－原著－

鼻中隔形成における一次繊毛の役割

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The role of primary cilia in nasal septum development

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令和６年９月30日受付　　令和６年10月15日受理

Keywords: Nasal septum development, Hedgehog signaling, canonical Wnt signaling, mouse

〈抄録〉

鼻中隔は中顔面の発生におけるペースメーカーとして機能することが知られており，そのメカニズムを理解することは非常に重要である。神経堤由来細胞における一次繊毛分子のコンデショナルノックアウトマウスでは，ヘッジホッグ（Hh）シグナル活性の変調により鼻中隔の重複が認められることが知られており，一次繊毛における Hh シグナル伝達が鼻中隔の発生に関与していることが示唆されているが，そのメカニズムは十分に解明されていない。本研究の目的は鼻中隔の発生と一次繊毛の役割の関係について詳細に検索することである。我々は，神経堤由来細胞における一次繊毛分子 *Ift88* のコンデショナルノックアウトマウス（*Ift88*$^{fl/fl}$*;Wnt1Cre* マウス）において，鼻中隔前部で Hh シグナルの上昇が起こっていることを発見した。上記の結果に加えて，ウィント（Wnt）シグナルのダウンレギュレーションも生じていた。神経堤由来細胞における Wnt シグナル伝達経路の条件付き欠失（*Ctnnb1*$^{fl/fl}$*;Wnt1Cre* マウス）も鼻中隔の重複を起こし，これは Hh シグナル伝達の上昇を伴っていた。従って，鼻中隔の発生において，Wnt シグナル伝達経路は Hh シグナル伝達の下流にある可能性が高い。鼻中隔の前方部分は，Hh シグナル伝達および Wnt シグナル伝達を制御することにより，鼻中隔の発生に重要な役割を果たしている。

ABSTRACT

The nasal septum is known to act as a pacemaker for midface development. Therefore, it is crucial to understand the mechanisms of nasal septum development. In conditional knockout mice for primary cilia molecules in neural crest-derived cells, it has been reported that the nasal septum was duplicated due to modulation of Hedgehog (Hh) signaling and this suggested that Hh signaling in primary cilia is involved in the nasal septum development. However, the mechanism has not been fully elucidated. Therefore, in this study, we aimed to clarify the relationship between the development of the nasal septum and the role of primary cilia. We found that upregulation of Hh signaling was occurred at the anterior part of the nasal septum in mice with conditional deletion of primary cilia molecule, *Ift88*, which is expressed in primary cilia of neural crest-derived cells (*Ift88*$^{fl/fl}$*;Wnt1Cre* mice). In addition to upregulated Hh signaling, canonical Wnt signaling was downregulated at the anterior part of the nasal septum in *Ift88*$^{fl/fl}$*;Wnt1Cre* mice. Conditional deletion of canonical Wnt signaling in neural crest-derived cells (*Ctnnb1*$^{fl/fl}$*;Wnt1Cre* mice) also led to duplication of the nasal septum, which was accompanied with the upregulation of Hh signaling. Thus, canonical Wnt signaling is likely downstream of Hh

signaling in nasal septum development. The anterior part of the nasal septum is crucial for nasal septum development through regulating Hh and canonical Wnt signaling.

INTRODUCTION

Approximately one-third of all birth defects include craniofacial abnormalities. Midfacial disorders are one of the major craniofacial anomalies, which encompass a spectrum of conditions. The nasal septum is one of the major structures in the midface. It has been shown that the nasal septum acts as a pacemaker for midfacial growth[1]. Therefore, it is crucial to understand the mechanisms of nasal septum development for not only clarifying the normal development of nasal structures but also establishing new regenerative therapy for nasal anomalies like cleft lip and palate patients. However, it is not fully understood.

