electrophoretic mobility-shift assay (EMSA) and Chromatin immunoprecipitation (ChIP) assays in the mouse dental pulp cells demonstrated direct functional binding of Runx3 to *Osterix* promoter. These results demonstrate the transcriptional regulation of *Osterix* expression by Runx3 during differentiation of dental pulp cells into odontoblasts in tooth development.

Short Title: Down-regulation of *Osterix* by Runx3

Keywords: Dental pulp cells, Runx3, Runx2, Osterix, Bone morphogenetic protein 2, tooth development

Abbreviations footnote: BMP, bone morphogenetic protein, RT-PCR, reverse transcription-polymerase chain reaction, Dspp, Dentin sialophosphoprotein, KLK4, Kallikrein 4, DPCs, dental pulp cells

INTRODUCTION

The transcriptional regulation of cell proliferation and differentiation by the Runt-related (RUNX) family of DNA-binding transcription factors is critical for both morphogenesis and regeneration. The regulatory function of Runx family on the promoters and enhancers of target genes where they associate with cofactors and other DNA-binding transcription factors to modulate gene expression is well known [1]. Runx family is composed of three members of Runx family designated Runx1/AML1/Cbfa2, Runx2/AML2/Cbfa1, Runx3/AML3/Cbfa3 [2, 3]. Although the Runx members share highly conserved DNA binding domains, they regulate distinct functions [4-7]. Runx1 is involved in regulation of hematopoiesis[8]. Runx2 are essential for bone and tooth development [9-11]. Runx3 is critical for gastric epithelial differentiation, neurogenesis of the dorsal root ganglia and T cell differentiation[8-10, 12-16].

Stringent control of gene activation and suppression is required for tooth development. The optimal gene expression during dentin formation is dependent on

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integration and regulation of signals that governs the commitment of stem/progenitor cells to pulp cell lineage and proliferation and differentiation into odontoblasts. Runx2 is essential for tooth formation. Molar development is arrested at the late bud stage in Runx2 homozygous mice [11], correlating with the intense expression of Runx2 in the dental mesenchyme during the bud and cap stages [17]. Runx3 is coexpressed in dental papilla at the cap and early bell stages with Runx2. Later Runx3 is restricted to the odontoblastic layer at the late bell stage while Runx2 is no longer detected [17]. Runx proteins might play a pivotal role in governing physiologically responsive control of dental genes.

Osterix, a zinc finger-containing transcription factor, is required for osteoblast differentiation and bone formation [18]. In *Osterix* null mice, no bone formation occurs, similar to the phenotypes in *Runx2* null mice [9, 18]. However, Runx2 is expressed without major alterations in *Osterix* null mice. In contrast, *Osterix* is not expressed in Runx2 null mice, demonstrating that Osterix acts downstream of Runx2 [18]. Recently transcriptional regulation of *Osterix* in cartilage by Runx2 has been suggested [19]. *Osterix* is expressed in mesenchymal cells of the tooth germ [18]. The expression of *Osterix* and its transcriptional regulation by Runx during tooth development have not been investigated.

In the present study, we investigated the expression of *Osterix* during tooth development, and demonstrated that *Osterix* was strictly expressed in odontoblastic layer at the bell and the differentiation stage, overlapping with *Runx3*. Therefore, the regulation of the expression of *Osterix* by Runx3 was further examined. Our results demonstrated that Runx3 directly binds to *Osterix* promoter and down regulates its expression in dental pulp cells.

EXPERIMENTAL

Cloning of the Osterix promoter

To clone the Osx promoter (nucleotide 66 to 1751; GenBank accession no. DQ229136), genomic DNA was isolated from the tail of ICR mouse. PCR was performed using two primers, Osterix promoter 5'-1:

