

Original Article

Upregulation of notch signaling in hBMSCs promotes the proliferation and differentiation of pre-osteoclasts

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Abstract: During bone remodeling, osteoblasts and osteoclasts have a mutual and coefficient influence on each other. However, the specific mechanisms by which they interact are unclear and need further exploration. We have demonstrated that the upregulation of Notch signaling in human bone marrow mesenchymal stem cells (hBMSCs) can improve cell proliferation and promote the differentiation of hBMSCs to osteoblasts. It also has an indirect effect on the proliferation and differentiation of pre-osteoclasts. Specifically, the upregulation of Notch signaling in the hBMSCs of OP (osteoporosis patients) induced expression and secretion of M-CSFs. The conditioned medium (CM) of OP-hBMSCs increased the number of multinuclear osteoclasts and the speed of proliferation after Notch upregulation. Furthermore, upregulation of Notch signaling increased the RANKL/OPG ratio and promoted osteoclastogenesis. Our study reveals the possible mechanism of the osteoblast-osteoclast interaction during the bone remodeling process and implicates the Notch signaling pathway as a potential target for the treatment of osteoporosis.

Keywords: Notch signaling, hBMSCs, osteoclast proliferation, osteoclast differentiation

Introduction

Osteoporosis is a common bone disease characterized by decreased bone mass and an increased risk of fracture. Osteoporotic fractures carry an enormous public health burden in that they are associated with mortality and morbidity [1, 2]. Osteoporosis causes bones to become so weak and brittle that a fall or even mild stresses, such as bending over or coughing, can cause a fracture. Osteoporosis is so common that approximately 1 out of 2 elderly Caucasian women have had a fracture, and 54 million Americans are currently living with or are at-risk for osteoporosis and low bone mass. Studies on the cells that control bone remodeling continue to yield insights into the underlying causes of osteoporosis and point to possible new therapeutic targets. However, the pathogenic mechanisms of osteoporosis have not been completely elucidated. It is still a prodigious challenge for clinicians to reduce the occurrence and development of osteoporosis.

Bone remodeling involves the coupling of osteoclastic bone resorption and osteoblastic bone formation, and abnormalities in this process lead to osteoporosis. Bone resorption is carried out by osteoclasts, which play a very important role in promoting bone remodeling and maintaining a balance throughout the process. Some studies showed that BMSCs have a profound effect on the cytogenesis, proliferation, differentiation, and function of osteoclasts by affecting signaling molecules, such as CXCL12/CXCR4 [3, 4]. In addition, Wang *et al.* found that CGRP signaling maintains bone mass by directly stimulating stromal cell osteoblastic differentiation and by inhibiting RANKL-induced NF-kappaB activation, osteoclastogenesis, and bone resorption [5]. Both of these studies identified RANKL, which is secreted by BMSCs, as the key regulator of osteoclastic differentiation and activation. However, the concrete molecular mechanism by which BMSCs and osteoclasts interact is still not clear.

Notch signaling is evolutionarily conserved and plays important roles in both developmental processes and adult mature tissue homeostasis. Additionally, Notch is an important signaling pathway in embryonic and postnatal bone development and in the pathogenesis of bone diseases. Loss-of-function studies of Notch signaling revealed an age-dependent osteoporotic phenotype in mice. The upregulation of Notch signaling may represent a potential approach for increasing bone formation over bone resorption as well as for inhibiting osteoclastogenesis. However, the effect of Notch on other cellular compartments, such as the mesenchymal stem cell pool, would have to be considered. Our previous *in vitro* study found that the expression of Notch signaling in the hBMSCs of healthy individuals is greater than that in the hBMSCs of osteoporotic patients [6]. After upregulating Notch signaling in the hBMSCs of osteoporotic patients by transfecting the cells with lentivirus (Lv) carrying NICD1, we observed an increase in the proliferation and osteogenic differentiation of hBMSCs [6]. However, it remains unknown whether upregulating Notch signaling in OP-hBMSCs could indirectly influence the proliferation and differentiation of osteoclasts and in turn regulate the process of osteoporosis.

To address this question, we transfected hBMSCs with lentivirus (Lv) carrying the Notch intracellular domain 1 (NICD1) and collected the conditioned medium (CM). After adding the necessary serum to the CM, we used the CM to culture RAW264.7 cells to model a co-culture environment. Using real-time PCR and ELISA, we detected the expression and secretion of M-CSF and RANKL/OPG in the hBMSCs pre- and post-transfection. CCK-8, colony-forming, and flow cytometry assays were used to detect the proliferation of RAW264.7 cells. Immunofluorescence (IF) was carried out to detect the translocation of the nuclear factor of activated T cells c1 (NFATc-1) in the osteoclasts. Real-time PCR was used to detect the expression of NFATc-1-related osteoclastogenesis marker genes, such as cathepsin K and OSCAR. Tartrate-resistant acid phosphatase (TRAP) was used to count the multinucleate osteoclasts to assess osteoclast differentiation. The purpose of this study was to examine whether the upregulation of Notch signaling in OP-hBMSCs is involved in osteoclast proliferation and differentiation by influencing M-CSF and RANKL/

OPG expression as well as the activity of the NFATc-1 transcription factor. Our findings provide insight into the role of abnormal bone remodeling in the pathogenesis of osteoporosis.

