Modulating Wnt Signaling Rescues Palate Morphogenesis in *Pax9* Mutant Mice

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Abstract

Cleft palate is a common birth defect caused by disruption of palatogenesis during embryonic development. Although mutations disrupting components of the Wnt signaling pathway have been associated with cleft lip and palate in humans and mice, the mechanisms involving canonical Wnt signaling and its regulation in secondary palate development are not well understood. Here, we report that canonical Wnt signaling plays an important role in *Pax9*-mediated regulation of secondary palate development. We found that cleft palate pathogenesis in *Pax9*-deficient embryos is accompanied by significantly reduced expression of *Axin2*, an endogenous target of canonical Wnt signaling, in the developing palatal mesenchyme, particularly in the posterior regions of the palatal shelves. We found that expression of *Dkk2*, encoding a secreted Wnt antagonist, is significantly increased whereas the levels of active β -catenin protein, the essential transcriptional coactivator of canonical Wnt signaling, is significantly decreased in the posterior regions of the palatal shelves in embryonic day 13.5 *Pax9*-deficient embryos in comparison with control littermates. We show that small molecule-mediated inhibition of Dickkopf (DKK) activity in utero during palatal shelf morphogenesis partly rescued secondary palate development in *Pax9*-deficient embryos. Moreover, we found that genetic inactivation of *Wise*, which is expressed in the developing palatal shelves and encodes another secreted antagonist of canonical Wnt signaling, also rescued palate morphogenesis in *Pax9*-deficient mice. Furthermore, whereas *Pax9^{del/del}* embryos exhibit defects in palatal shelf elevation/reorientation and significant reduction in accumulation of hyaluronic acid—a high molecular extracellular matrix glycosaminoglycan implicated in playing an important role in palatal shelf elevation—80% of *Pax9^{del/del};Wise^{-/-}* double-mutant mouse embryos exhibit rescued palatal shelf elevation/reorientation, accompanied by restored hyaluronic acid accumulation in the palatal mesenchyme. Together, these data identify a crucial role for canonical Wnt signaling in acting downstream of *Pax9* to regulate palate morphogenesis.

Keywords: cell signaling, cleft palate, craniofacial biology, transcription factors, *Sostdc1*, Wnt antagonist

Introduction

The mammalian secondary palate arises from the oral side of embryonic maxillary prominences and initially grows vertically to form a pair of palatal shelves flanking the developing tongue. As development proceeds, the palatal shelves undergo remodeling and reorient to the horizontal position above the tongue, subsequently fusing to form the roof of oral cavity (Lan et al. 2015; Li et al. 2017). These developmental processes, including palatal shelf growth, elevation/reorientation, and fusion, are often disrupted by genetic or environmental perturbations, resulting in a high frequency of cleft palate defects in humans (Dixon et al. 2011; Bush and Jiang 2012). Extensive genetic and developmental studies with multiple animal model systems have identified crucial roles of multiple signaling pathways and transcription factors in palate development (Bush and Jiang 2012; Lan et al. 2015). However, critical gaps remain in current knowledge regarding the molecular networks integrating various signaling pathways and transcription factors in regulation of palate development, and no effective strategy has been developed for prevention of cleft palate.

Several studies have demonstrated that *Shh*, *Bmp4*, and *Fgf10* signaling pathways act in a molecular network containing the *Msx1*, *Osr2*, and *Pax9* transcription factors in regulating palatal shelf growth (reviewed by Bush and Jiang 2012; Lan et al. 2015). *Shh* and *Fgf10* are expressed in palatal shelf

epithelium and mesenchyme, respectively, and function in a positive feedback loop during palatal shelf outgrowth (Rice et al. 2004; Lan and Jiang 2009). *Msx1* regulates anterior palatal mesenchyme proliferation through activation of *Bmp4* expression in the palatal mesenchyme (Zhang et al. 2002). *Osr2* and *Pax9* are 2 key regulators of palatogenesis expressed throughout the developing palatal mesenchyme (Peters et al. 1998; Lan et al. 2004; Zhou et al. 2013). Mouse embryos lacking *Pax9* function exhibit significantly reduced expression of *Bmp4*, *Fgf10*, *Msx1*, and *Osr2* messenger RNAs (mRNAs) in the palatal mesenchyme as well as significantly reduced expression of *Shh* mRNAs in the palatal epithelium. Remarkably, restoration of

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Osr2 expression in the palatal mesenchyme was able to partly rescue palatal morphogenesis in the absence of Pax9 function (Zhou et al. 2013), suggesting that modulation of signaling pathways downstream of Pax9 and *Osr2* could prevent cleft palate.

