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Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth

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Summary

The mammalian secondary palate arises by outgrowth from the oral side of the paired maxillary processes flanking the primitive oral cavity. Palatal growth depends on reciprocal interactions between the oral ectoderm and the underlying neural crest derived mesenchyme. Previous studies have implicated Sonic hedgehog (Shh) as an important epithelial signal for regulating palatal growth. However, the cellular and molecular mechanisms through which Shh regulates palatal development in vivo have not been directly analyzed, due in part to early embryonic lethality of mice lacking Shh or other essential components of the Shh signaling pathway. Using Cre/*loxP*-mediated tissue-specific inactivation of the *Smoothened* (*Smo*) gene in the developing palatal mesenchyme, we show that the epithelially expressed Shh signals directly to the palatal mesenchyme to regulate palatal mesenchyme cell proliferation through maintenance of *CyclinD1* and *CyclinD2* expression. Moreover, we show that Shh-Smo signaling specifically regulates the expression of the transcription factors Foxf1, Foxf2, and Osr2 in the developing palatal mesenchyme. Furthermore, we show that Shh signaling regulates *Bmp2*, *Bmp4*, and *Fgf10* expression in the developing palatal mesenchyme and that specific inactivation of *Smo* in the palatal mesenchyme indirectly affects palatal epithelial cell proliferation. Together with previous reports that the mesenchymally expressed Fgf10 signals to the palatal epithelium to regulate *Shh* mRNA expression and cell proliferation, these data demonstrate that Shh signaling plays a central role in coordinating the reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth.

Keywords

cleft palate; Fgf10; Osr2; Osr2-IresCre; palate development; Shh; Smo; tissue-specific gene inactivation

Introduction

The secondary palate arises from the medial side of the maxillary processes flanking the embryonic oral cavity. In mammals, the palatal processes initially grow vertically down the sides of the developing tongue. At a precise developmental stage the bilateral palatal shelves elevate to a horizontal position above the dorsum of the tongue and fuse with each other at the midline to form the intact secondary palate that separates the nasal cavity from the oral cavity (Ferguson, 1988). Disturbances of the growth, elevation or fusion of the palatal shelves result in cleft palate, one of the most common birth defects in humans.

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Classic organ culture assays and recent palatal explant studies with exogenous recombinant signaling molecules have demonstrated that growth and patterning of the developing palatal shelves depend on epithelial-mesenchymal interactions (Tyler and Koch, 1977; Ferguson and Honig, 1984; Zhang et al., 2002; Rice et al., 2004; Yu et al., 2005). During early palate development, expression of several signaling molecules and transcription factors, including Bmp4, Fgf10, Msx1, and Shox2, was found specifically restricted in the anterior palate (Zhang et al., 2002; Alappat et al., 2005; Yu et al., 2005; reviewed by Hilliard et al., 2005). Bmp4 and Msx1 appeared to function in a positive feedback loop to regulate mesenchyme proliferation in the anterior palate (Zhang et al., 2002). In addition, mesenchymal Bmp4 was required to maintain Shh expression in the anterior palatal epithelium (Zhang et al., 2002) and exogenous Shh protein was capable of stimulating palatal mesenchyme proliferation in palate explant cultures (Zhang et al., 2002; Rice et al., 2004). The mesenchymally expressed Fgf10 regulates palatal epithelial cell survival and proliferation (Rice et al., 2004; Alappat et al., 2005). Both Fgf10 and its epithelial receptor Fgfr2b are also required for maintenance of Shh expression in the palatal epithelium (Rice et al., 2004). These data suggest that Shh plays important roles in palatal growth and patterning.

Shh is a member of the Hedgehog family of secreted proteins and plays critical roles in diverse developmental processes, including left-right axis establishment, dorsal-ventral patterning of the neural tube, endoderm development, anterior-posterior patterning of the developing limb, brain development and patterning (reviewed by Ingham and McMahon, 2001; McMahon et al., 2003; Roessler and Muenke, 2003). Shh signals to recipient cells through binding to the Patched family of receptors, Ptch1 or Ptch2. In the absence of ligands, the Patched receptors inhibit the function of another transmembrane protein Smoothened (Smo), an obligatory component of the Hedgehog signaling pathway. Activation of Smo upon Shh signaling leads to activation of downstream genes by the Gli family transcription factors. Interestingly, *Ptch1* and *Gli1* are also among the downstream target genes of the Shh pathway, thus establishing a feedback regulatory loop (reviewed by Ingham and McMahon, 2001; McMahon et al., 2003).

Shh signaling plays essential roles in craniofacial development. Mutations in *SHH* in humans cause holoprosencephaly (reviewed by Cohen, 2004), whereas a targeted null mutation in *Shh* in mice resulted in severe cranial deficiencies (Chiang et al., 1996). Inhibition of Shh signaling in the chick facial primordia with a function-blocking antibody inhibited facial outgrowth, whereas ectopic application of active Shh protein caused facial overgrowth (Hu and Helms, 1999). Ahlgren and Bronner-Fraser (1999) showed that inhibition of Shh in the cranial mesenchyme caused extensive cell death. Thus, Shh signaling appears to regulate both proliferation and survival of the neural crest-derived cranial mesenchyme. However, the severe cranial deficiency in *Shh* null mutants and early embryonic lethality of mutant mouse embryos lacking either *Ptch1* or *Smo* prevented a direct genetic analysis of the molecular mechanisms involving Shh signaling in palate development (Chiang et al., 1996; Goodrich et al., 1997; Zhang et al., 2001).

The adaptation of the Cre/*loxP* system for temporally- and/or spatially-controlled gene inactivation in mice made it possible to systematically dissect the genetic pathways in specific developmental processes (Jiang and Gridley, 1997; Sauer, 1998). Whereas mice homozygous for null mutations in *Shh* failed to develop most facial structures (Chiang et al., 1996), the *K14-Cre;Shh*$^{c/n}$ mice, in which *Shh* was inactivated specifically in epithelial cells after E11.5, developed most facial structures but exhibited tooth developmental arrest (Dassule et al., 2000). The *K14-Cre* transgenic mice also expressed Cre in the oral epithelium, including in the palatal epithelium, during palatal outgrowth (Dassule et al., 2000; Vaziri Sani et al., 2005). About 85% of the *K14-Cre;Shh*$^{c/n}$ mice had cleft palate (Rice et al., 2004; Gritli-Linde, 2007). Unfortunately, there has been no report regarding

palate development in the *K14-Cre;Shh*$^{c/n}$ mutant mice other than the brief descriptions of the late-term cleft palate phenotype. Similarly, while mice lacking *Gli2* and mice with neural-crest specific inactivation of *Smo* have been reported to have palatal defects (Mo et al., 1997; Jeong et al., 2004), how the Shh signaling pathway regulates palate development remains to be elucidated. Here we show, through analysis of mice with specific inactivation of *Smo* in the palatal mesenchyme, that Shh signaling regulates expression of a number of signaling molecules and transcription factors in the palatal mesenchyme to coordinate the epithelial-mesenchymal interactions that control palatal outgrowth.

Materials and methods

Mouse strains

The *Osr2-IresCre* mice have been recently described (Lan et al., 2007). *Shh*$^{+/n}$ (*Shh*^{tm1Amc}/*J*), *Shh*$^{c/c}$ (*Shh*^{tm2Amc}/*J*) and *Smo*$^{c/c}$ (*Smo*^{tm2Amc}/*J*) mice have been described previously (St-Jacques et al., 1998; Dassule et al., 2000; Lewis et al., 2001; Long et al., 2001) and were purchased from the Jackson Laboratory (Bar Harbor, ME). The *R26R* reporter mice (Soriano, 1999) were also purchased from the Jackson Laboratory. *K14-Cre* transgenic mice (line 43, Andl et al., 2004) were acquired through a Material Transfer Agreement with Dr. Sarah Millar at the University of Pennsylvania and provided by Dr. Yang Chai (University of Southern California). *K14-Cre* transgenic mice were maintained by crossing to C57BL/6J mice. *Shh*$^{+/n}$ mice were also maintained by crossing to the C57BL/6J mice, whereas *Shh*$^{c/c}$, *Smo*$^{c/c}$, and *Osr2-IresCre* mice were maintained as homozygotes in their own strain background. Male *K14-Cre* transgenic mice were crossed to *Shh*$^{+/n}$ female mice to generate *K14-Cre;Shh*$^{+/n}$ males, which were subsequently crossed to *Shh*$^{c/c}$ mice to generate *K14-Cre;Shh*$^{c/n}$ embryos for analysis. Independently, *Osr2*$^{IresCre/IresCre}$ homozygous male mice were crossed to *Smo*$^{c/c}$ female mice to generate *Osr2-IresCre;Smo*$^{+/c}$ male mice, which were subsequently crossed to *Smo*$^{c/c}$ female mice to generate *Osr2-IresCre;Smo*$^{c/c}$ embryos for analysis. Although the *Ires-Cre* insertion in the *Osr2*^{IresCre} allele did not disrupt *Osr2* gene function (Lan et al., 2007), all experimental analysis of *Osr2-IresCre;Smo*$^{c/c}$ embryos used *Osr2-IresCre;Smo*$^{+/c}$ littermates as controls. Genotyping of mice and embryos were carried out by allele-specific PCR as previously described (Dassule et al., 2000; Long et al., 2001; Lan et al., 2007).

