Journal of Hard Tissue Biology 25[2] (2016) 195-204
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CODEN-JHTBFF, ISSN 1341-7 649

Original

Intracellular Signaling Pathway Activation via TGF-β Differs in the Anterior and Posterior Axis During Palatal Development

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(Accepted for publication, February 4, 2016)

Abstract: It is important to understand the different mechanisms involved in anterior hard and posterior soft palate development to prevent and treat patients with cleft palate. Genetic analyses of humans and gene-mutated mice with cleft palate have shown that TGF-β signaling has a critical role in palatogenesis. However, the intracellular signaling pathway of TGF-β during palatogenesis in the anterior-posterior axis has not yet been fully understood. In the present study, the expression patterns of intracellular molecules Smad2/3 and phospho-p38 (Pp38) were examined at embryonic days 13.5, 14.0, and 14.5 (E13.5, E14.0, and E14.5) in mice. It was found that Smad3 was activated in anterior palatal mesenchyme and in the medial edge epithelium (MEE) region, with TGF-β3 expressed at E13.5. On the other hand, Pp38 was more expressed in posterior palatal mesenchyme and strongly expressed in the entire palatal epithelium at E13.5. These opposing expression patterns between Smad3 and Pp38 in palatal mesenchyme were also observed at E14.0. Interestingly, Pp38 expression was inhibited in MEE from E14.0. Generally, from E14.5, the tissue specificities of hard and soft palate started showing their characteristics following the activation of cell differentiation in palatal mesenchyme, and the medial edge seam (MES) of the palatal epithelium started to disappear for fusion to occur. At this stage, Smad3 was also more expressed in anterior palatal mesenchyme, while expression of Pp38 was activated in posterior palatal mesenchyme. Pp38 expression was inhibited, but Smad3 was activated in the MES. These results suggest that TGF-β signaling plays various roles, such as in cell proliferation and differentiation of palatal mesenchyme and in the disappearance of the MES, through different intracellular signaling pathways in anterior-posterior palatal mesenchyme and epithelium.

Key words: TGF-β, Smad, p38, Palate, Development

Introduction

Cleft lip and/or palate is one of the most common congenital craniofacial defects in humans. These defects have been thought to result from genetic and/or environmental influences[1,2]. Cleft palate patients have various functional difficulties with occlusion, swallowing, and speech. Therefore, prevention of cleft palate is a very important issue.

Palatal development is a multistep process that involves palatal shelf growth, elevation, midline fusion of palatal shelves, and disappearance of the medial edge seam (MES)[3]. These morphological changes of palatogenesis commonly occur along the entire anterior-posterior axis, but different tissues are ultimately formed, such as the anterior bony hard palate and the posterior muscular soft palate. In human patients, there are variations, such as complete or partial cleft palate affecting only the posterior region. There is also submucous cleft palate with a defect of palatal muscle development. To understand the molecular mechanism that can explain the variations of cleft palate, it is important to investigate the characteristic signaling pathways in the anterior-posterior axis.

Transforming growth factor β (TGF-β) signaling plays a crucial role during palatogenesis[4]. For example, mice with loss of function of *Tgfb2* or *Tgfb3* display cleft palate, but their cleft palates are caused by different pathogenetic mechanisms in palatal mesenchyme and epithelium. TGF-β2 is expressed in the palatal mesenchyme, and the cleft palate in *Tgfb2* mutants was due to a growth defect of the palatal shelves[5]. However, TGF-β3 expression in medial edge epithelium (MEE) has been reported to

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induce programmed cell death or the epithelial-mesenchymal transition (EMT) leading to disappearance of MEE[6,7]. *Tgfb3*-knockout mice showed cleft palate because of failure to fuse by an adhesion defect of the MEE and disappearance of the MES[8,9]. The expression of TGF-β2 in palatal mesenchyme and TGF-β3 in palatal epithelium does not show a characteristic tendency in the anterior-posterior axis.

The role of TGF-β receptors has also been investigated during palatogenesis using conditional deletion of each receptor in palatal mesenchyme and epithelium, because *Tgfbr1* and *Tgfbr2* conventional knockout mice die at an early embryonic stage[10,11]. In mice with neural crest cell-specific deletion of TGF-β type I and II receptor genes, there is loss of function of receptors in palatal mesenchyme, *Wnt1-cre*;*Tgfbr1$^{fl/fl}$* and *Wnt1-cre*;*Tgfbr2$^{fl/fl}$*, and they show completely cleft palate[12-14]. Epithelial-specific deletion of TGF-β type I and II receptor genes, *K14-cre*;*Tgfbr1$^{fl/fl}$* and *K14-cre*;*Tgfbr2$^{fl/fl}$*, causes partial clefts in the posterior part of the palate (soft palate)[14,15].

