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Role of Indian Hedgehog Signaling in Palatal Osteogenesis

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Abstract

Introduction—Cleft lip and palate are common birth defects and represent a large biomedical burden. Through the use of animal models, the molecular underpinnings of cleft palate are becoming increasingly clear. Indian Hedgehog (Ihh) has been shown to be associated craniofacial development and active in the palatine bone. We hypothesize that Ihh activity plays a role in osteogenesis within the secondary palate and that defects in this pathway may inhibit the osteogenesis of the secondary palate.

Methods—Palates were isolated from wild type mice during the period of palate development (e9.5–e17.5). qRT-PCR was used for detecting gene expression during osteogenic differentiation and cellular differentiation (Shh, Ihh, Ptc1, Gli1, Gli2, Gli3, Runx2, Alp, Col1a1). Next, palates were analyzed by H&E, Aniline Blue, Pentachrome, and *In situ* Hybridization to assess osteogenesis of the palatal shelf and expression of hedgehog pathway genes. Finally, the palate of Indian Hedgehog null mice was analyzed to determine the effect of genetic deficiency on palatal development osteogenesis.

Results—Increased Indian Hedgehog and osteogenic signaling coincided with ossification and fusion of the palate in wild-type mice. This included a 5–150 fold peak in expression of Hedgehog elements, including *Ihh*, at e15.5 as compared to e9.5. Contrarily, loss of Indian Hedgehog by genetic knockout (*Ihh*^{−/−}) resulted in decreased secondary palate ossification.

Conclusion—Our results suggest a role for Hedgehog signaling during palatal ossification. The hedgehog pathway is activated during palatal fusion and deletion of Indian hedgehog leads to diminished ossification of the secondary hard palate.

Keywords

Palatal Development; Cleft palate; Indian Hedgehog; Osteogenesis

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Introduction

The incidence of cleft lip varies from 1 in 500 to about 1 in 1000 births, while isolated cleft palate has an incidence of approximately 1 in 1000 births.(1) The commonality of clefting creates an enormous biomedical burden. Affected children with cleft lip/palate require multiple operations beginning as early as 3 months of age with the goals of cleft closure and establishing normal facial form, normal midface maxillary position, proper dental occlusion, and preventing hearing loss. The molecular underpinnings of cleft palate are increasingly being understood complicated and clearly multifactorial.

Defects in signaling pathways, such as Wnt and NFAT have been shown to increase the propensity of clefting.(2-4) Another signaling pathway, the Hedgehog pathway is known to play a major role in craniofacial development, however its association with clefting remains unclear.(5) We set out to examine the potential role of Indian Hedgehog (Ihh) signaling pathway in palatal development. We hypothesized that Ihh activity may play a role in osteogenesis within the secondary palate and that defects in this signaling pathway may inhibit the osteogenesis of the secondary palate.

Hedgehog signaling has been shown to modulate mammalian skeletal development. The Hedgehog family of secreted proteins consists of Indian hedgehog (Ihh), Sonic hedgehog (Shh) and Desert hedgehog (Dhh).(6) Hedgehog signaling is initiated by the binding of the Hedgehog ligand to its transmembrane receptor Patched1 (Ptc1), which in turn, relieves suppression of the transmembrane protein Smoothened (Smo). Smo then activates an intracellular cascade that results in activation of Gli transcription factors.(7) Both Ihh and Shh have been shown to play a key role in skeletal development, and mutations in the Hedgehog pathway have been shown to lead to craniofacial malformations such as holoprosencephaly.(8-11)