The nasal septum is a long structure which consists of the septal cartilage anteriorly, the perpendicular plate of the ethmoid bone, and the vomer bone posteriorly. Primary cilia have been shown to regulate midface development through controlling Hedgehog (Hh) signaling[2, 3]. In Hh signaling, without ligands, the receptors Ptch1 and Ptch2 inhibit the signal activator Smo. Binding of ligands releases Smo from the inhibition, which lead to activation of Hh signaling. This activates the three transcription factors: Gli1, Gli2, and Gli3. Gli2 and Gli3 act as bifunctional transcription factors, either a repressor or activator, which depends on the context[4].

Here, we found that anterior part of the nasal septum likely determines nasal septum development, and Wnt signaling is also related to nasal septum development as downstream of Hh signaling through primary cilia.

MARETIALS & METHODS

Production and analysis of transgenic mice

All the experimental procedures involving animals were reviewed and approved by the Niigata University Institutional Animal Care and Use Committee (approval number SA00551). *R26SmoM2*^{i}, *Ctnnb1*$^{fl/fl}$, *Smo*$^{fl/fl}$, *Wnt1Cre*, and *Ift88*$^{fl/fl}$ mice were produced as described by Jeong et al., Messerschmidt et al., Jeong et al., Danielian et al. and Haycraft et al., respectively[5, 6, 7, 8]. Embryonic day 0 (E0) was taken to be midnight prior to finding a vaginal plug.

In situ hybridization

In situ hybridization was carried out for detecting the mRNAs by [^{35}S]UTP-labeled riboprobes as described previously[9].

Immunohistochemistry

For paraffin embedding, the craniums of mouse embryos were fixed in 4% paraformaldehyde for 24 hours at 4℃ and embedded and infiltrated in paraffin using standard protocols. Paraffin blocks were sectioned using a LEICA RM2235 rotary microtome. The nasal septum was sectioned at 7㎛ in the anterior direction. Sections were incubated at 4℃ overnight with primary antibodies to acetylated α-tubulin (Sigma-Aldrich) and Ptch1 (Abcam). Sections were then incubated with Alexa Fluor@ 488 conjugated antibody (ThermoFisher) for detecting primary antibody. Nuclei were stained with DAPI. α-tubulin: a marker for primary ciliary, Ptch1: a marker for Hh signaling.

RESULTS

Nasal septum phenotypes in *Ift88* mutant mice

It has been shown that primary cilia regulate the nasal septum. The disruption of primary cilia in neural crest-derived cells induces duplication of the nasal septum[3]. We also confirm same nasal septum phenotype in mice with conditional deletion of *Ift88* (primary cilia molecule) in neural crest-derived cells (*Ift88*$^{fl/fl}$*;Wnt1Cre* mice; Fig. 1A-1F). The disruption of primary cilia has been shown to be caused by upregulation of Hh signaling[3]. To confirm this, we generated mice with conditional upregulation of Hh signaling in neural crest-derived cells (*R26SmoM2;Wnt1Cre* mice). In *R26SmoM2;Wnt1Cre* mice, Hh signaling activity is upregulated in neural crest-derived cells, when *R26SmoM2* mice were crossed with Wnt1Cre mice. In fact, duplication of the nasal septum was observed in *R26SmoM2;Wnt1Cre* mice (Fig. 1G, 1H).

Initiation of nasal septum phenotypes in *Ift88* mutant mice

To identify when nasal septum phenotypes in *Ift88*$^{fl/fl}$*;Wnt1Cre* mice was emerged, we examined

