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# Primary cilia in murine palatal rugae development

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ABSTRACT

Periodic patterning of iterative structures is a fundamental process during embryonic development, since these structures are diverse across the animal kingdom. Therefore, elucidating the molecular mechanisms in the formation of these structures promotes understanding of the process of organogenesis. Periodically patterned ridges, palatal rugae (situated on the hard palate of mammals), are an excellent experimental model to clarify the molecular mechanisms involved in the formation of periodic patterning of iterative structures. Primary cilia are involved in many biological events, including the regulation of signaling pathways such as Shh and non-canonical Wnt signaling. However, the role of primary cilia in the development of palatal rugae remains unclear. We found that primary cilia were localized to the oral cavity side of the interplacode epithelium of the palatal rugae, whereas restricted localization of primary cilia could not be detected in other regions. Next, we generated mice with a placodal conditional deletion of the primary cilia protein *Ift88*, using *ShhCre* mice (*Ift88*$^{fl/fl}$*;ShhCre*). Highly disorganized palatal rugae were observed in *Ift88*$^{fl/fl}$*;ShhCre* mice. Furthermore, by comparative *in situ* hybridization analysis, many Shh and non-canonical Wnt signaling-related molecules showed spatiotemporal expression patterns during palatal rugae development, including restricted expression in the epithelium (placodes and interplacodes) and mesenchyme. Some of these expression were found to be altered in *Ift88*$^{fl/fl}$*;ShhCre* mice. Primary cilia is thus involved in development of palatal rugae.

## 1. Introduction

Many organs share the same molecular mechanisms and fundamental processes, as seen in the genetic commonality during developmental processes. Periodic patterning is a common structural element observed in many organs. Thus, clarifying the molecular mechanisms involved in periodic pattern formation promotes understanding of the process of organogenesis.

Palatal rugae are corrugated structures on the hard palate and are conserved in all mammals (Thomas, 1984; Takanosu et al., 1996; Sakamoto et al., 1989). They function in the tactile sensing of objects including food, assist in holding and crushing food, and aid in tongue placement during the production of certain speech sounds. The number and patterns of palatal rugae are known to be species-specific. In mice, there are eight or nine palatal rugae. Three transverse ridges called antemolar rugae are formed just behind the incisor teeth, which cross the midline. Five or six intermolar rugae are observed between the molar tooth fields. These rugae are shorter, more oblique, and do not cross the midline (Fig. 1A). Since palatal rugae are consistent in mice, development of murine palatal rugae is likely to be under strict genetic control (Sohn et al., 2011; Charles et al., 2007; Pantalacci et al., 2008, 2009; Lin et al., 2011; Porntaveetus et al., 2010; Welsh and O'Brien, 2009; Welsh et al., 2007; Economou et al., 2013; Lee et al., 2011). Thus, palatal rugae are an excellent experimental model to clarify the molecular mechanisms behind periodic patterning of iterative structures.

The first morphological sign of rugae development is local thickening of the palatal epithelium to form placodes while the underlying mesenchymal cells condense (Fig. 1B). Placodes can be detected as slight protrusions on the surface of the developing palate as the thickened epithelium protrudes over the surface. Subsequently, the placode regions bulge toward the oral cavity to form an overall corrugated appearance (Peterkova et al., 1987). Palatal rugae are sequentially added on the growing palate; their interposition appears to be dependent on activation-inhibition mechanisms. Thus, rugae development has been proposed as a simple tool to study regulation of patterning of serial structures (Pantalacci et al., 2008). We previously found that

