Activin and Bmp4 Signaling Converge on Wnt Activation during Odontogenesis

Journal of Dental Research
2017, Vol. 96(10) 1145–1152
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for Dental Research 2017
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DOI: 10.1177/0022034517713710
journals.sagepub.com/home/jdr

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Abstract

Previous studies show that both activin and Bmp4 act as crucial mesenchymal odontogenic signals during early tooth development. Remarkably, mice lacking activin- β A (*Inhba*^{-/-}) and mice with neural crest-specific inactivation of *Bmp4* (*Bmp4*^{ncx1/ncx2}) both exhibit bud-stage developmental arrest of the mandibular molar tooth germs while their maxillary molar tooth germs completed morphogenesis. In this study, we found that, whereas expression of *Inhba* and *Bmp4* in the developing tooth mesenchyme is independent of each other, *Bmp4*^{ncx1/ncx2}/*Inhba*^{-/-} compound mutant mice exhibit early developmental arrest of all tooth germs. Moreover, genetic inactivation of *Osr2*, a negative regulator of the odontogenic function of the Bmp4-Msx1 signaling pathway, rescues mandibular molar morphogenesis in *Inhba*^{-/-} embryos. We recently reported that *Osr2* and the Bmp4-Msx1 pathway control the bud-to-cap transition of tooth morphogenesis through antagonistic regulation of expression of secreted Wnt antagonists, including *Dkk2* and *Sfrp2*, in the developing tooth mesenchyme. We show here that expression of *Dkk2* messenger RNAs was significantly upregulated and expanded into the tooth bud mesenchyme in *Inhba*^{-/-} embryos in comparison with wild-type littermates. Furthermore, in utero treatment with either lithium chloride, an agonist of canonical Wnt signaling, or the DKK inhibitor IIC3a rescued mandibular molar tooth morphogenesis in *Inhba*^{-/-} embryos. Together with our finding that the developing mandibular molar tooth bud mesenchyme expresses significantly higher levels of *Dkk2* than the developing maxillary molar tooth mesenchyme, these data indicate that Bmp4 and activin signaling pathways converge on activation of the Wnt signaling pathway to promote tooth morphogenesis through the bud-to-cap transition and that the differential effects of loss of activin or Bmp4 signaling on maxillary and mandibular molar tooth morphogenesis are mainly due to the differential expression of Wnt antagonists, particularly *Dkk2*, in the maxillary and mandibular tooth mesenchyme.

Keywords: cell signaling, growth factors, genetics, morphogenesis, hypodontia, tooth development

Introduction

Tooth development is controlled by reciprocal signaling interactions between epithelial and mesenchymal tissues and has long been used as a model for understanding cell-cell signaling interactions that regulate mammalian organogenesis (Thesleff et al. 1995; Jernvall and Thesleff 2000). Tissue explant culture assays have shown that the odontogenic potential initially arises in oral ectoderm, where dental placodes form, and shifts to the prospective tooth mesenchyme at early bud stage, with tooth mesenchyme from bud and later stages capable of inducing tooth organogenesis even when recombined with embryonic nondental epithelium (Kollar and Baird 1970a, 1970b; Ruch et al. 1973; Mina and Kollar 1987; Lumsden 1988; Jernvall and Thesleff 2000). At bud stage, mesenchymal signals induce formation of the primary enamel knot (PEK), a signaling center consisting of nonproliferating epithelial cells in the distal tooth bud region that secretes many growth factors, including members of Bmp, Fgf, and Wnt families and Shh, which drives tooth morphogenesis through bud-to-cap transition and beyond (reviewed by Jernvall and Thesleff 2000; Thesleff 2003; Tucker and Sharpe 2004; Zhang et al. 2005; Lan et al. 2014).

