Shh signaling involved in *Pitchfork* regulated primary cilia disassembly during mouse palate development

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ABSTRACT

Shh signaling involved in *Pitchfork* regulated primary cilia disassembly during mouse palate development

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(Directed by Professor Hyoung-Seon Baik)

Pitchfork, a mouse embryonic node gene, is associated with ciliary targeting complexes located at the basal body during primary cilia disassembly. Various developmental disorders, such as cleft palate, and disorders of the lung, kidney and heart, have been known as an association with ciliary defects. Mammalian palate development is regulated by complex processes. Many cellular and molecular events, such as cell proliferation, apoptosis, cell migration and the epithelial mesenchymal transition, regulate proper palate development, and surely, some abnormalities in palate development lead to cleft palate. To determine the function of *Pitchfork* during palate development, we examined *Pitchfork* expression patterns and morphological changes in the developing secondary palate after Pitchfork over-expression. During periods E12.5 and E13.5 in mice, *Pitchfork* was highly expressed in the developing mouse secondary palate. Morphological differences were

observed *in vitro* in cultured palates in the Pitchfork over-expression group compared to the control group. Pitchfork over-expression induced primary cilia disassembly during palate development. *Shh* and *Ptch1* expression levels and palatine rugae morphology were altered in the over-expressed *Pitchfork* group during palate development. Therefore, the proper expression levels of *Pitchfork* may play a pivotal role in normal secondary palate morphogenesis.

Key words: Palate development, Primary cilia, *Pitchfork*, Cell proliferation, Apoptosis

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I. Introduction

1. Mouse secondary palate development

Palate development is one of the critical events in craniofacial morphogenesis. Outgrowth of maxillary processes initiates the development of mouse palatal shelves at E12.5, and at E13.5 palatal shelves are vertically positioned at each side of the tongue. The palatal shelves are elevate above the tongue (Fig. 1A) and make contact for palatal fusion between E14 and E14.5 (Fig. 1B) (Ferguson., 1988). At E14.5, the medial edge

epithelium (MEE) transforms into the midline epithelial seam (MES) which will be removed later. (Fig. 1C) (Johnston et al., 1995). The palatal shelves are completely fused at E15.5 with disappearing midline epithelial seam (Dixon et al., 2011; Gritli-Linde., 2007; Rot et al., 2013).

On the mouse secondary palate, nine palatine rugae are found (sakamoto et al., 1989). Three transverse ridges which just behind the incisor teeth are formed spanning the midline of the secondary palate. As opposed to the anterior three rugae, a further six rugae are observed around the molar tooth areas, which have an oblique arrangement and do not span the midline (Pantalacci et al., 2008). In the palatine rugaes, many nerve fibers that respond to touch and pressure on the palate are located at them (Kido et al., 2003; Nunzi et al., 2004; Porntaveetus et al., 2010).

In palate development, various cellular and molecular events, such as cell proliferation, apoptosis, cell migration and the epithelial mesenchymal transition, are involved (Parada et al., 2010; Shin et al., 2012). A number of complex networks of growth factors and transcription factors regulate the development of the secondary palate during mammalian embryogenesis. Previous studies have revealed that numerous transcription factors and signaling pathways, such as sonic hedgehog (Shh), Wnt and fibroblast growth factor (Fgf), play a pivotal role in normal palate development, including palatine rugae formation (Fig. 2) (Cobourne et al., 2012; Kemler et al., 2004; Lee et al., 2011; Lee et al., 2008; Lipinski et al., 2010; Rice et al., 2006). In addition, Alteration of primary cilia-related genes, such as oral-facial-digital syndrome type I (OFD1) and *Kif3a*, lead to primary cilia defects and various craniofacial disorders, including lateral cleft

palate; lobed, lipomas or hamartomas of the tongue; and hypodontia (Brugmann et al., 2010; Sukarova-Angelovska et al., 2012; Toriello et al., 1993).

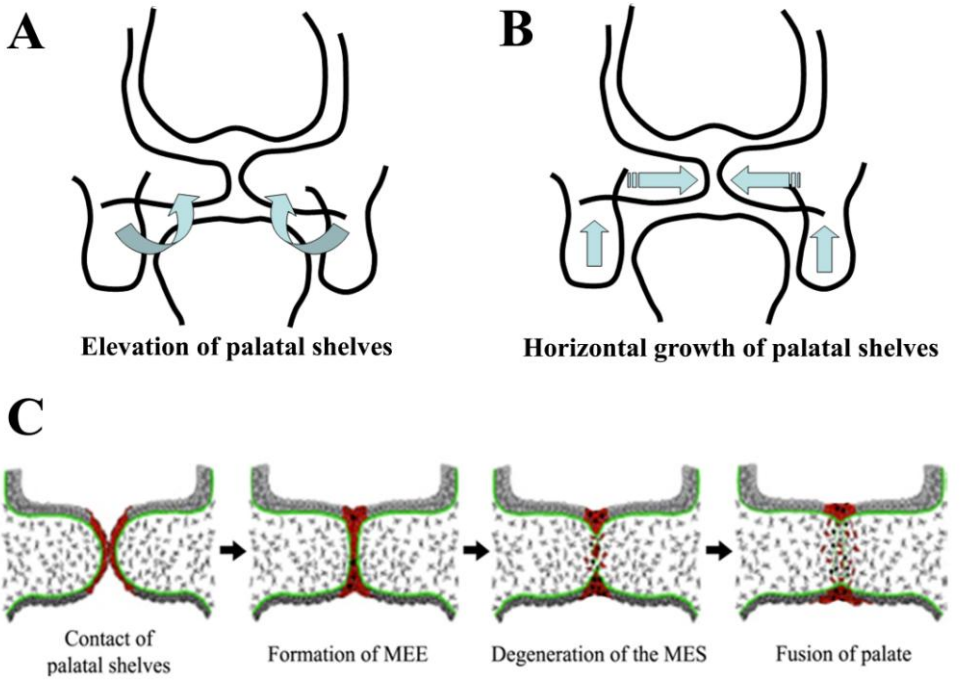

Figure 1. Morphological stages during mouse palate development. (A, B) At E13.5, the palatal shelves are vertically located on each side of the tongue, and at E14, the palatal shelves elevate to a horizontal position above the tongue. (C) The palatal shelves are make contact for palatal fusion between E14 and E14.5, and that completely fused at E15.5 with disappearing MES. *E* embryonic; *MEE* medial edge epithelium; *MES* midline epithelial seam (Takigawa et al., 2004).

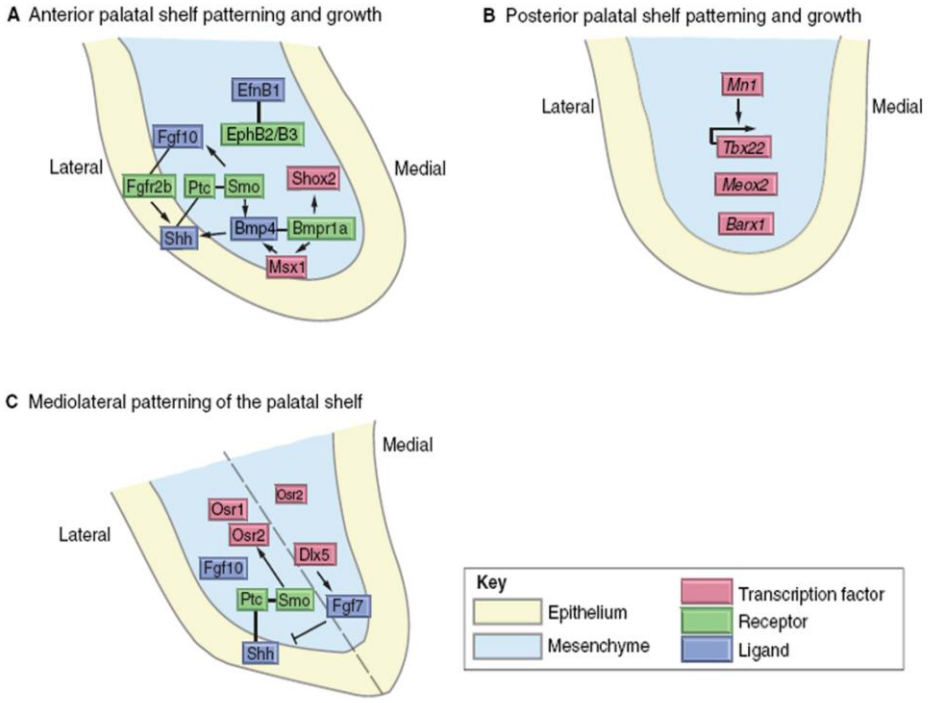

Figure 2. Molecular control of palatal shelf growth and patterning. (A) Signaling interactions controlling anterior palatal growth. Shh is expressed in the oral epithelium and binds to its receprot Ptch1 in the undrlying mesenchyme to permit Smo activation of palatal cell proliferation. (B) Genes involved in development of the posterior palate. (C) Pathways responsible for mediolateral patterning of the palatal shelves during vertical outgrowth (Bush and Jiang., 2012).