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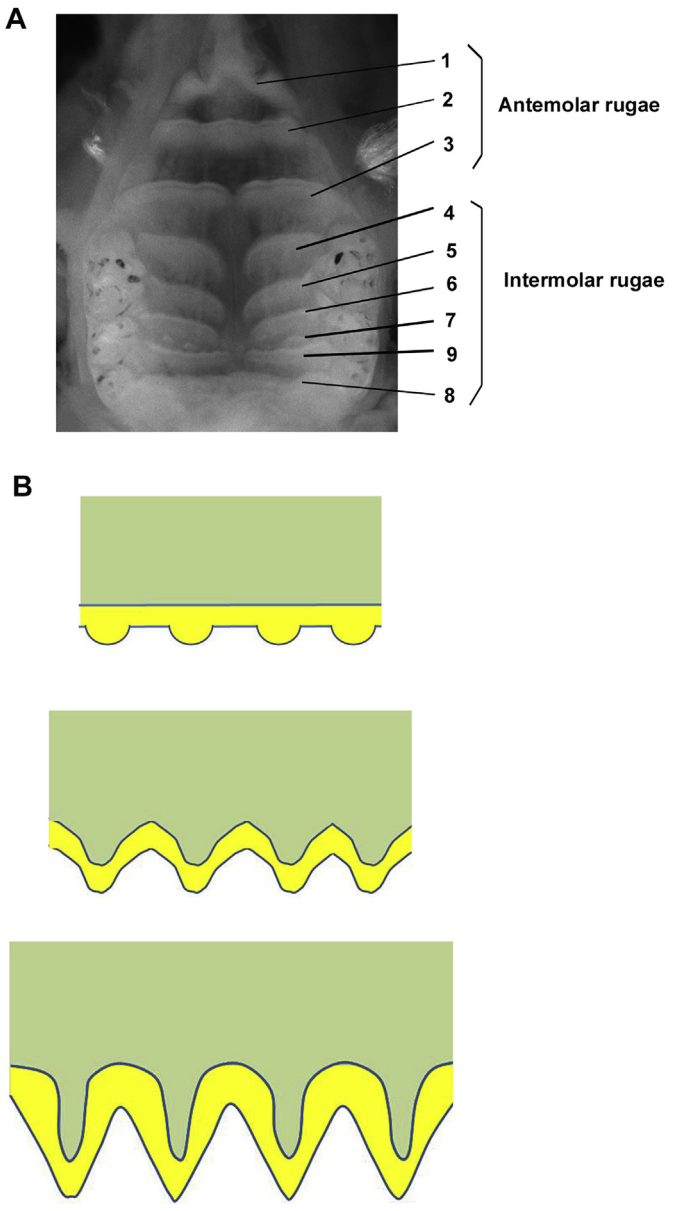


**Fig. 1.** Murine palatal rugae development. (A) SEM image of adult murine palatal rugae. (B) Diagrammatic representation of the developmental stages of palatal rugae formation (sagittal view). Mesenchyme (light green), Epithelium (yellow).

murine palatal rugae development is regulated by Fgf and Shh signaling through reaction-diffusion mechanisms (Economou et al., 2012) and that the interaction between *Wise* and *Lrp4* is involved in palatal rugae development by regulating the reaction-diffusion mechanisms through Shh and Fgf signaling (Kawasaki et al., 2018). However, molecular mechanisms behind palatal rugae development are not fully understood.

The primary cilium, a nonmotile organelle that exists on almost all somatic cell surfaces in vertebrates, has shown to play critical roles in many biological processes including the determination of left/right axis patterning and regulation of signaling pathways and sensory functions such as the detection of light, odor, fluid flow, osmolarity changes, and sound (Evans et al., 2006; Bisgrove and Yost, 2006; Zaghloul and Brugmann, 2011). The primary cilium consists of a membrane-bound cylinder surrounding nine doublet microtubules that extend from a centriole known as the basal body. Cilia are assembled and maintained by intraflagellar transport (IFT), in which multiple protein complexes

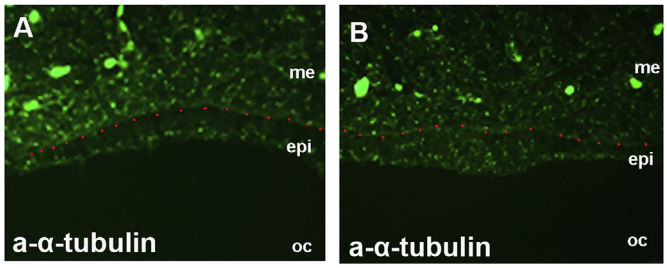


**Fig. 2.** The primary cilia in palatal rugae development. Sagittal sections showing immunohistochemistry for acetylated α-tubulin in interplacode (A) and placode (B) region at E14.5. Epithelium was outlined by red dots. me; mesenchyme, epi; epithelium, oc; oral cavity.

are moved bidirectionally along the axoneme by the coordination of IFT motors. In IFT, groups of protein particles are transported from the base to the tip of the cilia (anterograde transport) by kinesin II and from the tip to the base (retrograde transport) by cytoplasmic dynein 1b. The IFT particles are composed of at least 17 polypeptides that form 2 complexes: complexes A and B (Cole et al., 1998; Cole, 2003; Eggenschwiler and Anderson 2007). This study aims to investigate whether primary cilia are involved in palatal rugae development.

## 2. Results

### 2.1. Primary cilia in developing palatal rugae

To understand where the primary cilia are located in palatal rugae primordia, we examined acetylated α-tubulin in developing rugae. Acetylated α-tubulin immunoreaction was ubiquitously observed in mesenchyme of palatal rugae. On the other hand, in interplacode epithelium, acetylated α-tubulin was localized on the oral cavity side of epithelial cells (Fig. 2A). However, any restricted localization of acetylated α-tubulin could not be detected in placode epithelium (Fig. 2B).