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5'-TCTGTCCCTCAGTCCTGCTT-3'; Osterix promoter 3'-2: 5-GGGCAAGTTGTCAGAGCTTC-3'. The 1.7 kb PCR product was then subcloned into *Mlu*I/*Xho*I site of pGL3-promoter vector (Promega, Madison, WI, U.S.A.), named pOsx1.7-luc. To prepare the *MSCV-eGFP-Flag-Runx3* expression vector, following primers were used: Flag-Runx3-5': 5'-GGCAGATCTGCCACCATGGACTACAAGGACGATGACGACAAGGCTTCCAACAGCATCTTTG-3' and Flag-Runx3-3': 5'-ATATGAGCTCTCCCGCGTGGT-3' to generate a Runx3 fragment with FLAG motif at N-terminal. The 300 bp PCR product was cloned into the *Bgl*II-*Sac*I site in PSL1180 vector (GE Healthcare, Buckinghamshire, U.K.) and named Flag-Runx3-300bp-PSL1180. A 1.0 kb Runx3 fragment was digested with *Sac*I from *MSCV-eGFP-Runx3* plasmid (kindly provided by Dr. Taniuchi Ichiro, Laboratory of Transcriptional Regulation, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan) and subcloned into Flag-Runx3-300bp-PSL1180 vector, named Flag-Runx3-PSL1180. The 1.3 kb full length of Runx3 with Flag-tagged N-terminal was digested with *Bgl*II from Flag-Runx3-PSL1180 and subcloned into *MSCV-eGFP* vector, named with *MSCV-eGFP-Flag-Runx3*. The orientation of the inserts was confirmed by sequencing.

Site-directed mutagenesis

Three putative Runx2-binding sequence -1823 to -1817, -1776 to -1771 and -713 to -707 bp from the Cap site [19] were mutated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer's recommendations. We generated mutants as follows; 5'-AACCACA-3' at -1823/-1817 bp was changed into 5'-**G**A**G**C**T**CA-3', 5'-ACCACT-3' at -1776/-1771 bp was changed into 5'-**G**C**T**ACT-3' and 5'-AGTGGTT-3' at -713/-707 bp was changed into 5'-A**TA**GA**C**T-3'. The mutated nucleotides are indicated in bold. Mutations in single, double, and triple motifs were termed M1-M5 (Fig. 3B). Incorporation of the mutated substitution of all constructs were confirmed by sequencing.

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In situ hybridization

ICR Mouse embryos at 15.0 dpc, 17.0 dpc and postnatal day 1 were fixed in 4 % paraformaldehyde at 4 °C overnight. In situ hybridization was carried out as previously described [20]. Primers (Osterix-5'-1: 5'-GGTCCAGGCAACACACCTAC-3'; Osterix-3'-2: 5'-GGTAGGGAGCTGGGTTAAGG-3') were used to amplify the mouse *Osterix* cDNA. PCR product was ligated into pBluescript II SK (-) vector (Stratagene). Mouse *Runx3* cDNA was digested by *Eco*RI from mouse *MSCV-eGFP-Runx3* plasmid, then subcloned into pBluescript II SK (-) vector. All inserts were confirmed by sequencing. The following cDNAs were used to generate sense and antisense riboprobes using either T3 or T7 RNA polymerase: a 184 bp murine Osterix fragment, a 1.2 kb Runx3 fragment and a 1.2 kb Bmp2 fragment. In situ hybridization was performed as described previously [21]

Cell Culture and transfection studies

Mouse dental pulp cells (DPCs) were isolated from tooth germ at 17.0 dpc. mDPC and HEK293 cells (epithelial cell line derived from human kidney transformed embryonic cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, U.S.A.) supplemented with 100 units/ml penicillin G, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, U.S.A.) and 10% (v/v) fetal bovine serum (SAFC Biosciences, Lenexa, Kansas, U.S.A.). Experiments assessing promoter activity by luciferase were performed as follows. HEK293 cells ($1x10^5$) were plated in 24-well plates in antibiotics-free and serum-free DMEM one day before, and transiently transfected with 2 μg of each promoter/pGL3 luciferase reporter plasmids, 3 μg of expression plasmid, and 0.2 μg of SV-40 promoter construct (Promega) as an internal standardize control for transfection efficiency. Transfections were performed using 2 μl/well of Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. *MSCV-eGFP* plasmid was also transfected as control. After 4 h, the medium was changed into DMEM with 10% (v/v) foetal bovine serum and cultured

