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Sonic Hedgehog Signaling in Craniofacial Development

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Abstract

Mutations in *SHH* and several other genes encoding components of the Hedgehog signaling pathway have been associated with holoprosencephaly syndromes, with craniofacial anomalies ranging in severity from cyclopia to facial cleft to midfacial and mandibular hypoplasia. Studies in animal models have revealed that SHH signaling plays crucial roles at multiple stages of craniofacial morphogenesis, from cranial neural crest cell survival to growth and patterning of the facial primordia to organogenesis of the palate, mandible, tongue, tooth, and taste bud formation and homeostasis. This article provides a summary of the major findings in studies of the roles of SHH signaling in craniofacial development, with emphasis on recent advances in the understanding of the molecular and cellular mechanisms regulating the SHH signaling pathway activity and those involving SHH signaling in the formation and patterning of craniofacial structures.

Keywords

Shh; cholesterol; primary cilium; neural crest; palate; mandible; tongue

1. Introduction

Mutations in *SHH* and several other genes encoding components of the Hedgehog signaling pathway, including *PTCH*, *DISP1*, *GAS1*, *CDON*, and *GLI2*, have been associated with holoprosencephaly (HPE) syndromes, with craniofacial anomalies ranging in severity from cyclopia to facial cleft to midfacial and mandibular hypoplasia (Bae et al., 2011; Belloni et al., 1996; Ming et al., 2002; Ribeiro et al., 2010; Roessler et al., 1996; Roessler et al., 2003; Roessler et al., 2009a; Roessler et al., 2009b). HPE is one of the most severe

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birth defects and has an estimated prevalence of ~4.8–8.8 per 100,000 live births (Croen et al., 1996; Ming and Muenke, 1998; Olsen et al., 1997; Roach et al., 1975; Urioste et al., 1988). Mutations in *SHH* have been found in about 23% of HPE patients in clinical studies (Abramyan, 2019; Roessler et al., 1996; Roessler and Muenke, 1998), underscoring the essential roles of SHH signaling in craniofacial development.

Shh is one of three mammalian homologs of *Drosophila* Hedgehog, a secreted signaling protein initially discovered through its role in controlling segmental patterning of the *Drosophila* embryos (Nusslein-Volhard and Wieschaus, 1980; Pereira et al., 2014). The other vertebrate/mammalian HH homologs include desert hedgehog (Dhh) and Indian hedgehog (Ihh) (Ingham, 2022; McMahon et al., 2003). Signaling by all Hedgehog family members shares an evolutionarily conserved but unusual signal transduction cascade composed of a series of inhibitory interactions (Fig. 1A) (Ingham, 2022; Kong et al., 2019). The main cell surface receptor for Hedgehog family ligands is Patched (Ptch), a twelve-pass transmembrane protein, whose function is to inhibit the activity of a transmembrane G-protein coupled receptor (GPCR)-like protein called Smoothened (Smo) in the absence of Hedgehog ligands (Chen and Struhl, 1996; Denef et al., 2000; Goodrich et al., 1996; Jenkins, 2009; Quirk et al., 1997; Stone et al., 1996; Taipale et al., 2002). Binding of Hedgehog ligands to the Ptch receptor relieves its repression of Smo, which then transduces the signaling activity intracellularly to activate the Glioma-associated oncogene (Gli) family of transcription factors (Gli1, Gli2, and Gli3 in vertebrates) by overcoming the negative influences of Suppressor of Fused (Sufu) and Protein Kinase A (PKA) (Huangfu and Anderson, 2006; Tukachinsky et al., 2010). In the absence of Hedgehog signals, Gli2 and Gli3 directly interacts with Sufu and are phosphorylated by PKA, leading to their partial proteolysis into truncated repressor forms (GliR) that function to suppress transcriptional target genes. Activated Smo inhibits PKA and promotes additional modifications of the full-length Gli proteins into the transcriptional activator form (GliA), which translocates into the cell nucleus to drive the expression of downstream target genes (Hui and Angers, 2011) (Fig. 1A). Each step of the Hedgehog signaling pathway, from the biogenesis of the active ligands to ligand-receptor interactions, to Smo and Gli activation, is regulated by multiple biochemical and cellular processes (Ingham, 2022; Kong et al., 2019; Zhang and Beachy, 2023). Thus, in addition to mutations affecting the functions of the core components of the SHH signaling pathway, genetic and/or environmental perturbations of many biochemical or cellular processes can disrupt or alter SHH signaling pathway activity and result in congenital developmental disorders. Before discussing the roles of and mechanisms mediating SHH signaling in craniofacial development, we next briefly review the current understanding of the biochemical and cellular mechanisms regulating SHH signaling pathway activity. More extensive discussions of the biochemical and cellular mechanisms regulating the Hedgehog signaling pathway can be found in several recent review articles (Ingham, 2022; Kong et al., 2019; Zhang and Beachy, 2023) and references therein.

2. Biochemical and cellular mechanisms regulating SHH signaling activity

2.1 Biogenesis and secretion of the Shh ligand

The Hedgehog family genes encode large precursor proteins containing a signal peptide sequence at the N-terminus, which targets newly synthesized Hedgehog protein to the endoplasmic reticulum where they undergo autocleavage through a cholesterol-mediated nucleophilic attack to release the N-terminal signaling fragment with covalently linked cholesterol at its C-terminus (Fig. 1B) (Hall et al., 1995; Lee et al., 1994; Porter et al., 1996). The HH signaling fragment is further modified by palmitoylation at its N-terminus by the membrane-bound O-acyltransferase HHAT to generate the dually lipid-modified mature ligand (Fig. 1B) (Buglino and Resh, 2008; Jiang et al., 2021; Pepinsky et al., 1998). The cholesterol modification contributes to membrane anchoring and enhances the stability of the Hedgehog ligands, which is critical for both long-distance and local signal transduction (Gallet et al., 2006; Hu and Song, 2019; Ingham, 2000; Kaushal et al., 2022; Li et al., 2006; Peters et al., 2004). Whereas several studies showed that pharmacological inhibition of HHAT blocked SHH signaling *in vitro* (Petrova et al., 2013; Rodgers et al., 2016), Shh lacking palmitoylation was able to drive expression of a Hedgehog signaling reporter in mammalian cells with lower potency (Chen et al., 2004; Palm et al., 2013; Pepinsky et al., 1998). Mouse embryos lacking *Hhat* function ($Hhat^{creface/Creface}$, description of this and other mutant mouse lines discussed in this article are provided in Supplementary table 1) exhibit severe holoprosencephaly defects including impaired forebrain cleavage and midline facial anomalies (Dennis et al., 2012; Kurosaka et al., 2014). Expression of *Patch1*, a direct target of SHH signaling, was considerably reduced in the $Hhat^{creface/Creface}$ embryos, indicating that deficiency in Hhat-mediated palmitoylation diminished the gradient and range of Shh activity *in vivo* (Dennis et al., 2012).

The lipid modifications render the Hedgehog ligands highly hydrophobic and tethered to the membranes of the producing cells (Kong et al., 2019). Whereas the membrane-associated Hedgehog ligands can signal to neighboring cells through direct cell-cell interactions, long-range signaling requires release of the mature ligands from the cell membrane. The multipass transmembrane protein Dispatched (Disp), which has significant sequence identity to Ptch, functions specifically for Hedgehog ligand release through interacting with the cholesterol moiety (Burke et al., 1999; Caspary et al., 2002; Ma et al., 2002; Tukachinsky et al., 2012). In addition, the Signal Peptide CUB EGF-like domain-containing protein (Scube2), itself a secreted protein, is required for Hedgehog ligand release by interacting with and shielding the lipid moieties to facilitate the dispersal from the secreting cell and enable the mature lipidated ligands to pass through the aqueous extracellular environment (Fig. 1B) (Petrov et al., 2017; Tukachinsky et al., 2012).

2.2. Reception of the SHH signal at the cell surface

Hedgehog family ligands must bind to the Ptch receptor to trigger signaling in target cells (Marigo et al., 1996; Stone et al., 1996). Vertebrates have two Ptch homologs, with Ptch1 acting as the major regulator of SHH signaling and Ptch2 playing a partially redundant role in some developmental processes (Carpenter et al., 1998; Goodrich et al., 1997; Koudijs et al., 2008; Nieuwenhuis et al., 2006). Cryo-electromicroscopy studies of PTCH1 structures

indicate that PTCH1 binds to dually lipidated SHH ligand through two interfaces, forming a complex with 1SHH:2PTCH stoichiometry with one PTCH1 binding SHH at its calcium- and zinc-binding surface and the second PTCH1 molecule engaging both the N-terminal palmitoyl and C-terminal cholesterol moieties (Gong et al., 2018; Qi et al., 2018a; Qi et al., 2018b; Qian et al., 2019). Whereas Scube2 is required for SHH release from the secreting cells and shields the lipid moieties of secreted Shh for its transport through the aqueous extracellular environment, the Scube2-SHH complex has significantly reduced affinity for Ptch1 compared to the free lipidated SHH (Wierbowski et al., 2020). Thus, for optimal Ptch1 binding and pathway activation, secreted SHH needs to be dissociated from Scube2 protection. Several co-receptors for the Hedgehog ligands have been identified, including the GPI-linked membrane glycoprotein Gas1 (Growth arrestspecific1) (Martinelli and Fan, 2007), the Ig/fibronectin single-pass membrane-spanning cell adhesion proteins Cdon (cell adhesion associated, oncogene regulated) and Boc (brother of Cdon) (Kang et al., 2002; Okada et al., 2006; Tenzen et al., 2006). Biochemical studies in cultured cells showed that Scube2 binds strongly to Cdon and Boc but not to Ptch1 (Wierbowski et al., 2020). Whereas Gas1 does not bind Scube2, it binds to both the cholesterol and palmitoyl moieties (Wierbowski et al., 2020). A SHH-receptor interaction relay model has been proposed that the Cdon/Boc coreceptors recruit the Shh-Scube2 complex to the cell surface where the SHH ligand is handed off in a dual lipid-dependent manner to Gas1 and then to Ptch1 to initiate signaling (Wierbowski et al., 2020). Studies in cell culture and genetic studies in mice indicate that Cdon, Boc, and Gas1 act partly redundantly (Wierbowski et al., 2020). *Cdo* mutant ($Cdo^{-/-}$) mouse embryos display microform holoprosencephaly whereas *Boc* mutants are adult viable and fertile (Cole and Krauss, 2003; Okada et al., 2006). $Cdo^{-/-};Boc^{-/-}$ double mutant embryos exhibit severe neural tube patterning defects but many Shh-dependent neural progenitors are still established (Allen et al., 2011). Loss of *Gas1* also disrupted neural tube patterning although the defects were less severe than in $Shh^{-/-}$ embryos (Allen et al., 2007). Simultaneous inactivation of all three genes ($Gas1^{-/-};Cdo^{-/-};Boc^{-/-}$) recapitulated mouse phenotypes of complete loss of Hedgehog activity, indicating an obligatory requirement for these co-receptors and differential requirement for each in Hedgehog signaling (Allen et al., 2011).