Histology and in situ hybridization analyses

For histology, embryos were fixed in Bouin's fixative, dehydrated through graded alcohols, embedded in paraffin, sectioned at 7 mm thickness, and stained with hematoxylin and eosin. For in situ hybridization, embryos were fixed overnight at 4°C in 4% paraformaldehyde in PBS, dehydrated through graded alcohols, embedded in paraffin, sectioned at 5 mm thickness. In situ hybridization of tissue sections were performed as described previously (Zhang et al., 1999). At least three pairs of control and mutant embryos were analyzed for each developmental stage.

X-gal staining and skeletal analysis

Embryos were dissected in ice-cold PBS and stained with X-gal for β-galactosidase detection as described previously (Hogan et al., 1994). Cryosections were counterstained with eosin following X-gal staining. Skeletal preparations were made from newborn mice as described previously (Martin et al., 1995).

Detection of cell proliferation and immunohistochemical staining

For detection of cell proliferation in the palatal shelves, timed pregnant female mice were injected intraperitoneally on gestational day 12.5 or 13.5 with BrdU (Roche) labeling

reagent (45 μg/g body weight). One hour after injection, embryos were dissected, fixed, embedded in paraffin, and sectioned in the coronal plane for immunodetection of BrdU using the BrdU labeling and detection kit (Roche) as described previously (Lan et al., 2004). Following BrdU immunostaining, the embryonic sections were counterstained with nuclear fast red to visualize all cellular nuclei. Sections were selected from the middle of the anterior halves (corresponding to the anterior margin of the maxillary first molar tooth buds) and posterior regions (posterior to the maxillary first molar tooth buds) of the palatal shelves in comparable positions in the control and mutant embryos. Cell counts were recorded separately for the palatal epithelium and mesenchyme in each of the bilateral palatal shelves from five continuous sections of each region of the palate. The cell proliferation index was calculated as percentage of the cell nuclei with BrdU labeling. Data were collected from at least three pairs of mutant and control littermates. Students' *t*–test was used to analyze the significance of difference and a *P* value less than 0.05 was considered statistically significant.

Immunohistochemical detection of CyclinD1 was carried out with paraffin sections of paraformaldehyde fixed embryos using a rabbit polyclonal antibody against CyclinD1 (Thermo Scientific, Fremont, CA) at 1:200 dilution and detected using the Zymed Histostain Plus Kit (Zymed Laboratories Inc.) as previously described (Casey et al., 2006).

Results

K14-Cre mediated inactivation of *Shh* in the palatal epithelium does not completely abrogate Shh signaling during palatal outgrowth

It has been reported that *K14-Cre;Shh$^{c/n}$* mutant mice exhibited incomplete penetrance of cleft palate in mice (Rice et al., 2004; Gritli-Linde, 2007). To investigate the roles of Shh signaling in palate development, we generated *K14-Cre;Shh$^{c/n}$* mutant mice using a different *K14-Cre* transgenic mouse line (line 43, Andl et al., 2004) from that used in the previous studies, hoping to achieve higher penetrance of the cleft palate phenotype. However, we observed that about 70% (12 of 17) of our *K14-Cre;Shh$^{c/n}$* mutant mice exhibited cleft palate (Fig. 1B and data not shown). To examine whether Shh signaling plays a critical role in palatal outgrowth, we first compared cell proliferation during early palate development in four pairs of *K14-Cre;Shh$^{c/n}$* mutant and control littermates by examining BrdU incorporation at E13.5. Of the four *K14-Cre;Shh$^{c/n}$* mutant embryos, one displayed significant reduction in the numbers of BrdU labeled cells in both the palatal epithelium and mesenchyme (Fig. 1C, D), one displayed significant reduction in BrdU-labeled cells in the epithelium only, one displayed reduction in BrdU-labeled cells in the palatal mesenchyme only, and one had similar cell proliferation in both the palatal epithelium and mesenchyme with that in the control embryos (Supplementary Table 1). These data suggest that the inactivation of *Shh* in the palatal epithelium may be variable in the different *K14-Cre;Shh$^{c/n}$* mutant mice.

To examine the efficiency of *Shh* inactivation in the palatal epithelium in *K14-Cre;Shh$^{c/n}$* mutant mice, we compared expression of *Ptch1* and *Gli1*, two known target genes of Shh signaling, in the developing palatal shelves in the mutant and control littermates. At E13.5, both *Ptch1* and *Gli1* are expressed in a lateral-medial gradient in the mesenchyme of the developing palatal shelves in the control embryos (Fig. 1E, G). In the E13.5 *K14-Cre;Shh$^{c/n}$* mutant embryos, expression of both *Ptch1* and *Gli1* in the developing palatal shelves is downregulated but substantial amounts of *Ptch1* and *Gli1* mRNAs remained in the palatal mesenchyme in all three mutant embryos examined (Fig. 1F, H). In contrast, expression of both mRNAs is nearly completely downregulated in the developing tooth epithelium in the *K14-Cre;Shh$^{c/n}$* mutant embryos in comparison with control littermates (Fig. 1, E-H). Both *Ptch1* and *Gli1* are also highly expressed in the mesenchymal cells in the mandibular

ossification areas, which are regulated by Ihh instead of Shh and are not altered in the *K14-Cre;Shh$^{c/n}$* mutant embryos (Fig. 1, E-H). Since Shh expression has only been detected in the palatal epithelium but not in the palatal mesenchyme (Zhang et al, 2002; Rice et al., 2006), and since *Ihh* expression is restricted to the areas undergoing ossification whereas *Dhh* is not expressed in the developing palate (Rice et al., 2006), the sustained expression of *Ptch1* and *Gli1* in the palatal mesenchyme indicates that the inactivation of *Shh* in the palatal epithelium in the *K14-Cre;Shh$^{c/n}$* mutant embryos was incomplete, which is the most likely reason for the great variability in palatal mesenchyme proliferation at E13.5 and the incomplete penetrance of the cleft palate phenotype at birth.

Shh signals directly to the mesenchyme to regulate palatal outgrowth

To further investigate the molecular mechanisms involving Shh-Smo signaling in the epithelial-mesenchymal interactions regulating palate development, we decided to analyze mice with inactivation of *Smo* in the palatal mesenchyme using the recently generated *Osr2-IresCre* knockin mice (Lan et al., 2007). The *Osr2-IresCre* mice express Cre recombinase in the palatal mesenchyme from the onset of palatal outgrowth at E11.5 (Lan et al., 2007; Fig. 2A). Importantly, although *Osr2-IresCre* mice express Cre in several other tissues in the developing craniofacial complex (Lan et al., 2007; Supplementary Figure 1), Cre activity was restricted in the mesenchyme and was not detected in the epithelium of the developing secondary palate (Fig. 2B). In the *Osr2-IresCre;Smo$^{c/c}$* embryos, whereas *Ptch1* and *Gli1* were expressed in the palatal epithelial cells and several other craniofacial tissues at similar levels to that in control littermates (Supplementary Figure 2, A-D), expression of both genes was dramatically downregulated in the palatal mesenchyme by E13 (Fig. 2C-F). These data demonstrate that signaling downstream of Shh remained intact in the palatal epithelium and was efficiently blocked in the palatal mesenchyme in *Osr2-IresCre;Smo$^{c/c}$* mutant embryos. Examination of *Osr2-IresCre;Smo$^{c/c}$* mutant pups at birth revealed that 100% (n=22) of them had cleft palate (Fig. 3), indicating that Shh signaling in the palatal mesenchyme is required for palate development. In addition to the cleft palate defect, the *Osr2-IresCre;Smo$^{c/c}$* mutant mice exhibited open eyelids and smaller but thickened tympanic rings (Fig. 3B, F). Since neither the *Osr2-IresCre* heterozygous mice nor the *Osr2-IresCre;Smo$^{+/c}$* mice had these defects (Fig. 3A, C, E, and data not shown), they likely resulted from loss of Smo-mediated hedgehog signaling in the developing eyelids and other craniofacial tissues where Cre was also expressed (Supplementary Figure 1). Consistent with this hypothesis, expression of both *Ptch1* and *Gli1* was also downregulated in the developing eyelid tissues in the *Osr2-IresCre;Smo$^{c/c}$* mutant mice, in comparison with the control littermates (Supplementary Figure 2, A-D).

Cell proliferation is reduced in both the mesenchyme and epithelium in the developing palatal shelves of *Osr2-IresCre;Smo$^{c/c}$* mutants

To clarify the role of Shh-Smo signaling in the palatal mesenchyme, we carried out histological analyses of embryos throughout palate development from E12 to E16. As shown in Fig. 4, initial palatal growth was normal in the *Osr2-IresCre;Smo$^{c/c}$* mutants by E12.5 (Fig. 4A, B). When examined at E13.5, the palatal shelves of the *Osr2-IresCre;Smo$^{c/c}$* mutant embryos appeared slightly retarded (Fig. 4D) compared with the control littermates (Fig. 4C). At E14.5, the palatal shelves had elevated and initiated fusion at the midline in the control embryos (Fig. 4E) whereas the palatal shelves in the *Osr2-IresCre;Smo$^{c/c}$* mutant littermates were significantly retarded and failed to make contact (Fig. 4F). In some *Osr2-IresCre;Smo$^{c/c}$* mutant embryos, in addition to palatal shelf retardation, there was aberrant tissue morphogenesis associated with the nasopharynx and the developing tongue (Fig. 4H).

To investigate whether palatal shelf retardation in the *Osr2-IresCre;Smo$^{c/c}$* mutant embryos was due to impaired cell proliferation, we analyzed BrdU incorporation in E13 embryos.

