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Deletion of *Osr2* Partially Rescues Tooth Development in *Runx2* Mutant Mice

H.J.E. Kwon[1], E.K. Park[1,2], S. Jia[1*], H. Liu[1], Y. Lan[1,3], and R. Jiang[1,3]

Abstract

Tooth organogenesis depends on genetically programmed sequential and reciprocal inductive interactions between the dental epithelium and neural crest–derived mesenchyme. Previous studies showed that the Msx1 and Runx2 transcription factors are required for activation of odontogenic signals, including Bmp4 and Fgf3, in the early tooth mesenchyme to drive tooth morphogenesis through the bud-to-cap transition and that Runx2 acts downstream of Msx1 to activate *Fgf3* expression. Recent studies identified Osr2 as a repressor of tooth development and showed that inactivation of *Osr2* rescued molar tooth morphogenesis in the *Msx1*$^{-/-}$ mutant mice as well as in mice with neural crest–specific inactivation of *Bmp4*. Here we show that *Runx2* expression is expanded in the tooth bud mesenchyme in *Osr2*$^{-/-}$ mutant mouse embryos and is partially restored in the tooth mesenchyme in *Msx1*$^{-/-}$*Osr2*$^{-/-}$ mutants in comparison with *Msx1*$^{-/-}$ and wild-type embryos. Whereas mandibular molar development arrested at the bud stage and maxillary molar development arrested at the bud-to-cap transition in *Runx2*$^{-/-}$ mutant mice, both mandibular and maxillary molar tooth germs progressed to the early bell stage, with rescued expression of *Msx1* and *Bmp4* in the dental papilla as well as expression of *Bmp4, p21*, and *Shh* in the primary enamel knot in the *Osr2*$^{-/-}$*Runx2*$^{-/-}$ compound mutants. In contrast to the *Msx1*$^{-/-}$*Osr2*$^{-/-}$ compound mutants, which exhibit nearly normal first molar morphogenesis, the *Osr2*$^{-/-}$*Runx2*$^{-/-}$ compound mutant embryos failed to activate the expression of *Fgf3* and *Fgf10* in the dental papilla and exhibited significant deficit in cell proliferation in both the dental epithelium and mesenchyme in comparison with the control embryos. These data indicate that Runx2 synergizes with Msx1 to drive tooth morphogenesis through the bud-to-cap transition and that Runx2 controls continued tooth growth and morphogenesis beyond the cap stage through activation of *Fgf3* and *Fgf10* expression in the dental papilla.

Keywords: Bmp4, Fgf3, Fgf10, Msx1, odontogenesis, genetic interaction

Introduction

Tooth development involves morphogenesis of the tooth germ through bud, cap, and bell stages regulated by sequential and reciprocal interactions between the adjacent dental epithelium and mesenchyme (Thesleff and Sharpe 1997; Pispa and Thesleff 2003). The bud-to-cap transition is a critical step and is regulated by multiple transcription factors and signaling pathways (Tucker and Sharpe 2004; Jussila and Thesleff 2012). Expression of the Msx1 transcription factor is induced in the developing tooth mesenchyme by Bmp and Fgf signals from the dental epithelium (Vainio et al. 1993; Chen et al. 1996; Bei and Maas 1998; Tucker et al. 1998). *Msx1*$^{-/-}$ mutant mice exhibit developmental arrest of all tooth germs at the bud stage, accompanied by significantly reduced expression of *Bmp4* in the dental mesenchyme (Satokata and Maas 1994; Chen et al. 1996). Mice lacking the Pax9 transcription factor also exhibit tooth developmental arrest at the bud stage (Peters et al. 1998). *Pax9* expression is induced in the prospective tooth mesenchyme at the onset of tooth development by Fgf signaling, and Pax9 function is required for maintenance of both *Msx1* and *Bmp4* expression in the tooth mesenchyme (Neubüser et al. 1997; Peters et al. 1998; Mandler and Neubüser 2001; Zhou et al. 2011). Remarkably, addition of recombinant Bmp4 protein rescued *Msx1*$^{-/-}$ mutant mandibular first molar tooth germs to late bell stage in explant cultures (Bei et al. 2000; Chen et al. 1996), which suggested that Bmp4 is a major mesenchymal odontogenic signal downstream of Msx1 to drive tooth morphogenesis through the bud-to-cap transition (Maas and Bei 1997). However, mice with tissue-specific inactivation of the *Bmp4* gene in the early cranial neural crest cells, which showed absence of functional *Bmp4* mRNA expression in the tooth bud mesenchyme, exhibited bud-stage developmental arrest of mandibular molar tooth germs but developed maxillary molars and incisors to mineralized teeth, suggesting that other Msx1-dependent mesenchymal factors also play critical roles in the bud-to-cap transition (Jia et al. 2013).