2.3. Regulation of the functions of Ptch and Smo and crucial roles of cholesterol

Whereas early studies suggested that Ptch might regulate Smo through direct physical interaction (Stone et al., 1996), subsequent studies indicate that Ptch regulates Smo activity in a non-stoichiometric manner (Denef et al., 2000; Ingham, 2000; Taipale et al., 2002). The Ptch proteins have significant sequence homology with the resistance-nodulation-division (RND) family protein pumps that many bacteria use to efflux toxic molecules and antibiotics, and with Niemann-Pick C1 (NPC1) that transports cholesterol out of the lysosomes (Kong et al., 2019). Experiments in cell culture showed that the amino acid residues essential for the transporter function of bacterial RND proteins are conserved in and required for Hedgehog pathway inhibition by Ptch (Taipale et al., 2002). Both Ptch and Disp share sequence homology with the sterol sensing domain of NPC1, which together with the finding that Disp exports the Hedgehog ligands through binding to the cholesterol moiety (Burke et al., 1999; Caspary et al., 2002; Ma et al., 2002; Tukachinsky et al., 2012) suggest that Ptch may regulate Smo activity through transporting cholesterol or other sterols. On

the other hand, the discovery that the naturally occurring steroidal alkaloid cyclopamine can bind and inhibit Smo activity (Chen et al., 2002) and subsequent finding that oxysterols, derivatives of cholesterol, can activate the SHH signaling pathway in cell culture (Corcoran and Scott, 2006), suggest that the endogenous Smo regulator is a sterol lipid. Remarkably, crystal structures of the Smo protein revealed a cholesterol molecule bound to the same position that oxysterols were predicted to bind (Byrne et al., 2016). Mutations that prevent Smo binding to cholesterol impair Hedgehog signaling in cultured cells and in mouse embryos (Byrne et al., 2016; Xiao et al., 2017). Additional studies showed that cholesterol is sufficient to activate Smo signaling even in the absence of Hedgehog ligands (Huang et al., 2016; Luchetti et al., 2016). Zhang et al. (2018) showed that cholesterol activity in the inner leaflet of the plasma membrane is reduced by overexpression of PTCH1 but rapidly restored by Hedgehog stimulation (Zhang et al., 2018). More recently, Kinnebrew et al. showed that Ptch1 regulates Smo activity by controlling cholesterol binding to its extracellular cysteine-rich domain (Kinnebrew et al., 2022). Together, these studies provide compelling evidence that Ptch regulates Smo activity by controlling cholesterol availability.

How can Ptch keep cholesterol, the single most abundant lipid in the plasma membrane (Maxfield and van Meer, 2010; Mouritsen and Bagatolli, 2015), away from Smo to prevent inappropriate pathway activation? The solution to this dilemma is based on the key role of the primary cilium in Hedgehog signaling, with the ciliary membrane acting as a subcellular compartment within which the levels of accessible cholesterol can be modulated by Ptch without impacting global cellular cholesterol homeostasis (Ingham, 2022). It has been shown that the ciliary membrane contains lower levels of accessible cholesterol than the plasma membrane but the ciliary cholesterol levels are increased upon Hedgehog stimulation (Kinnebrew et al., 2019). Thus, Ptch would prevent Smo activation within the primary cilium while its inhibition by Shh binding would promote accumulation of active Smo. Whereas *Drosophila* Hedgehog signaling does not depend on the primary cilia, cholesterol represents a minor fraction of plasma membrane sterols in *Drosophila* cells and, hence, Hedgehog signaling activity in *Drosophila* could be regulated through Ptch-mediated regulation of cholesterol availability in the plasma membrane (Ingham, 2022; Kinnebrew et al., 2019).

This model that cholesterol is the endogenous Smo activating ligand regulated by Ptch provides new mechanistic insights into multiple developmental disorders associated with disruption of cholesterol biosynthesis (Kinnebrew et al., 2019; Porter and Herman, 2011). For example, mutations in the gene encoding the cholesterol biosynthetic enzyme 7-dehydrocholesterol reductase (DHCR7) cause Smith–Lemli–Opitz syndrome (SLOS) (OMIM #270400) (Wassif et al., 1998). SLOS patients exhibits craniofacial defects including cleft palate, syndactyly and polydactyly, and HPE (Kelley et al., 1996). Blassberg et al. (2016) showed that SHH signaling activity is reduced in *Dhcr7* deficient mouse embryonic fibroblasts (Blassberg et al., 2016). Two SLOS-like syndromes, known as Desmosterolosis (OMIM #602398) and Lathosterolosis (OMIM #607330), respectively, are caused by homozygous mutations in *SC5D*, encoding sterol-C5-desaturase, and *DHCR24*, encoding the 24-dehydrocholesterol reductase (Andersson et al., 2002; Brunetti-Pierri et al., 2002; Krakowiak et al., 2003; Waterham et al., 2001). Together with DHCR7, SC5D and DHCR24 catalyze the terminal steps of cholesterol biosynthesis (Porter and Herman,

2011). Cell culture assays showed that loss of function of each of these three genes impaired transcriptional induction of endogenous Gli1, a direct target of Hedgehog signaling (Dai et al., 1999; Kinnebrew et al., 2019). Interestingly, Hedgehog signaling in *Dhcr7*$^{-/-}$ cells, but not in *Dhcr24*$^{-/-}$ cells, could be rescued by exogenous cholesterol, suggesting that accumulation of some precursor sterols due to defects in terminal steps of cholesterol biosynthesis may bind to Smo and antagonize Hedgehog signaling (Kinnebrew et al., 2019; Nguyen et al., 2022; Porter and Herman, 2011). Cholesterol biosynthesis involves over 30 biochemical steps and mutations disrupting several other enzymes catalyzing cholesterol biosynthesis have been associated with distinct developmental disorders (Porter and Herman, 2011). Studies in zebrafish showed that disruption of *hmgcrb*, a homolog of the mammalian *Hmgcr* gene encoding 3-**h**ydroxy-3-**m**ethyl-**g**lutaryl-**C**oA **r**eductase, the first rate-limiting enzyme in the sterol biosynthesis pathway (Schumacher and DeBose-Boyd, 2021), resulted in orofacial clefts associated with reduction in expression of *Gli1* during early facial development (Signore et al., 2016). Interestingly, statins that are commonly used to treat hypercholesterolemia and dyslipidemia are structural analogs of HMG-CoA and competitive inhibitors of HMGCR (Istvan and Deisenhofer, 2001). Thus, better understanding of the effects on cholesterol metabolism and related treatment approaches on Hedgehog and other developmental signaling pathways will directly impact strategies for treatment and/or prevention of developmental disorders.

2.4 Intracellular transduction of SHH signaling and the roles of the primary cilium

Although evolutionarily conserved from *Drosophila* to human, Hedgehog signaling in vertebrates, but not in *Drosophila*, depends on the primary cilium, a non-motile microtubule-based flagellar projection on almost all vertebrate cells (Goetz and Anderson, 2010) (Fig. 1C–D). Remarkably, most of the core components of the vertebrate Hedgehog signaling pathway, including the Ptch receptors, Smo, Sufu, and the full-length Gli proteins, are found localized to the primary cilia although the mechanisms localizing these factors to the primary cilia are not well understood yet (reviewed in (Bangs and Anderson, 2017; Ingham, 2022)). Vertebrates have three Gli proteins; Gli2 and Gli3 are expressed in the absence of Hedgehog signaling whereas Gli1 is a direct transcriptional target of Hedgehog signaling and functions mainly as an activator to amplify Hedgehog signaling activity (Bai et al., 2002; Dai et al., 1999; Kong et al., 2019). It is important to note that Gli2 and Gli3 proteins exist in at least three activity states: proteolytically processed transcriptional repressors (Gli2R/Gli3R), transcriptionally inactive full-length Gli2/Gli3 proteins, and full-length transcriptional activators (Gli2A/Gli3A) (Kong et al., 2019). It is also important to know that the Gli proteins travel through the primary cilia whether the cells receive Hedgehog signaling or not (Kong et al., 2019). In the absence of Hedgehog signaling, Sufu binds to Gli2/3 proteins and the Sufu-Gli complex travels through the primary cilia where PKA specifically phosphorylates full-length Gli2/3 to initiate a pathway of proteasomal processing into the truncated GliR forms, which dissociate from Sufu and translocate into the nucleus to repress target gene expression (Ding et al., 1999; Humke et al., 2010; Kogerman et al., 1999; Niewiadomski et al., 2014; Tukachinsky et al., 2010; Tuson et al., 2011). PKA activity is regulated by GPCR signaling (Kong et al., 2019). The orphan rhodopsin GPCR protein Gpr161 is localized to the cilium and contributes to the local activation of PKA (Mukhopadhyay and Rohatgi, 2014; Mukhopadhyay et al., 2013). When

Hedgehog signaling is on, activated Smo accumulates in the ciliary membrane and inhibits PKA activity via its C-terminal PKA inhibitor motif (Happ et al., 2022). Activated Smo also promotes the dissociation of full-length Gli2/3 from Sufu, allowing formation of the Gli2/3A transcriptional activators (Kong et al., 2019). Previous studies showed that *Sufu*-deficient mouse embryos died at ~E9.5 with a ventralized neural tube reminiscent of global activation of SHH signaling but the $Sufu^{-/-}$ embryos exhibited barely detectable Gli2/3 proteins (Chen et al., 2009; Cooper et al., 2005; Svard et al., 2006). Chen et al. (2009) showed that Sufu maintains Gli2 and Gli3 protein levels through antagonizing Spop (speckle-type POZ protein)-mediated ubiquitination (Chen et al., 2009). In addition, Sufu can bind to and modulate Gli-mediated transcriptional activity in the nucleus (Lin et al., 2014). The atypical kinesin Kif7 is strongly enriched at the ciliary tip and plays a crucial role in regulating the processing and stability of Gli proteins (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009). The molecular mechanisms converting the full-length Gli proteins to GliA activators and translocating them into the nucleus are still not completely understood.

The assembly of cilia, and trafficking of proteins into and out of the cilia, are mediated by a two-way intraflagellar transport (IFT) system that consists of two IFT complexes, IFT-A and IFT-B (Chinipardaz et al., 2022; Nakayama and Katoh, 2020). The IFT-B complex together with the kinesin II motor proteins regulate anterograde trafficking from the base to the tip of the cilium whereas the IFT-A complex works with the dynein motor proteins to regulate retrograde transport from the tip to the base of cilium (Nakayama and Katoh, 2020). Disruption of the genes encoding IFT-B components, such as *Ift88* and *Ift172*, or disruption of *Kif3a*, resulted in cilial agenesis and loss of Shh-dependent ventral neural tube cell types (Huangfu and Anderson, 2005; Huangfu et al., 2003). Inactivation of genes encoding IFT-A components exhibited complex effects: mutations in several *IFT-A* genes, including *Ift122* and *Ift139*, caused bulged cilia and exhibited ligandindependent gain of SHH signaling in neural tube and limb development (Qin et al., 2011; Tran et al., 2008) whereas several other IFT-A mutations resulted in short bloated cilia with a highly disrupted axoneme and were associated with loss of SHH signaling activity in neural tube patterning (Bangs and Anderson, 2017). Production of both Gli2/3R repressors and Gli2/3A activators are affected when ciliogenesis is disrupted (Huangfu and Anderson, 2005; Liu et al., 2005), consistent with the cilium-dependence of PKA mediated phosphorylation of Gli2/3 and Smo-mediated Hedgehog signal transduction. Furthermore, mutations disrupting some ciliary proteins, such as Ift25 and Ift27, apparently did not affect the morphology of the primary cilia but caused mild defects in trafficking of Hedgehog signaling pathway components including Ptch1, Smo, Gli2, through the primary cilia (Eguether et al., 2014; Keady et al., 2012). Hence, the phenotypic effects of mutations in IFT components and other ciliary proteins are dependent on the developmental context of Hedgehog ligand and Gli2/3 expression, mutant effects on ciliary structure and/or function, and may result from the loss of GliR-mediated target gene repression, loss of Hedgehog signaling-dependent GliA-mediated target gene activation, or both.