Indeed, the *Osr2-IresCre;Smo$^{c/c}$* mutants consistently exhibited significant reductions in cell proliferation throughout the anterior-posterior axis in the palatal mesenchyme (Fig. 5). Interestingly, the *Osr2-IresCre;Smo$^{c/c}$* mutant embryos also exhibited significant reduction in cell proliferation in the anterior, but not in the posterior, palatal epithelia (Fig. 5C, F). Since these embryos had *Smo* inactivation in the palatal mesenchyme but not in the palatal epithelium, the significant reduction in cell proliferation in the anterior palatal epithelium suggests that Shh-Smo signaling controls expression of mesenchymal factors involved in the regulation of palatal epithelial proliferation.

Expression of *CyclinD1* and *CyclinD2* are downregulated in the developing palatal mesenchyme in *Osr2-IresCre;Smo$^{c/c}$* mutants

Shh signaling has been shown to regulate cell proliferation during many developmental processes through modulation of *CyclinD1* or *CyclinD2* expression (Kenney and Rowitch, 2000; Ishibashi and McMahon, 2002; Lobjois et al., 2004; Mill et al., 2005; Hu et al., 2006). Immunohistochemical staining using a CyclinD1 specific antibody showed that it is strongly expressed in both the palatal epithelium and mesenchyme at E13.5 in control embryos (Fig. 6A). Expression of CyclinD1 was substantially diminished in the palatal mesenchyme in *Osr2-IresCre;Smo$^{c/c}$* mutant littermates (Fig. 6B). *CyclinD2* is expressed strongly in the palatal epithelium and relatively weakly throughout the palatal mesenchyme in control embryos at E13.5 (Fig. 6C). In the *Osr2-IresCre;Smo$^{c/c}$* mutant embryos, whereas *CyclinD2* mRNA expression in the palatal epithelium was similar to that in the control embryos, its expression in the palatal mesenchyme was clearly reduced (Fig. 6D). Thus, Shh signaling regulates palatal mesenchyme cell proliferation at least in part through the maintenance of *CyclinD1* and *CyclinD2* expression.

Alterations in *Bmp2* and *Bmp4* expression in the developing palatal mesenchyme of *Osr2-IresCre;Smo$^{c/c}$* mutant embryos

In palatal explant culture assays, exogenous Shh protein induced *Bmp2* but not *Bmp4* mRNA expression in the anterior palate (Zhang et al., 2002). In E13.5 wild-type embryos, both *Bmp2* and *Bmp4* mRNAs are expressed in the distal mesenchyme underlying the medial edge epithelium in the anterior palate and neither is expressed in the posterior palatal mesenchyme (Zhang et al., 2002; Hilliard et al., 2005). *Bmp2* mRNA expression in the palatal mesenchyme was downregulated in *Osr2-IresCre;Smo$^{c/c}$* mutant embryos in comparison with that in control littermates (Fig. 7A, B), confirming the hypothesis that Shh-Smo signaling is required for maintenance of *Bmp2* mRNA expression in the anterior palatal mesenchyme in vivo. On the other hand, *Bmp4* expression in the anterior palatal mesenchyme was up-regulated, particularly in the lateral region of the anterior palatal mesenchyme, in *Osr2-IresCre;Smo$^{c/c}$* mutant embryos compared with control littermates (Fig. 7C, D). Expression of *Msx1*, a known down-stream target of Bmp4 signaling, was also up-regulated in the anterior palatal mesenchyme in the *Osr2-IresCre;Smo$^{c/c}$* mutant embryos (Fig. 7E, F). In contrast, expression of *Shox2*, encoding a transcription factor specifically expressed in and required for development of the anterior palate (Yu et al., 2005), was not significantly altered in the *Osr2-IresCre;Smo$^{c/c}$* mutant embryos compared with control littermates (Fig. 7G, H), indicating that the effects of *Smo* inactivation on the expression of *Bmp2*, *Bmp4*, and *Msx1* in the palatal mesenchyme are specific and not due to tissue retardation.

Downregualtion of expression of *Foxf1*, *Foxf2* and *Osr2* in the palatal mesenchyme of *Osr2-IresCre;Smo$^{c/c}$* mutant embryos

Shh-Smo signaling has been shown to directly regulate the expression of several members of the Forkhead Box (Fox) family of transcription factors, including Foxc2, Foxd1, Foxd2, Foxf1, and Foxf2, during early craniofacial development (Jeong et al., 2004). To better

understand the Shh signaling pathway in palate development, we examined expression of these *Fox* genes in the developing palatal shelves in the *Osr2-IresCre;Smo$^{c/c}$* mutant embryos and control littermates. Expression of *Foxc1*, *Foxc2*, and *Foxd2* was absent or very weak in the control palatal mesenchyme at E13.5 and was not altered in the *Osr2-IresCre;Smo$^{c/c}$* mutant littermates (data not shown). *Foxf1* mRNA was highly specifically expressed in the lateral palatal mesenchyme as well as in the lingual side of the molar tooth mesenchyme in the E13.5 control embryos (Fig. 8A, C). In the *Osr2-IresCre;Smo$^{c/c}$* littermates, *Foxf1* mRNA expression was specifically downregulated in the palatal mesenchyme in both the anterior and posterior palate whereas its expression in the tooth mesenchyme persisted (Fig. 8B, D). *Foxf2* mRNA was highly expressed in the palatal mesenchyme and in the developing tongue musculature at E13.5 in control embryos (Fig. 8E, G). Expression of *Foxf2* mRNA was specifically downregulated in the palatal mesenchyme, while strong expression persisted in the developing tongue, in the *Osr2-IresCre;Smo$^{c/c}$* littermates (Fig. 8F, H). Thus, similar to that in the early developing facial primordia, expression of *Foxf1* and *Foxf2* in the developing palatal mesenchyme are also positively regulated by Shh-Smo signaling.

To further investigate the molecular mechanisms of Shh-Smo signaling in palate development, we compared expression of several other mesenchymal transcription factors known to regulate palate development. *Osr2* encodes a zinc finger protein specifically required for palatal mesenchyme cell proliferation (Lan et al., 2001; Lan et al., 2004). Whereas *Osr2* mRNA expression was similar in the *Osr2-IresCre;Smo$^{c/c}$* mutant and control embryos at E12.5 (data not shown), there was a significant reduction in *Osr2* mRNA expression in the palatal mesenchyme in *Osr2-IresCre;Smo$^{c/c}$* mutant embryos by E13.5 (Fig. 9A, B). In contrast, *Osr2* mRNA expression in the mesenchyme lingual to the first molar tooth buds remained at similar levels in the control and mutant littermates (Fig. 9A, B). Moreover, expression of three other transcription factor genes, *Osr1*, *Pax9*, and *Tbx22*, were not significantly altered in the *Osr2-IresCre;Smo$^{c/c}$* mutant palate from that in the control littermates (Fig. 9C, D, and data not shown). Thus, Smo activity appears to be specifically required for the maintenance of *Osr2* mRNA expression in the palatal mesenchyme.

Shh-Smo signaling is required for maintenance of *Fgf10* expression in the palatal mesenchyme

Fgf10 is expressed in the palatal mesenchyme and regulates palatal epithelial cell proliferation (Rice et al., 2004). Interestingly, *Fgf10* mRNA is strongly expressed in the anterior half, with little expression in the posterior half, of the developing palatal shelves (Alappat et al., 2005; Welsh et al., 2007). We found that *Fgf10* mRNA expression in the developing anterior palatal mesenchyme was dramatically reduced in *Osr2-IresCre;Smo$^{c/c}$* mutant embryos by E13.5, in comparison with control littermates (Fig. 9E, F). Interestingly, *Fgf10* expression was also downregulated in the developing eyelid mesenchyme in *Osr2-IresCre;Smo$^{c/c}$* mutant embryos, in comparison with the control littermates (Supplementary Figure 2E, F). Expression of *Fgf10* in both the developing palate and eyelid overlapped with that of *Ptch1*, and the down-regulation of *Fgf10* expression also correlated with down-regulation of *Ptch1* in these tissues, in the *Osr2-IresCre;Smo$^{c/c}$* mutant embryos (Supplementary Figure 2A, B, E, F). Fgf10 also plays a critical role in regulating cell proliferation during eyelid development (Tao et al., 2005). Similar to *Osr2-IresCre;Smo$^{c/c}$* mutant mice, *K14-Cre;Shh$^{c/n}$* mutant mice exhibited open eyelids at birth (Dassule et al., 2000). Taken together, these data suggest that Fgf10 acts downstream of Shh signaling to regulate development of the eyelid and secondary palate.

Discussion

Despite significant advances in understanding of the molecular basis of palate development in recent years (reviewed by Gritli-Linde, 2007), it is not known what signals induce initial palatal outgrowth and how various molecular pathways are integrated to regulate the epithelial-mesenchymal interactions controlling palatal growth. *Shh* is expressed in the oral epithelium prior to palatal outgrowth and becomes restricted to the medial and lateral epithelium of the downward growing palatal shelves (Rice et al., 2006). In palatal explant culture assays, exogenous Shh protein increased palatal mesenchyme cell proliferation whereas a function-blocking Shh antibody inhibited palatal mesenchyme proliferation (Zhang et al., 2002). Whereas targeted null mutations in the *Shh* gene resulted in severe craniofacial defects in homozygous mutant mice, K14-Cre mediated inactivation of *Shh* in epithelial tissues, including oral ectoderm, caused incomplete penetrance of cleft palate (Rice et al., 2004; Gritli-Linde, 2007). We found that the incomplete penetrance of cleft palate in the *K14-Cre;Shh*$^{c/n}$ mutant mice was due to incomplete inactivation of Shh activity in the palatal epithelium. The different *K14-Cre* transgenic mouse strains resulted in different penetrance of the cleft palate phenotype in the *K14-Cre;Shh*$^{c/n}$ mutant mice, most likely due to transgene positional effects and different mouse strain background effects on *Cre* expression.

