

# Loss of Notch3 Signaling Enhances Osteogenesis of Mesenchymal Stem Cells from Mandibular Torus

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X.W. Dou<sup>1\*</sup>, W. Park<sup>1,2\*</sup>, S. Lee<sup>3</sup>, Q.Z. Zhang<sup>1</sup>, L.R. Carrasco<sup>1,4</sup>,  
and A.D. Le<sup>1,4</sup>

## Abstract

Mandibular torus (MT) is a common intraoral osseous outgrowth located on the lingual surface of the mandible. Histologic features include hyperplastic bone consisting of mature cortical and trabecular bone. Some theories on the etiology of MT have been postulated, such as genetic factors, masticatory hyperfunction, trauma, and continued growth, but the underlying mechanism remains largely unknown. In this study, we investigated the potential role of mesenchymal stem cells (MSCs) derived from human MT in the pathogenesis of bone outgrowth. We demonstrated that MT harbored a distinct subpopulation of MSCs, with enhanced osteogenic and decreased adipogenic differentiation capacities, as compared with their counterparts from normal jaw bone. The increased osteogenic differentiation of mandibular torus MSCs was associated with the suppression of Notch3 signaling and its downstream target genes, *Jag1* and *Hey1*, and a reciprocal increase in the transcriptional activation of *ATF4* and *NFATc1* genes. Targeted knockdown of *Notch3* expression by transient siRNA transfection promoted the expression of osteogenic transcription factors in normal jaw bone MSCs. Our data suggest that the loss of Notch3 signaling may contribute partly to bone outgrowth in MT, as mediated by enhanced MSC-driven osteogenic differentiation in the jaw bone.

**Keywords:** cell signaling, jaw bone anomalies, bone formation, gene expression, oral and maxillofacial surgery, adult stem cell(s)

## Introduction

Oral torus is an exostosis, or benign bony outgrowth, and mandibular torus (MT) is the most common torus on the lingual aspect, above the mylohyoid ridge (Choi et al. 2012). Usually, MT presents as a very slow and progressive growth that can stop spontaneously (Komori and Takato 1998). The prevalence of MT ranges from 0.54% to 64.4%, and varies among ethnic groups (Sirirungrojyng and Kerdpon 1999; García-García et al. 2010).

To date, the etiology of MT is still unknown. Several mechanisms have been proposed, including genetics, trauma, and dietary habits (Ladizinski and Lee 2014). Despite the most widely accepted theory of genetic etiology, torus has not always been reported as autosomal dominant (Eggen 1989; Bruce et al. 2004). Mechanical stress, especially occlusal force, is another important factor of MT formation. It has been reported that bone response and remodeling happen under functional stress (Frost 2004; Cortes et al. 2014), so MT is frequently observed in patients who have powerful masticatory muscles and parafunctional habits (Eggen 1989; Sirirungrojyng and Kerdpon 1999; Sonnier et al. 1999). Since MT often is asymptomatic, the treatment is not necessary except for prosthetic needs, autologous bone graft, and functional problems, such as food retention or phonetic disturbances and cancer-phobic patients (Barker et al. 2001; Proussaefs 2006).

Bone formation and resorption are coupled events during bone growth and remodeling (Zuo et al. 2012). Despite the fact that they originate from different cell lineages and possess opposite functions, osteoblasts, osteoclasts, and osteocytes interact and establish microscopic basic multicellular units to

<sup>1</sup>Department of Oral and Maxillofacial Surgery and Pharmacology, University of Pennsylvania School of Dental Medicine, Philadelphia, PA, USA

<sup>2</sup>Department of Advanced General Dentistry, College of Dentistry, Yonsei University, Seoul, South Korea

<sup>3</sup>Department of Endodontics, University of Pennsylvania School of Dental Medicine, Philadelphia, PA, USA

<sup>4</sup>Department of Oral and Maxillofacial Surgery, Penn Medicine Hospital of the University of Pennsylvania, Philadelphia, PA, USA

\*Authors contributing equally to this article

A supplemental appendix to this article is available online.

## Corresponding Authors:

A.D. Le, Department of Oral and Maxillofacial Surgery and Pharmacology, University of Pennsylvania School of Dental Medicine, 240 South 40th Street, Philadelphia, PA 19104, USA.  
Email: Anh.Le@uphs.upenn.edu

