Ectopic Hedgehog Signaling Causes Cleft Palate and Defective Osteogenesis

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Abstract

Cleft palate is a common birth defect that frequently occurs in human congenital malformations caused by mutations in components of the Sonic Hedgehog (*SHH*) signaling cascade. *Shh* is expressed in dynamic, spatiotemporal domains within epithelial rugae and plays a key role in driving epithelial-mesenchymal interactions that are central to development of the secondary palate. However, the gene regulatory networks downstream of Hedgehog (Hh) signaling are incompletely characterized. Here, we show that ectopic Hh signaling in the palatal mesenchyme disrupts oral-nasal patterning of the neural crest cell-derived ectomesenchyme of the palatal shelves, leading to defective palatine bone formation and fully penetrant cleft palate. We show that a series of Fox transcription factors, including the novel direct target *Foxl1*, function downstream of Hh signaling in the secondary palate. Furthermore, we demonstrate that Wnt/bone morphogenetic protein (BMP) antagonists, in particular *Sostdc1*, are positively regulated by Hh signaling, concomitant with downregulation of key regulators of osteogenesis and BMP signaling effectors. Our data demonstrate that ectopic Hh-Smo signaling downregulates Wnt/BMP pathways, at least in part by upregulating *Sostdc1*, resulting in cleft palate and defective osteogenesis.

Keywords: craniofacial abnormalities, sonic hedgehog, neural crest, bone morphogenetic proteins, micrognathism, wnt signaling pathway

Introduction

Specification, growth, elevation, adherence, and fusion of the palatal shelves are essential mechanisms involved in secondary palate formation (Mossey et al. 2009; Dixon et al. 2011). Disruption of these processes leads to cleft palate, a common congenital disorder that affects ~1:2,500 live births (Mossey et al. 2009). Cleft palate causes major morbidity through problems with feeding, speech, hearing, and social adjustment. Affected children require multidisciplinary care into adulthood at considerable cost to health care systems worldwide. The frequent occurrence and major burden imposed by cleft palate highlight the need to dissect the mechanisms underlying palatal development. Although substantial progress has been made identifying the mutations underlying syndromic forms of cleft palate, the developmental role of many of the mutated genes is unknown (Dixon et al. 2011).

Development of the mouse secondary palate mirrors that of humans; as a result, the mouse is the major model organism for analyzing palatogenesis (Bush and Jiang 2012). In mice, palatal shelves initiating from the maxillary processes on embryonic day (E) 11 grow lateral to the tongue during E12/E13 before reorientating above the tongue during E14. Subsequently, the medial edge epithelia of apposed shelves adhere to form a midline epithelial seam, which degenerates to allow mesenchymal continuity across the palate by E15. In parallel, the oral and nasal palatal epithelia differentiate into stratified, squamous, keratinizing, and pseudostratified, ciliated epithelia, respectively. Similarly, the palatal mesenchyme differentiates into bony and muscular elements forming the hard and soft palate, respectively. Reflecting these different developmental

fates, gene expression studies have revealed molecular heterogeneity along both the oral-nasal and anterior-posterior axes of the palatal shelves (Hilliard et al. 2005).

Mutations in components of the Hedgehog (Hh) signaling pathway underlie several human congenital malformations that are associated with cleft palate (Mansilla et al. 2006; Cohen 2010). *Shh* is expressed in epithelial rugae on the oral aspect of the palate, which initially define the anterior-posterior boundary of the palatal shelves and act as signaling centers that drive epithelial-mesenchymal interactions (Rice et al. 2004; Rice et al. 2006; Pantalacci et al. 2008; Lan and Jiang 2009). Thus, Shh signaling is linked spatiotemporally to both oral-nasal and anterior-posterior patterning of the secondary palate. Recent transgenic approaches to modulate Shh signaling within cranial neural crest cells (CNCCs) (Jeong et al. 2004), facial epithelia (Rice et al. 2004; Cobourne et al. 2009; Lan and Jiang 2009; Kurosaka et al. 2014), and secondary palate mesenchyme (Lan and Jiang 2009) have demonstrated the critical importance of this pathway to normal secondary palate development. Shh signaling regulates expression of the transcription