In summary, SHH signaling pathway is regulated at multiple steps (Fig. 1), from the biogenesis and cellular release of the active ligand, interaction with the receptor and co-receptors, activation of the obligate signaling transducer protein Smo, to multiple mechanisms regulating Gli protein processing and transcription factor activity as well

as Gli-independent mediators of downstream target gene regulation (for recent reviews, please see (Ingham, 2022; Kong et al., 2019; Zhang and Beachy, 2023)). Disruption at any of these steps results in dysregulation of target gene expression and may cause developmental defects including craniofacial malformations. It is important to emphasize that Hedgehog signaling is not a binary ON/OFF switch but rather is composed of a series of negative inhibitory interactions of which the outcome depends on the strengths and duration of signaling activity in target cells (Kong et al., 2019). Since the GliA and GliR share the same DNA binding domains, the ratio of GliA and GliR in the target cells is an important determinant for target gene expression. Even modest alterations in SHH signaling strength can lead to developmental defects (Nieuwenhuis and Hui, 2005). In the following sections, we review and summarize advances in the understanding of the roles of and mechanisms involving SHH signaling in major craniofacial developmental processes, including formation and patterning of the facial primordia, palate development, mandibular development and patterning, tongue organogenesis, taste bud formation and homeostasis, and tooth development.

3. SHH function in the formation of facial primordia

At the beginning of facial development in vertebrate embryos, cranial neural crest cells (CNCCs), a population of multipotent progenitor cells that give rise to most of the bones, cartilages, and connective tissues in the head, arise from the lateral edges of the anterior neural plate (Gammill and Bronner-Fraser, 2003; Morales et al., 2005; Steventon et al., 2005). These CNCCs delaminate and migrate ventrally underneath the surface ectoderm where epithelial-mesenchymal interactions between the surface ectoderm and CNCCs result in the formation and outgrowth of the embryonic facial primordia, including the frontonasal prominence (FNP) rostral to the primitive mouth, and the paired maxillary and mandibular arches lateral and caudal, respectively, to the primitive mouth. The FNP grows around the bilateral nasal placodes to form the medial nasal processes (MNP) and lateral nasal processes (LNP). Subsequent growth and convergence of the MNP, LNP, maxillary and mandibular processes result in the formation of the intact embryonic face. During the growth and convergence of the facial primordia, the CNCC-derived facial mesenchyme cells receive multiple signals, including members of the bone morphogenetic protein (BMP) family, fibroblast growth factor (FGF) family, WNT family, and SHH, from the surrounding epithelial cells that regulate their proliferation and differentiation (Helms and Schneider, 2003; Jiang et al., 2006; Santagati and Rijli, 2003). For example, upon arriving in mandibular arches, the CNCCs encounter Fgf8 expressed in the proximal mandibular arch epithelium and Bmp4 expressed by the distal mandibular arch epithelium (Haworth et al., 2004; Shigetani et al., 2000; Tucker et al., 1998; Tucker et al., 1999). Fgf8 and Bmp4 act to pattern the proximal-distal axis of the mandibular arch by activating expression of *Barx1* in the proximal domain and expression of *Msx1, Msx2*, and *Alx4*, in the distal domain, respectively (Barlow et al., 1999; Ferguson et al., 2000; Parada and Chai, 2015; Tucker et al., 1998). In addition, Fgf8 signalling patterns the rostral-caudal axis of the mandibular arch mesenchyme by restricting the expression of *Lhx6* and *Lhx8* in the rostral domain (Cobourne and Sharpe, 2003; Grigoriou et al., 1998; Tucker et al., 1999). Several members of the WNT family are also expressed in the early facial ectoderm and activates canonical

WNT/β-catenin signaling in both the epithelium and underlying mesenchyme of the MNP, LNP, and maxillary processes (Lan et al., 2006). Mutations in *WNT3* and *WNT9b* have been linked to non-syndromic cleft lip with or without cleft palate (Chiquet et al., 2008; Fontoura et al., 2015; Lu et al., 2015; Mostowska et al., 2012; Nikopensius et al., 2010). Mouse genetic studies showed that Wnt9b from the facial ectoderm signals to the maxillary and nasal mesenchyme to regulate the cell proliferation and ensure lip fusion (Jin et al., 2012; Juriloff et al., 2014; Lan et al., 2006). More information about the roles of BMP, FGF, and WNT signaling pathways in early facial morphogenesis can be found in several recent articles and references therein (Brewer et al., 2016; Graf et al., 2016; Nie et al., 2006; Ray et al., 2020; Reynolds et al., 2019; Reynolds et al., 2020; Stanier and Pauws, 2012; Ueharu and Mishina, 2023). In this review, we focus on discussing the cellular and molecular mechanisms mediating SHH signaling pathway function in regulating craniofacial development.

In both chick and mouse embryos, *Shh* is expressed in several epithelial regions during early facial morphogenesis, including neuroectoderm of the ventral forebrain, oral ectoderm, and pharyngeal endoderm (Jeong et al., 2004). SHH produced by the forebrain neuroepithelium induces *Shh* expression in the facial ectoderm (Marcucio et al., 2005). It has been shown that Shh-Gli3 signaling regulates primary mouth opening in both *Xenopus* and mouse embryos by controlling buccopharyngeal membrane dissolution (Tabler et al., 2014). In the frontonasal region, initial expression of Shh and Fgf8 in the stomodeal ectoderm defines a specific signaling center called frontonasal ectodermal zone (FEZ), with *Shh* expression in the ectodermal cells comprising the roof of the mouth and with *Shh*- and *Fgf8*-expressing cells forming a boundary that spans the mediolateral axis of the FNP (Hu and Marcucio, 2009a, b; Hu et al., 2003). Subsequently, *Fgf8* expression becomes restricted to the nasal pits at Hamburger Hamilton stage (HH) 20–21 in chick and at about E9.5 to E10.5 in mice (Hu and Marcucio, 2009a, b, 2012; Hu et al., 2003). BMP signaling is implicated in regulating the onset of *Shh* expression in the FEZ in chick embryos (Foppiano et al., 2007). Blocking BMP signaling using Noggin at HH~15/16 resulted in decreased *Shh* expression in the facial ectoderm and a lack of outgrowth of the treated FNP (Hu et al., 2015), but the source of the BMP signal and whether BMP signaling directly activates *Shh* expression in the facial ectoderm are not known. SHH from the ectoderm in turn regulates expression of several *Bmp* genes in the adjacent CNCC-derived facial mesenchyme (Hu and Marcucio, 2009a, b; Hu et al., 2015). The BMP-SHH signaling loop is critical for restricting *Fgf8* expression to the nasal pits to regulate regional cell proliferation in the FNP (Hu et al., 2015). In chick embryos, inhibiting SHH signaling by using cyclopamine or introduction of dominant-negative Ptch1, in the FNP at HH15 resulted in a decrease in cell proliferation leading to hypoplasia of the lateral edges of the FNP, a failure of the globular process to contact the lateral nasal and maxillary processes, and a cleft of the primary palate (Hu et al., 2015). Together, these findings indicate that interactions of SHH, BMP, and FGF8 signaling pathways form a highly coordinated molecular regulatory network to control early facial patterning and growth although the exact molecular mechanisms regulating the interactions of these signaling pathways remain to be elucidated.

Studies using function-blocking anti-SHH antibody or application of recombinant SHH protein in chick embryos indicated that Shh plays important roles for survival and

proliferation of early CNCCs (Ahlgren and Bronner-Fraser, 1999; Hu and Helms, 1999). Using tissue-specific inactivation of *Smo* in premigratory neural crest cells (*Wnt1Cre;Smo*$^{n/c}$) in mice, Jeong et al investigated the role of Hedgehog signaling activity in CNCC development and facial primordial outgrowth (Jeong et al., 2004). By E10.5, the *Wnt1Cre;Smo*$^{n/c}$ mouse embryos displayed smaller FNP and mandibular arches, associated with increased cell apoptosis and decreased proliferation of CNCC-derived facial mesenchyme (Jeong et al., 2004). Conversely, the *Wnt1-Cre;R26SmoM2* transgenic mouse embryos, which carry a Cre-activated transgene encoding a constitutively active form of Smo (Supplementary Table 1) specifically in the neural crest lineage, exhibited a mild hyperplasia of the facial processes by E10.5 (Jeong et al., 2004). Expression of several genes encoding the Forkhead box (Fox) containing transcription factors, including *Foxf1*, *Foxf2*, *Foxd1*, *Foxd2* and *Foxc2*, were reduced in the developing facial primordia in *Wnt1Cre;Smo*$^{n/c}$ embryos and expanded in *Wnt1-Cre;R26SmoM2* embryos (Jeong et al., 2004). Further studies showed that several Fox family transcription factors, including Foxf1 and Foxf2, play crucial roles in cell survival, tissue growth, and patterning of multiple facial developmental processes (Everson et al., 2017; Xu et al., 2019; Xu et al., 2016; Xu et al., 2022). Mice with tissue specific inactivation of both *Foxf1* and *Foxf2* in cranial neural crest cells (*Foxf1*$^{c/c}$*Foxf2*$^{c/c}$*Wnt1Cre*) display severe craniofacial defects resembling *Wnt1Cre;Smo*$^{n/c}$ embryos (Jeong et al., 2004; Xu et al., 2019). Furthermore, chromatin immunoprecipitation followed by high throughput sequencing analyses revealed direct binding of Gli3 protein at genomic sites in or around several Fox family genes, including *Foxf1* and *Foxf2*, in multiple tissues including in the developing face (Elliott et al., 2020; Hoffmann et al., 2014). Together, these results indicate that Foxf1 and Foxf2 function as important mediators of SHH signaling during craniofacial development.

Studies of two mutant mouse lines showed that SHH signaling interacts with WNT signaling to regulate upper lip fusion. The *Hhat*^{creface} transgenic allele disrupted the *Hhat* gene, encoding the acyltransferase that adds the palmitoyl moiety to the Hedgehog protein, and resulted in loss of SHH signaling in the facial primordia (Dennis et al., 2012; Kurosaka et al., 2014). The *Ptch1*^{wiggable} mutant mouse allele generates a truncated Ptch1 protein, leading to enhanced SHH signalling (Kurosaka et al., 2014). The *Hhat*^{creface};*Ptch1*^{wiggable} double mutant embryos displayed hypoplastic nasal process outgrowth and cleft lip with persistence of epithelial seam between the MNP and LNP (Kurosaka et al., 2014). Interestingly, analysis using the TOPgal transgenic reporter for detecting canonical WNT signaling activity (DasGupta and Fuchs, 1999) showed that WNT signaling activity in the developing facial primordia is reduced in the *Ptch1*^{wiggable} mutant embryos and increased in *Hhat*^{creface} embryos, the *Hhat*^{creface};*Ptch1*^{wiggable} double mutants exhibited restored TOPgal activity except in the lambdoidal region coinciding with the lack of apoptosis, suggesting that SHH signaling regulates lip fusion at least in part through restricting WNT signaling activity. The balance of SHH-WNT signaling is thought to maintain proper proliferation and apoptosis for removing the epithelial seam at lambdoidal region during lip fusion.