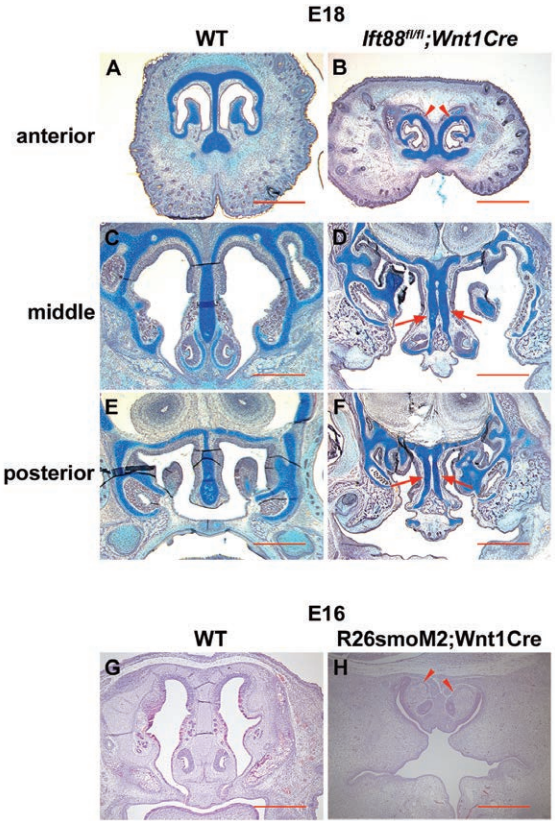

Figure 1. Nasal septum phenotypes in *Ift88*$^{fl/fl}$*;Wnt1Cre* mice. Frontal sections showing histology of nasal septum in wild-type (A, C, E, G), *Ift88*$^{fl/fl}$*;Wnt1Cre* (B, D, F) and *R26SmoM2;Wnt1Cre* (H) mice at E16.5 (G, H) and E18.5 (A-F). Arrows and arrowheads indicating duplicated nasal septum. *Scale Bar;500μm

expression of cartilage marker, *Sox9*. Significant changes of *Sox9* expression could not be detected in *Ift88*$^{fl/fl}$*;Wnt1Cre* mice at embryonic day (E) 11.5 (Fig. 2A-2F'). Duplication of *Sox9* was observed in *Ift88* mutant mice at El2.5 (Fig, 2G-2L'). Thus, El2.5 is likely crucial stage to regulate nasal septum development through primary cilia.

Primary cilia formation in *Ift88* mutant mice

It has been shown that deletion of *Ift88* disrupt of primary cilia formation[10]. To identify region of the nasal septum with disruption of primary cilia formation in *Ift88*$^{fl/fl}$*;Wnt1Cre* mice, acetylated α-tubulin (a marker for primary cilia) was examined. Disruption of primary cilia was observed in entire region of the nasal septum of *Ift88*$^{fl/fl}$*;Wnt1Cre* mice (Fig. 3).

Hh signaling in *Ift88* mutant mice

The disruption of primary cilia has been shown to induce upregulation of Hh signaling activity, which result in duplication of nasal septum[3]. However, the region with upregulation of Hh signaling activity has not been identified. Therefore, we examined Hh signaling marker, *Gli1*, at the anterior, middle, and posterior septal cartilage, since duplication has been occurred in septal cartilage, but not the ethmoid bone and the vomer bone. Upregulation of *Gli1* was observed at the anterior region, while middle and posterior part showed downregulation of *Gli1* expression (Fig. 4A-4F'). To understand whether downregulation of Hh signaling in middle and posterior part of *Ift88*$^{fl/fl}$*;Wnt1Cre* mice induce duplication of the nasal septum, we examined mice with conditional deletion of Hh signaling activity in neural crest-derived cells (*Smo*$^{fl/fl}$*;Wnt1Cre* mice), since Smo is essential molecule to induce Hh signaling activity. *Smo*$^{fl/fl}$*;Wnt1Cre* mice showed no obvious duplication of the nasal septum (Fig. 4G-4L). Thus, duplication of nasal septum was caused by upregulation of Hh signaling at the anterior part, but not downregulation of Hh signaling at the middle and posterior part.