[1]Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA
[2]Department of Oral Pathology, School of Dentistry, Kyungpook National University, Daegu, Republic of Korea
[3]Division of Plastic Surgery, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA
[*]Present address: School of Dentistry, University of Utah, Salt Lake City, UT, USA

Corresponding Author:
R. Jiang, Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, MLC 7007, Cincinnati, OH 45229, USA.
Email: Rulang.Jiang@CCHMC.org

The zinc finger transcription factor Osr2 is expressed in a buccolingual gradient in the developing tooth mesenchyme and inhibits Msx1-meditated propagation of mesenchymal odontogenic signals along the buccolingual axis (Zhang et al. 2009). *Osr2*$^{-/-}$ mutant mice exhibit de novo supernumerary tooth induction lingual to the normal molar tooth germs (Zhang et al. 2009). The induction of supernumerary teeth in *Osr2*$^{-/-}$ mutant embryos depended on Msx1 function as *Msx1*$^{-/-}$*Osr2*$^{-/-}$ double mutant mice did not form supernumerary tooth germs. Remarkably, however, in contrast to the bud-stage arrest of all tooth germs in *Msx1*$^{-/-}$ mutant mice, the maxillary and mandibular first molar tooth germs developed to late bell stage in the *Msx1*$^{-/-}$*Osr2*$^{-/-}$ double mutant mice (Zhang et al. 2009). Whereas *Bmp4* expression was partially restored in the tooth mesenchyme in *Msx1*$^{-/-}$*Osr2*$^{-/-}$ double mutant embryos compared with the loss of *Bmp4* expression in *Msx1*$^{-/-}$ tooth mesenchyme, we recently found that reducing *Osr2* gene dosage by 50% was able to rescue the mandibular first molar morphogenesis in mice with neural crest–specific deletion of *Bmp4* (Zhang et al. 2009; Jia et al. 2013). These results suggest that Msx1 and Osr2 antagonistically regulate other critical mesenchymal odontogenic factors, in addition to Bmp4, during early tooth development.

The *Runx2* gene encodes a runt-domain containing transcription factor that is essential for bone and tooth development (Otto et al. 1997; D'Souza et al. 1999; Ryoo and Wang 2006). *Runx2* is expressed in the dental mesenchyme at the bud and cap stages and mediates Fgf signaling from the dental epithelium to mesenchyme (D'Souza et al. 1999; Aberg, Wang, et al. 2004). *Runx2*$^{-/-}$ mice exhibit tooth developmental arrest at late bud stages (D'Souza et al. 1999), accompanied by significant reduction or loss of expression of *Fgf3* in the dental mesenchyme and of *Shh, Edar*, and *p21* in the enamel knot (Aberg, Wang, et al. 2004). Explant culture assays showed that Msx1 and Runx2 are required for Fgf-induced expression of *Fgf3* in the dental mesenchyme (Bei and Maas 1998; Kettunen et al. 2000; Aberg, Wang, et al. 2004). Since *Runx2* mRNA expression was significantly reduced in the tooth bud mesenchyme in *Msx1*$^{-/-}$ mutant embryos whereas *Msx1* expression in the tooth mesenchyme was unaltered in *Runx2*$^{-/-}$ mutant embryos, these data suggest that Runx2 acts downstream of Msx1 to activate *Fgf3* expression during early odontogenesis.

In this study, we investigated possible genetic interactions between *Osr2* and *Runx2* and compared molar tooth morphogenesis in *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutant mice with that in *Msx1*$^{-/-}$*Osr2*$^{-/-}$ mice. We found that, in contrast to bud-stage developmental arrest in *Runx2*$^{-/-}$ mutants, both upper and lower first molar tooth germs progressed past the cap stage, with obvious primary enamel knot formation, in *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutants. However, in contrast to *Msx1*$^{-/-}$*Osr2*$^{-/-}$ mutants, the *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutant embryos failed to activate the expression of *Fgf3* and *Fgf10* in the dental papilla and showed significant deficit in cell proliferation in both the upper and lower molar tooth germs. Our data provide new insight into the molecular mechanism through which Runx2 regulates odontogenesis.

Materials and Methods

Mouse Strains

Msx1$^{+/-}$, *Osr2*$^{+/-}$, and *Runx2*$^{+/-}$ mice, which have been described previously (Satokata and Maas 1994; Otto et al. 1997; Lan et al. 2004), were maintained in the CD1 background. All animal procedures were approved by the Cincinnati Children's Hospital Medical Center Institutional Animal Care and Use Committee.

Histology and In Situ Hybridization

Embryos were collected from timed pregnant females, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5- to 7-μm thickness, and stained with hematoxylin and eosin (Zhang et al. 2009). For in situ hybridization, paraffin sections were hybridized with DIG-labeled cRNA probes as described previously (Zhang et al. 2009).

Cell Proliferation and Cell Death Assays

BrdU was injected intraperitoneally into timed pregnant female mice at E14.5 (Sigma-Aldrich, St. Louis, MO, USA; 50 μg/g body weight), and embryos were harvested 1 h after injection. Paraffin sections were prepared as described above. BrdU-incorporated cells were detected by immunofluorescent staining using the Alexa Fluor 594–conjugated anti-BrdU antibody (Life Technologies, Carlsbad, CA, USA; 1:50). The cell proliferation index was defined as the percentage of BrdU-positive nuclei relative to Hoechst-stained nuclei in the dental epithelial and mesenchymal compartments, respectively. Cell proliferation data were statistically analyzed using Student's *t* test for pairwise comparison, with a *P* value less than 0.05 considered significant. Cell death was detected by using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA) following the manufacturer's instruction.

