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Olfactomedin-like protein OLFML1 inhibits Hippo signaling and mineralization in osteoblasts

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ABSTRACT

Congenital scoliosis is a lateral curvature of the spine that is due to the presence of vertebral anomalies. Although genetic and environmental factors are involved in the pathogenesis of congenital scoliosis, the specific cause of only a small number of individuals has been identified to date. We identified a *de novo* missense mutation in the olfactomedin-like 1 (*OLFML1*) gene by whole-exome sequencing of a patient with congenital scoliosis. Then, we carried out further functional investigation in mice. An assessment of the tissue distribution of *Olfml1* revealed it to be prominently expressed in developing skeletal tissues, specifically osteoblasts. Short hairpin RNA-mediated knockdown of Olfml1 in osteoblasts induced the translocation of Yes-associated protein (YAP) transcriptional coactivator from the cytoplasm to the nucleus, which accelerated the Hippo signaling pathway to promote osteoblast mineralization. In contrast, experimentally induced gain of function of Olfml1 retained YAP in the cytoplasm. There appears to exist a novel cell-autonomous mechanism by which osteoblasts avoid excess mineralization through Olfml1. Our results also indicate that mutation of *OLFML1* leads to impaired osteoblast differentiation and abnormal development of bone tissue.

1. Introduction

With an incidence of 0.5–1 per 1000 births, congenital scoliosis (CS) is characterized by a lateral curvature of the spine exceeding 10° that is due to the presence of one or more abnormally formed vertebrae [1,2]. Genetic inheritance is responsible for some congenital vertebral anomalies, such as spondylocostal dysostosis and spondylothoracic dysostosis [3]. Wu et al. reported that 11% of CS cases harbored a rare null mutation and/or a hypomorphic allele of *TBX6* [4]. Therefore, some CS cases may develop due to a TBX6 abnormality, although the genetic etiology of most CS patients remains unclear.

Olfactomedin-like 1 (OLFML1) is a member of the olfactomedin (OLF) protein family containing an olfactomedin-like domain in the C-terminus [5]. OLF family expression is restricted to certain tissues and has been implicated in both normal development and pathology [6–14]. OLF proteins contribute to the formation of extracellular matrix and play a role in developmental processes [13–16]. OLFML1 is considered a secreted glycoprotein that enhances cell cycle progression in human cancer cell lines *in vitro* [17]. However, its physiological roles require clarification [18].

This study examined a Japanese patient with CS and his parents by whole-exome sequencing (WES) to identify a *de novo* missense mutation in the *OLFML1* gene (Met250Leu; Supplemental Figure S1A–D). We evaluated a transcript abundance of *Olfml1* in newborn mice and found it to be predominantly expressed in osteoblasts. Experimentally induced gain or loss of Olfml1 function revealed that the protein suppressed the Hippo signaling pathway and inhibited mineralization in osteoblasts. Our data provide evidence of a novel cell-autonomous mechanism by which osteoblasts avoid excess mineralization through Olfml1.

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2. Material and methods

2.1. Mice

Male and female 6-week-old ddY mice were purchased from Japan SLC. Animal experiments were performed in compliance with the 3Rs, and all efforts were made to minimize suffering. All procedures for animal care were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals and approved by the Animal Management Committee of Matsumoto Dental University.

2.2. Cell cultures

Murine bone marrow macrophages and mature osteoclasts were prepared as described previously [19]. Briefly, bone marrow cells (1×10^7 cells) were obtained from 8 to 10-week-old male ddY mice and cultured in minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) and 100 μg ml^{-1} penicillin/streptomycin in the presence of Csf1 (50 ng ml^{-1}; Kyowa Kirin) for 1 day. Nonadherent cells containing osteoclast precursors were harvested for 2 days with Csf1 and used as bone marrow macrophages (BMMs). BMMs were cultured further with Csf1 and GST-Rankl (200 ng ml^{-1}; Oriental Yeast) for 3 days and the presence of mature osteoclasts was determined by tartrate-resistant acid phosphatase activity using Napthol-ASTR-phosphate as a substrate.

Primary osteoblasts derived from calvaria were prepared from P0.5 mice as described previously [20]. Cells were maintained in α-MEM supplemented with 10% FBS and 100 μg ml^{-1} penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. To induce osteoblastic differentiation, primary osteoblasts (2×10^4 cells cm^{-1}) were cultured in osteogenic medium containing 5 mM β-Glycerophosphate and 100 μg ml^{-1} ascorbic acid with or without 100 ng ml^{-1} BMP2 (R&D systems) using gelatin-coated 96-well plates. The cells were fixed after 5 or 20 days of cultivation and stained with alkaline phosphatase (ALP) or alizarin red S, respectively. The alizarin red S staining was released from the cell matrix by incubation in 3.5% cetyl-pyridinium chloride for 1 h. The amount of released dye was quantified by spectrophotometry at 555 nm (Synergy HTX; Bio-Tek).

