

MSX1 Drives Tooth Morphogenesis Through Controlling Wnt Signaling Activity

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Abstract

Tooth agenesis is a common structural birth defect in humans that results from failure of morphogenesis during early tooth development. The homeobox transcription factor *Msx1* and the canonical Wnt signaling pathway are essential for “bud to cap” morphogenesis and are causal factors for tooth agenesis. Our recent study suggested that *Msx1* regulates Wnt signaling during early tooth development by suppressing the expression of *Dkk2* and *Sfrp2* in the tooth bud mesenchyme, and it demonstrated partial rescue of *Msx1*-deficient molar teeth by a combination of DKK inhibition and genetic inactivation of SFRPs. In this study, we found that *Sostdc1*/Wise, another secreted Wnt antagonist, is involved in regulating the odontogenic pathway downstream of *Msx1*. Whereas *Sostdc1* expression in the developing tooth germ was not increased in *Msx1*^{-/-} embryos, genetic inactivation of *Sostdc1* rescued maxillary molar, but not mandibular molar, morphogenesis in *Msx1*^{-/-} mice with full penetrance. Since the *Msx1*^{-/-};*Sostdc1*^{-/-} embryos exhibited ectopic *Dkk2* expression in the developing dental mesenchyme, similar to *Msx1*^{-/-} embryos, we generated and analyzed tooth development in *Msx1*^{-/-};*Dkk2*^{-/-} double and *Msx1*^{-/-};*Dkk2*^{-/-};*Sostdc1*^{-/-} triple mutant mice. The *Msx1*^{-/-};*Dkk2*^{-/-} double mutants showed rescued maxillary molar morphogenesis at high penetrance, with a small percentage also exhibiting mandibular molars that transitioned to the cap stage. Furthermore, tooth development was rescued in the maxillary and mandibular molars, with full penetrance, in the *Msx1*^{-/-};*Dkk2*^{-/-};*Sostdc1*^{-/-} mice. Together, these data reveal 1) that a key role of *Msx1* in driving tooth development through the bud-to-cap transition is to control the expression of *Dkk2* and 2) that modulation of Wnt signaling activity by *Dkk2* and *Sostdc1* plays a crucial role in the *Msx1*-dependent odontogenic pathway during early tooth morphogenesis.

Keywords: cell signaling, growth factors, genetics, odontogenesis, hypodontia, congenital abnormalities

Introduction

To make a tooth, a tooth germ must undergo morphogenesis through the bud, cap, and bell stages, which involves reciprocal interactions between the dental epithelium and mesenchyme (Kwon and Jiang 2018). Initially, the oral epithelium forms a localized thickening, the dental placode, which continues to bud into the underlying mesenchyme and induces mesenchymal condensation around the bud. Prior to the cap stage, the dental mesenchyme induces formation of the primary enamel knot (PEK), which expresses *Shh* and members of the BMP, FGF, and WNT families, such as *Bmp2/4/7*, *Fgf3/4/9/20*, and *Wnt3/6/10a/10b*, in the distal end of the tooth bud and drives “bud to cap” morphogenesis. Extensive genetic studies demonstrated that tooth development is controlled by an intricate regulatory network consisting of transcription factors and the major signaling pathways: *Bmp*, *Fgf*, *Shh*, and *Wnt* (Lan et al. 2014). Perturbation in this regulatory network may cause disruption in morphogenesis, in many cases affecting PEK formation and bud-to-cap transition and resulting in tooth agenesis, which can severely affect oral health (Tan et al. 2016).

Transcription factor *Msx1* is essential in early tooth morphogenesis. With growth factor *Bmp4*, it forms the *Bmp4*-*Msx1* positive feedback pathway within the bud-stage dental

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mesenchyme and promotes bud-to-cap morphogenesis (Chen et al. 1996; Bei et al. 2000; Zhao et al. 2000). The canonical Wnt signaling pathway is also essential in early tooth morphogenesis. Binding of Wnt signals to their receptors Frizzled (Fzd) and Lrp5/6 causes stabilization and translocation of the β -catenin into the nucleus, where β -catenin interacts with DNA-bound Tcf/Lef transcription factors and activates transcription of target genes, such as *Lef1* or *Axin2* (Nusse and Clevers 2017). Mutations in *MSX1*, *WNT10A*, or *AXIN2* account for 46% of human tooth agenesis cases (Fournier et al. 2018), and genetic deletion of *Msx1* or *Lef1*, as well as tissue-specific inactivation of β -catenin in the dental epithelium or mesenchyme, causes developmental arrest at the bud stage in mice (Kratochwil et al. 1996; Liu et al. 2008; Chen et al. 2009). Overexpression of secreted Wnt antagonist Dickkopf 1 (*Dkk1*) in the epithelium consistently results in bud-stage arrest (Liu et al. 2008), while deletion of *Sostdc1*—otherwise known as *Wise*, another Wnt antagonist—induces formation of supernumerary teeth caused by dysregulated Wnt signaling activity in the rudimentary dental tissues (Ahn et al. 2010). *Dkk1* and *Sostdc1* antagonize canonical Wnt signaling by binding to Lrp5/6 and preventing Frz-Lrp5/6 complex formation (Nusse and Clevers 2017). Yet, forced activation of Wnt signaling in the oral epithelium, through constitutive stabilization of β -catenin, results in formation of multiple supernumerary teeth (Järvinen et al. 2006). However, similar activation of β -catenin in the mesenchyme induces ectopic tooth bud-like invaginations in the developing palate but do not result in supernumerary teeth (Chen et al. 2009).