In addition to roles in palatogenesis, *Msx1*, *Osr2*, and Pax9 are important regulators of tooth development (reviewed by Lan et al. 2014). Mice lacking either *Msx1* or Pax9 exhibit bud-stage developmental arrest of all tooth germs (Satokata and Maas 1994; Peters et al. 1998; Zhou et al. 2011). Mice lacking *Osr2* exhibit *Msx1*-dependent induction of supernumerary tooth formation (Zhang et al. 2009). *Msx1* and *Osr2* were recently shown to act antagonistically to regulate expression of several secreted Wnt antagonists, including *Dkk2* and *Sfrp2*, in developing tooth mesenchyme (Jia et al. 2016). Remarkably, genetic deletion of *Sfrp2/Sfrp3* with inhibition of Dickkopf (DKK) function partly rescued molar tooth morphogenesis in *Msx1*^{-/-} mice (Jia et al. 2016). Although many components of the Wnt signaling pathway are required for palatogenesis in humans and mice (reviewed by He et al. 2008; Chiquet et al. 2008; Menezes et al. 2010; Liu et al. 2015), the mechanisms involving Wnt signaling and the relationship between the *Msx1*, *Osr2*, Pax9 transcription factors and Wnt signaling in palate development are not well understood.

Here we report that Pax9-deficient mouse embryos exhibit decreased canonical Wnt signaling in the developing palatal shelves and that pharmacologic inhibition of DKK function or genetic inactivation of *Wise* (also known as *ectodin* or *Sostdc1*), which encodes a distinct Wnt antagonist (Itasaki et al. 2003; Laurikkala et al. 2003; Lintern et al. 2009; reviewed by Cruciat and Niehrs 2013), partly rescued secondary palate development in Pax9-deficient mice. Our data identify a novel role for Pax9 in integrating the Wnt signaling pathway in the regulation of palate development.

Materials and Methods

Mouse Strains

Pax9^{del/+} and *Wise*^{+/-} mice have been described (Ahn et al. 2010; Zhou et al. 2011). The *Pax9* and *Wise* genes are both located on mouse chromosome 12, separated by about 20 Mb. We crossed *Pax9*^{del/+} mice to *Wise*^{-/-} mice to generate *Pax9*^{del/+}; *Wise*^{+/-} double-heterozygous mice. We crossed *Pax9*^{del/+}; *Wise*^{+/-} mice to C57BL/6J inbred mice to select for *Pax9*^{del/+}; *Wise*^{+/-} progeny in which the 2 mutations are in cis on the same chromosome, resulting from meiotic recombination between the 2 loci. *Pax9*^{del/+} and *Pax9*^{del/+}; *Wise*^{+/-} mice were subsequently maintained by backcrossing to C57BL/6J mice and intercrossed to generate homozygous mutants for analyses. For timed pregnancies, embryonic day (E) 0.5 was designated as noon of the day when a vaginal plug was identified. This study was performed in strict accordance with the recommendations in the “Guide for the Care and Use of Laboratory Animals” by the National Institutes of Health. The animal use protocol was approved by the Institutional Animal Care and Use Committee of Cincinnati Children’s Hospital Medical Center. This study conformed with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines for preclinical animal studies.

Histology and In Situ Hybridization

Embryos were collected at desired developmental stages and processed for histology or in situ hybridization as described previously (Lan et al. 2001; Baek et al. 2011). For in situ hybridization, serial sections from the anterior, middle, and posterior regions of the secondary palate were processed as described previously with digoxigenin-labeled cRNA probes (Zhang et al. 1999).