In the canonical TGF-β signaling pathway, TGF-β stimulates the transduction of intracellular signals through the TβRI/TβRII receptor complex via phosphorylation and nuclear translocation of receptor-activated Smad2 or Smad3, and they form a complex with Smad4. It has been shown that the Smad2-dependent pathway has an important role in the palatal epithelium. For example, overexpression of *Smad2* in *Tgfb3* null mice rescued cleft palate[16]. Additionally, TGFβ3 inhibits E-cadherin expression through Smad2, Smad4, and the LEF1 complex leading to disappearance of the MES[17].

Recently, the importance of the non-canonical TGF-β signaling pathway during palatogenesis has been the focus of interest[18,19]. Interestingly, abnormal phosphorylation of p38 (Pp38) has been observed in palatal mesenchyme and epithelium in *Wnt1-cre*;*Tgfbr2* and *K14-cre*;*Smad4$^{fl/fl}$*, respectively[20,21]. This suggests that TGF-β can mediate downstream target genes in both palatal mesenchyme and epithelium through canonical and non-canonical signaling pathways. However, the function of non-canonical Smad-independent pathways in normal palatogenesis is still largely unknown.

In the present study, we hypothesized that intra-cellular TGF-β signaling differed along the anterior-posterior axis during palatogenesis, since the expression of ligands and receptors of TGF-β signaling did not show a specific pattern in the anterior-posterior axis. To examine the balance and dependency of canonical Smad-dependent or non-canonical Smad-independent pathways of TGF-β signaling, spatiotemporal expressions of Smad2, Smad3, and Pp38 were examined during palatogenesis in epithelial and mesenchymal tissues and compared along the anterior-posterior axis.

Materials and Methods

All procedures for animal care were reviewed and approved by the Animal Experiment Committee of Fukuoka Dental College, Fukuoka, Japan (No. 13032). ICR mice were prepared from embryonic stage 13.5 (E13.5) to 14.5 (E14.5). Tissue was fixed with 10% neutral buffered formalin or 4 % paraformaldehyde in PBS overnight at 4°C, embedded in paraffin, sectioned, and mounted using standard procedures.

Histological examination

Sections at each embryonic stage were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Azan staining for histological analysis.

Immunohistochemical analysis

All sections for immunohistochemistry were prepared adjacently to compare protein expressions. Antigen retrieval was performed by immersing the slides in citric acid buffer (pH 6.0) and boiled by microwave for 20 minutes or trypsin for 15 minutes at room temperature. Sections were treated with 4 % normal goat serum to avoid nonspecific reactions for 30 minutes. The primary antibodies used were rat anti-Ki67 (Dako Cytomation, Carpinteria, CA, USA), rabbit anti-Smad2 and anti-Smad3 (Abcam, Cambridge, UK), rabbit anti-phospho-p38 (Cell Signaling Technology, Danvers, MA, USA), rat anti-tenascin C (R&D Systems, Minneapolis, MN, USA), and rabbit anti-TGFβ3 (Santa Cruz Biotechnology, Burlingame, CA, USA). The immunoreaction was visualized with anti-IgG antibody conjugated with Alexa Fluor® 488 and 594 (Molecular probes, Eugene, OR, USA), followed by counterstaining with DAPI (Vector Laboratory, Burlingame, CA, USA). Stained sections were observed by immunofluorescence microscopy (KEYENCE, Osaka, Japan).

Statistical analysis

Statistical analyses of the scores of nuclei with positive signals for each protein in all cells (DAPI) were performed using the Mann-Whitney test in each region and developmental stage. Values are presented as means ± SD. A P-value of <0.01 was considered significant.

Results

Histological differences between anterior and posterior palates

The oral view of the palate in mice at E16.5 is shown; the secondary palate is completely fused from the anterior to the posterior palatal shelf (Fig. 1 A). To show the histological differences between the hard and soft palates, we selected the sections of the anterior hard palate and posterior soft palate. In the anterior hard palate, the maxillary bone and tooth germs were observed, while the palatine aponeurosis was clearly evident as a layer of aggregated cells in the posterior soft palate at E16.5 (Fig. 1 B, C). In the present study, anterior palate sections were also