2. Primary cilia structure and function

In mammals, large numbers of motile 9+2 cilia normally concentrate on the cell surface with an orchestrated wavelike fashion, and they are believed to involve in fluid and cell movement (Bisgrove et al., 2006). Compared with motile cilia, primary chilia is single immotile organelles from the apical surface of cells. Primary cilia are found on nearly all cell types in mammals, and the basal body, the axoneme and the ciliary membrane are three main components of primary cilia (Fig. 3) (Wheatley et al., 1996; Zaghloul et al., 2011). The basal body acts as the nucleation point when ciliogenesis occurred, and also mediates the cargo transport from the cytoplasm to the ciliary membrane (Marshall et al., 2008). The shuttle along the axoneme in which proteins transported to the cilium are a specialized system of transport known as intraflagellar transport (IFT) (Gerdes et al., 2009). Except the basal body and axoneme, the ciliary membrane is also very important for ciliary function, particularly in the reception and transduction of extracellular signaling cues (Madhivanan et al., 2012). Previous study have reported that primary cilia play crucial role in palate development such as oral-facial-digital syndrome type I (Toriello & Franco., 1993). A various developmental disorders such as lung, kidney and heart have been associated with ciliary defects (Lancaster., 2009; Patel et al., 2009). Moreover, mutations in proteins localized to cilia and ciliary basal bodies can cause rare recessive human disorders known as ciliopathies – complex syndromes that can involve cystic kidneys, obesity, mental retardation, blindness and various developmental malformations (Badano et al., 2006; Baker et al., 2009; Gerdes et al., 2009; Tobin et al., 2009).

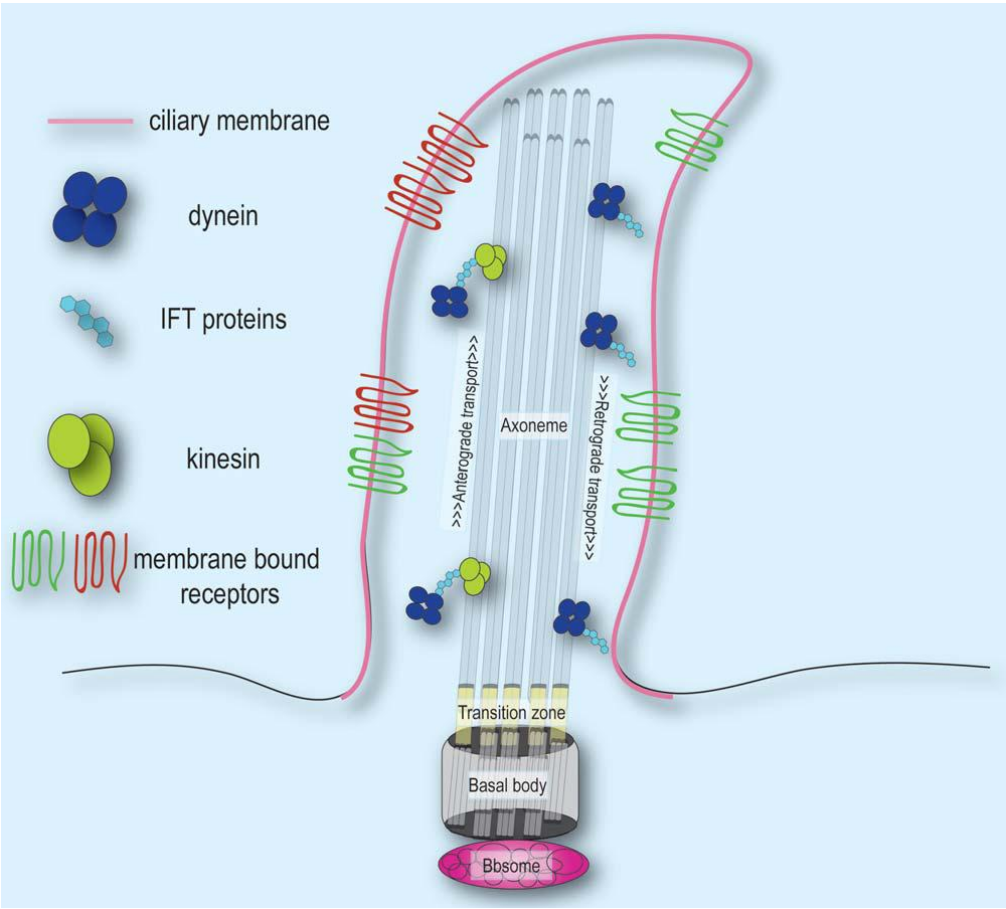

Figure 3. Schematic diagram of an extended primary cilium. (Zaghloul et al., 2011)

3. The role of *Shh* signaling pathway in primary cilia

The Sonic hedgehog (*Shh*) signaling pathway is implicated in a number of craniofacial disorders, and plays important role in tissue patterning and homeostasis in diverse species (Ingham et al., 2011; Metzis et al., 2013). The secreted protein Shh binds and inactivates Ptch1, allowing activation of a second transmembrane protein, smoothened (Smo). *Smo* then triggers target gene transcription through the *Gli* family of transcription factors. In the absence of signal, the transmembrane protein Patched1 (Ptch1) keeps the pathway turned off by inhibiting the function of Smo. Changes in both the phosphorylation and conformation of *Smo* are associated with the activation of *Shh* signaling (Chen et al., 2011). The mechanism by which *Shh* inhibits *Ptch1* and *Ptch1* inhibits *Smo* is still unkown in mammals (Du et al., 2013; Rohatgi et al., 2007; Zhang et al., 2011). The developmental genetic analyses and human genetic studies revealed the functions of mammalian primary cilia (Goetz et al., 2010). Primary cilia are microtubule-based organelles which serve as hubs for the transduction of various developmental signaling pathways including Shh, Wnt, Fgf, and PDGF (Berbari et al., 2009; Dorn et al., 2012; Hsiao et al., 2012; Nozawa et al., 2013; Oishi et al., 2006). It is interesting that embryos harboring mutations in genes necessary for cilia formation were defective in *Shh* signaling (Huangfu et al., 2003). Loss of primary cilia induce *Shh*, *Ptch* and *Gli1* activity during craniofacial morphogenesis (Brugmann et al., 2010; Zaghloul et al., 2011). Upon *Shh* pathway activation the composition of the cilia changes, *Smo* moves in to the cilia from the adjacent membrane in the place where *Ptch1* disappears from cilia (Sasai et al., 2012). Molecular mechanisms responsible for the removal of *Ptch1* and the accumulation of *Smo* are not known yet.