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Figure 1. *Osr2-IresCre;Smo^{+M2}* mice have fully penetrant cleft palate. (A–F) Histological analyses indicate mutant embryos have severe defects in secondary palate development. Mutant palatal shelves are smaller in size from E13.5, with marked reduction in vertical growth of the palatal shelves (A, B). Although mutant palatal shelves appear to reorientate by E14.5, they are abnormally shaped and far apart (C, D), resulting in a wide cleft of the secondary palate by E15.5 (E, F). Tooth germ development is also arrested at the bud stage in mutant embryos (C–F). ps, palatal shelf; t, tongue. Scale bars: A–H = 300 μ m.

factors *Foxf1*, *Foxf2*, and *Osr2* and the growth factors BMP2, BMP4, and *Fgf10* in palatal mesenchyme (Lan and Jiang 2009), but the Shh-induced pathways controlling epithelial-mesenchymal crosstalk remain incompletely characterized.

In this study, we investigated how ectopic Hh signaling affects normal secondary palate development. Using a gain-of-function mouse model to activate Smoothed (*Smo*) signaling in the palatal mesenchyme (*Osr2-IresCre;Smo^{+M2}*), we demonstrate that ectopic Hh-Smo signaling results in fully penetrant cleft palate and defective palatine bone formation. Using transcriptional profiling and expression analyses, we demonstrate Hh-Smo signaling is expanded and disrupts oral-nasal patterning by driving the expression of several transcriptional repressors and antagonists of Hh, Wnt, and bone morphogenetic protein (BMP) signaling pathways. We show Hh-Smo signaling upregulates several Fox transcription factors, including the direct transcriptional target *Foxl1*. Furthermore, we reveal the dual Wnt/BMP antagonist, *Sostdc1*, is expressed ectopically in the nasal mesenchyme, coincident with downregulation of nasally expressed master regulators of osteogenesis (*Sox9*, *Runx2*), bone-related extracellular matrix proteoglycans (*Dcn*, *Lum*), and BMP signaling effectors (pSmad 1/5/9). Our data suggest Hh-Smo signaling negatively regulates Wnt/BMP pathways by upregulating antagonists, resulting in cleft palate and defective osteogenesis.

Materials and Methods

Detailed methods are in the Appendix. Microarray data have been deposited in Array Express with the accession E-MTAB-5518.

Results

Osr2-IresCre;Smo^{+M2} Mice Have a Complete Cleft of the Secondary Palate

To investigate the gene regulatory networks downstream of the Hh-Smo signaling cascade, we used *Cre/loxP* to constitutively activate *Smo* (*SmoM2*) (Xie et al. 1998) in the palatal mesenchyme in vivo (*Osr2-IresCre*) (Appendix Fig. 1). *Osr2-IresCre;Smo^{+M2}* embryos, hereafter referred to as mutants, displayed a wide cleft of the secondary palate. Histological analysis of E13.5 mutant mice revealed smaller, abnormally shaped palatal shelves compared with their wild-type littermates, which was most pronounced in the anterior and mid-regions, indicating reduced palatal outgrowth (Fig. 1A, B). By E14.5, wild-type palatal shelves had reorientated above the tongue while those of mutant littermates had failed to elevate and were rounded in appearance, and tooth germ development was arrested at the bud stage (Fig. 1C, D; arrowheads). These anomalies were more pronounced by E15.5, when mutant embryos displayed a fully penetrant complete cleft of the secondary palate ($n = 15$) compared to the fused palate of wild-type littermates (Fig. 1E, F). To investigate the cause of smaller palatal shelves, we performed cell proliferation analysis using bromodeoxyuridine (BrdU) incorporation at E13.5 and revealed a significant proliferation defect in anterior and mid-palatal regions while the posterior palate was unaffected (Appendix Fig. 2).

Osr2-IresCre;Smo^{+M2} Mice Have Multiple Skeletal Defects

We analyzed alcian blue/alizarin red–stained skeletal preparations, which revealed defects in the viscerocranium of mutant embryos (Fig. 2A; E17, $n = 3$). The anterior midline structures of the premaxilla and posterior regions of the maxilla were absent in mutant embryos, along with the associated palatine processes, revealing the presphenoid, which is normally obscured (Fig. 2B). Mutant mandibles were shorter and showed anterior ossification defects with rudimentary condyloid processes and no defined coronoid processes (Fig. 2C).

