

## Hedgehog Signaling in Palatal and Facial Development

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### ABSTRACT

The Hedgehog (HH) signaling pathway has shown to be crucial in modulating mammalian skeletal and craniofacial development, mutations in which can lead to craniofacial malformations such as cleft lip/palate. With three known homologues, including Desert Hedgehog (DHH), Sonic Hedgehog (SHH), and Indian Hedgehog (IHH), only the latter two are expressed in the craniofacial complex. The focus of this review pertains to the role of SHH and IHH signaling in palatal and facial bone development, and the underlying differences between the two. While expression of both SHH and IHH is required for calvarial ossification and craniofacial development, they differ in where they are expressed and how they elicit their effects. SHH is not expressed in the facial mesenchyme, but rather in the frontonasal and maxillary cranial suture mesenchyme. IHH expression by contrast occurs on developing palatine bone and in

most studies pertains to endochondral ossification, playing a key role in inducing cell proliferation and differentiation.

**Key words:** Facial development; Palatal development; Sonic Hedgehog; Indian Hedgehog

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### INTRODUCTION

The Hedgehog (HH) signaling pathway is responsible for regulating the morphogenesis and development of many organs, including skeletal development in mammalian vertebrate organisms<sup>[1]</sup>. With three known homologues of the HH ligand, consisting of Desert Hedgehog (DHH), Sonic Hedgehog (SHH), and Indian Hedgehog (IHH), only the latter two are critical to embryonic skeletal development; knockout in murine models results in neonatal lethality and multiple craniofacial abnormalities such as holoprosencephaly and cleft lip/palate<sup>[2-7]</sup>. Despite remaining well conserved across their signaling cascades, the HH ligands have distinct domains of expression and function, which at times may overlap<sup>[8]</sup>.

The SHH signaling pathway is critical to embryonic development and is thought to depend upon epithelial-mesenchymal interactions<sup>[9]</sup>. SHH plays a distinct role in determining the organizational pattern of neural tubes, somites, and limbs<sup>[9]</sup>, is implicated in vertebrate organogenesis, and promotes adult stem cell proliferation in hematopoietic, mammary, and neural tissues<sup>[10]</sup>. SHH regulates osteoprogenitor cell differentiation by enhancing osteogenic differentiation and inhibiting adipogenic differentiation in multiple mesenchymal cell types<sup>[11]</sup>. Furthermore, SHH is involved in skeletogenesis and development of facial, appendicular, and axial skeletal patterns<sup>[9]</sup>. Thus, improper ossification occurs in developing

animals as a result of impaired SHH signaling and can lead to mutations such as cleft palate<sup>[12]</sup>.

Meanwhile, the IHH signaling pathway is involved in chondrocyte differentiation and proliferation, as well as maturation during endochondral ossification. IHH coordinates endochondral growth and morphogenesis via parathyroid hormone related-protein-dependent and -independent pathways<sup>[13]</sup>. Furthermore, in the context of palatal development, IHH expression has shown to be specific to the developing palatine bones<sup>[14,15]</sup>.

The focus of this review is to further elucidate how SHH and IHH elicit their effects on palatal and facial development, and the differences between the two in this context. While both also play a major role in fusion of cranial sutures and calvarial ossification, this is beyond the scope of this review (Please see<sup>[16]</sup> for a detailed review on HH signaling in cranial suture fusion).

## BRIEF REVIEW OF THE HEDGEHOG SIGNALING MECHANISM

The HH signaling pathway plays an essential role in mesodermal tissue patterning and differentiation<sup>[17,18]</sup>. Originally identified in *Drosophila melanogaster* as an embryonic segment polarity gene<sup>[19]</sup>, HH was later isolated in vertebrate organisms as a modulator of mammalian skeletal development<sup>[1]</sup>. Three homologues of the HH family include DHH, SHH, and IHH<sup>[8]</sup>. Though DHH is implicated in Leydig cell differentiation in the male reproductive system and Schwann cell function, its function is not well-studied in the context of bone biology<sup>[20-22]</sup>. Conversely, both SHH and IHH are critical to embryonic skeletal development. Knockout studies using murine models have resulted in neonatal lethality and multiple craniofacial abnormalities such as holoprosencephaly<sup>[2-7]</sup>. In addition, SHH regulates vertebrate organogenesis and promotes adult stem cell proliferation in hematopoietic, mammary, and neural tissues<sup>[23,24]</sup>, whereas IHH couples chondrogenesis to osteogenesis during endochondral bone formation primarily in the appendicular skeleton<sup>[25]</sup>.

