Specific roles of *Wnt11* and *Fgf8* for palatogenesis in mouse embryo

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Specific roles of *Wnt11* and *Fgf8* for palatogenesis in mouse embryo

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This certifies that the Master's Thesis of

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The Graduate School
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ABSTRACT

Specific roles of *Wnt11* and *Fgf8* for palatogenesis in mouse embryo

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During palatogenesis, many dynamic cellular and molecular events occur, including the elevation and fusion of the palate. These complex events are mediated by cell proliferation, cell death and epithelial-mesenchymal transformation (EMT). Failure of these processes can cause cleft palate, the most common birth defect in humans. A defective palate causes difficulties in swallowing, sucking and verbal pronunciation. To understand the precise progresses and causes of defect in palate development, respective expression patterns of signalling molecules and transcription factors, such as *Fgf8*, *Fgfr1b*, *Wnt7a*, *Wnt11* and *Lef1*, were investigated during early mouse secondary palate development. In order to reveal the mutual interactions between signalling molecules and transcription factors during early mouse secondary palate development, author employed the electroporation method in an *in vitro*

organ culture system. In addition, beads soaked with the Fgf receptor 1 (*Fgfr1*) inhibitor SU5402 were implanted into the *in vitro* organ cultures. The resulting alterations in expression patterns of signalling molecules and transcription factors caused by the electroporation of the various transcription factors and the implantation of the beads suggested that the process of early mouse secondary palate development is dependent on FGF, Wnt, and Lef signalling. To examine the signalling networks involved in the development of the secondary palate, cell death and proliferation were examined after the over-expression of signalling molecules and transcription factors such as Wnt-7a, Wnt-11 and Lef-1 by electroporation. The findings presented in this study provide evidence suggesting that the cellular and molecular mechanisms involved in secondary palate development, including elevation and fusion, are closely regulated through signalling by the Fgfs and Wnt family members.

Key words: palatogenesis, morphogenesis, *Fgf8*, *Fgfr1b*, *Wnt7a*, *Wnt11*, *Lef1*

Specific roles of *Wnt11* and *Fgf8* for palatogenesis in mouse embryo

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I. INTRODUCTION

Mammalian palatogenesis depends on cellular and molecular mechanisms that cause the elevation and fusion of palatal shelves. From the internal surfaces of the maxillary primordia, two palatal shelves appear when the development of the secondary palate is initiated. At embryonic day 13.5 (E13.5) in mouse, these palatal shelves are vertically located on each side of the tongue. At E14.0, the palatal shelves elevate to a horizontal position above the tongue.[1] After elevation, the palatal shelves make contact, adhere, and fuse with each other from the middle region to the anterior and posterior regions

through the processes of apoptosis, cell proliferation and epithelial-mesenchymal transformation (EMT) (Fig. 1).[2,3]

However, the mechanisms underlying palatogenesis have not been elucidated in detail. Author hypothesized that the development of palatal shelves would share similar processes in common with limb development because the morphological changes made during limb bud development involve similar signalling networks as those that appear to be involved in palatogenesis. The Wnt gene family encodes a conserved class of secreted signalling molecules and is considered one of the major gene families essential for proper embryonic patterning and organogenesis.[4] The discovery of the common origin of the *Drosophila* segment polarity gene *Wingless* and the murine proto-oncogene *Int-1* laid the keystone of a signalling pathway now commonly referred to as the canonical Wnt cascade.[5,6] The Wnt gene family has been subdivided into at least two classes.[7] Members of the *Wnt1* class (*Wnt1*, *Wnt3*, *Wnt3a*, *Wnt7a*, *Wnt7b* and *Wnt8a*) are effective activators of the canonical pathway, whereas members of the *Wnt5a* class (*Wnt4*, *Wnt5a* and *Wnt11*), with few exceptions, are poor activators of the canonical pathway.[4,8, 9, 10] Convergent extension has been observed in a number of developmental systems, including axis formation in *Xenopus*,[11, 12, 13,14] the sensory epithelium of the inner ear,[15] and neural tube closure.[16] In addition, *Wnt5a* and *Wnt11* have

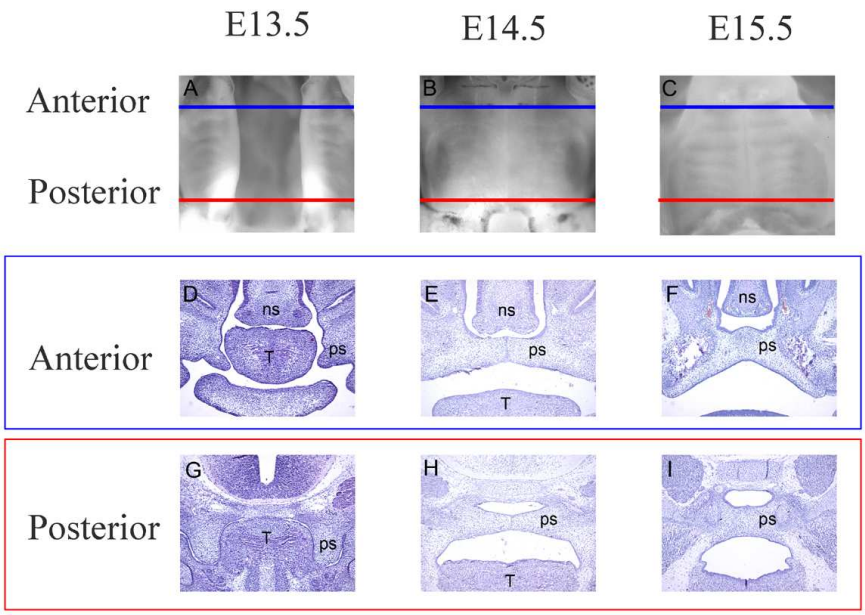


Figure 1. (A-C) Morphogenesis of the palatal shelf. Blue lines indicate the section plane across the anterior region and red lines indicate the posterior region of the secondary palate. (D) At E13.5, the anterior palatal shelf bends towards the ventral side of the tongue. (E, H) At E14.5, the palatal shelf has moved both anteriorly and posteriorly into a horizontal position. (F, I) At E15.5, complete fusion has occurred and no midline epithelial seam is now seen. The posterior palate is much flatter than anterior palate. (G) At E13.5, the posterior palatal shelf is at right angles to tongue and closely apposed to it. G, genioglossus muscle; ns, nasal septum; ps, palatal shelf; T, tongue.

been implicated in promoting the calcium pathway. This pathway activates protein kinase C and Cam kinase,[13,17] affecting cell adhesion[18] and inhibiting the canonical Wnt signalling pathway.[13,19] *Wnt11* and *Wnt7a* expression observed in embryonic chick heart appear to be consistent with the roles of these two Wnts in the differentiation of cardiac conduction tissues. Moreover, *Wnt11* and *Wnt7a* are up-regulated in association with endothelin-1.[11] *Lef1* is a member of the HMG box family and a nuclear mediator of Wnt signalling.[20] *Lef1* and *Tcf1* are co-expressed in the presomitic mesoderm of developing mice, and the targeted inactivation of both genes results in the lack of paraxial mesoderm and in the generation of multiple neural tubes, phenotypes that are identical to those of the *Wnt3a* null mutation.[21] *Lef1* is initially expressed in the thickened dental epithelium at E11 in the developing tooth germ. Loss of *Lef1* function results in mice lacking teeth. *Lef1* function is needed only transiently in the dental epithelium to control *Fgf4* expression in the enamel knot, thus relaying a Wnt signal reception to a cascade of FGF signalling activities.[22,23] The application of exogenous FGFs was able to rescue the phenotype of *Lef1* mutant teeth.[24 25]

Wnt signalling has been reported to be closely linked to Fgf signalling in the development of other organs, such as the digit. Fgf signalling controls the elongation of digits and tip formation. When Fgf expression is extinguished in

the ridge, the tip formation program, possibly involving Wnt signalling, is activated.[26] A growing number of genetic and environmental factors that are significant to the process of palate formation have recently been identified.[1]