Transcriptional Profiling Reveals Negative Regulators of Hh, Wnt, and BMP Pathways are Upregulated in Response to Persistent Hh Signaling

To gain insight into the gene regulatory networks affected by increased Hh-Smo signaling, we compared the transcriptomes of palatal shelves dissected from E13.5 wild-type and mutant embryos. Microarray analysis identified 580 differentially expressed genes ($P < 0.05$) (E-MTAB-5518; Appendix Table 1; Appendix Fig. 3A), of which 327 genes were upregulated in

response to increased Hh-Smo signaling. These included known direct targets (*Gli1*, *Ptch1*, *Ptch2*, *Hhip*) and several members of the Fox family of transcription factors (*Foxd1*, *Foxd2*, *Foxf1*, *Foxf2*, and *Foxl1*). Overrepresentation enrichment analysis indicated significant enrichment of gene ontology terms, including *mesenchyme development*, *receptor serine/threonine kinase signaling*, and *cell fate commitment* (Appendix Fig. 3B; Appendix Table 4). Further annotation of these gene groups revealed upregulation of several transcriptional repressors and antagonists of Hh (e.g., *Ptch1*, *Hhip*, *Cdon*), Wnt, and BMP (e.g., *Sostdc1*, *Twsg1*) signaling pathways.

Hh-Smo Direct and Downstream Targets Are Upregulated throughout the Palatal Mesenchyme

Subsequently, we investigated the expression of known Hh direct targets (*Gli1*, *Ptch1*) and candidate targets from the microarray analysis using a combination of whole-mount and section in situ hybridization. *Gli1* and *Ptch1* are normally expressed in rugae epithelium and the underlying mesenchyme on the oral side of the palate. However, their expression was expanded into the tooth germ and nasal mesenchyme of mutant embryos (Fig. 3A–H) while reduced expression was noted in epithelial rugae (Fig. 3C, D, G, H). Ectopic expression of *Gli1* and *Ptch1* was also observed in the mandibular mesenchyme (Fig. 3D, H; arrows), correlating with *Osr2-IresCre* expression (Appendix Fig. 1C).

Members of the Fox transcription factor family, including *Foxf1* and *Foxf2*, have been implicated downstream of Hh signaling during facial and secondary palate development (Lan and Jiang 2009; Nik et al. 2016; Xu et al. 2016). *Foxf2* was expressed throughout the anterior-posterior length of the oral palatal mesenchyme in wild-type embryos with increased expression underlying rugae (Fig. 3I, K). In contrast, *Foxf2* was markedly upregulated in the oral and nasal mesenchyme of mutant embryos (Fig. 3J, L). Transcriptional profiling identified *Foxl1* as the highest upregulated Fox factor (Appendix Table 1; Appendix Table 2) and has not been implicated in palate development. *Foxl1* was expressed in the oral palatal mesenchyme of wild-type embryos associated with rugae. However, in mutant embryos, *Foxl1* was markedly upregulated and expanded from the oral to nasal palatal mesenchyme in anterior and posterior regions of the palate (Fig. 3M–P).

Figure 2. *Osr2-IresCre;Smo^{+/M2}* mice have skeletal abnormalities and lack the palatine bones. (A–C) Whole-mount skeletal preparations stained with alcian blue and alizarin red reveal multiple defects in the mutant skeleton at E17.5. (A) Skeletal abnormalities include truncated fore- and hind-limbs with cartilage and ossification defects. (B) Detailed analysis of the craniofacial skeleton reveals multiple abnormalities of the viscerocranium. Mutant embryos demonstrate reduced ossification of the maxilla (mx) and premaxilla along with absence of the palatine and palatine processes of both the premaxilla and maxilla. (C) Mutant embryos also have a shorter mandible (green arrow, wild-type; red arrow, mutant) with rudimentary angular and condyloid processes and no defined coronoid process. Mutant mandibles also show reduced ossification anteriorly. agp, angular process; als, alisphenoid; bo, basiooccipital; bs, basosphenoid; cdp, condyloid process; crp, coronoid process; eo, exoccipital; fr, frontal bone; lo, lamina obturans; md, mandible; mx, maxilla; p, palatine bone; pmx, premaxilla; ppmx, palatine process of the maxilla; ppp, palatine process of the premaxilla; ps, presphenoid; ptg, pterygoid; sq, squamosal; sy, symphysis.

Similar to known Hh direct targets, ectopic expression of both Fox factors was observed in the lingual aspect of the mandibular mesenchyme (Fig. 3P; arrow).