In this study, we used the recently generated *Osr2-IresCre* knockin mice to genetically dissect the molecular mechanism involving Shh signaling in palate development. Although the *Osr2-IresCre* mice also express Cre in several other craniofacial tissues (Supplementary Figure 1), they express Cre throughout the palatal mesenchyme but not in the palatal epithelium (Fig. 2B). Indeed, we found that the *Osr2-IresCre;Smo*$^{c/c}$ mutant embryos exhibited dramatic reduction in expression of *Ptch1* and *Gli1* mRNAs in the palatal mesenchyme by E13. Interestingly, although *Osr2-IresCre;Smo*$^{c/c}$ mutant mice exhibited complete penetrance of cleft palate, their palatal shelves appeared morphologically normal prior to E13 and were elevated by E14.5, in contrast to the previously described severely retarded palatal shelves that failed to elevate in the *K14-Cre;Shh*$^{c/n}$ mutant mice (Rice et al., 2004; Gritli-Linde, 2007). These differences in palatal phenotype may be due to loss of Shh signaling in both the epithelium and mesenchyme in the *K14-Cre;Shh*$^{c/n}$ mutant mice compared with loss of Shh signaling only in the palatal mesenchyme in the *Osr2-IresCre;Smo*$^{c/c}$ mutant mice. It is also possible that the high penetrance of cleft palate phenotype in the *Osr2-IresCre;Smo*$^{c/c}$ mutant mice resulted from a combination of palatal specific defects and secondary effects of loss of Smo in other Cre-expressing craniofacial tissues. Nevertheless, the *Osr2-IresCre;Smo*$^{c/c}$ mutant mice provided an opportunity to clarify the role of Shh signaling in the reciprocal epithelial-mesenchymal interactions during palate development.

The epithelially expressed Shh signals directly to the palatal mesenchyme to regulate palatal outgrowth

In palatal explant culture assays, Zhang et al. (2002) showed that exogenous Shh protein induced *Bmp2*, but not *Bmp4* mRNA expression in the anterior palatal mesenchyme. Zhang et al. (2002) further showed that both exogenous Shh and Bmp2 proteins induced palatal mesenchyme proliferation and that exogenous Noggin protein blocked the effect of Shh on palatal mesenchyme proliferation, which led to the hypothesis that Bmp2 mediated the mitogenic effect of Shh signaling on the palatal mesenchyme. Whereas our data confirm a role for Shh-Smo signaling in maintenance of *Bmp2* expression in the anterior palatal mesenchyme, it should be noted that *Bmp2* expression in the anterior palatal mesenchyme is highly restricted to just subjacent to the medial edge epithelium and at very low abundance compared to its abundant expression in many other regions of the developing craniofacial complex (Supplementary Figure 3). Moreover, whereas Shh was shown to induce *Bmp2*

expression in the anterior but not the posterior palatal explants (Zhang et al., 2002), we found that mesenchymal cell proliferation was significantly reduced in both anterior and posterior regions of the developing palate in the *Osr2-IresCre;Smo*$^{c/c}$ mutant embryos, indicating that other factors also mediate the mitogenic effects of Shh signaling in the palatal mesenchyme. Furthermore, we found that the expression levels of CyclinD1 protein and of *CyclinD2* mRNA were both reduced in the palatal mesenchyme of *Osr2-IresCre;Smo*$^{c/c}$ mutant embryos, compared with the control littermates. Shh signaling has been shown to regulate *CyclinD1* and/or *CyclinD2* expression in multiple tissues and Gli proteins have been shown to bind to specific sites in the *CyclinD1* and *CyclinD2* gene promoters (Kenney and Rowitch, 2000; Long et al., 2001; Ishibashi and McMahon, 2002; Yoon et al., 2002; Mill et al., 2005; Hu et al., 2006). *Gli1*, *Gli2*, and *Gli3* are all expressed in the palatal mesenchyme during palatal outgrowth (Rice et al., 2006). Thus, it is likely that Shh-Smo signaling directly activates Gli-mediated transcriptional activation of *CyclinD1* and *CyclinD2* in the palatal mesenchyme to promote palatal outgrowth.

Foxf1, Foxf2, and Osr2 may be downstream effectors of Shh signaling during palatal outgrowth

During early craniofacial development, expression of several Fox family genes, including *Foxf1* and *Foxf2*, depended on Shh-Smo signaling (Jeong et al., 2004). *Foxf1* expression in the developing lung mesenchyme, a non-neural crest derived tissue, was also positively regulated by Shh signaling (Mahlapuu et al., 2001a). We found that *Foxf1* and *Foxf2* were expressed in the developing palatal mesenchyme in the control embryos and both were downregulated in the palatal mesenchyme of the *Osr2-IresCre;Smo*$^{c/c}$ mutant embryos. Whereas *Foxf1*$^{-/-}$ null mouse embryos died prior to craniofacial morphogenesis, *Foxf2*$^{-/-}$ mutant mice exhibited complete cleft palate (Mahlapuu et al., 2001b; Wang et al., 2003). Jeong et al. (2004) proposed a Fox code for mediating the roles of Shh-Smo signaling during early facial development. It is likely that Foxf1 and Foxf2 also mediate some of the effects of Shh-Smo signaling during palate development.

We found that *Osr2* mRNA expression was downregulated in the palatal mesenchyme of *Osr2-IresCre;Smo*$^{c/c}$ mutant mouse embryos. Although *Osr2* was not expressed in many tissues that receive active Shh signaling, such as in the ventral neural tube, *Osr2* mRNA expression overlapped with *Ptch1* mRNA expression during palate development, with each exhibiting a lateral-to-medial gradient as the palatal shelves grew vertically from E12 to E13.5 (Lan et al., 2001; 2004; Rice et al., 2006). To confirm that Shh signaling plays a role in the maintenance of *Osr2* mRNA expression in the developing palatal mesenchyme, we examined *Osr2* mRNA expression in *K14-Cre;Shh*$^{c/n}$ mutant embryos and found that it was also downregulated in the palatal mesenchyme in those embryos by E13.5 (data not shown). Osr2 is one of a few transcription factors with a proven role in regulating palatal mesenchyme cell proliferation (Lan et al., 2004). It is possible that Shh regulates palatal mesenchyme cell proliferation in part through maintenance of *Osr2* expression.

Shh signaling coordinates the reciprocal epithelial-mesenchymal interactions during palatal outgrowth

We found that *Osr2-IresCre;Smo*$^{c/c}$ mutant mice exhibited significant reduction in cell proliferation in the anterior palatal epithelium. Since Shh-Smo signaling remained intact in the palatal epithelium in these mutants, we hypothesized that Shh signaling controls the expression of a mesenchymal factor required for palatal epithelial cell proliferation. Rice et al. (2004) showed that *Fgf10*, expressed in the developing palatal mesenchyme, was required for palatal epithelial cell proliferation. Interestingly, *Fgf10* is normally strongly expressed in the anterior half, with very little expression in the posterior half, of the developing palate (Alappat et al., 2005; Hilliard et al., 2005; Welsh et al., 2007). We found

that expression of *Fgf10* mRNA in the anterior palatal mesenchyme was dramatically down-regulated in *Osr2-IresCre;Smo*$^{c/c}$ mutant embryos, suggesting that Shh-Smo signaling in the palatal mesenchyme secondarily affected palatal epithelial cell proliferation through regulation of *Fgf10* expression.

Shh has been shown to regulate *Fgf10* expression during development of several other vertebrate organs. Shh induced *Fgf10* mRNA expression in the chick limb bud (Ohuchi et al., 1997). Exogenous Shh also induced, and a function blocking Shh antibody inhibited, *Fgf10* mRNA expression in the developing genital tubercle mesenchyme (Haraguchi et al., 2001). We found, in addition to down-regulation of *Fgf10* in the palatal mesenchyme, *Osr2-IresCre;Smo*$^{c/c}$ mutant mice exhibited down-regulation of *Fgf10* expression in the developing eyelid mesenchyme. In contrast, Shh inhibited *Fgf10* expression in the developing lung mesenchyme and *Shh*$^{-/-}$ mutant mice exhibited more widespread expression of *Fgf10* mRNA in the developing lung mesenchyme (Bellusci et al., 1997; Litingtung et al., 1998). Thus, *Fgf10* expression may be either positively or negatively regulated by Shh signaling depending on the specific developmental context. It is possible that similar effectors downstream of Shh-Smo signaling exist in the developing eyelid, palate, limb bud, and genital tubercle mesenchyme for the maintenance of *Fgf10* mRNA expression. Interestingly, Rice et al. (2004) showed that exogenous Fgf10 protein induced *Shh* mRNA expression in the palatal epithelium of wild-type embryos and that *Shh* mRNA expression was reduced in the palatal epithelium in *Fgf10*$^{-/-}$ and *Fgfr2b*$^{-/-}$ mutant embryos. Whereas our data indicate secondary effects of *Smo* inactivation in the palatal mesenchyme on palatal epithelial cell proliferation, Rice et al. (2004) showed that cell proliferation was significantly reduced not only in the palatal epithelium but also in the palatal mesenchyme in *Fgf10*$^{-/-}$ and *Fgfr2b*$^{-/-}$ mutant mice. Taken together, these data suggest that Shh and Fgf10 function in a positive feedback loop to regulate palatal outgrowth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Ahlgren SC, Bronner-Fraser M. Inhibition of sonic hedgehog signaling in vivo results in craniofacial neural crest cell death. Curr. Biol 1999;9:1304–1314. [PubMed: 10574760]