Canonical Wnt signaling in *Ift88* mutant mice

Hh and Wnt pathways are the two major evolutionarily conserved signaling pathways

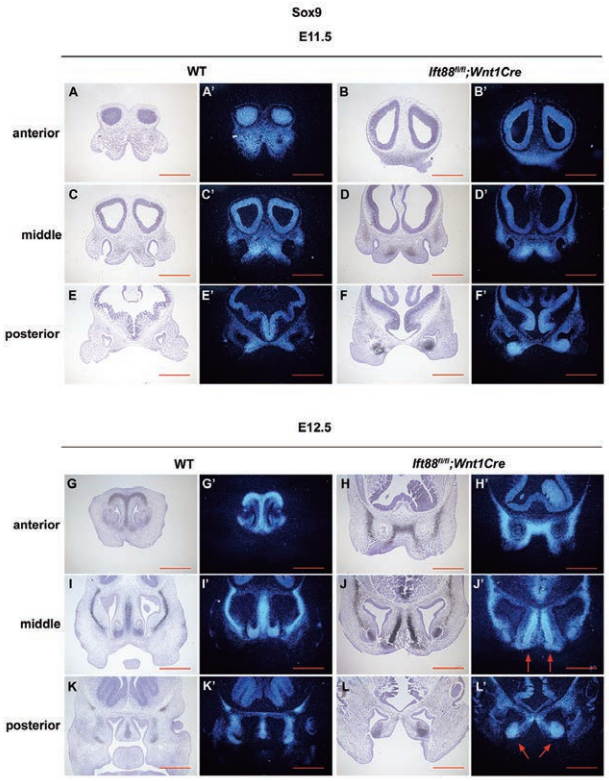

Figure 2. *Sox9* expression in *Ift88*$^{fl/fl}$*; Wnt1Cre* mice. Frontal sections showing *in situ* hybridization of *Sox9* in wild-type (A, A', C, C', E, E', G, G', 1, 1', K, K') and *Ift88*$^{fl/fl}$*; Wnt1Cre* (B, B', D, D', F, F', H, H', J, J', L, L') at E11.5 (A-F') and E12.5 (G-L'). Left panel; bright field, right panel; dark filed. Arrows indicating duplication of *Sox9* expression. *Scale Bar;500μm

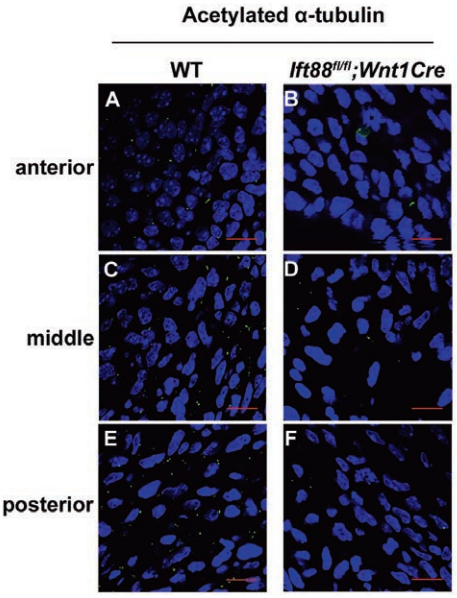

Figure 3. Primary cilia in *Ift88*$^{fl/fl}$*; Wnt1Cre* mice. Frontal sections showing immunohistochemistry of acetylated α-tubulin in wild-type (A, C, D and *Ift88*$^{fl/fl}$*; Wnt1Cre* (B, D, F) mice. at E12.5. *Scale Bar;10μm