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for an additional 44 h. Cells were then lysed, and luciferase activity was determined using a Dual Luciferase Report Assay kit as instructed by the manufacturer (Promega). All activities were normalized against co-transfected internal control plasmid pRL-SV40 (Promega). For overexpression experiments, 4 x 10^6 DPCs were transfected by 8 µg of expression plasmid using ECM 830 Electroporator (BTX, San Diego, CA, U.S.A.) following the manufacturer's instructions, then plated on collagen type I-coated 35 mm dish (Iwaki, Chiba, Japan). After 4 h, the medium was changed into DMEM with 10% (v/v) foetal bovine serum. Cells were harvested at 0h, 24h, and 48h after transfection. The cell viability was determined with trypan blue soon after transfection, and the efficiency was estimated by fluorescent microscopy 24 hours after transfection with the plasmid vector *AFP* (kindly provided by Dr. Hidesato Ogawa, Graduate School of Biological Sciences, Nara Institute of Science and Technology, Japan).

Real time reverse transcriptase polymerase-chain reaction (RT-PCR) analysis

Total RNA was extracted by using Trizol (Invitrogen), and 2 µg of freshly isolated RNA was reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) following the manufacturer's recommendations. The resulting cDNA was then amplified by Real Time RT-PCR with Light Cycler-FastStart DNA master SYBR Green I (Roche Diagnostics, Mannheim, Germany). The primers used in this study are presented in Table 1.

Preparation of nuclear extracts

Nuclear extract was isolated as previously reported [22]. Briefly, mouse DPCs was washed with 10 ml of PBS, scraped in 1.5 ml ice cold PBS, and centrifuged at 100 ***g*** for 5 min. The pellet was suspended in 1 ml of PBS and centrifuged again at 660 ***g*** for 15 sec. After resuspension in cold buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1mM dithiothreitol and 0.5 mM PMSF) on ice for 15 min. The cell membranes were lysed by Nonidet P40 at a final concentration of 0.5 %, centrifuged at 660 ***g*** for 30 sec, and the pelleted nuclei were resuspended in

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cold buffer C (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 1 mM PMSF). The nuclear protein was extracted by shaking at 4 °C for 15 min, centrifuged at 15,000 *g* for 5 min, and the supernatant fractions were collected. The protein content of the nuclear extracts was determined using the Bradford protein analysis method [23].

Electrophoretic mobility shift assay (EMSA)

Individual oligonucleotides were annealed to equimolar amounts of their complementary strands (Wild type, Osterix-gel-WT-5'-1: 5'-CAGATCTCTAATTAGTGGTTTGGGGTTTGTTCCTTTTC-3' and Osterix-gel-WT-3'-2: 5'-GAAAAGGAACAAACCCCAAACCACTAATTAGAGATCTG-3'; mutant, Osterix-gel-MT-5'-1: 5'-CAGATCTCTAATTATAGACTTGGGGTTTGTTCCTTTTC-3' and Osterix-gel-MT-3'-2: 5'-GAAAAGGAACAAACCCCAAGTCTATAATTAGAGATCTG-3') by heating to 95 °C for 5 min and slowly cooling to room temperature. DIG Gel Shift Kit, 2nd generation (Roche Diagnostics) was used in electrophoretic mobility shift assay according to the manufacturer's protocol. Briefly, wild type double-stranded oligonucleotide probes were labeled with digoxigenin-11-ddUTP at 3'-ends. The labelled probes (20 fmol) were added to 10 μg nuclear extracts in a binding buffer (20 mM Hepes, pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.2 % (w/v) Tween 20, 30 mM KCl, 25 ng/μl poly d (I-C), 25 ng/μl poly d (A-T) and 50 ng/μl poly L-lysine) at room temperature for 30 min. For competition experiments, 125-fold unlabelled cold oligonucleotides were added in the mixture. After incubation, the protein–DNA complexes were separated by 6% acrylamide native polyacrylamide gel electrophoresis, transferred to a nylon membrane (Whatman Inc., New Jersey, U.S.A.) by contact-blotting, and detected by the DIG-detection kit. Antibody against Runx3 (Active Motif, Carlsbad, CA, U.S.A.) was added to examine specificity of the protein-DNA complex.