Among signaling molecules expressed in the early dental epithelium and mesenchyme, Bmp4 exhibits an expression pattern that coincides with the shift of odontogenic potential from epithelium to mesenchyme during tooth bud formation (Vainio et al. 1993). Bmp signaling activates expression of *Msx1*, encoding a homeodomain-containing DNA-binding transcription factor, in developing tooth mesenchyme (Vainio et al. 1993; Chen et al. 1996; Tucker et al. 1998). Mice lacking *Msx1* exhibit tooth developmental arrest at bud stage, with significantly reduced *Bmp4* messenger RNA (mRNA) expression in

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A supplemental appendix to this article is available online.

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the tooth mesenchyme (Satokata and Mass 1994; Chen et al. 1996; Tucker et al. 1998). Remarkably, addition of recombinant Bmp4 protein rescued morphogenesis of *Msx1*^{-/-} molar tooth germs to bell stage in explant cultures (Bei et al. 2000). However, in contrast to the developmental arrest of all tooth germs in *Msx1*^{-/-} mice, only mandibular molar development was arrested at bud stage in mice lacking Bmp4 expression in the dental mesenchyme (Jia et al. 2013; Jia et al. 2016), suggesting that another mesenchymal factor partly complemented Bmp4 function in maxillary molar and incisor development.

Wnt signaling is another key molecular pathway driving tooth organogenesis. In the absence of Wnt activation, β -catenin is phosphorylated in the cytoplasm by GSK3 β and targeted for degradation. Wnt activation of the frizzled and Lrp5/6 co-receptors leads to inhibition of GSK3 β , resulting in stabilization and translocation of β -catenin into the nucleus, where it activates target gene expression (MacDonald et al. 2009). Tissue-specific inactivation of β -catenin in either the embryonic oral epithelium or early tooth mesenchyme caused tooth developmental arrest at bud stage (Andl et al. 2002; Liu et al. 2008; Chen et al. 2009). On the other hand, constitutive stabilization of β -catenin in the mouse embryonic oral epithelium caused supernumerary tooth formation (Järvinen et al. 2006; Wang et al. 2009). However, constitutive stabilization of β -catenin could not induce tooth formation in the absence of BMP receptor-Ia in the dental epithelium (O'Connell et al. 2012), indicating that BMP and Wnt signaling acts synergistically to induce tooth organogenesis.

Recent studies show that the Bmp4-*Msx1* pathway regulates expression of secreted Wnt antagonists during early tooth development. Multiple secreted Wnt inhibitors, including Dickkopf family members Dkk2 and Dkk3, as well as secreted frizzled-related proteins Sfrp1, Sfrp2, Sfrp3, are expressed in developing tooth mesenchyme (Sarkar and Sharpe 1999; Fjeld et al. 2005; Jia et al. 2013; Jia et al. 2016). Inactivation of Bmp4 in the neural crest lineage (*Bmp4*^{ncko}) caused increased expression of Dkk2 mRNAs in the developing tooth mesenchyme (Jia et al. 2013). Remarkably, in utero treatment with a small-molecule inhibitor of DKKs, IIC3a (Li et al. 2012), rescued mandibular molar morphogenesis in *Bmp4*^{ncko/ncko} embryos (Jia et al. 2016). Further studies showed that expression of Dkk2 and Sfrp2 in the developing tooth mesenchyme depended on the function of Osr2, a zinc finger transcription factor that interacts with *Msx1* and antagonizes its function in tooth development (Zhang et al. 2009; Zhou et al. 2011; Jia et al. 2016). Whereas *Msx1*^{-/-} embryos exhibit increased expression of Dkk2, Sfrp1, and Sfrp2 mRNAs in the tooth bud mesenchyme, genetic inactivation of Sfrp2 and Sfrp3 in combination with IIC3a treatment partly rescued molar tooth morphogenesis in *Msx1*^{-/-} embryos (Jia et al. 2016).