Immunofluorescence Detection

For detection of phospho-Smad1/5/9, tissue sections were stained with the rabbit monoclonal antibody D5B10 (1:100; catalog 13820, Cell Signaling Technology) following standard protocols (Xu et al. 2014). For detection of hyaluronic acid (HA), tissue sections were incubated with biotin-labeled HABP (1:200; catalog 385911-50UG, Millipore) and detected with Texas red-conjugated streptavidin (1:200; SA-5006, Vector Labs). Images in red fluorescent protein (RFP) single channel were used to analyze fluorescence intensity over the palatal shelf area with ImageJ. Data were analyzed with Student’s *t* test, and *P* < 0.05 was considered statistically significant.

Quantitative RT-PCR Analysis

Quantitative RT-PCR (reverse transcription quantitative real-time polymerase chain reaction, abbreviated as RT-qPCR) was performed as previously described (Zhou et al. 2013). The sequences of gene-specific primers are provided in the Appendix Table. The relative levels of mRNAs in each sample were normalized to that of *Hprt* mRNAs. Student’s *t* test was used to analyze differential expression data.

Western Blot Assays

E13.5 palate shelves were dissected and lysed in radioimmunoprecipitation assay (RIPA) buffer (150mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH 8.0, and Roche Protease Inhibitor Cocktail) and sonicated 6 times (pulses: 1 s on, 1 s off). The mouse monoclonal antibodies to active β -catenin (1:1,000; clone 8E7, EMD Millipore) and β -actin (1:2,000; clone 8H10D10, Cell Signal Technologies) were used to detect the specific proteins. The intensity of detected bands was quantified with Photoshop Histogram Analysis from 3 independent experiments and the data statistically analyzed with Student’s *t* test.

Results

Pax9 Mutant Embryos Exhibit Decreased Canonical Wnt Signaling Activity in the Developing Palatal Shelves

Since *Pax9*^{del/del} embryos exhibit significantly reduced expression of *Bmp4* mRNAs in the developing palatal shelves (Zhou et al. 2013) and since BMP signaling induces *Wise* mRNA expression in other developmental contexts (Itasaki et al. 2003;

Laurikkala et al. 2003; Lintern et al. 2009), we compared *Wise* mRNA expression in developing palatal shelves in *Pax9^{del/del}* embryos and their control littermates. By in situ hybridization analysis, we found that *Pax9^{del/del}* embryos had an obvious decrease in *Wise* mRNA expression in the anterior and posterior regions of the palatal shelves by E13.5 (Fig. 1A–H). RT-qPCR analysis of manually microdissected E13.5 palatal shelves confirmed that expression of *Wise* mRNAs was significantly reduced in *Pax9^{del/del}* embryos in comparison with *Pax9^{del/+}* littermates (Fig. 1I).

Since *Wise* antagonizes Wnt signaling in vivo (Ahn et al. 2010), we investigated whether Wnt signaling activity is altered in the *Pax9^{del/del}* palatal shelves. Surprisingly, we found that expression of *Axin2*, a direct target of canonical Wnt signaling (Jho et al. 2002), was reduced in the palatal mesenchyme, particularly in the posterior regions, in E13.5 *Pax9^{del/del}* embryos compared with control littermates (Fig. 1J–M). RT-qPCR analysis confirmed that *Axin2* mRNA expression was significantly reduced in posterior palatal tissues, whereas the levels of *Axin2* mRNAs in the anterior half of the palatal shelves were modestly reduced, though the change was not statistically significant, in the E13.5 *Pax9^{del/del}* embryos as compared with *Pax9^{del/+}* littermates (Fig. 1N). To further validate that canonical Wnt signaling activity in the palatal shelves is affected in *Pax9^{del/del}* embryos, we took advantage of an antibody that specifically recognizes the Wnt-activated signaling form of β -catenin (van Noort et al. 2002). Western blot analysis showed that the posterior regions of palatal shelves contain significantly more accumulation of active β -catenin than anterior palatal tissues in E13.5 wild-type embryos (Appendix Fig. 1). Remarkably, the levels of active β -catenin were significantly reduced in the posterior palatal tissues in E13.5 *Pax9^{del/del}* embryos as compared with *Pax9^{del/+}* littermates (Fig. 1O, P).