### 2.2. The palatal rugae in Ift88 mutant mice

The *Ift88* gene encodes a protein required for IFT and the formation of primary cilia (Murcia et al., 2000). Recently, *Ift88* has shown to be involved in palate development (Tian et al., 2017; Watanabe et al., 2019). To clarify whether primary cilia regulate palatal rugae development, we generated *Ift88*$^{fl/fl}$*;ShhCre* mice. *Shh*-expressing cells are known to play a critical role in palatal rugae development (Economou et al., 2012). *Shh* has shown to be expressed in the whole placodes of developing palatal rugae (Fig. 3A and B). *Ift88* is therefore deleted from all palatal rugae placodes in *Ift88*$^{fl/fl}$*;ShhCre* mice. In *Ift88* mutants, highly disorganized palatal rugae (e.g. waved, folded, supernumerary, lacked, shortened and fragmented palatal rugae) was observed in the intermolar rugae, although palatal rugae phenotypes were varied between *Ift88* mutant mice (Fig. 3D). Unlike the intermolar rugae, no obvious anomalies were found in the antemolar rugae.

### 2.3. Expression of Shh signaling-related molecules in palatal rugae development

Primary cilia are structures only 200–300 nm in diameter, and a few microns in length, making it slight technically difficult to capture the images of ciliary proteins such as IFT proteins even in cultured cells (Hua and Ferland, 2017). Shh signaling is activated within the primary cilia and is known to regulate palatal rugae development (Sohn et al., 2011; Charles et al., 2007; Pantalacci et al., 2008, 2009; Lin et al., 2011; Porntaveetus et al., 2010; Welsh and O'Brien, 2009; Welsh et al., 2007; Economou et al., 2013; Lee et al., 2011). Unlike ciliary proteins, Shh-related molecules can be detected by immunohistochemistry and *in*

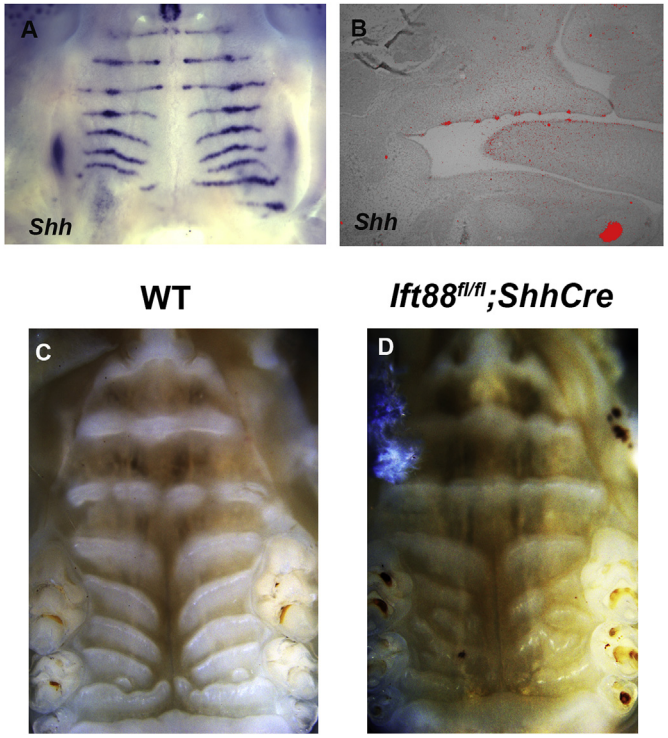


**Fig. 3.** Palatal rugae phenotypes in *Ift88* mutant mice. Whole mount (A) and sagittal sections (B) showing *in situ* hybridization of *Shh* at E14.5. Oral views of palatal rugae in adult wild-type (C) and *Ift88*$^{fl/fl}$*;ShhCre* mice (D).

*situ* hybridization as *in vivo* gene and protein expression analyses. However, the expression of Shh signaling-related molecules in palatal rugae development is not fully understood. We therefore examined the expression of Shh signaling-related molecules in palatal rugae to further understand the temporo-spatial involvement of primary cilia in palatal rugae development. *Ptch1*, a receptor of Shh signaling, was expressed in mesenchyme underneath the placodes at E14.5, whereas *Ptch1* expression was observed in both the placodes and mesenchyme underneath the placodes at E16.5 (Fig. 4A and B). Another receptor of Shh, *Ptch2*, was also expressed in mesenchyme underneath the placodes at E14.5 (Fig. 4C). Unlike *Ptch1*, *Ptch2* showed expression in the interplacode epithelium at E16.5 (Fig. 4D). We next examined the transcription factors activated by Shh signaling: *Gli1*, *Gli2*, and *Gli3*. Likewise *Ptch1*, *Gli1* exhibited expression in mesenchyme underneath the placodes at E14.5 and expression in the placodes and mesenchyme underneath the placodes at E16.5 (Fig. 4E and F). *Gli2* was ubiquitously expressed in developing rugae; however, expression in mesenchyme underneath the placodes at E14.5 and in the placodes and mesenchyme underneath the placodes at E16.5 was slightly stronger than expression in other regions (Fig. 4G and H). *Gli3* was ubiquitously expressed in developing rugae (Fig. 4I and J). *Smo* (an essential effector of the Shh pathway) also showed ubiquitous expression in developing rugae (Fig. 4K, L).