2.3. Quantitative reverse transcription-PCR (qPCR) assay

Lysis of tissues harvested from newborn mice (P0.5), BMMs, mature osteoclasts, and primary osteoblasts was performed in TRIzol Reagent (Invitrogen), and RNA was extracted using NucleoSpin® RNA Mini Kits (Takara Bio). Complementary DNA was synthesized from 1 μg of total RNA, an oligo(dT) primer, a random primer, and reverse transcriptase (ReverTra Ace; Toyobo). Each primer set was purchased from Takara Bio and shown in Supplementary Table S1. qPCR was performed using PowerUp™ SYBR Green Master Mix (Applied Biosystems) and a StepOnePlus machine and analyzed by StepOne software (Applied Biosystems). Individual mRNA expression levels were calculated with the $\Delta\Delta C_T$ method and normalized to the expression of the house-keeping gene *hypoxanthine phosphoribosyl transferase* (*Hprt1*).

2.4. Adenovirus-mediated knockdown

Short hairpin RNAs (shRNAs) strands were designed to target mouse *Olfml1* (NM_172907.3) using an shRNA sequence design tool made by Takara Bio. Double-stranded oligonucleotides of the target were inserted into pSIREN-Shuttle vectors, and PCR fragments containing a U6 promoter and the target sequences were cloned further in the pAdenoX vector. The linearized vectors were transfected into human embryonic kidney 293T (HEK293T) cells to produce adenoviruses. The target sequences of *Olfml1* were as follows: sh*Olfml1*_#1; 5′-CTCTCATCGAATCACCTGT-3′ and sh*Olfml1*_#2; 5′-GCCATGGTACACTACATCTAC-3'. The shRNA targeting luciferase was used as a control shRNA, with a targeting sequence of 5′-GTGCGTTGCTAGTACCAAC-3' (shControl).

2.5. Cell viability assay

Calvaria-derived osteoblasts were enriched in 96-well plates and infected with adenoviruses expressing shControl or sh*Olfml1*_#1 and #2. After 48 h, cell numbers were counted using a Cell Counting Kit-8 (Dojindo Laboratories).

2.6. Immunoblotting assay

Cell lysates preparation and SDS-polyacrylamide gel electrophoresis/immunoblotting analysis were performed according to a standard protocol. Proteins were harvested in cell lysis buffer supplemented with proteinase inhibitor cocktail (Sigma-Aldrich, 1:100). Immunoblotting was performed using the following antibodies: anti-Yes-associated protein (YAP) rabbit mAb (#14074; Cell Signaling Technology, 1:1000), anti-transcriptional co-activator with PDZ-binding motif (TAZ) rabbit mAb (ab84927; abcam, 1:1000), anti-α tubulin mouse mAb (CP06; Calbiochem, 1:1000), anti-Myc-tag mouse mAb (M192-3; MBL, 1:5000), donkey anti-rabbit IgG-HRP (NA934V; GE Healthcare, 1:5000), and goat anti-mouse IgG-HRP (170−6516; Bio-Rad Laboratories, 1:10000).

2.7. Overexpression of Olfml1 in ST2 cells

A plasmid vector expressing murine Olfml1 with a Myc-tag driven by the CMV promoter was obtained from OriGene (Olfml1-Myc-pCMV6-Entry; MR202820). The plasmid was digested with BamH1 and Not1 restriction enzymes, and we cloned the mCherry sequence (Clontech) into the vector instead of the Olfml1 sequence (mCherry-pCMV6-Entry). These plasmid vectors were transfected into ST2 cells using Xtreme Gene 9 (Roche) according to the manufacturer's instructions and incubated for 24 or 48 h.

2.8. Immunocytochemistry

Primary osteoblasts and ST2 cells on gelatin-coated cover glasses were used for the analysis. The cells were fixed with 4% solution of paraformaldehyde in PBS and blocked with 1% bovine serum albumin and 0.1% Triton X-100 in PBS for 1 h at room temperature. Each samples was incubated overnight at 4 °C with primary antibodies. After washing 3 times with PBS, the cells were incubated with secondary antibodies for 2 h at room temperature. The samples were washed, stained with TO-PRO®-3 (Thermo Fisher Scientific), and analyzed using a confocal laser scanning system (LSM 510; CarlZeiss). The images were captured with Zen acquisition and analysis software (Carl Zeiss). Immunocytochemistry was performed using the following antibodies: anti-YAP rabbit mAb (#14074; Cell Signaling Technology, 1:100), anti-Myc-tag mouse mAb (M192-3; MBL, 1:200), donkey anti-rabbit IgG NL557-conjugated pAb (NL004; R&D Systems, 1:200), donkey anti-rabbit IgG FITC-conjugated pAb (A120-201F; Bethyl Laboratories, 1:100), and donkey anti-mouse IgG Cy3-conjugated pAb (AP192C; Merck Millipore, 1:100).

2.9. Statistical analysis

All values represent the means and standard error. Statistical analysis was performed using GraphPad Prism7 software. When

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