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Runx2, Osx, and *Dspp* in Tooth Development

ABSTRACT

The transcription factors *Runx2* and *Osx* are necessary for osteoblast and odontoblast differentiation, while *Dspp* is important for odontoblast differentiation. The relationship among *Runx2*, *Osx,* and *Dspp* during tooth and craniofacial bone development remains unknown. In this study, we hypothesized that the roles of *Runx2* and *Osx* in the regulation of osteoblast and odontoblast lineages may be independent of one another. The results showed that *Runx2* expression overlapped with *Osx* in dental and osteogenic mesenchyme from E12 to E16. At the later stages, from E18 to PN14, *Runx2* and *Osx* expressions remained intense in alveolar bone osteoblasts. However, *Runx2* expression was down-regulated, whereas *Osx* expression was clearly seen in odontoblasts. At later stages, *Dspp* transcription was weakly present in osteoblasts, but strong in odontoblasts where *Osx* was highly expressed. In mouse odontoblast-like cells, *Osx* overexpression increased *Dspp* transcription. Analysis of these data suggests differential biological functions of *Runx2*, *Osx,* and *Dspp* during odontogenesis and osteogenesis. *Abbreviations:* E, embryonic day; PN, post-natal day; *Dspp*, dentin sialophosphoprotein; *Osx*, Osterix.

KEY WORDS: *Runx2, Osx, Dspp,* odontoblast, osteoblast, tooth development.

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INTRODUCTION

Tooth development involves sequential and reciprocal interactions between dental epithelial and mesenchymal cells and proceeds through a series of cytodifferentiations in specific spatial-temporal patterns. Epithelial and mesenchymal cells differentiate into ameloblasts and odontoblasts, respectively (Linde and Goldberg, 1993). Odontoblasts synthesize and secrete collagenous and non-collagenous proteins (NCPs) to form dentin extracellular matrix (DECM). Among NCPs, dentin sialophosphoprotein (*Dspp*) is a phosphorylated protein representing a major component of non-collagenous DECM, highly expressed in odontoblasts and essential for dentinogenesis (D'Souza *et al.,* 1997; Butler, 1998). Mutations of *Dspp* gene are associated with various forms of dentin genetic disorders (Xiao *et al.,* 2001; Rajpar *et al.,* 2002; Kim and Simmer, 2007). Dentinogenesis is a complex process in which multiple signaling pathways converge to induce dentin formation and is controlled by many growth and transcription factors (Thesleff, 2003).

Transcription factor *Runx2* is necessary for osteoblast and odontoblast differentiation and regulates many bone- and tooth-related gene expressions. *Runx2* determines the lineage of osteoblasts and odontoblasts from mesenchymal cells (Ducy *et al.,* 1997; Lian *et al.*, 2006). The temporal-spatial *Runx2* expression pattern during bone and tooth formation has been described (Jiang *et al.,* 1999; Bronckers *et al.,* 2001; Yamashiro *et al.,* 2002). For example, *Runx2*-deficient mice showed impaired tooth formation, progressing only to the cap/early bell stages (D'Souza *et al.,* 1999). Moreover, persons with *Runx2* gene mutations display dental disorders, with supernumerary teeth, abnormal tooth eruption, and tooth hypoplasia (Komori *et al.,* 1997; Lee *et al.,* 1997; Mundlos *et al.,* 1997).