The HH signaling pathway occurs via a three-step mechanism which is generally conserved across all three homologues<sup>[26]</sup>. First, activation of the pathway occurs via autocatalytic cleavage, followed by modification of the C and N-termini with cholesterol and palmitoyl acid respectively<sup>[27-29]</sup>. Hedgehog acyltransferase (HHAT) is essential to the post-translational palmitoylation of HH, and HH secretion is downregulated in the absence of this enzyme<sup>[11]</sup>. The conversion of HH to a multimeric form is critical to increasing its membrane solubility and ability to undergo paracrine signaling<sup>[26]</sup>. Next, the activated HH is then released via Dispatched (DISP), a 12-pass transmembrane protein from the signaling cell, thus enabling it to bind to its receptor Patched (PTCH) on the receiving cell<sup>[30]</sup>. This binding disinhibits and releases Smoothed (SMO), a 7-pass transmembrane protein that activates downstream target genes through interaction with transcription factors from the glioblastoma gene product family (Gli)<sup>[31]</sup>. Consequently, HH-Gli signaling modulates lineage commitment and cell fate during differentiation primarily through transcription<sup>[32]</sup>.

## HEDGEHOG SIGNALING IN PALATAL DEVELOPMENT

The primary palate comprises all structures anterior to the incisive foramen including the lip, nostril sill, alveolus and hard palate anterior to the incisive foramen. The medial and lateral nasal

prominences of the frontonasal process migrate and fuse with the maxillary prominence to form the primary palate in humans during weeks 4-7 of gestation. The secondary palate includes the hard palate posterior to the incisive foramen as well as the entire soft palate. Migration and fusion of the lateral palatal process of the maxillary prominence form the secondary palate between 5-12 weeks of gestation in humans. At 8 weeks of gestation the lateral palatal processes are vertical and as the tongue drops, the palatal processes rotate into a horizontal position and fuse from anterior to posterior. The right lateral palatal process becomes horizontal before the left explaining the greater incidence of deformities on the left side. The cause of aberrations in these developmental processes are still not completely understood and recent studies have explored the role of HH signaling in cleft palatal deformities. HH signaling has been shown to play a crucial role in skeletogenesis, and is specifically implicated in palatal development<sup>[33-35]</sup>. For example, Morava *et al* examined a case study of a 14-month-old girl with submucous cleft palate and found a duplication of the chromosome region 7q35-qter located near the SHH gene on 7q36, suggesting that HH regulates palatogenesis<sup>[6]</sup>. Several studies have also investigated how the HH signaling pathway affects cranial neural crest cells (CNCC), which serve as the progenitors of palatal bones. Jeong *et al* found that deletion of *Smo* in murine embryonic CNCC results in craniofacial abnormalities such as an incomplete nasal septum, absent vomer and hyperplasia<sup>[36]</sup>. Furthermore, SHH rescues CNCC from ethanol-induced cell death and the associated craniofacial growth defect<sup>[37]</sup>. Thus, these early studies suggest that HH signaling plays a role in palatogenesis, likely through affecting the osteoprogenitor cells.

Although both SHH and IHH homologues play a significant role in palatal development, it is primarily SHH that regulates palatal growth. Rice *et al.* showed that prior to palatal shelf elevation, SHH signaling regulates the growth and morphogenesis of the palatal shelves and is limited to thickened palatal oral epithelium<sup>[14]</sup>. After palatal shelf elevation, SHH is strongly expressed in the oral epithelium in a pattern of stripes that correspond to the future rugae palatine<sup>[14,33]</sup>. SHH is also an important epithelial signal for regulating palatal growth, which is dependent upon reciprocal interactions between the oral ectoderm and underlying neural-crest-derived mesenchyme<sup>[34]</sup>. Specifically, SHH-Smo signaling modulates the expression of transcription factors *Foxf1a*, *Foxf2*, and *Osr2*, and *Bmp2*, *Bmp4*, and *Fgf10*, which are present in the palatal mesenchyme and affect palatal epithelial cell proliferation<sup>[34]</sup>. Moreover, mice lacking SHH signaling develop secondary cleft palate<sup>[33]</sup>.

In another study, Sasaki *et al* implicated the involvement of *Shh* and *Pax9* in transforming growth factor-beta (*TGF-β3*)-regulated normal palatal fusion by investigating the expression of both genes during normal mouse palate development and in the developing cleft palates of *TGF-β3* null embryos 38. Mice lacking *TGF-β3* exhibit incomplete failure of palatal shelf fusion and thus are a suitable model to examine the molecular cause of isolated cleft palate<sup>[39]</sup>. Compared to wild-type mice, *Pax9* expression was reduced in the palatal medial edge at the critical time of palatal fusion (embryonic days 14.5-15.5), and *Shh* expression was reduced throughout embryonic days 12.5-15.5 in the palates of *TGF-β3* null mice<sup>[38]</sup>. Thus, this experiment indicates that the correlation between the expression of *TGF-β3*, *Pax9*, and *Shh* is crucial for normal cleft palate formation.

