Full Title: **Ectopic Hedgehog signalling 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 (S*HH*) signalling cascade. *Shh* is expressed in dynamic, spatio-temporal 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) signalling are incompletely characterised. Here, we show that ectopic Hh signalling 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 signalling in the secondary palate. Furthermore, we demonstrate that Wnt/BMP antagonists, in particular *Sostdc1*, are positively regulated by Hh signalling, concomitant with down-regulation of key regulators of osteogenesis and BMP signalling effectors. Our data demonstrate that ectopic Hh-Smo signalling down-regulates Wnt/BMP pathways, at least in part by up-regulating *Sostdc1*, resulting in cleft palate and defective osteogenesis.

Keywords

Shh; Wnt; BMP; cleft palate; osteogenesis

Introduction

Specification, growth, elevation, adherence and fusion of the palatal shelves are essential mechanisms involved in secondary palate formation (Dixon et al., 2011; Mossey et al., 2009). Disruption of these processes leads to cleft palate, a common congenital disorder that affects ~1:2500 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 healthcare 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 analysing palatogenesis (Bush and Jiang, 2012). In mice, palatal shelves initiate from the maxillary processes on embryonic day (E)11 grow lateral to the tongue during E12/E13 before re-orientating 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, keratinising 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) signalling pathway underlie several human congenital malformations which are associated with cleft palate (Cohen, 2010; Mansilla et

al., 2006). *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 signalling centres that drive epithelial-mesenchymal interactions (Lan and Jiang, 2009; Pantalacci et al., 2008; Rice et al., 2004; Rice et al., 2006). Thus, Shh signalling is linked spatio-temporally to both oral-nasal and anterior-posterior patterning of the secondary palate. Recent transgenic approaches to modulate Shh signalling within cranial neural crest cells (CNCC) (Jeong et al., 2004), facial epithelia (Cobourne et al., 2009; Kurosaka et al., 2014; Lan and Jiang, 2009; Rice et al., 2004), and secondary palate mesenchyme (Lan and Jiang, 2009) have demonstrated the critical importance of this pathway to normal secondary palate development. Shh signalling regulates expression of the transcription factors Foxf1, Foxf2, Osr2, and the growth factors BMP2, BMP4 and Fgf10 in palatal mesenchyme (Lan and Jiang, 2009) but the Shh-induced pathways controlling epithelial-mesenchymal cross-talk remain incompletely characterised.

In this study, we investigated how ectopic Hh signalling affects normal secondary palate development. Using a gain-of-function mouse model to activate Smoothened (*Smo*) signalling in the palatal mesenchyme (*Osr2-IresCre*;*Smo*$^{+/M2}$), we demonstrate that ectopic Hh-Smo signalling results in fully penetrant cleft palate and defective palatine bone formation. Using transcriptional profiling and expression analyses, we demonstrate Hh-Smo signalling is expanded and disrupts oral-nasal patterning by driving the expression of several transcriptional repressors and antagonists of Hh, Wnt and BMP signalling pathways. We show Hh-Smo signalling up-regulates 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 down-regulation of nasally-expressed master regulators of osteogenesis (*Sox9*, *Runx2*), bone-related extracellular matrix proteoglycans (*Dcn*, *Lum*) and BMP signalling effectors (pSmad 1/5/9). Our data suggest Hh-Smo signalling negatively regulates Wnt/BMP pathways by up-regulating antagonists, resulting in cleft palate and defective osteogenesis.

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 signalling 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 re-orientated above the tongue while those of mutant littermates had failed to elevate, 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 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 analysed 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 up-regulated in response to persistent Hh signalling.

To gain insight into the gene regulatory networks affected by increased Hh-Smo signalling, 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 up-regulated in response to increased Hh-Smo signalling. 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*). Over-representation Enrichment Analysis indicated significant enrichment of Gene Ontology terms including 'mesenchyme development', 'receptor serine/threonine kinase signalling' and 'cell fate commitment' (Appendix Fig. 3B; Appendix Table 4). Further annotation of these gene groups revealed up-regulation of several transcriptional repressors and antagonists of Hh (eg. *Ptch1*, *Hhip*, *Cdon*), Wnt and BMP (eg. *Sostdc1*, *Twsg1*) signalling pathways.