Alappat SR, Zhang Z, Suzuki K, Zhang X, Liu H, Jiang R, Yamada G, Chen YP. The cellular and molecular etiology of the cleft secondary palate in *Fgf10* mutant mice. Dev. Biol 2005;277:102–113. [PubMed: 15572143]

Andl T, Ahn K, Kairo A, Chu EY, Wine-Lee L, Reddy ST, Croft NJ, Cebra-Thomas JA, Metzger D, Chambon P, et al. Epithelial Bmpr1a regulates differentiation and proliferation in postnatal hair follicles and is essential for tooth development. Development 2004;131:2257–2268. [PubMed: 15102710]

Bellusci S, Grindley J, Emoto H, Itoh N, Hogan BL. Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. Development 1997;124:4867–4878. [PubMed: 9428423]

Casey LM, Lan Y, Cho E-S, Maltby KM, Gridley T, Jiang R. Jag2-Notch1 signaling regulates oral epithelial differentiation and palate development. Dev. Dyn 2006;235:1830–1844. [PubMed: 16607638]

Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Wesphal H, Beachy P. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature 1996;383:407–413. [PubMed: 8837770]

Cohen, MM. SHH and holoprosencephaly.. In: Epstein, CJ.; Erickson, RP.; Wynshaw-Boris, A., editors. Inborn Errors of Development: The Molecular Basis of Clinical Disorders of Morphogenesis. Oxford University Press; 2004. p. 240-248.

Dassule HR, Lewis P, Bei M, Maas R, McMahon A. Sonic hedgehog regulates growth and morphogenesis of the tooth. Development 2000;127:4775–4785. [PubMed: 11044393]

Ferguson MWJ. Palate Development. Development [suppl] 1988;103:41–60.

Ferguson MWJ, Honig LS. Epithelial-mesenchymal interactions during vertebrate palatogenesis. Curr. Top. Dev. Biol 1984;19:137–164. [PubMed: 6389026]

Goodrich LV, Milenkovic L, Higgins KM, Scott MP. Altered neural cell fates and medulloblastoma in mouse patched mutants. Science 1997;277:1109–1113. [PubMed: 9262482]

Gritli-Linde A. Molecular control of secondary palate development. Dev. Biol 2007;301:309–326. [PubMed: 16942766]

Gritli-Linde A, Bei M, Maas R, Zhang XM, Linde A, McMahon AP. Shh signaling within the dental epithelium is necessary for cell proliferation, growth and polarization. Development 2002;129:5323–5337. [PubMed: 12403705]

Haraguchi R, Mo R, Hui C, Motoyama J, Madino S, Shiroishi T, Gaffield W, Yamada G. Unique functions of sonic hedgehog signaling during external genitalia development. Development 2001;128:4241–4250. [PubMed: 11684660]

Hilliard SA, Yu L, Gu S, Zhang Z, Chen YP. Regional regulation of palatal growth and patterning along the anterior-posterior axis in mice. J. Anat 2005;207:655–667. [PubMed: 16313398]

Hogan, B.; Beddington, R.; Costantini, F.; Lacy, E. 2nd ed.. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, New York: 1994. Manipulating the Mouse Embryo: A Laboratory Manual..

Hu D, Helms JA. The role of sonic hedgehog in normal and abnormal craniofacial morphogenesis. Development 1999;126:4873–4884. [PubMed: 10518503]

Hu MC, Mo R, Bhella S, Wilson CW, Chuang PT, Hui CC, Rosenblum ND. GLI3-dependent transcriptional repression of Gli1, Gli2 and kidney patterning genes disrupts renal morphogenesis. Development 2006;133:569–578. [PubMed: 16396903]

Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. Genes Dev 2001;15:3059–3087. [PubMed: 11731473]

Ishibashi M, McMahon AP. A sonic hedgehog-dependent signaling relay regulates growth of diencephalic and mesencephalic primordial in the early mouse embryo. Development 2002;129:4807–4819. [PubMed: 12361972]

Jeong J, Mao J, Tenzen T, Kottmann AH, McMahon AP. Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia. Genes Dev 2004;18:937–951. [PubMed: 15107405]

Jiang R, Gridley T. Gene targeting: Things go better with Cre. Curr. Biol 1997;7:R321–323. [PubMed: 9115382]

Kenney AM, Rowitch DH. Sonic hedgehog promotes G(1) cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. Mol. Cell. Biol 2000;20:9055–9067. [PubMed: 11074003]

Lan Y, Kingsley PD, Cho E-S, Jiang R. *Osr2*, a new mouse gene related to *Drosophila odd-skipped*, exhibits dynamic expression patterns during craniofacial, limb, and kidney development. Mech. Dev 2001;107:175–179. [PubMed: 11520675]

Lan Y, Ovitt CE, Cho ES, Maltby KM, Wang Q, Jiang R. Odd-skipped related 2 (*Osr2*) encodes a key intrinsic regulator of secondary palate growth and morphogenesis. Development 2004;131:3207–3216. [PubMed: 15175245]

Lan Y, Wang Q, Ovitt CE, Jiang R. A unique mouse strain expressing Cre recombinase for tissue-specific analysis of gene function in palate and kidney development. Genesis 2007;45:618–624. [PubMed: 17941042]

Lewis PM, Dunn MP, McMahon JA, Logan M, Martin JF, St-Jacques B, McMahon AP. Cholesterol modification of sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1. Cell 2001;105:599–612. [PubMed: 11389830]

Litingtung Y, Lei I, Westphal H, Chiang C. Sonic hedgehog is essential to foregut development. Nat. Genet 1998;20:58–61. [PubMed: 9731532]

Lobjois V, Benazeraf B, Bertrand N, Medevielle F, Pituello F. Specific regulation of cyclins D1 and D2 by FGF and Shh signaling coordinates cell cycle progression, patterning, and differentiation during early steps of spinal cord development. Dev. Biol 2004;273:195–209. [PubMed: 15328007]

Long F, Zhang XM, Karp S, Yang Y, McMahon AP. Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. Development 2001;128:5099–5108. [PubMed: 11748145]

Mahlappu M, Enerback S, Carlsson P. Haploinsufficiency of the forkhead gene Foxf1, a target for sonic hedgehog signaling, causes lung and foregut malformations. Development 2001a;128:2397–2406.

Mahlappu M, Ormestad M, Enerback S, Carlsson P. The forkhead transcription factor Foxf1 is required for differentation of extraembryonic and lateral plate mesoderm. Development 2001b; 128:155–166.

Martin JF, Bradley A, Olson EN. The paired-like homeobox gene Mhox is required for early events of skeletogenesis in multiple lineages. Genes Dev 1995;9:1237–1249. [PubMed: 7758948]

McMahon AP, Ingham PW, Tabin CJ. Developmental roles and clinical significance of hedgehog signaling. Curr. Top. Dev. Biol 2003;53:1–114. [PubMed: 12509125]

Mill P, Mo R, Hu MC, Dagnino L, Rosenblum ND, Hui CC. Shh controls epithelial proliferation via independent pathways that converge on N-Myc. Dev. Cell 2005;9:293–303. [PubMed: 16054035]

Mo R, Freer AM, Zinyk DL, Crackower MA, Michaud J, Heng HH, Chik KW, Shi XM, Tsui LC, Chen SH, et al. Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. Development 1997;124:113–123. [PubMed: 9006072]

Ohuchi H, Nakagawa T, Yamamoto A, Araga A, Ohata T, Ishimaru Y, et al. The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. Development 1997;124:2235–2244. [PubMed: 9187149]

Ormestad M, Astorga J, Landgren H, Wang T, Johansson BR, Miura N, Carlsson P. *Foxf1* and *Foxf2* control murine gut development by limiting mesenchymal Wnt signaling and promoting extracellular matrix production. Development 2005;133:833–843. [PubMed: 16439479]

Rice R, Connor E, Rice DPC. Expression patterns of Hedgehog signaling pathway members during mouse palate development. Gene Expression Patterns 2006;6:206–212. [PubMed: 16168717]

Rice R, Spencer-Dene B, Connor EC, Gritli-Linde A, McMahon AP, Dickson C, Thesleff I, Rice DP. Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate. J. Clin. Invest 2004;113:1692–1700. [PubMed: 15199404]

Roessler E, Muenke M. How a Hedgehog might see holoprosencephaly. Hum. Mol. Genet 2003;12:R15–25. [PubMed: 12668593]

Sauer B. Inducible gene targeting in mice using the Cre/*lox* system. Methods 1998;14:381–392. [PubMed: 9608509]

Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat. Genet 1999;21:70–71. [PubMed: 9916792]

St-Jacques B, Dassule HR, Karavanova I, Botchkarev VA, Li J, Danielian PS, McMahon JA, Lewis PM, Paus R, McMahon AP. Sonic hedgehog signaling is essential for hair development. Curr. Biol 1998;8:1058–1068. [PubMed: 9768360]

Tao H, Shimizu M, Kusumoto R, Ono K, Noji S, Ohuchi H. A dual role of Fgf10 in proliferation and coordinated migration of epithelial leading edge cells during mouse eyelid development. Development 2005;132:3217–3230. [PubMed: 15958512]