Deficiency in *Msx1* or *Bmp4* in the dental mesenchyme leads to significant reduction of the expression of Wnt-target genes *Lef1* and *Tcf7* (Jia et al. 2016), whereas *Lef1* deficiency or dental mesenchyme-specific deletion of β -catenin does not alter mesenchymal *Msx1* or *Bmp4* expression (Kratochwil et al. 1996; Chen et al. 2009). A systems biology study identified Wnt and Bmp as the two major mediators of dental epithelial-mesenchymal signaling (O'Connell et al. 2012). These studies suggest that *Msx1* is positioned upstream of Wnt signaling activation in early tooth development. Our recent study, based on dental mesenchyme-specific RNA-seq analysis, showed that expression of *Dkk2* and Secreted frizzled-related protein 2 (*Sfrp2*), encoding distinct secreted antagonists of Wnt signaling, was significantly increased in the *Msx1*^{-/-} mutant embryonic tooth mesenchyme (Jia et al. 2016). However, neither treatment with the DKK inhibitor IIC3a nor genetic inactivation of *Sfrp2* was able to rescue tooth morphogenesis in *Msx1*^{-/-} embryos, whereas 30% of *Msx1*^{-/-};*Sfrp2*^{+/-};*Sfrp3*^{+/-} embryos treated with IIC3a showed partial rescue of the maxillary molar only (Jia et al. 2016). Thus, the molecular mechanism mediating *Msx1* function in tooth morphogenesis and the relationship between *Msx1* and Wnt signaling during tooth development require investigation. In this study, we show that complete inactivation of *Dkk2* alone was able to rescue maxillary molar morphogenesis in the *Msx1*^{-/-} mice at high penetrance, which identifies *Dkk2* as a crucial downstream target gene in the *Msx1*-mediated odontogenic pathway. Furthermore,

although expression of *Sostdc1* in the developing tooth mesenchyme was not increased in the *Msx1*^{-/-} embryos, we found 1) that inactivation of *Sostdc1* also rescued maxillary molar morphogenesis in *Msx1*^{-/-} mice and 2) that inactivation of *Dkk2* and *Sostdc1* rescued tooth development in the maxillary and mandibular molars in *Msx1*^{-/-} mice with full penetrance. These results indicate that *Msx1* drives early tooth morphogenesis from the bud to cap stage primarily through controlling Wnt signaling activity.

Materials and Methods

Mouse Strains

Msx1^{+/-}, *Sostdc1*^{+/-}, and *Dkk2*^{+/-} mice were used for generating compound mutants (Satokata and Maas 1994; Li et al. 2005; Ahn et al. 2010). Mice were maintained in a CD1 outbred background. A total of 178 mouse samples were used. At least 3 mice, male and female, from embryonic day 12.5 (E12.5) to birth were examined per experiment. Tooth development beyond birth was not examined because *Msx1*^{-/-} mice develop cleft palate and die at birth. All protocols were prepared before the study and approved by the Institutional Animal Care and Use Committee at the University at Buffalo and Cincinnati Children's Hospital Medical Center. This study is compliant with the ARRIVE guidelines (Animal Research Reporting of In Vivo Experiments). Additional detailed information about the mice and the experimental procedures is included in the Appendix.

Results

Expression of *Lef1* and *Sostdc1* during Tooth Morphogenesis in the *Msx1*-Deficient Maxillary and Mandibular Molars

To address the knowledge gap on the relationship between *Msx1* and the Wnt signaling pathway during early tooth development, we analyzed whether expression of *Sostdc1* during tooth development is affected by *Msx1*. We characterized *Sostdc1* mRNA expression during tooth morphogenesis in comparison with that of *Lef1*, a representative Wnt signaling target gene (Kratochwil et al. 1996; Sasaki et al. 2005). When compared with the strong expression in the control embryonic tooth germs, *Lef1* expression was markedly reduced at E12.5 to E14.5 in *Msx1*^{-/-} molars (Fig. 1A–C, A'–C'). In contrast, *Sostdc1* signals showed a moderate and gradual decrease in *Msx1*^{-/-} molars, especially in the mandible (Fig. 1D–F, D'–F'). Real-time quantitative polymerase chain reaction (qRT-PCR) results confirmed the dramatic decrease in *Lef1* and moderate decrease in *Sostdc1* in the *Msx1*^{-/-} embryos in both jaws at the bud-to-cap transition (E14.0; Fig. 1G). Interestingly, *Sostdc1* and *Lef1* exhibited complementary expression patterns. In the distal tip of the tooth germ, *Sostdc1* signals were weaker, whereas *Lef1* signals were strong and concentrated, as similarly shown in the PEK (Fig. 1A–C, D–F, black arrowheads).