To understand why *Pax9^{del/del}* embryos exhibit decreased canonical Wnt signaling activity in the palatal shelves despite the decrease in *Wise* expression, we investigated whether expression of *Dkk1* and *Dkk2*, which encode another class of secreted antagonists of canonical Wnt signaling (reviewed by Cruciat and Niehrs 2013), was altered during palate development in *Pax9^{del/del}* embryos. By in situ hybridization and RT-qPCR analyses, we found that expression of *Dkk2* mRNAs was significantly increased in posterior regions of palatal mesenchyme in E13.5 *Pax9^{del/del}* embryos in comparison with control littermates (Fig. 1Q–W). Thus, the decrease in canonical Wnt signaling activity in *Pax9^{del/del}* palatal shelves correlated with the increase in *Dkk2* expression.

Inhibition of DKK Activity In Utero Partly Rescued Palate Morphogenesis in *Pax9^{del/del}* Mice

Recent studies have shown that treatment of pregnant mice with IIC3a, a small molecule inhibitor that competitively blocks DKK binding to the Wnt coreceptors Lrp5/6 (Li et al. 2012), could effectively inhibit *Dkk2* function in embryonic tooth development (Jia et al. 2016; Kwon et al. 2017). We injected pregnant *Pax9^{del/+}* female mice intraperitoneally with IIC3a

(catalog 317701, EMD Millipore; 10 mg/kg of body weight, stock solution is 20 mg/mL in dimethyl sulfoxide [DMSO]) once a day at gestational days 12.5, 13.5, and 14.5, to test whether IIC3a-mediated inhibition of DKK activity could rescue palate morphogenesis in *Pax9^{del/del}* embryos. Whereas neither DMSO nor IIC3a treatment had any detectable effect on palate development in the wild-type and *Pax9^{del/+}* embryos and although all *Pax9^{del/del}* pups treated with DMSO still exhibited cleft palate (Fig. 2A–H), 7 of 11 (64%) IIC3a-treated *Pax9^{del/del}* pups exhibited secondary palate that was fused in the middle and posterior regions, with only a partial cleft between the anterior secondary palate and the primary palate (Fig. 2I–L). In contrast to the partial rescue of palate morphogenesis, all IIC3a-treated *Pax9^{del/del}* pups still had bud-stage tooth developmental arrest similar as untreated or DMSO-treated *Pax9^{del/del}* pups (Fig. 2C, G, K).

Genetic Inactivation of *Wise* Restored Wnt Signaling Activity in the Developing Palatal Shelves and Rescued Palate Development in *Pax9^{del/del}* Embryos

Since *Wise* mutant mice exhibit increased canonical Wnt signaling activity in several developing tissues (Ahn et al. 2010; Ahn et al. 2013; Ellies et al. 2014), we investigated whether genetic inactivation of *Wise* could restore Wnt signaling activity in the developing palatal shelves in *Pax9^{del/del}* embryos. In contrast to the complete penetrance of cleft palate in *Pax9^{del/del}* embryos (Fig. 3E–H), 70% of *Pax9^{del/del};Wise^{-/-}* embryos (14 of 20), examined after E16.5, exhibited fused secondary palate (Fig. 3I–L). All *Pax9^{del/del};Wise^{-/-}* embryos had irregular palatal rugae in comparison with the wild-type embryos (Fig. 3A, I), which is likely due to a requirement for *Wise* function in ruga patterning as previously reported (Welsh and O'Brien 2009). In addition, all *Pax9^{del/del};Wise^{-/-}* embryos exhibited bud-stage tooth developmental arrest similar to *Pax9^{del/del}* embryos (Fig. 3C, G, K; Appendix Fig. 2).

We next compared expression of *Axin2* mRNAs in the developing palatal shelves in *Pax9^{del/del};Wise^{-/-}* mutant and *Pax9^{del/+};Wise^{+/-}* control littermates. By in situ hybridization, we found that expression of *Axin2* mRNAs was consistently increased in the anterior and posterior regions of the palatal mesenchyme in E13.5 *Pax9^{del/del};Wise^{-/-}* embryos as compared with their *Pax9^{del/+};Wise^{+/-}* littermates (Fig. 4A–D). RT-qPCR analysis of manually microdissected palatal shelves also showed a modest, albeit statistically not significant, increase in *Axin2* mRNA levels in E13.5 *Pax9^{del/del};Wise^{-/-}* embryos (Fig. 4E). Furthermore, in contrast to the significantly reduced levels of active β -catenin protein in E13.5 *Pax9^{del/del}* palatal shelves versus *Pax9^{del/+}* littermates (Fig. 1O, P), we found that the palatal shelves of E13.5 *Pax9^{del/del};Wise^{-/-}* embryos and their *Pax9^{del/+};Wise^{+/-}* littermates had similar amounts of active β -catenin protein (Fig. 4F, G). Together, these results indicate that complete loss of *Wise* function restored canonical Wnt signaling in *Pax9^{del/del}* palatal mesenchyme to the level comparable to that in *Pax9^{del/+};Wise^{+/-}* control samples.