In addition to Bmp4, activin-A has been identified as an important mesenchymal odontogenic signal. Mice lacking activin- β A, encoded by the *Inhba* gene, exhibit mandibular molar arrest at bud stage, but their maxillary molar teeth develop normally (Ferguson et al. 1998). Expression of *folistatin* (*Fst*) and *Irx1*, 2 downstream target genes of activin signaling, were significantly downregulated in both maxillary

and mandibular molar tooth germs in *Inhba*^{-/-} embryos, which led to the hypothesis that maxillary and mandibular molar tooth morphogenesis involves distinct genetic pathways (Ferguson et al. 1998; Ferguson et al. 2001). In this study, we show that *Bmp4*^{ncko/ncko} *Inhba*^{-/-} compound mutant mice exhibit early bud developmental arrest of all tooth germs, whereas genetic inactivation of *Osr2* rescues mandibular molar morphogenesis in *Inhba*^{-/-} embryos. Moreover, we demonstrate that *Inhba*^{-/-} embryos exhibit significantly increased expression of Dkk2 in the tooth bud mesenchyme and that in utero treatment with IIC3a also rescues mandibular molar morphogenesis in *Inhba*^{-/-} embryos. These data indicate that the activin and Bmp4 signaling pathways converge on the regulation of Wnt pathway activation in both maxillary and mandibular molar tooth germs to control early tooth morphogenesis.

Materials and Methods

Animals

Bmp4^{fl/fl}, *Inhba*^{+/-}, *Osr2*^{+/-}, and *Wnt1Cre* transgenic mice and generation of *Bmp4*^{fl/fl}; *Wnt1Cre* (*Bmp4*^{ncko/ncko}) compound mutant mice were previously described (Matzuk et al. 1995; Danielian et al. 1998; Lan et al. 2004; Jia et al. 2013). These mice were maintained in a CD1 mixed background. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Cincinnati Children's Hospital Medical Center. This study conformed with ARRIVE guidelines for preclinical animal studies.

Laser Capture Microdissection and Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

Embryo heads microdissected at E13.5 were frozen in Tissue-Tek OCT compound. Specimens were cryosectioned, and molar tooth mesenchyme tissues were isolated by laser capture microdissection (LCM) (Appendix Fig. 1) as described previously (Jia et al. 2016). Tissues were pooled from 3 embryos of the same genotype, and total RNAs were extracted by using the RNeasy Micro Kit (Qiagen). Complementary DNAs were analyzed using real-time polymerase chain reaction (PCR) using the C1000 Touch Thermal Cycler (Bio-Rad) and Advanced SYBR Green Supermix (Bio-Rad). PCR experiments were carried out in duplicate. Student's *t* test was used to analyze pairwise differential expression, and *P* values <0.05 were considered statistically significant.

Histology, In Situ Hybridization, and Immunofluorescence Assays

Embryos were collected from timed pregnant females, fixed in 4% paraformaldehyde, dehydrated through graded ethanol series, embedded in paraffin, and sectioned at a 7- μ m thickness. For histological analysis, sections were stained with hematoxylin and eosin as described previously (Chen et al.

2009). Section in situ hybridization was performed as described in Zhang et al. (1999). Cryosectioned embryo heads were used for immunofluorescence staining using a rabbit monoclonal antibody for pSmad1/5/9 (D5B10; Cell Signaling Technology) as previously described (Zhou et al. 2011).

Lithium Chloride and IIC3a Treatments

Lithium chloride (LiCl) was injected intraperitoneally into pregnant mice (6–12 $\mu\text{mol/g}$ body weight) once every 24 h from gestational days 11 to 14. NaCl solution was used as negative control for LiCl treatment. IIC3a (Millipore) was dissolved in dimethyl sulfoxide (DMSO) and injected intraperitoneally into pregnant mice (10–25 $\mu\text{g/g}$ body weight) once every 24 h from gestational days 11 to 13 or 14. Control mice were injected with the same concentration of DMSO. Embryos were harvested at E13.5 or E18.5 for in situ hybridization and histological analyses, respectively.