Tyler MS, Koch WE. In vitro development of palatal tissues from embryonic mice: II. Tissue isolation and recombination studies. J. Embryol. Exp. Morphol 1977;38:37–48. [PubMed: 886249]

Vaziri Sani F, Hallberg K, Harfe BD, McMahon AP, Linde A, Gritli-Linde A. Fate-mapping of the epithelial seam during palatal fusion rules out epithelial-mesenchymal transformation. Dev. Biol 2005;285:490–495. [PubMed: 16109396]

Wang T, Tamakoshi T, Uezato T, Shu F, Kanzaki-Kato N, Fu Y, Koseki H, Yoshida N, Sugiyama T, Miura N. Forkhead transcription factor Foxf2 (LUN)-deficient mice exhibit abnormal development of secondary palate. Dev. Biol 2003;259:83–94. [PubMed: 12812790]

Welsh IC, Hagge-Greenberg A, O'Brien TP. A dosage-dependent role for Spry2 in growth and patterning during palate development. Mech. Dev 2007;124:746–761. [PubMed: 17693063]

Yoon JW, Kita Y, Frank DJ, Majewski RR, Konicek BA, Nobrega MA, Jacob H, Walterhouse D, Iannaccone P. Gene expression profiling leads to identification of GLI1-binding elements in target genes and a role for multiple downstream pathways in GLI1-induced cell transformation. J. Biol. Chem 2002;277:5548–5555. [PubMed: 11719506]

Yu L, Alappat S, Song S, Yan Y, Zhang M, Zhang X, Jiang G, Zhang Y, Zhang Z, Chen YP. Shox2-deficient mice exhibit a rare type of incomplete clefting of the secondary palate. Development 2005;132:4393–4406.

Zhang XM, Ramalho-Santos M, McMahon AP. Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R symmetry by the mouse node. Cell 2001;105:781–792. [PubMed: 11440720]

Zhang YD, Zhao X, Hu Y, St. Amand TR, Ramamurthy R, Qiu MS, Chen YP. *Msx1* is required for the induction of *Patched* by *Sonic hedgehog* in the mammalian tooth germ. Dev. Dyn 1999;215:45–53. [PubMed: 10340755]

Zhang Z, Song Y, Zhao X, Zhang X, Fermin C, Chen Y. Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. Development 2002;129:4135–4146. [PubMed: 12163415]

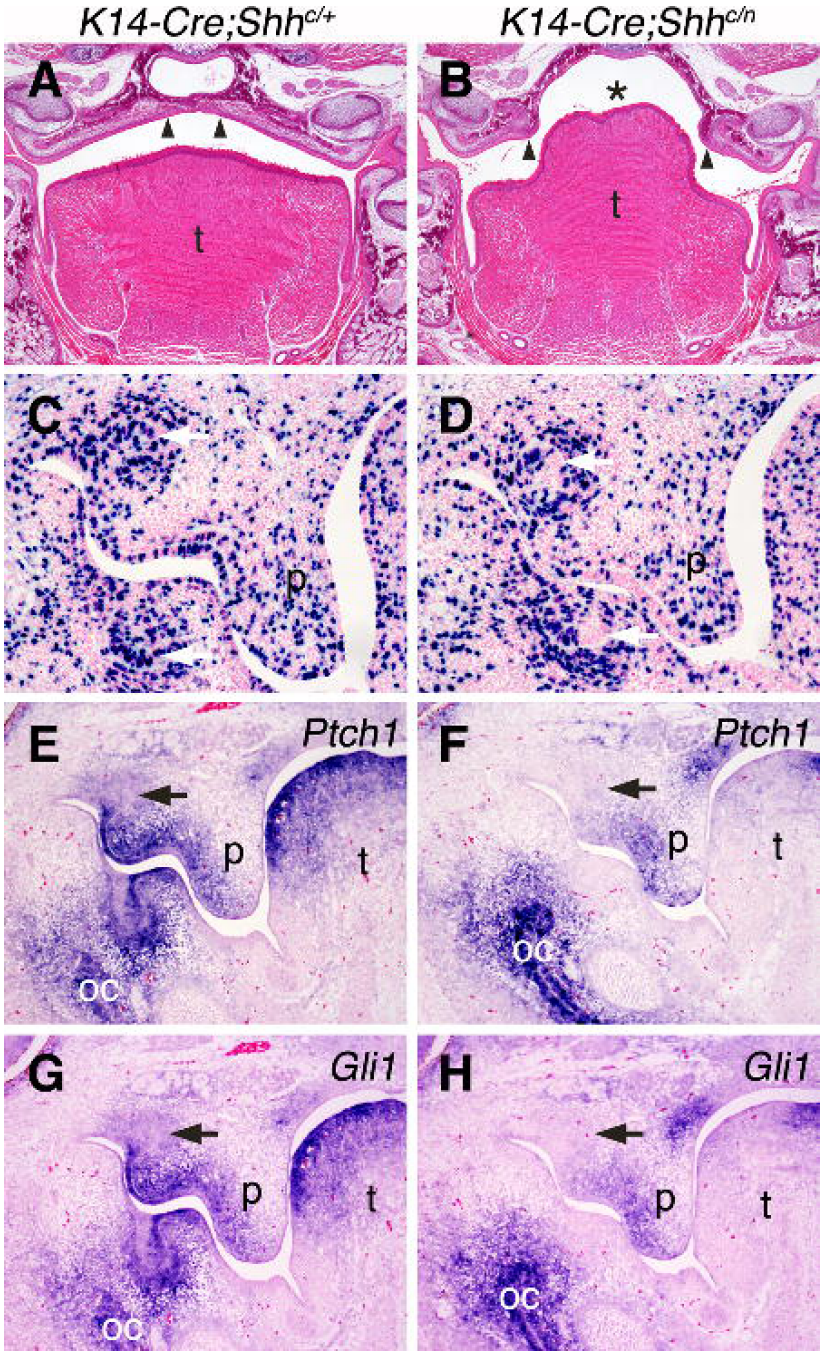

Fig. 1.

Partial inactivation of *Shh* in the developing palate in *K14-Cre;Shh*$^{c/n}$ mutant mice. (A, B) Frontal sections of E17.5 *K14-Cre;Shh*$^{c/+}$ (A) and *K14-Cre;Shh*$^{c/n}$ (B) mutant mouse heads showing cleft palate in the *K14-Cre;Shh*$^{c/n}$ mutant. Arrowheads point to palatal shelves, and the asterisk in B marks the cleft between the bilateral palatal shelves in the mutant. (C, D) At E13.5, the percentage of BrdU labeled cells are reduced in the palatal epithelium and mesenchyme in some *K14-Cre;Shh*$^{c/n}$ mutant embryos (D) in comparison with *K14-Cre;Shh*$^{c/+}$ littermates (C). White arrows point to the first molar tooth buds in which cell proliferation is also reduced in the *K14-Cre;Shh*$^{c/n}$ mutant embryo in comparison with *K14-Cre;Shh*$^{c/+}$ littermate. (E, F) *Ptch1* mRNA expression is reduced in the oral epithelium and

palatal mesenchyme in *K14-Cre;Shh*$^{c/n}$ mutant embryos (F) in comparison with *K14-Cre;Shh*$^{c/+}$ littermates (E). (G, H) *Gli1* mRNA expression was also downregulated in the oral epithelium and palatal mesenchyme in the *K14-Cre;Shh*$^{c/n}$ mutant embryos (H) in comparison with *K14-Cre;Shh*$^{c/+}$ littermates (G) at E13.5. BrdU staining and mRNA signals were detected in blue color. Black arrows in E - H point to the maxillary molar tooth buds. oc, mandibular ossification center; p, palatal shelf; t, tongue.

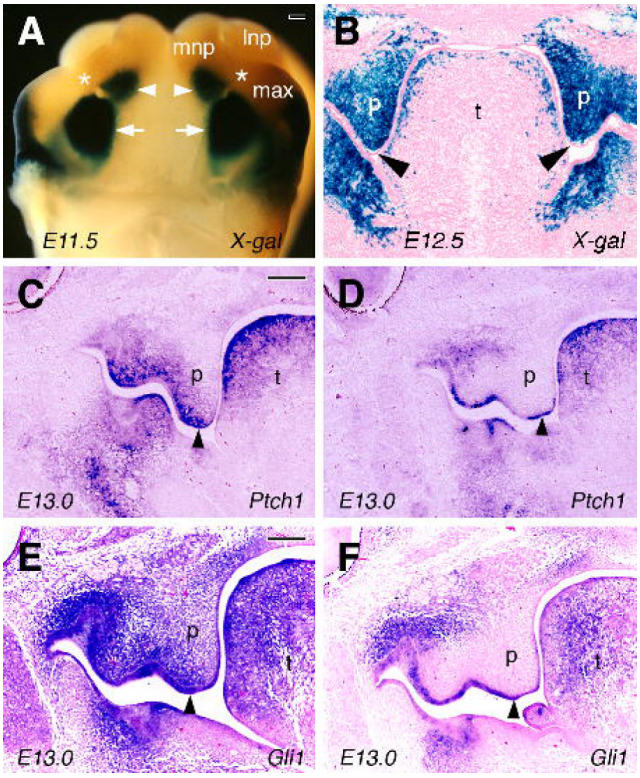

Fig. 2.