Fibroblast growth factor 8 (FGF8), in particular, appears to play an important role in the developing nervous system, although the mechanism(s) by which it acts have not been elucidated fully. FGF8 was first identified as a mitogen,[27] and some data support a mitogenic role for FGF8 in neural tissues.[28,29,30] Previous studies have shown that FGF8 strongly induces *Pax9* and have reported that *Pax9* deficiency results in unelevated palatal shelves owing to a mechanical hindrance.[31,32] *Fgf8* plays important roles in many different organs such as the limb and the brain.[33,34] The expression patterns of the FGF receptor (FGFR) gene indicate that nephrogenic progenitors and their derivatives are likely to be competent to respond to the various FGF ligands.[35] *Fgfr1* is uniquely expressed at high levels in the cortical zone,[35,36] where nephrogenic progenitors require *Fgf8* for survival.[37] Moreover, beak truncation in the cleft primary palate is due to an epithelial defect in the frontonasal mass that is coincident with a failure to down-regulate expression of *Fgf8*.[38] A recent study also revealed that *Fgf8* expression is closely related to Wnt signalling in the developing kidney.[37]

In this study, author examined the expression patterns of *Wnt11*, *Wnt7a*, *Lef1*, *Fgf8* and *Fgfr1b* in the developing secondary palate. In order to investigate the interactions between *Wnt11*, *Wnt7a*, *Lef1*, *Fgf8* and *Fgfr1b*, electroporation of constructs for each gene and the implantation of beads soaked with SU5402, an Fgfr1 inhibitor, were performed. The results of this study revealed that *Wnt11* expression is related to the palatal fusion processes via apoptosis. Interactions between *Fgf8*, *Fgfr1b* and *Wnt11* were especially important in the patterning, cell differentiation and morphogenesis of the palate.

II. MATERIALS AND METHODS

1. Animals

Adult ICR mice were housed in a temperature-controlled room (22°C) 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 embryos were obtained from time-mated pregnant mice. Embryonic day 0 (E0) was designated as the day a vaginal plug was confirmed. Embryos at each developmental stage (daily intervals from E13.5–E15.5) were used in this study.

2. *In vitro* organ culture

The palatal shelves were isolated from E13.5 mouse maxilla. They were then cultured in medium without Fetal Bovine Serum (FBS) at 37℃ and 5% CO_2 for 48 hours using a slight modification of the culture method reported by Trowell.[39] The culture medium was replaced every 24 hours. The culture medium (DMEM/F12, Gibco) was supplemented with 20 μg/ml ascorbic acid (Sigma) and 1% penicillin/streptomycin. Tissues were then fixed and processed for *in situ* hybridizations and immunohistochemistry. At least 30 specimens were examined in each experiment.

3. Expression constructs

pEGFP-N1 was made by excising the fluorescent protein (Clontech). The pEGFP-N1 vector has previously been optimized for brighter fluorescence. *Wnt11*, *Wnt7a* and *Lef1* were inserted into the pEGFP-N1 vector. Plasmid DNA was purified using a plasmid purification kit (Qiagen) and dissolved in T1/4E (Tris-HCl (pH 8.0) 10 mM, EDTA 0.25 mM).

4. Electroporation of organ cultures

DNA (1 μg/μl) in PBS buffer was injected into the palate mesenchyme using a microcapillary needle, and 20 ms current pulses of 25 volts were applied using an electroporator. Before injection, fast green 1/10,000 (Sigma) was added to the DNA solution for visualization within the tissue.

5. Bead implantation

AG-1X2 (Bio-Rad Laboratories) formate-derived beads were incubated in 10 mM SU5402 for 1 hour at room temperature. Beads were implanted on the palatal shelf at E13.5. After 48 hours of culture *in vitro*, the palate was used for whole-mount *in situ* hybridization.

6. Immunohistochemistry and TUNEL assay

The specimens were embedded in OCT compound using conventional methods. Specimens were cut to a thickness of 7 μm. The tissues were incubated with the primary antibody, mouse monoclonal antibody against Proliferating Cell Nuclear Antigen (PCNA, Neo Markers), at 4°C overnight. After washing with PBS, the specimens were reacted with two consecutive incubations with the biotinylated goat anti-mouse immunoglobulins and a streptavidin peroxidase at room temperature for 10 minutes each. Finally, the specimens were visualized using a DAB reagent kit (Zymed). TUNEL assay was performed using the '*in situ* cell apoptosis detection kit' (Trevigen, Inc.) following the manufacturer's instructions. 7 μm sections were treated with Proteinase K (in 10 mM Tris–HCl, pH 8.0) at a concentration of 20 μg/ml for 15–20 minutes at room temperature. The samples were incubated with the labeling reaction mixture at 37°C for 1 hour and Strep-HRP solution for 10 minutes at 37°C. DAB was used as substrate solution to detect the sites of *in situ* apoptosis using a light microscope.

7. Whole mount *in situ* hybridization

Specimens were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS). *In situ* hybridization was carried out by treating the

specimens with 20 μg/ml proteinase K for 3 minutes at room temperature.[40] Anti-sense RNA probes were labelled with digoxigenin (Roche). After *in situ* hybridization, specimens were cryosectioned at a thickness of 30 μm.

III. RESULTS

1. Expression pattern of *Wnt11* and *Wnt7a* in the developing mouse palate

Wnt11 and *Wnt7a* expression were examined by *in situ* hybridization at E13.5 and E14.5 (Figs. 2A-L). At E13.5, *Wnt11* was weakly expressed in the anterior to middle region of the secondary palate. Strong *Wnt11* expression was detected from the posterior region of secondary palate to the soft palate (Fig. 2A). Frontal sectioning showed *Wnt11* was weakly expressed in the mesenchyme of the palate around the Medial Edge Epithelium (MEE) region (Fig. 2C). On the other hand, strong expression of *Wnt11* was detected in the epithelium of the posterior palate from the nasal to the oral side. Weak *Wnt11* expression was also observed in the nasal side of the mesenchyme (Fig. 2E). At E14.5, *Wnt11* was detected along the region of midline fusion. In the middle part of the palate showed the first fusion point, *Wnt11* expression was observed along the fusion line. While the expression range was extended from the middle to the anterior and posterior portions of the palate (Fig. 2B), expression of *Wnt11* was detected only in the epithelium of the palate after frontal sectioning (Figs. 2D and F). *Wnt11* was also expressed on the oral side of the

midline epithelial seam (MES). MES was then transformed into mesenchyme. At E13.5, *Wnt7a* was strongly expressed in the anterior to middle region of the palatal shelves but was clearly not detected from the posterior part of the secondary palate to the soft palate region (Fig. 2G). Frontal sections showed that *Wnt7a* was weakly expressed around the MEE region but strongly expressed in the mesenchyme away from the MEE (Fig. 2I). In the posterior region, *Wnt7a* was not detected in either the epithelium or mesenchyme (Fig. 2K). At E14.5, expression of *Wnt7a* was observed in the anterior to posterior region of the palate except at the end of the secondary palate and soft palate forming region (Fig. 2H). After sectioning, strong *Wnt7a* expression was observed in the mesenchyme from the nasal to the oral side of the palate (Fig. 2J). However, in the posterior region, *Wnt7a* expression was not detected in either the epithelium or the mesenchyme (Fig. 2L).

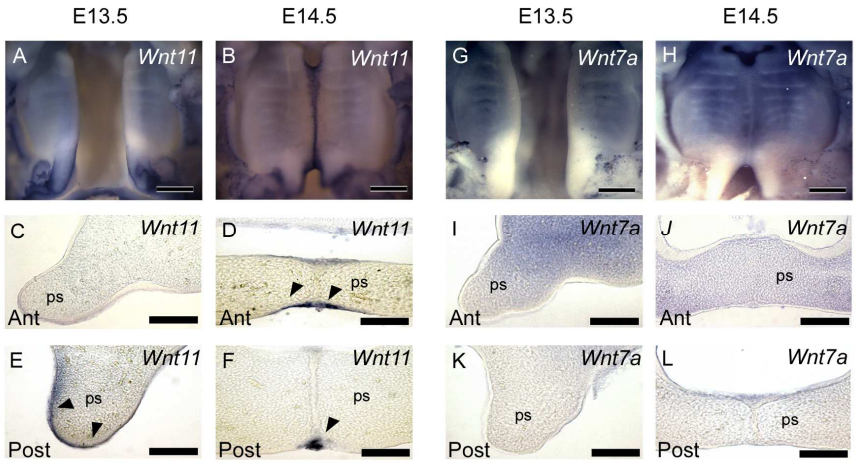


Figure 2. Expression patterns of *Wnt11* and *Wnt7a* in the developing palate. (A) *Wnt11* expression was detected by whole-mount *in situ* hybridization and then (C, E) in sections. All sections shown were made through a frontal section. (A, E) *Wnt11* expressed in the posterior region but (C) not in the anterior region of the palatal shelf at E13.5. (E) Strong expression was detected in the palate epithelium (arrowheads). (B, D, F) At E14.5, *Wnt11* expression was restricted along the fusion line of the palate. (D, F) Expression of *Wnt11* was observed in the palate epithelium in both the anterior and posterior regions (arrowheads). (G) *Wnt7a* expression was detected by *in situ* hybridization and observed in the anterior to middle region of the palatal shelf. (I) *Wnt7a* expression was detected in the mesenchyme away from the medial edge epithelium but (K) not in the posterior region. (H) At E14.5, *Wnt7a* expression was observed in anterior to middle region of the palate. (J) Expression of *Wnt7a* was restricted in the anterior mesenchyme of the palate but (L) not in the posterior. ps, palatal shelf; Scale bars in A, B, G, H = 500 μm; C-F, I-L = 200 μm.