As described above, SHH signaling depends on the primary cilium. Mutations in genes encoding components of the primary cilia cause a class of genetic disorders collectively referred to as ciliopathies, of which many exhibit craniofacial developmental defects (Ferkol and Leigh, 2012). Genetic studies in mice showed that mutations in most of the genes

encoding major components of the ciliary IFT machinery or ciliary structural proteins cause disruption of ciliogenesis, resulting in homozygous embryonic lethality at midgestation associated with context-dependent loss or gain of SHH signaling (reviewed in (Bangs and Anderson, 2017)). Interestingly, disruption of the IFT-B genes *Ift25* and *Ift27* in mice did not overtly disrupt the morphology and structure of the primary cilia but still caused neonatal death with craniofacial defects including hypotelorism, micrognathia, and cleft palate (Eguether et al., 2014; Keady et al., 2012). The developmental defects in $Ift25^{-/-}$ and $Ift27^{-/-}$ embryos suggest decreased SHH signaling activity. Indeed, upon stimulation with Shh or a Smo agonist, the $Ift25^{-/-}$ and $Ift27^{-/-}$ mutant mouse embryonic fibroblast cells exhibited aberrant accumulation of Ptch1 and Gpr61 and reduced Gli2 in the primary cilia, suggesting that these ciliary proteins play highly specialized roles in intraflagellar transport of the Hedgehog pathway components (Eguether et al., 2014; Keady et al., 2012). Tissue specific inactivation of *Kif3a*, encoding a subunit of the kinesin-II IFT motor for anterograde transport in the cilium, in CNCCs caused gain of SHH signaling activity during midfacial development and resulted in hypertelorism and a midfacial cleft in mice (Brugmann et al., 2010). Chang et al. (2016) showed that the ciliopathic midfacial defects in the $Kif3a^{f/f};Wnt1Cre$ and $Ift88^{f/f};Wnt1Cre$ mice occur primarily via a de-repression mechanism due to loss of the Gli2R and Gli3R repressors (Chang et al., 2016).

In addition to the IFT machinery, many other proteins play crucial roles in building the primary cilia and their disruption also affect SHH signaling (Bangs and Anderson, 2017). The chick $talpid^2$ mutation disrupts the *C2cd3* gene, encoding structural protein required for the centriole to dock on the ciliary vesicle (Ye et al., 2014), and caused facial cleft due to disruption of ciliogenesis (Chang et al., 2014). Post-translational processing of Gli2 and Gli3 was disrupted in the developing facial prominences *in* $talpid^2$ mutant embryos, resulting in increased accumulation of full-length Gli2 and Gli3 proteins and decreased level of the Gli3R repressor, which correlated with the increased activation/de-repression of the SHH pathway in the developing FNP (Chang et al., 2014). Another ciliopathic mutant mouse, the $Fuz^{-/-}$ mutant mouse, displays high arched palate and a single calvarial bone pair due to expanded CNCCs (Tabler et al., 2013; Tabler et al., 2016). Remarkably, tissue-specific inactivation of *Fuz* in the CNCCs did not recapitulate the $Fuz^{-/-}$ craniofacial defects, indicating that the effects of *Fuz* loss of function on CNCCs is cell non-autonomous. Expanded *Fgf8* expression was found in the hindbrain and early facial ectoderm associated with reduced Gli3R activity in the $Fuz^{-/-}$ embryos (Tabler et al., 2013; Tabler et al., 2016). $Gli3^{xt\text{-}J/xt\text{-}J}$ mutants display expanded neural crest cells in the frontal bone mesenchyme and developing larynges similar with the $Fuz^{-/-}$ mutants, suggesting that the expanded *Fgf8* expression in the $Fuz^{-/-}$ embryos was due to loss of cilia-mediated production of Gli3R (Tabler et al., 2016; Tabler et al., 2017). Genetically deleting one allele of *Fgf8* ameliorated the maxillary and skull phenotypes, indicating that excessive Fgf8 was responsible for the expanded neural crest cells in the $Fuz^{-/-}$ embryos (Tabler et al., 2013; Tabler et al., 2016). Furthermore, mouse embryos deficient in *Ofd1*, encoding a centriole-associated protein that colocalizes with C2CD3, exhibited strikingly similar maxillary hyperplasia and expanded *Fgf8* expression in the facial ectoderm similarly as in $Fuz^{-/-}$ embryos (Tabler et al., 2013). These results identify a crucial role of ciliummediated production of Gli3R and its repression of *Fgf8* expression in the embryonic facial ectoderm in the regulation of facial

primordial outgrowth (Tabler et al., 2013). Together with other studies that demonstrated crosstalk of SHH, BMP, and FGF8 signaling pathways during facial primordia outgrowth as discussed above, these results indicate that primary cilium mediated GLI processing is a key regulator of the molecular network controlling facial primordial growth.

4. SHH function in palate development and patterning

In mammals, the palate separates the oral and nasal cavities and plays crucial roles in feeding, breathing, and speech. In mouse, the development of secondary palate begins as a pair of outgrowths from the oral side of the paired maxillary at around E11.5 to a pair of palatal shelves that initially grow vertically downward flanking the developing tongue. At around E14.0, the palatal shelves reorient to the horizontal position above the tongue, grow towards and subsequently fuse with each other at the midline. The secondary palate also fuses anteriorly with the primary palate and nasal septum to form the intact roof of the oral cavity (Fig. 2A–F) (reviewed by (Bush and Jiang, 2012; Chai and Maxson, 2006; Dixon et al., 2011; Lan et al., 2015).

SHH signalling plays critical roles in regulating growth, patterning, and fusion of palatal shelves. At the onset of palatal outgrowth, *Shh* expression was detected throughout the early oral epithelium (Rice et al., 2006). During palatal shelf growth, *Shh* expression becomes restricted to the oral side palatal epithelium, at high levels in the ectodermal ridges forming the palatal rugae in the anterior region and in the sensory papilla in the posterior region of the palatal shelves (Fig. 2A–F) (Pantalacci et al., 2008; Welsh and O'Brien, 2009). *Ptch1* and *Gli1*, known target genes of SHH signalling, are expressed in palatal epithelial and mesenchyme cells, indicating that SHH signalling pathway is active in both the palatal epithelium and mesenchyme (Economou et al., 2012; Lan and Jiang, 2009; Rice et al., 2006). In palatal shelf explants culture, adding exogenous SHH induced, whereas adding anti-SHH antibody inhibited, cell proliferation in the palatal mesenchyme (Han et al., 2009; Rice et al., 2004; Zhang et al., 2002). Tissue-specific inactivation of *Smo* in the CNCCs (*Smo*$^{n/c}$*;Wnt1Cre*) caused complete agenesis of the secondary palate (Jeong et al., 2004). Deletion of *Shh* in the oral epithelium after E11 (*Shh*$^{c/n}$*;K14-Cre*) caused an incomplete penetrance of cleft palate (Lan and Jiang, 2009; Rice et al., 2004). Loss of function of *Gas1* also resulted in an incomplete penetrance of cleft palate phenotype associated with reduced cell proliferation in developing palatal shelves. Deleting one *Shh* allele or inactivation of *Boc* increased the penetrance and severity of cleft palate in *Gas1* mutant mice (Seppala et al., 2007; Xavier et al., 2016). Tissue-specific inactivation of *Smo* in the palatal mesenchyme (*Smo*$^{n/c}$*;Osr2-IresCre*) resulted in complete penetrance of cleft palate with reduced cell proliferation (Lan and Jiang, 2009). Together, these results indicate that SHH signalling plays a key role in palatal shelf outgrowth.

A series of mouse genetic studies uncovered a positive feedback regulatory loop between *Shh* expression in the palatal epithelium and *Fgf10* expression in the palatal mesenchyme. Mice lacking either Fgf10 or Fgfr2b, an epithelium-specific isoform of the FGF receptor, exhibited cleft palate with failure of maintenance of *Shh* expression in the palatal epithelium (De Moerlooze et al., 2000; Rice et al., 2004). In palatal explant culture assays, agarose beads containing recombinant FGF10 protein induced *Shh* expression in wildtype palatal

shelves but not in the *Fgfr2b*$^{-/-}$ palatal shelves (Rice et al., 2004), indicating that FGF10-FGFR2b signaling is required for maintaining *Shh* expression during palatal shelf outgrowth. Expression of *Fgf10* in the palatal mesenchyme was significantly downregulated in the *Smo*$^{n/c}$*;Osr2-IresCre* as well as in *Osr2*$^{-/-}$ embryos (Lan and Jiang, 2009; Zhou et al., 2013). *Osr2* expression is also reduced in the *Smo*$^{n/c}$*;Osr2-IresCre* palatal mesenchyme and *Osr2*$^{-/-}$ mice displayed cleft palate with reduced palatal shelf growth, suggesting that Osr2 acts down-stream of SHH signaling and regulates the expression of *Fgf10* (Lan and Jiang, 2009; Lan et al., 2004). How SHH signaling regulates, directly or directly, the expression of *Osr2* in the developing palatal mesenchyme remains to be determined. Interestingly, mice lacking the Pax9 transcription factor exhibited cleft palate and the *Pax9*$^{-/-}$ embryos had reduced expression of both *Osr2* and *Fgf10* in the palatal mesenchyme (Peters et al., 1998; Zhou et al., 2013). Restoring *Osr2* expression in the palatal mesenchyme in *Pax9* mutants by inserting the *Osr2* cDNA into the *Pax9* locus partly restored *Fgf10* expression and rescued palatal shelf growth and fusion (Zhou et al., 2013). Together, these studies identified a positive regulatory cascade of Shh-Osr2/Pax9-Fgf10-Shh mediating the reciprocal epithelial-mesenchymal interactions driving palatal shelf growth (Fig. 2G).

In contrast to the positive regulation of *Shh* expression in the palatal epithelium by Fgf10, *Fgf7* is also expressed in the palatal mesenchyme, but it inhibited *Shh* expression in palatal explant culture assays (Han et al., 2009; Rice et al., 2004). Interestingly, *Dlx*5$^{-/-}$ embryos exhibited reduced *Fgf7* expression in the palatal mesenchyme and expansion of *Shh* expression to the nasal side palatal epithelium. Mice lacking Msx1, a transcription factor with restricted expression in the anterior palatal mesenchyme, exhibited reduced palatal shelf growth accompanied by significant reduction in *Shh* expression in the anterior palatal epithelium (Zhang et al., 2002). Zhang et al. (2002) showed that *Msx1*$^{-/-}$ embryos exhibited reduced expression of *Bmp4* in the anterior palatal mesenchyme and that ectopic expression of *Bmp4* driven by the *Msx1* promoter rescued the cleft phenotype of *Msx1*$^{-/-}$ mutants accompanied by restored palatal cell proliferation and the expression of *Shh* (Zhang et al., 2002). Remarkably, inactivation of *Dlx5* in *Msx1*$^{-/-}$ mutants also rescued palatal shelf growth and fusion in the *Dlx5*$^{-/-}$*Msx1*$^{-/-}$ embryos compared with cleft palate in the *Msx1*$^{-/-}$ mutants (Han et al., 2009). These results indicate that multiple molecular pathways converge on the regulation of *Shh* expression during palate development to regulate palatal shelf growth and patterning (Fig. 2G).