Results

Runx2 *Expression in the Developing Tooth Mesenchyme Is Regulated by Antagonistic Actions of Msx1 and Osr2*

To understand possible interactions between Osr2 and Runx2 during tooth development, we first examined *Runx2* expression during tooth development in the control and *Osr2*$^{-/-}$ mutant embryos. *Runx2* was expressed in the dental mesenchyme and in osteogenic mesenchyme in the control embryos at the bud (E13.5) and late cap (E15.0) stages (Fig. 1A, C). In the *Osr2*$^{-/-}$ embryos, *Runx2* mRNA expression was up-regulated in the mesenchymal cells lingual to the tooth bud as well as in the palatal mesenchyme (Fig. 1B, D). In contrast, the *Osr2* mRNA expression pattern in the developing tooth germs was similar in the control and *Runx2*$^{-/-}$ embryos (Fig. 1E, F). Since *Runx2* mRNA expression in the developing tooth mesenchyme was

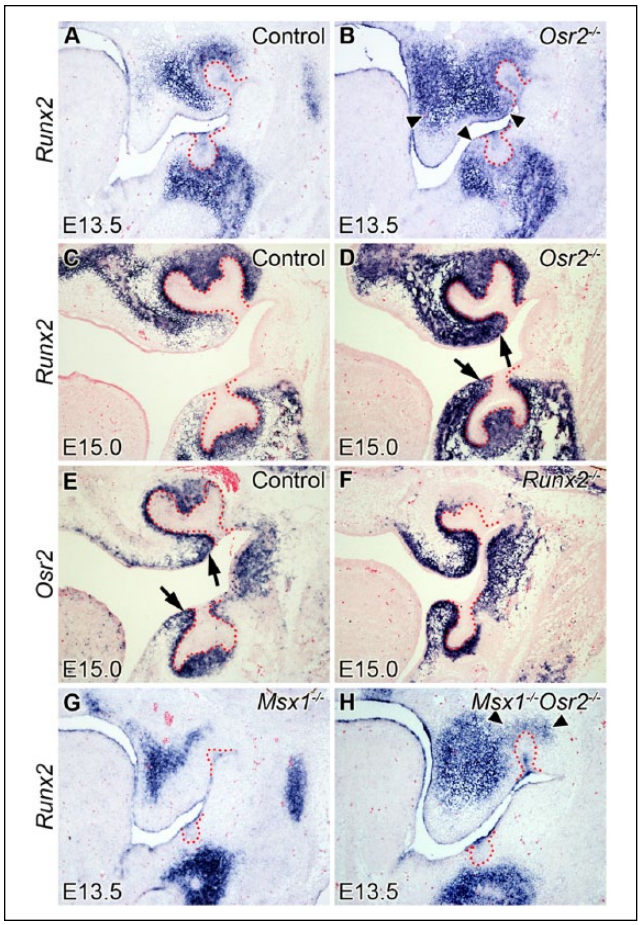

Figure 1. Expression of *Runx2* and *Osr2* in the tooth germ in control, *Osr2*$^{-/-}$, *Runx2*$^{-/-}$, *Msx1*$^{-/-}$, and *Msx1*$^{-/-}$*Osr2*$^{-/-}$ mutant mouse embryos. (**A–H**) Frontal sections at E13.5 (A, B, G, H) and E15.5 (C–F). (A, C) *Runx2* is expressed in both bone-forming regions and dental mesenchyme in the control group. (B, D) *Runx2* is expressed in both bone-forming regions and dental mesenchyme, and it is expanded to the palatal shelf and lingual mesenchyme (arrowheads, arrows) in the *Osr2*$^{-/-}$ group. (E, F) At E15.0, *Osr2* is expressed in the dental mesenchyme and also in the mesenchymal cells surrounding the dental stalk (arrows) in the control group (E); *Osr2* expression pattern is similar to the control group in the *Runx2*$^{-/-}$ group (F). (G, H) *Runx2* is expressed only in the bone-forming regions but not in the dental mesenchyme in the *Msx1*$^{-/-}$ group at E13.5 (G); *Runx2* is expressed in both bone-forming regions and the maxillary dental mesenchyme (arrowheads) in the *Msx1*$^{-/-}$*Osr2*$^{-/-}$ group (H).

reduced in the *Msx1*$^{-/-}$ embryos (Aberg, Wang, et al. 2004; Fig. 1G), we examined *Runx2* mRNA expression in the *Msx1*$^{-/-}$*Osr2*$^{-/-}$ mutant embryos and found that it was partially restored in the maxillary tooth mesenchyme in these double mutants (Fig. 1H). These results suggest that Msx1 and Osr2 act antagonistically to regulate *Runx2* expression during early tooth development.

Deletion of Osr2 *Substantially Rescued Tooth Morphogenesis in the* Runx2*-Deficient Mice*

To investigate whether Osr2 and Runx2 interact to regulate tooth development, we generated and analyzed *Osr2*$^{-/-}$*Runx2*$^{-/-}$ double mutant mice. At E18.5, whereas *Runx2*$^{-/-}$ embryos

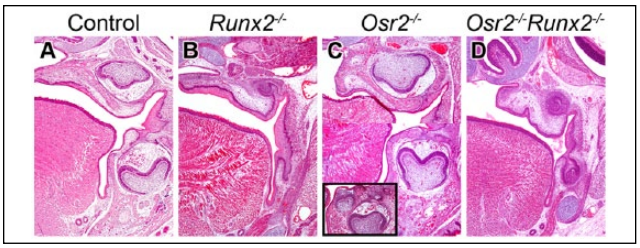

Figure 2. Tooth morphogenesis in the first molar at E18.5. (**A–D**) Frontal sections of control (A), *Runx2*$^{-/-}$ (B), *Osr2*$^{-/-}$ (C), and *Osr2*$^{-/-}$*Runx2*$^{-/-}$ (D) mutant mouse embryos. (A, C) Controls and *Osr2*$^{-/-}$ mutants exhibit bell stage tooth germs, whereas supernumerary teeth appear in the lingual region in the *Osr2*$^{-/-}$ mutants (C, box). (B) Tooth germ is arrested at the bud stage in the *Runx2*$^{-/-}$ mutants. (D) Tooth morphogenesis is substantially rescued, showing a bell shape, in the *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutants. Supernumerary tooth is not found in the lingual region.