2. *Fgf8*, *Fgfr1b* and *Lef1* expression patterns during palatogenesis

Fgf8 and *Fgfr1b* have similar expression patterns during early mouse palatogenesis (Figs. 3A, B, G and H). At E13.5, *Fgf8* was expressed from the anterior to the middle region of the mouse secondary palate (Fig. 3A). Frontal sections showed that *Fgf8* was expressed in the mesenchyme of the palate. *Fgf8* was expressed more strongly away from the MEE region than in proximity to the MEE region (Fig. 3C). On the other hand, *Fgf8* was not detected in the posterior region of the secondary palate (Fig. 3E). At E14.5, *Fgf8* was expressed throughout the anterior and posterior regions of the secondary palate, except in cells at the posterior end of the secondary palate and in cells of the soft palate (Fig. 3B). After frontal sectioning, *Fgf8* was found to be strongly expressed in the mesenchyme of the anterior region (Fig. 3D). In contrast, expression of *Fgf8* was not observed in the posterior region in either the epithelium or the mesenchyme (Fig. 3F). At E13.5, *Fgfr1b* was expressed in the anterior to middle region but not in the posterior region (Fig. 3G). Frontal serial sections showed strong expression of *Fgfr1b* only in the mesenchyme of the palatal shelves (Fig. 3I). However, in the posterior region, *Fgfr1b* expression was not observed (Fig. 3K). At E14.5, expression of *Fgfr1b* was detected from the anterior to posterior region. Similar to expression

patterns of *Fgf8*, *Fgfr1b* expression was not detected in the region from the end of secondary palate to the soft palate. After frontal sectioning, strong expression of *Fgfr1b* was observed only in the mesenchyme of the palate (Fig. 3J) but was not detected in the posterior region (Fig. 3L). At E13.5, *Lef1* expression was detected in the anterior to middle region of the secondary palate (Fig. 3M). Frontal sectioning showed that *Lef1* was only expressed in the mesenchyme of anterior palate (Fig. 3O) but not expressed in the posterior region (Fig. 3Q). Interestingly, at E14.5, the *Lef1* expression pattern was completely different from E13.5. Expression of *Lef1* was condensed into the rugae-forming region (Fig. 3N). In addition, *Lef1* expression was shifted from the mesenchyme to the epithelium in the anterior region (Fig. 3P). In the posterior region, *Lef1* expression was observed for the first time at E14.5 and observed only in the palate epithelium (Fig. 3R).

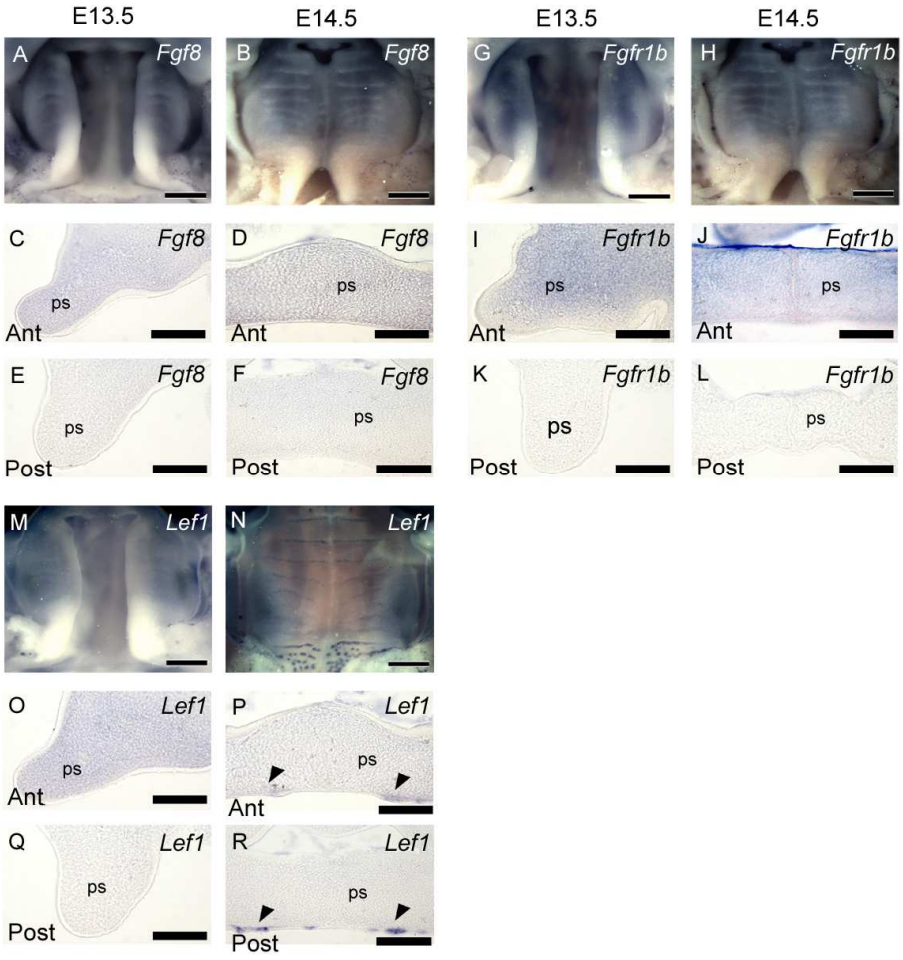

E13.5
E14.5
E13.5
E14.5
A Fgf8
B Fgf8
G Fgfr1b
H Fgfr1b
C Fgf8
ps
Ant
D Fgf8
ps
Ant
I Fgfr1b
ps
Ant
J Fgfr1b
ps
Ant
E Fgf8
ps
Post
F Fgf8
ps
Post
K Fgfr1b
ps
Post
L Fgfr1b
ps
Post
M Lef1
N Lef1
O Lef1
ps
Ant
P Lef1
ps
Ant
Q Lef1
ps
Post
R Lef1
ps
Post

Figure 3. Expression patterns of *Fgf8*, *Fgfr1b* and *Lef1* in the developing palate. (A-R) *Fgf8*, *Fgfr1b* and *Lef1* expression was detected by whole mount *in situ* hybridization. (A, B) Both at E13.5 and E14.5, *Fgf8* was expressed in the anterior to middle region of the palate. (C, D) Expression of *Fgf8* was observed in the palate mesenchyme in anterior the region but (E, F) not in the posterior region. (G, H) Expression of *Fgfr1b* was also detected in the anterior to middle region both at E13.5 and E14.5. (I, J) *Fgr1b* was expressed in the anterior region of the palate mesenchyme but (K, L) not in the posterior region. AT E13.5, (M, O) *Lef1* was expressed in the interior to middle region of the palate mesenchyme but (Q) not in the posterior region. (N) At E14.5, *Lef1* expression was restricted to the rugae-forming region in the secondary palate. (P, R) Expression of *Lef1* was observed in the epithelium of the palate rugae region. All sections shown were made through a frontal section. ps, palatal shelf; Scale bars in A, B, G, H, M, N = 500 μm; C-F, I-L, O-R = 200 μm.

