The Effects of Osterix on the Proliferation and Odontoblastic Differentiation of Human Dental Papilla Cells

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Abstract

Introduction: Dental papilla cells (DPCs) are precursors of odontoblasts and have the potential to differentiate into odontoblasts. Osteoblasts and odontoblasts have many common characteristics. Osterix (Osx) is essential for osteoblast differentiation. However, no information is available for the effects of Osx on the odontoblastic differentiation of DPCs. The purpose of this study was to investigate the effects of Osx on the proliferation and odontoblastic differentiation of DPCs. **Methods:** An immortalized human dental papilla cell (hDPC) line was used. Osx was stably overexpressed or knocked down in hDPCs with infection of lentiviral particles to determine its biological effects on hDPCs. The proliferation of cells was measured by the 5-ethynyl-2′-deoxyuridine incorporation assay and direct cell counting. Expressions of dentin sialophosphoprotein, nestin, dentin matrix protein 1, and alkaline phosphatase were detected by real-time polymerase chain reaction to determine the odontoblastic differentiation of cells. The mineralization ability of cells was evaluated by von Kossa staining and alkaline phosphatase activity assay. **Results:** Overexpression of Osx retarded the proliferation of hDPCs, whereas knockdown of Osx increased the cell proliferation. Overexpression of Osx promoted the odontoblastic differentiation of hDPCs by up-regulating odontoblastic differentiation genes and increased the mineralization ability of hDPCs. Knockdown of Osx down-regulated odontoblastic differentiation genes and decreased the mineralization ability of hDPCs. **Conclusions:** Osx might function as a potential regulator for the proliferation and odontoblastic differentiation of hDPCs. *(J Endod 2014;40:1771–1777)*

Key Words

Dental papilla cells, dentin sialophosphoprotein, 5-ethynyl-2′-deoxyuridine, lentivirus, osterix, short hairpin RNA

Odontogenesis is the result of the reciprocal interactions between ectoderm-derived oral epithelium and the underlying cranial neural crest–derived mesenchymal cells leading to the terminal differentiation of odontogenic cells (1). Tooth development initiates from thickening of the oral epithelium. After bud stage, tooth development reaches the cap stage when odontogenic mesenchymal cells become partly surrounded by the epithelium and form the dental papilla (2, 3). During the late bell stage of tooth development, only the dental papilla cells (DPCs) immediately underlying the enamel epithelium differentiate into odontoblasts, whereas the rest of the DPCs remain undifferentiated and form the dental pulp in the future (4). It is now generally accepted that DPCs are precursors of odontoblasts and have the potential to differentiate into odontoblasts (5, 6). Although the differentiation process from DPCs to odontoblasts has been reported to be regulated by complex signaling pathways, including many growth factors (bone morphogenetic protein, fibroblast growth factor, Wingless-Int, and so on) and transcription factors (msh homeobox 1, paired box 9, runt-related transcription factor 2, and so on) (2, 7), the molecular mechanisms controlling odontoblastic differentiation of DPCs are yet not understood.

Osterix (Osx or Sp7) is a zinc finger–containing transcription factor encoded by the Sp7 gene expressed in all osteoblasts during embryonic development and is essential for osteoblast differentiation and bone formation (8). Osx regulates the expression of important bone-related genes including bone sialoprotein and osteocalcin (9, 10). In Osx-null mice, preosteoblasts are arrested in their differentiation and unable to express genes characteristic of the osteoblast phenotype, which results in the abolishment of cortical bone and bone trabeculae formation (8). Besides osteoblasts, Osx is also expressed in chondrocytes (11) and involved in chondrogenic gene activation as a positive regulator of the chondrocyte differentiation (12). Furthermore, Osx is an essential transcription factor in the cell-fate decision process during osteogenesis by determining mesenchymal cells become chondrocytes or osteoblasts through the activation of cell type–specific genes (13).

Both osteoblasts and odontoblasts originate from mesenchymal cells and share many common characteristics (14). However, no information is available regarding the effects of Osx on the odontoblastic differentiation potential of DPCs. To understand the role of Osx during tooth development, the Osx and dentin sialophosphoprotein (Dspp) messenger RNA (mRNA) expression patterns at different stages of tooth development were studied using *in situ* hybridization by Chen et al (14). They found the following: at the early bud stage, Osx expression was weakly detected in the mesenchymal condensates of forming teeth; at the cap stage, Osx mRNA was expressed in mesenchymal cells in dental papilla, and no Dspp signal was detected; at the bell stage, Osx transcripts were highly expressed in differentiating odontoblasts and dental pulp cells, and there was

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0099-2399/$ - see front matter

http://dx.doi.org/10.1016/j.joen.2014.04.012

a weak Dspp signal in these cells; and from embryonic day 18 to postnatal day 14, Osx expression became more intense in odontoblasts and dental pulp cells, and during this period the Dspp mRNA signal was highly expressed in differentiating and differentiated odontoblasts. Taken together, Osx expression was initiated in early dental mesenchyme, remained intense in odontoblasts and dental pulp cells, and overlapped with Dspp expression at later stages (14), suggesting that Osx may play a role in regulating odontoblastic differentiation of DPCs.

Thus, the purpose of the present investigation was to evaluate the effects of Osx on odontoblastic differentiation of DPCs. An immortalized human dental papilla cell (hDPC) line was used, and Osx was stably overexpressed or knocked down in hDPCs with infection of lentiviral particles to determine its roles on their proliferation and odontoblastic differentiation.