Materials and methods

Materials

Alpha-modified Eagle's minimal essential medium (a-MEM) was purchased from Thermo scientific (Beijing, China). Plastic dishes were obtained from Costar (NY, USA). Fetal bovine serum (FBS) was purchased from Gibco Life Technologies (Grand Island, NY, USA). The antibody against NFATc-1 (MA3-024) was purchased from Pierce Life Technologies, which is part of Thermo Scientific (Rockford, USA). Penicillin-Streptomycin was purchased from Solarbio (Beijing, China). The other materials were of the highest grade commercially available.

Cell culture and differentiation

Primary hBMSCs were obtained from bone marrow aspirates from 8 healthy individuals (Normal hBMSCs) (bone mineral density, BMD: T-score \geq -1SD) or 8 patients with primary osteoporosis (OP-hBMSCs) (BMD: T-score \leq -2.5SD) after they provided informed consent to the research protocol. The ethical approval for this procedure was obtained from the ethics committee of the Fourth Military Medical University (20110405-5). hBMSCs were harvested in a sterile environment and cultured as described previously [7].

Lentiviral transfer vectors carrying human NICD1 ORF (+921~+2409 AA) were generated. Transgenes were amplified from a human complementary DNA library (MegaMan; Stratagene, La Jolla, CA, USA) and directionally inserted into the GV205 vector (NICD), which was purchased from GeneChem, Shanghai. An Ubi-MCS-3FLAG internal-site fragment was added to monitor the transfection efficiency. An empty vector was used as a negative control (empty Lv). Virus vector particles were obtained by transiently transfecting 293T cells with the transfer vector and packaging plasmids as previously described [8-10]. The primary hBMSCs were transduced once they had reached confluency with a 1:10 dilution of the neat virus vector supernatant at 37°C for 12 hours. The transduction

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efficiency was quantified by real-time PCR and western blotting.

Mouse macrophage RAW264.7 cells were obtained from ATCC (Manassas, Virginia, USA). hBMSCs were cultured in a-MEM supplemented with 20% fetal bovine serum (FBS) at 37°C under a humidified atmosphere of 5% CO₂. The conditioned medium (CM) was harvested, centrifuged to remove cell debris, filtered through a 0.22-µm MILLEX GP filter unit (MILLIPORE, Ireland), and stored at -80°C.

Real-time PCR

Total RNA was extracted using an OMEGA E.Z.N.A. Total RNA Kit I (Norcross, GA, USA). Real-time PCR of each gene was performed in triplicate for at least three independent experiments. Real-time PCR was performed with a Bio-RAD CFX-96 Real-time PCR system (Philadelphia, PA, USA) using SYBR Premix Ex Taq™ II (Takara Bio, Dalian, China). The sequences of the primers were as follows: Human GAPDH (NM_002046.4): (forward, 491) 5'-AGAAGGCTGGGGCTCATTTG-3', (reverse, 729) 5'-AGGGGCCATCCACAGTCTTC-3'; Human M-CSF (NM_172211.3): (forward, 755) 5'-ACTCTCTTTGAGGCTGAAGAGC-3', (reverse, 982) 5'-TTGCAATC AGGCTTGGTAC-3'; Human RANKL (NM_003701): (forward, 513) 5'-GCCTTTCAAGGAGCTGTGCAA-3', (reverse, 608) 5'-ATCTAACCATGAGCCATCCACCAT-3'; Human OPG (NM_002546.3): (forward, 821) 5'-CATGTCTTTGGTCTCCTGCTA-3', (reverse, 1057) 5'-CTGTGTTGCCGTTTTATCCTCT-3'; Mouse GAPDH (NM_008084.2): (forward, 761) 5'-TGTGTCCGTCGTGGATCT-3', (reverse, 890) 5'-TTGCTGTTGAAGTCGAGGAG-3'; Mouse Cathepsin K (NM_007802): (forward, 121) 5'-CACCCAGTGGGAGCTATGGAA-3', (reverse, 243) 5'-GCCTCCAGGTTATGGGCAGA-3'; Mouse OSCAR (NM_175632): (forward, 116) 5'-TGCATGCGTGCTGACTTC-3', (reverse, 223) 5'-AAGGT-CACGTTGATCCCAGGAG-3'.

ELISA

ELISAs were carried out using an ELISA Kit purchased from AMEKO (Lianshuo Biological Technology Co, Ltd, Shanghai) according to the manufacturer's instructions. Briefly, 50 µl of the CM from hBMSCs and OP-hBMSCs were separately dispensed into a polystyrene ELISA plate. Biotinylated and HRP-conjugated anti-

bodies were subsequently added and the plates were incubated for 60 min at 37°C. After washing 5 times, chromogen solutions A and B were added and the plates were incubated for 15 mins at 37°C. Finally, a stop solution was added to each well. The reaction was measured spectrophotometrically at 450 nm within 15 min.

CCK-8

RAW264.7 cells were treated with different CM collected from hBMSCs and OP-hBMSCs. To measure cell growth, a CCK-8 kit (Dojindo Molecular Technologies, Kumamoto, Japan) was used according to the manufacturer's protocol, and the cells were measured at a wavelength of 450 nm in an enzyme-linked microplate reader (MK-3, LabSystems Dragon, Beijing, China).

Colony formation assay

Briefly, RAW264.7 cells were plated in 6-well plates at a density of 20-40 cells/well in CM. The experiment was repeated three times. The colony formation assay was carried out as described previously [11].