Figure 1. Changes in expression of Wnt signaling components in the developing palatal shelves of *Pax9^{del/del}* mutant embryos. **(A–D)** Whole mount view of patterns of *Wise* mRNA expression in the palatal shelves of *Pax9^{del/+}* control and *Pax9^{del/del}* mutant embryos at E12.5 and E13.5. Arrows point to the medial edge of the palatal shelves. **(E–H)** Frontal sections showing *Wise* mRNA expression patterns in the anterior and posterior regions of palate shelves in E13.5 *Pax9^{del/+}* control and *Pax9^{del/del}* mutant embryos. **(I)** RT-qPCR analysis of the levels of expression of *Wise* mRNAs in E13.5 palatal shelves in *Pax9^{del/+}* control and *Pax9^{del/del}* mutant embryos ($n = 5$). **(J–M)** Frontal sections showing *Axin2* mRNA expression patterns in the anterior and posterior regions of palate shelves in E13.5 *Pax9^{del/+}* control and *Pax9^{del/del}* mutant embryos. Green arrows point to the domains of *Axin2* mRNA expression in the palatal mesenchyme. Black arrowheads point to *Axin2* mRNA expression in the tooth germs. **(N)** RT-qPCR analysis of the levels of expression of *Axin2* mRNAs in the anterior and posterior regions, respectively, of the palatal shelves in E13.5 *Pax9^{del/+}* control and *Pax9^{del/del}* mutant embryos ($n = 3$). **(O)** Western blot analysis of the levels of active β -catenin proteins in the posterior halves of palatal shelves from E13.5 *Pax9^{del/+}* control and *Pax9^{del/del}* mutant embryos. The levels of β -actin in each sample were detected as an internal loading control. **(P)** Relative active β -catenin band intensity on Western blots normalized against β -actin ($n = 3$ samples each for *Pax9^{del/+}* and *Pax9^{del/del}* embryos). **(Q, T)** Whole mount view of patterns of *Dkk2* mRNA expression in the palate of E12.5 *Pax9^{del/+}* control and *Pax9^{del/del}* mutant embryos. **(R, S, U, V)** Frontal sections showing *Dkk2* mRNA expression patterns in the anterior and posterior regions of palate shelves of E13.5 *Pax9^{del/+}* control and *Pax9^{del/del}* mutant embryos. Black arrowheads point to *Dkk2* mRNA expression in the tooth germs. **(W)** RT-qPCR analysis of the levels of *Dkk2* mRNAs in the anterior and posterior regions, respectively, of the palatal shelves from E13.5 *Pax9^{del/+}* control and *Pax9^{del/del}* mutant embryos ($n = 3$). E, embryonic day; ns, not significant; p, palatal shelf; t, tongue. Error bars represent SD. * $P < 0.05$. ** $P < 0.01$.

Figure 2. Inhibition of DKK activity in utero partly rescued palate development in *Pax9*^{del/del} mutant embryos. (A, E, I) Whole mount view of the palate in WT, DMSO-treated *Pax9*^{del/del}, and IIIC3a-treated *Pax9*^{del/del} pups at P0. Arrowhead in panel I points to cleft between the primary and anterior secondary palates. (B–D, F–H, J–L) Representative frontal sections from the anterior, middle, and posterior regions of the secondary palate in WT, DMSO-treated *Pax9*^{del/del}, and IIIC3a-treated *Pax9*^{del/del} embryos at E16.5. Of 11 IIIC3a-treated *Pax9*^{del/del} pups, 7 exhibited secondary palate that was fused in the middle and posterior regions, whereas the other 4 still had cleft palate. Asterisk indicates cleft palate in panels E–H. Arrows point to molar tooth germs. E, embryonic day; P0, postnatal day; p, palatal shelf; t, tongue; WT, wild type.