### 2.4. Expression of non-canonical Wnt signaling-related molecules in palatal rugae development

Wnt signaling is divided into two pathways: canonical and non-canonical (Gómez-Orte et al., 2013; Grigoryan et al., 2008). It has been shown that canonical Wnt signaling plays a critical role in palatal rugae development, since mutation of β-catenin results in no palatal rugae formation (Lin et al., 2011). Many non-canonical Wnt signaling-related molecules are known to be localized within the primary cilia (Goggolidou and Wilson, 2016; Kim and Park, 2016). However, it is unclear whether these non-canonical Wnt signaling-related molecules

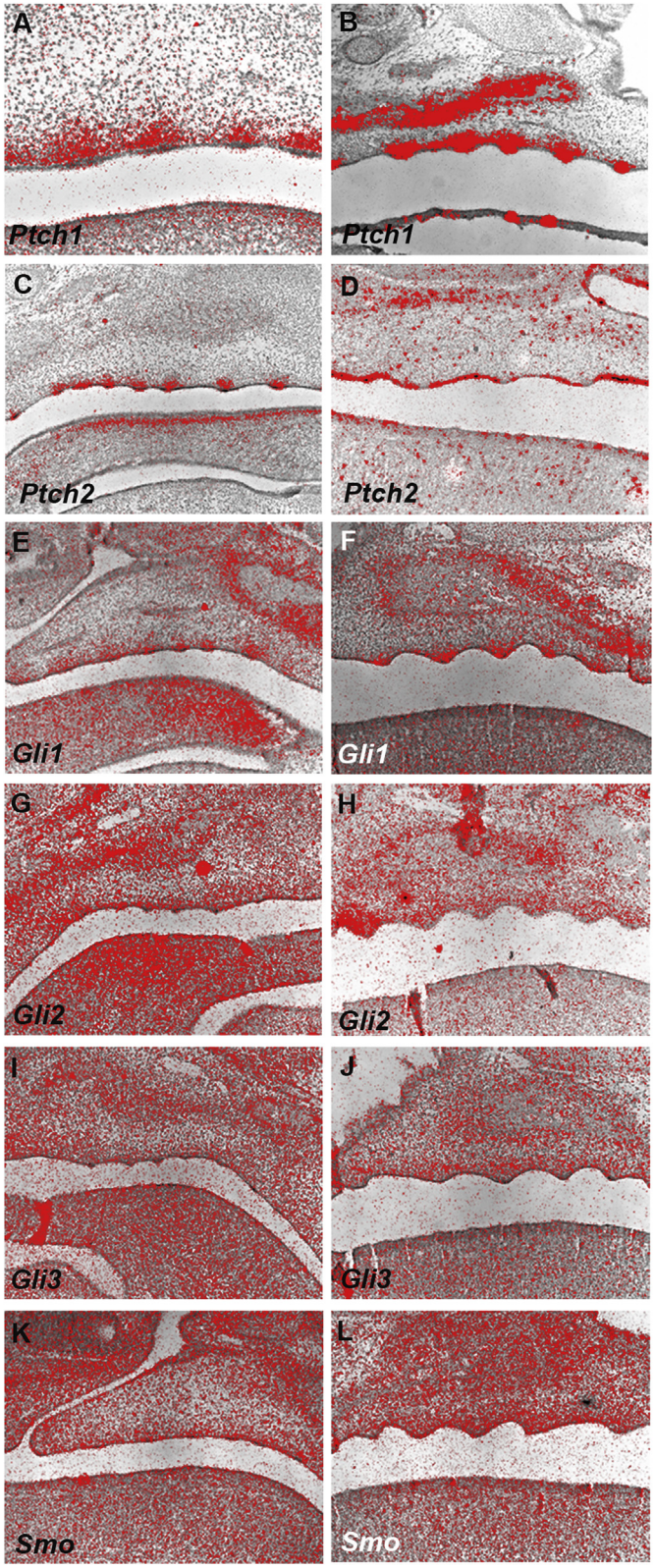


**Fig. 4.** Shh signaling-related molecules in palatal rugae development. Sagittal sections showing *in situ* hybridization of *Ptch1* (A, B), *Ptch2* (C, D), *Gli1* (E, F), *Gli2* (G, H), *Gli3* (I, J) and *Smo* (K, L) at E14.5 (A, C, E, G, I, K) and E16.5 (B, D, F, H, J, L).

are expressed in palatal rugae development. We therefore performed comparative *in situ* hybridization analysis of non-canonical Wnt-related genes during palatal rugae development. *Wnt1*, *Wnt4*, *Wnt5*, *Wnt6*, and