Flow cytometry

Briefly, adherent cells in the culture plate were gently washed with PBS and were detached with a 0.05% Trypsin-EDTA solution (Sigma, St Louis, MO, USA) at 37°C for five minutes. Although it was difficult to detach intact osteoclasts, a portion of the osteoclasts could be analyzed by flow cytometry on day 5. After washing, the cells were analyzed on a flow cytometer (FACScaliber; BD Bioscience, San Jose, CA, USA).

Immunocytochemistry

RAW264.7 cells were added to sterile chamber slides (Thermo, Rockford, USA) at a density of 3×10³ cells/well. After 3-5 days, the coverslips were incubated for 30 min with an anti-NFATc-1 antibody diluted 1:150 in blocking solutions. The next day, the coverslips were incubated for 30 min with an Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) secondary antibody diluted 1:500 in 4% BSA. After incubation with 10 µg/ml DAPI for 5 min, the coverslips were mounted with PermaFluor aqueous mounting medium (Lipshow, Pittsburgh, PA).

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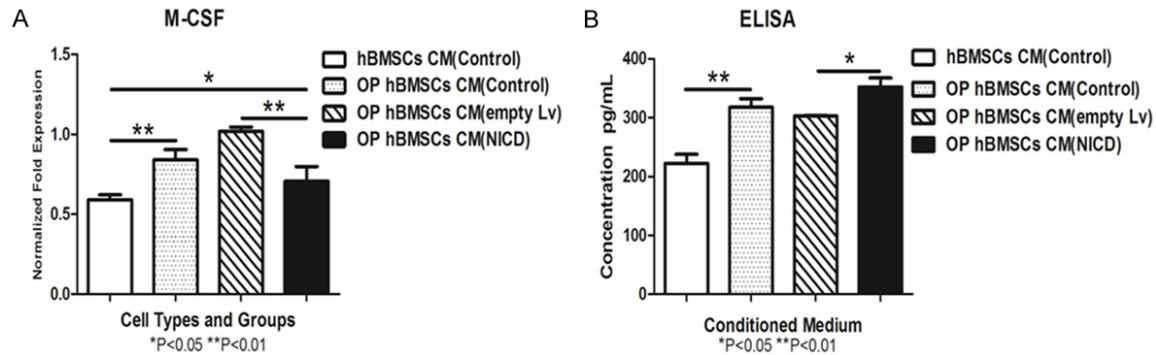


Figure 1. A. Upregulating Notch signaling in OP-hBMSCs promotes the expression and secretion of M-CSF. A. The M-CSF's expression in the hBMSCs. The expression of M-CSF, which was determined by RT-PCR on the 9th day was obviously increased in OP-hBMSCs before NICD fragment was transfected (**). In OP-hBMSCs, the level of M-CSF was down-regulated in OP-hBMSCs when NICD fragment was transfected (*), but it was still higher than which in the hBMSCs before NICD fragment was transfected (*). B. The M-CSF secretion of the hBMSCs. The secretion of M-CSF was obviously increased in OP-hBMSCs before NICD fragment was transfected (**). The secretion of M-CSF was obviously increased in OP-hBMSCs before the NICD fragment was introduced by transfection (**). In OP-hBMSCs, the level of M-CSF was down-regulated 5 days after the NICD fragment was introduced by transfection (*), but it was still higher than it was in the hBMSCs before the NICD fragment was introduced by transfection (*).

TRAP staining and the counting of TRAP-positive cells

Cells were plated at a concentration of 3.16×10^3 cells/cm² in the presence of CM. The medium was changed every day. After 5 days in culture, the cells were fixed and stained using the TRAP staining kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. TRAP-positive cells with more than three nuclei were considered to be osteoclast-like cells. The number of osteoclast-like cells was counted under a light microscope.

Statistical analysis

All of the data are expressed as the mean \pm SD, and a minimum of three independent experiments were performed for each assay. Analysis of variance (ANOVA) was used for statistical analysis. Significant differences are indicated with "**", and *P* values less than 0.05 are considered different. Statistical significance is indicated with "***", and *P* values less than 0.01 are considered significant.

Results

Upregulating notch signaling in OP-hBMSCs promotes the expression and secretion of M-CSF

M-CSF is an osteoblast-dependent cytokine that can promote the proliferation of osteoclast

precursor cells. In our study, we found that the expression of M-CSF, which was determined by RT-PCR on the 9th day, was obviously increased in OP-hBMSCs (0.59-fold) compared to hBMSCs (0.84-fold, *P*<0.01) before the NICD fragment was introduced by transfection. Although upregulating Notch signaling in the OP-hBMSCs (0.71-fold) decreased the expression of M-CSF (*P*<0.01), M-CSF expression in these cells was still higher than in the hBMSCs (0.59-fold, *P*<0.05) before the NICD fragment was introduced by transfection (**Figure 1A**). Because M-CSF is a secreted cytokine, its function is more related to its secretion than its expression. Therefore, to measure M-CSF secretion from hBMSCs, we used ELISA to examine the concentration of M-CSF in CM. The data showed that before transfection with NICD1, the M-CSF secretion from the OP-hBMSCs (318.0 ± 14.29 pg/ml) was more than that from the hBMSCs (222.4 ± 15.33 pg/ml, *P*<0.01). However, compared to the NICD (352.2 ± 15.33 pg/ml) and empty Lv groups (302.9 ± 1.219 pg/ml, *P*<0.05) of OP-hBMSCs, there was no significant difference on days 1-3. Upregulating Notch signaling increased M-CSF secretion on day 5 (**Figure 1B**).