showed tooth developmental arrest with absence of condensed dental mesenchyme (Fig. 2B) and *Osr2*$^{-/-}$ embryos had supernumerary tooth germ lingual to the first molar (Fig. 2C), *Osr2*$^{-/-}$ *Runx2*$^{-/-}$ double mutant embryos had smaller than normal molar tooth germs resembling a bell shape, with mesenchymal cells condensed inside the bell to form the dental papilla (Fig. 2D).

Molecular marker analysis showed that the *Runx2*$^{-/-}$ mutant tooth germs had reduced expression of *Msx1* and *Bmp4* in the tooth mesenchyme and lack of *Shh* expression in the mandibular molar epithelium at E15.5, in comparison with the control littermates (Fig. 3A, B, E, F, I, J). The *Osr2*$^{-/-}$ littermates showed increased expression of all of these 3 markers (Fig. 3C, G, K). Although the molar tooth germs in the *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutant embryos were much smaller in size compared with those in the control and *Osr2*$^{-/-}$ littermates, both *Msx1* and Bmp4 were strongly expressed in the molar mesenchyme while *Shh* was strongly expressed in the distal molar epithelium that corresponds to the primary enamel knot in these double mutant embryos (Fig. 3D, H, L). Furthermore, whereas the primary enamel knot marker *p21* was not detected in the molar tooth germs in the E15.5 *Runx2*$^{-/-}$ mutant embryos, in comparison with the control and *Osr2*$^{-/-}$ embryos, robust expression of *p21* was detected in both the maxillary and mandibular molar tooth germs in the *Osr2*$^{-/-}$*Runx2*$^{-/-}$ double mutant embryos (Fig. 3M–P). These molecular markers confirm that molar tooth morphogenesis in the *Runx2*$^{-/-}$ mutant embryos was partially rescued by deletion of *Osr2*.

Runx2 Is Required for Fgf3 *and* Fgf10 *Expression in the Dental Papilla*

Despite the formation of the primary enamel knot, the *Osr2*$^{-/-}$ *Runx2*$^{-/-}$ mutant molar tooth germs appeared significantly smaller at both E18.5 (Fig. 2) and E15.5 (Fig. 3). To investigate whether the reduced tooth germ size was due to aberrant cell death or defective cell proliferation, we performed TUNEL and BrdU-labeling assays at E14.5. No significant differences in the distribution of TUNEL-positive cells were detected in the tooth germs of the different genotypes except that the