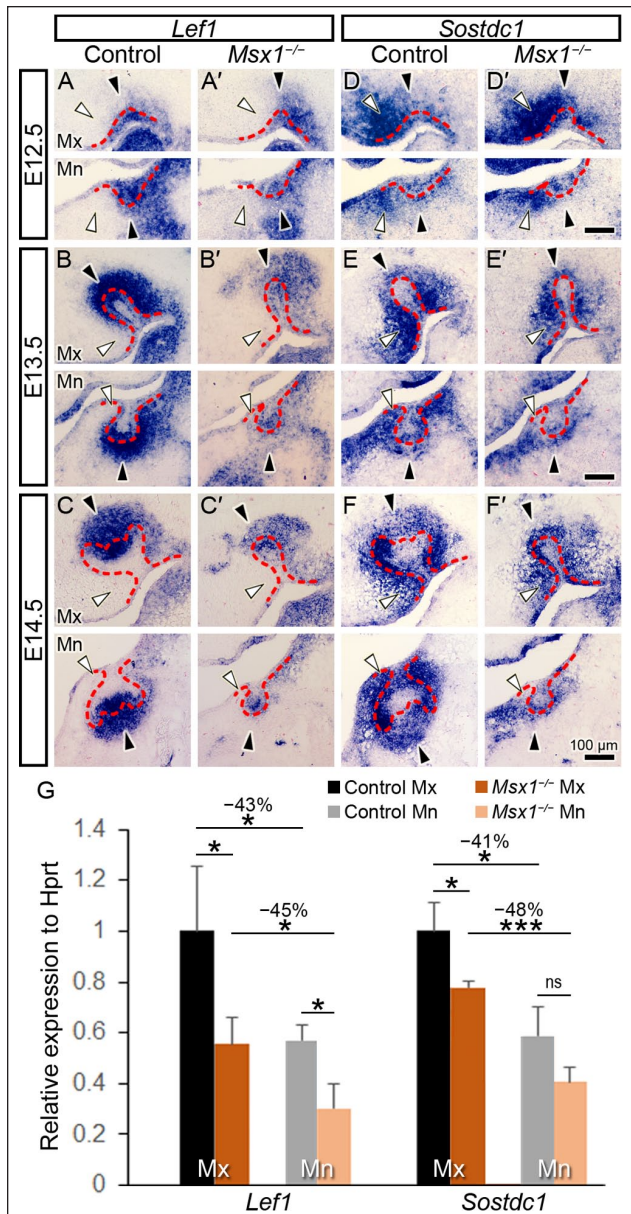


Figure 1. *Lef1* and *Sostdc1* expression in normal and *Msx1*^{-/-} tooth morphogenesis. (A–F; A'–F') Expression pattern of *Lef1* and *Sostdc1* in frontal sections through the molar tooth germs in the control (A–F) and *Msx1*^{-/-} (A'–F') groups. Representative time points for early tooth development were examined: the placode (embryonic day 12.5 [E12.5]), bud (E13.5), and cap (E14.5) stages. In each panel, a white horizontal line divides the upper jaw (maxilla, Mx) and the lower jaw (mandible, Mn); the left side is palatal/lingual and right side is buccal. White arrowhead marks the palatal/lingual mesenchyme; black arrowhead marks the distal end of the tooth germ; red dashed line marks the basal lamina between the dental epithelium and mesenchyme. Scale bars, 100 μ m. *n* = 4 for panels A–C and A'–C'; *n* = 10 for panels D–F and D'–F'. (G) Real-time quantitative polymerase chain reaction of *Lef1* and *Sostdc1* in Mx and Mn molar tooth germs in the control and *Msx1*^{-/-} groups at bud-to-cap transition (at E14.0). Results are expressed as fold change \pm SD relative to control Mx. *n* = 3 for each group. Student *t* test. **P* \leq 0.05. ****P* \leq 0.001. ns, not significant.

However, *Sostdc1* signals were strongest whereas *Lef1* signals were markedly decreased or even absent in the palatal (for maxilla) or lingual (for mandible) mesenchyme (Fig. 1A–C,

D–F, white arrowheads). Whereas the complementary patterns of *Lef1* and *Sostdc1* expression in the developing tooth germs are consistent with previous findings that *Sostdc1* antagonized canonical Wnt signaling during tooth development (Ahn et al. 2010), the moderately decreased expression of *Sostdc1* in the *Msx1*^{-/-} tooth germs indicate that expression of *Sostdc1* is regulated differently from that of *Dkk2* and *Sfrp2*, which exhibited significant upregulation in the *Msx1*^{-/-} tooth mesenchyme (Jia et al. 2016).

Deletion of *Sostdc1* Partially Rescues Tooth Development in *Msx1*-Deficient Maxillary Molars

Although *Sostdc1* expression was not suppressed by *Msx1*, we hypothesized that *Sostdc1* might contribute to the suppression of canonical Wnt signaling in the *Msx1*^{-/-} tooth germs. To test this, we examined whether *Sostdc1* deletion could rescue tooth development in *Msx1*^{-/-} mice. In contrast to the bud-stage arrest in all *Msx1*^{-/-} molars, *Msx1*^{-/-};*Sostdc1*^{-/-} compound mutants showed full penetrance (100%) of rescued maxillary molar, which advanced to the bell stage at birth (Fig. 2, Table). Control and *Msx1*^{-/-};*Sostdc1*^{-/-} maxillary molars exhibited histodifferentiation of the dental epithelium and mesenchyme into ameloblasts and odontoblasts, respectively, a key feature of bell-stage morphogenesis (Fig. 2A'–C'). To rule out the possibility that the rescued molar is a supernumerary tooth, which can ectopically form in *Sostdc1*^{-/-} mice (Ahn et al. 2010), we examined its anteroposterior position through whole-mount analysis of *Shh* mRNA expression, which marked the tooth germs and the palatal rugae that served as anteroposterior landmarks. Sagittal sections through the first and second molars were also analyzed, where the maxillary nerve (V2) and optic nerve marked the first and second molar positions, respectively (Fig. 2A–C, G–I). Our data indicated that the rescued *Msx1*^{-/-};*Sostdc1*^{-/-} molars were first molars. However, the rescue remained “partial” because of the marked reduction in the anteroposterior width and height (Fig. 2J).

Forced activation of canonical Wnt signaling in the oral epithelium can induce supernumerary teeth in *Msx1*^{-/-} mice, manifested as multiple ectopically positioned odontoma-like tissues that form without going through the normal “bud to cap to bell” morphogenesis (Wang et al. 2009). We assayed for various odontogenic molecular markers to verify whether the rescue of the *Msx1*^{-/-};*Sostdc1*^{-/-} molar involved normal bud-to-cap morphogenesis. At early cap stage (E14.25), expression of PEK markers *Shh*, *Fgf4*, and *Fgf3* was markedly downregulated in the maxillary and mandibular molars in the *Msx1*^{-/-} mutants, and their expression was considerably restored in the *Msx1*^{-/-};*Sostdc1*^{-/-} double mutants but only in the maxillary molars (Fig. 3A–I). Of note, *Fgf3* expression in the dental mesenchyme showed similar marked downregulation in the maxillary and mandibular *Msx1*^{-/-} molars and was rescued only in the maxillary molars in the *Msx1*^{-/-};*Sostdc1*^{-/-} embryos (Fig. 3G–I). These results indicate that the *Msx1*^{-/-};*Sostdc1*^{-/-} maxillary molar developed through bud-to-cap morphogenesis and PEK formation similar to control mice. To examine whether the rescue of maxillary molar morphogenesis involved recovery of Wnt signaling, we examined *Lef1* expression at the