Osterix (*Osx* or *Sp7*) is another osteoblast-specific transcription factor and is expressed in tooth germ mesenchymal cells (Nakashima *et al.,* 2002). Genetic studies have shown that cortical bone and bone trabeculae formation is abolished in *Osx* knock-out mice. Also, in *Osx* null mice, expression of type I collagen and osteoblast marker genes is reduced in mesenchymal cells. However, *Runx2* expression in *Osx* null mice is unaffected, whereas no *Osx* transcripts are detected in skeletal elements of *Runx2* null mice, indicating that *Osx* acts as a downstream gene of *Runx2* in the osteoblast differentiation signaling pathway (Nakashima *et al.,* 2002). Recent studies have found that the effect of *Osx* on its target genes is involved in other signaling pathways, independent of *Runx2* (Lee *et al.,* 2003; Ulsamer *et al.,* 2008). Although osteoblasts and odontoblasts originate from mesenchymal cells and have several common characteristics, bone and dentin display some different physical and biological functions. Thereby, the molecular mechanisms regulating the expression of tooth-/bone-related genes in odontoblasts, especially in the later stages of tooth development, somehow differ from those of

osteoblasts (Chen *et al.,* 2005; James *et al.,* 2006). Previously, we and other laboratories observed differential *Runx2* expression patterns between alveolar bone osteoblasts and dental cells during tooth formation (Bronckers *et al.,* 2001; Yamashiro *et al.,* 2002; Chen *et al.,* 2005). However, the *Osx* expression pattern during tooth development has not been described. Furthermore, the relationship among *Runx2*, *Osx,* and *Dspp* during development of teeth and craniofacial bones remains unclear. In this study, we investigated the spatial-temporal expression patterns of *Runx2*, *Osx,* and *Dspp* at different stages of development of teeth and craniofacial bones, as well as studied the effect of *Osx* on *Dspp* transcription in mouse odontoblast-like cells.

MATERIALS & METHODS

Animals and Tissue Preparation

All experimental procedures involving the use of animals were approved by the University of Texas Health Science Center at San Antonio (UTHSCSA), TX. ICR mice were purchased from Harlan-Laboratory Animals Inc. (Indianapolis, IN, USA). Mouse tissues were dissected and fixed in 4% paraformaldehyde overnight. After demineralization in 15% EDTA, samples were dehydrated in increasing concentrations of ethanol, embedded in paraffin, sectioned, and prepared for *in situ* hybridization assay.

In situ Hybridization

^{32}P-rUTP-labeled antisense riboprobes corresponding to *Runx2* (Chen *et al.,* 2002), *Osx* (Nakashima *et al.,* 2002), and *Dspp* (Chen *et al.,* 2005) were generated. The *in situ* hybridization was performed as described previously (Gluhak-Heinrich *et al.*, 2003).

Cell Culture

Mouse immortalized odontoblast-like (MO6-G3) cells were grown at 33ºC under 5% CO_2 in α-MEM with 10% fetal calf serum, 100 units/mL penicillin/streptomycin, 50 µg/mL ascorbic acid, and 10 mM Na β-glycerophosphate (MacDougall *et al.,* 1995). Mouse bone marrow stromal ST2 cells and primary calvarial osteoblasts isolated at post-natal day 3 from C57BL6 mice (Harlan-Laboratory Animals Inc.) were grown in α-MEM supplemented with 10% fetal calf serum, 100 units/mL penicillin/streptomycin, at 37°C under 5% CO_2.

Immunohistochemistry

MO6-G3 cells were cultured on glass slides and fixed with methanol/acetone (1:1). Cells were treated with 10% normal donkey or goat serum for 60 min at room temperature. Immunohistochemistry was performed with a 1:100 dilution of primary polyclonal antibodies specific for *Dsp* and *Osx* (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). A negative control of mouse IgG I was purchased from Dakocytomation (Carpinteria, CA, USA). The cells were incubated at 4ºC overnight and then washed, followed by incubation with the secondary antibodies (goat-anti-rabbit or donkey-anti-goat, 1:3000) with Alexa Fluo® 488 (Molecular Probes, Eugene, OR, USA). Images of Alexa Fluo® 488 staining were obtained at the Core Optical Imaging Facility at UTHSCSA, under the same parameters, with a Nikon inverted microscope.