Ihh has also been shown to play a key role in skeletogenesis, specifically with regards to periosteum induced osteoblast differentiation.(12) Ectopic expression of Ihh has been shown to promote osteoblast differentiation.(13) Most studies on Ihh and skeletal development, however, focus on long bone or endochondral ossification.(13-16) A paucity of research exists with regards to the effect of Ihh on intramembranous ossification, the mechanism active during palatal development. A case report of a child with a gene deletion in the location of the hedgehog gene demonstrated a submucous cleft palate further supporting the idea that the hedgehog pathway may play a crucial role in the skeletal development of the palate rather than in fusion of the mucous portion.(11) Deletion of Smo, has also been shown to result in facial deformities.(17) Further studies of Smo deficient mice demonstrated decreased, proliferation and differentiation of cranial neural crest cells, the progenitors of the palatal bones.(17,18) Though Smo can be activated by Shh or Ihh, only Ihh was shown in previous studies to be upregulated in the developing palatine bones.(19)

Herein, we analyze the correlation between Ihh and palatal osteogenesis. We quantified Hedgehog signaling expression over palatal development by qRT-PCR. Next, we utilize Ihh^{-/-} transgenic mice to determine the role of Hh signaling in defining the osteogenic capacity of the palatal shelves. The combination of these techniques suggested that Ihh signaling derived from palatal bone may induce palatal ossification.

Methods

Animals

Animals were maintained in the Stanford Animal Care Laboratory and received food and water ad libitum. For all experiments, animals were bred over night and the day of vaginal

was considered as e0.5 day of gestation. Time-gestations of mice were obtained from Charles River Laboratories (Wilmington, Mass.). *Ihh*^{+/-} mice were mated to generate *Ihh*^{-/-} embryos on a C57B6 background. The tails of the embryos were used for genotyping by PCR as previously described (12). To generate *Ihh*^{+/-};*Ptc-lacZ* mice, *Ihh*^{+/-} mice were crossed with *Ptc-lacZ* mice that contain an insertion of the lacZ transgene in one patched 1 (*Ptc1*) locus (20). *Ihh*^{+/-};*Ptc-lacZ* mice were mated with *Ihh*^{+/-} mice to obtain *Ihh*^{-/-};*Ptc-lacZ* embryos. All procedures were approved by the Stanford University Animal Care and Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals.

Palatal shelf dissection

Wild-type pups were harvested on embryonic (e) days e9.5, e11.5, e13.5, e15.5 and e17.5 of gestation. Embryos were harvested and immediately placed on ice in cold, sterile, phosphate-buffered saline. Microdissection was performed on the individual embryos to isolate and remove the palatal shelves. Once dissected, palatal shelves were snap-frozen in liquid nitrogen for gene expression analysis.

RNA Isolation and Real-Time PCR

Once dissected, wild type palates (e9.5-17.5) were pooled (N=3 distinct pooled samples, consisting of 3-4 microdissected palates per sample), and homogenized by sonification. RNA was isolated per the manufacturer's instructions (RNeasy Kit, Qiagen Sciences, Maryland), genomic DNA was removed (DNA-free kit, Ambion, Austin, TX), and a total of 1 ug RNA was reverse-transcribed (Taqman Reverse Transcription Reagents, Applied Biosystems, Foster City, CA). Quantitative real-time PCR was carried out using the Applied Biosystems Prism 7900HT Sequence Detection System and Power Sybr Green Mastermix (Applied Biosystems, Foster City, CA). Specific primers were designed based on PrimerBank sequences (<http://pga.mgh.harvard.edu/primerbank/>). Sequences are shown in Table 1. All reactions were performed in triplicate; relative values were calculated to housekeeping gene (*GAPDH*) and presented as means and standard deviations.

Tissue Processing and Histology

For histological analysis, sections were prepared from tissues fixed overnight in 0.4% PFA, decalcified in 19% EDTA (pH 7.4) at 4°C and dehydrated through graded ethanol for paraffin embedding. For histological staining, 5 µm paraffin-embedded tissue sections were stained with Aniline Blue, and Movat's Pentachrome bone stain. Alkaline phosphatase staining was performed using NTMT ALP buffer (5M NaCl, 1M Tris, 1M MgCl₂, Tween20), and nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-indolyl-phosphatase (BCIP) for color (Roche, Indianapolis, IN). *In situ* hybridization was performed on select slides for mouse *Ptc1*, *Ihh*, *Runx-2*, *Ocn* and *Bmp-2* as previously described.(21) Non-specific binding was minimized by high stringency hybridization conditions, for all assays sense probes were used side-by-side with minimal background.