4. The function of primary ciliary related gene-*Pitchfork* in mammals

A mouse embryonic node gene named *Pitchfork*, which is localized on chromosome 3, has two transcript variant. The length of *Pitchfork* mRNA is 925bp, including six exons (CH466608.2: 6693507..6693575; 6694648..6694765; 6695904..6696034; 6696748..6696846; 6697475..6697620; 6697704..6697764). *Pitchfork* accumulates at the basal body and ciliary necklace specifically during the early phase of cilia assembly and disassembly (Kinzel et al., 2010). *Pitchfork* appears with chordates and is expressed specifically in the organizer regions of embryonic organizing activities (EOA), which are important for embryonic patterning and are a source of differentiation and proliferation signals, for example, the mouse node, the apical ectodermal ridge, the vertebrate of the neural tube, and the growth zone of the embryonic limb bud (Kinzel et al., 2010). Therefore, Pitchfork may play important role during mammalian embryonic organogenesis. Previous study have reported that the *Pitchfork* haploinsufficiency in $Pifo^{lacZ/+}$ mouse lead to a unique node cilia duplication phenotype, heart failure and left-right asymmetry defects. This cilia duplication phenotype highlights the fact that Pitchfork is a cilia disassembly protein which play a very important role in organizer regions of EOA by specifically controling basal body detachment as well as centrosome duplication and ciliary retraction (Kinzel et al., 2010). Based on previous study, *Pitchfork* plays pivotal role in cilia formation. Furthermore, cilia deficiency is associated with various human diseases, such as ciliay dysfunction syndromes, polycystic kidney, male infertility, craniofacial abnormalities (Badano et al., 2006; Brugmann et al.,

2010; Michaud et al., 2006). *Pitchfork* potentially participate in human congenital disease and ciliary dysfunction syndromes. Therefore, it is important to understand the function of *Pitchfork* on primary cilia disassembly. *Pitchfork* was localized from apical to the adherens junction of the primary cilium base, and is co-localizes with *Kif3a* in mouse ventral node pit cells (Kinzel et al., 2010). Loss of the intraflagellar transport protein (IFT), *Kif3a*, can induce primary cilia disassembly and secondary lateral cleft palate accompanying the widened frontonasal prominence. In vertebrate, *Kif3a* loss of function leads to altered *Shh* and Wnt signaling expression levels, and cell proliferation during craniofacial development (Brugmann et al., 2010). Therefore, *Pitchfork* may be involved in regulating a variety of gene signaling pathway during craniofacial development.

To confirm the relationship between palate development and *Pitchfork*, we firstly examined *Pitchfork* expression patterns at E12.5, E13.5 and E14.5 by *in situ* hybridization clearly. In addition, Pitchfork over-expression was used to understand the function of *Pitchfork* during mouse palate development. Over-expressed *Pitchfork* induced abnormal secondary palate structure and regulated the genes (*Shh* and *Ptch1*) related to palate development by primary cilia disassembly. Our findings revealed that the proper expression level of *Pitchfork* is necessary for normal secondary palate development.

Table 1. Introduction of *Pitchfork* gene

Function	During primary cilia disassembly, involved in cilia disassembly. Required specifically to control cilia retraction as well as the liberation and duplication of the basal body/centrosome. May act by stimulating AURKA activity at the basal body in a cell cycle-dependent manner.
Subunit structure	Interacts with proteins involved in ciliary transport, including ARL13B, CETN1, KIF3A, RAB6A, RAB8A, TUBB1 and TUBG1. Interacts with AURKA.
Subcellular location	Isoform 1: Golgi apparatus › Golgi stack. Golgi apparatus › trans-Golgi network. Isoform 2: Nucleus. Cytoplasm. Cytoplasmic vesicle. Note: Accumulates specifically at the basal body and ciliary necklace during the early steps of cilia assembly and disassembly, when structural, functional and regulatory proteins are delivered to cilia. At S phase, accumulates in vesicles and declines during mitosis. In node pit cells, found close to the ciliary membrane along the axoneme. In spermatocytes, localizes to particles along the stabilized microtubules of tails.
Tissue specificity	Expressed in tissues rich in ciliated cells, such as lung, kidney, vas deferens and testis. Both isoforms 1 and 2 are expressed in testis.
Developmental stage	At 7.75 dpc, expression restricted to the ventral node monociliated pit cells. Not expressed in other tissues at detectable levels until 9.5 dpc. At 10.5 dpc, expressed in motor neurons in the ventral neural tube and in the apical ectodermal ridge of lim buds.

www.uniprot.org (Kinzel et al., 2010)

II. MATERIALS AND METHODS

All experiments complied with the guidelines of the Intramural Animal Use and Care Committee, Yonsei University College of Dentistry.

1. Animals

Institute of Cancer Research (ICR) mice (Koatech Co, Pyeongtaek, Korea) was used in this study. Adult ICR mice were housed in a temperature-controlled room (22℃) under artificial illumination (Lights on from 05:00 to 17:00) and at 55% relative humidity with access to food and water ad libitum. The embryonic day 0 (E0) was designated as the day on which a vaginal plug was detected. Mouse embryos at each developmental stages E12.5，E13.5, E14.5 were used in this study.

2. Immunohistochemistry and TUNEL assays

Histochemical and immunohistochemical tissue analysis was performed as described previously. The specimens were embedded in wax compound using conventional methods. Sections (4 μm thickness) of the specimens were incubated with 1st antibody at 4℃ overnight. The specific primary antibodies were used an enhanced

green fluorescent protien EGFP (dilution, 1:100; cat. No. NB 110-75115; Novus Biologicals, Canada) and Ki-67 (dilution, 1:100; cat. No. M 3060; Spring Bioscience Corp, USA). After washing with PBS, the specimens were allowed to react with biotinylated goat anti mouse immunoglobulins and streptavidin peroxidase at room temperature for two consecutive 10 min incubations. Finally, the specimens were visualized using a 3, 30-diaminobenzidine (DAB) reagent kit (Zymed). A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using an in situ cell apoptosis detection kit (Trevigen) according to the manufacturer's instructions. The 4μm thick sections were treated with 20 μg/ml proteinase K [in 10mM Tris–Hcl (pH 8.0)] for 15 min at room temperature. The samples were incubated with the labeling reaction mixture at 37℃ for 1 hr and horseradish peroxidase (HRP) -streptavidin solution for 10 min at room temperature. DAB was used as a substrate solution to detect the sites of *in situ* apoptosis. Tissue sections (4 μm thick) were stained with hematoxylin and eosin (H&E) and observed.

3. Whole-mount *in situ* hybridization (WISH)

Specimens were fixed overnight in 4% paraformaldehyde in phosphate buffered saline (PBS). For *in situ* hybridization, the specimens were treated with 20 μg/ml proteinase K for 6 min at room temperature. Antisense RNA probes were labeled with digoxigenin (Roche). After *in situ* hybridization, the specimens were frozen-sectioned at a thickness of 12 μm. At least 30 specimens from each stage were examined.

The primer sequences of the genes are as follows:

Pitchfork, 5´-CCCTGGGTGTTATGCAGCAG-3´

5´-CTGGACACGAAATGGGCAGA-3´;

Shh, 5´-TCCAAAGCTCACATCCACTG-3´

5´-AGCGTCTCGATCACGTAGAA-3´;

Ptch1, 5´-CCACCTGGACTCTGGCTCCTT-3´

5´-CCTCCACCTTTGAGTCCCTCCT-3´.

4. *Pitchfork* lentiviral vector infection and in vitro organ culture

To construct lentivirus transfer plasmid pCDH-*Pitchfork*, the sequences expressing *Pitchfork* plus kozak sequence are generated by PCR using E13 ICR mice palates cDNA as template. The primers are as follow: *Pitchfork* forward primer (5´-CTAGCTAGCATGAACACGGAGGAAATACC-3´), *Pitchfork* reverse primer (5´-CGCGGATCCTCACTGGTAATATAGGCTAAAG-3´).

Two micrograms of the resulting lentivirus transfer plasmids pCDH-Pitchfork and pCDH together with 1.5 g psPAX2 vector and 0.5 g pMD2.G vector, respectively, were co-transfected into HEK-293T cells (Invitrogen) in 10 cm plate. After 72 hr of incubation at 37℃ and 5% CO2, the supernatant was collected and virus particles were concentrated with Amicon® Ultra-15 Centrifugal Filter Devices by centrifugation at 3000 rpm for 30 mins.