Results

Bmp4 and Inhba Are Independently Expressed in the Developing Tooth Mesenchyme and Act Synergistically to Drive Tooth Morphogenesis

To investigate possible interactions between Bmp4 and activin signaling during tooth development, we first compared *Bmp4* expression during tooth development in *Inhba*^{-/-} embryos and control littermates. *Bmp4* mRNAs were similarly expressed in tooth bud mesenchyme in control and *Inhba*^{-/-} embryos (Fig. 1A, B). We also compared the expression of *Inhba* mRNAs in *Bmp4*^{ncko/ncko} embryos and control littermates and did not find any significant difference (Fig. 1C, D). Moreover, immunofluorescence staining for pSmad1/5/9 showed that BMP signaling activity was not altered in *Inhba*^{-/-} tooth germs (Appendix Fig. 2). These data indicate that Bmp4 and activin-A do not regulate each other's expression during early tooth development.

To gain better understanding of the molecular mechanisms mediating activin signaling regulation of tooth development, we generated and analyzed tooth development in *Bmp4/Inhba* compound mutants. Whereas *Bmp4*^{ncko/ncko} and *Bmp4*^{ncko/+} *Inhba*^{-/-} embryos showed continued maxillary molar morphogenesis beyond bud stage (Fig. 2B, C, F, G, J, K), all *Bmp4*^{ncko/ncko} *Inhba*^{-/-} mutants ($n = 8$) exhibited bud-stage arrest of maxillary molars (Fig. 2D, H, L) and all incisor tooth germs

Figure 1. Comparison of *Bmp4* and *Inhba* messenger RNA (mRNA) expression in the developing tooth bud mesenchyme in control, *Inhba*^{-/-}, and *Bmp4*^{ncko/ncko} embryos. **(A)** Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) analysis of *Bmp4* mRNAs in E13.25 maxillary (Max) and mandibular (Man) molar mesenchyme in the control and *Inhba*^{-/-} embryos. **(B)** In situ hybridization analysis of *Bmp4* mRNA expression in frontal sections through the first molar tooth germs in E13.25 control and *Inhba*^{-/-} embryos. **(C)** Quantitative real-time RT-PCR analysis of *Inhba* mRNAs in E13.5 maxillary (Max) and mandibular (Man) molar mesenchyme in control and *Bmp4*^{ncko/ncko} embryos. **(D)** In situ hybridization analysis of *Inhba* mRNA expression in frontal sections through the first molar tooth germs in E13.5 control and *Bmp4*^{ncko/ncko} embryos. Red dashed lines mark the boundary between the tooth bud epithelium and mesenchyme; mandibular region is shown below the maxillary region; lingual side is to the left in panels B and D. Error bars represent standard error of the mean in panels A and C. Panels B and D are representative results of each group ($n = 8$ for each group).

(Appendix Fig. 3), with their mandibular molar tooth germs arrested at early bud formation (Fig. 2D, H, L). These data indicate that Bmp4 and activin signaling act synergistically to regulate organogenesis of both the maxillary and mandibular teeth.

Genetic Inactivation of Osr2 Rescued Mandibular Molar Tooth Morphogenesis in *Inhba*^{-/-} Mice

The *Osr2* transcription factor is known to suppress *Msx1*-mediated propagation of *Bmp4* expression in the developing tooth mesenchyme (Zhang et al. 2009; Zhou et al. 2011). We investigated whether inactivating *Osr2* could affect mandibular molar development in *Inhba*^{-/-} mice. At E16.5, whereas *Inhba*^{-/-} embryos exhibit bud-stage arrest of mandibular molar tooth germs compared to wild-type littermates (Fig. 3A, B), mandibular first molar tooth germs in *Inhba*^{-/-} *Osr2*^{+/-} ($n = 6$) and *Inhba*^{-/-} *Osr2*^{-/-} ($n = 4$) embryos progressed to cap and early bell stages, respectively (Fig. 3C, D). By E18.5, whereas mandibular first molar tooth buds were still developmentally arrested in *Inhba*^{-/-} embryos ($n = 8$) (Fig. 3F), all *Inhba*^{-/-} *Osr2*^{+/-} ($n = 10$) and *Inhba*^{-/-} *Osr2*^{-/-} ($n = 6$) embryos showed bell-stage maxillary and mandibular first molar tooth germs (Fig. 3G, H). However, the mandibular molar tooth germs in *Inhba*^{-/-} *Osr2*^{+/-} and *Inhba*^{-/-} *Osr2*^{-/-} embryos showed abnormal cusp patterning (compare Fig. 3G and H with Fig. 3E). Moreover, expression of *Fst* and *Irx1* mRNAs in the molar tooth epithelium was similarly reduced in E13.5 *Inhba*^{-/-} and