Upregulating notch signaling in OP-hBMSCs indirectly promotes the proliferation of osteoclastic precursor cells

After detecting the expression and secretion of M-CSF, we needed to determine whether upreg-

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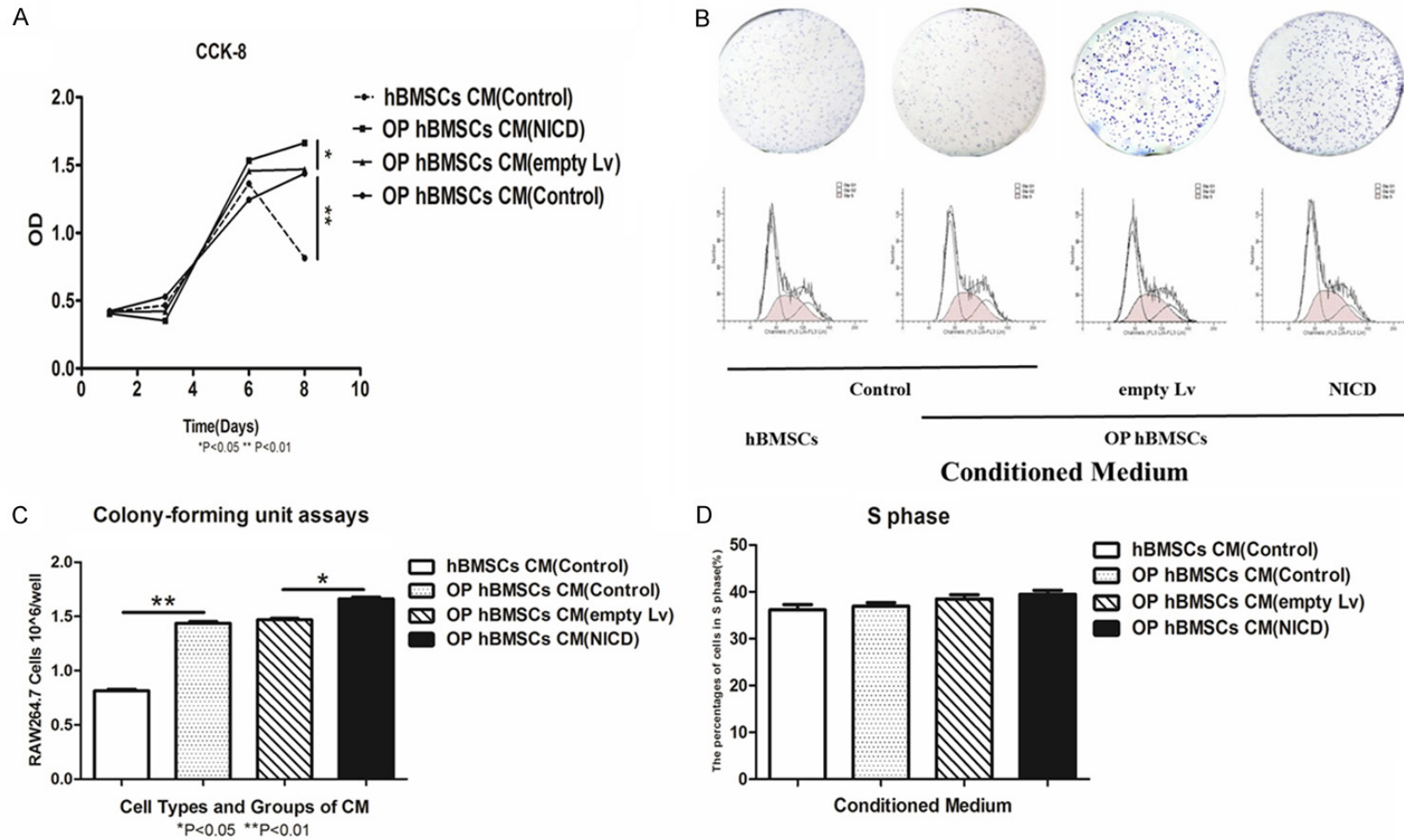


Figure 2. Upregulating notch signaling in OP-hBMSCs indirectly promotes the proliferation of osteoclastic precursor cells. CCK-8 and colony formation assays to detect changes in osteoclast proliferation and flow cytometry to determine the cell cycle distribution of osteoclasts. (A) CCK-8 assay. The number of RAW264.7 cells was obviously increased after culture with CM from OP-hBMSCs before the NICD fragment was introduced by transfection (**). Upon culture with CM from OP-hBMSCs and after transfection with the NICD fragment, the number of RAW264.7 cells was increased (*). Both general observation (B, upper panel) and the calculation of cell density under a microscope (C), showed a similar result. Upregulating Notch signaling in OP-hBMSCs indirectly induced the proliferation of RAW264.7 cells (*). Before the NICD fragment was introduced by transfection, proliferation was more significantly increased following culture with CM from OP-hBMSCs (**). In contrast, neither the cell cycle distribution map (B, lower panel) nor the bar graph showing the percentage of S phase cells (D) showed any statistically significant differences.