Palatal shelves were isolated from E13.5 mouse maxilla and cultured in a medium without FBS at 37℃ and in 5% CO_2 for 48 hr using a slightly modified culture

method reported by Trowell (Taya et al., 1999). A 0.04-mm gap between the palatal shelves was created in the in vitro organ culture such that the shelves could proliferate to achieve fusion. The culture medium, specifically, DMEM/F12 (Gibco) supplemented with 20 µg/ml ascorbic acid (Sigma) and 1% penicillin/streptomycin, was replaced every 24 hr. To increase the infection effciency of lentiviral vector, 10 µg/ml of polybrene (Sigma) is added into the culture medium. Next, 100 µl of concentrated Pitchfork-expressing lentivirus was added in 1 ml culture medium (Fig. 4).

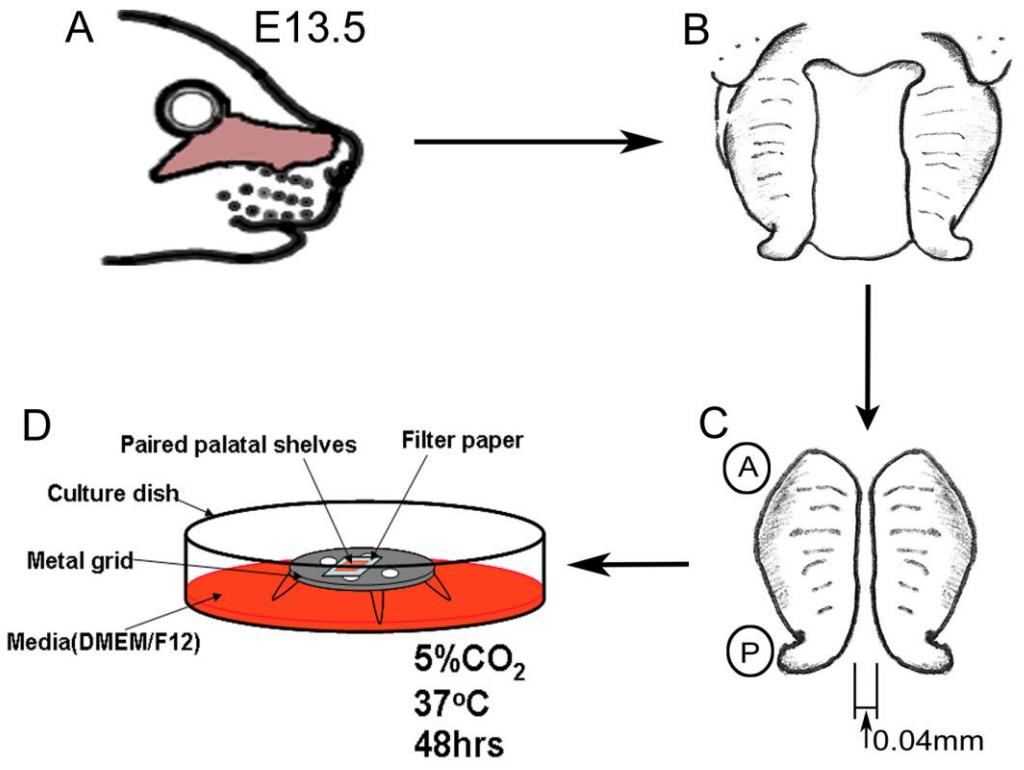

Figure 4. *In vitro* culture method for dissected mouse palate. (A, B) Palatal shelves were harvested from E13.5 mouse embryos. (C) Two palatal shelves were then placed on

filter paper. Previous reports showed that the fusion of palatal shelves could be brought about even from transgenic mice with a cleft palate, by simply placing the shelves together *in vitro* (Zhang et al., 2002). To avoid this kind of fusion, a 0.04 mm gap between the palatal shelves was created in the *in vitro* culture so that the shelves needed to proliferate in order to achieve fusion. (D) Paired palatal shelves were incubated for 48 hr with DMEM/F12 under 5% CO_2 and 37℃ conditions (Dr. Jong-Min Lee provided this diagram). Ⓐ anterior; Ⓟ posterior.

5. Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from the cultured palates using Trizol according to manufacturer's instruction. For cDNA synthesis, reverse transcription was performed using M-MuLV reverse transcriptase (New England BioLabs). Real-time quantitative PCR (RT-qPCR) was performed using Thermal Cycler Dice™ Real Time System TP800 (Takara) with SYBR Premix EX Taq™ (Takara). The amplification program consisted of 40 cycles of denaturation at 95℃ for 5 sec, annealing at 55℃ for 20 sec, and extension at 72℃ for 20 sec. The results of RT-qPCR for each sample were normalized by B2M (Beta-2-Microglobulin). RT-qPCR also was used to detect the Pitchfork. The data were analyzed with the Thermal Cycler Dice™ Real Time System analysis software (Takara). The results were expressed as normalized ratios. The primer sequences of the genes are as follows:

B2M, 5´-GGGAAGCCGAACATACTGAA-3´

5´-TCACATGTCTCGATCCCAGT-3´;

Pitchfork, 5´-GAGTGCAGCAAAGGGTGA-3´

5´-ACACGGCTACGATGCTTTCT-3´;

Shh, 5´-CGGACCTTCAAGAGCCTTACC-3´

5´-GCATAGCAGGAGAGGAATGC-3´;

Ptch1, 5´-TCCAGACATCAGCCTCCCTTG-3´

5´-GCCTCTCCTCACATTCCACGTC-3´.

6. 5E1 drug delivery

Drug delivery was preformed as previously described (Lee et al., 2011). A monoclonal antibody (mAb) 5E1 (an IgG1 monoclonal antibody against Shh protein) was obtained from hybridoma cells at the Developmental Studies Hybridoma Bank. Cultured E13.5 ICR mice palates were treated with 5E1 (130 μg/ml) or PBS (100 μl/ml) in culture medium for 48hr.

III. Results

1. Expression pattern of *Pitchfork* during mouse palate development

In detailed analysis of *Pitchfork* expression was examined in the mouse secondary palate, using whole-mount *in situ* hybridization at E12.5, E13.5 and E14.5 (Fig. 5). At E12.5, *Pitchfork* was expressed from the anterior to the middle regions of the palatal shelves, but not expressed in the posterior region (Fig. 5a). *Pitchfork* was strongly expressed in the underlying mesenchyme just beneath palatal epithelium, but not in the palatal epithelium (Fig. 5d, g). At E13.5, *Pitchfork* was strongly expressed from the anterior to the middle regions in the vertically shaped developing palate shelves. However, *Pitchfork* was not expressed in the posterior region (Fig. 5b). At high magnification, *Pitchfork* was strongly expressed in the palatal mesenchymal cells underlying the palatal epithelium, but not expressed in the palatal epithelium (Fig. 5e, h). *Pitchfork* expression was observed throughout the anterior to posterior region of the fused secondary palate (Fig. 5c). Faint expression of *Pitchfork* was detected in the palatal mesenchyme. Interestingly, strong expression of *Pitchfork* was observed at the epithelium in the lateral side of the developing nasal cavity (Fig. 5f, i).