Similarly, Zhang *et al* used the *Msx1*-deficient mice model to study the genetic regulation of mammalian palatogenesis through the SHH signaling pathway<sup>[40]</sup>. *Msx1*-deficient mice exhibit severe craniofacial abnormalities including cleft secondary palate, an absence of alveolar processes, and arrest of tooth development at the bud stage<sup>[41]</sup>.

Likewise, mutations in the human *MSX1* gene are associated with isolated non-syndromic cleft palate and tooth agenesis<sup>[42]</sup>. Zhang *et al* has shown that *Msx1* is required for the expression of both *Bmp4* and *Bmp2* in the palatal mesenchyme and *Shh* in the medial edge epithelium (MEE)<sup>[40]</sup>. Furthermore, disruption of *Msx1* function impairs cell proliferation in the palatal mesenchyme, which leads to formation of cleft secondary palate<sup>[40]</sup>. However, ectopic expression of *Bmp4* in *Msx1* knockout mice restores *Bmp2* and *Shh* expression, rescues the cleft palate phenotype, and recovers normal cell proliferation in the palatal mesenchyme<sup>[40]</sup>. Thus, *Bmp4* functions upstream of *Bmp2* and *Shh* to regulate palate development and is able to bypass the requirement for *Msx1*<sup>[40]</sup>. Further *in vitro* analysis revealed that *Shh* derived from the MEE activates expression of *Bmp2* and stimulates cell proliferation<sup>[40]</sup>. Thus, this study has elucidated how *Msx1* regulates *Bmp* and *Shh* signaling, which in turn affects mammalian palatogenesis.

Likewise, correlation has also been observed between IHH and palatal osteogenesis. Levi *et al* found that HH pathway activity colocalizes with osteogenic gene expression of during palatal development<sup>[35]</sup>. Specifically, upregulation of HH ligand *Ihh* and HH targets *Ptch1*, *Gli1*, and *Gli2* coincided with increased expression of Alkaline Phosphatase and *Type I Collagen* in the palatal mesenchyme and developing palatine bones<sup>[35]</sup>. Furthermore, *Ihh* null mice displayed impaired palatal osteogenesis, as indicated through decreased expression of osteogenic markers *Runx2* and *Osteocalcin* and decreased ossification of the palatine bone compared to wild-type mice<sup>[35]</sup>.

## HEDGEHOG SIGNALING IN FACIAL BONE DEVELOPMENT

Craniofacial development is a delicate developmental processes requiring the control of cell commitment, proliferation, and differentiation. SHH and IHH are expressed in the craniofacial complex and are both integral to the process of calvarial ossification. The frontonasal prominence of the forebrain results in nasal and olfactory placodes that become medial and lateral processes. The mandibular arch bifurcates to form mandibular processes that move toward the midline to form the lower mouth. When the facial process fail to fuse, craniofacial clefts can result.

With regards to the craniomaxillofacial skeleton, SHH mediates the development of mid- and upper-face, frontonasal, and maxillary processes<sup>[43]</sup>. In many studies, similarities were found between the patterning of SHH and BMP expression during neonatal craniofacial development<sup>[44,45]</sup> as well as with the transcription factor Muscle Segment Homeobox (MSX)-2, a transcriptional repressor of neural crest-derived cells<sup>[46,47-49]</sup>. In fact, SHH may increase mesenchymal proliferation via promotion of MSX2, a homeobox gene present in osteoblastic cells, in concert with BMP4 signaling<sup>[50]</sup>. Interestingly, Chong *et al* found that when SHH signaling is blocked in the brain but SHH is also applied early to the frontonasal ectodermal zone, the formation of the face and upper jaw is significantly improved. This suggests that the SHH signaling, which mediates brain-to-face interaction, is time-dependent<sup>[51]</sup>.

Various other studies have also demonstrated that SHH signaling is essential for early craniofacial development. Cobourne *et al* investigated the molecular basis of craniofacial defects seen in nevoid basal cell carcinoma syndrome (NBCCS) using a transgenic mouse model expressing *Shh* in basal epithelium under Keratin 14-promoter<sup>[52]</sup>. NBCCS is an autosomal dominant or spontaneous disorder characterized by multiple cutaneous basal cell