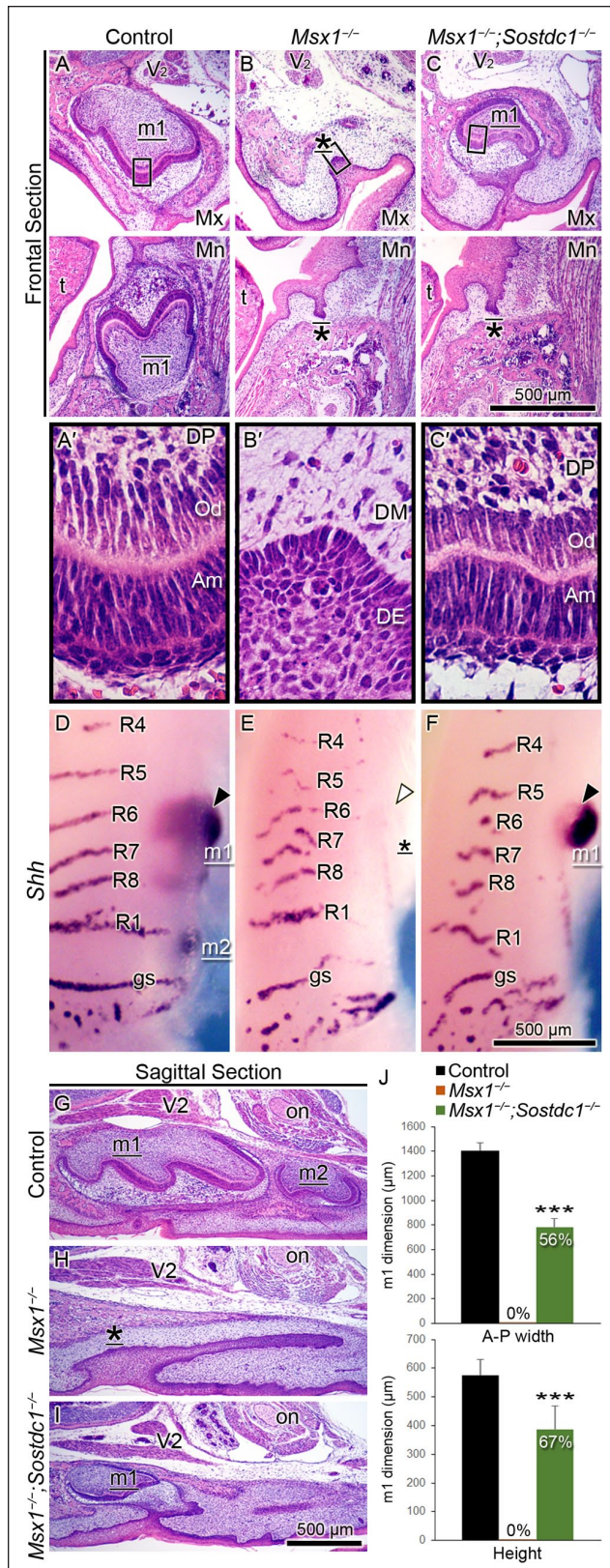


Figure 2. *Sostdc1* deletion partially rescues tooth development in *Msx1*^{-/-} maxillary first molars. (A–C; A'–C') Hematoxylin and eosin-stained molar tooth germs are shown in frontal sections through the molar tooth germs at birth (P0). (A–C) White horizontal lines divide

Table. Developmental Rescue of *Msx1*-Deficient Teeth and Palate by Deletion of *Dkk2* and *Sostdc1*.

Genotype	Mandible		
	Maxilla Molar	Molar	Palate
Control ^a	21/21	21/21	21/21
<i>Msx1</i> ^{-/-}	0/7	0/7	0/7
<i>Msx1</i> ^{-/-} ; <i>Dkk2</i> ^{+/+} ; <i>Sostdc1</i> ^{-/-b}	9/9	0/9	0/9
<i>Msx1</i> ^{-/-} ; <i>Dkk2</i> ^{-/-} ; <i>Sostdc1</i> ^{+/+b}	5/6	1/6	0/6
<i>Msx1</i> ^{-/-} ; <i>Dkk2</i> ^{+/+} ; <i>Sostdc1</i> ^{-/-}	6/6	0/6	6/6
<i>Msx1</i> ^{-/-} ; <i>Dkk2</i> ^{-/-} ; <i>Sostdc1</i> ^{+/+}	3/3	3/3	3/3
<i>Msx1</i> ^{-/-} ; <i>Dkk2</i> ^{-/-} ; <i>Sostdc1</i> ^{-/-}	6/6	6/6	0/6

All mice were part of the same breeding scheme. Rescue was determined at E15.5 or later. Tooth rescue was defined as tooth germs that developed to the cap stage or beyond; palate rescue was defined as complete fusion of palatal shelves. All rescues were bilateral. Numbers represent mouse counts: numerator is the rescue count; denominator is the total count.

^aControl group includes wild type, *Msx1*^{+/+}, *Msx1*^{+/+};*Dkk2*^{+/+}, *Msx1*^{+/+};*Sostdc1*^{+/+}, and *Msx1*^{+/+};*Dkk2*^{-/-};*Sostdc1*^{+/+} genotypes.

^bFor consistency, *Msx1*^{-/-};*Sostdc1*^{-/-} and *Msx1*^{-/-};*Dkk2*^{-/-} were indicated as *Msx1*^{-/-};*Dkk2*^{+/+};*Sostdc1*^{-/-} and *Msx1*^{-/-};*Dkk2*^{-/-};*Sostdc1*^{+/+}, respectively.