L.R. Carrasco, Department of Oral and Maxillofacial Surgery and Pharmacology, University of Pennsylvania School of Dental Medicine, 240 South 40th Street, Philadelphia, PA 19104, USA.  
Email: Lee.Carrasco@uphs.upenn.edu

orchestrate bone remodeling (Zanotti and Canalis 2012; Zuo et al. 2012). At the molecular level, several major signals and transcription factors—including Hedgehog, wingless-type MMTV integration site family member (Wnt), bone morphogenetic protein (BMP), fibroblast growth factor (FGF), and Notch signals—have been implicated in the regulation of differentiation and function of bone cells (Long 2012). Evolutionarily conserved Notch signaling plays a critical role during embryonic development and tissue renewal. There are 4 receptors (Notch 1 to 4) and 5 Delta/Serrate/Lag-2 ligands (Jagged 1 [JAG1] and Jagged 2; Delta-like 1, 3, and 4). In the canonical signaling pathway, Notch receptors are cleaved by  $\gamma$ -secretase complex, leading to the release of the Notch intracellular domain (NICD), which regulates the downstream gene expression (Kopan and Ilagan 2009; Zanotti and Canalis 2012). The *Notch3* gene was initially reported as being expressed in proliferating neuroepithelium, while targeted deletion of murine *Notch3* does not lead to embryonic lethality possibly due to its more restricted tissue distribution (Bellavia et al. 2008). Numerous studies have shown that deregulated Notch3 signaling is closely linked to the pathogenesis of several diseases, such as heart failure (Ragot et al. 2016), pulmonary arterial hypertension (Li et al. 2009), renal disease (El Machhour et al. 2015), and tumorigenesis (Serafin et al. 2011). Furthermore, several studies have shown that Notch canonical signaling plays a critical role in prenatal skeletal development and postnatal bone remodeling by regulating the proliferation and differentiation of bone cells (Engin and Lee 2010). Dysregulation of Notch signaling is associated with numerous developmental and postnatal skeletal diseases, such as Alagille syndrome, brachydactyly and spondylocostal dysostosis, and Hajdu-Cheney syndrome (Zanotti and Canalis 2012). Previous studies have also shown that Notch-Hey1 or Notch-Hes signaling contributes to impaired osteoblast differentiation/maturation or osteogenesis of bone marrow mesenchymal stem cells (MSCs) (Zamurovic et al. 2004; Hilton et al. 2008; Fei et al. 2015).

Here, we reported the isolation and characterization of MSCs derived from human mandibular torus (T-MSCs) and normal jaw bone (JB-MSCs) and explored their potential role and mechanisms in the pathogenesis of MT. In addition, we demonstrated the functional role of Notch3 signaling in the regulation of osteogenesis of T-MSCs and JB-MSCs as a potential mechanism of bone overgrowth in MT.

## Materials and Methods

### Subjects and Isolation and Culture of Cells

All tissues were collected from generally healthy patients (48 to 65 y of age) after informed consents were obtained following the protocol approved by the Institutional Review Board of University of Pennsylvania. Normal jaw bone tissues ( $n = 5$ ) were collected from patients undergoing extraction of impacted third molars. MT tissues ( $n = 5$ ) were obtained during surgical removal of MT for prosthodontic restoration. Bone tissues were treated aseptically, cut into small pieces, and digested at 37 °C for 2 h in sterile 1× phosphate-buffered solution (PBS)

containing 4 mg/mL of collagenase IV (Gibco). The dissociated cell suspension was filtered through a 70- $\mu$ m cell strainer (Falcon) and plated on nontreated 10-cm Petri dishes in  $\alpha$ -Minimum Essential Medium ( $\alpha$ -MEM; Gibco) containing 10% fetal bovine serum (FBS; Gibco), 100 U/mL of penicillin, 100  $\mu$ g/mL of streptomycin, 2 mM of L-glutamine, 100 mM of nonessential amino acid, and 550  $\mu$ M of 2-mercaptoethanol (Sigma-Aldrich). Nonadherent cells were removed after 3 d, and the cells were washed with PBS. The medium was exchanged twice weekly. At 70% to 80% confluence, the fibroblast-like colonies were harvested with 0.05% trypsin-EDTA (Gibco). The subcultures were continuously maintained at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere.