Since Wise can antagonize Bmp and Wnt signaling (Itasaki et al. 2003; Laurikkala et al. 2003; Lintern et al. 2009), we analyzed whether loss of *Wise* function affected BMP signaling in *Pax9*^{del/del} palatal shelves. By in situ hybridization and RT-qPCR analyses, we found that *Bmp4* mRNA expression was significantly reduced in *Pax9*^{del/del}; *Wise*^{-/-} palatal shelves versus *Pax9*^{del/+}; *Wise*^{+/-} littermates, similar to the reduction in *Bmp4* expression in *Pax9*^{del/del} palatal shelves (Fig. 4H–N). Moreover, we found that the E13.5 *Pax9*^{del/del} and *Pax9*^{del/del}; *Wise*^{-/-} embryos exhibit similarly reduced levels of pSmad1/5/9 in the palatal mesenchyme (Fig. 4O–T). Together, these results suggest that *Wise* does not have a detectable effect on canonical BMP signaling in the developing palatal mesenchyme.

Differences in Palatal Shelf Morphogenesis in *Pax9*^{del/del} and *Pax9*^{del/del}; *Wise*^{-/-} Embryos

Previous studies have shown that mouse embryos homozygous for each of 2 distinct *Pax9*-null alleles exhibit delay in palatal shelf elevation/reorientation to the horizontal position above the tongue (Peters et al. 1998; Zhou et al. 2013). By E14.5, the

palatal shelves had reoriented to the horizontal position and often initiated fusion at the midline in wild-type and *Pax9*^{del/+} embryos (Fig. 5A–C), whereas the palatal shelves in *Pax9*^{del/del} littermates were still oriented vertically (Fig. 5D–F). Remarkably, both palatal shelves had reoriented to the horizontal position in 4 of 5 E14.5 *Pax9*^{del/del}; *Wise*^{-/-} embryos (Fig. 5G–I), suggesting that reduction in Wnt signaling is at least part of the mechanism underlying the impairment of palatal shelf morphogenesis in *Pax9*^{del/del} embryos.

It has been hypothesized that HA, an extracellular glycosaminoglycan that is capable of binding a large amount of water, accumulates at higher levels in specific regions of the palatal mesenchyme and generates osmotic pressure to drive palatal shelf reorientation (Brinkley and Morris-Wiman 1987; reviewed by Ferguson 1988; Li et al. 2017). We found that *Pax9*^{del/del} embryos had significantly reduced HA accumulation in the middle and posterior regions of palatal mesenchyme at E13.5 when compared with control littermates (Fig. 5J, K, M). Remarkably, the palatal mesenchyme in E13.5 *Pax9*^{del/del}; *Wise*^{-/-} embryos had HA accumulation restored to similar levels as in the *Pax9*^{del/+}; *Wise*^{+/-} control embryos (Fig. 5L, M),

Figure 3. Genetic inactivation of *Wise* rescued palate morphogenesis in *Pax9^{del/del}* mice. (A, E, I) Whole mount view of the palate in WT, *Pax9^{del/del}*, and *Pax9^{del/del}; Wise^{-/-}* pups at P0. (B–D, F–H, J–L) Representative frontal sections from anterior, middle, and posterior regions of the secondary palate in E16.5 WT, *Pax9^{del/del}*, and *Pax9^{del/del}; Wise^{-/-}* embryos. Of 20 *Pax9^{del/del}; Wise^{-/-}* pups examined after E16.5, 14 exhibited fused palate, whereas the other 6 *Pax9^{del/del}; Wise^{-/-}* pups had cleft palate. Asterisk indicates the cleft palate in panels E–H. Arrows point to the molar tooth germs. E, embryonic day; P0, postnatal day; p, palatal shelf; t, tongue; WT, wild type.

although they still showed a palatal shelf shape defect as in *Pax9^{del/del}* embryos—that is, the lack of the lateral indentation between the palatal shelf and maxillary molar tooth germ (Fig. 5J–L). These results indicate that *Pax9* plays a crucial role in palatal shelf elevation/reorientation through regulation of Wnt signaling activity in the palatal mesenchyme.