Whole mount skull preparation

To evaluate the palate phenotype of *Ihh*^{-/-} embryos whole mount bone and cartilage staining was performed as previously described.(21) Specimens were fixed in 100% ethanol for 48 h, transferred to acetone for 48 h, stained in 0.15% alcian blue in 20% glacial acetic / 80% ethanol for 24 h, fixed in ethanol for 24 h and cleared in 1% aqueous KOH for 24-31 h prior to staining in 0.005% alizarin red in 1% KOH for 15 h. The tissue was cleared through 20% glycerol 1% KOH for 24-72h, followed by increasing concentrations of glycerol in dH₂O up to 70%.

Detection of *Ptc*-LacZ transgene expression

Expression of the *Ptc-lacZ* transgene was compared in serial coronal sections between *Ihh*^{-/-} embryos and *Ihh*^{+/+} littermates. Briefly, sections were fixed in 1% formaldehyde, 0.2% glutaraldehyde in PBS for 10 min at 4°C, washed twice in 0.01% sodium deoxycholate, 0.02% Nonidet P-40 in PBS at room temperature, and stained in 1% deoxycholate, 2% Nonidet P-40, 2mM MgCl₂, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mg/mL Xgal in PBS at 37°C.

Statistical analyses

Means and standard deviations were calculated from all numerical data. In graphs, all bars represent means whereas all error bars represent one standard deviation. Statistical analyses were performed using the Welch's two-tailed t-test when comparing two groups with unequal standard deviations. When comparing multiple groups, a one-way ANOVA test without replication was performed, followed by post-hoc analysis using the Bonferroni correction to adjust for multiple comparisons. *P<0.05 was considered significant, unless otherwise stated.

Results

Osteogenic Gene Expression Increases with Palate Embryonic Development

We first set out to define and quantify osteogenic gene expression during mouse palatal development and ossification. To do this, palatal shelves were harvested at stratified embryonic timepoints (from embryonic age (e)9.5-17.5) by microdissection and qRT-PCR analysis as described in the methods above. Genes associated with early osteogenesis were analyzed, including Runt-related transcription factor-2 (*Runx2/Cfba1*), Alkaline phosphatase, and *Type I Collagen*. Overall, a significant increase in gene expression was observed during ossification events, beginning at e13.5 and continuing until e17.5 (Figure 1A,B). *Alkaline Phosphatase* and *Type I Collagen* increased between e13.5 and e17.5 (Figure 1A,B). *Runx2/Cfba1*, the known master transcription factor for osteogenesis, increased overtime starting at e11.5 with a peak in expression at e15.5 (Figure 1B). These gene expression profiles corresponded with secondary palate development, which begins at e11.5, undergoes condensation at e12.5 and ossification at e13.5 and continues through e17.5.

Hedgehog pathway activity colocalizes with Osteogenic Gene Expression during Palatal Development

Next, we analyzed Hedgehog pathway activity during palatal development. First, *in situ* hybridization for hedgehog responsive element *Ptc1* was performed (Figure 2A). *Ptc1* expression correlated with newly ossified bone as marked by pentachrome and aniline blue staining in the palatine bones (Figure 2A). To further validate the presence of Hh activity in the developing palatine bones we analyzed *Ihh* and *Shh* levels within the palatal mesenchyme via qRT-PCR. *Ihh* expression was significantly increased between e13.5 and e17.5, with peak expression at e15.5 (Figure 2B), whereas *Shh* was significantly upregulated at e15.5 and e17.5 (Figure 2C). Upregulation of the Hedgehog ligands was accompanied by increased expression of Hedgehog targets including, *Ptc1*, *Gli1* and *Gli2* (Figure 2C). *Gli1* and *Gli2* demonstrated a significant upregulation between e11.5 and e17.5, with peak expression at e15.5 (Figure 2C), whereas *Ptc1* demonstrated a significant upregulation at e15.5 and e17.5 (Figure 2C). *Gli3* did not reach statistical significance (Figure 2C). This is what we expected given that *Gli1* and *Gli2* are known to be activators and *Gli3* is known to be a repressor of Hedgehog pathway activity. In summary, Hedgehog pathway activity was