Effect of *Osx* on *Dspp* and *Runx2* Expression

To assess the effect of *Osx* on *Dspp* and *Runx2* expression, we transfected MO6-G3 and ST2 cells with either *Osx* or an empty expression vector. After 48 hrs, RNA was isolated with the use of a RNA STAT-60 kit (Tel-Test, Inc. Friendswood, TX, USA), treated with DNase I, and purified with the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). For quantitative real-time PCR (qRT-PCR), amplification reactions were assayed in real-time on an ABI 7500 (Applied Biosystems, Foster City, CA, USA) with SYBR Green chemistry as described previously (Chen *et al.,* 2005). Differential *Dspp* and *Runx2* expressions between *Osx* and mock expression in MO6-G3 and ST2 cells were calculated and normalized to SYBR activity. We used analysis of variance with a *t* test to determine significant differences between the control and treated groups. All values were expressed as means ± SD from 3 independent experiments performed in triplicate.

RESULTS

Expression Patterns of *Runx2*, *Osx*, and *Dspp* in Developing Teeth

To understand the relationship among *Runx2*, *Osx,* and *Dspp* during tooth and craniofacial bone development, we studied *Runx2*, *Osx,* and *Dspp* mRNA expression patterns at different stages of tooth and craniofacial bone development, using an *in situ* hybridization assay. At E12, *Runx2* and *Osx* expression was intense in the mesenchymal condensates of forming bones and teeth in the developing maxillary and mandibular arches (Appendix Figs. 1B, 1C). Overall, *Osx* expression levels were relatively weaker than those of *Runx2,* while *Dspp* transcripts were not detectable at E12 (Appendix Fig. 1D).

At the cap stage (E14), *Runx2* mRNA was highly expressed in mesenchymal cells in alveolar bone, dental papilla, and follicle (Fig. 1B). *Osx* was almost co-expressed with *Runx2* in these same areas (Fig. 1C). However, *Runx2* and *Osx* mRNA expression was barely seen in dental epithelium. In addition, there was no *Dspp* signal in dental and osteogenic mesenchyme (Fig. 1D).

At the bell stage (E16), both *Runx2* and *Osx* transcripts were expressed in differentiating osteogenic mesenchyme, ameloblasts, odontoblasts, and dental pulp cells (Figs. 1F, 1G). At this stage, there was a weak *Dspp* signal in odontoblasts, ameloblasts, dental pulp cells, and surrounding tissues (Fig. 1H).

At E18, *Runx2* expression was dramatically down-regulated in the odontoblasts, ameloblasts, and dental pulp cells, except for cells near the mesenchyme within alveolar bone in the developing incisor and molar. Its signal was apparent in differentiating alveolar bone osteoblasts (Fig. 1J, Appendix Figs. 1F, 1J). *Osx* expression in the alveolar bone osteoblasts overlapped with *Runx2* expression. However, *Osx* expression remained intense in