Materials and Methods

All procedures were approved by the Ethics Committee on Human and Animal Research, Wuhan University, Wuhan, China.

Cell Culture of hDPCs

An immortalized hDPC line previously established (15) was used. Cells were cultured in maintenance medium containing Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin (all from Gibco, Grand Island, NY). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

For differentiation and mineralization experiments, cells were cultured in odontoblastic induction medium (ie, Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 10 nmol/L dexamethasone, 10 mmol/L beta-glycerolphosphate, and 50 μg/mL ascorbic acid [all from Sigma-Aldrich, St Louis, MO]).

Immunofluorescence

Cells grown on glass coverslips were fixed with 4% paraformaldehyde for 10 minutes and permeabilized with 0.5% Triton-X (Sigma-Aldrich) for 15 minutes. Nonspecific binding of antibodies was blocked by 10% goat serum for 30 minutes. Samples were incubated overnight at 4°C with primary anti-Osx antibody (Santa Cruz Biotechnology, Dallas, TX). After washing with phosphate buffered saline (PBS), secondary immunoglobulin G antibody conjugated to Alexa-Fluor 568 (Invitrogen, Grand Island, NY) was added and incubated for 1 hour. 4',6-diamidino-2-phenylindole (DAPI) was used for nuclear counterstain.

Lentivirus Vector Construction

Packaging plasmid psPAX2 and envelop plasmid pMD2.G as well as vector plasmid pLL3.7 were used. pLL3.7 contains an enhanced green fluorescence (EGFP) reporter gene promoted by cytomegalovirus (CMV) promoter. pLL3.7-Osx lentivirus vector was constructed to overexpress Osx. Briefly, the full-length human Osx complementary DNA (cDNA) promoted by CMV promoter was subcloned into the pLL3.7 vector between the HpaI and XhoI enzyme sites. Sequencing was used to confirm the identity and orientation of the vector.

Generation of Recombinant Lentivirus and Infection of hDPCs

The production of recombinant Osx lentivirus was achieved by transient cotransfection of 3 plasmids into human embryonic kidney 293T (HEK293 T) cells. Briefly, cells were transfected using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to manufacturer's instructions with a total of 10 μg plasmids DNA (4 μg packaging plasmid psPAX2, 1 μg envelop plasmid pMD2.G, and 5 μg vector plasmid pLL3.7-Osx [pLL3.7 empty vector for control cells]). Supernatant containing viral particles was collected 48–72 hours after transfection and filtered through a 0.45-μm filter (Millipore, Billerica, MA). The viral titers were determined by infection of 293T cells with serial dilutions of the lentiviral supernatants and flow cytometric analysis of the percentage of EGFP-expressing cells.

For infection of cells, hDPCs at 70%–80% confluence were exposed to viral supernatant for 6 hours in the presence of polybrene (Millipore) at a final concentration of 8 μg/mL; after this, cells were maintained in fresh medium. After incubation for 24 hours, fresh viral suspensions were added for an additional 6 hours with subsequent change of the medium. At that time, the cells were determined as day 0. Cells with successful infection of Osx were designated as hDPC/Osx; control cells infected by empty vector and wild-type hDPCs were designated as hDPC/pLL3.7 and hDPC/wt, respectively.

Detection of Integration of CMV-Osx Gene by Polymerase Chain Reaction

Genomic DNA was isolated from cells using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). In pLL3.7-Osx plasmid, human Osx cDNA was promoted by CMV promoter. Thus, to confirm integration, the following construct-specific primers amplifying both CMV and Osx were used: forward: 5′-CGCAAATGGGCGGTAGGCGTG-3′ and reverse: 5′-AGACACTGGGCAGACAGTC AGAAGA-3′. The sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were the same as those used in real-time polymerase chain reaction (PCR) shown in Supplemental Table 1 (Supplemental Table 1 is available online at www.jendodon.com).

Lentivirus-mediated Infection of Osx shRNA

To achieve the stable knockdown of endogenous Osx, Osx shRNA(h) and negative control shRNA lentiviral particles were purchased from Santa Cruz Biotechnology. Infection was performed as mentioned earlier. Cells infected with Osx shRNA and control shRNA were designated as hDPC/shRNA and hDPC/Scrambled, respectively.

Cell Growth Analysis

To determine cell proliferation, cells were seeded into 6-well plates at 2×10^4 cells per well. Cells were detached from the culture wells with 0.05% trypsin/EDTA (Gibco) and counted daily using a hemocytometer for up to 19 days.

5-ethynyl-2′-deoxyuridine Incorporation Assay

Cell proliferation ability was also detected by the 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay with Click-iT EdU HCS Assays (Invitrogen) (16). All the procedures were applied according to the manual. Briefly, cells at a density of 2×10^4 cells per well in 48-well plates were serum starved for 24 hours and incubated with EdU for 6 hours, and then the EdU incorporation was detected by the Click-iT reaction cocktail. After that, nuclei were stained with Hoechst 3342. The number of EdU-positive cells and total cells was counted in 3 different view fields. The ratio of the EdU-positive cell number to the total cell number was calculated as the proliferation index.

Real-time Reverse-transcription PCR

Cells were cultured in odontoblastic induction medium for 0, 7, and 14 days. Total RNA from cells was isolated using TRIzol Reagent (Invitrogen). cDNA synthesis was performed using the Reverse Transcription System (Promega). PCR amplification was performed with