Figure 2. Bmp4 and activin-A act synergistically to drive tooth germ morphogenesis. (A–L) Hematoxylin and eosin–stained frontal sections through the first molar tooth germs from E13.5 to E16.5 ($n = 8$ for each group). (A, E, I) Control embryos show normal bud, cap, and bell stages of tooth development at E13.5, E14.5, and E16.5, respectively. (B, F, J) $Bmp4^{nccko/nccko}$ embryos show bud-stage arrest of the mandibular molar tooth germs (arrowheads). (C, G, K) $Bmp4^{nccko/+}; Inhba^{-/-}$ embryos show bud-stage arrest of the mandibular molar tooth germs (arrowheads). (D, H, L) $Bmp4^{nccko/nccko}; Inhba^{-/-}$ compound mutants show bud-stage arrest of the maxillary molar tooth germs and even earlier developmental arrest of the mandibular molar tooth germs (arrowheads).

$Inhba^{-/-}; Osr2^{-/-}$ embryos compared to control littermates (Appendix Fig. 4). These results indicate that reducing *Osr2* function could rescue mandibular molar development through the bud-to-cap transition in $Inhba^{-/-}$ embryos, but activin signaling has an *Osr2*-independent role in dental epithelial differentiation and cusp patterning.

Expression of *Dkk2* Is Significantly Upregulated in the Tooth Bud Mesenchyme in $Inhba^{-/-}$ Embryos

We recently reported that *Dkk2* is expressed at significantly higher levels in the mandibular molar mesenchyme than in the maxillary molar mesenchyme in wild-type embryos and that its expression is significantly upregulated in both maxillary and mandibular molar tooth bud mesenchyme in $Bmp4^{nccko/nccko}$ embryos (Jia et al. 2013). In addition, we showed that *Msx1* and *Osr2* have opposing effects on expression of *Dkk2*, *Sfrp1*, and *Sfrp2* in the developing tooth bud mesenchyme, with expression of each of these 3 Wnt antagonist genes significantly upregulated in the tooth bud mesenchyme in $Msx1^{-/-}$ embryos (Jia et al. 2016). To investigate whether activin signaling also affects expression of these Wnt antagonists in

the developing tooth mesenchyme, we isolated E13.5 maxillary and mandibular molar mesenchyme, respectively, from $Inhba^{-/-}$ and control (wild-type and heterozygous) littermates using LCM and carried out quantitative real-time reverse transcription (RT)–PCR analyses. We confirmed that expression of *Dkk2* mRNAs in the mandibular molar tooth bud mesenchyme is more than 2-fold of that in the maxillary tooth mesenchyme in control embryos (Fig. 4A). Moreover, expression of *Dkk2* mRNAs is significantly increased in both maxillary and mandibular tooth bud mesenchyme in $Inhba^{-/-}$ embryos (Fig. 4A). Expression of *Sfrp1* and *Sfrp2* mRNAs, respectively, is not significantly different in the maxillary and mandibular molar mesenchyme in control embryos, with expression of *Sfrp2* mRNAs increased about 2-fold in the mandibular molar mesenchyme in $Inhba^{-/-}$ embryos compared to control littermates (Fig. 4A). In situ hybridization analysis showed that *Dkk2* mRNAs were preferentially expressed in the oral mesenchyme lingual to developing tooth buds and excluded in the condensing tooth mesenchyme surrounding the distal tooth bud epithelium in control embryos (Fig. 4B). Expression of *Dkk2* mRNAs expanded into the distal tooth mesenchyme in both maxillary and mandibular tooth germs in $Inhba^{-/-}$ embryos (Fig. 4C).