detected in the palatal mesenchyme and developing palatine bones during palatal development and correlated with markers of osteogenic differentiation.

Indian Hedgehog Null Mice have Decreased Osteogenic Gene Expression

Results from our histology, *in situ* hybridization and RT-PCR as well as previous studies suggest that *Ihh* might play a role in the developing palatine bones(19). We therefore inquired as to the specific signal of *Ihh* in secondary palate ossification. To answer this question, we examined the palatal phenotype of the *Ihh* null mouse (Figure 3).

In order to appreciate differences in osteogenic activity, we assessed alkaline phosphatase activity on histological sections of e16.5 wild-type and *Ihh*^{-/-} palates (Figure 3A). A notable decrease in alkaline phosphatase activity in the palatal shelves was observed in the *Ihh*^{-/-} mice when compared to their wild-type littermates.

In addition to the differences we observed in alkaline phosphatase activity, we also found relative differences in level of gene expression of known osteogenic markers at e16.5 by *in situ* hybridization (Figure 3B-D). Expression of *Runx2* (Figure 3B), *Osteocalcin* (*Ocn*) (Figure 3C), and *Bmp2* (Figure 3D) transcripts within the palatine bone and surrounding areas, were appreciably decreased in *Ihh*^{-/-} palates when compared to wild-type littermates. *In situ* hybridization of *Ihh* was performed, which confirmed that *Ihh* is expressed in high levels in the palatal shelf and palatine bone in the wild-type mice (Figure 3E, **left column**). As expected, *Ihh* was not expressed in the *Ihh*^{-/-} mice (Figure 3E, **right column**), further confirming the genotype of the null mutant mice.

Indian Hedgehog Null Mice have Impaired Palatal Osteogenesis

To detect palatal ossification, whole mount bone and cartilage staining was performed (Figure 4A). An appreciable decrease in size of palatine bones was observed in the *Ihh*^{-/-} mice as compared to *Ihh*^{+/+} littermates at e16.5 and e17.0 (Figure 4A). Upon close examination of the e17.0 null mouse, one can see both decreased osteogenesis by alizarin red stain and a cleft in the midline of the palatine bone (second column, Figure 4A).

To visualize *Ihh* activity in *Ihh* null mice, we crossed *Ihh*^{+/-} into the *Ptc-LacZ* background (20) (Figure 4B). As expected, Xgal staining was reduced in the palate of *Ihh*^{-/-} *Ptc-LacZ* mice, most notably at the ossification fronts of the palatine bone. Staining was equivalent between *Ihh*^{+/+} and *Ihh*^{-/-} mice in other areas, such as the oral epithelium (Figure 4B).

Discussion

In humans, it is estimated that 70% of cases of cleft lip and palate are nonsyndromic with complex inheritance patterns (22). Significant research efforts have been made to identify specific gene loci that are linked to cleft palate formation. Thus far, some progress has been made through the identification of TGF- α , TGF- β 3 and MSX1, however, these have not been related to specific genetic variations with identifiable functional consequences(23,24). MSX1 has led to the most fruitful findings as heterozygous MSX1 mutations have been shown to result in cleft lip and palate as well as tooth agenesis(25). Syndromic causes of cleft palate with osteochondrodysplasias such as Stickler Syndrome have been linked to mutations in COL1A1 and COL1A2(26).