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Chromatin immunoprecipitation (ChIP) assay

Mouse DPCs were treated for 10 min of 1% formaldehyde and washed by ice cold PBS, 3 times, harvested and centrifuged at 100 *g* for 5 min. The pellet was suspended in 200 μl of SDS lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 1 % (w/v) SDS, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin) and incubated on ice for 20 min. The sample was sonicated for 7.5 min (power high, on 30 sec, off 1 min) using a Bioruptor (Cosmo Bio, Tokyo, Japan) to produce soluble chromatin, with average size at 500 bp. The chromatin sample was then diluted nine-fold in ice cold ChIP dilution buffer (50 mM Tris-HCl, pH 8.0, 167 mM NaCl, 1.1 % (v/v) Triton X-100, 0.11 % (w/v) sodium deoxycholate, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin). From the diluted sample 200 μl was removed to keep as input fraction at 4 °C. The rest of the sample was precleaned for 6 h using 60 μl of salmon sperm DNA/protein G Sepharose beads at 4 °C, centrifuged at 10,000 *g* for 10 sec, and the supernatant was collected. Twenty microgram of rabbit anti-Runx3 polyclonal antibody (Active Motif, Carlsbad, CA, U.S.A.) or 10 μg of goat anti-mouse Runx2 polyclonal antibody (Santa Cruz, CA, U.S.A.) was added and incubated overnight at 4 °C. To collect the immunocomplex, 60 μl of salmon sperm DNA/protein G Sepharose beads were added to the samples for 3 h at 4 °C. The beads were washed once in each of the following buffers, in order: low salt, high salt, and LiCl wash solution; it was then washed twice in TE buffer. The bound protein-DNA immunocomplexes were eluted twice with 200 μl of ChIP direct elution buffer (10 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM EDTA, pH 8.0, 0.5 % (w/v) SDS) and subjected to reverse crosslinking at 65 °C for 6 h. The reverse crosslinked chromatin DNA was further purified by 50 μg/ml proteinase K digestion at 55 °C for 1 h and phenol-chloroform extraction. DNA was then precipitated in ethanol and dissolved in 20 μl of TE buffer. Two microliters of DNA were used for each RT-PCR with primers Osx-ChIP-F: 5'-GAGTGTCGTCCCCAATCC-3' and Osx-ChIP-R: 5'-CTGCTACCACCGAGGCTG-3', yielding a 120-bp product. For a negative control of ChIP assay of Runx3 or Runx2, another 1×10^7 mouse DPCs was treated as

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the same way but with 20 μg rabbit IgG or 10 μg goat IgG. Input (1/20) was used as the positive control of RT-PCR.

Statistics

Statistical analyses were performed using Student's unpaired *t*-test. Each experiment was performed at least twice, and the representative data were presented as means ± S.D. of independent replicates ($n \geq 3$).

RESULTS

Expression of Runx3, Runx2, Osterix and Bmp2 during tooth development

In the developing tooth, *Runx3* was detected in the dental papillae at the late cap stage (15.0 dpc). *Runx3* was progressively restricted to the odontoblastic layer of tooth germ from the bell stage (17.0 dpc) to the differentiation stage, postnatal day 1 (P1) during terminal differentiation of odontoblasts (Figs.1A-D). In contrast, *Osterix* was first detected in the odontoblastic layer at 17.0 dpc, and was a more pronounced at P1 and P4 (Figs.1E-H), overlapped with *Runx3* expression. In the odontoblasts, *Bmp2* also was strongly expressed at P1 (Fig. 1O) but not *Runx2* (Fig. 1K). No positive signal was detected when using sense probe.

Expression of Runx3 and Osterix during differentiation of the dental pulp cells into odontoblasts in vitro

We next determined whether the mouse DPCs *in vitro* have the similar expression patterns of *Runx3 and Osterix* as those *in vivo*, RT-PCR was performed to examine gene expression of *Runx3, Osterix,* and odontoblast markers, *dentin sialophosphoprotein* (*Dspp*), *enamelysin* and *kallikrein 4* (*KLK4*) during culture (Fig. 2A). *Dspp* and *KLK4* were first detected clearly on day 21 and *enamelysin* on day 28, showing spontaneous differentiation of the DPCs into odontoblasts. *Runx3* expression was weakly detected on day 1, and increased further on day 21. *Osterix* expression was first detected on day 21 (Fig. 2A). These results correlated with *in vivo* expression during tooth development, suggesting that the DPCs might be useful for

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study on the regulation of expression of *Osterix* by Runx3 at the stage before terminal differentiation of odontoblasts.