### Immunophenotyping of MSCs by Flow Cytometric Analysis

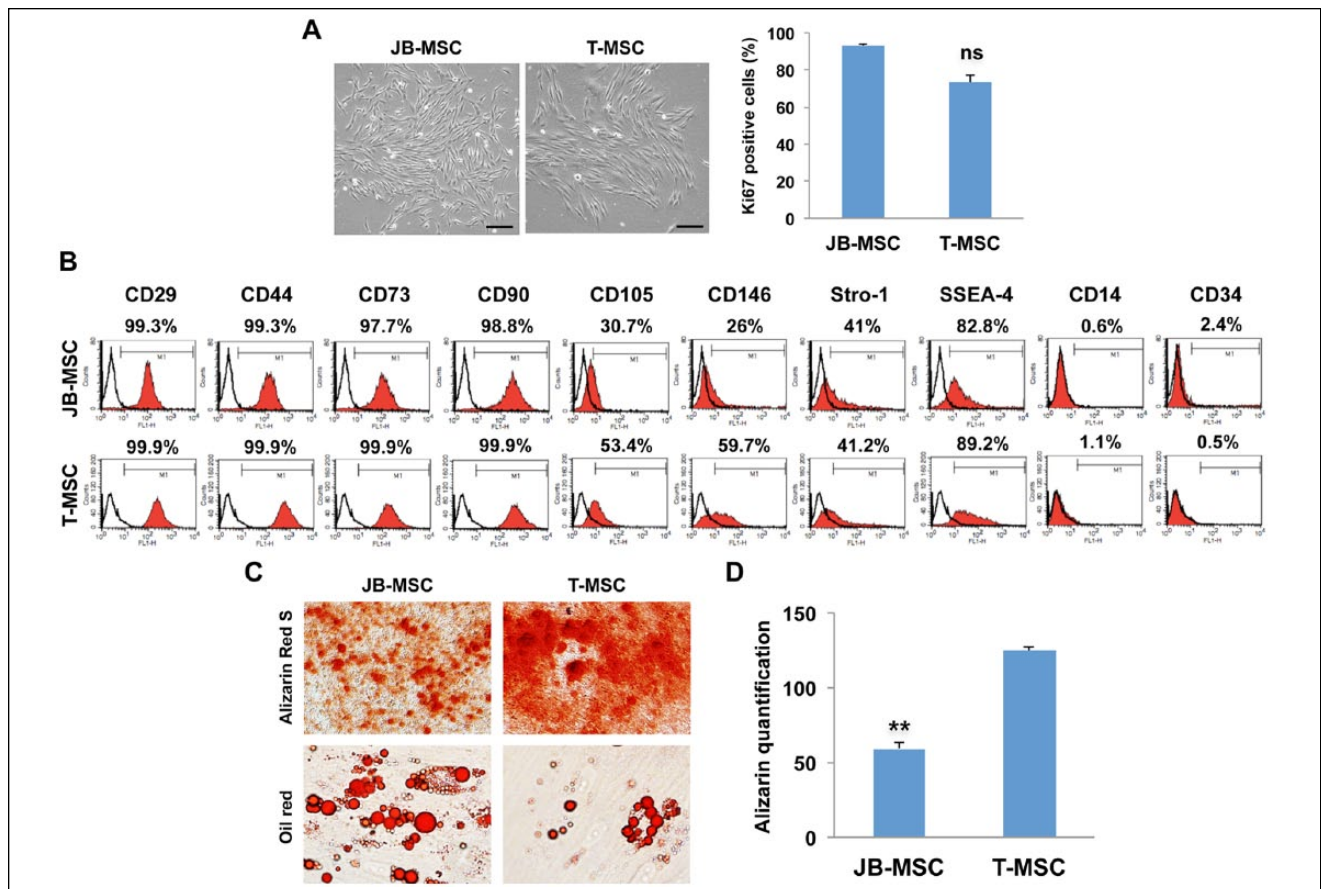
JB-MSCs and T-MSCs ( $1 \times 10^5$ ) were immunolabeled with specific mouse monoclonal antibodies for human CD29, CD44, CD73, CD90, CD105, stro-1 (BioLegend), CD14, CD34, CD146 (BD Biosciences), and stage-specific embryonic antigen (SSEA)-4 (R&D) at 4 °C for 1 h. Mouse isotype antibodies served as controls (BD Biosciences). After washing with PBS, cells were incubated with fluorescein-isothiocyanate-conjugated secondary antibodies for 30 min in the dark. Cells were analyzed on a BD LSR II flow cytometer (BD Bioscience).

### In Vitro Multipotent Differentiation Assay

**Osteogenic Differentiation.** JB-MSCs and T-MSCs were plated at  $5 \times 10^5$  cells per well in 6-well plates in MSC growth medium and allowed to adhere. When 100% confluency was achieved, osteogenic inductive medium ( $\alpha$ -MEM supplemented with 10% FBS, 50 U/mL of penicillin, 50  $\mu$ g/mL of streptomycin, 100 nM of dexamethasone, 100  $\mu$ M of L-ascorbic acid 2-phosphate, and 10 mM of  $\beta$ -glycerophosphate) was replaced and changed every 3 d. After differentiation periods (9 d and 3 wk), cell cultures were fixed and assayed for mineralized deposits of calcium by alizarin red S staining (Fisher Scientific). Cells in negative control groups were grown in  $\alpha$ -MEM with 10% FBS for 9 d.

**Adipogenic Differentiation.** JB-MSCs and T-MSCs ( $5 \times 10^5$  cells respectively) were seeded in 6-well plates in MSC growth medium and allowed to adhere. When 80% to 90% confluency was reached, adipogenic inductive medium ( $\alpha$ -MEM supplemented with 10% FBS, 50 U/mL of penicillin, 50  $\mu$ g/mL of streptomycin, 100  $\mu$ M of L-ascorbate-2-phosphate, 100 nM of dexamethasone, 0.45 mM of 3-isobutyl-1-methylxanthine, and 60  $\mu$ M of indomethacin) was replaced and changed every 3 d for 2 wk. Oil red O staining (Sigma-Aldrich) was performed to detect intracellular lipid vacuoles characteristic of adipocytes.