Elevated Hh-Smo signaling also upregulated several Wnt/BMP antagonists. We investigated the expression of *Sostdc1*, a dual Wnt/BMP secreted antagonist with reported roles in the spatial patterning of teeth and rugae (Ahn et al. 2010; Cho et al. 2011; Lee et al. 2011). *Sostdc1* was expressed in interrugae domains in the anterior palatal epithelium and the posterior palate (Fig. 3Q, S) (Welsh and O'Brien 2009; Lee et al. 2011). In mutant embryos, *Sostdc1* was expressed ectopically in the lingual and nasal mesenchyme of the palate while expression in the posterior palate and mandible was also upregulated (Fig. 3R). Real-time quantitative polymerase chain reaction (qPCR) confirmed significant upregulation of all these genes in the palatal shelves of mutant embryos (Fig. 3U).

Sequential Rugae Interposition Is Blocked and *Shh* Is Downregulated in *Osr2-IresCre; Smo^{+/-M2}* Embryos

Whole-mount analysis of *Shh* targets indicated reduced numbers of rugae in mutant embryos at E13.5 (Fig. 3; arrowheads). Subsequently, *Shh* expression from E13.5 to E15.5 showed the sequential addition of up to 8 rugae in wild-type embryos (Appendix Fig. 4A–C), while mutants developed only 3 rugae (Appendix Fig. 4D–F). Furthermore, at E15.5 *Shh* expression was secondarily downregulated in mutant rugae, while expression persisted in wild-type embryos (Appendix Fig. 4D, H).

Extracellular Matrix Proteoglycans in the Nasal Palatal Mesenchyme Are Downregulated

Transcriptional profiling indicated that the extracellular matrix proteins decorin (*Dcn*), lumican (*Lum*), and keratocan (*Kera*) were amongst the most significantly downregulated genes (Appendix Table 3; Appendix Fig. 2A). These matricellular proteins are members of the small leucine-rich proteoglycan (SLRP) family with multiple roles in osteogenesis (Raouf et al. 2002; Waddington et al. 2003). Expression analyses in wild-type embryos showed *Dcn* and *Lum* were restricted to the nasal mesenchyme of the palate. In agreement with the microarray analysis, expression of both genes was downregulated in mutant palatal mesenchyme while expression elsewhere was unaffected (Fig. 4A–H). Similarly, *Dlx5*, a factor crucial for osteoblast differentiation (Acampora et al. 1999), is expressed in the anterior nasal mesenchyme of wild-type palatal shelves (Fig. 4I, K) but was downregulated in the palatal mesenchyme of mutant embryos (Fig. 4J, L). Real-time qPCR confirmed the reduction of *Dcn* and *Lum*, but *Dlx5* was not significant (Fig. 4M).

Master Regulators of Osteogenesis Are Downregulated

Subsequently, we analyzed the expression of key transcriptional effectors of osteogenesis, *Sox9* and *Runx2*, which are regulated by Wnt/Bmp crosstalk (Gaur et al. 2005; Pan et al. 2008). Immunohistochemical analyses of *Sox9* and *Runx2* at E13.5 revealed both proteins were expressed in overlapping domains within the nasal palatal mesenchyme (Fig. 5A, C, E). *Sox9* was also highly expressed in developing craniofacial cartilages (Fig. 5A, E). The expression of both proteins was dramatically downregulated in the nasal mesenchyme while expression elsewhere was unaffected (Fig. 5B, D, F). Similarly, at E14.5, *Sox9* and *Runx2* were expressed in overlapping domains in the reorientated nasal mesenchyme directly beneath the midline epithelial seam (Fig. 5G, I, K) while the expression of both proteins was markedly downregulated in the nasal mesenchyme of mutant embryos (Fig. 5H, J, L). *Sox9* and *Runx2* are critical factors in orchestrating multiple steps of intramembranous ossification, and their expression in wild-type palatal mesenchyme defines the nasal mesenchymal contribution to palatal growth, fusion, and bone formation (Fig. 5A, C, E, G, I,

K). Since *Sox9* and *Runx2* were downregulated and *Sostdc1* is a BMP antagonist (Wu et al. 2008), we analyzed whether BMP signaling was affected in mutant embryos. Immunofluorescence for pSmad 1/5/9 revealed the effectors of BMP signaling were also downregulated in the nasal mesenchyme at E13.5 and the future palatine bone regions at E14.5 (Fig. 5M–P).