3. Expression patterns of *Wnt11*, *Wnt7a*, *Lef1*, *Fgf8* and *Fgfr1b* in *in vitro* organ cultures of the palate

In order to confirm the conditions for the *in vitro* organ culture, author examined the gene expression pattern of *Wnt11*, *Wnt7a*, *Lef1*, *Fgf8* and *Fgfr1b* after 48 hours in culture (Figs. 5A, B, C, J and K). *Wnt11* expression was detected in the posterior region and the fusion region along the midline (Fig. 5A). Frontal sectioning showed that *Wnt11* expression was observed in the epithelium at the fusion region (Fig. 5D). In contrast, *Wnt11* was expressed in the posterior region of the mesenchyme (Fig. 5G). The *Wnt7a* expression pattern was different from that of *Wnt11*. *Wnt7a* expression was detected in the anterior to middle region of the cultured palate (Fig. 5B). In the anterior region, expression of *Wnt7a* was observed in the mesenchyme (Fig. 5E), while in posterior region, *Wnt7a* expression was not detected in either the epithelium or the mesenchyme (Fig. 5H). *Lef1* expression was detected in the rugae-forming region of the palate (Fig. 5C). After frontal sectioning, expression of *Lef1* was detected in the epithelium not only in the anterior region, but also in the posterior region (Figs. 5F and I). *Fgf8* and *Fgfr1b* were expressed in almost same region, which was anterior to the middle region (Figs. 5J and K). *Fgf8* and *Fgfr1b* were detected only in the mesenchyme after frontal sectioning

(Figs. 5L and M). In the posterior region, *Fgf8* was weakly expressed in the mesenchyme of the nasal side of the palate (Fig. 5N). Expression of *Fgfr1b* was only weakly observed in the mesenchyme region (Fig. 5O).

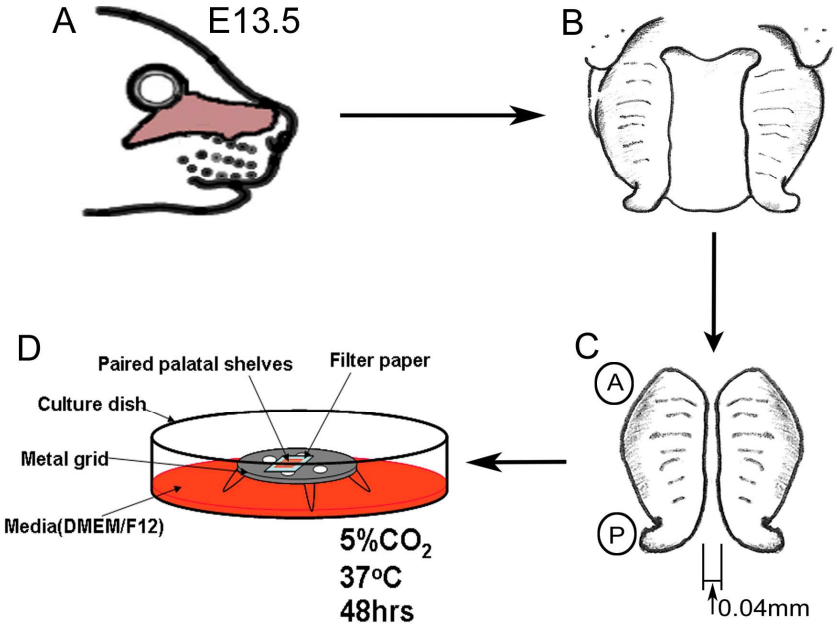
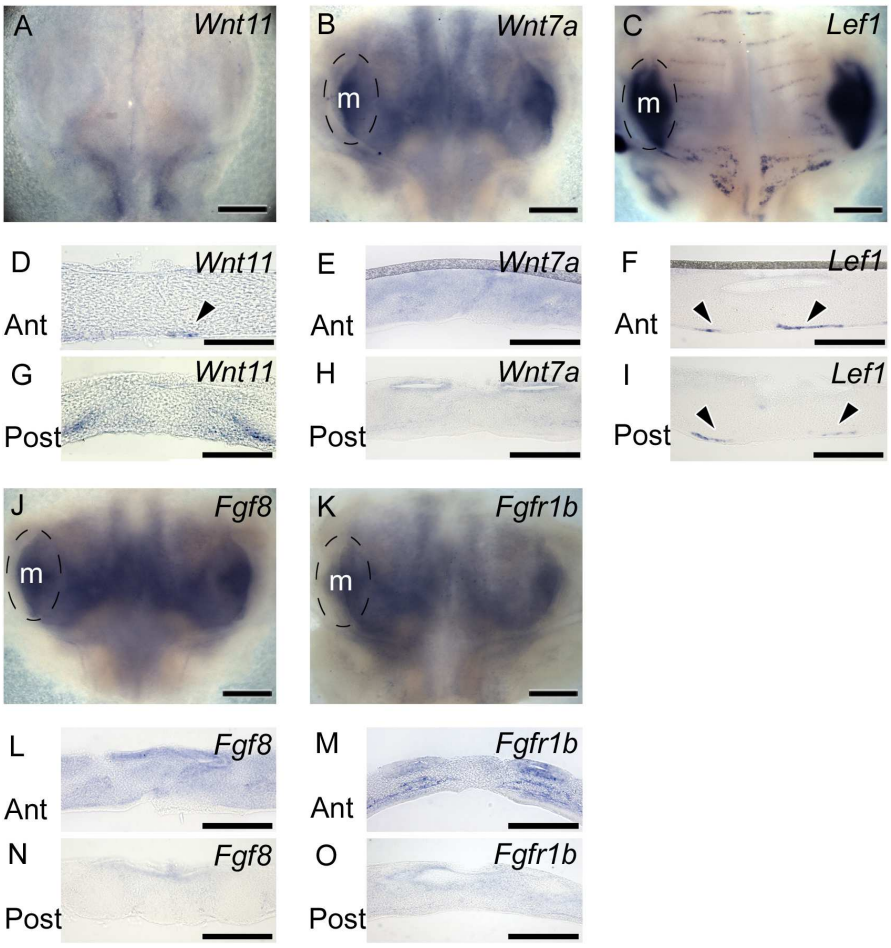


Figure 4. *in vitro* culture method for dissected mouse palate. (A, B) Palatal shelves were harvested from E13.5 mouse embryos. (C) Two palatal shelves were then placed on filter paper. Previous reports showed that the fusion of palatal shelves could be brought about even from transgenic mice with a cleft palate, by simply placing the shelves together *in vitro*.[31] 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 hours with DMEM/F12 under 5% CO_2 and 37^{O}C conditions.


A
Wnt11
B
Wnt7a
m
C
Lef1
m
D
Wnt11
Ant
E
Wnt7a
Ant
F
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Ant
G
Wnt11
Post
H
Wnt7a
Post
I
Lef1
Post
J
Fgf8
m
K
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m
L
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Ant
M
Fgfr1b
Ant
N
Fgf8
Post
O
Fgfr1b
Post

Figure 5. Expression patterns of *Wnt11*, *Wnt7a*, *Fgf8*, *Fgfr1b* and *Lef1* in *in vitro* cultured palates. (A-C, J, K) Expression patterns were examined by whole-mount *in situ* hybridization and (D-I, L-O) all sections shown were made through a frontal section. (A, D) *Wnt11* expression was detected in the epithelium of the fusion line (arrowhead) and (A, G) in the posterior region palate mesenchyme. (B, E) Expression of *Wnt7a* was observed in the anterior to middle region of the palate mesenchyme but (H) not in the posterior region. (C, F, I) *Lef1* expression was restricted in the rugae-forming palate epithelium (arrowheads). (J, L) Expression of *Fgf8* was detected in the anterior to middle region of the palate mesenchyme but (J, N) not in the posterior region. (K, M) *Fgfr1b* is strongly expressed in the anterior region palate mesenchyme and (K, O) weakly expressed in the nasal side of the posterior palate mesenchyme. Black dotted circle, first molar region; m, molar; A, B, C, J, K = 500 μm; D-I, L-O = 200 μm.