Figure 3. Genetic inactivation of *Osr2* rescued mandibular molar morphogenesis in *Inhba*^{-/-} embryos. (A–H) Hematoxylin and eosin–stained frontal sections through the maxillary and mandibular first molar tooth germs at E16.5 and E18.5, respectively. At E16.5, wild-type embryos show both maxillary and mandibular first molar tooth germs at early bell stage (A). *Inhba*^{-/-} embryos show bud-stage developmental arrest of the mandibular molar ($n = 6/6$) (B). In *Inhba*^{-/-}*Osr2*^{+/-} ($n = 6/6$) and *Inhba*^{-/-}*Osr2*^{-/-} ($n = 4/4$) embryos, the mandibular first molar tooth germs progressed to late cap and early bell stages, respectively (C, D). At E18.5, wild-type embryos show both maxillary and mandibular first molar at bell stage (E). *Inhba*^{-/-} embryos show bud-stage developmental arrest of the mandibular first molar tooth germs ($n = 8$) (F). Both *Inhba*^{-/-}*Osr2*^{+/-} ($n = 10/10$) and *Inhba*^{-/-}*Osr2*^{-/-} ($n = 6/6$) embryos show mandibular first molar tooth germs at bell stage (G, H). Arrowheads point to the mandibular first molar tooth germs. A white line separates the pictures for maxillary and mandibular molar tooth germs in panels D–H because the 2 pictures are taken from distinct sections of a series from the same embryo. Scale bars, 200 μ m.

Pharmacological Activation of Canonical Wnt Signaling Pathway or Inhibition of DKKs Rescued Mandibular Molar Development in *Inhba*^{-/-} Mice

To investigate the possibility that mandibular molar developmental arrest in *Inhba*^{-/-} embryos is due to inhibition of Wnt signaling, we tested whether activation of canonical Wnt signaling could rescue mandibular molar tooth morphogenesis in *Inhba*^{-/-} mice. We treated pregnant mice bearing *Inhba*^{-/-} embryos with intraperitoneal injection of LiCl, a simple salt that inhibits the kinase activity of Gsk3 β and thus increases intracellular β -catenin (Klein and Melton 1996; Song et al. 2009; Zeilbeck et al. 2014), from gestational days 11 to 14. As control, NaCl treatment did not significantly affect development of the embryonic tooth germs in control and *Inhba*^{-/-} embryos (Appendix Fig. 5A, B). Whereas LiCl treatment did not significantly affect tooth morphogenesis in control embryos (Appendix Fig. 5C), both maxillary and mandibular first molar tooth germs progressed to bell stage by E18.5 in 7 of 15

LiCl-treated *Inhba*^{-/-} embryos ($n = 14/30$ mandibular first molars) (Appendix Fig. 5D).

We then investigated whether inhibiting DKK activity directly could rescue mandibular molar morphogenesis in *Inhba*^{-/-} embryos by treating pregnant *Inhba*^{+/-} mice with intraperitoneal injection of IIC3a from gestational days 11 to 14. Remarkably, mandibular molar tooth germs progressed to bell stage by E18.5 in 4 of 8 IIC3a-treated *Inhba*^{-/-} embryos (Fig. 5C, D), with restored expression of Wnt target genes *Lef1* and *Axin2* in the mandibular molar mesenchyme at E13.5 (Fig. 5E–L). These data indicate that an important role for activin signaling in early tooth development is to regulate expression of Wnt antagonists, particularly *Dkk2*, in the developing tooth mesenchyme.

Discussion

Previous studies of *Inhba*^{-/-} mice led to the hypothesis that development of maxillary and mandibular molar teeth involves

Figure 4. Comparison of *Dkk2*, *Sfrp1*, and *Sfrp2* messenger RNA (mRNA) expression in the developing tooth mesenchyme in E13.5 *Inhba*^{-/-} mutants and their control littermates. **(A)** Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) analysis of *Dkk2*, *Sfrp1*, and *Sfrp2* mRNAs in the maxillary (Max) and mandibular (Man) molar mesenchyme. Error bars represent standard error of the mean. *******P* < 0.05. **(B, C)** Frontal sections showing expression patterns of *Dkk2* mRNAs in and around the maxillary (upper) and mandibular (lower) molar tooth mesenchyme in the control (B) and *Inhba*^{-/-} (C) embryos (*n* = 6). Red dashed lines mark the boundary between the tooth bud epithelium and mesenchyme; lingual side is to the left; arrowheads point to the first molar tooth germs in panels B and C.