Collectively, these results demonstrate that ectopic Hh-Smo signaling in the nasal mesenchyme downregulates BMP signaling, concomitant with upregulation of Wnt/BMP antagonist *Sostdc1*, resulting in defective osteogenesis and cleft palate.

Foxl1 Is a Direct Target of *Gli1* in the Secondary Palate

To determine if *Sostdc1* and *Foxl1* are direct targets of Hh signaling, we analyzed the promoters (–1 kb) of these genes for candidate Gli binding sites. No Gli sites were found near *Sostdc1*. However, we identified 2 highly conserved candidate binding sites in the *Foxl1* promoter (–237 and –371 from the TSS), with 1 mismatch from the Gli consensus (Appendix Fig. 5A), in regions of accessible chromatin (Appendix Fig. 5B). Gli1 chromatin immunoprecipitation (ChIP)–qPCR analyses of E13.5 palatal shelves demonstrated significant enrichment of Gli1 on the *Foxl1* promoter and also known direct targets, *Ptch1* and *Gli1* (Appendix Fig. 5C). This is the first report to demonstrate *Foxl1* is a direct target of Gli1 in vivo.

Discussion

Spatiotemporal Hh-Smo signaling defines a gene regulatory network that patterns the oral axis of the secondary palate. Recent research has established that epithelial *Shh* expressed within rugae (Pantalacci et al. 2008) signals to the underlying mesenchyme to activate *Smo* (Rice et al. 2004; Lan and Jiang 2009) and direct gene expression and cell fate through Gli transcription factors. Rice and colleagues (2004) showed that *Shh* signaling is crucial for palate development as disruption of Fgf10-Fgfr2b-*Shh* mesenchymal-epithelial signaling results in cleft palate, while targeted loss of the key *Shh* transducer, *Smo*, in palatal mesenchyme also results in cleft palate (Lan and Jiang 2009). Conversely, transgenic expression of *Shh* in all epithelial tissues results in a severe craniofacial phenotype with cleft palate (Cobourne et al. 2009). However, the molecular mechanisms downstream of Hh-Smo signaling within the secondary palate remain poorly characterized.

Mutant mouse studies to uncover the molecular mechanisms driving secondary palate development are often confounded by early embryonic lethality or gross craniofacial abnormalities. However, the recent generation of *Osr2-IresCre* mice (Lan et al. 2007) allows tissue-specific manipulation of genes involved in palate development. While the use of Cre-based mouse models to interrogate gene function is an aggressive tool that can disrupt normal physiological gene expression, such an approach has been used successfully to uncover targets of Hh signaling in the early embryonic face, limb, palate, and brain (Jeong et al. 2004; Vokes et al. 2008; Heine and Rowitch

Figure 3. Hedgehog direct and downstream targets are upregulated in *Osr2-IresCre;Smo^{+/M2}* embryos. Whole-mount (A, B, E, F, I, J, M, N, Q, R) and in situ (C, D, G, H, K, L, O, P, S, T) hybridization sections for direct targets *Gli1* (A–D) and *Ptch1* (E–H) demonstrate they are associated with rugae on the oral side of E13.5 wild-type palates (A, C, E, G). *Gli1* and *Ptch1* are upregulated and expanded from the oral to nasal mesenchyme of mutant embryos (B, D, F, H). *Foxf2* is expressed in the oral mesenchyme along the anterior-posterior length of the palate while *Foxl1* is strongly expressed in the mesenchyme underlying rugae (I, K, M, O). Upregulation and expansion of both Fox factors are seen in mutant palates (J, N, L, P). Expression of *Sostdc1* is observed in interrugae domains of the epithelium and in the posterior palate. In contrast, *Sostdc1* is ectopically expressed in the nasal mesenchyme and upregulated in the posterior palate of mutants (Q–T). Ectopic expression of all targets is seen in the mandibular mesenchyme of mutant embryos (D, H, L, P, T; arrows). (M) Real-time quantitative polymerase chain reaction analysis of E13.5 palatal shelves confirms upregulation of all targets (* $P < 0.05$, ** $P < 0.01$; Mann-Whitney U test, $n = 5$). md, mandible; ps, palatal shelf; t, tongue. Scale bars: C, D, G, H, K, L, O, P, S, T = 100 μ m.