bud-to-cap transition (at E14.0). When compared with the marked reduction of *Lef1* signals in the *Msx1*^{-/-} molar, where residual *Lef1* was higher in the maxilla than in the mandible, *Msx1*^{-/-};*Sostdc1*^{-/-} maxillary molars showed considerable restoration of *Lef1* expression in the dental epithelium and mesenchyme (Fig. 3J–L). qRT-PCR analysis showed that as compared with the significantly downregulated *Lef1* expression in the *Msx1*^{-/-} molars in both jaws, *Msx1*^{-/-};*Sostdc1*^{-/-} molars showed significant recovery (+48%) in the maxilla but not the mandible (Fig. 3P). When testing whether the rescue involved downregulation of ectopic *Dkk2* expression, we found that ectopic *Dkk2* signals in the *Msx1*^{-/-} molar mesenchyme versus the control molars remained upregulated in the *Msx1*^{-/-};*Sostdc1*^{-/-} molars, in the maxilla and mandible, which was confirmed with qRT-PCR. Taken together, our results indicate that although *Msx1*^{-/-};*Sostdc1*^{-/-} embryos exhibited increased expression of *Dkk2* in the tooth mesenchyme similar to that of *Msx1*^{-/-} embryos, the complete inactivation of *Sostdc1* restored

the maxilla (Mx) and mandible (Mn); the left side is palatal/lingual and the right side is buccal. (A'–C') High-magnification images of corresponding boxed areas in panels A–C show the histodifferentiation of the ameloblasts (Am), odontoblasts (Od), and dental papilla (DP), as well as the underdifferentiated dental epithelium (DE) and dental mesenchyme (DM). (D–F) Whole-mount visualization of tooth germs in the Mx jaw at embryonic day 16.5 by analyzing *Shh* mRNA detection, where upper is anterior and lower is posterior. Black and white arrowheads mark *Shh*-positive and *Shh*-negative first molar tooth germs, respectively. (G–I) Hematoxylin and eosin-stained molar tooth germs at birth are shown in sagittal sections, where left is anterior and right is posterior. Landmarks used for identification of the tooth germs are the maxillary nerve (V₂), which is the second branch of the trigeminal nerve, the fifth cranial nerve, and the optic nerve (on). Tooth germ is labeled m1 (first molar), m2 (second molar), or * (for developmentally arrested tooth bud). These labels are underlined (for Mx) or overlined (for Mn). t, tongue; R, palatal ruga; gs, "geschmacksstreifen" (taste stripes). Scale bars, 500 μm. n = 8 for each of panels A–C; n = 6 for each of panels D–F; n = 10 for each of panels G–I. (J) Measurements of the anteroposterior (A-P) width and height of the tooth germ, showing reduction by 44% in A-P width and by 33% in height. Results are expressed as mean ± SD. n = 10 for each group. Student t test. ***P ≤ 0.001.

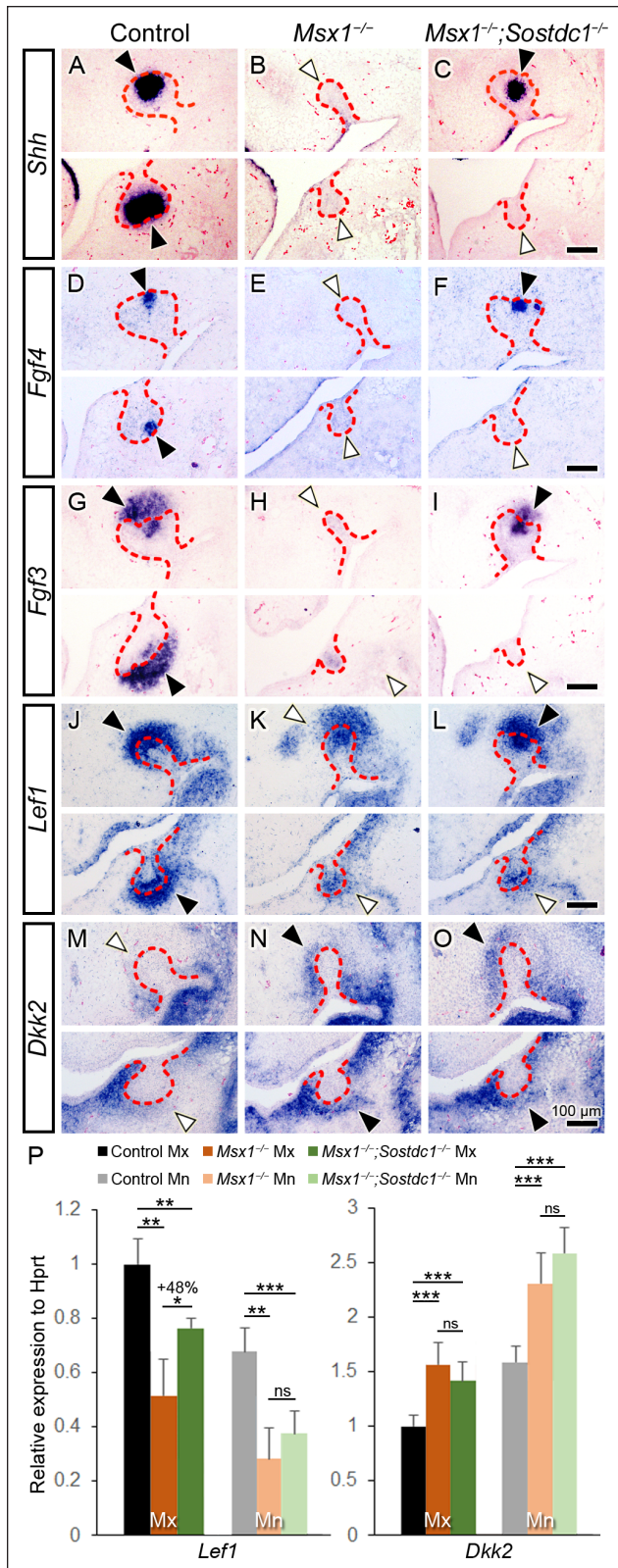


Figure 3. *Sostdc1* deletion partially restores Wnt signaling and bud-to-cap transition in *Msx1*^{-/-} maxillary molars. (A–O) Molecular marker assay in control, *Msx1*^{-/-}, and *Msx1*^{-/-};*Sostdc1*^{-/-} tooth development at around the bud-to-cap transition (embryonic day 13.75 [E13.75]) to

Wnt signaling activity to a sufficient level to drive tooth morphogenesis through the bud-to-cap transition in the *Msx1*^{-/-}; *Sostdc1*^{-/-} maxillary molar germs as compared with *Msx1*^{-/-} maxillary molars.