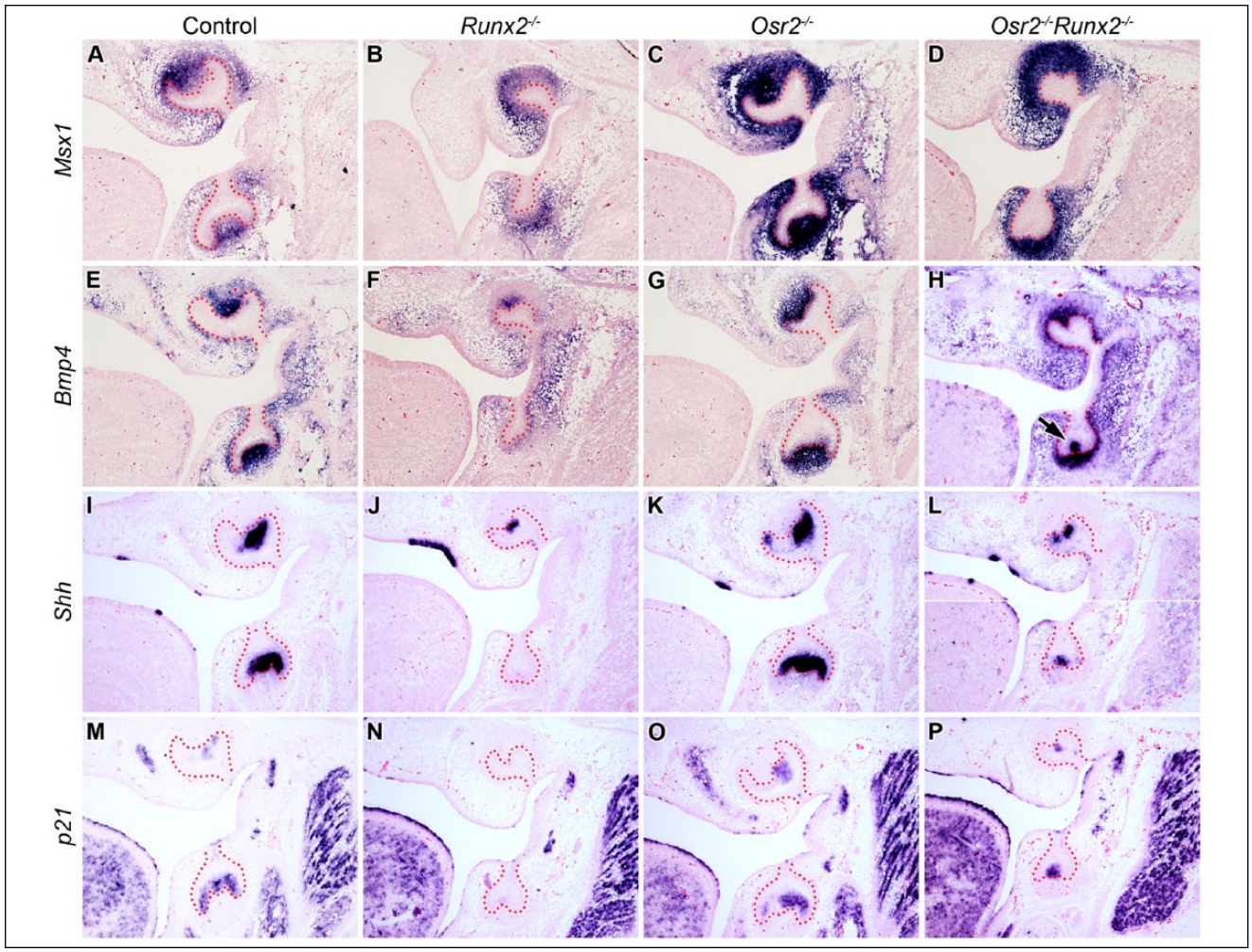

Figure 3. Expression of odontogenic markers in control, *Runx2*$^{-/-}$, *Osr2*$^{-/-}$, and *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutant mouse embryos at E15.5. (**A–H**) Down-regulation of *Msx1* and *Bmp4* in the dental mesenchyme in *Runx2*$^{-/-}$ mutants (B, F) was rescued in the *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutants (D, H). Epithelial *Bmp4* was also rescued in the enamel knot in *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutants (H, arrow). (**I–P**) Down-regulation of *Shh* and *p21* in the enamel knot in *Runx2*$^{-/-}$ mutants (J, N) was rescued in the *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutants (L, P).

control, *Osr2*$^{-/-}$, and *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutant tooth germs all showed specific TUNEL-positive cells in the primary enamel knot, whereas the *Runx2*$^{-/-}$ mutant mandibular molar tooth germs did not have the primary enamel knot structure (data not shown). The BrdU-labeling assay showed that the cell proliferation index for the epithelium was similar in the control and *Osr2*$^{-/-}$ tooth germs but reduced in *Runx2*$^{-/-}$ and *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutants (Fig. 4A–E). The deletion of *Osr2* did not significantly change the dental epithelial proliferation compared with *Runx2*$^{-/-}$ single mutants. In the mesenchyme, the proliferation index from lowest to highest was as follows: *Runx2*$^{-/-}$, *Osr2*$^{-/-}$*Runx2*$^{-/-}$, control, and *Osr2*$^{-/-}$ tooth germs (Fig. 4A–D, F). Thus, *Osr2* deletion significantly improved cell proliferation in the *Runx2*-deficient mesenchyme, although the growth of the *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutant tooth germs was still significantly retarded compared with the control tooth germs. These results suggest that Runx2 is required for activation of important growth factors in the tooth mesenchyme even in the absence of Osr2-mediated suppression.

It has been shown that the tooth bud developmental arrest in the *Runx2*$^{-/-}$ mutant mouse embryos was accompanied by failure of activation of *Fgf3* expression in the developing tooth mesenchyme (Aberg, Wang, et al. 2004). We found that both *Runx2*$^{-/-}$ and *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutant molar tooth germs failed to activate *Fgf3* expression at E15.5, in comparison with the robust *Fgf3* expression in the dental papilla in the control and *Osr2*$^{-/-}$ mutant embryos (Fig. 5A–D). In contrast, whereas the *Msx1*$^{-/-}$ mutant molar tooth germs failed to activate *Fgf3* expression, the *Msx1*$^{-/-}$*Osr2*$^{-/-}$ double mutant molar tooth germs showed partially restored *Fgf3* expression in comparison with the control and *Osr2*$^{-/-}$ tooth germs (Fig. 5E–H). *Fgf3* mutations in mice or humans were associated with abnormalities in tooth crown size or cusp patterning (Wang et al. 2007; Charles et al. 2009). However, the differences in *Fgf3* expression alone could not account for the differences in molar tooth morphogenesis between the *Osr2*$^{-/-}$*Runx2*$^{-/-}$ and *Msx1*$^{-/-}$*Osr2*$^{-/-}$ mutant embryos since mice lacking *Fgf3* were able to form the full mouse dentition (Mansour et al. 1993; Wang et al. 2007). A related