Figure 4. Extracellular matrix proteoglycans are downregulated in *Osr2-IresCre;Smo^{+/M2}* embryos. Whole-mount (A, B, E, F, I, J) and in situ (C, D, G, H, K, L) hybridization sections for the extracellular matrix genes *Dcn* (A–D), *Lum* (E–H), and the homeobox transcription factor *Dlx5* (I–L) demonstrate *Dcn* and *Lum* are expressed in the nasal mesenchyme throughout the anterior-posterior length of the palatal shelves in E13.5 wild-type embryos, while *Dlx5* is also expressed in the nasal mesenchyme in the anterior region of the palatal shelves (A, C, E, G, I, K). Mutant embryos show loss of expression of these genes in the palatal shelves (B, D, F, H, J, L). (M) Real-time quantitative polymerase chain reaction data confirm significantly reduced levels of messenger RNA for *Dcn* and *Lum* (* $P < 0.05$, Mann-Whitney U test, $n = 4$). md, mandible; ps, palatal shelf; t, tongue. Scale bars: C, D, G, H, K, L = 100 μ m.

2009; Lan and Jiang 2009). In this study, we generated a palate-specific *Smo* gain-of-function mouse model by targeting constitutively active *Smo* (Xie et al. 1998) to the palatal mesenchyme. We found that mutant embryos were characterized by a fully penetrant wide cleft of the secondary palate with various skeletal defects. Taken together, this clearly illustrates that a precise level of Hh-Smo signaling is required for normal palate development.

Patterning of the secondary palate is complex, with molecular heterogeneity along both the oral-nasal and anterior-posterior axes (Hilliard et al. 2005). We and others (Rice et al. 2006; Han et al. 2009; Lan and Jiang 2009) have shown that effectors of Hh-Smo signaling are expressed on the oral side of the palate while the nasal side is reportedly characterized by transforming growth factor β (TGF β)/BMP mediators (Iwata et al. 2011; Parada and Chai 2012). We demonstrated that elevated Hh-Smo

signaling resulted in upregulation and expansion of direct and downstream targets of the Hh pathway within the palatal mesenchyme. Using transcriptional profiling and gene ontology analyses, we identified and characterized several upregulated transcriptional repressors and Wnt/BMP antagonists, particularly *Foxf2*, *Foxl1*, and *Sostdc1*.

Members of the Fox transcription factor family (*Foxd1*, *Foxd2*, *Foxc2*, *Foxf1*, and *Foxf2*) are dependent on *Smo* signaling in the early developing face, leading to the suggestion that Fox factors are the mediators of Hh-Smo signaling (Jeong et al. 2004). Indeed, loss of *Smo* in the palatal mesenchyme also results in downregulation of *Foxf1* and *Foxf2* (Lan and Jiang 2009). Our data demonstrate that *Foxf1*, *Foxf2*, and the novel target *Foxl1* are all robustly upregulated in response to increased Hh-Smo signaling within the palate, confirming these as *Smo*-dependent targets. Mutations in *FOXF2* have

Figure 5. Master regulators of osteogenesis are downregulated in the nasal palatal mesenchyme of *Osr2-IresCre;Smo^{+/-M2}* embryos. (A–F) Immunostaining for Sox9 (A, B; red) and Runx2 (C, D; green) in adjacent sections at E13.5 indicates both markers are expressed in overlapping domains in the nasal mesenchyme of wild-type palatal shelves (A, C, E; arrows). Sox9 is also highly expressed in the developing nasal cartilage (arrowheads) and extends into the mesenchyme beneath the medial edge epithelia (A, E) while Runx2 is also expressed in the odontogenic mesenchyme (C, E). Conversely, in mutant palatal shelves, expression of both markers is absent from the nasal palatal mesenchyme while expression in the nasal cartilage (arrowheads) and odontogenic mesenchyme is unaffected (B, D, F). (G–L) At E14.5, Sox9 is expressed in the mesenchyme beneath the midline epithelial seam (G, K), while Runx2 has a characteristic expression pattern in the future bone condensations of the wild-type palate (I, K). Both markers are excluded from the mesenchyme along the oral aspect of the horizontal palate (G, I, K). In contrast, expression of both markers is absent from the palatal mesenchyme of the mutant palate while expression in the nasal cartilages (arrowheads) and associated structures is unaffected (H, J, L). pSmad 1/5/9 is expressed in the nasal mesenchyme at E13.5 and future palatine bone mesenchyme at E14.5 in wild-type embryos (M, N; red) but is absent from these regions in mutant embryos (O, P). Autofluorescence of red blood cells is identified by triple immunofluorescent images (M–P; yellow). md, mandible; ps, palate; t, tongue. Scale bars: A–P = 100 μ m.