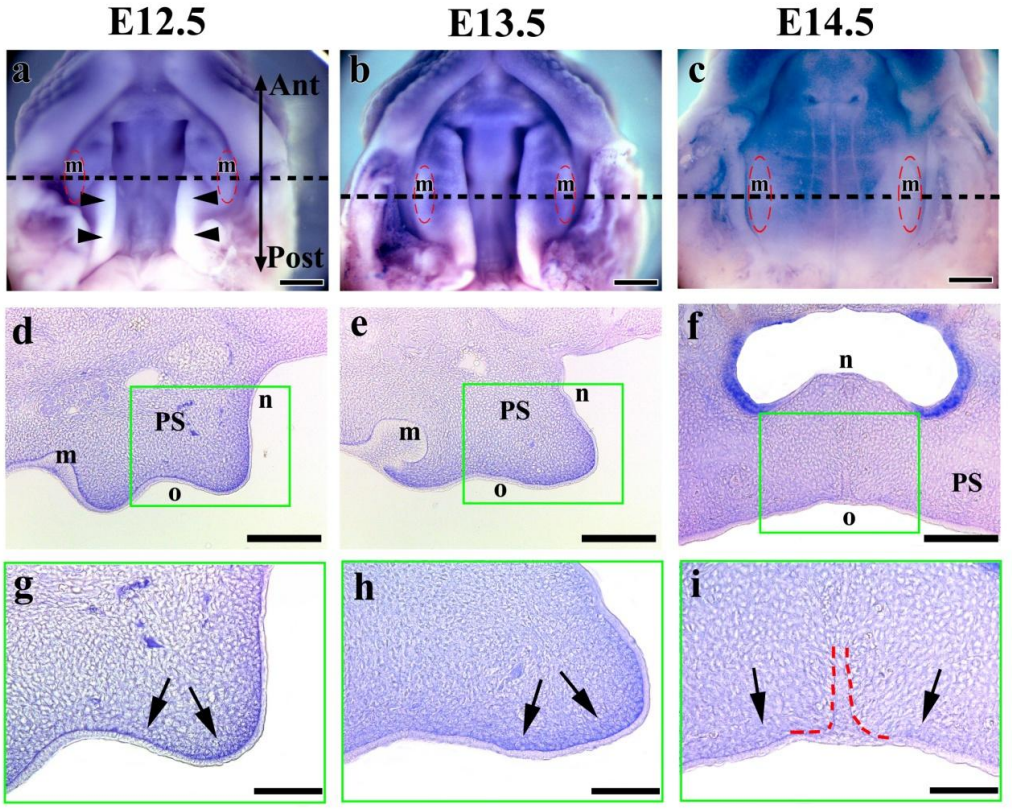

Figure 5. Expression pattern of *Pitchfork* in developing palatal shelves. a-i The expression pattern of *Pitchfork* is detected by whole-mount *in situ* hybridization and subsequent sectioning. **a**, **d**, **g**, *Pitchfork* is expressed anterior to the middle side of the palatal shelves. *Pitchfork* was strongly expressed in the mesenchyme along the epithelium of the palatal shelf at E12.5. **b**, **e**, **h**, *Pitchfork* is strongly expressed from the anterior to the middle region of the palatal shelves and in the palatal mesenchyme, especially the underlying mesenchyme just beneath the epithelium at E13.5. **c**, **f**, **i**, After the palatal shelves make contact, *Pitchfork* is faintly expressed in palatal mesenchyme at E14.5, but not in the oral side epithelium. *black dotted line* section plane; *black bilateral arrow*

anterior-posterior axis; *black arrows* strong expression region of *Pitchfork*; *black arrowheads* posterior region; *green box* high magnification region of *Pitchfork*; *red dotted line* midline epithelial seam (MES) region; *red dotted circle* molar regions; *Ant* anterior; *m* molar; *n* nasal side; *o* oral side; *Post* posterior; *PS* palatal shelf; *scale bars* **a**, **b**, **c** 500 µm; **d**, **e**, **f** 200 µm; **g**, **h**, **I** 50 µm.

2. Morphological changes after pitchfork over-expression during palate development

To determine the function of *Pitchfork* during secondary palate development, *Pitchfork* was over-expressed in the palatal shelves at E13.5 and then examined after culturing for 48hr (Fig. 6). Morphological differences between the control (Fig. 6a) and the Pitchfork over-expressed palates (Fig. 6b) were observed as *in-vitro* culture system for 48 hr. The vertical length (fusion region of nasal to oral cavity palatal epithelium) of the developing palate was increased (98%) in the Pitchfork over-expression group compared to the control group (N=25) (Fig. 6c).To confirm successful transfection of the control (lentiviral empty vector-pCDH) (Fig. 2d) and the *Pitchfork* lentiviral vector (Fig. 6e), immunohistochemistry was performed using the EGFP antibody. EGFP was detected in both epithelium and mesenchyme of the lentiviral-infected palate. RT-qPCR results showed that the *Pitchfork* expression level was significantly increased after Pitchfork over-expression (N=17) (Fig. 6f). Scanning electron microscopy confirmed primary cilia formation after Pitchfork over-expression (Fig. 6g, h). Large numbers of primary cilia were detected in the control group (Fig. 6g). However, the number of primary cilia was dramatically reduced in the Pitchfork over-expression group compared to the control group strikingly (Fig. 6h). The horizontal length (lateral edge of developing first molars) of the developing palate was increased (56.3%) in the Pitchfork over-expression group (Fig. 6j, l) compared to the control group (measure the horizontal length of palate at E13.5+0hr and E13.5+48hrin the same specimen) (Fig. 6i, k), but the anterior to posterior

length was not significantly changed. Statistical analysis revealed that cultured palate was expanded in the Pitchfork over-expression group compared to the control group (N=16) (Fig. 6m).

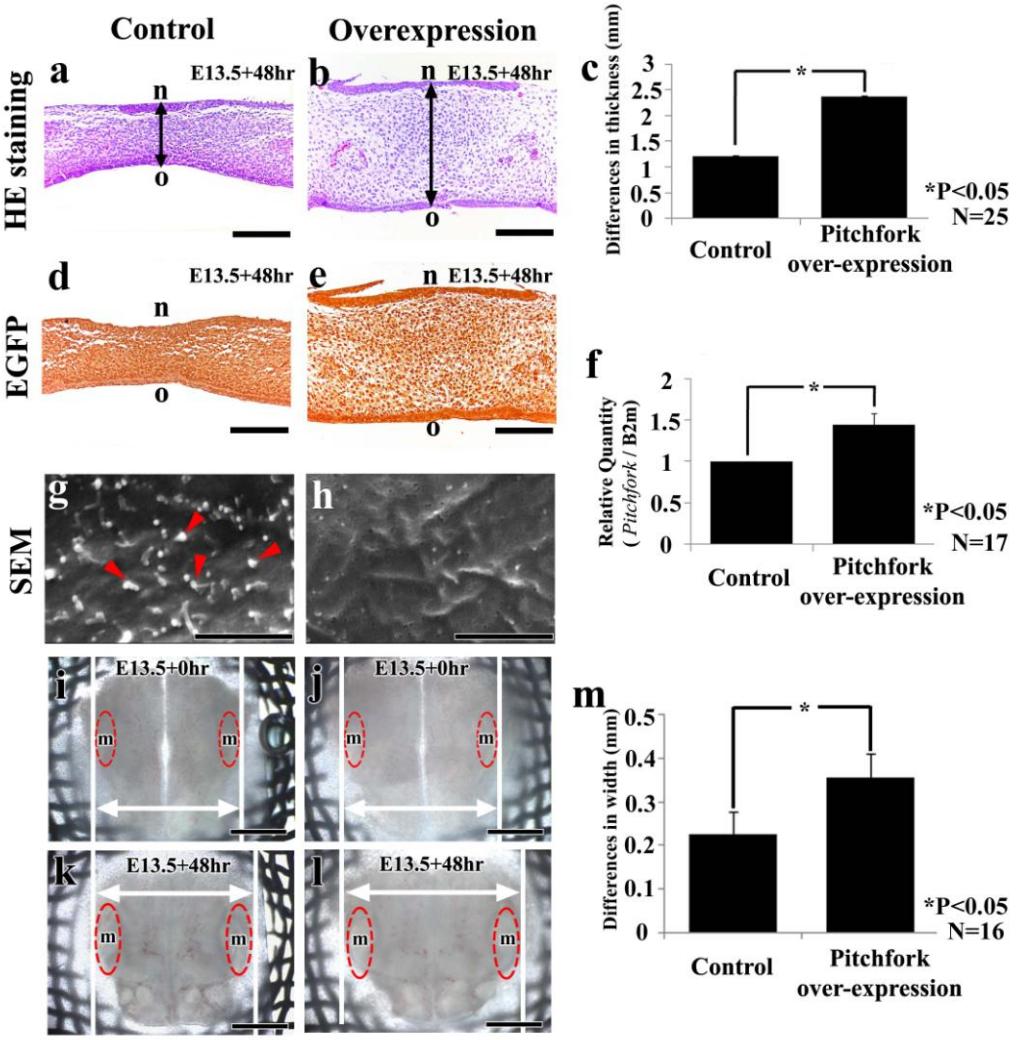

Figure 6. Morphological changes in the developing palate after Pitchfork over-expression. **a**, **b** The vertical length of the developing palate was increased in the Pitchfork over-expression group compared to the control group. **c** Statistical analysis revealed that the cultured palate is thickened in the Pitchfork over-expression group compared to the control group. **d**, **e** Immunohistochemistry is performed using an