Expression of the *Foxf1* and *Foxf2* genes in the developing palatal mesenchyme depends on SHH signaling (Jeong et al., 2004; Lan and Jiang, 2009). *Foxf2*$^{-/-}$ mouse embryos exhibited reduced palatal shelf growth whereas mice with neural crest-specific inactivation of both *Foxf1* and *Foxf2* (*Foxf1*$^{c/c}$*Foxf2*$^{c/c}$*Wnt1Cre*) exhibited rudimentary palatal shelves, resembling the severe palatal growth defect observed in *Smo*$^{n/c}$*;Wnt1Cre* embryos (Jeong et al., 2004; Xu et al., 2016). Through RNA-seq analysis, we found that *Foxf2*$^{-/-}$ embryos exhibited aberrantly increased expression of multiple genes, including *Fgf18*, in the developing palatal shelves (Xu et al., 2016). The domains of ectopic *Fgf18* expression in the palatal mesenchyme in *Foxf2*$^{-/-}$ embryos correlated with where *Shh* expression was downregulated in the palatal epithelium. Furthermore, *Foxf1*$^{c/c}$*;Foxf2*$^{c/c}$*;Wnt1Cre* embryos exhibited ectopic *Fgf18* expression throughout the palatal mesenchyme and complete loss of *Shh* expression in the palatal epithelium (Xu et al., 2016). Addition of recombinant Fgf18

protein to palatal explant cultures inhibited *Shh* mRNA expression in the palatal epithelium (Xu et al., 2016). Together, these data indicate that the Foxf1/Foxf2 transcription factors act downstream of SHH signaling in the palatal mesenchyme to regulate palatal shelf growth and feedback to maintain *Shh* expression in the palatal epithelium by preventing ectopic activation of *Fgf18* expression (Fig. 2G).

Whereas several FGFs act antagonistically to restrict *Shh* expression to the oral side of the palatal epithelium during palatal shelf growth (Fig. 2G), studies of gain-of-function mouse models further demonstrated the importance of spatiotemporally regulated SHH signalling activity in palatogenesis. Li et al. analyzed the effects of persistent activation of Smo-mediated Hedgehog signalling throughout the palatal epithelium in *K14-Cre;R26SmoM2* embryos and showed that they developed submucous cleft palate with persistence of the medial edge epithelium (MEE) (Li et al., 2018). Cobourne et al. showed that *K14-Shh* transgenic mouse embryos, expressing *Shh* throughout the palatal epithelium, also exhibited failure of palatal shelf fusion (Cobourne et al., 2009). These data indicate that Shh is a central node integrating multiple signaling pathways in the palate development regulatory network and that SHH signaling activity is spatiotemporally precisely controlled for proper palatal shelf growth and palatal fusion.

In addition to regulating palatal shelf growth and fusion, the crosstalk between SHH and FGF signaling pathways play a crucial role in the formation and patterning of the palatal rugae, the periodic epithelial ridges on the oral side of the hard palate that are involved in sensing and holding food (Economou et al., 2012). Both *Fgf10*$^{-/-}$ and *Fgfr2b*$^{-/-}$ mice lack palatal rugae (Hosokawa et al., 2009; Welsh and O'Brien, 2009). Mice lacking both Spry1 and Spry2, intracellular antagonists of FGF signaling, showed highly disorganized palatal rugae (Economou et al., 2012). Epithelium-specific inactivation of *Shh* also resulted in highly disorganized rugae. In palatal explant cultures, inhibiting FGF signalling or enhancing SHH signaling inhibited ruga formation, whereas antagonizing SHH signalling by using cyclopamine resulted in dramatic broadening of the *Shh*-expressing domain at 24 hours of treatment. These results suggest that the formation of palatal rugae is regulated by a Turing-type activator-inhibitor reaction-diffusion mechanism, with FGF signaling acting as an activator whereas SHH signaling acts as an inhibitor to regulate the periodic ruga formation (Economou et al., 2012) (Fig. 2H). Interestingly, deletion of *β-Catenin* in *Shh*-expressing palatal epithelial cells resulted in complete loss of palatal rugae formation accompanied by down-regulation of *Shh* and *Ptch1* expression (Lin et al., 2011). On the other hand, mice lacking Sostdc1, a secreted antagonist of BMP and WNT signaling (Ahn et al., 2010; Itasaki et al., 2003; Laurikkala et al., 2003), and mice lacking Lrp4, an antagonist of Lrp6-mediated WNT signaling (Li et al., 2010), displayed disorganized palatal rugae (Kawasaki et al., 2018). Together, these data indicate with both FGF and WNT signaling pathways interact with SHH signaling to regulate palatal rugae formation and patterning.

5. SHH function in mandible development and jaw patterning

5.1 SHH signaling in regulation of mandibular patterning

The mandible is a complex structure, composed of bone, cartilage, teeth, muscle, bone marrow, nerves, vascular and connective tissues. Most of the mandibular tissues arise

from the paired first pharyngeal arches in the early embryo. At around E9.5 in mouse, the mandibular processes become morphologically distinguishable from the more dorsally located maxillary processes. During early mandible development, *Shh* is expressed in the oral epithelium and anterior pharyngeal endoderm whereas *Ptch1* is expressed in both oral epithelium and the underlying mandibular mesenchyme, indicating that SHH signals to both cell types (Fig. 3A and B) (Jeong et al., 2004; Xu et al., 2019). Tissue-specific inactivation of *Shh* in the oropharyngeal epithelium (*Shh*$^{fx/-}$*;Nkx2.5Cre*) or tissue-specific inactivation of *Smo* in either the pre-migratory CNCCs (*Smo*$^{n/c}$*;Wnt1Cre*) or CNCC-derived distal mandibular arch mesenchyme (*Smo*$^{n/c}$*;Hand2Cre*) in mouse embryos resulted in increased apoptosis and decreased cell proliferation in the distal mandibular mesenchyme by E10.5, resulting in micrognathia and aglossia at birth (Billmyre and Klingensmith, 2015; Jeong et al., 2004; Xu et al., 2019).

Through unbiased single-cell RNA-seq analysis followed by *in situ* hybridization analysis, we found that SHH and BMP4 signalling pathways are activated in a complementary pattern along the oral and aboral axis in the developing mandibular arch. *Shh* mRNA is expressed in the oropharyngeal side of the mandibular epithelium at E10.5. Several down-stream target genes of SHH signalling, including *Foxf1*, *Foxf2* and *Ptch1*, are expressed in the oropharyngeal side of the mandibular arch mesenchyme cells. BMP4 signalling target genes, including *Msx1*, *Msx2* and *Alx4*, are expressed in an aboral-to-oral gradient in the mandibular arch mesenchyme (Fig. 3A–D). Tissue specific inactivation of *Smo* in CNCC-derived mesenchyme in the distal mandibular mesenchyme (*Smo*$^{c/c}$*;Hand2Cre*) caused expansion of BMP4 signalling, indicated by phosphorylated Smad1/5/9, to throughout the oral-aboral axis of the distal mandibular mesenchyme. These mutant mice displayed duplication of the dentary bone in the oral side of the mandible at the expense of tongue formation. Furthermore, tissue specific inactivation of *Foxf1* and *Foxf2* in the CCNC-derived mesenchyme (*Foxf1*$^{c/c}$*;Foxf2*$^{c/c}$*;Wnt1Cre*) resulted in similar mandibular defects as the *Smo*$^{c/c}$*;Hand2Cre* mutants, including orally expanded BMP signalling, ectopic bone formation at the oral side of mandible, and oral tongue agenesis, indicating that *Foxf1* and *Foxf2* are key mediators of SHH signalling in regulating the oral-aboral patterning of mandible (Fig. 3E) (Xu et al., 2019).

5.2 SHH signaling in regulation of cell survival in the developing mandibular arch

We have shown that disruption of SHH signalling in the mandibular mesenchyme (*Smo*$^{c/c}$*;Hand2-Cre*) resulted in increased apoptosis associated with expanded BMP signalling in the distal mandible and that genetically deleting one copy of the *Bmp4* gene in the *Smo*$^{c/c}$*;Hand2-Cre* mutants significantly reduced apoptosis in the mandibular mesenchyme (Xu et al., 2019). Billmyre and Klingensmith (2015) showed that inhibition of p53 reduced mandibular mesenchyme apoptosis in the *Shh*$^{fx/-}$*;Nkx2.5Cre* embryos (Billmyre and Klingensmith, 2015). It has also been shown that ectopic activation of BMPR1A signalling in CNCCs caused a significant up-regulation of p53 and p53-mediated apoptosis (Hayano et al., 2015). Together, these results suggest that SHH-SMO signalling regulates mandibular mesenchyme cell survival through inhibition of BMP signalling induced p53-mediated cell apoptosis.

It has been shown that deletion of *Islet1* (*Isl1*), which encodes an LIM homeodomaincontaining transcription factor, in the *Shh*-expressing oropharyngeal epithelial cells (*ShhCre;Isl1*$^{fl/fl}$) resulted in distal mandible truncation (Li et al., 2017), a similar phenotype as in the *Smo*$^{c/c}$*;Hand2-Cre* mice (Xu et al., 2019). The *ShhCre;Isl1*$^{fl/fl}$ embryos exhibited increased apoptosis and decreased cell proliferation in the distal mandibular arch mesenchyme at E10.5 and E11.5, respectively, with concomitant downregulation of expression of *Shh*, *Ptch1*, *Gli1* and of a group of *Fox* genes (Li et al., 2017). Interestingly, expression of several WNT antagonists was upregulated in the distal mandibular arch in the *ShhCre;Isl1*$^{fl/fl}$ embryos. Cre/loxP-induced reactivation of β-Catenin in the *Shh*-expressing cells in the *Isl1* mutant embryos restored *Shh* expression, resulting in restored cell survival and partially rescued distal mandible in the *ShhCre;Isl1*$^{fl/fl}$*;Ctnnb*$^{ex(3)fl/+}$ embryos (Li et al., 2017). Together, these data indicate that the Isl1 transcription factor regulates WNT signaling to control expression of *Shh* in the oropharyngeal epithelium and SHH signaling interacts with BMP4 signaling to regulate survival and patterning of the CNCC-derived mandibular mesenchyme.

5.3 Role of the primary cilia in SHH signaling regulation of mandibular development

Genetic inactivation of several genes encoding components of the primary cilia in CNCCs also caused mandibular defects (Ashe et al., 2012; Brugmann et al., 2010; Eguether et al., 2014; Keady et al., 2012; Kitamura et al., 2020; Millington et al., 2017; Schock et al., 2017). Mice lacking Ift27 and mice with tissue specific inactivation of *Kif3a* (*Kif3a*$^{fl/fl}$*;Wnt1Cre*) exhibited micrognathia and aglossia/microglossia (Brugmann et al., 2010; Eguether et al., 2014; Millington et al., 2017; Schock et al., 2017). Deletion of *Ift88* in CNCCs (*Ift88*$^{fl/fl}$*;Wnt1Cre*) resulted in micrognathia, oral tongue agenesis and formation of ectopic mandibular bones, similar as in *Smo*$^{fl/fl}$*;Wnt1Cre* mutant mice (Kitamura et al., 2020). Notably, in contrast to the gain of SHH signaling activity due to loss of Gli2R/Gli3R-mediated repression in the developing frontonasal primordia in various ciliopathic mutant mouse lines (Chang et al., 2016), as described earlier, the mandibular defects in these ciliopathic mutant mouse lines primarily reflect a requirement for the primary cilia mediated Smo and Gli activation, further highlighting the context-dependent effects of ciliary function in the regulation of SHH signaling.