**Western Blotting.** Equal amounts of protein extracts (30  $\mu$ g) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 10% polyacrylamide gels



**Figure 1.** Characteristics of mesenchymal stem cells from jaw bone (JB-MSCs) and mandibular torus (T-MSCs). **(A)** After culture for 7 to 10 d, the adherent cells from normal jaw bone and mandibular torus formed colonies and displayed fibroblast-like morphologies and similar proliferative activities as determined by immunostaining of Ki67 expression. **(B)** The expression of MSC-associated cell surface markers was determined by flow cytometric analysis. **(C, D)** In vitro osteogenic and adipogenic differentiation of JB-MSCs and T-MSCs were determined by alizarin red S and oil red O staining, respectively. Scale bars, 50  $\mu$ m. The data are expressed as the mean  $\pm$  SD of 3 independent experiments (\*\* $P < 0.01$ ). ns, no significant difference.

and transferred to nitrocellulose membrane. Bands were detected immunologically with polyclonal antibodies against Notch1, Notch2, Notch3, or Notch4 (1:1000; Cell Signaling). Monoclonal antibody against mouse  $\beta$ -actin (Santa Cruz) was used as a loading control. The binding of all antibodies was detected with an enhanced chemiluminescence (ECL) detection system (Thermo Scientific).

**Quantitative Real-time Polymerase Chain Reaction.** Total RNA was isolated from cultured cells with the RNeasy Mini kit (Qiagen). cDNA was synthesized with Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) according to the instructions of the manufacturer. Quantitative real-time polymerase chain reaction was performed with the ABI Prism 7900 HT Sequence Detection System. All reactions were performed in triplicate. The lists of all primers are summarized in the Appendix Table. All primers were synthesized by Integrated DNA Technologies.

**Transfection with siRNA.** Twenty-four hours before siRNA transfection, JB-MSCs were seeded in 6-well plates containing fresh medium without antibiotics. The cells were then transfected with

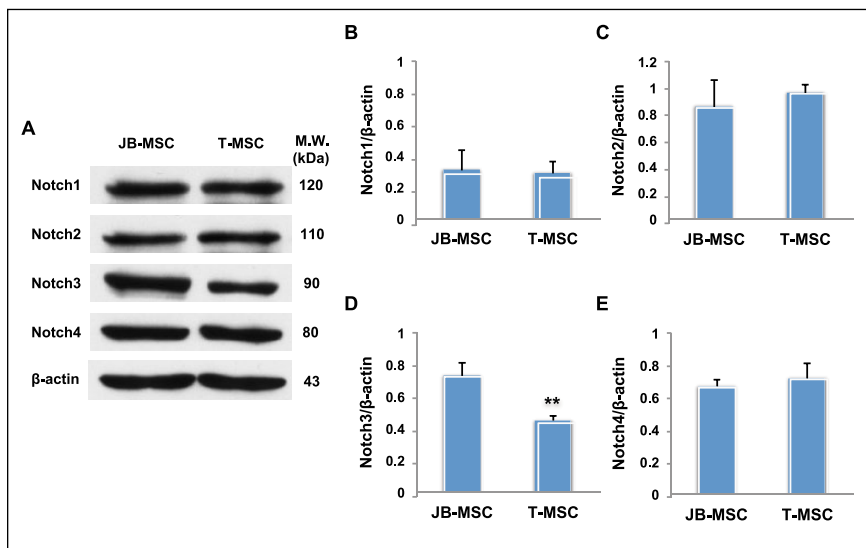
siRNAs against human *Notch3* gene and a nontarget siRNA control with Lipofectamine RNAi MAX (Invitrogen), as described in the manufacturer's instruction. The sequences for downregulating *Notch3* in JB-MSCs were as follows: *Notch3* siRNA-1, 5'-GAGCCAAUAAGGACAUGC A-3'; *Notch3* siRNA-2, 5'-U AUAGGUGUUGACGCCAUCCACGCA-3'; nontarget control siRNA, 5'-UUCUCCGAACGUGUCACGU-3'. The cells were harvested at 48 h posttransfection for further analysis.

**Statistical Analysis.** Data obtained from 3 independent experiments were presented as mean  $\pm$  SD and analyzed by unpaired *t* test. *P* values  $< 0.05$  were considered statistically significant.