4. Expression of *Wnt7a*, *Lef1*, *Fgf8* and *Fgfr1b* after Wnt-11 over-expression

Wnt-11 was over-expressed using electroporation within the red dotted circle (Figs. 7A-H). After Wnt-11 over-expression, *Fgf8* expression was inhibited in the anterior region (Fig. 7A). But in the posterior region, over-expression of Wnt-11 did not affect the expression of *Fgf8* (Fig. 7E). Wnt-11 over-expression led to the inhibition of *Fgfr1b* expression in the anterior and posterior regions (Fig. 7B). Expression of *Fgfr1b* was also weakly interrupted by over-expression of Wnt-11 in the posterior region (Fig. 7F). Over-expression of Wnt-11 inhibited *Wnt7a* expression in the anterior region (Fig. 7C) but did not inhibit *Wnt7a* expression in the posterior region (Fig. 7G). *Lef1* expression was interrupted by Wnt-11 over-expression in the anterior region of the secondary palate. *Lef1* expression in the rugae-forming line was also disrupted after Wnt-11 over-expression in the anterior region (Fig. 7D). Likewise, *Lef1* expression was inhibited by over-expression of Wnt-11 (Fig. 7H). These results showed that over-expression of Wnt-11 inhibited *Fgf8*, *Fgfr1b*, *Wnt7a* and *Lef1* expression at one or both sides between the anterior and posterior regions of the palate.

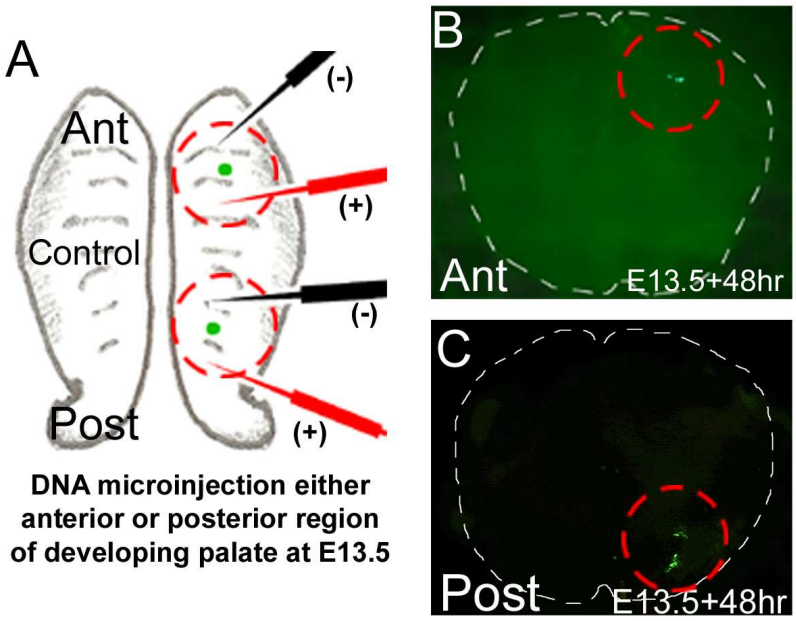


Figure 6. Electroporation method for gene over-expression in cultured palates. Electroporation was targeted to either the anterior (Ant) or posterior (Post) palate in each case. All specimens were electroporated at E13.5 and then incubated for 48 hours. (A) Schematic diagram showing DNA microinjection into the dissected mouse palate at E13.5. (B, C) After 48 hours culture, each gene was successfully transfected as indicated by GFP expression. White dotted lines indicate the palate.

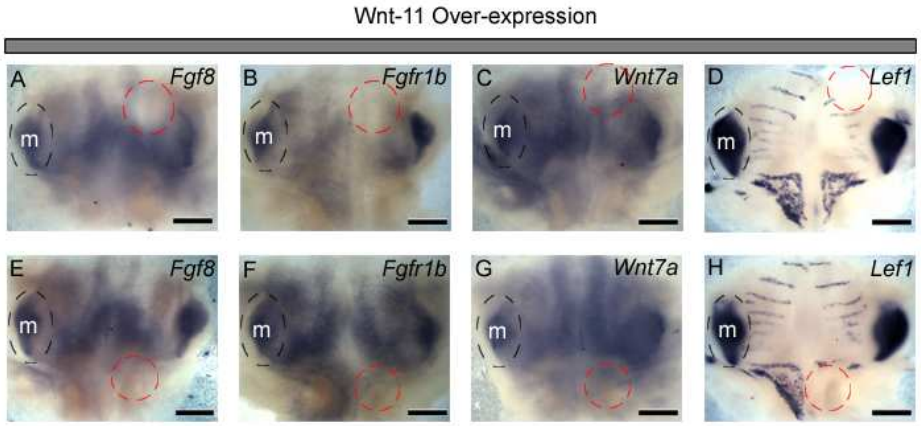


Figure 7. Over-expression of Wnt-11 and its resulting effect on gene expression. (A-H) Alterations in *Fgf8*, *Fgfr1b*, *Wnt7a* and *Lef1* expression patterns after Wnt-11 over-expression were detected by whole-mount *in situ* hybridization. (A-D) After Wnt-11 over-expression, gene expression was down-regulated in the anterior region. (E, G) *Fgf8* and *Wnt7a* were not affected by Wnt-11 over-expression in the posterior region. (F, H) Wnt-11 over-expression inhibited the expression of *Fgfr1b* and *Lef1* in the posterior region. (I) *Wnt11* inhibited the expression of *Fgf8*, *Fgfr1b*, *Wnt7a* and *Lef1*. Black dotted circle, first molar region; m, molar; red dotted circle, DNA injected region; Scale bars in A-H = 500 μm.

5. Expression of *Wnt11*, *Lef1*, *Fgf8* and *Fgfr1b* after Wnt-7a over-expression

The expression patterns of *Fgf8*, *Fgfr1b*, *Wnt11* and *Lef1* were changed after Wnt-7a was over-expressed in the *in vitro* palate cultures (Figs. 8A-H). *Fgf8* was expressed in the anterior to middle region of the palate. However, the over-expression of Wnt-7a inhibited *Fgf8* expression in the anterior region (Fig. 8A). Interestingly, following *Wnt7a* over-expression in the posterior region, *Fgf8* expression was disrupted in the anterior region of the palate (Fig. 8E). Expression of *Fgfr1b* was interrupted by Wnt-7a over-expression in both the anterior and posterior regions of the palate (Figs. 8B and F). In contrast, the anterior expression of *Wnt11* was induced by Wnt-7a over-expression (Fig. 8C). In the posterior region, the expression pattern of *Wnt11* was changed after Wnt-7a over-expression but was not completely diminished (Fig. 8G). *Lef1* expression was disrupted after Wnt-7a over-expression in both the anterior and posterior regions (Figs. 8D and H). In the anterior region, *Lef1* expression was reduced (Fig. 8D), while in the posterior region, expression of *Lef1* was inhibited after Wnt-7a over-expression (Fig. 8H). When Wnt-7a was over-expressed in the cultured palates, inhibition of *Fgf8*, *Fgfr1b* and *Lef1*, as well as the induction of *Wnt11* were detected.

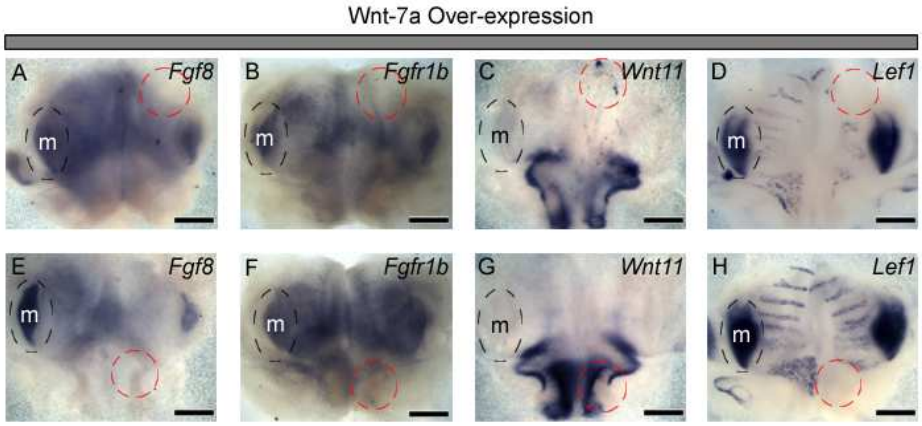


Figure 8. Over-expression of Wnt-7a and its resulting effect on gene expression. (A-H) Alterations in *Fgf8*, *Fgfr1b*, *Wnt11* and *Lef1* expression patterns after Wnt-7a over-expression were detected by whole-mount *in situ* hybridization. (A, B, D) After Wnt-7a over-expression, gene expression was down-regulated in the anterior region. (C) *Wnt11* expression was up-regulated by Wnt-7a over-expression in the anterior region. (E) Over-expressed Wnt-7a in the posterior region disrupted the anterior expression of *Fgf8*. (F, H) Wnt-7a over-expression inhibited the expression of *Fgfr1b* and *Lef1* in the posterior region. (G) The *Wnt11* expression pattern was changed but not completely diminished by Wnt-7a over-expression in the posterior region. (I) *Wnt7a* inhibits the expression of *Fgf8*, *Fgfr1b* and *Lef1* but up-regulates *Wnt11*. Black dotted circle, first molar region; m, molar; red dotted circle, DNA injected region; Scale bars in A-H = 500 μm.