Osr2-IresCre mediated inactivation of *Smo* efficiently blocks Hedgehog signaling in the palatal mesenchyme by E13. (A) Whole mount X-gal staining of an E11.5 *Osr2-IresCre;R26R* embryo showing specific Cre-mediated activation of lacZ expression in the primordia of the primary (arrowheads) and secondary palate (arrows). Asterisk marks site of fusion between the medial nasal and the maxillary processes. (B) X-gal stained frontal section of E12.5 *Osr2-IresCre;R26R* embryo showing lacZ activity throughout the palatal mesenchyme but absent in the palatal epithelium (arrowheads). (C, D) By E13.0, *Ptch1* mRNA expression was dramatically down-regulated in the palatal mesenchyme and persisted in the palatal epithelium (arrowhead) in the *Osr2-IresCre;Smo*$^{c/c}$ embryo (D) in comparison with control littermate (C). (E, F) *Gli1* mRNA expression was also dramatically down-regulated in the palatal mesenchyme and persisted in the palatal epithelium (arrowhead) in the *Osr2-IresCre;Smo*$^{c/c}$ mutant embryo (F) in comparison with the control littermate (E) by E13.0. lnp, lateral nasal process; max, maxillary process; mnp, medial nasal process; p, palatal shelf; t, tongue. Scale bar, 100 μm.

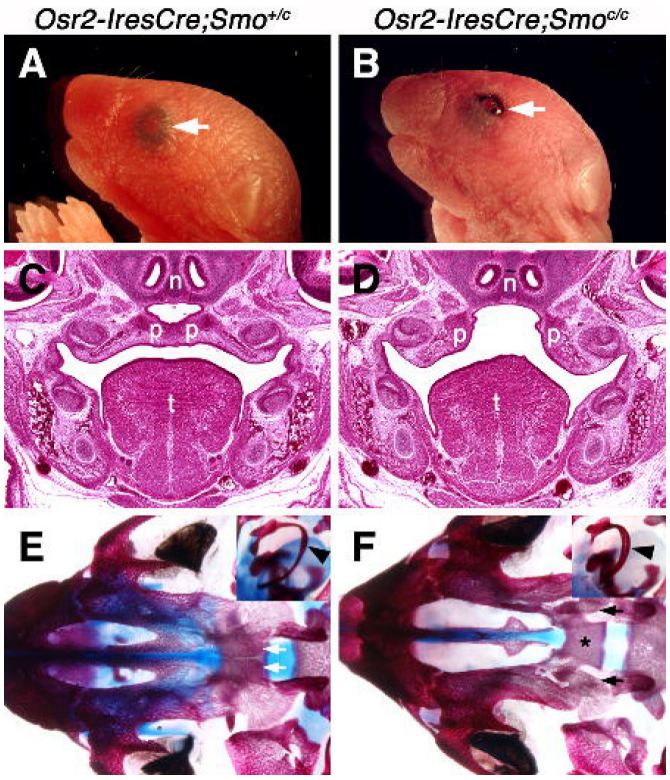

Fig. 3.

Osr2-IresCre;Smo$^{c/c}$ mutant mice exhibit cleft palate and open eyelids at birth. (A, B) Neonatal control (A) and *Osr2-IresCre;Smo*$^{c/c}$ mutant (B) mice showing open eyelids (arrow) at birth in the mutant. (C, D) Frontal sections of E17.5 control (C) and *Osr2-IresCre;Smo*$^{c/c}$ mutant (D) embryos showing cleft palate in the mutant. (E, F) Skeletal preparations of control (E) and *Osr2-IresCre;Smo*$^{c/c}$ mutant (F) neonatal mice showing widely separate palatine bones (arrows) in the mutant. The insets show high magnification views of the tympanic rings (arrowheads) in the *Osr2-IresCre;Smo*$^{+/c}$ (E) and *Osr2-IresCre;Smo*$^{c/c}$ mutant (F) skeletons. Bones are stained red and cartilage blue. Asterisk in F marks the presphenoid bone of the cranial base, which is hidden under the palatine bones from the oral view of the skeleton in the control mouse in E but fully exposed in the *Osr2-IresCre;Smo*$^{c/c}$ mutant skeleton due to cleft palate. n, nasal septum; p, palatal shelf; t, tongue.

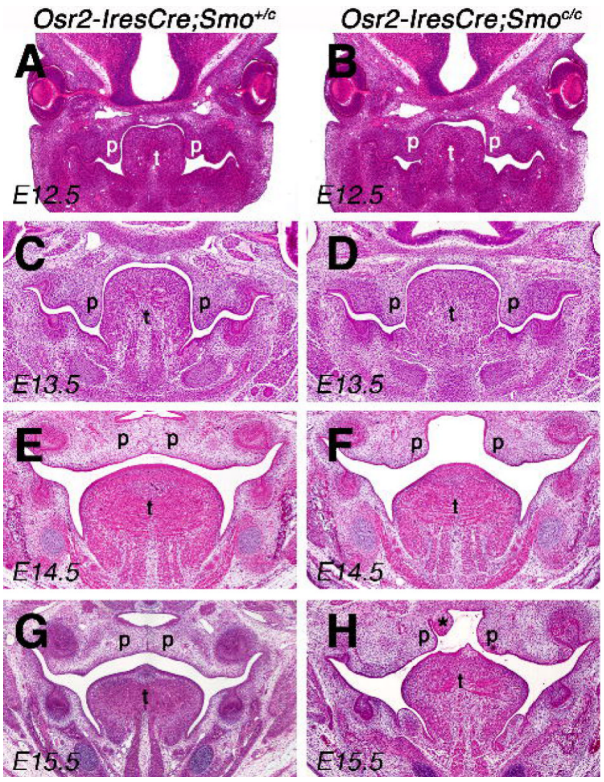

Fig. 4.

Histological analyses of palate development in the control and *Osr2-IresCre;Smo*$^{c/c}$ mutant embryos. (A, B) HE-stained frontal sections of E12.5 *Osr2-IresCre;Smo*$^{+/c}$ (A) and *Osr2-IresCre;Smo*$^{c/c}$ mutant (B) embryos showed comparable initial outgrowth of palatal shelves. (C, D) At E13.5, the palatal shelves of *Osr2-IresCre;Smo*$^{c/c}$ mutant embryo (D) appeared slightly retarded distally in comparison with the *Osr2-IresCre;Smo*$^{+/c}$ embryo (C). (E, F) At E14.5, while the palatal shelves had elevated and initiated fusion at the midline in the control embryo (E), the palatal shelves of the *Osr2-IresCre;Smo*$^{c/c}$ mutant embryo (F) appeared severely retarded and failed to contact each other. (G, H) Some *Osr2-IresCre;Smo*$^{c/c}$ mutant embryos exhibited tissue protrusion into the nasopharynx, shown in H (marked by an asterisk), which was never observed in control embryos. p, palatal shelf; t, tongue.

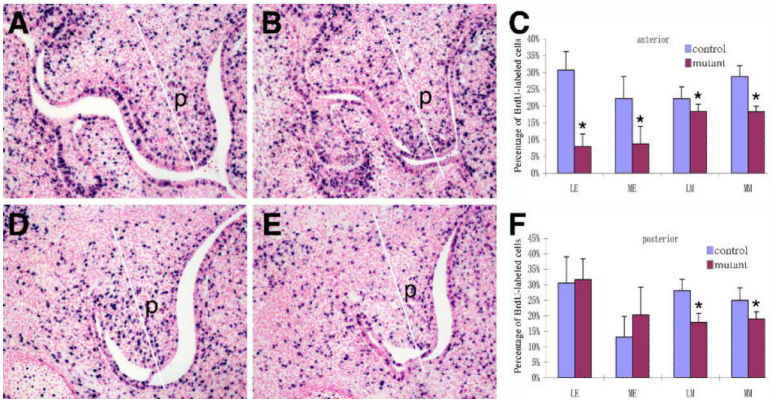

Fig. 5.

Osr2-IresCre;Smo$^{c/c}$ mutant embryos exhibited defects in palatal shelf growth at E13.5. Frontal sections through the palatal regions of BrdU labeled *Osr2-IresCre;Smo*$^{+/c}$ (A, D) and *Osr2-IresCre;Smo*$^{c/c}$ mutant (B, E) embryos were stained with anti-BrdU antibody. The labeled cell nuclei were stained blue. The white line in each panel divided each image of the palatal shelf to medial and lateral halves for the calculation of the percentage of BrdU labeled nuclei in those regions separately. A and B show typical sections through the middle of the anterior half of the palatal shelves whereas D and E are from the posterior third of the palatal shelves. (C, F) Comparison of the percentage of BrdU labeled cells in the anterior (C) and posterior (F) regions of developing palate in the *Osr2-IresCre;Smo*$^{+/c}$ (control) and *Osr2-IresCre;Smo*$^{c/c}$ (mutant) embryos. Standard deviation values were used for the error bars. Asterisk denotes a significant reduction in the percentage of BrdU labeled cells in the mutant ($p < 0.05$). p, palatal shelf.