## Results

### Characterization of MSCs from Human Normal Jaw Bone and MT

After culture for 7 to 10 d, the adherent MSCs from normal jaw bone (JB-MSCs) and mandibular torus (T-MSCs) formed colonies and displayed fibroblast-like morphologies (Fig. 1A). In



**Figure 2.** Expression of Notch signals in mesenchymal stem cells from jaw bone (JB-MSCs) and mandibular torus (T-MSCs). (A) The expression of Notch1, Notch2, Notch3, and Notch4 proteins in JB-MSCs and T-MSCs was determined by Western blot analysis. (B–E) The ratio of Notch1, 2, 3, 4/β-actin expression in JB-MSCs and T-MSCs was determined through densitometric analysis. The data are expressed as the mean ± SD of 3 independent experiments (\*\* $p < 0.01$ ).

all MT samples examined, the derived JB-MSCs and T-MSCs showed similar proliferative activities by immunostaining of nuclear Ki67 expression (Fig. 1A). Flow cytometric analysis of representative JB-MSCs and T-MSCs showed that both types of cells were negative or very low for CD14 and CD34 (hematopoietic stem cell markers) but positive for CD29, CD44, CD73, and CD90 (positive markers of MSCs) and stem cell-related markers Stro-1 and SSEA-4 (Fig. 1B). Both CD105 and CD146 were relatively more abundant in T-MSCs; however, their significance remains unknown. Interestingly, in vitro differentiation assays showed that T-MSCs exhibited increased osteogenic differentiation capabilities and decreased potentials for adipogenic differentiation as compared with JB-MSCs (Fig. 1C, D). These results suggest that normal jaw bone and MT harbor distinct subpopulation of MSCs (i.e., JB-MSCs and T-MSCs, respectively) that possess different differentiation capabilities.

### Downregulation of Notch3 Protein Expression in T-MSCs

Previous studies have shown that Notch plays a central role in skeletal development and bone remodeling and that dysregulation of Notch signaling is associated with developmental and postnatal skeletal diseases (Zanotti and Canalis 2012). We next examined the expression of Notch family members in JB-MSCs and T-MSCs. Using Western blot analysis, we showed that the baseline expression of Notch1, Notch2, and Notch4 was similar in JB-MSCs and T-MSCs (Fig. 2A–C, E). However, in comparison with JB-MSCs, T-MSCs expressed a significantly lower level of Notch3 protein (41.66% of decrement; Fig. 2A, D). These results suggest that suppressed Notch3 may play a role in the biological function of T-MSCs.

### Downregulation of Notch3 Signals Is Conversely Correlated with Osteogenic Gene Expressions in T-MSCs and JB-MSCs

To explore the relationship between Notch3 signaling and osteogenic differentiation potentials of JB-MSCs and T-MSCs, we examined the mRNA expression of *Jag1*, a surface ligand of Notch receptors; *Hey1* and *Hey2*, 2 family members of bHLH transcription factors of Notch signaling; *Osterix*, *Dlx5*, activating transcription factor 4 (*ATF4*), and nuclear factor of activated T-cell (*NFATc*), genes that play an important role in the regulation of different stages of osteogenesis (Deng et al. 2008; Samee et al. 2008; Long 2012). As shown in Figure 3, a reduced expression of Notch signals, including *Notch3*, *Jag1*, *Hey1*, and

*Hey2*, was consistently observed in T-MSCs in comparison with JB-MSCs (Fig. 3A–D). In accordance, the downregulation of Notch signals in T-MSCs was coupled with a decreased expression of *Osterix* and *Dlx5* genes (Fig. 3E, F), 2 transcription factors that play an important role in the early formation and proliferation of premature and immature osteoblasts (Komori and Takato 1998). In contrast, the expression of *ATF4* and *NFATc1* genes—2 transcription factors that play a critical role in the late differentiation and maturation of osteoblasts (Komori and Takato 1998; Long 2012)—was significantly increased in T-MSCs versus JB-MSCs (Fig. 3G, H). These data suggest that the suppressed Notch3 signaling might play a role in augmenting the osteogenic capability of T-MSCs, at least in part, by promoting the expression of *ATF4* and *NFATc1* genes.

### Effect of Loss of Notch3 Signals on Osteogenic Differentiation of JB-MSCs

Using in vitro osteogenic assay, we showed that upon exposure to osteogenic induction condition, JB-MSCs gradually lost Notch3 signals; the decrease in Notch3 is associated with the appearance of mineralization on day 9 (Fig. 4A, B). To confirm the direct contribution of Notch3 in osteogenic differentiation, we performed transient loss of function of Notch3 in JB-MSCs by using specific siRNAs. Our results showed that transfection of 2 *Notch3* siRNAs efficiently knocked down Notch3 expression at the mRNA and protein levels in JB-MSCs (Fig. 4C, D, E), which correlated with a significant increase in the mRNA expression of bone sialoprotein, a marker of bone formation (Fig. 4D, E). Mechanistically, knockdown of Notch3 expression in JB-MSCs led to a significantly reduced expression of the downstream transcriptional factor *Hey1* but not *Hey2* (Fig. 5A–C) and, correspondingly, the expression of *Osterix* (Fig.