6. Expression of *Wnt11*, *Wnt7a*, *Fgf8* and *Fgfr1b* after Lef-1 over-expression

Lef-1 was over expressed using the electroporation method within the red dotted circles (Figs. 9A-H). *Fgf8* expression was inhibited by the over-expression of Lef-1 in the anterior region (Fig. 9A). However, in the posterior region, over-expressed Lef-1 did not affect the expression of *Fgf8* (Fig. 9E). After Lef-1 over-expression, *Fgfr1b* expression was strongly inhibited in the anterior region of the palate (Fig. 9B), and disrupted in the posterior region (Fig. 9F). *Wnt11* expression was up-regulated by the over-expression of Lef-1 in the anterior region (Fig. 9C). In contrast, alteration of *Wnt11* expression was not observed in the posterior region after Lef-1 over-expression (Fig. 9G). *Wnt7a* expression was interrupted by Lef-1 over-expression (Fig. 9D); however, over-expressed Lef-1 did not affect to expression of *Wnt7a* in the posterior region (Fig. 9H). These results indicate that the over-expression of *Lef1* inhibited the expression of *Fgf8*, *Fgfr1b* and *Wnt7a*. In contrast, the expression of *Wnt11* was up-regulated by the over-expression of Lef-1.

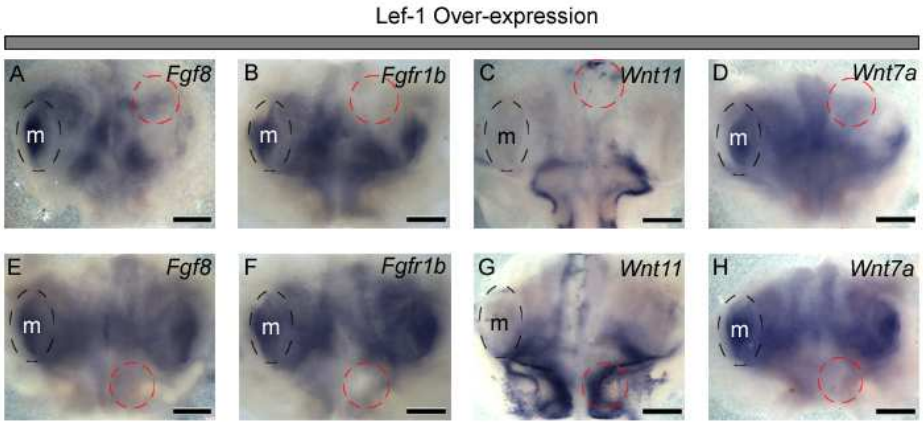


Figure 9. Over-expression of Lef-1 and its resulting effect on gene expression. (A-H) Alterations in *Fgf8*, *Fgfr1b*, *Wnt11* and *Wnt7a* expression patterns after Lef-1 over-expression were detected by whole-mount *in situ* hybridization. (A, B, D) After Lef-1 over-expression, gene expression was down-regulated in the anterior region. (C) *Wnt11* expression was up-regulated by Lef-1 over-expression in the anterior region. (E, G, H) Lef-1 over-expression did not inhibit the expression of *Fgfr8*, *Wnt11* and *Wnt7a* in the posterior region. (F) Over-expressed Wnt-7a disrupted the expression of *Fgfr1b* in the posterior region. (I) *Lef1* inhibited the expression of *Fgf8*, *Fgfr1b* and *Wnt7a* but up-regulated *Wnt11*. Black dotted circle, first molar region; m, molar; red dotted circle, DNA injected region; Scale bars in A-H = 500 μm.

7. Alteration of gene expression patterns after the implantation of SU5402-soaked beads

In order to confirm that *Fgfr1b* could induce or inhibit *Fgf8*, *Wnt11* and *Lef1* expression, beads soaked with the Fgf receptor 1 inhibitor, SU5402, were implanted during *in vitro* organ culture (Figs. 10A, B and C). The *Fgf8* expression level was clearly down-regulated after 48 hours in culture after the implantation of SU5402 soaked beads (Fig. 10A). After frontal sectioning, mesenchymal expression of *Fgf8* was not detected (Fig. 10D). In contrast, the expression of *Wnt11* was up-regulated after SU5402 soaked bead implantation (Fig. 10B). Frontal sectioning showed that inhibition of the Fgf receptor induced *Wnt11* expression in palate epithelium (Fig. 10E). Expression of *Lef1* was also induced in the vicinity of the SU5402 soaked beads (Fig. 10C). Frontal sectioning revealed that epithelial expression of *Lef1* was up-regulated by SU5402 (Fig. 10F). From these results, author determined that the expression of *Wnt11* and *Lef1* was inhibited and that *Fgf8* was induced by *Fgfr1b*.

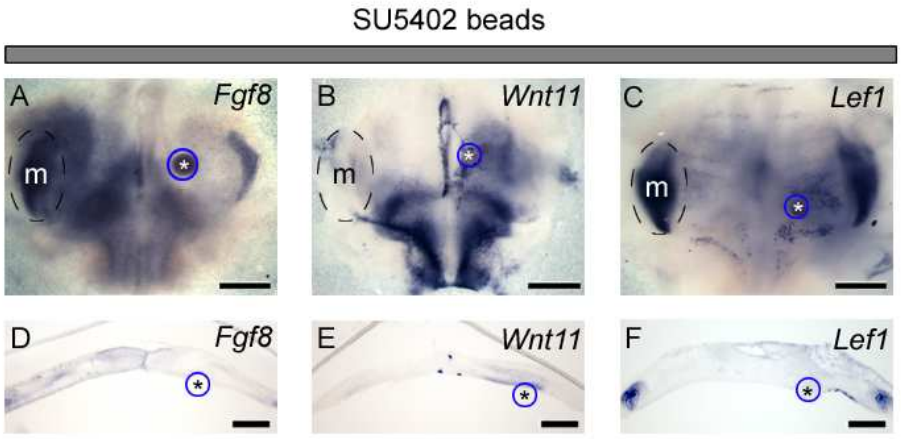
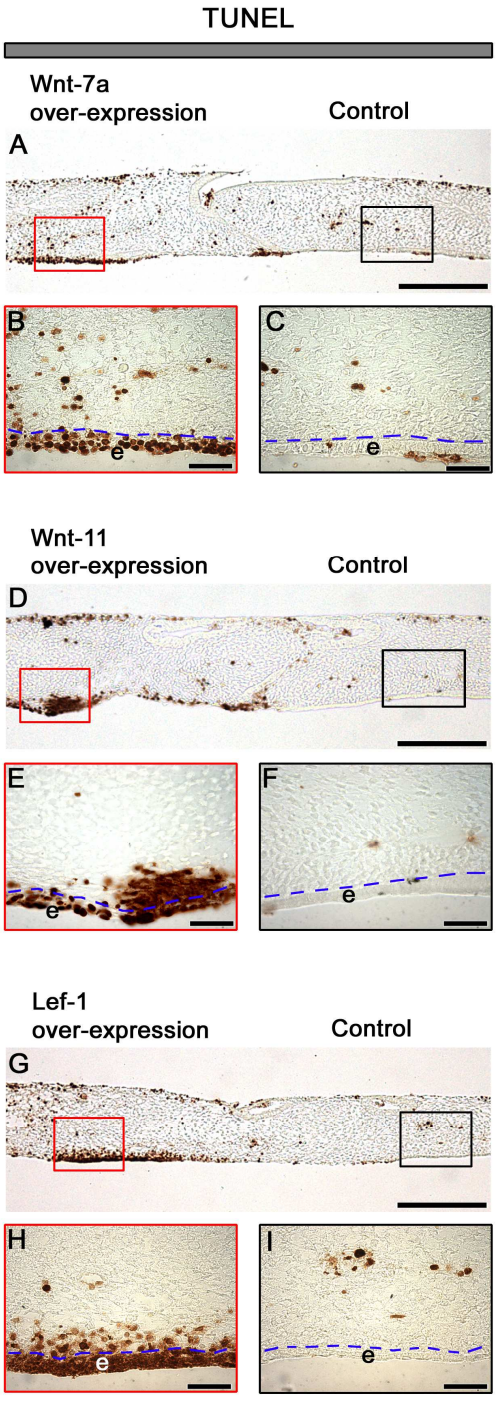


