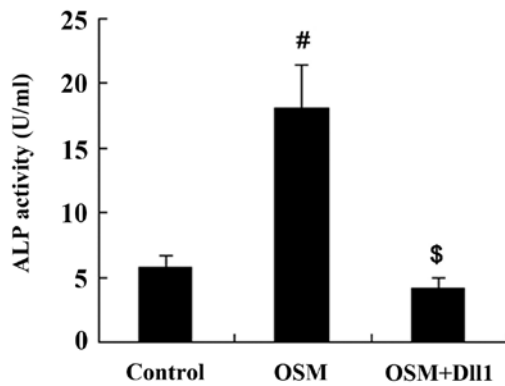
the Dll1 overexpression vector for 48 h, the expression levels of Dll1, Notch1 and Hes1 in the MC3T3-E1 cells treated

Figure 8. Activation of Notch signaling attenuates the effects of OSM on ALP activity. [#]P<0.01 vs. control group; [$]P<0.01 vs. OSM group. OSM, oncostatin M; ALP, alkaline phosphatase; Dll1, Delta-like 1.
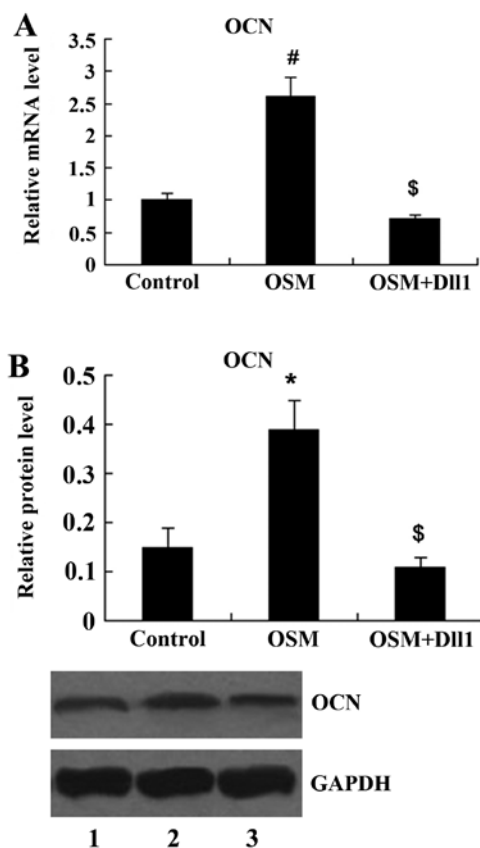

Figure 9. Activation of Notch signaling attenuates the effects of OSM on OCN (A) mRNA and (B) protein expression. [*]P<0.05 and [#]P<0.01 vs. control group; [$]P<0.01 vs. OSM group. Lane 1, control; lane 2, OSM; lane 3, OSM + Dll1 OSM, oncostatin M; OCN, osteocalcin; Dll1, Delta-like 1.

treated with 50 ng/ml OSM for 3 days following transfection with the Dll1 overexpression vector. Subsequently, the cells were harvested for the measurement of ALP activity and OCN expression. The results revealed that both ALP activity and OCN expression in the MC3T3-E1 cells were increased following treatment with OSM. However, the activation of Notch signaling attenuated the effects of OSM on MC3T3-E1 cell differentiation; compared with the OSM group, both ALP

activity and OCN expression were decreased in the OSM + Dll1 group (P<0.01; Figs. 8 and 9).

Discussion

Previous studies have suggested that OSM contributes to the pathogenesis of OA. It has been demonstrated that OSM over-expression in the mouse knee joint induces changes to the joint that resemble OA, including cartilage destruction and periosteal bone formation similar to osteophytes (14,15). In the present study, we found that OSM was detected in the synovial tissue of the knee joint, and the expression level of OSM was higher in patients with knee OA compared with the controls. This suggests that OSM contributes to the development of knee OA.

OSM has been shown to stimulate osteoblast differentiation by stromal cells and to reduce the ability of stromal cells to differentiate into adipocytes (16,17). It also drives the formation of osteoclasts, particularly under pathological conditions. It has been documented that osteoblasts play essential roles in bone remodeling in arthritis (18). In the present study, we performed *in vitro* experiments using MC3T3-E1 cells (mouse osteoblasts) to investigate the role of OSM on MC3T3-E1 cell proliferation and differentiation. It was demonstrated that OSM affected MC3T3-E1 cell proliferation in a concentration-dependent manner. The results of specific ALP activity and OCN, as indicators of osteogenic differentiation revealed that OSM induced MC3T3-E1 differentiation. These findings demonstrate that OSM induces bone formation by increasing osteoblast cell proliferation and differentiation.

Notch signaling is a highly conserved pathway (19) regulated by interactions between neighboring cells. It is crucial for the regulation of a number of cellular processes, including proliferation, differentiation, apoptosis and cell death during embryogenesis, as well as the development and renewal of adult tissues (20,21). Notch signaling has also been implicated in regulating articular cartilage homeostasis during adult life (22,23). It has been demonstrated that several Notch signaling molecules are abundantly expressed in OA (23), and Notch signaling is activated in OA cartilage (24). Our findings suggest a connection between OSM and Notch signaling. We found that the expression of Notch ligand, receptor and target gene, including Dll1, Notch1 and Hes1 was decreased following treatment with OSM in a time-dependent manner in the MC3T3-E1 cells. We then investigated the hypothesis that Notch signaling plays a role in the effects of OSM on MC3T3-E1 cell proliferation and differentiation.

Dll1 is able to activate the Notch1 receptor, leading to the activation of endogenous Hes1 genes (25) and several studies have demonstrated the inhibitory effects of Notch1 on osteo-blastic cell differentiation (26-28). In the present study, the Dll1 overexpression vector was used to activate Notch signaling and the results revealed that the activation of Notch signaling attenuated the effects of OSM on MC3T3-E1 cell proliferation and differentiation.

In conclusion, elevated levels of OSM in the synovial tissue may induce bone formation by increasing osteoblast cell prolif-eration and differentiation. OSM exerts an inhibitory effect to Notch signaling, and the OSM-induced MC3T3-E1 prolifera-tion and differentiation may be reversed by the activation of Notch signaling. It may prove useful if future directions in

the research and treatment of OA focus on the upstream and downstream molecules of Notch signaling that modulate the initiation and progression of OA.

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