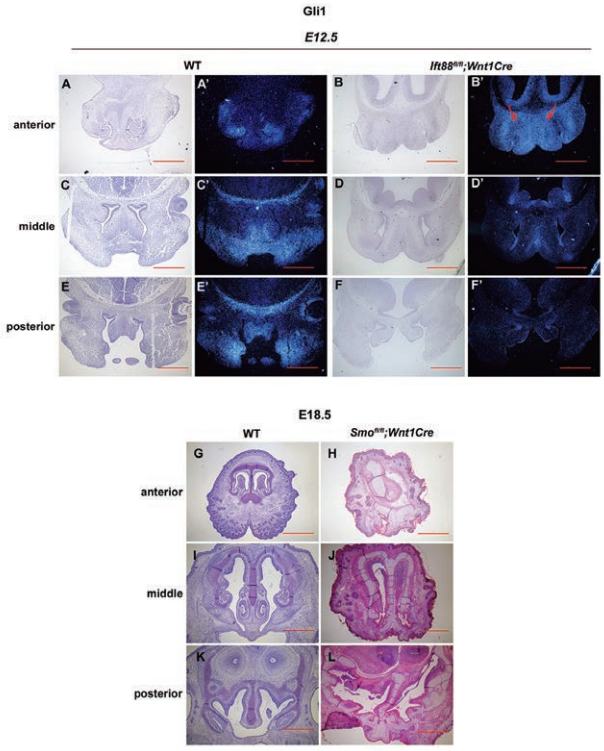

Figure 4. Hh signaling in *Ift88*$^{fl/fl}$*; Wnt1Cre* mice. (A-F') Frontal sections showing *in situ* hybridization of *Gli1* in wild-type (A, A', C, C', E, E') and *Ift88*$^{fl/fl}$*; Wnt1Cre* (B, B', D, D', F, F') mice at E12.5. Left panel; bright field, right panel; dark field. Arrows indicating upregulation of *Gli1* expression. (G-L) Frontal sections showing histology in wild-type (G, I, K) and *Smo*$^{fl/fl}$*;Wnt1Cre* (H, J, L) mice at E18.5. *Scale Bar;500μm

interacting each other that regulate embryonic development and adult tissue homeostasis[11, 12]. In addition, primary cilia have also been shown to be involved in canonical Wnt signaling activity[13]. To understand whether primary cilia is also involved in nasal septum development through canonical Wnt signaling, we examined canonical Wnt signaling marker, *Axin2* in *Ift88*$^{fl/fl}$*;Wnt1Cre* mice. Downregulation of *Axin2* was observed at all anterior, middle, and posterior part of the nasal septum (Fig. 5A-5F'). In order to understand whether downregulation of canonical Wnt signaling activity at the anterior nasal septum is involved in duplication of the nasal septum, we generated mice with conditional deletion of canonical Wnt signaling activity in neural crest-derived cells (*Ctnnb1*$^{fl/fl}$*;Wnt1Cre* mice), since Ctnnb1 is essential molecule to induce canonical Wnt signaling activity. *Ctnnb1*$^{fl/fl}$*;Wnt1Cre* mice showed duplication of the nasal septum (Fig. 5G, 5H). To further understand whether canonical Wnt signaling is