In mice, secondary palatal development begins on e12.5. The maxillary processes give rise to bilateral palatal shelves, which consist of a mesodermal core surrounded by neural crest and subsequently by pharyngeal endoderm and ectoderm. These palatal shelves initially grow vertically downward, flanking the tongue. On e14.5, the initial reflex opening and closing movements of the mouth occur, which subsequently permit descent of the tongue

and the horizontal alignment of the palatal shelves. By e15.5, palatal fusion is complete and mesenchymal condensation followed by osteogenic differentiation occurs. Hedgehog ligands such as *Ihh* have been shown to localize to areas of the palate from e11.5-14.5 (19).

Targets and effectors of the Hedgehog pathway are expressed in the palate epithelium and mesenchyme, and *Ihh* is specifically expressed in developing palatine bones(19,27). During skeletogenesis, Indian Hedgehog plays a role in endochondral ossification (6,28). In addition, *Ihh* has been implicated in intramembranous ossification, although disagreement exists on their precise roles (29,30). Specifically, *Ihh*^{-/-} mice have been shown to demonstrate cranial base synchondrosis and brachycephaly but there was no comment on abnormal mandibular development or tongue descent that might prevent palatal fusion (28).

Though previous studies have shown that *Shh* inhibition causes a cleft palate and that *Ihh* is localized in the palatine bone (19), previous studies have not investigated the effect of a genetic knockout of *Ihh* on palatal clefting(27). We hope this current study sheds some light on the involvement of the hedgehog pathway, and specifically *Ihh* in the secondary hard palate.

Hedgehog signaling exerts pleiotropic effects through regulation of the cell cycle,(31) cell differentiation,(32) and alteration of cell survival(33) which have been shown to play a key role in limb development (13-16). Increased Hedgehog signaling promotes osteogenesis in various bone-forming cells *in vitro*(34-37). We present an analysis of the effects of Hedgehog signaling in intramembranous ossification as seen in palatal development. First, we demonstrated osteogenic gene expression during normal palatal development. Next we showed an increase in Hedgehog activity during palatal development *in vivo*. Finally, we demonstrate mice lacking *Ihh* have deficiencies in osteogenic gene expression and osteogenesis within the secondary palate. Taken together, these data provide evidence that *Ihh* signaling favors the differentiation of palatal tissue to an osteogenic lineage and suggest a mechanism whereby cell fate can be directed to further study palatal development. Submucous cleft palate, a subset of a cleft palate where there is fusion of the soft palate but incomplete fusion of the hard palate that may represent a similar phenotype to the one seen in our *Ihh* null mice.

Conclusion

Osteogenic genes are upregulated during palatal development and this coincides with an upregulation of *Ihh*. *Ihh* may play a role in the osteogenic differentiation of the secondary palate and the *Ihh* null mouse represents a model of incomplete hard palate ossification.

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Figure 1.

Palatal osteogenic gene expression. (A-B) Quantitative assessment of osteogenic gene expression overtime throughout embryonic palatal development, normalized to *Gapdh* (including Alkaline Phosphatase, Runx2/Cbfa1, and Type I Collagen, respectively). Asterisks indicate statistical significance compared to level of gene expression at E9.5 for a given gene. (N=3 distinct pooled samples, consisting of 3-4 microdissected palates per sample), * $p < 0.05$.

Figure 2.

In vivo Hedgehog signaling gene expression. (A) Expression of *Ptc1* in palatine bones by *in situ*. (Left) H&E is provided for orientation with a box signifying the palatine bone (pb) within the palatal shelf. From left to right, H&E, *Ptc1 in situ* hybridization in which signal appears purple, aniline blue staining in which osteoid appears blue, and pentachrome staining in which bone appears yellow. (B-C) Expression of signaling components was examined by real-time PCR, normalized to *Gapdh*. (B) *Indian Hedghog (Ihh)* expression (C) *Sonic Hedgehog (Shh)*, *Patched1 (Ptc1)*, *Gli1*, *Gli2* and *Gli3* expression. Asterisks indicate statistical significance compared to level of gene expression at E9.5 for a given gene. N=10 palates per timepoint, * $p < 0.05$.