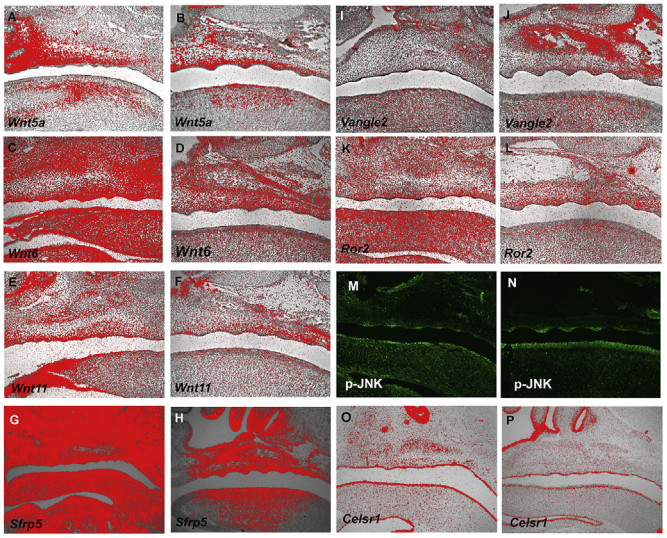


**Fig. 5.** Non-canonical Wnt signaling-related molecules in palatal rugae development. Sagittal sections showing *in situ* hybridization of *Wnt5a1* (A, B), *Wnt6* (C, D), *Wnt11* (E, F), *Sfrp5* (G, H), *Vangle2* (I, J), *Ror2* (K, L), *Celsr1* (O, P) and immunohistochemistry for p-JNK (M, N) at E14.5 (A, C, E, G, I, K, M, O) and E16.5 (B, D, F, H, J, L, N, P).

*Wnt11* are known to be ligands involved in non-canonical Wnt signaling. *Wnt1* and *Wnt4* could not be detected in developing palatal rugae (data not shown). *Wnt5a* was expressed in palatal rugae mesenchyme at both E14.5 and E16.5 (Fig. 5A and B). *Wnt6* exhibited ubiquitous expression in developing rugae (Fig. 5C and D). *Wnt11* was expressed in rugae epithelium at E14.5 and weakly expressed in mesenchyme at E16.5 (Fig. 5E and F). Sfrp is a secreted protein that binds to Wnt ligand to prevent the activation of both canonical and non-canonical Wnt signaling. *Sfrp*5 was ubiquitously expressed in developing rugae at both E14.5 and E16.5 (Fig. 5G and H). *Vangl2* is known to be a core component of non-canonical Wnt signaling pathway, and it could not be detected in developing rugae at E14.5 (Fig. 5I, Gómez-Orte et al., 2013). However, *Vangle2* showed weak expression in mesenchyme underneath the placodes at E16.5 (Fig. 5J). *Ror2* acts as a receptor or coreceptor for *Wnt5a* to mediate Wnt5a-induced activation of the non-canonical Wnt pathway (Gómez-Orte et al., 2013). *Ror2* expression was observed in rugae mesenchyme at both E14.5 and E16.5 (Fig. 5K, L). JNK is known to mediate non-canonical Wnt signaling and is phosphorylated when non-canonical Wnt signaling is activated (Gómez-Orte et al., 2013). Phosphorylated JNK was observed in the placodes at both E14.5 and E16.5 (Fig. 5M, N). *Celsr1* and *Celsr2* are key components of non-canonical Wnt signaling (Gómez-Orte et al., 2013). *Celsr1* was expressed in the epithelium at both E14.5 and E16.5 (Fig. 5O, P). *Celsr2* expression could not be detected in the developing rugae at either E14.5 or E16.5 (data not shown).

### 2.5. Expression of molecules-related Shh and/or non-canonical Wnt signaing in palatal rugae development

To further understand Shh and non-canonical Wnt signaing in palatal rugae development, we also examined molecules involved in Shh and/or non-canonical Wnt signaling in developing palatal rugae. *Notch* is related to Shh and non-canonical Wnt signaing during organogenesis (Alfred and Vaccari, 2018; Yabut et al., 2015). *Notch1* was expressed in the interplacode epithelium at both E14.5 and E16.5 (Fig. 6A and B). *Pitx2*, *Pax9*, and *Barx1* have also been shown to be related to non-canonical Wnt signaling during tooth development (Peng et al., 2010). *Pitx2* expression was observed in palatal rugae epithelium including the interplacode and placodes (Fig. 6C and D). *Pax9* expression could not be detected at E14.5 but was observed in mesenchyme at E16.5 (Fig. 6E and F). *Barx1* was expressed in the interplacode epithelium at E14.5 and E16.5, and in the mesenchyme at E16.5 (Fig. 6G and H).