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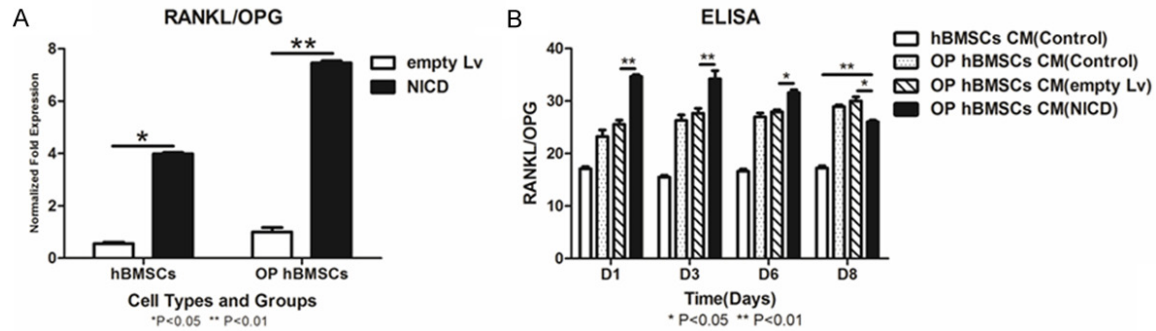


Figure 3. Upregulating Notch signaling in hBMSCs indirectly promotes the expression and secretion of RANKL/OPG. RANKL/OPG expression and secretion. A. In the hBMSCs, the RANKL/OPG expression in the NICD group was more than that in the empty Lv group (*). There was a more significant increase in the OP-hBMSCs (**). B. The RANKL/OPG secretory ratio of the OP-hBMSCs displayed significant differences on day 1 and day 3 (**). On day 6, there was no significant increase in the RANKL/OPG secretory ratio of the OP-hBMSCs (*). However, the ratio was still higher than that in the hBMSCs before the NICD fragment was introduced by transfection (**).

ulating Notch signaling in OP-hBMSCs could indirectly affect the proliferation of RAW264.7 cells. We used the CCK-8 assay to detect the proliferation of RAW264.7 cells cultured with CM. A comparison of the NICD OP-hBMSC group (1.697 ± 0.009) with the empty Lv OP-hBMSC group (1.469 ± 0.025 , $P < 0.05$) revealed an increase in the NICD group (Figure 2A). The data verified the former results regarding M-CSF secretion.

Second, we used the colony formation assay to detect changes in RAW264.7 proliferation. Both general observation (Figure 2B, upper panel) and the calculation of cell density under a microscope (Figure 2C) showed similar results. Before the cells were transfected with the NICD fragment, RAW264.7 proliferation was significantly increased upon culture with the CM from OP-hBMSCs ($P < 0.01$). The upregulation of Notch signaling in OP-hBMSCs could indirectly induce the proliferation of RAW264.7 cells ($P < 0.05$).

Finally, we used flow cytometry to monitor cell cycle progression in each group. We determined the percentage of cells in the S phase. Both the cell cycle distribution map (Figure 2B, lower panels) and the bar graph showing the percentage of cells in the S phase (Figure 2D) showed that there were no statistically significant differences among the groups. This is in contrast to the results from the colony formation assay. One reason for this discrepancy may be that the simple measurement of the percentage of cells in the S phase does not truly

reflect cell proliferation. A more precise measure is the ratio of S phase to G2 phase cells.

Upregulating Notch signaling in hBMSCs indirectly promotes the expression and secretion of RANKL/OPG

RANKL and OPG are osteoblast-dependent cytokines that influence the differentiation of the osteoclastic precursor cells to osteoclasts. They are a pair of emulative ligand-receptors. The ratio of RANKL/OPG is indicative of their effect on osteoclast differentiation. In the hBMSCs, the RANKL/OPG in the NICD group (3.987-fold) was greater than that in the empty Lv group (0.559-fold, $P < 0.05$). There was a more significant increase in the OP-hBMSCs (NICD group 7.46-fold, empty Lv 1-fold, $P < 0.01$) (Figure 3A).

Like M-CSF, RANKL and OPG are secreted cytokines. Therefore, the ELISA results were more important and definitive than the data obtained from real-time PCR. The data from each group before the NICD fragment was introduced by transfection showed that in OP patients, the secretion of RANKL/OPG was more than in healthy individuals (Figure 3B) and that the difference persisted throughout the 8 days of the experiment. We then compared the secretion of RANKL/OPG in the different groups of osteoporosis patients at different times to investigate the effect of upregulating Notch signaling on RANKL/OPG secretion. The data suggested that the RANKL/OPG secretory ratios of the OP-hBMSC groups displayed significant differ-