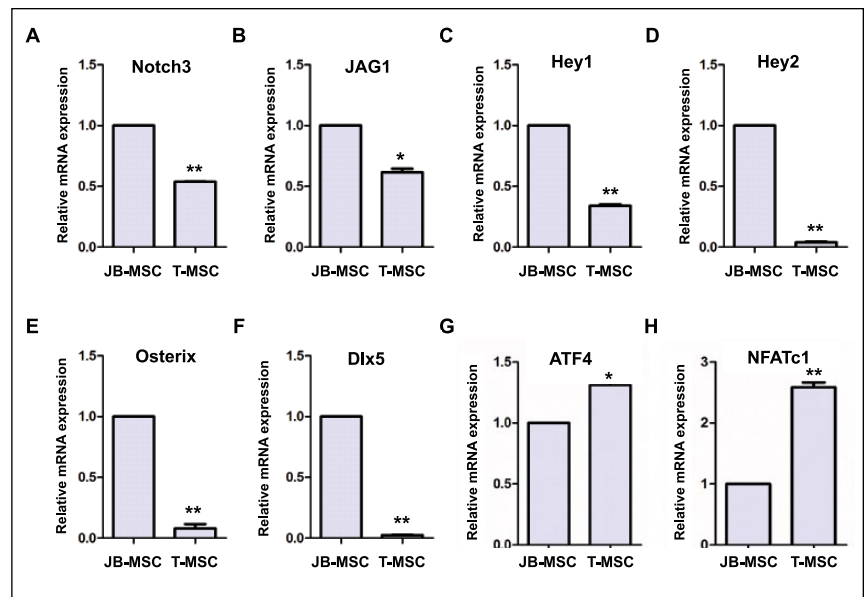
5D). On the contrary, knockdown of Notch3 expression simultaneously led to an increased expression of *AFT4* and *NFATc1* (Fig. 5E, F) but had no obvious effects on the expression of *Runx2* and *Dlx5* (Fig. 5G, H). These results further support the notion that loss of function of Notch3 signals may lead to enhanced osteogenic differentiation of JB-MSCs, a characteristic phenotype conferred by T-MSCs.

## Discussion

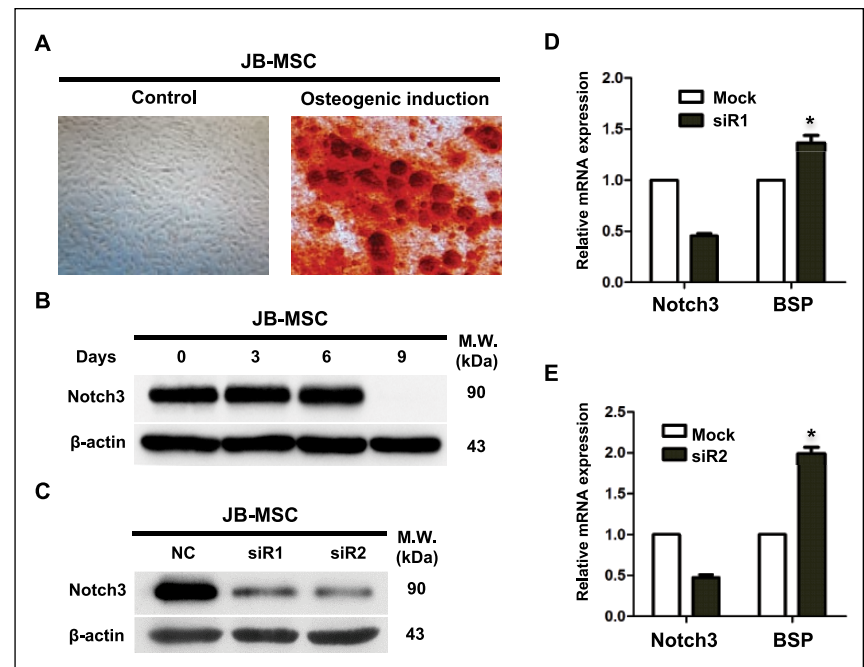
MSCs represent a unique subpopulation of stromal progenitor cells with multipotent and self-renewing capabilities, which exist not only in bone marrow but in almost all nonskeletal tissues. Beyond the potential use of MSCs in tissue regeneration and cell-based therapy of autoimmune and inflammatory diseases, dysregulated phenotypes and functions of MSCs may play an important role in the pathogenesis of skeletal and nonskeletal diseases. Most recently, we identified a subpopulation of MSCs from keloids, a benign skin tumor (Zhang et al. 2009). These MSCs possess distinctive properties in comparison to normal MSCs and may play an important role in the pathogenesis of these benign tumors.

MT constitutes the most common oral exostoses that do not have cartilage involvement, owing to their anatomic location. Even though several hypotheses, including genetic and environmental causes, have been proposed for the etiology of this benign bony outgrowth, there is still a lack of basic knowledge about the cellular and molecular mechanisms underlying its pathogenesis. In the present study, we have demonstrated for the first time, to our knowledge, that MT harbors a distinct subpopulation of MSCs (T-MSCs) that display enhanced osteogenic and decreased adipogenic differentiation capabilities as compared with their counterparts derived from normal jaw bone (JB-MSCs), suggesting that dysregulated functions of MSCs may contribute to the formation of MT.