5.4 SHH signaling and Meckel's cartilage formation

SHH signalling has also been implicated to play an important role in Meckel's cartilage differentiation. During mandible development, a subset of the CNCC-derived mandibular mesenchyme cells condense and then differentiate into chondrocytes to form Meckel's cartilage, a rod-like structure providing shape to the lower jaw (Yuan and Chai, 2019). *Shh*$^{fx/-}$*;Nkx2.5Cre* mutant mice displayed complete loss of Meckel's cartilage (Billmyre and Klingensmith, 2015). Cyclopamine treated mouse mandibular explants exhibited a stage-dependent inhibition of Meckel's cartilage differentiation, and this phenotype could be rescued by exogenous Fgf8 protein treatment (Melnick et al., 2005). In chick embryos, overexpression of *Shh* in the non-oral ectoderm resulted in the formation of ectopic cartilage associated with expansion of *Fgf8* expression (Haworth et al., 2007). Moreover, grafting Shh-expressing cells into the first pharyngeal arch of early chick embryos induced mirror-image duplication of the mandible, including the Meckel's cartilage (Brito et al., 2008).

However, *Smo*$^{c/c}$*;Wnt1Cre* and *Smo*$^{c/c}$*;Hand2Cre* mutant mice developed distally truncated Meckel's cartilage, likely due to aberrant apoptosis of the distal mandibular mesenchyme prior to Meckel's cartilage formation (Jeong et al., 2004; Xu et al., 2019). These results suggest that SHH signalling regulates development of Meckel's cartilage through an indirect mechanism, possibly through regulation of *Fgf8* expression in the mandibular arch epithelium.

6. SHH function in tongue organogenesis

The mammalian tongue is composed of mesoderm derived muscles, CNCCs-derived connective tissues, and non-keratinized epithelium. The anterior two-thirds of the tongue arises from the mandibular arch whereas the posterior third of the tongue is derived from the 3rd and 4th pharyngeal arches. At the initiation stage of tongue development, around E10.5 in mouse, the tongue primordium consists of CNCC-derived mesenchymal cells covered by a layer of oral epithelium. By E11.5, myogenic precursor cells from the occipital somites had migrated into the tongue primordium and subsequently give rise to the intrinsic muscular structures as the CNCC-derived mesenchymal cells develop into connective tissues including tendons and ligaments (Noden and Francis-West, 2006; Parada and Chai, 2015; Parada et al., 2012).

Several different mutant mouse models with disruption of SHH signaling during early mandibular development exhibited tongue agenesis (Billmyre and Klingensmith, 2015; Jeong et al., 2004; Xu et al., 2019). In the *Smo*$^{n/c}$*;Wnt1Cre* and *Smo*$^{c/c}$*;Hand2Cre* embryos, *Myf5*-expressing myogenic progenitor cells failed to migrate into the tongue primordium (Jeong et al., 2004; Xu et al., 2019). The *ShhCre;Isl1*$^{fl/fl}$ mice exhibited tongue agenesis associated with loss of *Shh* expression in the mandibular arch epithelium and failure of migration of myogenic precursor cells into the tongue primordium (Zhang et al., 2022). Transgenic expression of *Ihh* in the mandibular arch epithelium was able to partly rescue tongue formation in the *ShhCre;Isl1*$^{fl/fl}$ mice (Zhang et al., 2022). Tissue-specific inactivation of both *Foxf1* and *Foxf2* in CNCCs resulted in oral tongue agenesis and severe muscular defects in the remaining rudimentary tongue (Xu et al., 2019). The *Myf5*-expressing myogenic precursor cells migrated into the mandibular arch by E10.5 but failed to further migrate anteriorly into the tongue primordium in the *Foxf1*$^{c/c}$*Foxf2*$^{c/c}$*Wnt1-Cre* embryos (Xu et al., 2019; Xu et al., 2022). We recently showed that Hedgehog-Foxf1/Foxf2 signaling in the CNCC derived tongue mesenchyme is required for activation of expression of HGF, a growth factor required for migration of myoblasts into the tongue primordium (Xu et al., 2022). Zhang et al. (2022) found that the *ShhCre;Isl1*$^{fl/fl}$ embryos exhibited decreased *Shh* expression and aberrant migration of *Cxcl12*+ mesenchyme cells in the tongue primordium (Zhang et al., 2022). Further study suggested that SHH signaling acts upstream of WNT5a signaling to attract *Cxcl12*+ tongue mesenchymal cells to help guide tongue myoblast migration (Zhang et al., 2022). Altogether, these results indicate that SHH signaling regulates the expression of key chemotactic signals in the CNCC-derived tongue mesenchyme to direct myogenic precursor migration and initiation of tongue myogenesis.

Following tongue initiation and myoblast arrival in the tongue primordium, expression of *Shh* is maintained in the epithelium of the primordial tongue and later becomes restricted

to the fungiform papillae of the anterior tongue by E12.5 (Jung et al., 1999). Temporally induced inactivation of *Shh* after E10.5 in mouse embryos did not affect initial myoblast migration into the tongue primordium but caused disruption and disorganization of the intrinsic muscles, suggesting that SHH signaling continues to regulate tongue myogenesis and tongue muscle organization after initial tongue formation (Okuhara et al., 2019). We recently reported that the *Foxf1*$^{c/c}$*;Foxf2*$^{c/+}$*;Wnt1Cre* and *Foxf1*$^{c/+}$*;Foxf2*$^{c/c}$*;Wnt1Cre* mutant mouse embryos exhibit disrupted lingual septum tendon formation, leading to an absence or disorganization of specific intrinsic muscles in the tongue (Xu et al., 2022). ChIP-seq analysis identified *Hgf, Tgfb2* and *Tgfb3* among others, as candidate direct target genes regulated by Foxf1 and Foxf2 in the developing tongue (Xu et al., 2022). *Tgfb2* and *Tgfb3* expression, and the levels of pSmad2/3, were significantly reduced in the lingual tendon progenitor cells in mouse embryos with temporally induced inactivation of *Shh* gene at E10.5 as well as in the *Foxf1*$^{c/c}$*;Foxf2*$^{c/+}$*;Wnt1Cre* and *Foxf1*$^{c/+}$*;Foxf2*$^{c/c}$*;Wnt1Cre* mutant embryos (Okuhara et al., 2019; Xu et al., 2022). Together, these studies identified a SHH/SMO-Foxf1/Foxf2-TGFβ molecular cascade regulating lingual tendon formation and indirectly regulating tongue muscular organization.

7. SHH function in taste bud formation and homeostasis

In addition to its great ability to move in all directions to perform essential coordinated functions in feeding, speech, and vocalization, the tongue is a major sensory organ. The dorsal mucosal surface of the tongue contains numerous papillae and taste buds, which sense and transduce five basic tastes (sweet, bitter, salt, sour, and umami) to signal palatability, nutritional value, and/or danger of substances in the oral cavity (Chaudhari and Roper, 2010; Golden et al., 2021). There are three types of taste papillae in mammals on the tongue surface: the fungiform papillae, the foliate papillae, and the circumvallate papilla. Taste buds are modified lingual epithelial cells that first form during embryogenesis from focal epithelial thickenings, called taste placodes, and are continually renewed postnatally from mitotically active basal keratinocytes (Barlow, 2022; Castillo et al., 2014; Golden et al., 2021). Multiple studies have demonstrated that SHH signaling is a principal and essential regulator of taste bud formation and patterning as well as taste cell differentiation and homeostasis (Barlow, 2022; Golden et al., 2021; Mistretta and Kumari, 2019). *Shh* is broadly expressed in the embryonic tongue epithelium prior to taste placode formation but its expression becomes restricted to the placode cells and subsequently restricted to the taste-fated cells within growing taste papillae (Bitgood and McMahon, 1995; Hall et al., 1999; Jung et al., 1999). Lineage tracing studies indicate that *Shh*-expressing embryonic taste placode cells differentiate into the first taste receptor cells around birth but do not contribute to other cells in the taste papillae (Thirumangalathu et al., 2009). Bead implantation studies in embryonic tongue explants showed that exogenous SHH repressed taste placode development, whereas pharmacological inhibition or genetic inactivation of SHH signaling resulted in more and enlarged taste buds (Hall et al., 2003; Iwatsuki et al., 2007; Mistretta et al., 2003). Further investigation revealed that SHH signaling restricts taste placode fate by antagonizing retinoic acid (RA) signaling through maintaining the expression of RA degrading enzymes, Cyp26a1 and Cyp26c1 (El Shahawy et al., 2017). Furthermore, mouse genetic studies showed that taste placode formation requires canonical WNT signaling

within the embryonic tongue epithelium (Iwatsuki et al., 2007). In mouse embryonic tongue explants, activation of canonical WNT signaling promoted focal expression of *Shh* (Iwatsuki et al., 2007). On the other hand, exogenous SHH inhibited WNT signaling activity whereas blocking SHH signaling caused expanded WNT target gene expression in the taste primordia (Iwatsuki et al., 2007). SHH and non-canonical WNT signaling pathways have been involved in the formation of circumvallate papilla (Kim et al., 2009). In an *in vitro* culturing system, Kim et al., showed that SHH signaling regulates cell proliferation for taste bud and von Ebner's gland formation, while WNT11/Rock1 signaling modulates cytoskeleton formation for proper structure formation of circumvallate papilla (Kim et al., 2009). Together, these results indicate that the interplay of SHH, RA and WNT signaling pathways control the formation and patterning of lingual taste buds and papillae (Barlow, 2022).

Whereas SHH signaling functions as a repressor of taste bud formation in the embryonic tongue, recent studies show that SHH signaling is required for taste cell renewal and promotes taste receptor cell differentiation in adults (Castillo et al., 2014; Castillo-Azofeifa et al., 2017; Kumari et al., 2015). Genetic lineage tracing studies showed that the embryonic Shh-expressing placode-derived taste receptor cells are lost from taste buds within a few months postnatally and that adult taste progenitors arise from $Krt14^+$ epithelial stem cells after birth (Okubo et al., 2009; Thirumangalathu et al., 2009). Pharmacological inhibition or conditional epithelium-specific inactivation of Smo in adult mice resulted in rapid loss of taste buds due to disruption of taste cell renewal but had no discernable effects on nontaste filiform papillae (Castillo-Azofeifa et al., 2017; Kumari et al., 2015; Kumari et al., 2017), indicating that Smo-mediated Hedgehog signaling in the lingual epithelial cells is selectively required for taste cell differentiation. However, whereas *Shh* expression is detected in postmitotic taste precursor cells derived from $K5^+$ resident epithelial stem cells in the lingual epithelium (Golden et al., 2021; Miura et al., 2006; Miura et al., 2001; Miura et al., 2014), conditional deletion of *Shh* in the $K5^+$ lineage resulted in reduction in *Shh* expression in the taste precursor cells but did not affect taste bud renewal (Castillo-Azofeifa et al., 2017). Castillo-Azofeifa et al. (2017) found that sensory neurons innervating the taste buds express *Shh* and that SHH supplied by the taste nerves and local taste epithelium act in concert to support continued taste bud differentiation (Castillo-Azofeifa et al., 2017). Whereas recent studies have identified Sox2 and Foxa2 as transcription factors acting downstream of SHH signaling in taste bud renewal (Castillo-Azofeifa et al., 2018; Golden et al., 2021), the exact molecular mechanisms mediating SHH signaling function in taste bud renewal remain to be elucidated.