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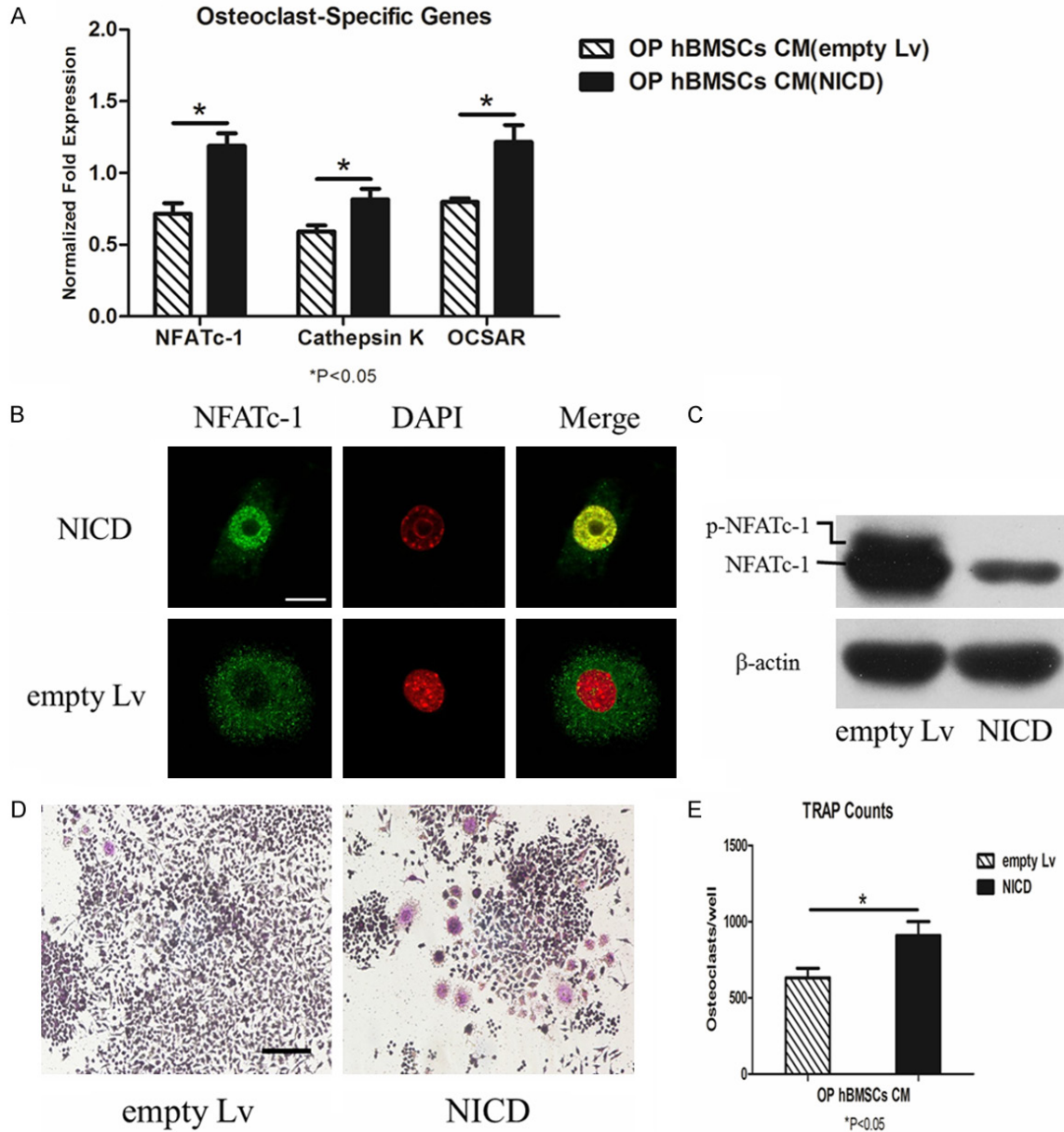


Figure 4. Upregulating Notch signaling in OP-hBMSCs indirectly promotes the differentiation of osteoclastic precursor cells. **A.** The expression of the NFATc-1-related osteoclastogenesis marker genes cathepsin K and OSCAR. In the OP patients, the expression of NFATc-1, cathepsin K, and OSCAR was higher in RAW264.7 cells treated with the conditioned medium from cells in the NICD group compared with those treated with conditioned medium from cells in the empty Lv group (*). **B, C.** NFATc-1 translocation in RAW264.7 cells. **B.** NFATc-1 expression was localized to the cytoplasm in the cells in the empty Lv group (bottom panel). In RAW264.7 cells treated with the conditioned medium from cells in the NICD group, intense green fluorescent staining for NFATc-1 was observed in the nucleus (upper panel). The bar indicates 20 μ m. **C.** The conditioned medium from the Notch group cells induced the de-phosphorylation of NFATc-1 in RAW264.7 cells. This was followed by nuclear translocation. In contrast, phosphorylated NFATc-1 (p-NFAT1c1), which appears as a band of approximately 120 kDa, was still detected in RAW264.7 cells treated with the conditioned medium from cells in the empty Lv group. **D, E.** TRAP staining and the number of TRAP-positive multinuclear cells. Osteoclastogenesis in RAW264.7 cells induced by CM. The cells were cultured and then stained for TRAP. The TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclasts. The bar indicates 200 μ m. **D.** Treatment with conditioned medium from cells in the NICD group induced the formation of TRAP-positive multinuclear osteoclasts. However, fewer effects were observed in cells treated with the conditioned medium from the empty Lv group. **E.** The number of osteoclasts after culture. The data represent the mean \pm SEM for the cultures. The statistical analysis was performed using Student's t-test. In the OP-hBMSCs, the number of TRAP-positive multinuclear RAW264.7 cells following treatment with the conditioned medium from cells in the NICD group cells was higher than that after treatment with conditioned medium from cells in the empty Lv group (*).

ences at the earlier time points (day 1 and day 3), but less differences on day 6. On day 8, the secretion of RANKL/OPG in the NICD group (26.037 ± 0.9163) was lower than that in the empty Lv group (30.0217 ± 1.984 , $P < 0.05$), but it was still higher than that in the hBMSCs before transfection with the NICD fragment (17.2373 ± 0.8249 , $P < 0.01$). Therefore, we surmised that upregulating Notch signaling could induce osteoclast differentiation in healthy individuals but not in osteoporosis patients.