Figure 3.

Histology of Indian Hedgehog $^{-/-}$ phenotype at e16.5. (A) Alkaline phosphatase stain of wild-type (left) and $Ihh^{-/-}$ littermates (right) at e16.5. There is greater alkaline phosphatase activity (more purple staining) in the palatal shelf near the palatine bone (pb) in wild-type palates than in Ihh null mutants. (B-D) *In situ* hybridization for the osteogenic genes *Runx2*, *Ocn* and *Bmp2*, respectively (signal is dark purple). These osteogenic genes are more highly expressed in the wild-type than in the $Ihh^{-/-}$ palates within and around the palatine bone. The dotted lines in the first and third columns indicate the area shown in higher magnification in columns 2 and 4, respectively. (E) *In situ* hybridization for *Ihh*, showing no expression in the Ihh null mutant (right column) and expression in the palatal shelf and palatine bone in the wild-type littermates (left column). The tongue (t) and nasal cavity (nc) are labeled for orientation purposes. Control experiments using sense probes were performed for all genes (data not shown).

Figure 4.

Ihh^{-/-} palatal phenotype. (A) Bone and cartilage whole mount staining among wild-type (left) and *Ihh*^{-/-} littermates (right). White dotted lines indicate the location of the palatine bones (pb). Additionally, the basisphenoid (bs), vomer (v), and maxilla (m) are labeled for orientation purposes. (Top Row) e16.5 with a larger palatine bone in the *Ihh*^{+/+} than the *Ihh* null (marked with white dotted line). (Bottom Row) e17.0 whole mount stain close up of the palatine bone in the center. Again, dotted line demonstrates area of palatine bone (pb). Note the midline cleft in the *Ihh*^{-/-} palatine bone. (B) Location of Hedgehog responsiveness among palates of wild-type (left) and *Ihh*^{-/-} littermates (right) at e16.5 and e18.5.

Representative coronal sections of Xgal staining are shown in a *Ptc/LacZ* reporter mouse. Staining appears blue. Palatine bone (pb) is outlined with a dotted white line with the tongue (t) labeled below. Images are taken at 10 $\times$ magnification. Note the wider area with decreased LacZ staining in the midline of the *Ihh*^{-/-} palates between the palatine bones at e16.5 and e18.5.

TABLE 1

Quantitative PCR Genes and Primer Sequences

Gene Name	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
<i>Alkaline Phosphatase</i>	GTTGCCAAGCTGGGAAGAACAC	CCCACCCCGCTATTCCAAAC
<i>Gapdh</i>	AGGAGTATATGCCCCGACGTG	TCGTCCACATCCACACTGTT
<i>Gli1</i>	TCGACCTGCAAACCGTAATCC	TCCTAAAGAAGGGCTCATGGTA
<i>Gli2</i>	GGCAGGGGTATCCACAGA	AGAAGGACCCATGTTGGAGTC
<i>Gli3</i>	CACAGTCTAGGCGACTG	CTGCATAGTGATTGCGTTTCTTC
<i>Ihh</i>	GCTTCGACTGGGTGTATTACG	GCTCGCGGTCCAGGAAAAT
<i>Ptc1</i>	GCCAAGCCCTAAAAAAT	ACCACAATCAATCTCCTG
<i>Runx2/Cbfa1</i>	CGGTCTCCTTCCAGGATGGT	GCTTCCGTCAGCGTCAACA
<i>Shh</i>	AAAGCTGACCCTTTAGCCTA	TTCGGAGTTTCTTGTGATCTTCC
<i>Type I Collagen</i>	AACCCGAGGTATGCTTGATCT	CCAGTTCTTCATTGCATTGC