8. SHH signaling in tooth development

Tooth development begins as a thickening of the oral epithelium, called dental lamina, at around E11 in mice. The dental lamina proliferates and protrudes into the underlying CNCC-derived mesenchyme and induces the mesenchyme to condense around the epithelial bud from E12 to E13. At around E13.5, cells at the tip of the epithelial tooth buds form an epithelial signaling center, termed the primary enamel knot, which secretes a number of growth factors including Shh (Dassule et al., 2000; Seppala et al., 2017). The interactions between tooth bud epithelium and the surrounding mesenchyme drive further

tooth morphogenesis through the “cap” and “bell” stages. As development proceeds, the epithelial cells in contact with the dental mesenchyme differentiate into ameloblasts and their adjacent mesenchymal cells differentiate into odontoblasts, and further develop into mature tooth (Cobourne and Sharpe, 2005; Lan et al., 2014; Thesleff and Sharpe, 1997; Tucker and Sharpe, 2004).

During initiation stage of tooth development, the dental epithelium goes through a process of thickening (to form vertically- oriented cells), stratification (to form multiple cells layers), and invagination to become an epithelial bud. *Shh* is one of the earliest markers of the dental epithelial cells (Cobourne et al., 2001; Keranen et al., 1999). The expression domain of *Shh* in the dental epithelium is complementary with the expression domain of *Wnt7b* in the oral epithelium (Sarkar et al., 2000). Transient ectopic expression of *Wnt7b* in the dental epithelium in mandibular arch explants led to down-regulation of *Shh* and *Ptch1* expression, and arrest of tooth development at the initiation stage (Sarkar et al., 2000). In the Wnt7b-infected mandibular arch explants, treatment with SHH-soaked beads restored tooth development (Sarkar et al., 2000). Furthermore, blocking SHH signaling using 5E1 anti-Shh antibody from E10.5 caused largely reduced dental epithelial thickening and arrested tooth development in mouse (Cobourne et al., 2001). These results led to a hypothesis that Wnt7b interacts with Shh to maintain the boundary of oral-dental epithelium during tooth initiation (Sarkar et al., 2000). *In vivo* mouse genetic studies further revealed the importance of SHH signaling in tooth development. Enhanced level of Shh in basal epithelium driven by a Keratin-14 promoter (*K14-Shh*) results in tooth development arrest at the bud stage, secondary to a lack of cell proliferation in this region (Cobourne et al., 2009). The *Gli2/Gli3* double homozygous ($Gli2^{-/-};Gli3^{-/-}$) mutants did not develop any normal teeth indicating an essential role of SHH signaling pathway during tooth development (Hardcastle et al., 1998). Bead implantation experiments showed that exogenous Shh treatment stimulates, whereas blocking of SHH signaling by cyclopamine inhibits, dental epithelial invagination (Li et al., 2016). Interestingly, inhibition of SHH signaling by cyclopamine starting from E11.5 did not prevent stratification of the dental placode but resulted in shallower and wider tooth buds (Li et al., 2016). In contrast, inhibition of FGF signaling in the same context caused failure of stratification in the dental placodal area. On the other hand, FGF10 treatment at E11.5 induced a prominent thickening of the epithelium. Together, these results suggest that SHH signaling drives cell rearrangement in the prospective dental epithelial tissue for tooth bud formation after FGF signaling induced stratification of the dental placode (Li et al., 2016).

Following tooth bud formation, *Shh* expression becomes restricted to the tip of the epithelial bud at around E13.5 and in the primary enamel knot at the cap stage (Dassule et al., 2000). At the bell stage, *Shh* mRNA is expressed in the stellate reticulum, stratum intermedium, pre-ameloblasts, and differentiating ameloblasts. Shh protein, *Ptch1* and *Gli1* can be observed in a broader domain in the dental epithelium and the dental papilla, indicating that SHH signaling may exert a long-range activity (Gritli-Linde et al., 2002; Gritli-Linde et al., 2001). In mice, tissue specific inactivation of *Shh* in dental epithelium ($Shh^{c/n};K14\text{-}Cre$) shortly after ingrowth of the tooth bud resulted in a cap stage tooth rudiment in which the morphology is severely disrupted (Dassule et al., 2000). Although the enamel knot still formed in these mutant mice, the overall size of the tooth is reduced, and the lingual

epithelial invagination and the dental cord are absent (Dassule et al., 2000). This study revealed that Shh regulates growth and shape of the tooth. Further study found *Ptch1* is more strongly expressed in the lingual side of the cap stage tooth germ (Dassule et al., 2000). Epithelial specific inactivation of *Smo* in $Smo^{c/n}$;*K14-Cre* mutant mice resulted in altered proliferation, differentiation, and polarization of ameloblasts (Gritli-Linde et al., 2002), indicating that SHH signaling regulates the growth and polarization of the dental epithelium. On the other hand, the $Smo^{c/n}$; *Wnt1-Cre* mice lack lower incisors and developed only one central upper incisor and malformed small molar tooth germs (Jeong et al., 2004), indicating that SHH signaling plays primary roles in both epithelial and mesenchymal cells to control tooth morphogenesis.

SHH signaling is also plays crucial roles in tooth root formation in postnatal mice. *Shh* is expressed in Hertwig's epithelial root sheath (HERS), a bilayered tissue formed from the inner and outer enamel epithelia, during early root morphogenesis (Liu et al., 2015; Nakatomi et al., 2006). *Ptch2*, a receptor of SHH signaling was also found in HERS (Nakatomi et al., 2006), while *Gli1* and *Ptch1* were detected in the proliferative dental mesenchyme in addition to the HERS (Liu et al., 2015; Nakatomi et al., 2006). Inhibition of SHH signaling with HH inhibitor or constitutive activation of SHH signaling genetically (*Gli1-creERT2;R26SmoM2*$^{fl/fl}$) caused decreased proliferation and shorter roots (Liu et al., 2015). $Nfic^{-/-}$ mice exhibited defect in root formation accompanied by increase SHH signaling (Liu et al., 2015; Steele-Perkins et al., 2003). Treatment with HH inhibitors partially restored root development in $Nfic^{-/-}$ mice (Liu et al., 2015). Interestingly, Shh is also shown to act upstream of *Nfic* and downstream of BMP/TGFβ-SMAD signaling in HERS controlling tooth root formation (Huang et al., 2010). Moreover, the mesenchymal dysplasia (*mes*) mice, which express a mutant Ptch1 protein with an abnormal C-terminus, have short tooth roots and reduced proliferation around the HERS (Nakatomi et al., 2006). Together, these studies highlight an important role for SHH signalling in regulating tooth root formation.

In mice, stem cells are maintained in the incisor tooth germs throughout life, resulting in continuous growth of the incisors (Feng et al., 2011; Harada et al., 1999). SHH signaling plays important roles in regulating the maintenance of both epithelial and mesenchymal stem cells in the mouse incisor tooth germs. Previous studies used Gli1, which is a transcription factor and down-stream target of SHH signaling pathway, as a marker of epithelial and mesenchymal stem cells in postnatal mouse incisors (Seidel et al., 2010; Zhao et al., 2014). The epithelial stem niche is located in the cervical loop at the proximal end at the labial side of the incisor (Seidel et al., 2010). Inhibition of SHH signaling pathway disrupts generation of ameloblasts from stem cells, indicating that SHH signaling is essential for generation of ameloblasts (Seidel et al., 2010). Gli1+ cells were also found in the developing molar but decreased significantly after birth (Li et al., 2015). BMP/SMAD4 signaling is implicated in inhibiting the activity of SHH signaling and controlling epithelial stem cell maintenance during molar development (Li et al., 2015). For the incisor mesenchymal stem cell niche, Shh secreted by sensory nerves supports the maintenance of the Gli1-positive stem cells surrounding the arterioles near the cervical loop region and regulates their differentiation into odontoblast and other incisor mesenchyme cells (Zhao et al., 2014).

9. SHH signaling and fetal alcohol exposure

Interactions between environmental stresses and SHH signaling have been shown to contribute to the etiology of craniofacial defects in humans and mice. Alcohol is a common teratogen that could induce a variety of birth defects from exposure during pregnancy (Jones and Smith, 1973). Many of the fetal alcohol syndrome defects, including HPE and other craniofacial defects, have been similarly observed in animal models with dysregulation of SHH signaling. Previous study in chicken showed that embryonic exposure to ethanol during early CNCC migration resulted in increased CNCC apoptosis and decrease in size of the frontonasal mass (Ahlgren et al., 2002). The craniofacial growth defects can be rescued by application of exogenous Shh ligand (Ahlgren et al., 2002). Using zebrafish models, Li et al. showed that alcohol exposure during gastrulation caused inhibition of cholesterol modification of Shh, impaired Hedgehog signal transduction, and a dose-dependent spectrum of defects including HPE (Li et al., 2007). Adding cholesterol rescued the loss of SHH signaling activity and alleviated the developmental defects in the alcohol-treated fish (Li et al., 2007). These studies suggest a role of dysregulation of SHH signaling in mediating the fetal alcohol induced birth defects.

Haploinsufficiency of several SHH pathway components cause structural birth defects in humans. The patients often display incomplete phenotypic penetrance and highly variable expressivity (Solomon et al., 2012), suggesting roles of gene-gene and/or environment-gene interactions. Studies have shown that alcohol exposure synergize with several mutations in SHH pathway components, resulting in significantly enhanced penetrance and severity of birth defects. In mice on the 129S6 background, inactivation of *Cdon* (*Cdon*$^{-/-}$) or in utero ethanol exposure alone resulted in little or no HPE related phenotype, but together they caused inhibition of SHH signaling and a broad spectrum of HPE phenotypes (Hong and Krauss, 2012). Deletion of one *Ptch1* allele rescued ethanol induced HPE phenotype in *Cdon*$^{-/-}$ mice, confirming that the alcohol induced HPE is associated with reduced SHH signaling activity (Hong and Krauss, 2013). Recently, the interaction between *Cdon* loss-of-function mutation and ethanol exposure was shown to regulate the activity of SHH signaling through a Nodal signaling-associated molecular mechanism (Hong et al., 2020). In wildtype C57BL/6J mice, ethanol exposure in utero alone induced a low penetrance of HPE. However, ethanol exposure significantly enhanced the HPE phenotype in *Shh*$^{+/-}$ and *Gli2*$^{+/-}$ mice, respectively, in the C57BL/6J background (Kietzman et al., 2014). Together, these results highlight the role of environmental factors interacting with genetic mutations disrupting SHH signaling in causing the broad spectrums of HPE phenotypes in human patients.