Runx3 down-regulates Osterix expression in the mouse dental pulp cells

To examine whether *Osterix* expression was regulated by *Runx3*, *MSCV-eGFP-Flag-Runx3* was transfected by electroporation into the mouse DPCs. Electroporation at three square-wave pulses at a frequency of 1 Hz, with a pulse length of 99 μsec and 1350 V, provided an optimal method for gene transfer *in vitro*. The cell viability was nearly 70% as determined with trypan blue, and the efficiency was nearly 35% as estimated by fluorescent microscopy. Real-time RT-PCR showed that the enhanced expression of *Runx3* mRNA, nearly 3 fold increase in the DPCs with *MSCV-eGFP-Flag-Runx3* than in control DPCs with *MSCV-eGFP* 24 hours after transfection (data not shown). Runx3 mRNA, however, were reduced to the almost same level as that of control 48 hours after transfection. On the contrary, *Osterix* expression was reduced in 25% 48 hours after transfection with *MSCV-eGFP-Flag-Runx3* compared with control transfection (Fig. 2B). These results suggest that Runx3 negatively regulates *Osx* expression in the DPCs.

Runx3 down-regulates the Osterix promoter activity in HEK293

A recent study has shown that *Runx2* specifically up-regulated *Osterix* promoter activity in C3H10T1/2 and ATDC5 cells, mesenchymal cell lines of bone and cartilage respectively [19]. There has been no report, however, concerning *Osterix* regulation by Runx3 so far. Runx3 shares highly conserved DNA binding domains with Runx2. Both *Runx2* and *Runx3* promoters have putative Runx binding sites that are fully conserved in sequence and location [24]. Therefore, cross-regulation between Runx2 and Runx3 might be plausible. To avoid this possible endogenous effect, HEK293 cells, in which neither *Runx2* nor *Runx3* are expressed (Fig. 3A), was used to examine transcriptional activity of Runx3.

Three putative Runx binding sites were identified on -1823 bp to -1817 bp (site 1,

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ACCACA), -1776 bp to -1771 bp (site 2, ACCACT) and -713 bp to -707 bp (site 3, AGTGGTT) from the cap site by computer analysis of the *Osterix* promoter (Fig. 3B). A wild type-luciferase reporter plasmid containing all the three putative Runx binding sites were compared in the *Osterix* promoter activity with five different mutant plasmids (M1-M5) in which some of the three putative sites were mutated (Fig. 3B). The wild type-reporter transfected with Runx3 reduced the *Osterix* promoter activity to nearly 55%. Transfection of the mutant reporters in which the site 1 and/or site 2 were mutated resulted in almost the same reduced activity as that of the wild type. In contrast, when used the mutant reporters in which the site 3 were mutated (M4 and M5), only a weak repression (90% activity) was detected (Fig. 3B). These results suggest that the site 3 is essential for *Osterix* promoter activity. To confirm this, shorter wild type- and mutant plasmids only containing the site 3 were used. The *Osterix* promoter activity was significantly reduced by the wild type, while not affected by the mutant (Fig. 3C). These results suggest that the site 3 is essential for *Osterix* promoter activity.

Characterization of the Runx3 protein binding to the site 3

To determine whether transcriptional repression of *Osterix* is due to direct binding of Runx3 to the site 3, electrophoretic mobility-shift assays using nuclear extracts from the mouse DPCs were performed. As shown in Fig. 4A, 38 bp end-labelled oligonucleotide containing the site 3 (-713 bp to -707 bp) of the *Osterix* promoter formed a DNA/protein complex (lane 2, arrowhead). The complex was completely competed by a 125-fold excess of unlabelled wild type-oligonucleotide (lane 3). An oligonucleotide in which the site 3 was mutated did not affect this binding (lane 4). Furthermore, antibody against Runx3 bound to the DNA/protein complex (lane 5, arrow), indicating the specificity of the DNA/protein complex. No band was not be detect when only nuclear extract loaded (lane 6).