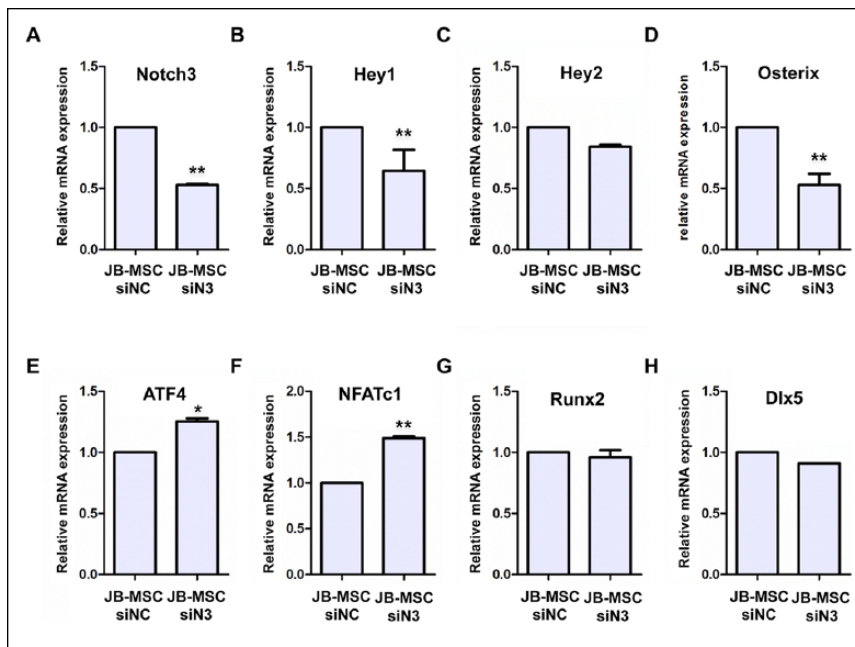
Notch signaling governs cell fate determination, proliferation, differentiation, and apoptosis (Long 2012; Chen et al. 2014). In recent years, accumulating



**Figure 3.** Osteogenic gene expressions in mesenchymal stem cells from jaw bone (JB-MSCs) and mandibular torus (T-MSCs). (A–H) The transcriptional expression of *Notch3*, *Jag1*, *Hey1*, *Hey2*, *Osterix*, *Dlx5*, *ATF4*, and *NFATc1* genes was determined by quantitative real-time polymerase chain reaction. The data are expressed as the mean  $\pm$  SD of 3 independent experiments (\* $P$  < 0.05, \*\* $P$  < 0.01).



**Figure 4.** Correlation between Notch3 expression and osteogenic differentiation of mesenchymal stem cells from jaw bone (JB-MSCs). (A) JB-MSCs were cultured under osteogenic induction condition for 9 d, and the mineralization was determined by alizarin red S staining. (B) JB-MSCs were cultured under osteogenic induction condition for different periods, and the expression of Notch3 protein was determined by Western blot analysis. (C) *Notch3* expression was knocked down by siRNA transfection (siR1, siR2) in JB-MSCs for 48 h, while transfection with a nonspecific siRNA (NC) was used as a control. (D, E) JB-MSCs were transfected with siRNAs against *Notch3* (siR1 and siR2) for 48 h, and the expression of bone sialoprotein (BSP) mRNA was determined by quantitative real-time polymerase chain reaction. The data are expressed as the mean  $\pm$  SD of 3 independent experiments (\* $P$  < 0.05).



**Figure 5.** Effect of *Notch3* gene expression on osteogenic markers in mesenchymal stem cells from jaw bone (JB-MSCs). JB-MSCs were transfected with siRNAs against *Notch3* (siN3) for 48 h, while transfection with a nonspecific siRNA (siNC) was used as a control. (A–H) The expression of *Notch3*, *Hey1*, *Hey2*, *Osterix*, *ATF4*, *NFATc1*, *Runx2*, and *Dlx5* mRNA was determined by quantitative real-time polymerase chain reaction. The data are expressed as the mean  $\pm$  SD of 3 independent experiments (\* $P < 0.05$ , \*\* $P < 0.01$ ).