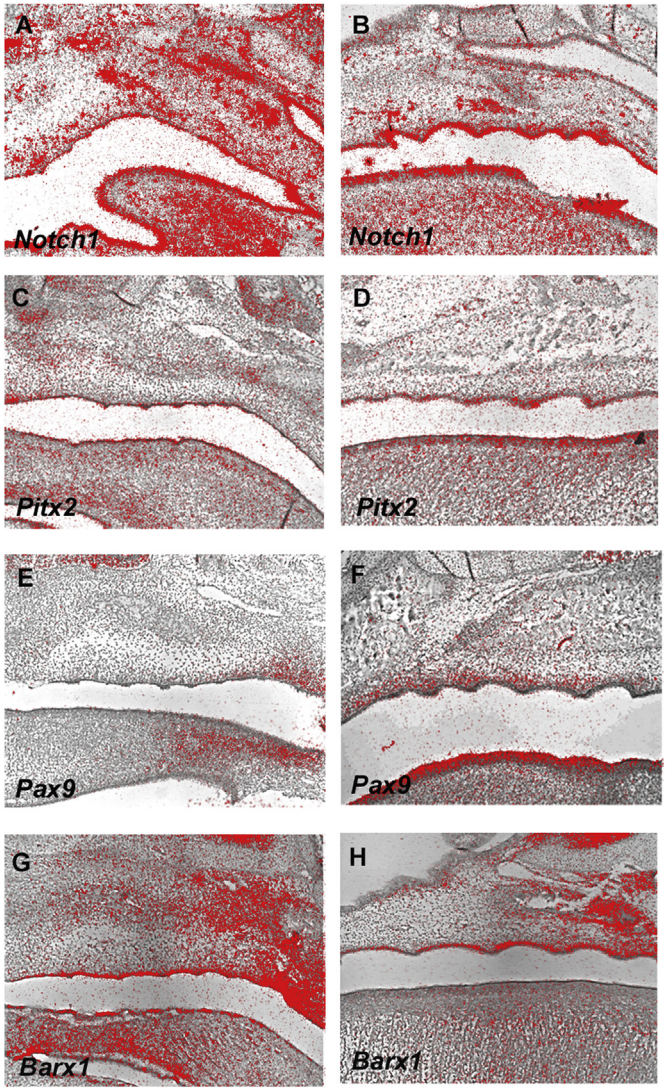


**Fig. 6.** Molecules-related non-canonical Wnt and/or Shh signaling in palatal rugae development. Sagittal sections showing *in situ* hybridization of *Notch1* (A, B), *Pitx2* (C, D), *Pax9* (E, F) and *Barx1* (G, H) at E14.5 (A, C, E, G) and E16.5 (B, D, F, H).

### 2.6. Molecular changes in Ift88 mutant mice

Acetylated α-tubulin immunoreaction was absent from *Ift88* mutant placode epithelial cells, although domains of stabilized microtubules were still present (Fig. 7B). These were consistent with results of previous report (Haycraft et al., 2005). To understand Shh and non-canonical Wnt signaling in *Ift88* mutant mice, *in situ* hybridization was performed. *Shh* expression was slightly downregulated in *Ift88*$^{fl/fl}$*;ShhCre* mice, and activity of Shh signaling pathway was decreased in *Ift88* mutant mice (Fig. 7D and F). In addition, *Ift88*$^{fl/fl}$*;ShhCre* mice exhibited reduced *Ror2* expression in palatal rugae development (Fig. 7H).

## 3. Discussion

In 1952, Turing proposed a simple model that two morphogens diffusing through a tissue could create self-regulating periodic patterns known as the reaction-diffusion model (Turing, 1952). Simulations of