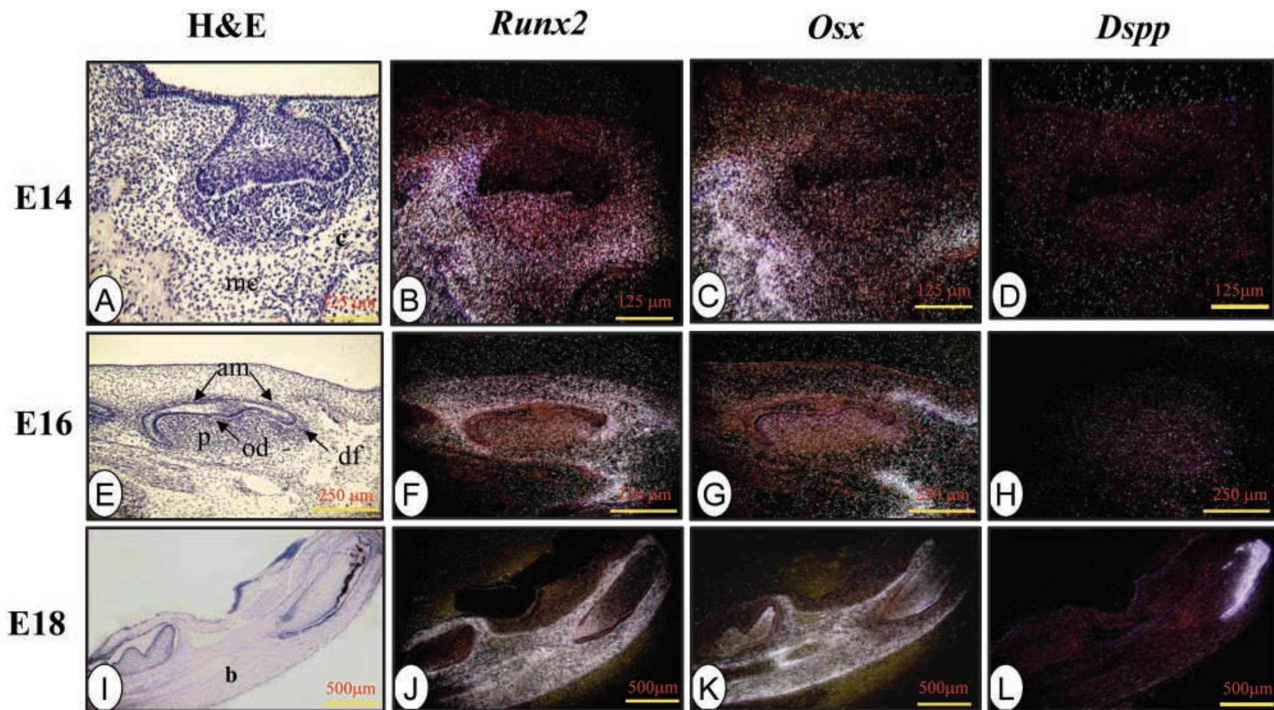

Figure 1. *Runx2*, *Osx,* and *Dspp* expression patterns in developing teeth and surrounding tissues from E14 to E18. *Runx2* mRNA was clearly detected in dental and osteogenic mesenchyme from E14 to E16, but its expression was down-regulated in odontoblast, ameloblast, and dental pulp cells at E18 of the mandibular incisor and first molar. *Osx* expression mostly overlapped with *Runx2* expression from E14-16. At E18, its expression was intense in ameloblasts, odontoblasts, and dental pulp cells. *Dspp* transcripts were absent at E14, but its transcript signals were slightly detected in dental and osteogenic mesenchyme at E16. At E18, *Dspp* transcripts were evident in odontoblasts and pre-ameloblasts in the mandibular incisor and first molar. am, ameloblasts; b, alveolar bone; c, Meckel's cartilage; de, dental epithelium; df, dental follicle; dp, dental papilla; mc, mesenchymal condensates; od, odontoblasts; p, dental pulp cells.

odontoblasts and dental pulp cells (Fig. 1K, Appendix Figs. 1G, 1K). At this stage, the *Dspp* mRNA signal was clear in differentiating and differentiated odontoblasts, and in pre-ameloblasts in the incisor and molar (Fig. 1L, Appendix Figs. 1H, 1L).

At PN 1, *Runx2* mRNA was highly expressed in osteoblasts, but its signal was weakly detected in odontoblasts and dental pulp cells in the developing incisor and molar (Fig. 2B, Appendix Fig. 2B). In contrast, *Osx* transcripts were intense in odontoblasts and dental pulp cells, in addition to its expression in osteoblasts (Fig. 2C, Appendix Fig. 2C). At this stage, *Dspp* signal was apparent in differentiating and differentiated odontoblasts and differentiating ameloblasts, but its signal was weakly detected in osteoblasts (Fig. 2D, Appendix Fig. 2D). At PN 5, *Runx2*, *Osx,* and *Dspp* expression patterns were similar to those at PN1. However, the *Osx* signal was more intense in odontoblasts where *Dspp* mRNA was highly expressed (Figs. 2G, 2H).