carcinomas, odontogenic keratocysts, skeletal anomalies, and facial dysmorphology including cleft lip and palate<sup>[53,54]</sup>. It is caused by a mutation in the *PTCH1* gene on chromosome 9q22.3-q31 in humans, which encodes the principle receptor for the Hedgehog signaling pathway<sup>[55,56]</sup>. The five year survival rate is only approximately 50%<sup>[57]</sup>. Cobourne *et al* showed that increased Hedgehog signal transduction in K14-*Shh* mice influences cell fate within the craniofacial region by affecting the processes of palatogenesis and odontogenesis<sup>[52]</sup>. Increased *Shh* activity in the medial edge epithelium of the palate prevents apoptosis and subsequently hinders palatal shelf fusion, whereas high levels of *Shh* in odontogenic epithelium is associated with lack of cell proliferation and arrested tooth development at the bud stage<sup>[52]</sup>. Thus, appropriately regulated Hedgehog signaling is crucial towards proper craniofacial development, as hallmarks of NBCCS such as orofacial clefting and hypodontia can both result from increased *Shh* signaling activity within embryonic epithelial tissues<sup>[52]</sup>.

IHH expression, by contrast, has been fully defined in its function to induce cell proliferation and differentiation, found mainly on the osteogenic fronts of the calvarial bones. This finding is consistent with limb development where IHH plays a greater role at the site of growth plates and cartilage formation and whereas its role in developed osteoblasts is less significant. IHH regulates chondrocyte differentiation and stimulates endochondral bone formation<sup>[26]</sup>. IHH stimulates the proliferation of chondrocytes at the growth plate and further in development, osteoblast differentiation. In addition, it also regulates chondrocyte hypertrophic differentiation through a negative feedback loop involving IHH-parathyroid hormone-related protein. As well, IHH promotes ossification and fusion of the cranial and palatine bones; IHH expression on the bony interfaces of synostotic rabbits may support the role of IHH in premature suture fusion<sup>[58]</sup>. Studies have suggested that IHH lies upstream of BMP2/4 signaling and may a part of the regulatory network that controls BMP expression, thereby influencing cranial ossification<sup>[59]</sup>. In aggregate, both SHH and IHH are found to be crucial regulators of osteogenesis and, therefore, of importance in early craniofacial development.

## CLINICAL IMPLICATIONS

Disruption of SHH signaling in humans is associated with a number of craniofacial defects, such as holoprosencephaly (loss of function) and nevoid basal cell carcinoma syndrome (gain of function)<sup>[60,61]</sup>. In clinical studies involving familial forms of holoprosencephaly, SHH gene mutations are found in as much as 23% of affected families, significantly more than the 1% of non-syndromic mutations<sup>[61,62]</sup>. Results from a recent study by Kurosaka *et al* strongly suggest proper SHH signaling is essential for neural crest cell patterning during normal nasal process growth and fusion, especially via *Tfap2a*, a transcription factor well known for its critical roles during craniofacial development<sup>[63-65]</sup>. It is worth noting that disruption of *TFAP2A* can result in branchio-oculo-facial syndrome in humans, a congenital disease seen with cleft lip/palate<sup>[66]</sup>. Aberrations at any stage in SHH signaling can result in cleft lip/palate, thus underscoring the signaling complexity necessary for normal nasal process growth and fusion.

Clinical studies have also shown that mutations in the IHH gene are associated with malformations resulting in syndactyly and craniosynostosis<sup>[67]</sup>. Additional mutations in IHH can also lead to acrocapitofemoral dysplasia, an autosomal recessive skeletal dysplasia that results in shortened or absent middle phalanges<sup>[43,68,69]</sup>. Interestingly, Wei *et al* demonstrated that increased signaling of

IHH correlated with the severity of osteoarthritis and expression of markers of chondrocyte hypertrophy<sup>[70]</sup>. Thus, for the clinician, IHH may be a potential therapeutic target of interest to prevent osteoarthritis progression. Deletion of the IHH gene is not currently a therapeutic option in humans, as it has proven lethal in animals<sup>[71,72]</sup>; however, RNA interference serves as a more suitable method, while also avoiding the severe side effects caused by chemical inhibitors<sup>[72-78]</sup>.

## CONCLUSION

While highly conserved across the different homologues, the HH family of proteins have distinct domains of expression and function, although not without overlap, in the context of palatal and facial development. In the context of skeletogenesis, both SHH and IHH have specific roles in endochondral ossification, and new studies even suggest a role for both in intramembranous ossification as well<sup>[46]</sup>. Thus, both are required for the process of ossification, although they differ in terms of location and how they elicit their effects. SHH expression is lacking in the facial mesenchyme, and instead is localized to the frontonasal and maxillary cranial suture mesenchyme. By contrast, IHH expression occurs primarily on developing palatine bone, and in most studies is placed in the context of endochondral ossification, playing a key role in inducing cell proliferation and differentiation. Finally, aberrations in the HH signaling pathway results in malformations in craniofacial development, studies of which provide further insight into the patterning and expression profile of the HH pathway.

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## CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

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