Hh-Smo direct and downstream targets are up-regulated 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* hybridisation. *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 down-stream of Hh signalling 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 up-regulated in the oral and nasal mesenchyme of mutant embryos (Fig. 3J, L). Transcriptional profiling identified *Foxl1* as the highest up-regulated 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 up-regulated and expanded from the oral to nasal palatal mesenchyme both in anterior and posterior regions of the palate (Fig. 3M-P). 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 signalling also up-regulated 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 inter-rugae domains in the anterior palatal epithelium and the posterior palate (Fig. 3Q, S) (Lee et al., 2011; Welsh and O'Brien, 2009). 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 up-regulated (Fig. 3R). Real-time qPCR confirmed significant up-regulation of all these genes in the palatal shelves of mutant embryos (Fig. 3U).

Sequential rugae interposition is blocked and Shh is down-regulated 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 - E15.5 showed the sequential addition of up to eight rugae in wild-type embryos (Appendix Fig. 4**A-C**), while mutants developed only three rugae (Appendix Fig. 4**D-F**). Furthermore, at E15.5 *Shh* expression was secondarily down-regulated in mutant rugae, while expression persisted in wild-type embryos (Appendix Fig. 4D,H).

Extracellular matrix proteoglycans in the nasal palatal mesenchyme are down-regulated

Transcriptional profiling indicated that the extracellular matrix proteins decorin (*Dcn*), lumican (*Lum*) and keratocan (*Kera*) were amongst the most significantly down-regulated 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 down-regulated 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 down-regulated in the palatal mesenchyme of mutant embryos (Fig. 4J, L). Real-time qPCR confirmed the reduction of *Dcn* and *Lum*, however *Dlx5* was not significant (Fig. 4M).

Master regulators of osteogenesis are down-regulated

Subsequently, we analysed 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 down-regulated 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 re-orientated nasal mesenchyme directly beneath the midline epithelial seam (Fig. 5G, I, K) while the expression of both proteins was markedly down-regulated 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 down-regulated and Sostdc1 is a BMP antagonist (Wu et al., 2008), we analysed whether BMP signalling was affected in mutant embryos. Immunofluorescence for pSmad 1/5/9 revealed the effectors of BMP signalling were also down-regulated in the nasal mesenchyme at E13.5 and the future palatine bone regions at E14.5 (Fig. 5M-P).

Collectively, these results demonstrate ectopic Hh-Smo signalling in the nasal mesenchyme down-regulates BMP signalling, concomitant with up-regulation 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 signalling, we analysed the promoters (-1 kb) of these genes for candidate Gli binding sites. No Gli sites were found near *Sostdc1.* However, we identified two highly conserved candidate binding sites in the *Foxl1* promoter (-237 and -371 from the TSS), with one mismatch from the Gli consensus (Appendix Fig. 5A), in regions of accessible chromatin (Appendix Fig. 5B). Gli1 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

Spatio-temporal Hh-Smo signalling defines a gene regulatory network which 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 (Lan and Jiang, 2009; Rice et al., 2004) and direct gene expression and cell fate through Gli transcription factors. Rice and colleagues showed that Shh signalling is crucial for palate development as disruption of Fgf10-Fgfr2b-Shh mesenchymal-epithelial signalling results in cleft palate (Rice et al., 2004), 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 down-stream of Hh-Smo signalling within the secondary palate remain poorly characterised.

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 are an aggressive tool which can disrupt normal physiological gene expression, such an approach has been used successfully to uncover targets of Hh signalling in the early embryonic face, limb, palate and brain (Jeong et al., 2004; Vokes et al., 2008; Lan and Jiang, 2009; Heine and Rowitch, 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 characterised by a fully penetrant wide cleft of the secondary palate with various skeletal defects. Taken together, this clearly illustrates a precise level of Hh-Smo signalling 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 (Han et al., 2009; Lan and Jiang, 2009; Rice et al., 2006), have shown that effectors of Hh-Smo signalling are expressed on the oral side of the palate whilst the nasal side is reportedly characterised by TGFβ/BMP mediators (Iwata et al., 2011; Parada and Chai, 2012). We demonstrated that elevated Hh-Smo signalling resulted in up-regulation and expansion of direct and down-stream targets of the Hh pathway within the palatal mesenchyme. Using transcriptional profiling and gene ontology analyses, we identified and characterised several

up-regulated transcriptional repressors and Wnt/BMP antagonists in particular *Foxf2, Foxl1* and *Sostdc1*.