evidence has implicated the critical role of Notch-mediated signaling in normal bone remodeling and homeostasis. It has been reported that osteo- and chondroprogenitors require Notch signaling to maintain multipotency, proliferation, and hypertrophy and to suppress premature and terminal differentiation into osteoblasts and chondrocytes (Chen et al. 2014). Several lines of evidence have demonstrated the expression of Notch1 and Notch2 by skeletal cells, which appear to mediate the effects of Notch signaling on bone microarchitecture and homeostasis (Engin et al. 2008; Hilton et al. 2008; Zanotti and Canalis 2014). It has been shown that activation of Notch signaling in the early phases of osteoblastic differentiation leads to reduce cancellous bone volume and disorganized deposition of woven bone (Engin et al. 2008; Zanotti et al. 2008; Tao et al. 2010) and elevated Notch-Hey1 or Notch-Hes signals result in impaired osteogenesis of bone marrow MSCs (Zamurovic et al. 2004; Hilton et al. 2008; Fei et al. 2015). In addition, a persistent Notch activation in MSCs has been shown to contribute to decreased osteoblast differentiation associated with rheumatoid arthritis (Zhang et al. 2014), and galectin-3 secreted in the bone microenvironment niche inhibits osteoblast differentiation through Notch1 signaling (Nakajima et al. 2014). However, inactivation of Notch signaling in osteoblasts results in increased trabecular bone volume due to enhanced osteoblastogenesis (Engin et al. 2008).

In the present study, we demonstrated that JB-MSCs and T-MSCs expressed similar baseline levels of Notch1, Notch2, and Notch4 signals; however, a relatively lower level of Notch3 signal was expressed in T-MSCs. Furthermore, we

showed that transient knockdown of *Notch3* expression increased the expression of osteogenic differentiation markers in JB-MSCs, suggesting that loss of function of Notch3 signals may play an important role in torus formation through promoting osteogenic differentiation of T-MSCs. Collectively, these data highlight the significance of Notch signaling pathways in the physiology and pathology of human bones. Of note, several lines of evidence have shown that causative mutations in exons 3 and 4 of the *Notch3* gene result in a gain or loss of a cysteine residue within the extracellular domain, which leads to an inherited disorder: cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (Mosca et al. 2014). It would be worthwhile to determine in a future study whether the lower/absent Notch3 signal in MT is caused by a pathogenic polymorphism or a mutation in the *Notch3* gene.

Binding of Notch receptors with cognate ligands leads to the proteolytic cleavage of the NICD, which translocates to the nucleus and transcriptionally activates the downstream target genes, including those encoding for the transcriptional repressors hairy enhancer of split (Hes) and Hes related with YRPW motif (Hey; Kopan and Ilagan 2009). To date, it is still unknown how Notch signaling pathways are involved in the regulation of osteoblast differentiation and bone formation. Runt-related transcription factor 2 (Runx2), one of the major osteogenic transcription factors, is essential for early but not the late stage of osteoblast differentiation, and it needs to be suppressed for the terminal differentiation of osteoblast into mature osteocytes (Liu and Lee 2013). Previous studies have shown that NICD and Hey1 can bind and inhibit the transactivation of Runx2, thus impairing osteoblast differentiation (Long 2012). However, in the present study we found that T-MSCs expressed a level of *Runx2* gene similar to that in JB-MSCs (data not shown) and that knockdown of *Notch3* gene expression had no obvious effects on *Runx2* expression in JB-MSCs. There is also evidence showing that Notch suppresses Wnt signaling that is required for osteoblastogenesis (Hilton et al. 2008; Zanotti et al. 2008). *ATF4*, another osteogenic transcription factor, plays an important role in the differentiation of more mature osteoblast cells. It can directly regulate the expression of osteocalcin and receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) and promote amino acid import to ensure proper protein synthesis by osteoblast (Long 2012). NFATc1, another key osteogenic transcription factor, governs the differentiation and function of osteoblasts, whereby the osteogenic differentiation is inhibited in *Nfatc1*- and *Nfatc2*-deficient cells (Koga et al. 2005). Later studies

indicated that *NFATc1<sup>Inuc</sup>* mice display massive osteoblast overgrowth and enhanced osteoblast proliferation and that viable *NFATc1*-deficient mice have defects in skull bone formation (Winslow et al. 2006). Furthermore, recent studies have shown that Notch inhibited ATF4 transactivation and NFATc1 transcription, while NFATc1 reciprocally inhibited the transactivation of Notch target genes, suggesting that Notch and NFATc1 form a regulatory network to regulate the differentiation of osteoblasts (Zanotti et al. 2011). Herein, we showed that T-MSCs constitutively express a higher level of *ATF4* and *NFATc1* than JB-MSCs, which is conversely correlated with the decreased expression of Notch3 in T-MSCs; knockdown of Notch3 expression led to increased expression of *ATF4* and *NFATc1* in JB-MSCs.

These findings suggest that a dysregulated Notch3-NFATc1 axis may play an important role in the regulation of aberrant osteogenic differentiation of JB-MSCs, thus contributing to the development of MT. However, further studies are warranted to establish stable Notch3-deficient JB-MSCs through appropriate genetic approaches to verify the long-term effects of loss of Notch3 on osteogenesis of JB-MSCs and the molecular mechanisms in detail.

### Author Contributions

X.W. Dou, contributed to conception, design, data acquisition, analysis, and interpretation, critically revised the manuscript; W. Park and S. Lee, contributed to data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; Q.Z. Zhang, A.D. Le, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; L.R. Carrasco, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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