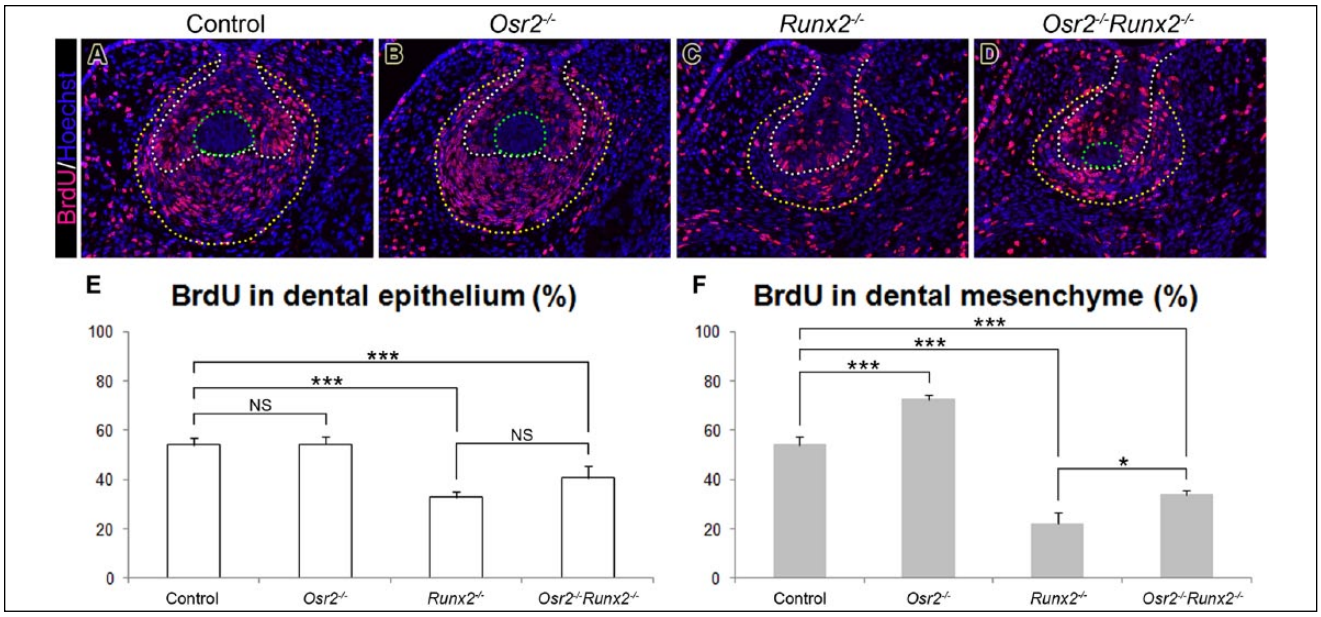

Figure 4. Comparative analysis of dental cell proliferation in the control, *Osr2*$^{-/-}$, *Runx2*$^{-/-}$, and *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutant mouse embryos at E14.5. (**A–D**) Representative immunofluorescent images of the mandibular molar tooth germs in control (A), *Osr2*$^{-/-}$ (B), *Runx2*$^{-/-}$ (C), and *Osr2*$^{-/-}$*Runx2*$^{-/-}$ (D) embryos. The white dotted line outlines the basement membrane in between the dental epithelium and mesenchyme. The yellow dotted line marks the outer margin of the tooth mesenchyme. The primary enamel knot is circled by the green dotted line in each of the control (A), *Osr2*$^{-/-}$ (B), and *Osr2*$^{-/-}$*Runx2*$^{-/-}$ (D) tooth germs. (**E, F**) Percentage of BrdU-labeled cells in the dental epithelium (E) and mesenchyme (F). Error bar represents SD. ***$P < 0.001$; *$P < 0.05$; NS, not significant.

fibroblast growth factor, *Fgf10*, is weakly expressed in the developing tooth mesenchyme at the bud stage but is strongly up-regulated in the dental papilla by the cap stage during tooth development (Aberg, Wang, et al. 2004). Although mice lacking *Fgf10* also had apparently normal prenatal tooth development, mice deficient in both *Fgf3* and *Fgf10* had tooth developmental arrest at the bud stage (Harada et al. 2002; Wang et al. 2007). We found that both *Runx2*$^{-/-}$ and *Osr2*$^{-/-}$*Runx2*$^{-/-}$ embryos failed to activate *Fgf10* expression in the tooth mesenchyme, in contrast to the robust *Fgf10* expression in the dental papilla in the control and *Osr2*$^{-/-}$ littermates (Fig. 5I–L). Since Fgf3 and Fgf10 have been shown to act synergistically to regulate molar tooth size (Wang et al. 2007), the lack of activation of both Fgf3 and Fgf10 in the tooth germs accounts for the significantly reduced rate of dental cell proliferation and small tooth germ size in the *Osr2*$^{-/-}$*Runx2*$^{-/-}$ embryos.

Discussion

Classic tissue recombination studies more than 40 y ago showed that the developing tooth mesenchyme from the bud to bell stages was able to induce and instruct complete tooth morphogenesis even when recombined with embryonic nondental epithelium (Kollar and Baird 1970a, 1970b). The molecular nature and mechanisms of regulation of this "mesenchymal odontogenic potential" are still not completely understood, however. Mutant mouse studies showed that mice lacking either Msx1 or Runx2 had tooth developmental arrest at early to late bud stages and that neither *Msx1*$^{-/-}$ nor *Runx2*$^{-/-}$ mutant tooth bud mesenchyme could support tooth organogenesis when recombined with wild-type embryonic dental epithelium (Satokata and Maas 1994; Bei and Maas 1998; D'Souza et al. 1999; Aberg, Cavender, et al. 2004; Aberg, Wang, et al. 2004), indicating that both Msx1 and Runx2 are important for activation of the mesenchymal odontogenic potential. Aberg, Wang, et al. (2004) showed that *Runx2* mRNA expression was significantly reduced in the molar tooth mesenchyme in E14 *Msx1*$^{-/-}$ embryos and suggested that Runx2 acts downstream of Msx1 to activate *Fgf3* expression during the bud-to-cap transition. However, the reduction of *Fgf3* expression alone in the tooth mesenchyme could not account for the bud-stage developmental arrest in the *Runx2*$^{-/-}$ mice since *Fgf3*-null mice had full dentition with only minor abnormalities in tooth crown size (Wang et al. 2007). Extensive molecular marker studies showed that most of the important tooth mesenchyme factors were expressed normally in the *Runx2*$^{-/-}$ mutant tooth germs at the bud stage (Aberg, Wang, et al. 2004). Thus, the molecular mechanism of Runx2-mediated tooth development remains to be elucidated. In this study, we show that deletion of *Osr2* partially rescued *Runx2*$^{-/-}$ molar tooth morphogenesis to the early bell stage. Our data provide new insights into the role of Runx2 in tooth development.