Discussion

Mice lacking *Pax9* function exhibit multiple defects in palate development, including aberrant shape of palatal shelves, reduced palatal mesenchyme proliferation, and defect in palatal shelf elevation/reorientation (Peters et al. 1998; Zhou et al. 2013), which provide an excellent model for studying molecular and cellular mechanisms of palatogenesis. In this study, we found that *Pax9^{del/del}* mouse embryos exhibit decreased canonical Wnt signaling activity in developing palatal shelves and that modulating Wnt signaling through pharmacologic inhibition of DKK activity or genetic inactivation of *Wise* partly rescued palate morphogenesis in *Pax9^{del/del}* mice. Our data significantly improve understanding of the roles of canonical

Wnt signaling in palatogenesis and identify a previously unknown link between *Pax9* and Wnt signaling in palate development.

Previous studies have established that palate development involves differential molecular patterning and regionalized control of growth along the anterior-posterior axis (recently reviewed by Lan et al. 2015). It has been shown that expression of the *BATGAL* transgenic reporter of canonical Wnt signaling activity is restricted to the anterior palatal mesenchyme and absent from the posterior palatal mesenchyme (He et al. 2011; Liu et al. 2015). We found that *Axin2*, a known endogenous target of canonical Wnt signaling (Jho et al. 2002), is expressed in both anterior and posterior palatal mesenchyme in wild-type mouse embryos and its expression is significantly reduced in posterior regions of the palatal mesenchyme in *Pax9^{del/del}* embryos (Fig. 1J–N). Moreover, we found that the signaling form of β -catenin protein was accumulated at much higher levels in the posterior than anterior regions of the palatal shelves in E13.5 wild-type embryos (Appendix Fig. 1) and was significantly reduced in the posterior palatal tissues in E13.5 *Pax9^{del/del}* embryos (Fig. 1O, P). The decrease in expression of *Axin2*

Figure 4. Genetic inactivation of *Wise* restored canonical Wnt signaling activity in the developing palatal shelves in *Pax9^{del/del}* embryos. **(A–D)** Frontal sections of E13.5 palatal shelves showing *Axin2* mRNA expression pattern in the anterior and posterior regions of palate shelves in *Pax9^{del/+};Wise^{+/-}* control and *Pax9^{del/del};Wise^{-/-}* double-mutant embryos. Green arrows point to the domains of *Axin2* mRNA expression in the anterior palatal mesenchyme. Black arrowheads point to *Axin2* mRNA expression in the tooth germs. **(E)** RT-qPCR analysis of the levels of expression of *Axin2* mRNAs in the anterior and posterior regions of the palatal shelves in E13.5 *Pax9^{del/+};Wise^{+/-}* control and *Pax9^{del/del};Wise^{-/-}* double-mutant embryos ($n = 3$). **(F)** Western blot detection of active β -catenin in the posterior palatal shelves in E13.5 *Pax9^{del/+};Wise^{+/-}* control and *Pax9^{del/del};Wise^{-/-}* mutant embryos. The levels of β -actin were detected as an internal loading control. **(G)** Relative band intensity of active β -catenin protein on Western blots was analyzed by ImageJ ($n = 3$) and normalized to that of β -actin. **(H–M)** Frontal sections showing *Bmp4* mRNA expression patterns in the anterior and posterior regions of palatal shelves in E13.5 WT, *Pax9^{del/del}*, and *Pax9^{del/del};Wise^{-/-}* embryos. **(N)** RT-qPCR analysis of the levels of expression of *Bmp4* mRNAs in E13.5 palatal shelves in *Pax9^{del/+}* control and *Pax9^{del/del}* mutant embryos, as well as *Pax9^{del/+};Wise^{+/-}* control and *Pax9^{del/del};Wise^{-/-}* mutant embryos ($n = 5$ for each genotype). **(O–T)** Immunofluorescence detection of pSmad1/5/9 proteins in the posterior and anterior regions of the palatal shelves from E13.5 WT, *Pax9^{del/del}*, and *Pax9^{del/del};Wise^{-/-}* embryos. Yellow arrows point to comparable regions of the posterior palatal mesenchyme, whereas white arrows point to comparable regions of anterior palatal mesenchyme. Error bars represent SD. E, embryonic day; ns, not significant; p, palatal shelf; t, tongue; WT, wild type. *** $p < 0.001$.

mRNAs and active β -catenin protein in E13.5 *Pax9^{del/del}* palatal shelves correlated with a significant increase in expression of *Dkk2* mRNAs in the posterior palatal mesenchyme (Fig. 1Q–W).