been associated with cleft palate (Jochumsen et al. 2008) while mice deficient in either *Foxf1* or *Foxf2* are born with cleft palate (Lan and Jiang 2009; Nik et al. 2016; Xu et al. 2016). We identified and characterized the expression of the novel *Foxl1* in the secondary palate and demonstrated that *Foxl1* is a direct target of Gli1. Although *Foxl1*^{−/−} mice are viable, most mutant mice die before weaning, attributed to impaired development of the gastrointestinal tract (Kaestner et al. 1997). However, secondary palate formation has not been investigated in these mice and may be a contributing factor. Alternatively, other Fox family members may compensate for the loss of *Foxl1*. In support of this hypothesis, *Foxf1* and *Foxf2* have similar functions in the developing stomach and intestine (Madison et al. 2009), while partial functional redundancy between *Foxf1* and *Foxf2*

has been demonstrated in the secondary heart field (Hoffmann et al. 2014) and palate (Xu et al. 2016). The function of Foxl1 during palate development remains unknown, but studies in other tissues have revealed Foxl1 (in addition to Foxf1 and Foxf2) can indirectly affect epithelial proliferation via modulation of Wnt– β -catenin signaling (Kaestner et al. 1997; Perreault et al. 2001; Madison et al. 2009). Therefore, we postulate that Foxl1 may play a role in coordinating palatal growth via epithelial-mesenchymal feedback.

Expansion of Hh-Smo signaling into the nasal mesenchyme resulted in a gain of oral gene expression concomitant with a loss of nasal gene expression, resulting in impaired osteogenesis of the palatine bones. We showed downregulation of extracellular matrix proteoglycans in the palatal mesenchyme,

which play multiple roles in osteogenesis (Raouf et al. 2002; Waddington et al. 2003). Furthermore, we confirmed Sox9 and Runx2 were downregulated along with a failure of BMP signaling in the nasal mesenchyme of the palate, coincident with ectopic expression of the dual Wnt/BMP antagonist, *Sostdc1*. Ectopic expression of *Sostdc1* in CNCCs directly antagonizes BMP-induced osteogenesis, resulting in cleft palate (Wu et al. 2008). Taken together, our data suggest that ectopic *Sostdc1* driven by expanded Hh-Smo signaling, at least in part, underlies the failure of BMP signaling and osteogenesis defects in mutant embryos.

During tooth and lip development, Shh negatively regulates Wnt signaling (Ahn et al. 2010; Kurosaka et al. 2014), while *Sostdc1* knockout mice have elevated Wnt signaling and supernumerary teeth (Zhang et al. 2009; Ahn et al. 2010; Cho et al. 2011). Our transcriptome data identified several upregulated Wnt antagonists, suggesting that Wnt signaling may be affected by ectopic Hh-Smo signaling. Furthermore, we noted that epithelial *Gli1* and *Ptch1* were reduced at E13.5 and *Shh* expression was secondarily downregulated in rugae at E15.5, which we suggest is due to epithelial-mesenchymal negative feedback mechanisms. Negative feedback via *Sostdc1* has been suggested for tooth and rugae patterning (Ahn et al. 2010; Lee et al. 2011). Thus, we speculate a Wnt-Hh-Sostdc1 negative feedback loop may also be present during secondary palate development. However, it is likely that other Wnt/BMP antagonists act in concert with *Sostdc1* to reinforce Wnt and BMP antagonism. In support of this hypothesis, pharmacological inhibition or genetic inactivation of Wnt antagonists rescued cleft palate in *Pax9*^{-/-} embryos (Jia et al. 2017; Li et al. 2017). Further work is needed to elucidate if *Sostdc1* and other Wnt/BMP antagonists are direct targets of Hh-Smo signaling during secondary palate development.

In this study, we identify *Foxl1* as a direct target of Gli1 in vivo. To delineate all Hh-Smo direct from downstream targets on a genome-wide scale, ChIP-seq data sets for the Gli transcription factors (Gli1, Gli2, and Gli3) on secondary palate tissue would enable the direct regulatory networks to be uncovered and will be addressed in future studies.

Author Contributions

M.J. Dixon, contributed to conception, design, and data interpretation, drafted and critically revised the manuscript; N.L. Hammond, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; K.J. Brookes, contributed to design and data interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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