enhanced green fluorescent protein (EGFP) antibody to determine transfection efficiency. EGFP is detected in both the epithelium and mesenchyme of lentiviral-infected palates. **f** Gene expression analysis of *Pitchfork* by RT-qPCR. The levels of *Pitchfork* is significantly increased after its over-expression in palates at E13.5 and then examined after culturing for 48 hr. **g**, **h** Scanning electron microscopy (SEM) confirm the condition of the primary cilia. **i**-**m** The width (E13.5+0hr and E13.5+48hr organ culture in the same specimen) of developing palate is increased in the Pitchfork over-expression group compared to the control group. *black bilateral arrows* palate tissue thickness; *white bilateral arrows* palate tissue width; *red arrowheads* primary cilia; *red dotted circle* molar regions; *m* molar; *N* number of specimens examined in each stage; *n* nasal cavity; *o* oral cavity; *Scale bars* **a**, **b**, **d**, **e** 200 μm; **g**, **h** 2 μm; **i**, **j**, **k**, **l** 500 μm; *P<0.05 as determined by ANOVA.

3. The effect of *Pitchfork* on cell proliferation and apoptosis during palate development

To examine the effect of over-expressed *Pitchfork* on cell proliferation and apoptosis during palate development, we performed immunohistochemistry using the Ki-67 antibody and TUNEL assay after Pitchfork over-expression (Fig. 7). In the control group, proliferating cells were randomly scattered in the mesenchyme of the secondary palate (Fig. 7a). However, in the Pitchfork over-expression group, proliferating cells were observed in the mesenchyme around the midline epithelial seam region (Fig. 7b). Statistical analysis showed that Ki-67 positive cells increased (37.2%) in the mesenchymal cells of the Pitchfork over-expression group compared to the control group (N=22) (Fig. 7c). TUNEL positive cells were observed in the mesenchyme of the secondary palate in the control group (Fig. 7d). However, there were fewer apoptotic cells in the mesenchyme in the Pitchfork over-expression group. Almost no apoptotic cells were detected in the developing palate mesenchyme in the Pitchfork over-expression group (Fig. 7e). Statistical analysis showed that the number of TUNEL positive cells was reduced (80.5%) in the mesenchymal cells of the Pitchfork over-expression group compared to the control group (N=22) (Fig. 7f).

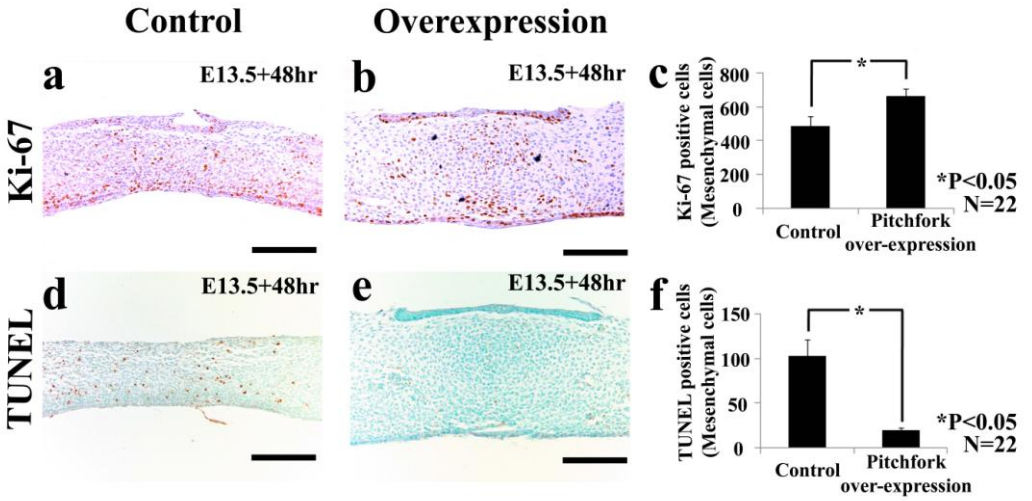

Figure 7. Alteration of cell proliferation and apoptosis after Pitchfork over-expression in cultured palates (at E13.5 + 48 hr). a, **b**, **d**, **e** To determine the effect of *Pitchfork* on cell proliferation and apoptosis during palate development, immunohistochemistry using the Ki-67 antibody and TUNEL assay are examined after Pitchfork over-expression. **a**, **b, c** The number of Ki-67 proliferating positive cells is higher in the mesenchymal cells of the Pitchfork over-expression group than the control. **d**, **e**, **f** After Pitchfork over-expression, the number of apoptotic cells is lower in the mesenchymal cells of the Pitchfork over-expression group than the control. *N* Number of specimens examined in each stage; *Scale bars* **a**, **b**, **d**, **e** 200 μm; **P*<0.05 as determined by ANOVA.

4. Alteration of *Shh* and *Ptch1* expression by Pitchfork over-expression during palate rugae formation

To identify the relationship between *Pitchfork* and *Shh*, as well as *Pitchfork* and *Ptch1* during palate development, we performed *in situ* hybridization of *Shh* and *Ptch1* after Pitchfork over-expression (Fig. 8). In the control group, *Shh* was expressed along the rugae lines (Fig. 8a). In sagittal sections, *Shh* was strongly expressed at the epithelial tip of the palatine rugae (Fig. 8c). The *Shh* expression pattern was not changed after Pitchfork over-expression (Fig. 8b). However, Pitchfork over-expression induced up-regulated *Shh* expression in the developing rugae compared to the control group (Fig. 8d). Moreover, the thickness of the palatine rugae increased after Pitchfork over-expression. RT-qPCR showed that *Shh* expression level was significantly increased after Pitchfork over-expression (N=15) (Fig. 8i). *Ptch1* was expressed along the rugae lines (Fig. 8e) and was strongly expressed in the palatal mesenchyme just beneath palatine rugae epithelium in the control group (Fig. 8g). The *Ptch1* expression pattern was not changed after Pitchfork over-expression (Fig. 8f). However, *Ptch1* expression was up-regulated in the developing palatine rugae compared to controls (Fig. 8h). Compared to the controls, the expression level of *Ptch1* was significantly increased in the Pitchfork over-expression group (N=15) (Fig. 8j). These results indicate that over-expressed *Pitchfork* lead to thickened rugae (Fig. 8d, h) compared to the control group (Fig. 8c, g). To investigate relationships between *Shh* signaling and *Pitchfork*, we treated 5E1, antibody against *shh* (Cho et al., 2011) in E13.5 palate then for 48hr cultured. Compared to the control group,

Ptch1 expression level was reduced in 5E1 treated group (N=15) (Fig. 8k), and *Pitchfork* expression level was significantly increased in 5E1 treated group (N=15) (Fig. 8l). These results indicate that *Pitchfork* may related to *Shh* and *Ptch1* during mouse secondary palate development. We performed immunohistochemistry using Ki-67 antibody and TUNEL assay after Pitchfork over-expression during palatine rugae formation. Compared to the control group, the number of Ki-67 positive proliferating cells was higher in the palatine rugae after Pitchfork over-expression (Fig. 8m, n). Statistical analysis showed that Ki-67 positive cells increased (43.2%) in the palatine rugae of the Pitchfork over-expression group compared to the control group (N=15) (Fig. 8o). In the control group, large number of the TUNEL positive cells were observed in the palatine rugae (Fig. 8p). However, apoptotic cells were markedly reduced in the developing palatine rugae in Pitchfork over-expression group (Fig. 8q). Statistical analysis showed that the number of TUNEL positive cells was reduced (78.5%) in the palatine rugae of the Pitchfork over-expression group compared to the control group (N=15) (Fig. 8r).