Figure 5. IIC3a treatment in utero rescued mandibular molar tooth morphogenesis and Wnt signaling activity in *Inhba*^{-/-} embryos. **(A–L)** Frontal sections through the maxillary and mandibular first molar tooth germs in *Inhba*^{+/+} control (A, C, E, G, I, K) and *Inhba*^{-/-} (B, D, F, H, J, L) embryos treated with DMSO (A, B, E, F, I, J) or IIC3a (C, D, G, H, K, L). **(A–D)** Hematoxylin and eosin (H-E) staining at E18.5. *n* = 6 for *Inhba*^{+/+} embryos treated with DMSO. Rescue rate is 7/16 with IIC3a treatment. A white line separates the pictures for maxillary and mandibular molar tooth germs in panel A because the 2 pictures are taken from distinct sections of a series from the same embryo. **(E–L)** Messenger RNA (mRNA) expression of *Lef1* (E–H) and *Axin2* (I–L) (*n* = 2 embryos for each genotype for each probe). Red dashed lines mark the boundary between the tooth bud epithelium and mesenchyme. Arrowheads point to the mandibular first molar tooth germ in *Inhba*^{-/-} mutants; lingual side is to the left; scale bars, 200 μ m.

fundamentally distinct molecular mechanisms (Ferguson et al. 1998; 2001). *Inhba* mRNAs are expressed strongly in the early developing tooth mesenchyme of all tooth germs, and expression of several downstream target genes, including *Fst* and *Irx1*, was dramatically reduced in both maxillary and mandibular molar tooth germs in *Inhba*^{-/-} embryos (Ferguson et al. 1998, 2001). Further studies ruled out the possibility that continued maxillary molar morphogenesis in *Inhba*^{-/-} embryos was due to functional complementation by another activin-like ligand (Ferguson et al. 2001). However, comparative expression analysis showed normal expression patterns of a large number of important tooth developmental genes in *Inhba*^{-/-} embryos (Ferguson et al. 1998, 2001), and the molecular mechanism mediating the differential requirement for activin signaling in maxillary and mandibular molar morphogenesis has remained a mystery.

We recently reported that *Bmp4*^{*ncko/ncko*} mice exhibit bud-stage developmental arrest of mandibular molars while their maxillary molars continued to develop to mineralized teeth (Jia et al. 2013). Through RNA-seq analysis of LCM-isolated E13.5 tooth mesenchyme, we found that the mandibular molar mesenchyme expresses significantly higher levels of *Dkk2* mRNAs than the maxillary molar mesenchyme in wild-type embryos and that *Dkk2* expression is significantly increased in both maxillary and mandibular molar mesenchyme in *Bmp4*^{*ncko/ncko*} embryos (Jia et al. 2013). Subsequently, we demonstrated that pharmacological activation of canonical Wnt signaling by in utero treatment with LiCl or IIC3a partly rescued mandibular molar morphogenesis in *Bmp4*^{*ncko/ncko*} mice (Jia et al. 2016). These results indicate that higher levels of Wnt antagonists in the mandibular molar mesenchyme could account for the differential effects of loss of mesenchymal *Bmp4* on maxillary and mandibular molar organogenesis. In this study, we found that, although expression of *Bmp4* in the developing tooth mesenchyme is unaffected in *Inhba*^{-/-} embryos, *Dkk2* expression was significantly increased in both maxillary and mandibular molar mesenchyme in *Inhba*^{-/-} embryos. Moreover, we found that expression of *Inhba* in the tooth bud mesenchyme is unaffected in *Bmp4*^{*ncko/ncko*}

embryos. These results suggest that activin and Bmp4 signaling regulate *Dkk2* expression in the developing tooth mesenchyme independently. However, in contrast to *Inhba*^{-/-} and *Bmp4*^{ncko/ncko} mutants, which exhibit continued morphogenesis of maxillary molars, all *Bmp4*^{ncko/ncko} *Inhba*^{-/-} embryos exhibit bud-stage arrest of maxillary molar tooth germs and even earlier developmental arrest of mandibular molar tooth germs. These results indicate that Bmp4 and activin signaling act synergistically and partly redundantly to regulate early morphogenesis of both maxillary and mandibular molar tooth germs.