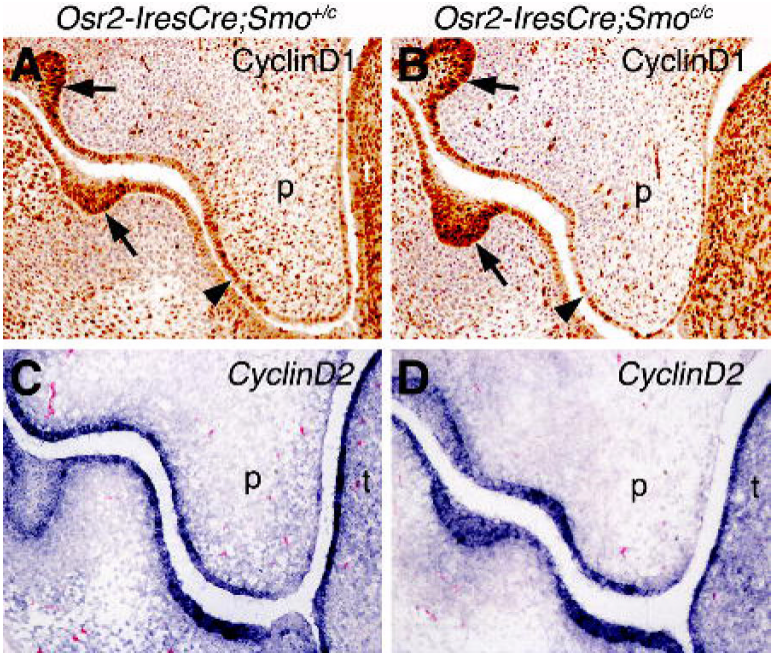

Fig. 6.

Comparison of CyclinD1 and *CyclinD2* expression in the developing palate in *Osr2-IresCre;Smo*$^{+/c}$ (A, C) and *Osr2-IresCre;Smo*$^{c/c}$ mutant (B, D) embryos at E13.5. (A, B) CyclinD1 immunostaining shown in brown color. CyclinD1 expression in the tooth bud epithelium (arrows) served as internal controls because *Osr2-IresCre* was not expressed in this tissue. Arrowheads point to the palatal epithelium, which appeared thinner in the *Osr2-IresCre;Smo*$^{c/c}$ mutant embryos compared to the control littermates. (C, D) In situ hybridization detection of *CyclinD2* mRNA (in blue color). p, palatal shelf.

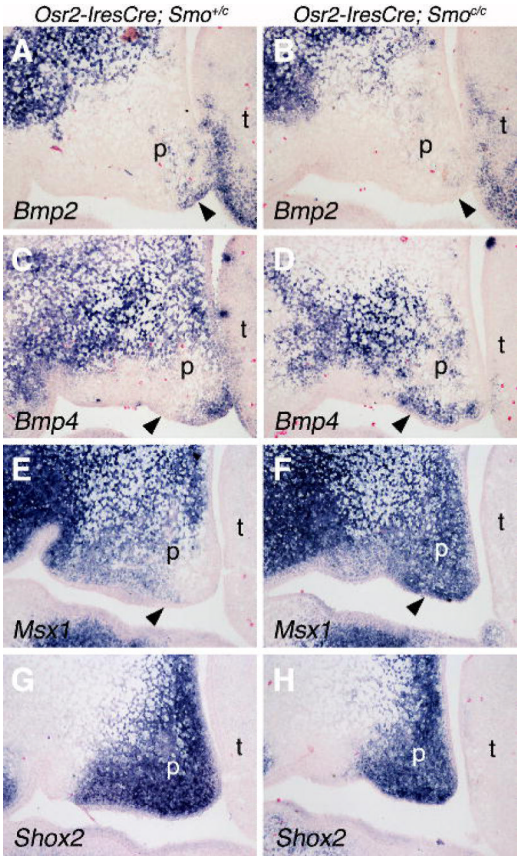

Fig. 7.

Comparison of *Bmp2* (A, B), *Bmp4* (C, D), *Msx1*(E, F) and *Shox2* (G, H) mRNA expression in the anterior palate of *Osr2-IresCre;Smo*$^{+/c}$ (A, C, E, G) and *Osr2-IresCre;Smo*$^{c/c}$ mutant (B, D, F, H) embryos at E13.5. Arrowheads in A-F point to differences in expression in the control and mutant palatal shelves. In comparison with the control embryos, *Bmp2* mRNA was downregulated whereas *Bmp4* and *Msx1* mRNAs were up-regulated in the anterior palatal mesenchyme. (G,H) *Shox2* mRNA expression was unaltered in the *Osr2-IresCre;Smo*$^{c/c}$ mutant palate. p, palatal shelf; t, tongue.

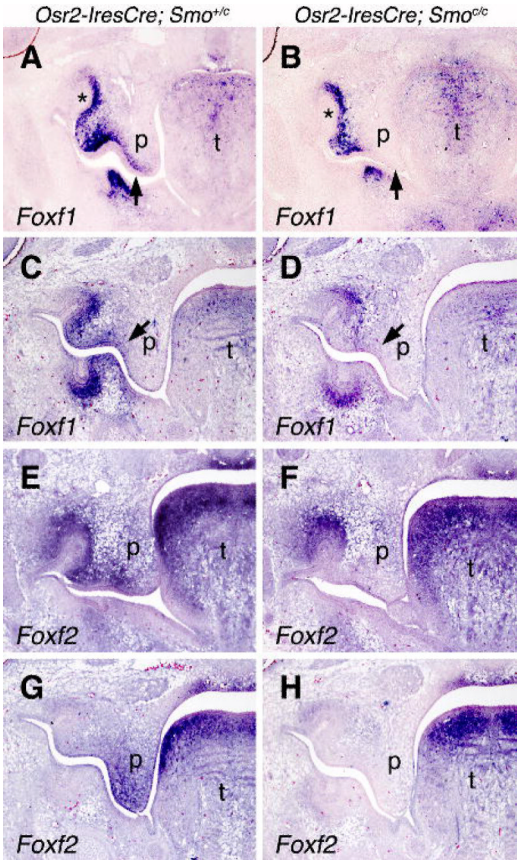

Fig. 8.

Foxf1 and *Foxf2* mRNA expression in the developing palate were downregulated in the *Osr2-IresCre;Smo*$^{c/c}$ mutant embryos. (A) At E13.5, *Foxf1* mRNA was expressed in the anterior palatal mesenchyme underlying the lateral and medial edge epithelium (arrow) in the control embryos. (B) *Foxf1* mRNA expression was specifically downregulated in the palatal mesenchyme but its expression in the tooth mesenchyme was not affected in the *Osr2-IresCre;Smo*$^{c/c}$ mutant embryos. Asterisks in A and B mark the maxillary first molar tooth buds. (C, D) At E13.5, *Foxf1* mRNA expression in the posterior palate was restricted to more lateral and proximal cells in the control embryo (C) and this domain of expression (arrows in C and D) was also downregulated in the *Osr2-IresCre;Smo*$^{c/c}$ mutant embryos (D). (E - H) *Foxf2* mRNA expression exhibited a lateral-medial gradient in both the anterior and posterior palate in the control embryos (E, G) and was downregulated in both the anterior and posterior palatal mesenchyme in the *Osr2-IresCre;Smo*$^{c/c}$ mutant embryos (F, H). p, palatal shelf; t, tongue.

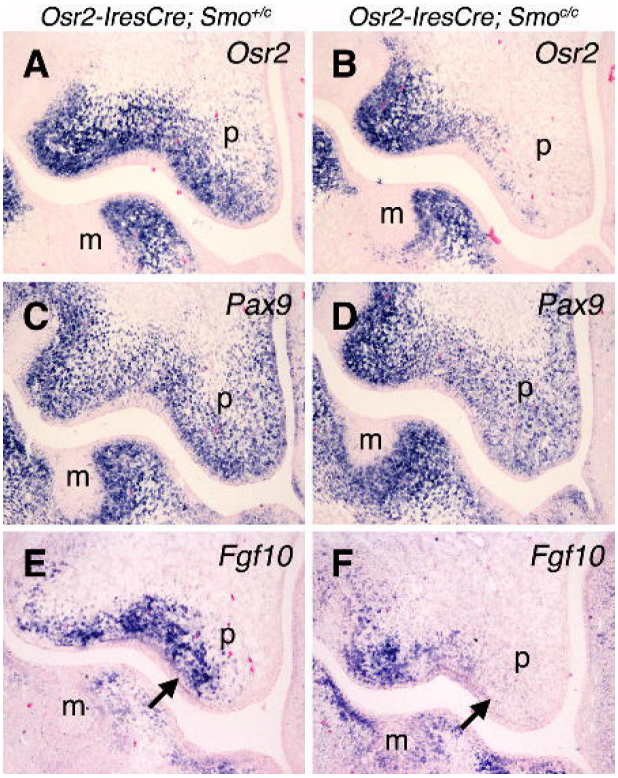

Fig. 9.

Comparison of expression of *Osr2*, *Pax9*, and *Fgf10* in the developing palate of the control and *Osr2-IresCre;Smo*$^{c/c}$ mutant embryos. (A) At E13.5, *Osr2* mRNA was strongly expressed in the mesenchyme lingual to the first molar tooth buds and exhibited a lateral-medial gradient in the palatal mesenchyme in control embryos. (B) In the *Osr2-IresCre;Smo*$^{c/c}$ mutant embryos at E13.5, *Osr2* mRNA expression was specifically downregulated in the palatal mesenchyme but the domain of expression in the lingual tooth mesenchyme was unaffected. (C, D) *Pax9* mRNA expression in the developing palate and tooth mesenchyme was not affected in the *Osr2-IresCre;Smo*$^{c/c}$ mutant embryos. (E, F) *Fgf10* expression in the anterior palatal mesenchyme (arrows) was significantly downregulated in the *Osr2-IresCre;Smo*$^{c/c}$ mutant embryos (F) in comparison with the control embryos (E). m, mandibular first molar tooth bud; p, palatal shelf.