Upregulating notch signaling in OP-hBMSCs indirectly promotes the differentiation of osteoclastic precursor cells

We next investigated whether upregulating Notch signaling results in the differential expression of NFATc-1-related genes. Total RNA was extracted from RAW264.7 cells treated with the conditioned medium from the normal and OP-hBMSCs of each group. Real-time PCR was performed for NFATc-1-related genes that are involved in osteoclast differentiation. The data showed that the expression of NFATc-1, cathepsin K, and OSCAR were higher in RAW264.7 cells treated with the conditioned medium from cells in the NICD group than in those treated with the CM from cells in the empty Lv group (**Figure 4A**).

During osteoclastogenesis, an activated form of NFATc-1 is known to translocate from the cytoplasm to the nucleus of osteoclast precursor cells. Therefore, we investigated whether the conditioned medium from the OP-hBMSCs of each group could stimulate NFATc-1 translocation. Following the treatment of RAW264.7 cells with conditioned medium for 5 days, the cells were fixed, permeabilized, and stained with an anti-NFATc-1 antibody and DAPI. We observed a green fluorescent signal predominantly in the cytoplasm, indicating that NFATc-1 expression was localized to the cytoplasm of cells in the empty Lv group (**Figure 4B**, bottom panel). When we treated RAW264.7 cells with the conditioned medium from cells in the NICD group, intense green fluorescent staining of NFATc-1 was observed in the nucleus (**Figure 4B**, upper panel). Nuclear staining with DAPI was visualized as a red fluorescent signal. In the merged image, we observed multiple yellow fluorescent signals restricted to the nuclei of cells treated with the conditioned medium from the NICD group but not from the empty Lv

group, thereby confirming NFATc-1 localization in the nuclei of these cells. The de-phosphorylation of NFATc-1 allows NFATc-1 to translocate to and be activated in the nucleus [12, 13]. To further examine the phosphorylation status of NFATc-1, the cell lysate was collected and subjected to western blot analysis using a NFATc-1-specific antibody. **Figure 4C** shows that the conditioned medium from cells in the NICD group induced the de-phosphorylation of NFATc-1 in RAW264.7 cells and that this in turn resulted in nuclear translocation. In contrast, phosphorylated NFATc-1 (p-NFAT1c1), which appears as a band of approximately 120 kDa, was still detected in RAW264.7 cells treated with the conditioned medium from cells in the empty Lv group. These differences were more obvious in the healthy individuals than in the OP patients (data not shown).

Finally, to examine the effect of upregulating Notch signaling in OP-hBMSCs on the induction of TRAP-positive multinuclear cells, we treated RAW264.7 cells with the conditioned medium from the normal hBMSCs or the OP-hBMSCs of each group. Treatment with the conditioned medium from cells in the NICD group induced the formation of TRAP-positive multinuclear osteoclasts. However, less of an effect was observed in cells treated with the conditioned medium from cells in the empty Lv group (**Figure 4D**). **Figure 4E** shows the number of TRAP-positive multinuclear cells in each well of a 6-well plate. The number of TRAP-positive multinuclear RAW264.7 cells was higher when the cells were treated with the conditioned medium from the NICD group (909.36 ± 32.71) than when they were treated with the conditioned medium from the empty Lv group (631.5 ± 21.77 , $P < 0.05$).

Discussion

Notch has a dual function in bone development in that it regulates both osteoblastogenesis and osteoclastogenesis. Cells in the osteoclast and osteoblast lineages communicate with each other through cell-to-cell contact. Although osteoblast-dependent activation of osteoclasts through Notch signaling has been demonstrated, further investigation is needed to determine whether there is reverse signaling between these two types of bone cells. We present two major findings that address this issue. First, we show that upregulating Notch

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signaling in OP-hBMSCs induces the expression and secretion of M-CSFs and that after upregulation, the CM of OP-hBMSCs increases the number of multinuclear osteoclasts as well as their proliferation. Second, we demonstrate that the upregulation of Notch signaling also induces the RANKL/OPG ratio. The CM induces the translocation of NFATc-1, the expression of NFATc-1-related osteoclastogenesis marker genes, and the number of TRAP-positive multinuclear cells and eventually promotes pre-osteoclast differentiation. These results provide insight into the role that the Notch signaling plays in the relationship between hBMSCs and osteoclasts.

hBMSCs control bone metabolism not only by synthesizing bone matrix proteins and regulating mineralization but also by orchestrating the process of bone resorption through the regulation of osteoclastogenesis [14, 15]. Notch signaling regulates osteoblastic differentiation in a cell type- and cell stage-dependent manner [16, 17]. Previously, transient Notch activation had been suggested to stimulate the osteoblastic differentiation of MC3T3-E1 cells. However, some studies suggested that Notch signaling impaired the osteoblastic differentiation of the Kusa, MC3T3, and ST-2 cell lines [17, 18]. Matthew J. Hilton demonstrated the opposite effects of Notch signaling on osteogenesis during the different stages of mouse development [19]. These opposing results may be due to the different cell lines used in the studies. Hilton's studies were mostly performed using an osteoblast precursor or murine stromal cell line instead of hBMSCs. Our study examined the indirect effect of upregulating Notch signaling on the function of osteoclastic precursor cells (RAW264.7) in hBMSCs derived from healthy individuals and osteoporosis patients.