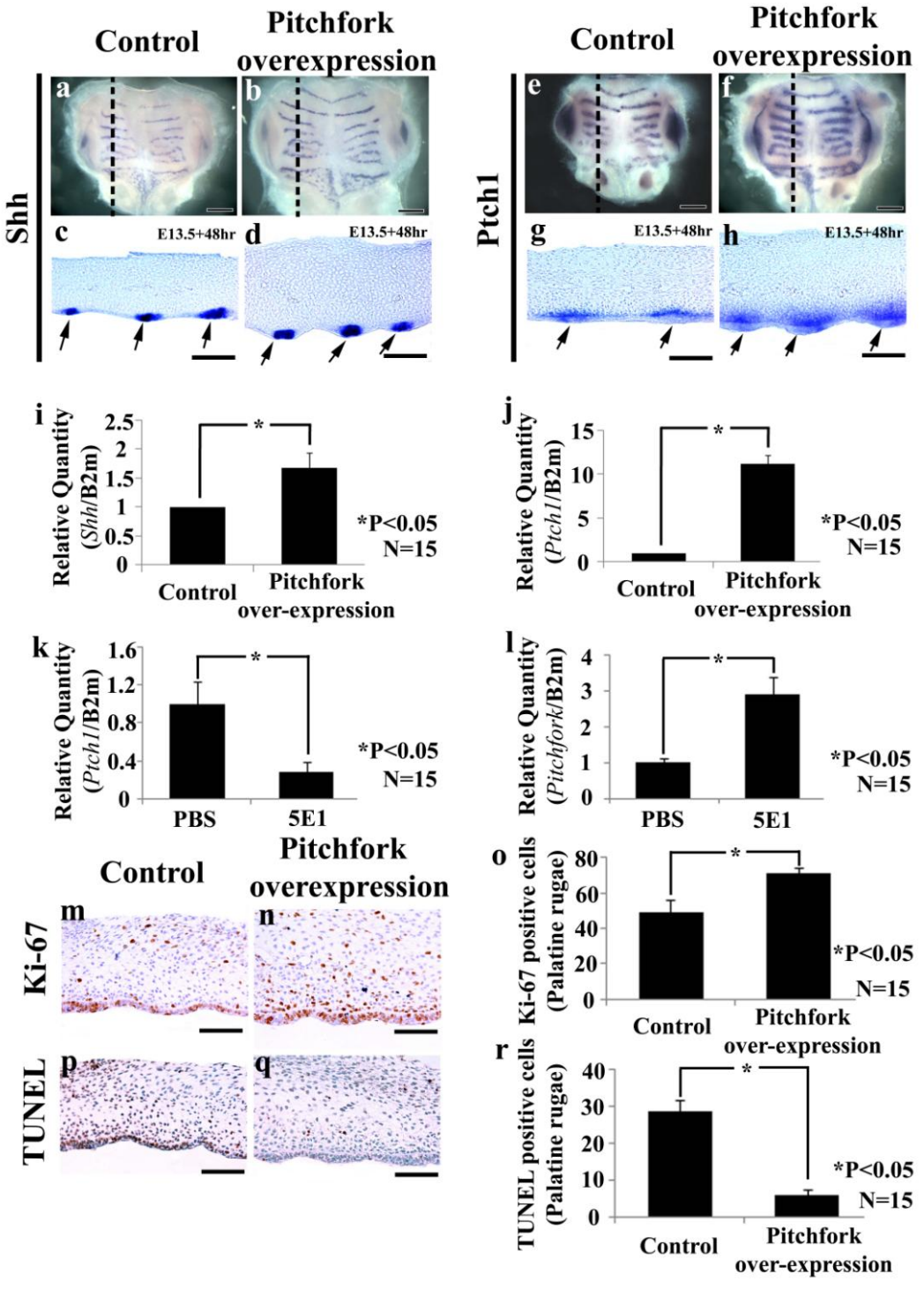
Control
Pitchfork overexpression
Shh
Ptch1
a
b
c
d
e
f
g
h
E13.5+48hr
i
j
k
l
Relative Quantity (Shh/B2m)
Relative Quantity (Ptch1/B2m)
Relative Quantity (Pitchfork/B2m)
*P<0.05
N=15
Control
Pitchfork over-expression
PBS
5E1
m
n
o
p
q
r
Ki-67
TUNEL
Ki-67 positive cells (Palatine rugae)
TUNEL positive cells (Palatine rugae)

Figure 8. Alteration of gene expression in the developing palatine rugae after Pitchfork over-expression. a-d The *Shh* expression pattern was not changed after Pitchfork over-expression. However, Pitchfork over-expression induced thickened *Shh* expression in the developing rugae compared to the control group. **e-h** To examine *Ptch1* expression patterns in the cultured palate for Pitchfork over-expression and control, *Ptch1* expression is expanded and thickened in the developing palatine rugae compared to the controls. **i, j** RT-qPCR is performed after Pitchfork over-expression to determine the relationships between *Pitchfork* and *Shh* and *Ptch1*. Compared to the control, *Shh* and *Ptch1* expression levels are significantly increased after Pitchfork over-expression. **k**, **l** 5E1 is treated at E13.5 palate then examined RT-qPCR after culturing for 48 hr. Compared to the control group, *Ptch1* expression level is reduced in 5E1 treated group, and *Pitchfork* expression level is significantly increased after 5E1 treatment. **m**, **n**, **o** The number of Ki-67 positive proliferating cells is higher in the palatine rugae of the Pitchfork over-expression group than the control group. **p**, **q**, **r** After Pitchfork over-expression, the number of apoptotic cells is lower in the palatine rugae of the Pitchfork over-expression group than the control. *black dotted line* section plane; *black arrows* strong expression region; *N* Number of specimens examined in each stage; *scale bars* **a**, **b**, **e**, **f** 500 μm; **c**, **d**, **g**, **h**, **m**, **n**, **p**, **q** 200 μm; *P<0.05 as determined by ANOVA.

5. Molecular and cellular effects of *Pitchfork* during palate development

The mechanism of the molecular and cellular effects of *Pitchfork* is regulating *Shh* signaling, cell proliferation and apoptosis during mouse secondary palate development (Fig. 9). *Pitchfork* plays important role during palatine rugae formation by positively controls of the *Shh* and *Ptch1* expression in palate development (Fig. 9A). At the Pitchfork over-expression during mouse secondary palate development, which Positively control of the cell proliferation and negatively control of the apoptosis lead to increasing vertical and horizontal length (Fig. 9B).

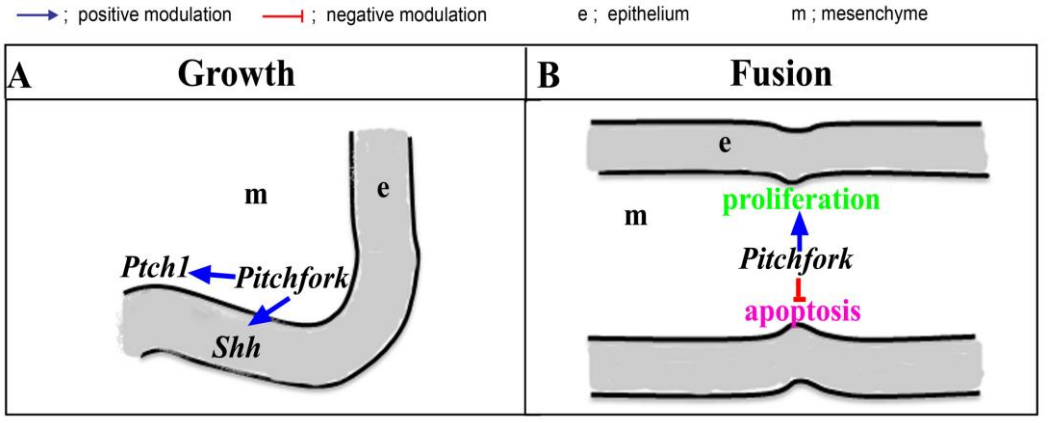

Figure 9. Schematic diagram of the molecular and cellular effects of *Pitchfork* as a crucial regulator during mouse secondary palate development.