Deletion of *Dkk2* Partially Rescues Tooth Development in *Msx1*-Deficient Maxillary Molars

Whereas our previous study showed that intraperitoneal injection of pregnant *Msx1*^{+/-} females with the DKK inhibitor IIC3a from gestational day 11 through 13 could not rescue tooth morphogenesis in *Msx1*^{-/-} embryos, we recently obtained *Dkk2*^{-/-} mice to directly examine whether *Dkk2* is a key downstream target gene of the *Msx1*-mediated odontogenic pathway. At later stages when molar tooth development advanced to the early (E15.5) and late (E17.5) bell stages in control mice, the *Msx1*^{-/-};*Dkk2*^{-/-} double mutants showed rescued maxillary molars at high penetrance (5 of 6 samples), with 1 of 6 samples also showing mandibular molar development to the cap stage but not as advanced as the bell-stage morphogenesis in the maxillary molars (Fig. 4A–D, B'; Table). We analyzed the molecular markers for PEK formation (*Shh*) and Wnt signaling activity (*Lef1*) at the bud-to-cap transition. *Shh* expression, which was present in the control and absent in the *Msx1*^{-/-} molars in both jaws, was restored in the *Msx1*^{-/-};*Dkk2*^{-/-} in the maxillary molar germs (Fig. 4E–G). *Lef1* expression, which was strong in the control but markedly reduced in the *Msx1*^{-/-} molars in both jaws, showed considerable recovery in the *Msx1*^{-/-};*Dkk2*^{-/-} maxillary molar and a minor recovery in the mandibular molar (Fig. 4H–J). These results indicate that the significantly increased expression of *Dkk2* in the *Msx1*^{-/-} tooth mesenchyme (Fig. 3M, N, P; Appendix Fig. 1) is a major contributor to the bud stage developmental arrest of the *Msx1*^{-/-} tooth germs.

Combined Inactivation of *Dkk2* and *Sostdc1* Rescues Development of Maxillary and Mandibular Molars in *Msx1*-Deficient Mice

Given that *Sostdc1* and *Dkk2* regulate canonical Wnt signaling in a similar fashion and that inactivation of either *Sostdc1* or

E14.25). Primary enamel knot markers *Shh* (A–C) and *Fgf4* (D–F) and a marker for primary enamel knot and dental mesenchyme, *Fgf3* (G–I), at the early cap stage (at E14.25). (J–L) Wnt signaling marker *Lef1* at bud-to-cap transition (at E14.0). (M–O) Secreted Wnt inhibitor *Dkk2* at the late bud stage (at E13.75). In each panel, a white horizontal line divides the maxillary (Mx) and mandibular (Mn) molars; the left side is palatal/lingual and the right side is buccal. The distal end of a tooth germ is marked with an arrowhead: black, stronger mRNA expression; white, weaker. The red dashed line marks the boundary between the dental epithelium and mesenchyme. Scale bars, 100 μ m. $n = 4$ for panels A–O. (P) Real-time quantitative polymerase chain reaction of *Lef1* and *Dkk2* expression in Mx and Mn molar tooth germs at bud-to-cap transition (at E14.0). Results are expressed as fold change \pm SD relative to control Mx. $n = 3$ –5 for *Lef1* expression; $n = 4$ –6 for *Dkk2* expression. Student t test. * $P \leq 0.05$. ** $P \leq 0.01$. *** $P \leq 0.001$. ns, not significant.