First, our data indicate that Runx2 regulates tooth morphogenesis through the bud-to-cap transition by modulating the antagonistic interactions of the Msx1 and Osr2 transcription factors. A critical step in tooth morphogenesis during the bud-to-cap transition is formation of the primary enamel knot in the tooth bud epithelium, which occurs at about E13.5 in mouse embryos. The primary enamel knot cells express multiple signaling molecules, including *Shh, Fgf4, Bmp4, Wnt10a*, and

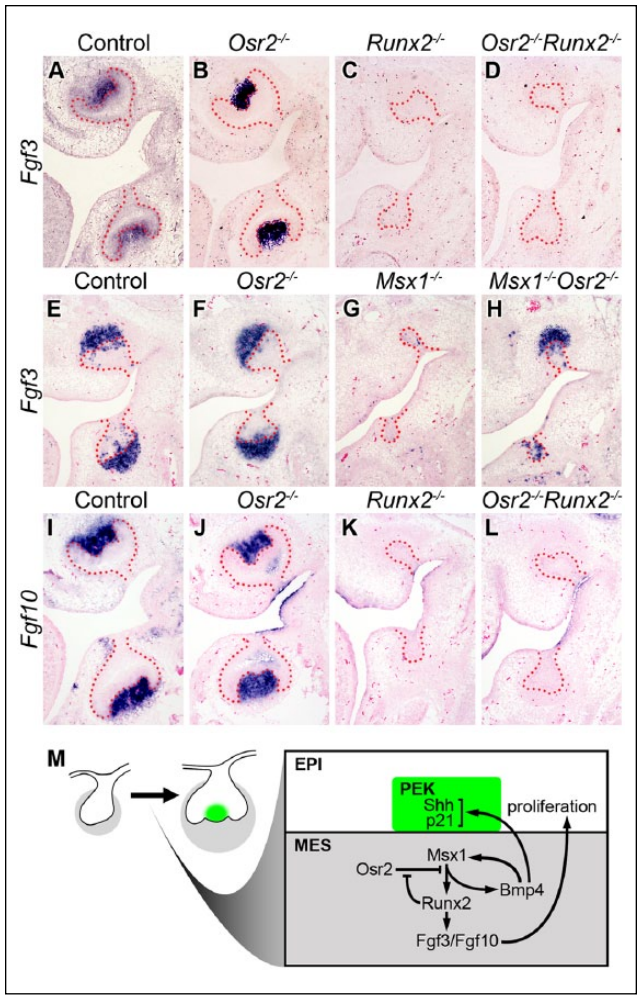

Figure 5. Expression of *Fgf3* and *Fgf10* in control, *Osr2*$^{-/-}$, *Runx2*$^{-/-}$, *Osr2*$^{-/-}$*Runx2*$^{-/-}$, *Msx1*$^{-/-}$, and *Msx1*$^{-/-}$*Osr2*$^{-/-}$ mutant mouse embryos at E14.5–E15.5. (**A–D**) *Fgf3* was present in the dental mesenchyme in controls and *Osr2*$^{-/-}$ mutants at E15.5 (A, B). Absence of *Fgf3* expression in the *Runx2*$^{-/-}$ mutants (C) was not rescued in the *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutants (D). (**E–H**) *Fgf3* was present in the dental mesenchyme in controls and *Osr2*$^{-/-}$ mutants at E14.5 (E, F). Absence of *Fgf3* in the *Msx1*$^{-/-}$ mutants (G) was rescued in the *Msx1*$^{-/-}$*Osr2*$^{-/-}$ mutants (H). (**I–L**) *Fgf10* was present in the dental mesenchyme in controls and *Osr2*$^{-/-}$ mutants at E15.5 (I, J). Absence of *Fgf10* expression in the *Runx2*$^{-/-}$ mutants (K) was not rescued in the *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutants (L). (**M**) Schematic diagram illustrating the bud and cap stage tooth germs, with the deduced molecular regulatory network involving Runx2, Msx1, Osr2, Bmp4, Fgf3, and Fgf10, during the bud-to-cap transition. EPI, dental epithelium; MES, dental mesenchyme (marked in gray); PEK, primary enamel knot (marked in green).