IV. DISCUSSION

1. *Pitchfork* plays an important role during palate development by regulating primary cilia disassembly

Palate development is a complex mechanism that involves vertical outgrowth, elevation, horizontal growth, adhesion and fusion (Bush et al., 2012). During the palate development, correct gene expression is necessary for proper palate structure (Martinez-Alvarez et al., 2004). Alteration of primary cilia-related genes, such as oral-facial-digital syndrome type I (OFD1) and *Kif3a*, lead to primary cilia defects and various craniofacial disorders, including lateral cleft palate; lobed, lipomas or hamartomas of the tongue; and hypodontia (Brugmann et al., 2010; Sukarova-Angelovska et al., 2012; Toriello et al., 1993). *Pitchfork* is a mouse embryonic node gene associated with ciliary targeting complexes located at the basal body during primary cilia disassembly (Kinzel et al., 2010). Previous study have reported that the Pitchfork haploinsufficiency in $Pifo^{lacZ/+}$ mouse lead to heart failure and left-right asymmetry defects by primary cilia disassembly (Kinzel et al., 2010). Based on previous studies, *Pitchfork* may be expressed and play an important role during mouse palate development. In our results, during the period of vertical outgrowth and elevation during palate development, *Pitchfork* was strongly expressed in the underlying mesenchyme just beneath the epithelium (Fig. 5). After the palatal shelves make contact, *Pitchfork* was reduced in the midline epithelial seam region, and strongly

expressed in the lateral nasal cavity epithelium. Therefore, *Pitchfork* may play an important role during the growth of the mouse secondary palate, in elevation and make contact between the two palatal shelves. To understand the mechanism of *Pitchfork* during palate development, we examined morphological changes during palate development after Pitchfork over-expression. Over-expressed *Pitchfork* in both the epithelium and mesenchyme, which is due to the limitation of lentivirus infection. For the over-expression of *Pitchfork in vitro* cultured palatal shelves, it is difficult to specifically over-express the *Pitchfork* just in epithelium or mesenchyme these two neighboring tissue layers. But considering the pivotal role of sequential and reciprocal interactions between epithelium and mesenchyme in palatal development (Zhang et al., 2002), and the expression of primary cilia by most mammalian cell types (McMurray et al., 2013), therefore, that the proper expression of *Pitchfork* play an important role in palatal cilia formation. Over-expressed *Pitchfork* leads to disassembly of primary cilia in cultured palatal surfaces (Fig. 6). Previous studies confirmed that non-functional primary cilia induce orofacial expansion and lead to secondary lateral cleft palate during mouse palate development (Brugmann et al., 2010; Ferrante et al., 2006). In our results, the vertical length and horizontal length of the developing palate were increased in the Pitchfork over-expression group compared to the control group. These results indicate that Pitchfork over-expression induced primary cilia disassembly and lead to morphological differences during palate development. To further validate and investigate the effects of *Pitchfork* in palate development, we confirmed cell proliferation and apoptosis, essential for normal palate development (Shin et al., 2012). The number of Ki-67 positive cells was

higher in the mesenchyme around the MES region in the Pitchfork over-expression group compared to the control group. However, TUNEL positive cell number was decreased in the palate after Pitchfork over-expression compared to the control group (Fig. 7). These results indicate that induced cell proliferation and reduced apoptosis lead to increasing vertical and horizontal length of secondary palate by over-expressed *Pitchfork* during mouse palate development.

In this study, we focused on the role of *Pitchfork* in palate development by the regulation of primary cilia formation. Pitchfork over-expression during palate development lead to morphological changes not only by regulating primary cilia disassembly but also by regulating cell proliferation and apoptosis.

2. Alteration of palatine rugae formation by Pitchfork over-expression

Previous studies have reported that *Shh* and *Ptch1* play an important role during rugae formation (Lee et al., 2011). In this study, we examined relationships between *Pitchfork* and *Shh*, as well as *Pitchfork* and *Ptch1* during palate development (Fig. 8). In our results, Pitchfork over-expression did not alter the expression pattern of *Shh* and *Ptch1*. However, Pitchfork over-expression induced up-regulated *Shh* and *Ptch1* expression in the developing palatine rugae compared to the control group. Previous studies indicate that primary cilia mutations result in an up-regulation of *Shh* signaling (Brugmann et al., 2010; Tran et al., 2008). Our results showed that reduced primary cilia were observed after over-expression of *Pitchfork* and lead to an up-regulation of *Shh* signaling pathway activity. The primary cilia is an organelle that protrudes from the cell surface, and *Ptch* is localized within it (Rohatgi et al., 2007). In addition, *Ptch* has been shown to be located mostly in intracellular vesicles (Martin et al., 2001; Polkinghorn et al., 2007). Therefore, over-expressed *Pitchfork* may lead to over-expression of *Ptch1* in intracellular vesicles. We also found that Pitchfork over-expression induced thickening of the palatine rugae more than in the control, but did not change rugae patterning. The number of Ki-67 positive cells was higher in the palatine rugae in Pitchfork over-expression group compared to the control group. However, TUNEL positive cell number was decreased in the palatine rugae after Pitchfork over-expression compared to the control group. According to these results, *Pitchfork* plays an important role during

palatine rugae formation by positive control of Shh and Ptch1 expression and controlling cellular event such as cell proliferation and apoptosis. Previous studies have reported that the epithelial-mesenchymal interaction regulates mammalian palate development. *Bmp2* is strongly expressed palatal mesenchyme. Epithelial expression of *Shh* induces *Bmp2* expression in the palatal mesenchyme and the induction of *Bmp2* increases cell proliferation during palate development (Zhang et al., 2002). Therefore, Pitchfork over-expression may regulate the epithelial-mesenchymal interaction by controlling the *Shh* signaling pathway, leading to morphological changes in secondary palate structure. It is expected that the results of this study will help to enhance our understanding of mammalian secondary palate development.

V. Conclusion

Pitchfork is expressed in mesenchyme throughout the entire palate development process. Our results revealed that *Pitchfork* plays an important role during palate development by the regulation of primary cilia formation. Pitchfork over-expression during palate development lead to morphological changes not only by regulating primary cilia disassembly but also by regulating cell proliferation and apoptosis. Over-expressed *Pitchfork* controlled the *Shh* signaling pathway, leading to morphological changes in secondary palatine rugae structure by regulate the epithelial-mesenchymal interaction.

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국문요약

Pitchfork 유전자가 mouse 구강구개측 발육에서 Shh signaling 과 Primary cilia 분해에 대한 조절

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Pitchfork 는 Cilia 와 연관되는 유전자이며 이는 Primary cilia 의 Basal body 에 위치하여 있고 Primary cilia 분해에 관여한다. 포유류의 배아 발생과정에서 Cilia 의 결함은 구개열 뿐만 아니라 폐, 신장, 심장 등과 같은 부분의 여러가지 질병을 초래 할 수 있다고 알려져 있다. 포유류의 구개는 발생과정 동안에 세포증식, 세포사멸, 세포이동, 상피−간엽 변환과 같은 다양한 세포 대사 과정이 관여한다. 또한 구개의 발생은 상피와 간엽의 여러 유전자들의 유기적인 상호작용에 의해 일어난다. 본 연구에서는 생쥐 입천장 발생에서 Pitchfork 의 기능을 알아보기 위하여 발현양상과 Pitchfork 과발현 시 입천장 형태학적 변화에 대한 영향을 확인하였다. 배아발생 12.5 일과 13.5 일째에서 Pitchfork 유전자는

강하게 발현되는 양상을 보였다. 대조군과 비교하였을 때 Pitchfork 과발현 그룹에서 현저한 형태학 적 변화를 관찰할 수 있었다. 생쥐 입천장발생에서 Pitchfork 유전자의 과발현은 Primary cilia 의 분해를 촉진시킬 뿐만 아니라 입천장주름발생에서 중요한 역할을 하는 Shh, Ptch1 의 발현양도 변화시켰다. 따라서 생쥐 입천장 발생에서 적절한 양의 Pitchfork 유전자의 발현은 정상적인 입천장 형태를 유지하는데 중요한 역할을 할 것으로 판단된다.

핵심되는말: 구개발생, Primary cilia, Pitchfork, 세포증식, 세포사멸