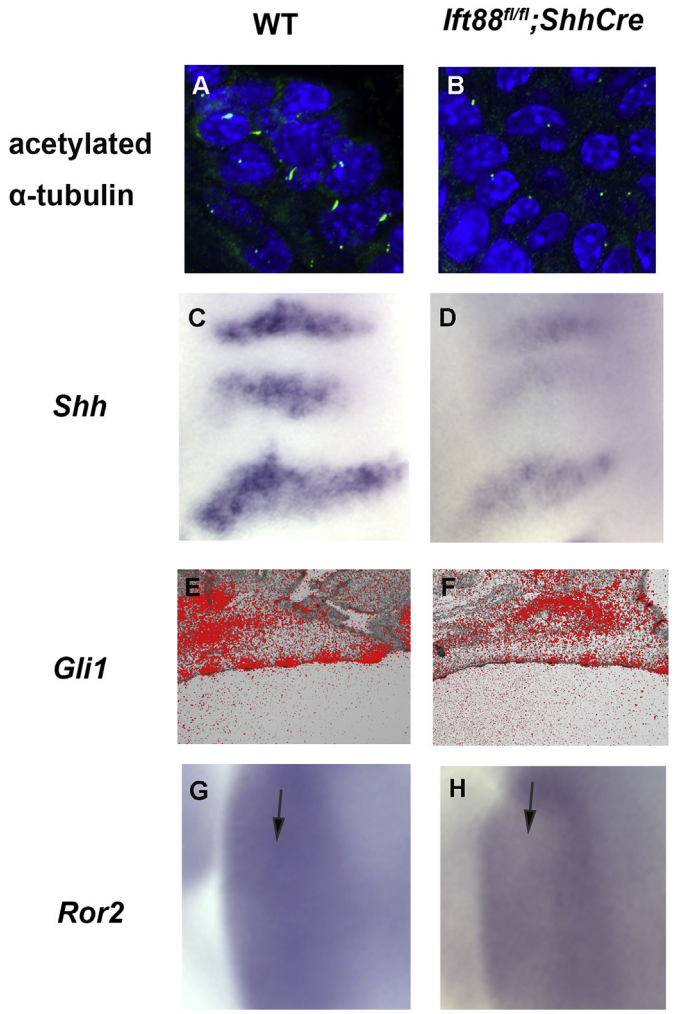


**Fig. 7.** *Shh* and *Ror2* expression in *Ift88* mutant mice. Sagittal sections showing immunohistochemistry for acetylated α-tubulin in placode epithelium of wild-type (A) and *Ift88*$^{fl/fl}$*;ShhCre* mice (B) at E14.5. Whole mount showing *in situ* hybridization of *Shh* (C, D) and *Ror2* (G, H) in palatal rugae of wild-type (C, G) and *Ift88*$^{fl/fl}$*;ShhCre* mice (D, H) at E14.5. Arrows indicating *Ror2* expression domains. Sagittal sections showing *in situ* hybridization of *Gli1* (E, F) of wild-type (E) and *Ift88*$^{fl/fl}$*;ShhCre* mice (F) at E15.5.

these mechanisms replicate many biological pattern types, such as fish stripes, digits, and feather and hair spacing (Kondo and Miura, 2010; Asai et al., 1999; Miura et al., 2006; Jiang et al., 1999; Sick et al., 2006). In fish stripe formation, the disruption of these mechanisms lead to disorganized pattern of these stripes (e.g. waved, supernumerary, shortened and fragmented stripes; Kondo and Asai, 1995, Kondo and Miura, 2010; Asai et al., 1999). We have previously shown that palatal rugae develop by a Turing-type reaction-diffusion mechanism, and disruption of the mechanism leads to disorganized palatal rugae (Economou et al., 2012). In *Ift88* mutant mice, we also observed disorganized palatal rugae which included many phenotypes such as waved, folded, supernumerary, lacked, shortened and fragmented palatal rugae. These data indicated that *Ift88* is likely to be involved in palatal rugae development through Turing reaction-diffusion model.

We have previously shown that Turing-type reaction-diffusion mechanism in palatal rugae development relies on Shh (as an inhibitor) and Fgf (as an activator) signaling for appropriate structure organization. Alteration of Shh and Fgf signaling results in disorganized rugae (Economou et al., 2012). We observed downregulation of *Shh* expression and disorganized palatal rugae in *Ift88*$^{fl/fl}$*;ShhCre* mice, suggesting that *Ift88* regulate palatal rugae development through Shh signaling.

In wild-type mice, *Shh* shows restricted expression in entire placodes of the palatal rugae. It has been reported that *Shh* mutant mice exhibit disorganized rugae in both the antemolar and intermolar regions (Economou et al., 2012). However, in *Ift88* mutant mice, disorganized rugae were observed only in the intermolar rugae (not in the antemolar rugae), even with the deletion of *Ift88* from all *Shh*-positive cells. Thus, primary cilia in placodes of palatal rugae partially regulate in development of palatal rugae.

In palatal rugae development, Turing-type reaction-diffusion mechanism depends on the interaction between Shh and Fgf signaling. Fgf signaling is activated in interplacode epithelium, while *Shh* is expressed in placode epithelium (Porntaveetus et al., 2010). In addition, *Lrp4* is expressed in a complementary pattern to *Wise* expression (putative ligand for *Lrp4*) in palatal rugae development, representing *Lrp4* expression in developing rugae placode epithelium and *Wise* in the interplacode epithelium (Ohazama et al., 2008, Kawasaki et al., 2019). Moreover, it has been shown that Bmp signaling is activated in rugae placode, while it was inhibited in interplacode epithelium due to the presence of its inhibitor (Kawasaki et al., 2019). It is thus likely that there is the interaction between interplacode and placode epithelium in palatal rugae development. Disorganized palatal rugae was caused by *Ift88* deletion only from placodes (*Ift88*$^{fl/fl}$*;ShhCre* mice), suggesting that the interaction between interplacode and placode epithelium play a critical role in palatal rugae development. We also found that the primary cilia were localized on the oral cavity side of interplacode epithelium, while placode epithelium did not exhibit any restricted localization of acetylated α-tubulin. Primary cilia are thus likely to possess distinct role between placode and interplacode epithelium.