Bmp4 is known to act in a positive feedback loop with *Msx1* (Chen et al. 1996; Bei et al. 2000). We recently showed that the *Msx1* and *Osr2* transcription factors interact and act antagonistically to regulate *Dkk2* and *Sfrp2* expression (Zhou et al. 2011; Jia et al. 2016). In this study, we found that genetic inactivation of 1 *Osr2* allele is sufficient to rescue mandibular molar morphogenesis through the bud-to-cap transition in *Inhba*^{-/-} embryos, and mandibular molar morphogenesis progressed to bell stage by E18.5 in *Inhba*^{-/-} *Osr2*^{-/-} compound mutants. Since *Osr2*^{-/-} embryos exhibit increased expression of *Bmp4* in the developing tooth mesenchyme (Zhang et al. 2009), one possibility is that the increase in *Bmp4* expression is responsible for driving mandibular molar morphogenesis through the bud-to-cap transition in *Inhba*^{-/-} *Osr2*^{+/-} and *Inhba*^{-/-} *Osr2*^{-/-} embryos. Alternatively, *Osr2* might directly interact with components of the activin signaling pathway to regulate *Dkk2* expression. Further studies are necessary to elucidate the molecular mechanisms acting downstream of activin signaling to regulate *Dkk2* expression during tooth development.

Our findings that in utero treatment with the DKK inhibitor IIC3a rescued mandibular molar morphogenesis in both *Inhba*^{-/-} and *Bmp4*^{ncko/ncko} mice (Jia et al. 2016 and this study), indicate that the differences in maxillary and mandibular molar tooth phenotypes in these mutant mice, respectively, are due, at least in part, to intrinsic differences in the levels of Wnt antagonists, particularly *Dkk2*, expressed in the developing tooth mesenchyme. Whereas the molecular mechanism underlying the differential levels of *Dkk2* expression in the maxillary and mandibular molar mesenchyme remains to be resolved, our results do not support the hypothesis that development of maxillary and mandibular molar teeth involves fundamentally distinct genetic pathways. Rather, the odontogenic activity of either activin or Bmp4 is sufficient to overcome the inhibitory effects of relatively low levels of Wnt antagonists in the maxillary molar mesenchyme, but both pathways are required to overcome high levels of Wnt inhibition in the mandibular molar tooth germs to drive their morphogenesis through bud-to-cap transition. Although we did not directly measure Wnt signaling activity in the tooth germs in *Bmp4*^{ncko/ncko} *Inhba*^{-/-} embryos, it is most likely that combined loss of both activin and Bmp4 signaling causes more dramatic inhibition of Wnt signaling in all tooth germs than that in the single mutants. Indeed, the early tooth developmental arrest phenotype of the *Bmp4*^{ncko/ncko} *Inhba*^{-/-} embryos is remarkably similar to those of mouse embryos with either overexpression of *Dkk1* or tissue-specific inactivation of β -catenin in the early oral epithelium (Andl et al. 2002; Liu et al. 2008). Together, these results

indicate that activin-A and Bmp4 act partly redundantly and converge on the regulation of expression of secreted Wnt antagonists during early tooth development. Furthermore, rescue of mandibular molar morphogenesis in both *Inhba*^{-/-} and *Bmp4*^{ncko/ncko} mice by IIC3a treatment suggests that small-molecule inhibitors of Wnt antagonists could be useful for treatment of congenital tooth agenesis in humans.

Author Contributions

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

Acknowledgments

This work was supported by National Institutes of Health (NIH) National Institute of Dental and Craniofacial Research (NIDCR) grant R01DE018401 to R.J. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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