Figure10. Effect of SU5402 on the expression of *Fgf8*, *Wnt11* and *Lef1*. (A-C) 0.5 mg/ml SU5402-soaked beads (asterisk) were implanted into the palate at E13.5 and the specimens were then incubated for 48 hours. (A, D) Expression of *Fgf8* was clearly reduced by SU5402 (compared to the c ontrol side). (B, E) Expression of *Wnt11* was strongly up-regulated in th e epithelium and weakly in the mesenchyme by the implantation of SU5 402 soaked beads. (C, F) Lef1 expression was also induced by SU5402 in the palate epithelium. (G) *Fgfr1b* inhibited the expression of *Wnt11* and *Lef1* and up-regulated *Fgf8*. Black dotted circle, first molar region; m, mol ar; red dotted circle, DNA injected region; Scale bars in A-F = 500 μm.

8. Assessing cell death and cell proliferation in palates in which Wnt-7a, Wnt-11 and Lef-1 were over-expressed

Cell death was examined in control specimens and in specimens over-expressing Wnt-7a, Wnt-11 and Lef-1 (Figs. 11A-I). Significantly strong positive reactions for cell death were detected on the side of the palate in which Wnt-7a, Wnt-11 and Lef-1 were over-expressed compared to the control side of the E13.5 palate after being cultured for 48 hours. In addition, many TUNEL-positive cells were observed in the epithelium of the middle line region that reported the fusion line of the palate (Figs. 11A, D and G). On the Wnt-7a over-expressing side, a multitude of TUNEL-positive cells were detected especially within the epithelium (Fig. 11B). On the control side, a small number of TUNEL-positive cells were detected in both the epithelium and the mesenchyme (Fig. 11C). Many more TUNEL-positive cells were observed on the Wnt-11 over-expressing side compared with the control side (Fig. 11D). On the Wnt-11 over-expressing side, strong positive reactions for cell death were detected in the epithelium and underlying mesenchyme (Fig. 11E). In contrast, few TUNEL-positive cells were observed on the control side (Fig. 11F). Many more TUNEL-positive cells were also observed in the region of Lef-1 over-expression (Fig. 11H) than were observed on control side (Fig.

11G). After Wnt-7a, Wnt-11 and Lef-1 were over-expressed, the number of PCNA-positive cells was not altered in the experimental side compared with the control side (Figs. 12A-C).


TUNEL
Wnt-7a
over-expression
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A
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Lef-1
over-expression
Control
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Figure 11. TUNEL assay on frontal sections after over-expression studies in cultured palates. (A-I) Palates with Wnt-7a, Wnt-11 and Lef-1 over-expression exhibit an increased level of cell death. (B, C) Over-expressed Wnt-7a induced cell apoptosis in cultured palate epithelium compared with control side. (E, F) After Wnt-11 over-expression, the number of TUNEL positive cells was remarkably increased in the epithelium and in the mesenchyme beneath the epithelium than in the control side. (H, I) Over-expressed Lef-1 also induced cell death in developing palate. e, epithelium; m, molar. Scale bars in A, D, G = 200 μm; B, C, E, F, H, I = 20 μm.

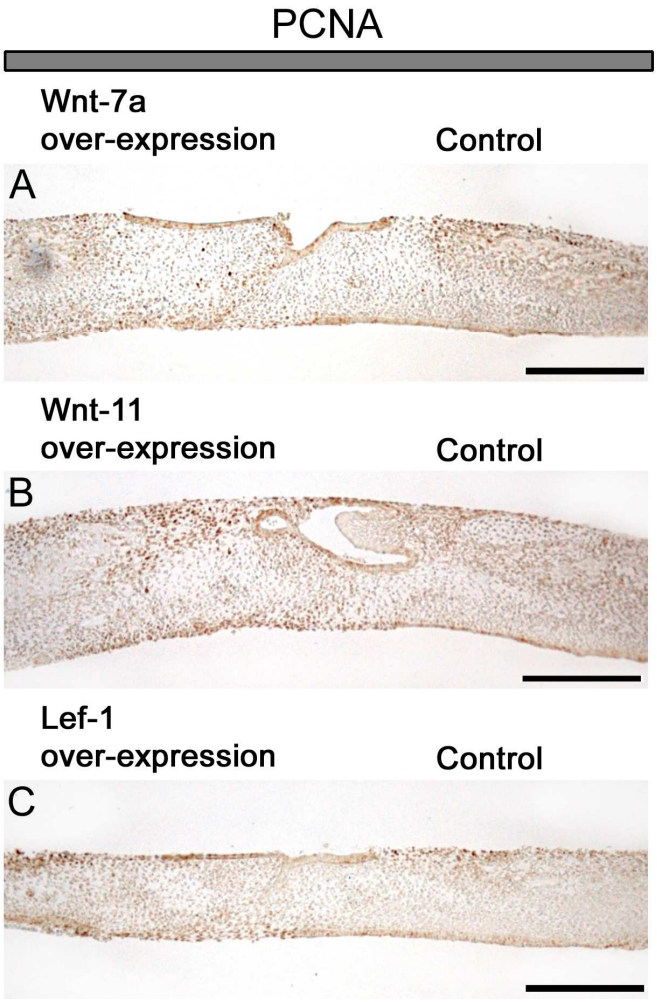

Figure 12. PCNA staining on frontal sections after over-expression studies in cultured palates. (A-C) Different numbers of PCNA-positive cells were not detected between the over-expressed side and the control side. Scale bars in A, B, C = 200 μm.

IV. DISCUSSION

1. *Wnt7a, Fgf8* and *Fgfr1b* genetically interact during mouse palatogenesis

In the limb bud, specific expression of *Fgf8* in the apical ectodermal ridge (AER) is required for limb patterning.[41,42] When *Fgf4* and *Fgf8* expression are deleted in the AER, development of the limb is completely disrupted, indicating a genetic redundancy in the functions of *Fgf4* and *Fgf8*.[43,44] *Fgf10* signalling from the mesenchyme has also been reported to regulate the epithelial expression of *Shh*, which signals back to the underlying mesenchyme. This signalling network also leads to changes in palatal size and shape.[45] At E13.5 in the palate, *Fgf8, Fgfr1b* and *Lef1* expression patterns are similar to that of *Wnt7a* (Figs. 3A, G and M). At E14.5, the expression pattern of *Fgf8, Fgfr1b* and *Wnt7a* are co-localized from the anterior region to the middle region of the secondary palate (Figs. 3B and H). From these results, author hypothesized that the function of *Wnt7a* is closely related to that of *Fgf8* and *Fgfr1b* during palate development. In addition, author also hypothesized that *Fgf8* and *Fgfr1b* induces cell proliferation and hard palate formation. The relationship between Fgf and Wnt signalling has previously been reported in the developing digit.[26] Moreover, *Lef1* may be involved in the regulation of palate development along with the expression of other genes at E13.5. However at E14.5, after contact between the palatal shelves, *Lef1* appears to play a role in rugae formation by a mechanism distinct from E14.5 such as epithelial thickening (Figs. 3M and N).