It is important to understand how Shh and Fgf signaling are controlled in palatal rugae development, since Turing-type reaction-diffusion mechanism relies on Shh and Fgf signaling in palatal rugae development. We reported that molecules related to Fgf signaling show a dynamic spatiotemporal expression pattern in palatal rugae (Porntaveetus et al., 2010). Data presented in this study also indicated that Shh signaling activity is controlled by spatiotemporal expression patterns of many Shh-related genes in palatal rugae development. Interestingly, some ligands, *Shh* and *Wnts* are expressed in epithelium, while their receptors, *Ptch1* and *Ror2* are expressed in mesenchyme. Fgf-related molecules also exhibit similar expression pattern (Porntaveetus et al., 2010). Epithelial-mesenchymal interactions are known to play a critical role in the development of many organs including the palate. In addition to the interaction between placode and interplacode epithelium, it is likely that a Turing-type reaction-diffusion mechanism is also via epithelial-mesenchymal interactions during palatal rugae development.

In addition to Shh and Fgf signaling, the canonical Wnt pathway is also known to be essential for palatal rugae development, since the lack of canonical Wnt signaling leads to no palatal rugae formation (Lin et al., 2011). Also, in addition to canonical Wnt signaling, non-canonical Wnt signaling has also been shown to play critical roles in organogenesis and be involved in primary cilia function (Gómez-Orte et al., 2013; Grigoryan et al., 2008). We found that non-canonical Wnt signaling-related molecules were also found to exhibit dynamic spatiotemporal expression patterns in palatal rugae development. Moreover, *Ror2* expression were downregulated in *Ift88* mutant mice. These indicated that primary cilia is involved in regulating non-canonical Wnt signaling in palatal rugae development. Non-canonical Wnt signaling acts independently of β-catenin and is further divided into several pathways such as the PCP pathway and the Wnt-$Ca^{2+}$ pathway. The PCP pathway regulates cell polarity in morphogenetic processes including neural tube closure and gastrulation. In this study, *Ift88* mutant mice showed disorganized rugae. It is possible that the function of the primary cilia in palatal rugae development depends on non-canonical Wnt signaling to regulate cell polarity, which is directly linked to regulating the shape and position of palatal rugae. The disruption of these functions are likely to lead to waved and folded palatal rugae. The

palatal rugae function in the tactile sensing of objects including food, assist in holding objects, and aid in tongue placement during the production of certain speech sounds, while interacting with nerves. In fact, the expression of many nerve-related molecules has been identified in palatal rugae development (Nunzi et al., 2004; Ichikawa et al., 2001; Kido et al., 2003). Innervation has been shown to initiate in the palatal rugae around E16.5. *Vangle2* expression becomes obvious in developing rugae at E16.5. Activation of a non-canonical Wnt pathway through *Vangle2* is known to be involved in innervation (Gómez-Orte et al., 2013; Ghimire et al., 2018). It is possible that establishing innervation is regulated by a non-canonical Wnt pathway in the palatal rugae.

Some animals including snakes possess teeth in the presumptive palate region. Some ancient animals also have teeth on the presumptive palate (Diedrich, 2010). Furthermore, teeth have been regenerated by using murine palatal tissue (Nakagawa et al., 2009), and palatal teeth have been reported in some mutant mice (Liu et al., 2014; Zhou et al., 2011). The molecules we examined in this study, including *Notch1*, *Pitx2*, *Pax*9, *Barx1*, and *Shh*, are known to be important genes in tooth development. It is possible that palatal rugae are remnants of evolutionarily lost palatal teeth.

## 4. Materials and methods

### 4.1. Production and analysis of transgenic mice

*Ift88*$^{fl/fl}$ and *ShhCre* mice were produced as described by Haycraft et al. (2007), Harfe et al. (2004), respectively. CD-1 mouse was used as wild-type mice for expression profile. Mouse heads were fixed in 4% paraformaldehyde (PFA), wax embedded and serially sectioned at 7 μm. Sections were split over 5–10 slides and prepared for immunohistochemistry or radioactive *in situ* hybridization.

### 4.2. In situ hybridization

Radioactive *in situ* hybridization with [35S]UTP-labeled riboprobes was carried out as described previously by Ohazama et al. (2008).

### 4.3. Immunohistochemistry analysis

After deparaffinization of sections, tissues were treated with proteinase K and then incubated with an antibody to acetylated-a-tubulin (Sigma) and phosphorylated-JNK (cell Signaling). As a negative control, normal rabbit serum or normal goat serum were used instead of primary antibody. Tyramide signal amplification system was performed (Parkin Elmer Life Science) for detecting phosphorylated-JNK.

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