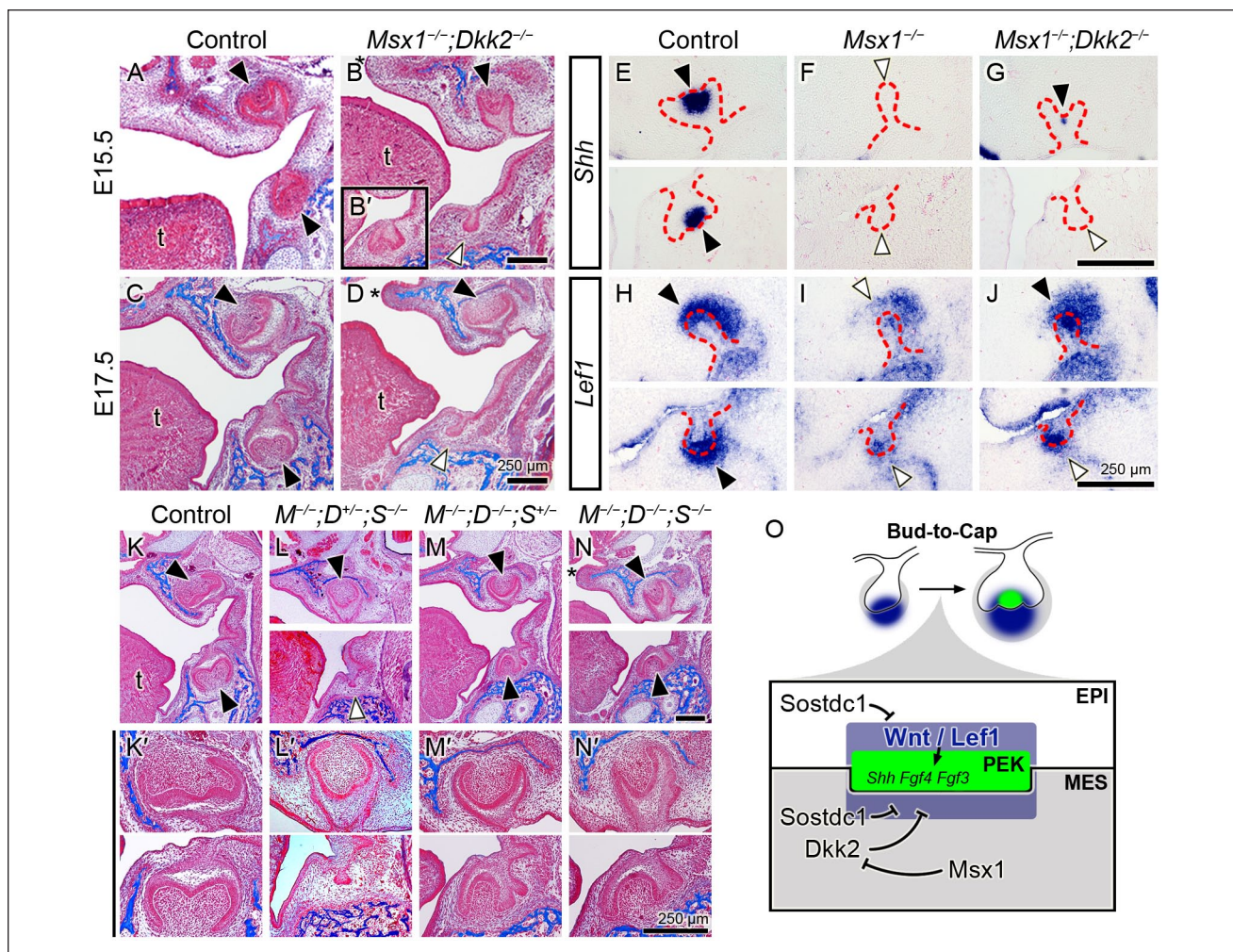


Figure 4. Compound deletion of *Dkk2* and *Sostdc1* rescues tooth development in *Msx1*^{-/-} maxillary and mandibular molars. (A–D) Trichrome-stained mouse embryo heads from control and *Msx1*^{-/-};*Dkk2*^{-/-} groups, shown in frontal sections through the molar tooth germs at embryonic days 15.5 (E15.5) and E17.5. (B, D) Deletion of *Dkk2* partially rescues tooth development in the *Msx1*^{-/-} maxillary molar. (B') Inset in panel B is from a different embryo, showing a rare case of rescue in the mandibular molar. (E–J) Molecular marker assay in control, *Msx1*^{-/-}, and *Msx1*^{-/-};*Dkk2*^{-/-} tooth development. (E–G) Primary enamel knot (PEK) marker *Shh* at the early cap stage (at E14.25). (H–J) Wnt signaling marker *Lef1* at bud-to-cap transition (at E14.0). Trichrome-stained frontal sections of embryo heads (K–N) and high magnification of their molar tooth germs (K'–N') at E18.5. M, D, and S stand for *Msx1*, *Dkk2*, and *Sostdc1*. In each panel, the left side is palatal/lingual and the right side is buccal. In panels A–D and K–N, the black arrowhead indicates morphogenesis beyond the bud-to-cap transition, and the white arrow indicates bud-stage arrest. In panels E–J, the distal end of the tooth germ is marked with an arrowhead: black, stronger mRNA expression within the 3 genotypes; white, weaker. Red dashed lines mark the boundary between the dental epithelium and mesenchyme. A white horizontal line in panels E–J, L, N, and K'–N' divides the maxilla and mandible, imaged from distinct sections from the same embryo. Asterisk in panels B, D, and N marks cleft palate. t, tongue. Scale bar, 250 μ m. *n* = 5, 2, 3, 1 for panels A–D; *n* = 4 for panels E–G; *n* = 6 for panels H–J; *n* = 4, 2, 3, 2 for panels K–N. (O) Schematic diagram illustrating the deduced molecular regulatory network involving *Msx1*, *Dkk2*, *Sostdc1*, and Wnt signaling/*Lef1* at the bud-to-cap transition.

Dkk2 resulted in rescue of the development of the maxillary molar but not as effectively that of the mandibular molar in the *Msx1*^{-/-} embryos, we next investigated whether combined inactivation of *Dkk2* and *Sostdc1* could rescue mandibular molar morphogenesis in the *Msx1*^{-/-} embryos. Similar to the *Msx1*^{-/-};*Sostdc1*^{+/-} mice, all *Msx1*^{-/-};*Dkk2*^{+/-};*Sostdc1*^{+/-} mice showed full penetrance of rescue only in the maxillary molars. In contrast, *Msx1*^{-/-};*Dkk2*^{+/-};*Sostdc1*^{+/-} and *Msx1*^{-/-};*Dkk2*^{-/-};*Sostdc1*^{+/-} mice showed full penetrance of rescue of the maxillary and mandibular molars (Fig. 4K–N, K'–N'; Table). With

our previous report showing significantly increased expression of *Sfrp1* and *Sfrp2*, in addition to *Dkk2*, in the mandibular molar mesenchyme in *Msx1*^{-/-} embryos (Jia et al. 2016), these data indicate that *Msx1* drives tooth morphogenesis through the bud-to-cap transition primarily through regulating canonical Wnt signaling activity by controlling expression of the secreted Wnt antagonists. Of note, *Msx1*^{-/-} mice exhibited complete penetrance of cleft palate and tooth bud arrest (Satokata and Maas 1994), and *Msx1*^{-/-};*Dkk2*^{+/-};*Sostdc1*^{+/-} and *Msx1*^{-/-};*Dkk2*^{+/-};*Sostdc1*^{+/-} exhibited successful palate fusion;

however, all *Msx1*^{-/-};*Dkk2*^{-/-};*Sostdc1*^{-/-} exhibited cleft palate without any substantial improvement, despite mice with each of these 3 compound mutant genotypes exhibiting rescued maxillary molars. This indicates that palate development and maxillary molar morphogenesis are independently regulated by *Msx1* interaction with the Wnt signaling pathway.

Discussion

The current study advances our knowledge of the molecular mechanism underlying tooth development by demonstrating *Dkk2* and *Sostdc1* as effectors of *Msx1* in early tooth morphogenesis. We provide for the first time genetic evidence that *Msx1* promotes Wnt signaling to drive tooth morphogenesis by suppressing *Dkk2* expression in the dental mesenchyme, demonstrated by the ectopic *Dkk2* expression in the *Msx1*^{-/-} tooth bud mesenchyme and the developmental rescue of *Msx1*^{-/-};*Dkk2*^{-/-} molar teeth (Fig. 4O). In addition, this study identifies a previously unknown role of *Sostdc1* in the tooth developmental arrest in *Msx1*^{-/-} mice. In contrast to *Dkk2* and *Sfrp2*, whose expression was significantly increased in the molar tooth mesenchyme in *Msx1*^{-/-} embryos (Jia et al. 2016), we found that expression of *Sostdc1* was moderately decreased in the *Msx1*^{-/-} molar germs, as compared with control embryos. Previous studies have shown that BMP4 induced *Sostdc1* expression in cultured tooth bud explants (Laurikkala et al. 2003) and that *Bmp4* expression was reduced in the *Msx1*^{-/-} molar mesenchyme (Chen et al. 1996; Jia et al. 2013). Thus, the moderate decrease in *Sostdc1* expression in *Msx1*^{-/-} molar germs was likely a secondary consequence of reduced *Bmp4* activity.