2. Putative regulation of Fgfs during palatogenesis

Epithelial-mesenchymal interactions regulate early secondary palate development. *in vitro* tissue dissociation and recombination studies have shown that the mesenchyme specifies the different specialized palate epithelia, which consist of the oral, nasal, and medial edge epithelia.[46] In the palate, *Wnt11* expression was up-regulated by the over-expression of Wnt-7a and Lef-1 (Fig. 7) and by the implantation of SU5402 soaked beads (Fig. 10). Thus, the epithelial *Wnt11* expression seen in the fusion line of the palate could be regulated by *Wnt7a* and *Lef1* expression in the palate. Moreover, *Fgfr1b* could negatively regulate epithelial *Wnt11* in the anterior to middle region of the palate except at the fusion line. Wnt-11, Wnt-7a and Lef-1 over-expression clearly down-regulated expression levels of *Fgfr1b* and *Fgf8* in the developing palate. These results suggest that Fgf and Wnt negatively regulate each other during normal palatogenesis. In addition, *Fgf8* was not expressed in regions implanted with SU5402 soaked beads (Fig. 10A). This result suggests that *Fgf8* and *Fgfr1b* interact with one another genetically. In contrast, inhibition of *Fgfr1b* stimulated the epithelial expression of *Lef1* (Fig. 10C). This result shows that mesenchymal *Fgfr1b* negatively regulates epithelial *Lef1* during early palatogenesis. Overall, several factors play pivotal roles in mediating tissue interactions, leading to the activation or inhibition of gene expression in the adjacent tissues.[47]

3. *Wnt11* is possibly involved in palatal fusion

Wnt11 has essential roles in vertebrate development, including the orientation of cell division planes,[48] convergent extension movements in zebrafish and *Xenopus*, heart formation in *Xenopus* and chick,[49,50,51,52] and branching morphogenesis in the kidney of the mouse.[36,53,54] In this study, author described the *Wnt11* expression pattern in palatal shelves in mouse embryos and showed that it is expressed in the fusion line region of the palate epithelium at E14.5. One possibility for the role of *Wnt11* is that its expression would be important in positively regulating the process of palatal fusion. The fact that *Wnt11* is expressed on the fusion line is also consistent with the idea that its expression is closely related with palatal fusion. Many mechanisms are involved in palatal fusion, including the epithelial-mesenchymal transformation (EMT).[55] *Wnt11* was found to be expressed in the oral side of the epithelium but not in the mesenchyme (Figs. 2D and F). This result showed that *Wnt11* is plays an important role in fusion during palatogenesis.

4. Wnt family induces cell death during palatogenesis

Previous studies have shown that palate mesenchyme maintains its potential for cell proliferation and apoptosis in the epithelium through the action of the epidermal growth factor on its receptor, which is located in the epithelium.[56,57] Members of the Wnt family are reported to not only positively but also negatively regulate cell death. WNT7b was reported to mediate programmed cell death by inducing macrophages in the vasculature patterning.[58] To investigate the role of *Wnt7a*, *Wnt11* and *Lef1* during palatogenesis, author examined cell death and proliferation in *in vitro* organ cultures of the mouse palate after *Wnt7a*, *Wnt11* and *Lef1* over-expression. Apoptosis positive cells were observed in the developing palate after Wnt-7a, Wnt-11 and Lef-1 over-expression (Figs. 12). TUNEL staining supported the conclusion that expression of *Wnt7a*, *Wnt11* and *Lef1* are closely related to cell death in secondary palate development. Thus, cell death could be involved in the regulation of the epithelial mesenchymal transformation during palatal fusion.

V. CONCLUSION

Palatogenesis is a complex event and is often disrupted, leading to the generation of the congenital defect known as cleft palate. Consequently, the events and mechanisms responsible for the development of the palate have been extensively studied, although some controversy remains.[59]

In this study, the functions of the Fgfs and Wnt family members, which play important roles in organogenesis, were examined by electroporation and bead implantation methods via organ culture to define the underlying mechanisms involved in secondary palate development. Mesenchymally-expressed *Wnt7a*, *Wnt11* and *Lef1* maintain down-regulation of *Fgf8* and *Fgfr1b* expression in the mesenchyme during secondary palate development. In addition, inhibition of Fgfr1 by SU5402 showed that *Wnt11* and *Lef1* expression were induced in the palate epithelium. These results imply that Fgfs and Wnts negatively regulate each other directly or indirectly. Overall, proliferation, induced by *Fgf8* and apoptosis, mediated by *Wnt11* are pivotal mechanism for achieving the precise morphologenesis of the secondary palate (Fig. 13).

1. Cell proliferation is regulated, in part, by *Fgf8* and *Fgfr1b* during palatogenesis.

2. Wnt family members potentially regulate palatal fusion by mediating cell death.

3. The pattern of *Lef1* expression, which is localized in the rugae forming region, suggests that *Lef1* could potentially be involved in rugae development.

4. Interactions between several key genes such as the Fgfs and Wnt family members are needed for proper formation of the palate during embryonic development.

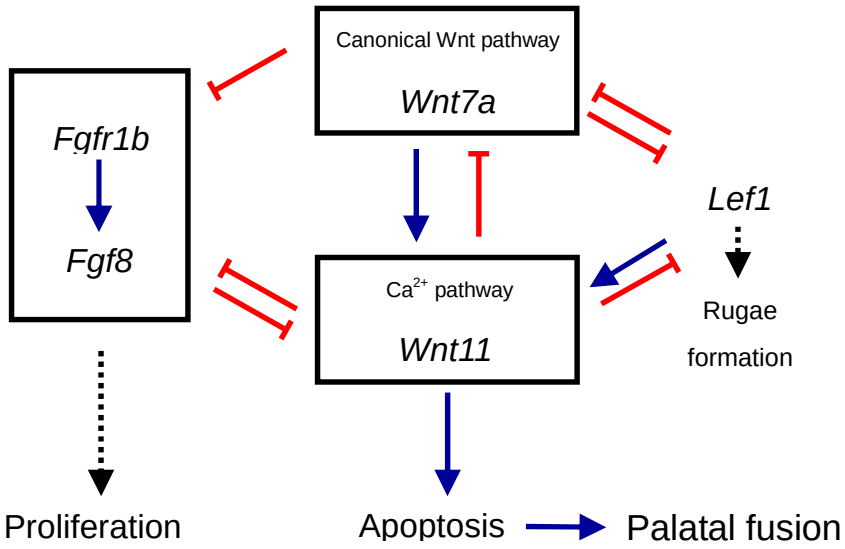

Figure 13. A model for Wnt and Fgf function in palatogenesis.

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ABSTRACT(IN KOREAN)

흰쥐 입천장 형성 동안의 *Wnt11* 과 *Fgf8* 의 역할 및 기능 구명

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이 종 민

입천장은 발음을 하거나 음식물을 삼키거나 빨거나 하는 기능을 수행하는 구강 구조물로서 발생단계 동안의 이차구개판의 상승, 융합 등의 정확한 형태학적 변화가 있어야 그 기능을 제대로 수행할 수 있다. 이차구개판의 형태형성 및 발생단계는 다양한 유전자 간의 상호작용과 외배엽-중배엽의 상호작용 등으로 설명이 가능한데, 이러한 발생과정에 문제가 생기게 되면 인간에 있어 가장 높은 빈도로 발생하는 유전적 질병의 하나인 입천장 갈림증이 유발되기도 한다. 본 연구에서는 정상적인 입천장 발생의 분자, 세포학적 변화 양상과 입천장 갈림증의 원인을 규명하기 위하여 다른 기관 형성에서 중요한 역할을 하는 것으로 알려진 신호전달물질과 전사인자인 *Fgf8*, *Fgfr1b*, *Wnt7a*, *Wnt11*, *Lef1* 등의 발현 양상을 초기 이차구개 발생 동안 확인하였다. 또한 이들의 상호작용 및

전달체계를 확인하기 위하여 전기유전자도입법 (Electroporation)을 이용하여 체외조직배양중인 입천장의 편측 이차구개판에 과 발현 시킨 후 그 영향을 대조군과 비교 확인 하였다. *Fgfr1* 의 억제제인 SU5402 를 체외배양하는 이차구개판에 처리하여 변화 양상을 확인 하였다. 또한 이차구개판 발생동안의 *Wnt7a*, *Wnt11* 그리고 *Lef1* 의 영향을 알아보기 위하여 과 발현 후, 세포의 증식과 사멸을 확인하였다. 그 결과 입천장 발생과정 동안에 Fgfs 는 세포의 증식과 관련되어있고 Wnt family 는 세포의 사멸과 관련되어있을 가능성을 확인하였으며 발생 동안 관찰되는 상승과 융합을 포함한 형태학적 변화는 Fgfs 와 Wnt family 의 상호작용과 깊은 연관성이 있음을 확인할 수 있었다.

핵심되는 말: 이차구개발생, 형태형성, *Fgf8*, *Fgfr1b*, *Wnt7a*, *Wnt11*, *Lef1*