Next, we carried out ChIP assay to test if Runx3 specifically binds to the element *in vivo*. The mouse DPCs were cross-linked and immunoprecipitated by Runx3

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antibody. The presence of the *Osterix* promoter DNA was detected by PCR using primers flanking the site 3 (-713 bp to -707 bp) (Fig.4B), indicating that Runx3 binds to the site 3 of *Osterix* promoter specifically and functionally. The use of Runx2 antibody resulted in the similar result (Fig.4B), suggesting Runx3 and Runx2 both bind to the site 3 *in vivo*.

DISCUSSION

During systematic in situ hybridization study of tooth development *Osterix* mRNA was first detected in terminally differentiating odontoblasts, and colocalized with *Runx3*, suggesting a potential role for both in odontoblast differentiation. *Runx3* over-expression resulted in down-regulation of *Osterix* in the mouse dental pulp cells (DPCs). This suggests that *Osterix* might be a downstream target of *Runx3* in tooth development. *Osterix* null mice [18] have a similar phenotype to the *Runx2* null mice [9, 10], in which both intramembranous and endochondral bone are not formed due to the lack of osteoblast differentiation. Whereas *Osterix* is not expressed in the *Runx2* null mutants, *Runx2* expression is not changed in the *Osterix* null mutants [18]. These genetic studies have placed *Osterix* downstream to *Runx2* [18]. The precise regulatory role of Runx2 in *Osterix* expression is not clear. A recent study has shown that -737 bp fragment of *Osterix* promoter is up-regulated upon *Runx2* over-expression in ATDC5 chondroprogenitor cells and the function of -737 bp fragment was confirmed by site-directed mutagenesis experiments [19]. Furthermore, this functional binding site is conserved among mouse, rat and human, showing conservation of the DNA binding site [19]. However, no information is available on the regulation of *Osterix* expression by Runx3. Therefore, we have performed transient co-transfection, electrophoretic mobility shift and ChIP assays to investigate the relationship between Runx3 and Osterix in dental pulp cells. Structural dissection of the proximal regulatory region of the *Osterix* gene revealed the presence of three putative Runx-binding sites. Only the site 3 (-713 bp to -707 bp) of these sites, was preferentially and functionally occupied by Runx3. The disruption of site 3 leads to increased *Osterix* promoter activity in HEK293 cells, in which both Runx2 and Runx3 are not expressed endogenously. These results indicate that *Osterix* expression is negatively regulated by Runx3. Furthermore, our electrophoretic mobility shift and ChIP assays confirmed that Runx3 directly down-regulates *Osterix* expression in

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dental pulp cells prior to terminal differentiation into odontoblasts. It is noteworthy that Runx3 negatively regulates *CD36* expression in myeloid cells [25] and suppresses gastric epithelial cell growth [26], implying a general role for Runx3 in transcriptional repression.

The distinct roles of Runx2 and Runx3 in odontoblast differentiation are not clear. Previous research indicated that tooth development was disrupted in the cap/early bell stages in the *Runx2* null mice and no overt differentiation of odontoblasts was observed [11, 27]. There was no conspicuous phenotype in teeth of *Runx3* null mice [17]. In *Runx2* null mice *Runx3* expression was dramatically enhanced in the mesenchyme of upper molars, and they differentiated into odontoblasts [27]. Our electrophoretic mobility shift and ChIP assays have shown that not only Runx2 but also Runx3 binds to the site 3 of *Osterix* promoter. *Runx3* over-expression resulted in down-regulation of *Osterix* in dental pulp cells. The *Osterix* promoter activity was down-regulated by *Runx3* transfection in HEK293 cells. These results suggested that the *Osterix* expression is cooperatively regulated by Runx2 and Runx3 sharing the same binding site on *Osterix* promoter. Thus, Runx3 might cooperate with Runx2 to regulate *Osterix* expression during odontoblast differentiation. The role of Osterix in tooth development is not clear. In skeletal development, Runx2, Runx3 and Osterix play a pivotal role in osteoblast differentiation and hypertrophic chondrocyte maturation [28, 29]. Osterix may play a role in segregation of osteoblast and chondrocyte lineages [29, 30]. *Runx2* and *Runx3* are co-expressed in early stage of tooth development. There is overlapping expression of *Osterix with Runx3* but not *Runx2* in terminal differentiation of odontoblasts. Therefore, *Osterix* in tooth development may play a role in lineage commitment of odontoblasts. The diverse transcriptional outcomes of Runx activity are dependent on context [1]. Runx family acts as organizing factors on the promoter of target genes where they associate with coactivators and other DNA-binding transcription factors including Smads [1]. Repression of *Osterix* by Runx3 in dental pulp cells is an example of context dependent regulation of lineage commitment. Thus, there might be cooperative