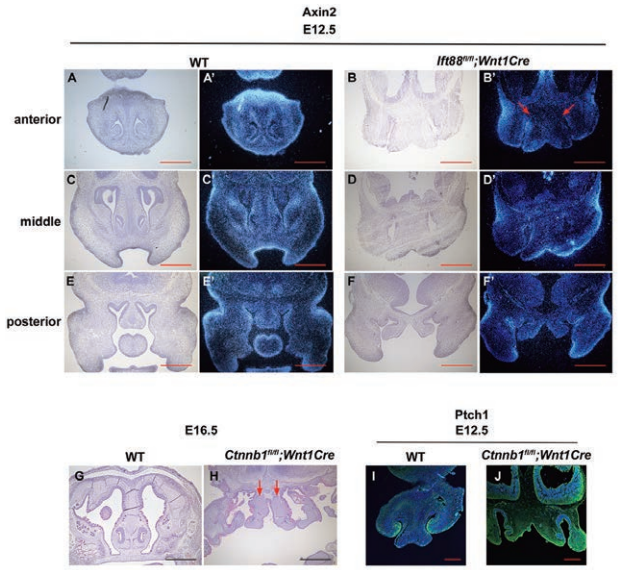

Figure 5. Canonical Wnt signaling in *Ift88*$^{fl/fl}$*;Wnt1Cre* mice. (A-F') Frontal sections showing in situ hybridization of *Axin2* in wild-type (A, A', C, C', E, E') and *Ift88*$^{fl/fl}$*;Wnt1Cre* mice (B, B', D, D', F, F') at E12.5. Left panel; bright field, right panel; dark field. Arrows indicating downregulation of *Axin2* expression. (G, H) Frontal sections showing histology in wild-type (G) and *Ctnnb1*$^{fl/fl}$*;Wnt1Cre* (H) mice at E16.5. Arrows indicating duplicated nasal septum. (I, J) Frontal sections showing immunohistochemistry of Ptch1 in wild-type (I) and *Ctnnb1*$^{fl/fl}$*;Wnt1Cre* mice (J) at E12.5. *Scale Bar;500μm

related to Hh signaling in nasal septum development, we examined Hh signaling activity in *Ctnnb1*$^{fl/fl}$*;Wnt1Cre* mice. Ptch1 (marker of Hh signaling) expression was likely increased in *Ctnnb1*$^{fl/fl}$*;Wnt1Cre* mice (Fig. 5. J).

DISCUSSION

Conditional mutation of primary cilia protein, *Kif3a*, in neural crest-derived cells has been shown to induce duplication of the nasal septum[3]. The phenotype is caused by upregulation of Hh signaling due to increased active form of Gli2. In fact, mice with increased Hh signaling (*R26SmoM2;Wnt1Cre* mice) was found to induce duplication of nasal septum. Hh signaling was upregulated in *Ift88*$^{fl/fl}$*;Wnt1Cre* mice, which was restricted in the anterior part of the nasal septum, although lack of primary cilia observed in all anterior, middle, and posterior part of the nasal septum. Unlike the anterior part of the nasal septum, Hh signaling activity was downregulated in the middle and posterior part of the nasal septum. No duplication of nasal septum was observed in mice with lack of Hh signaling (*Smo*$^{fl/fl}$*;Wnt1Cre* mice), indicating that changes of Hh signaling activity in the middle and posterior part of nasal septum unlikely affect nasal septum development. Only anterior part of the nasal septum may act as a pacemaker for midface development, although the nasal septum is long structure. Duplication of the nasal septum is caused by upregulated Hh signaling in *Kif3a* and *Ift88*$^{fl/fl}$ mutant mice, and *R26SmoM2;Wnt1Cre* mice also showed duplication of the nasal septum. However, the morphology of the nasal septum is different between *Ift88*$^{fl/fl}$*;Wnt1Cre* and *R26SmoM2;Wnt1Cre* mice. Hh signaling is upregulated in all neural crest-derived cells of *R26SmoM2;Wnt1Cre* mice, while it is occurred in some neural crest-derived cells of *Ift88*$^{fl/fl}$*;Wnt1Cre* mice. These suggest that in addition to anterior part of the nasal septum, some neural crest-derived cells in other region are probably involved in nasal septum formation.

We also found canonical Wnt signaling was downregulated in *Ift88*$^{fl/fl}$*;Wnt1Cre* mice, and mice with conditional inactivation of canonical Wnt signaling in neural crest-derived cells showed duplication of nasal septum. It has been shown that there is interact between Wnt and Hh signaling in other organogenesis[14, 15]. Furthermore, we found upregulation of Hh signaling in *Ctnnb1*$^{fl/fl}$*;Wnt1Cre* mice. These indicate that upregulation of Hh signaling result in downregulation of canonical Wnt signaling in *Ift88*$^{fl/fl}$*;Wnt1Cre* mice. Thus, canonical Wnt signaling is likely downstream of Hh signaling in nasal septum development.

Acknowledgments

This research was funded by the Japan Society for the Promotion of Science (JSPS;22K17248)

Competing interests

The authors declare no competing interests.

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