Similar to PN5, at PN 8 to 14, *Runx2* mRNA was weakly detected in odontoblasts and dental pulp cells in developing molars, whereas its signal was apparently seen in alveolar bone osteoblasts, cells within the cemento-enamel junction, and roots (Fig. 2J, Appendix Figs. 2F, 2J, 2N, 2R). Unlike *Runx2*, high *Osx* expression was still present in odontoblasts and dental pulp cells. *Osx* gene expression was also seen in bone, cemento-enamel junction, and roots overlapped with *Runx2* expression (Fig. 2K, Appendix Figs. 2G, 2K, 2O, 2S). *Dspp* transcripts from PN 8 to PN14 were dominantly expressed in odontoblasts (Fig. 2L, Appendix Figs. 2H, 2L, 2P, 2T). Notably, high *Osx* and *Dspp* expression levels overlapped in odontoblasts at the later stages of tooth development. *Runx2* expression in mouse osteoblast cell lines was higher than in the mouse odontoblast-like cells (Appendix Fig. 3).

Expression of *Osx* and *Dspp* in Mouse Odontoblast-like Cells

To assess cellular localization of *Dspp* and *Osx* expression, we used mouse odontoblast-like cells to detect *Osx* and *Dspp* expression by immunohistochemistry. *Dspp* was expressed in the cytoplasm in MO6-G3 cells, while *Osx* signal was found in the nucleus and cytoplasm (Fig. 3F).

Effect of *Osx* Overexpression on *Dspp* Expression

Since expression of *Osx* and *Dspp* genes was observed in mouse odontoblasts (Figs. 1, 2), we further examined whether forced expression of *Osx* is able to regulate *Dspp* gene expression in mouse odontoblast-like cells. *Osx* overexpression caused a 2.7-fold increase in *Dspp* mRNA levels compared with the control