The research examining the role of Notch in the proliferation and differentiation of osteoclasts was conducted in a similar manner. In this study, we demonstrated that upregulating Notch signaling in hBMSCs increased M-CSF and RANKL/OPG expression. In agreement with our real-time PCR results, we also showed that the concentration of M-CSF and RANKL/OPG in the culture medium was increased in the NICD group compared to the control group. This, combined with the results of the CCK-8, colony formation, and flow cytometry assays suggested that upregulating Notch signaling in hBM-

SCs indirectly promotes the proliferation of osteoclastic precursors. The results from the immunocytochemistry and TRAP staining and the number of TRAP-positive cells further supported the hypothesis that upregulating Notch signaling in hBMSCs indirectly promotes osteoclast differentiation. The promotion of both proliferation and differentiation was more obvious in healthy individuals than in osteoporosis patients. In contrast, Shuting Ba and Raphael Kopan, *et al.* [20] suggested that Notch signaling inhibits the response to M-CSF-induced precursor proliferation, which is likely to contribute to the stimulated osteoclastogenesis evident in the Notch1, 2, 3OC^{-/-} osteoclast lineage cells. Notch deficiencies in either osteoclast or osteoblast lineage cells optimally promote osteoclast formation. On the other hand, they considered that the deletion of Notch1-3 promotes osteoclast differentiation. There are two explanations for the differences between their findings and ours. First, we used cells from different species. They used Notch knock-out mice as the source of osteoblasts and osteoclasts, whereas we used humans as the source. Considering the differences in Notch function in the different species, the different developmental processes, and the different cells, variability in the results is expected. Second, a portion of their conclusion was obtained from experiments focused on Notch signaling in the osteoclast. In contrast, we paid more attention to the upregulating of Notch signaling in hBMSCs and its indirect effect on osteoclastogenesis. Based on the above two points, the differences were understandable.

The balance of the RANKL/OPG ratio is known to be crucial for bone homeostasis [21-23] because the ratio of RANKL/sRANKL to OPG produced by osteoblasts modulates osteoclast differentiation and activity [23, 24]. Increases in the ratio of RANKL/sRANKL to OPG are expected to favor osteoclastogenesis, while decreases should favor the inhibition of osteoclastogenesis. The results from our present study indicate that Notch signaling can regulate the expression and production of RANKL/sRANKL and OPG and lead us to hypothesize that Notch signaling in hBMSCs is involved in osteoclastogenesis. RANKL/sRANKL-RANK signaling induces the expression of various transcription factors, including NFATc-1, that can act as positive modulators of osteoclast differentiation [21, 25, 26]. In this study, we have

shown that the de-phosphorylation and translocation of NFATc-1 were induced by treatment with conditioned medium from cells in the NICD group but not from the conditioned medium from cells in the empty Lv group. This result indicates that upregulating Notch signaling in osteoblasts promotes its ability to induce NFATc-1 de-phosphorylation and translocation in osteoclast precursors. NFATc-1 induces the expression of its target genes by binding to NFAT-binding sites in the promoter region of genes, such as TRAP, cathepsin K, and OSCAR, that are important for osteoclast differentiation or function [27, 28]. A positive feedback loop exists during late-stage osteoclastogenesis, in which NFATc-1 induces OSCAR expression leading to the activation of NFATc-1 [28, 29]. Consistent with previous reports, we showed that the conditioned medium from cells in the NICD group induced NFATc-1, cathepsin K, and OSCAR expression following NFATc-1 translocation. Moreover, the number of TRAP-positive multinuclear cells induced by the conditioned medium from cells in the NICD group was higher than that induced by the conditioned medium from cells in the empty Lv group. These results indicate that upregulating Notch signaling in osteoblasts has a positive effect on osteoclastogenesis through the upregulation of NFATc-1-related osteoclast marker genes.

In our previous study, we concluded that in osteoporosis patients, upregulating Notch signaling in hBMSCs promoted cell proliferation and induced the differentiation of osteoblasts. In the present study, we showed that upregulating Notch signaling in hBMSCs promoted the proliferation and differentiation of osteoclasts by regulating M-CSF, RANKL, and OPG expression. The effects on proliferation and differentiation were more pronounced in the healthy individuals than in the OP patients. Ongoing studies in our lab have shown that upregulating Notch signaling in the bone marrow cavity of mice can increase bone mass. These observations may provide a new therapeutic target for osteoporosis that could promote osteoblastogenesis while at the same time reducing osteoclastogenesis. Our findings imply that upregulating Notch signaling in hBMSCs exerts complicated and pleiotropic effects on osteoblastogenesis and osteoclastogenesis, respectively. Therefore, further studies are needed to investigate the detailed molecular mechanism of the

Notch signaling-mediated crosstalk between hBMSCs and osteoclasts.

In conclusion, we demonstrated that upregulating Notch signaling increased M-CSF and the ratio of RANKL/sRANKL to OPG at both the mRNA and protein levels in hBMSCs. Moreover, this upregulation of Notch signaling in hBMSCs promoted the proliferation and differentiation of osteoclasts. Our present study provides the first line of evidence that Notch signaling might be a new therapeutic target for osteoporosis.

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Disclosure of conflict of interest

None.

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