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interactions among BMPs, Smads, Runx2 and Runx3 in the regulation of *Osterix* expression during dental pulp cell differentiation into odontoblasts.

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TABLE 1 Primers for RT-PCR

Name		5'- Sequence -3'	Product size (bp)	Accession number
beta-actin	Forward	AAATCGTGCGTGACATCAAA	178	X03765
	Reverse	AAGGAAGGCTGGAAAAGAGC		
Runx3	Forward	GGTTCAACGACCTTCGATTC	180	NM_019732
	Reverse	AGGCCTTGGTCTGGTCTTCT		
Runx2	Forward	CAGACCAGCAGCACTCCATA	178	NM_009820
	Reverse	CAGCGTCAACACCATCATTC		
Osterix	Forward	GGTCCAGGCAACACACCTAC	178	AF184902
	Reverse	GGTAGGGAGCTGGGTTAAGG		
Dspp	Forward	GGAACTGCAGCACAGAATGA	199	NM_010080
	Reverse	CAGTGTTCCCCTGTTCGTTT		
Enamelysin	Forward	CGACAATGCTGAGAAGTGGA	180	NM_013903
	Reverse	CCCTTTCACATCATCCTTGG		
Klk4	Forward	TTGCAAACGATCTCATGCTC	228	NM_019928
	Reverse	TGAGGTGGTACACAGGGTCA		

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Figure 1 Expression of *Runx3, Osterix, Runx2* and *Bmp2* by *in situ* hybridization during tooth development in the mouse

(A-D) *Runx3* was progressively restricted to the odontoblastic layer of tooth germ starting from the bell stage (17.0 dpc) to the differentiation stage (post natal stage day 1 (P1)) during terminal differentiation of odontoblasts. (E-H) *Osterix* was first detected weakly in the odontoblastic layer at 17.0 dpc, and was a more pronounced at P1, overlapping with *Runx3* expression. (I-L) *Runx2* was not expressed in odontoblast layer after P1. (M-P) *Bmp2* was strongly expressed in the odontoblasts at P1. Arrowheads indicate the positive signals in the odontoblastic layer. dp, dental papillae; ol, odontoblast layer. Bar = 200 μm

Figure 2 Down-regulation of *Osterix* expression by Runx3 in mouse dental pulp cells *in vitro*.

(A) mRNA expression of *Runx3*, *Osterix*, and differentiation markers of odontoblasts, *dentin sialophosphoprotein* (*Dspp*), *enamelysin* and *kallikrein-4* (*KLK4*) in mouse DPCs during culture. (B) *Osterix* expression was down-regulated in mouse DPCs at 48 hours after *Runx3* transfection. The experiment was repeated twice with similar results.

Figure 3 Down-regulation of *Osterix* promoter activity by Runx3 in human embryonic kidney 293 (HEK293) cells

(A) Determination of endogenous expression of *Runx3, Runx2* and *Osterix* in mouse DPCs but not in HEK293 cells. (B) Wild type (WT) and different mutation (MT) of *Osterix* promoter plasmids were analyzed 48 hours after co-transfection with *MSCV-eGFP-Flag-Runx3* into HEK293 cells. (C) Wild type (WT) or mutation (MT) with shorter *Osterix* promoter plasmids containing -713 to -707 (site 3) was co-transfected with *MSCV-eGFP-Flag-Runx3* into HEK293 cells. The activities were determined after 48 hours and normalized against co-transfected internal control plasmid (pRL-SV40). The values represent means ± S.D. of four individual samples. The experiment was repeated twice with similar results. ** $P<0.01$ compared with the

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