Remarkably, IIC3a treatment in utero rescued development and fusion of the palatal shelves in the middle and posterior regions in *Pax9^{del/del}* embryos (Fig. 2). Together, these results

Figure 5. Inactivation of *Wise* rescued palatal shelf elevation/reorientation in *Pax9^{del/del}* embryos. (A–I) Representative frontal sections from anterior, middle, and posterior regions of the secondary palate in E14.5 WT, *Pax9^{del/del}*, and *Pax9^{del/del};Wise^{-/-}* embryos. Arrows point to the molar tooth germs. (J–L) Representative frontal sections from the posterior region of the palatal shelves of E13.5 WT, *Pax9^{del/del}*, and *Pax9^{del/del};Wise^{-/-}* embryos showing fluorescent staining of hyaluronic acid (red color). White dashed line marks the proximal boundary of the palatal shelf area used for quantification of fluorescence intensity. (M) Quantification of mean fluorescence intensity of hyaluronic acid staining in the WT, *Pax9^{del/del}*, and *Pax9^{del/del};Wise^{-/-}* samples ($n = 4$ for each genotype). Fluorescence intensity of hyaluronic acid staining was normalized against the area of palatal shelves. Error bars represent SD. p, palatal shelf; t, tongue; WT, wild type. * $P < 0.05$. ** $P < 0.01$.

indicate that canonical Wnt signaling is active in the anterior and posterior regions of the developing palatal shelves and that Pax9-regulated canonical Wnt signaling is crucial for palate morphogenesis, particularly in the middle and posterior regions.

During preparation of this manuscript, another laboratory independently found that the expression levels of several Wnt antagonists were greatly increased in the *Pax9* mutant embryos. These authors were able to rescue embryonic palatogenesis in vivo using 2 DKK antagonists (R. D'Souza, personal communication). Results from both our studies are hence encouraging and suggest that the WNT pathway is a good target for future therapeutic interventions.

Uniquely in this study, we also used a genetic approach to delete another WNT antagonist, *Wise* (Ahn et al. 2010). *Wise* mRNAs were downregulated by 26% in the developing palatal tissues in *Pax9^{del/del}* embryos (Fig. 1I). However, this decrease in expression was not enough to prevent clefting. As we found, the full deletion of *Wise* was necessary to offset the inhibition of canonical Wnt signaling in *Pax9^{del/del}* palatal mesenchyme, likely caused by the increase in *Dkk2* expression. The inactivation of both *Wise* alleles restored canonical Wnt signaling in the palatal tissues and rescued palate morphogenesis in *Pax9^{del/del}* embryos.

The increased *Dkk2* expression in the palatal mesenchyme caused significant inhibition of canonical Wnt signaling in *Pax9^{del/del}* embryos despite the decrease in *Wise* expression. These results suggest that *Dkk2* is a more potent inhibitor of canonical Wnt signaling than *Wise*. Alternatively, but not exclusively, the increase in *Dkk2* expression and decrease in canonical Wnt signaling might have occurred earlier in palate development in *Pax9^{del/del}* embryos whereas the downregulation of *Wise* occurred later due to changes in expression of other genes, such as *Bmp4*. Indeed, our data show that *Wise* mRNAs were still robustly expressed in the *Pax9^{del/del}* palatal shelves at E12.5 (Fig. 1B) but were significantly downregulated in the *Pax9^{del/del}* palatal shelves by E13.5 (Fig. 1C–I). Further studies are needed to elucidate the detailed molecular mechanisms involving Pax9-mediated regulation of canonical Wnt signaling during palate development.

Author Contributions

C. Li contributed to design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; Y. Lan

contributed to conception, design, data analysis, and interpretation, critically revised the manuscript; R. Krumlauf contributed to design and critically revised the manuscript; R. Jiang contributed to conception, design, data analysis and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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