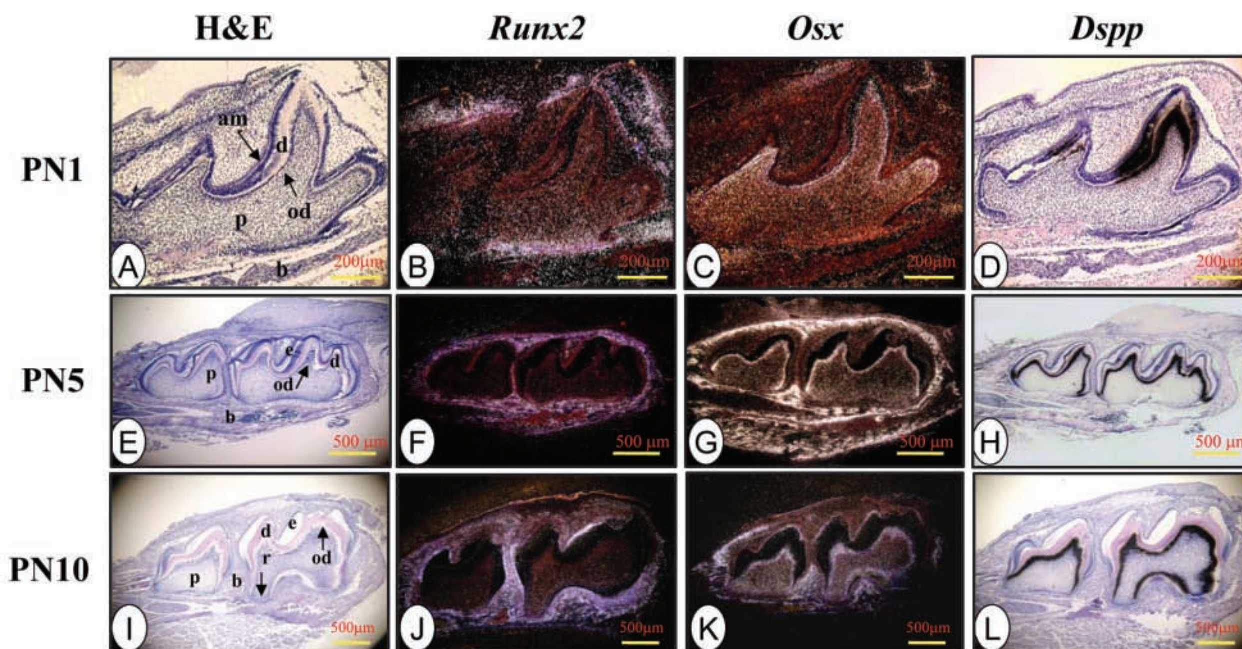

Figure 2. *Runx2*, *Osx,* and *Dspp* expression patterns in developing teeth and surrounding tissues at post-natal days (PNs) ranged from PN 1 to PN 10. *Runx2* mRNA was detected in osteogenic mesenchyme and down-regulated in ameloblasts, odontoblasts, and dental pulp cells. In contrast, *Osx* expression was seen in odontoblasts and dental pulp cells. *Dspp* mRNA was strongly expressed in odontoblasts and weakly expressed in ameloblasts at post-natal (PN) days 5 and 10 during tooth development.

group by qRT-PCR analysis (Fig. 4A), indicating that *Osx* enhances *Dspp* transcription in mouse odontoblast-like cells. In contrast, there was no change in *Runx2* expression levels in *Osx*-transfected odontoblast-like and osteoblast cells (Figs. 4B, 4D).

DISCUSSION

In this present study, we investigated *Runx2*, *Osx,* and *Dspp* gene expression patterns at different stages of mouse tooth development using an *in situ* hybridization assay. At E12, *Runx2* transcripts were seen in dental and osteogenic mesenchyme in developing maxillary and mandibular bones. With tooth development at the cap and bell stages, *Runx2* signaling was detected in mesenchymal cells within the alveolar bone, dental papilla, and follicle. At E16, *Runx2* transcripts were expressed in ameloblasts, odontoblasts, and dental pulp cells, in addition to the osteogenic mesenchyme, but its signal was remarkably down-regulated in ameloblasts, odontoblasts, and dental pulp cells at E18 and the later stages during tooth development. For the *Osx* gene, its expression pattern was similar to that of *Runx2* from E12 to E16. Unlike *Runx2* expression, *Osx* transcripts remained intense in odontoblasts and dental pulp cells at the late stages, from E18 to PN14. In particular, *Osx* expression was apparent in odontoblasts in which *Dspp* was highly expressed. This suggests that the mechanisms regulating osteoblast and odontoblast lineages differentially involve *Runx2* and *Osx*. *Runx2* null mice have shown a complete arrest of tooth development in cap/early bell stages prior to odontoblast differentiation (D'Souza *et al.,* 1999). Furthermore, *Runx2* up-regulated *Dspp* transcription in mouse pre-odontoblast-like cells, but down-regulated *Dspp* activity in mouse odontoblast-like cells (Gaikwad *et al.,* 2001; Chen *et al.,* 2005). Therefore, *Runx2* may be involved in tooth development prior to the bell stage, but not the later stages. Differential effects of *Runx2* on dentin matrix protein 1 (*Dmp1*) gene expression during bone and tooth development have been reported (Feng *et al.,* 2002). Like *Dspp*, *Dmp1* is one member of the SIBLING gene family (Fisher and Fedarko, 2003). These investigators found that *Runx2* expression overlapped with *Dmp1* expression at E14.5-E19.5 in normal developing bones and teeth. *Dmp1* expression was absent in developing bones in *Runx2* null mice, whereas *Dmp1* expression in developing teeth was unaffected. Based on different *Runx2* and *Osx* expression patterns during tooth development, we hypothesized that *Runx2* regulates bone and tooth development at the early stages (E12-16). *Runx2* may regulate bone and tooth development directly or *via* a *Runx2*-related signaling pathway such as *Osx*. With tooth development, *Runx2* expression was down-regulated in ameloblasts, odontoblasts, and dental pulp cells. Thus, *Runx2* may not be involved in odontoblast and dental pulp cell differentiation and the regulation of dental gene expression such as *Dspp* at the later stages of tooth formation. Recently, it was observed that *Runx2* inhibits the terminal differentiation of odontoblasts and *Dspp* expression in *Runx2* transgenic mice (Miyazaki *et al*., 2008). In contrast, *Osx* continues to be expressed in these cells and may induce cell differentiation and tooth-related gene expression at the later stages of tooth development *via* a *Runx2*-independent signaling pathway (Celil and Campbell, 2005; Ulsamer *et al.,* 2008). Furthermore, our study showed that *Osx* overexpression