Wnt10b, which drive growth and morphogenesis of both the dental epithelium and mesenchyme to and through the cap stage (Jernvall and Thesleff 2000). In contrast to the *Msx1*$^{-/-}$ mutant mice, which exhibit tooth bud arrest and lack of primary enamel knot formation in all tooth germs, *Runx2*$^{-/-}$ mutant embryos showed expression of several enamel knot markers, including *Shh, p21, Fgf4, Edar*, and *Bmp4*, in the maxillary first molars but not in the mandibular molar tooth buds (Chen et al. 1996; Bei and Maas 1998; Aberg, Wang, et al. 2004). This difference in maxillary and mandibular molar tooth phenotypes was initially explained by possible partial complementation of Runx2 function by Runx3 since *Runx3* expression was shown to be up-regulated in the maxillary but not mandibular molar mesenchyme in *Runx2*$^{-/-}$ embryos (Aberg, Wang, et al. 2004). However, subsequent studies showed that the primary enamel knot also formed in the maxillary molar tooth germs in *Runx2*$^{-/-}$*Runx3*$^{-/-}$ double mutants (Wang et al. 2005), leaving the molecular basis of tooth developmental arrest in *Runx2*$^{-/-}$ embryos unresolved. We recently showed that mice with neural crest–specific inactivation of the *Bmp4* gene had mandibular molar bud arrest while their maxillary molars and incisors continued morphogenesis (Jia et al. 2013). Remarkably, deletion of one *Osr2* allele rescued mandibular molar tooth morphogenesis, whereas a reduction of *Msx1* by 50% neutralized this effect, in the neural crest–specific *Bmp4*-mutant mice (Jia et al. 2013; Lan et al. 2014). In this study, we found that, whereas *Runx2*$^{-/-}$ mutant molar tooth mesenchyme exhibited reduced expression of *Msx1* and *Bmp4* mRNAs, both *Osr2*$^{-/-}$ and *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutant embryos had increased expression of *Msx1* and *Bmp4* mRNAs in the tooth mesenchyme compared with the control embryos (Fig. 3A–H). Since Osr2 physically interacts with Msx1 and antagonizes Msx1-mediated activation of the mesenchymal odontogenic factors, including Bmp4 (Zhang et al. 2009; Zhou et al. 2011), and since Bmp4 is able to induce *Msx1* expression in the dental mesenchyme through a positive feedback loop (Chen et al. 1996; Bei et al. 2000) as well as to induce expression of *Shh* and *p21* in the dental epithelium (Jernvall et al. 1998; Chen et al. 2000), these results suggest that Runx2 normally acts to attenuate Osr2-mediated suppression of Msx1 function during the bud-to-cap transition (Fig. 5M). In the absence of Runx2 function, stronger than the normal level of Osr2-mediated repression of Msx1 function causes reduction in Bmp4 and other Msx1-dependent odontogenic factors in the developing tooth mesenchyme, leading to tooth developmental arrest in bud-to-cap transition in *Runx2*$^{-/-}$ mutant mice. In the *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutant embryos, the lack of Osr2-mediated repression causes increased expression of Msx1-dependent mesenchymal odontogenic factors, including Bmp4, which induces primary enamel knot formation and drives successful transition of the molar tooth germs from the bud to cap stage.

Second, our data demonstrate that Runx2 function is required for activation of expression of both *Fgf3* and *Fgf10* in the dental papilla. Previous studies showed that Runx2 is required for Fgf4 induction of *Fgf3* expression in the tooth mesenchyme (D'Souza et al. 1999; Aberg, Wang, et al. 2004). Since *Fgf10* is only weakly expressed in the tooth mesenchyme at the bud stage and since the developmentally arrested *Runx2*$^{-/-}$ tooth germs also had weak *Fgf10* expression at E14, initial *Fgf10* expression in the tooth bud mesenchyme is not Runx2-dependent (Aberg, Wang, et al. 2004). However, *Fgf10* expression is dramatically up-regulated in the tooth mesenchyme by the cap stage during normal tooth development (Aberg, Wang, et al. 2004b), but it was not detected in the E15.5 *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutant tooth germs that have progressed to the cap stage (Fig. 5L). The lack of *Fgf10* expression in the E15.5 *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutant tooth germs when the control and *Osr2*$^{-/-}$ mutant dental mesenchyme had robust *Fgf10* expression

strongly suggests that Runx2 mediates the up-regulation of *Fgf10* expression during the bud-to-cap transition. It has been shown that Fgf10 significantly stimulated tooth epithelial proliferation (Kettunen et al. 2000). Cell proliferation was significantly reduced in the *Runx2*$^{-/-}$ and *Osr2*$^{-/-}$*Runx2*$^{-/-}$ tooth germs by E14.5, in comparison with wild-type littermates (Wang et al. 2005; Fig. 4). Moreover, Fgf3 and Fgf10 have been shown to act partly redundantly to regulate molar tooth size (Wang et al. 2007). Thus, the inability of the tooth mesenchyme to up-regulate either *Fgf3* or *Fgf10* expression is likely a major contributor to the growth retardation of the molar tooth germs in the *Osr2*$^{-/-}$*Runx2*$^{-/-}$ mutant embryos.

Taken together, our data indicate that Runx2 acts downstream of and interacts with the Msx1-Osr2 antagonistic pair of transcription factors to regulate the mesenchymal odontogenic activity and that Runx2 plays an additional role in mediating up-regulation of mesenchymal Fgf signals to control tooth germ growth and morphogenesis beyond the cap stage.

Author Contributions

H.J.E. Kwon, R. Jiang, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; E.K. Park, Y. Lan, contributed to conception, design, data acquisition, analysis, and interpretation, drafted the manuscript; S. Jia, H. Liu, contributed to data acquisition, analysis, and interpretation, drafted the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work.

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