Members of the Fox transcription factor family (*Foxd1, Foxd2, Foxc2, Foxf1 and Foxf2*) are dependent on *Smo* signalling in the early developing face, leading to the suggestion that Fox factors are the mediators of Hh-Smo signalling (Jeong et al., 2004). Indeed, loss of *Smo* in the palatal mesenchyme also results in down-regulation of *Foxf1* and *Foxf2* (Lan and Jiang, 2009). Our data demonstrate that *Foxf1*, *Foxf2* and the novel target *Foxl1* are all robustly up-regulated in response to increased Hh-Smo signalling within the palate, confirming these as *Smo*-dependent targets. Mutations in *FOXF2* have 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 characterised 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 *Foxl1* 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, however, studies in other tissues have revealed Foxl1 (in addition to Foxf1 and Foxf2) can indirectly affect epithelial proliferation via modulation of Wnt-β-catenin signalling (Kaestner et al., 1997; Madison et al., 2009; Perreault et al., 2001). Therefore we postulate Foxl1 may play a role in coordinating palatal growth via epithelial-mesenchymal feedback.

Expansion of Hh-Smo signalling 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 down-regulation 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 down-regulated along with a failure of BMP signalling 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 antagonises BMP-induced osteogenesis, resulting in cleft palate (Wu et al., 2008). Taken together, our data suggest that ectopic *Sostdc1* driven by expanded Hh-Smo signalling, at least in part underlies the failure of BMP signalling and osteogenesis defects in mutant embryos.

During tooth and lip development, Shh negatively regulates Wnt signalling (Ahn et al., 2010; Kurosaka et al., 2014), while *Sostdc1* knockout mice have elevated Wnt signalling and supernumerary teeth (Ahn et al., 2010; Cho et al., 2011; Zhang et al., 2009). Our transcriptome data identified several up-regulated Wnt antagonists, suggesting that Wnt signalling may be affected by ectopic Hh-Smo signalling. Furthermore, we noted that epithelial *Gli1* and *Ptch1* were reduced at E13.5 and *Shh* expression was secondarily down-regulated 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 signalling during secondary palate development.

In this study we identify *Foxl1* as a direct target of Gli1 *in vivo*. In order to delineate all Hh-Smo direct from down-stream targets on a genome-wide scale, ChIP-seq datasets 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.

Materials and Methods

Detailed Methods are in the Appendix. Microarray data has been deposited in ArrayExpress with the accession E-MTAB-5518.

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Figure Legends

Fig. 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 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, 300μm.

Fig. 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. md, mandible; mx, maxilla; pmx, premaxilla; p, palatine bone; ppp, palatine process of the palatine; ppmx, palatine process of the maxilla; pppmx, palatine process of the premaxilla; als, alisphenoid; fr, frontal bone; ps, presphenoid; bs, basosphenoid; bo, basioccipital; eo, exoccipital; agp, angular process; cdp, condyloid process; crp, coronoid processes.

Fig. 3. Hedgehog direct and down-stream targets are up-regulated in *Osr2-IresCre*;*Smo*$^{+/M2}$ embryos. Whole-mount (**A,B,E,F,I,J,M,N,Q,R**) and section *in situ* (**C,D,G,H,K,L,O,P,S,T**) hybridisation 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 up-regulated 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**). Up-regulation and expansion of both Fox factors is seen in mutant palates (**J,N,L,P**). Expression of *Sostdc1* is observed in inter-rugae domains of the epithelium and in the posterior palate In contrast, *Sostdc1* is ectopically expressed in the nasal mesenchyme and up-regulated 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 qPCR analysis of E13.5 palatal shelves confirms up-regulation of all targets ($p<0.05$ *, $p<0.01$ **; Mann-Whitney U test, n=5). ps, palatal shelf; t, tongue; md, mandible. Scale bars: C,D,G,H,K,L,O,P,S,T 100 μm.

Fig. 4. Extracellular matrix proteoglycans are down-regulated in *Osr2-IresCre;Smo*$^{+/M2}$ embryos. Whole-mount (**A,B,E,F,I,J**) and section *in situ* (**C,D,G,H,K,L**) hybridisation 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 qPCR data confirm significantly reduced levels of mRNA for *Dcn* and *Lum* ($p<0.05$ *, Mann-Whitney U test, n=4). ps, palatal shelf; t, tongue; md, mandible. Scale bars: C,D,G,H,K,L 100 μm.

Fig. 5. Master regulators of osteogenesis are down-regulated 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 indicate 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**). Auto-fluorescence of red blood cells is identified by triple immunofluorescent images (**M-P**; yellow). ps, palate; t, tongue; md, mandible. Scale bars: A-P, 100 μm.