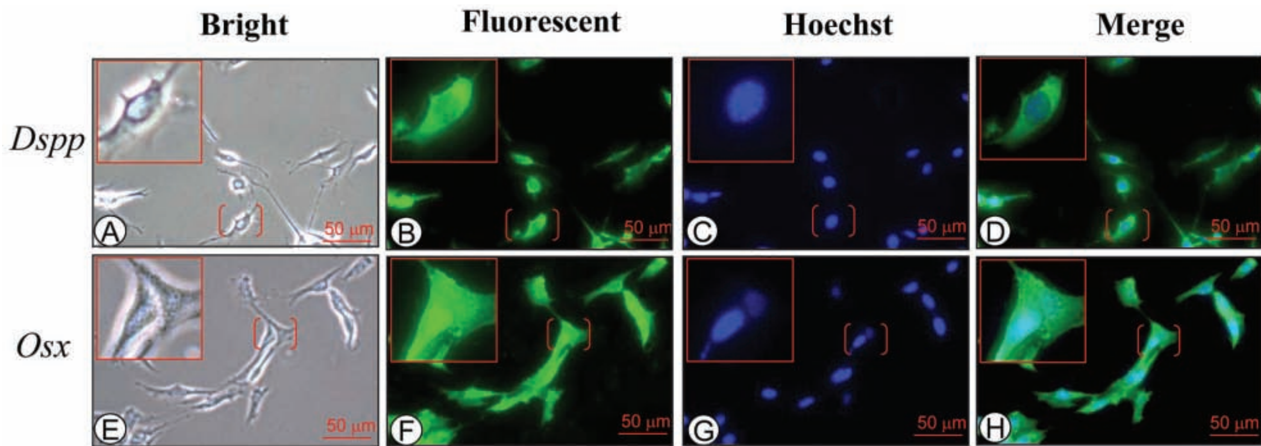

Figure 3. *Osx* and *Dspp* expression in mouse odontoblast-like cells. Immortalized mouse odontoblast-like cells (MO6-G3) were photographed by light microscopy **(A,E)**. Expression of *Osx* and *Dspp* proteins in MO6-G3 cells was analyzed by immunostaining with primary anti-*Dsp* or anti-*Osx* antibody (**B** and **F**, respectively). Cells were stained with Hoechst for the nucleus **(C,G)**. Panels **D** and **H** are composites of B-C and F-G, respectively. *Dspp* signal was detected in the cytoplasm in the mouse odontoblast-like cells. *Osx* protein was expressed in both the nucleus and cytoplasm.