Sostdc1 was initially proposed to function as an inhibitor of *Bmp* signaling in tooth development (Laurikkala et al. 2003). However, extensive genetic interaction assays with various signaling pathway components, including *Lrp5/6* (Wnt), *Bmpr1a* (*Bmp*), and *Fgf10* or *Fgfr1/2* (*Fgf*), indicate that *Sostdc1* regulates tooth development primarily through antagonizing *Lrp5/6*-mediated canonical Wnt signaling (Ahn et al. 2010). The complementary expression patterns of *Sostdc1* and *Lef1* during normal tooth morphogenesis (Fig. 1) and the recovery of *Lef1* expression in the molar germs in the *Msx1*^{-/-};*Sostdc1*^{-/-} and *Msx1*^{-/-};*Dkk2*^{-/-} mouse embryos (Figs. 3 and 4) indicate that the rescue of tooth development in these mice are mediated through recovery of Wnt signaling activity. Although expression of *Sostdc1* expression was moderately decreased whereas that of *Dkk2* was significantly increased in the *Msx1*^{-/-} molar germs, genetic inactivation of *Sostdc1* or *Dkk2* similarly rescued maxillary molar morphogenesis in the *Msx1*^{-/-} mice. We previously showed that expression of *Sfrp2* was also significantly increased in the *Msx1*^{-/-} molar mesenchyme and that genetic inactivation of *Sfrp2* and *Sfrp3* combined with treatment with the DKK inhibitor IIC3a was able to partly rescue maxillary molar development (Jia et al. 2016). Together, these results indicate that the bud-stage tooth development arrest in the *Msx1*^{-/-} embryos resulted from the combined inhibitory

effects on Wnt signaling activity by several Wnt antagonists, including *Dkk2*, *Sfrp2*, and *Sostdc1*, and that *Msx1* controls tooth development through the bud-to-cap transition primarily through regulating expression of these genes encoding secreted Wnt antagonists.

Tooth agenesis and orofacial clefts are two of the most common craniofacial birth defects that occasionally co-occur in patients as well as in animal models (Phan et al. 2016). While cleft palate may result from primary defects in the developing palatal shelves or secondary effects of structural abnormality in surrounding structures (Bush and Jiang 2012), *Msx1* plays critical primary roles in regulating palate and tooth development (Zhang et al. 2002). We observed that *Msx1*^{-/-};*Dkk2*^{+/-};*Sostdc1*^{-/-} and *Msx1*^{-/-};*Dkk2*^{-/-};*Sostdc1*^{+/-} mice exhibited fused secondary palate, in contrast to the full penetrance of cleft palate in *Msx1*^{-/-} mice. While this study has not specifically investigated the relationship between *Msx1* and *Dkk2*/*Sostdc1* in palate development, the rescue of palate morphogenesis in *Msx1*^{-/-};*Dkk2*^{+/-};*Sostdc1*^{-/-} and *Msx1*^{-/-};*Dkk2*^{-/-};*Sostdc1*^{+/-} mice suggests that the combined inactivation of *Dkk2* and *Sostdc1* in the developing palate also modulates *Msx1*-dependent developmental processes during palate morphogenesis. Although *Dkk2*^{-/-} or *Sostdc1*^{-/-} mutant mice do not show cleft palate, recent studies have implicated misregulation of *Dkk2* and combined activities of *Dkk2* and *Sostdc1* in contributing to cleft palate in the *Pax9*^{-/-} mutant mice through inhibition of Wnt signaling (Jia et al. 2017; Li et al. 2017).

Remarkably, our study reveals that the molecular mechanisms underlying tooth development are not identical between the maxillary and mandibular molars. An asymmetrical tooth rescue between jaws in the *Msx1*^{-/-};*Sostdc1*^{-/-} and *Msx1*^{-/-};*Dkk2*^{-/-} mice was similarly observed in our previous rescue study in the *Msx1*^{-/-};*Sfrp2*^{-/-};*Sfrp3*^{-/-} mouse treated with IIC3a. This may be explained, at least partly, through the difference in the intrinsic level of certain tooth developmental antagonists, such as *Dkk2*, *Osr2*, *Sfrp1/2*, and *Wif1*, between the maxillary and mandibular molars, which may set a different molecular environment for tooth development, possibly leading to a different susceptibility to certain gene mutations between the jaws (Jia et al. 2013; Kwon et al. 2017). From a clinical perspective, this study may provide a key understanding of why certain teeth in humans, such as the permanent second premolars or maxillary lateral incisors, are more susceptible than other teeth to genetic mutations and show a higher prevalence of tooth agenesis (Fournier et al. 2018). It would be meaningful to dissect the molecular landscape of different teeth within the same organism to gain insights into the site-specific developmental programs employed in organogenesis. Our rescue studies also identify secreted Wnt antagonists as potential therapeutic targets for preventing or correcting congenital dental anomalies. The recent development of a monoclonal antibody therapy to treat osteoporosis by targeting sclerostin (SOST), a paralog of SOSTDC1 that similarly antagonizes canonical Wnt signaling through *Lrp5/6* disruption (MacNabb et al. 2016), sets a good example of how our research can provide

the groundwork for developing new strategies for resolving birth defects and for regenerative therapies.

Author Contributions

J.-M. Lee, C. Qin, R. Jiang, H.-J.E. Kwon, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; O.H. Chai, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript; Y. Lan, contributed to conception, design, data analysis, and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Declaration of Conflicting Interests


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