10. Concluding remarks and Perspective

Previous research, especially studies using animal models, revealed essential functions of SHH signaling and interactions between SHH and other signaling pathways during craniofacial development. These studies have provided important insights into the mechanisms underlying *SHH* related diseases. However, several gaps remain in our knowledge about the molecular mechanisms involving SHH signaling in craniofacial development and patterning. **1)** From the onset of facial development, dynamic and

tissue specific activation of SHH and other signaling pathways, including members of the FGF, BMP and WNT families, are repeatedly deployed to drive tissue morphogenesis. The molecular mechanisms controlling the specific and dynamic expression patterns of these signaling molecules remains largely unknown. The tissue specific and precise transduction of SHH signaling activity also needs to be further dissected. For example, previous studies have shown that deleting two or more Hedgehog co-receptors caused more severe phenotype than single gene inactivation in certain tissues. More detailed and tissue-specific studies of the co-receptors and modifiers of SHH will advance our knowledge about the precise regulation of SHH signaling activity. **2)** During several craniofacial developmental processes, SHH signaling plays key roles in maintaining cell survival and regulating tissue growth and patterning. Previous studies have revealed several important down-stream mediators of SHH signaling at different steps of craniofacial morphogenesis. For example, the Fox family transcription factors are known to be important down-stream mediators. Studies on tissue-specific mutant animal models of Fox genes have provided valuable information between SHH signaling mis-regulation and the related phenotypes. However, much remain to be understood about how disruption of SHH signaling lead to the various phenotypes in mutant animal models and human patients. Future studies involving genome wide screening down-stream targets of SHH signaling at multiple steps of craniofacial development and tissue morphogenesis, including comprehensive single cell genomic/transcriptomic analyses, followed by functional studies of the candidate mediators and effectors of SHH signaling will help fill the gap. **3)** Dynamic and highly coordinated interactions between SHH and other signaling pathways have been observed throughout craniofacial development. The positive and negative cross-regulations between SHH and other signaling pathways, involving multiple cell types including CNCCs, orofacial epithelium, and mesoderm derived tissues such as myogenic progenitors, form a complex signaling network driving the developmental processes forming different craniofacial tissues. The mechanisms underlying these cross-regulations are still not well understood. Nowadays cutting-edge technologies such as single cell genomic and proteomic approaches are starting to be widely used in biological and medical studies. Combining these technologies with CRISPR genome edited animal models will help us achieve a better understanding of molecular and cellular mechanisms underlying the precise control of SHH signaling activity, and the gene regulatory networks involving SHH signaling in the multiple stages of craniofacial morphogenesis. Together, these efforts will provide the foundation for new development and improvement of strategies for treatment and prevention of craniofacial birth defects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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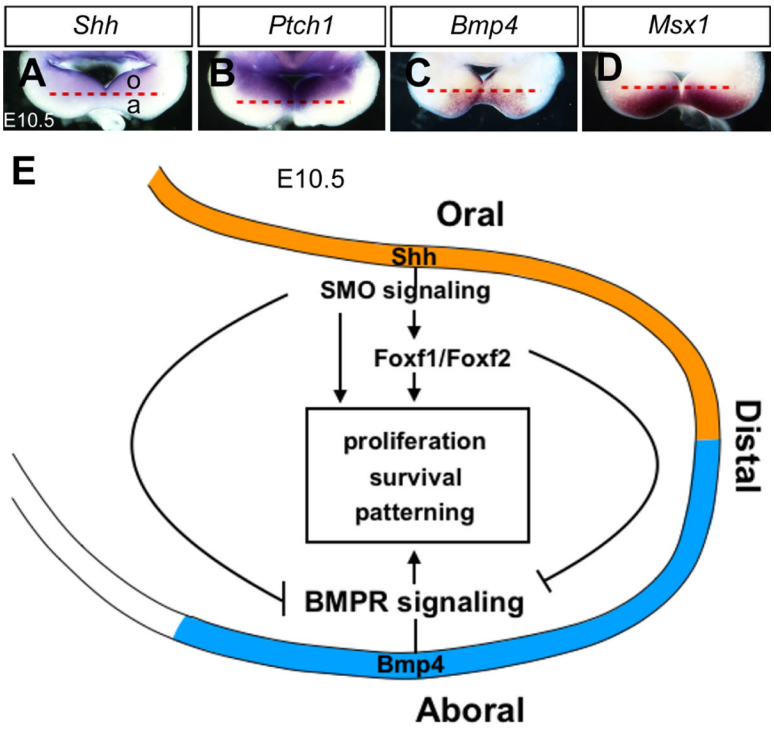

Fig. 1.

Overview of the Shh signaling pathway. **(A)** A simplified schematic of the core steps of the Shh signaling pathway. Shh ligands bind to its receptor Ptch, resulting in relief of Ptch-mediated inhibition of Smo. Activated Smo transduces the signaling activity intracellularly to activate the Gli family of transcription factors through inhibition of PKA and Sufu. *Gli1* and *Ptch1* are among the direct transcriptional target genes of the Gli transcription factors. **(B)** Biogenesis of the Shh ligand. The *Shh* mRNA encodes a large precursor protein, which undergoes auto-cleavage to release the N-terminal signaling domain with a covalently linked cholesterol moiety at the C-terminus followed by addition of palmitoylation at the N-terminal residue. The dually lipidated mature Shh ligand is released from the signalling cell through the functions of Disp and Scube2. **(C, D)** Hedgehog signal transduction in vertebrates requires the primary cilium. In the absence of active Hedgehog ligand, Ptch accumulates and inhibits Smo in the primary cilium. PKA and Sufu serve as major negative regulators of Hedgehog signaling and promote Gli proteins to undergo proteolysis into transcriptional repressor (GliR) (C). Binding of Shh ligands to Ptch and co-receptors induces Ptch removal from the ciliary membrane and accumulation of Smo in the ciliary membrane. Activated Smo promotes the transport of the GliSufu complex to the tip of the primary cilium and inhibits PKA. Full-length Gli proteins are post-translationally modified in the primary cilium to the active forms (GliA). GliA then translocates into the nucleus and drives target gene transcription (D).

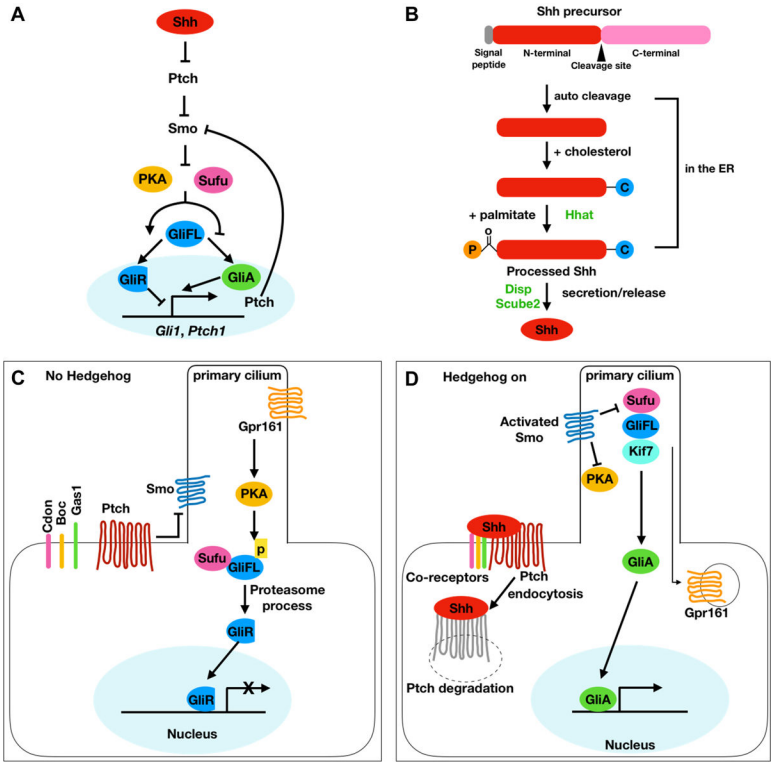

Fig. 2.

SHH signaling plays a central role in regulating palatal shelf growth and patterning. **(A-F)** Whole mount *in situ* hybridization detection of *Shh* mRNAs showing *Shh* was initially expressed throughout the oral epithelium at the onset of palatal outgrowth at E11.5 and becomes restricted to the epithelial ridges that develop into the palatal rugae on the oral side of the palatal shelves. Dashed lines in **(A-D)** outline developing palatal shelves. Dashed lines in **(E-F)** outline the fused palates. r1–r8 mark the individual rugae in the order of their formation. gs indicates geschmacksstreifen. Arrowheads indicate the sensory papillae. The double headed arrow in **(A)** shows the anterior-posterior axis of developing palate. **(G)** Schematic diagram of a coronal section of the developing palatal shelf at E13.5, with the oral side to the left and nasal side to the right, to depict the molecular network involving Shh in the reciprocal epithelial-mesenchymal interactions regulating palatal shelf growth and patterning. The Shh-Osr2-Fgf10-Shh (shown in pink) cascade positively maintains the expression of *Shh* in oral side of palatal epithelium. The Dlx5-Fgf7 (shown in blue) cascade prevents Shh expression at the nasal side of palatal epithelium. The Msx1-Bmp4 (shown in green) feed-back loop has been shown to positively regulate the expression of Shh in the anterior palatal shelves. The Shh-Foxf-Fgf18-Shh circuit has also been shown to maintain the expression of Shh in the palatal epithelium. **(H)** Schematic diagram showing the cross regulation of SHH, FGF and WNT signaling during palatal ruga formation. Palatal ruga formation is regulated by a Turing-type activator-inhibitor reaction-diffusion mechanism, with FGF signaling acting as an activator whereas SHH signaling acting as an inhibitor to regulate the periodic ruga formation. WNT signaling is also required to maintain Shh expression and ruga formation.

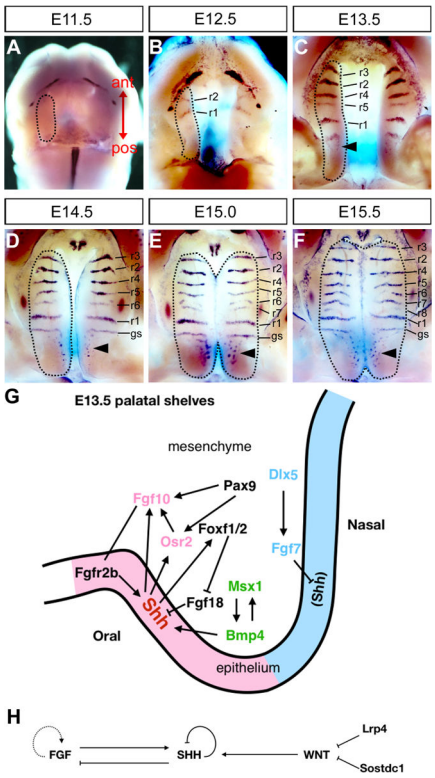

Fig. 3.

SHH signaling and mandible development. **(A-D)** Whole mount rostral views of E10.5 embryonic mouse mandibular arches showing the patterns of expression of *Shh* **(A)**, *Ptch1* **(B)**, *Bmp4* **(C)** and *Msx1* **(D)** mRNAs. The mRNA signals are shown in purple/brown color. o, oral, and a, aboral domains of develop mandibular arch. **(E)** Schematic diagram depicting the crucial roles of SHH signaling in regulating mandibular arch growth and oral-aboral patterning through antagonizing the BMP signaling pathway at E10.5. *Shh* mRNA is expressed in the oropharyngeal side of the mandibular epithelium at E10.5. *Foxf1* and *Foxf2*, known downstream targets of SHH signaling, are expressed in the oropharyngeal side of the mandibular arch mesenchyme cells. BMP4 signalling activated in an aboral-to-oral gradient in the mandibular arch mesenchyme. SHH signaling antagonize BMP4 signaling and regulate the growth, cell survive and patterning of developing mandibular arch.