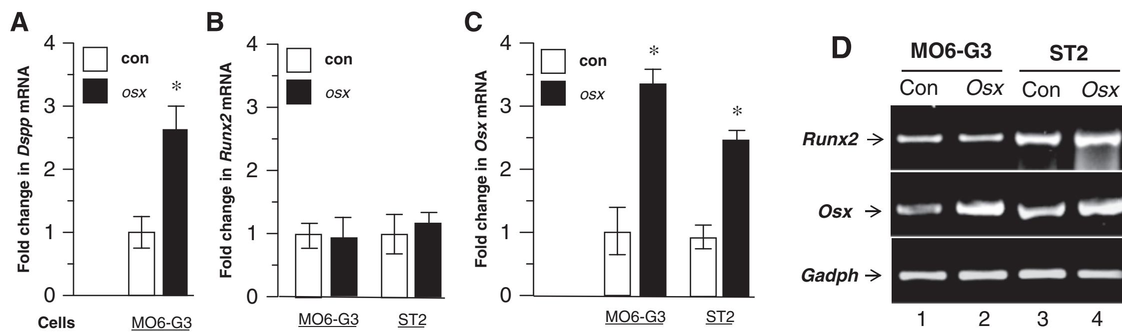

Figure 4. Effect of *Osx* on *Dspp* and *Runx2* expressions. Mouse odontoblast-like and osteoblast cells were transfected with either *Osx* or empty expression plasmid as a control. After 48-hour transfection, total RNA was used for qRT-PCR and semi-quantitative PCR. The primer sequences used for qRT-PCR were as follows: *Dspp*, forward 5′-AACTCTGTGGCTGTGCCTCT-3′ and reverse 5′-TATTGACTCGGAGCCATTCC-3′; *Runx2*, forward 5′-AGTGCTCTAACCACAGTCCATGCA-3′ and reverse 5′-TACAAACCATACCCAAGTACCTGTTT-3′; *Osx*, forward 5′-ATGGCGTCCTCTCTGCTTGA-3′ and reverse 5′-TTGAGAAGGGAGCTGGGTAG-3′; and cyclophilin, forward 5′-GGTGACTTCACACGCCATAA-3′ and reverse 5′-CATGGCCTCCACAATATTCA-3′. The mRNA expression without *Osx* overexpression (empty vector transfectant) in MO6-G3 or ST2 cells is designated as a 1.0-fold increase. Expression levels of *Runx2*, *Osx,* and *Dspp* mRNAs in MO6-G3 or ST2 cells with *Osx* transfectants are represented as fold-changes in relation to the control. All data are represented by mean ± SD from 3 independent experiments performed in triplicate. *P values lower than 0.05 were considered statistically significant. For semi-quantitative PCR, a pair of primers of the glyceraldehyde 3-phosphate dehydrogenase (GADPH) gene was used as an internal positive control, as follows: forward 5′-CCATGGAGAAGGCCGGG-3′ and reverse 5′-CAAAGTCATGGATGACC-3′. The primers of *Runx2* and *Osx* are described above. Quantitative RT-PCR was used to detect mRNA expression levels of *Dspp* (**A**), *Runx2* (**B**), and *Osx*(**C**) in MO6-G3 or ST2 cells with or without Osx transfection. **D**. Semi-quantitative PCR analysis of *Runx2*, *Osx,* and GADPH mRNA expressions in MO6-G3 and ST2 cells transfected with or without *Osx* expression vector.

resulted in a *Dspp* transcription increase in mouse odontoblast-like cells, but had no effect on *Runx2* expression in both the mouse odontoblast-like and osteoblast cells. This suggests that *Osx* directly affects *Dspp* expression independent of *Runx2.*

In addition, our study demonstrated that *Runx2* and *Osx* were highly expressed in osteogenic mesenchyme through all stages of craniofacial bone development. This suggests that both *Runx2* and *Osx* are involved in osteoblast differentiation at the early and later stages of osteogenesis, independently or in concert (Nakashima *et al.,* 2002; Celil and Campbell, 2005; Ulsamer *et al.,* 2008).

Finally, *Runx2* and *Osx* were expressed in tooth roots, suggesting that they may be involved in tooth eruption and movement (Zou *et al.,* 2003).

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