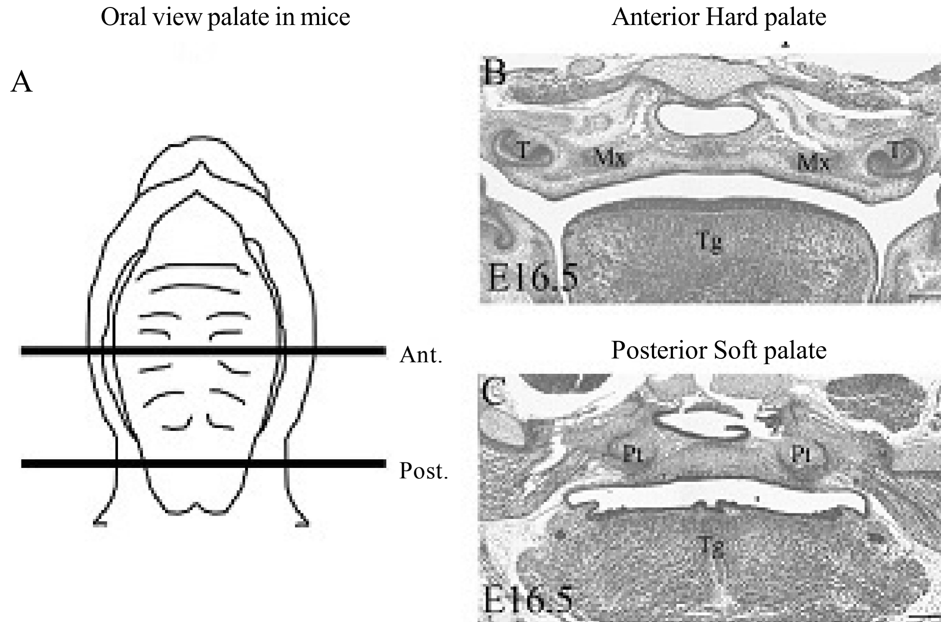

Figure 1.Schematic drawing of the palate in mice (A). The upper line indicates the anterior region of the palate (B), and the lower line indicates the posterior region of the palate (C). H&E staining of the anterior and posterior palates at E16.5 (B, C). Mx: maxillary bone, T: tooth germ, Tg: tongue, Pt: pterygoid process. Scale bar: 100 μm.

chosen to include maxillary bone and tooth germs at E13.5 and E14.5. Posterior palate sections were chosen by including the lateral pterygoid process without any bone and tooth germ at E13.5 and E14.5.

Histological views with H&E staining of the anterior and posterior palates are shown at E13.5 and E14.5; from E13.5 to E14.5, the shape of the palatal shelves changed dramatically, from the vertical position beside the tongue to horizontally above the tongue (Fig. 2 A, B, C, D). To compare the different component of palatal mesenchyme between the anterior and posterior palates, PAS and Azan staining were performed to observe glycogen deposition and fiber structure components, respectively. PAS-positive staining was observed in the foundation of the palatal shelves in the anterior palate, and it was slightly weak in the posterior palate. The tip of the palatal shelves of the anterior palate was slightly stained with PAS at E13.5 and E14.5 (Fig. 2 E, F, G, H). The clear fiber structure with Azan staining was not observed in palatal mesenchyme in both the anterior and posterior palates at E13.4 and E14.5 (Fig. 2 I, J, K, L). These results suggest that the histological differences between anterior and posterior were not detected in palatal mesenchyme at E13.5 and E14.5. Tenascin C expressed in mesenchyme during palatogenesis. At E13.5, there was a clear difference in the intensity and range of tenascin C expression between the anterior and posterior palates. Tenascin C was not expressed in the mesenchyme in the anterior palate, but it was expressed in posterior palatal mesenchyme at E13.5 (Fig. 2 M, N). At E14.5, tenascin C was weakly expressed in the anterior palatal mesenchyme and part of the basal membrane (Fig. 2 O). Almost all of the mesenchymal tissue expressed tenascin C in the posterior palate, and the expression range and intensity were clearly greater in the posterior palate at E14.5 than in the anterior palate (Fig. 2 O, P). Immunohistochemical analysis of Ki67 expression was performed to compare cell proliferation in the anterior-posterior axis. Cell proliferation activity in palatal mesenchyme was significantly higher in the posterior palate than in the anterior palate, both at E13.5 and E14.5 (Fig. 2 Q, R, S, T, and Figure 3). Taken together, the developmental processes, such as cell proliferation and ECM deposition, were already different between the anterior and posterior palates from E13.5.

Smad2/3 and Pp38 MAPK kinase activities at E13.5

During palatogenesis, TGF-β signaling is involved in the appropriate growth of the palatal shelves and disappearance of the MES. Immunohistochemical analysis of Smad2, Smad3, and Pp38 expressions was performed to see the canonical and non-canonical TGF-β signaling pathways in the anterior-posterior axis. The canonical TGF-β signaling pathway, which phosphorylates Smad2 and Smad3, was activated in palatal mesenchyme at E13.5. Cells with Smad2 expression in nuclei were observed ubiquitously in palatal epithelium and mesenchyme (Fig. 4 A, E). Interestingly, Smad3 was mainly expressed in the mesial side of palatal mesenchyme, and it was in close proximity to the MEE region that expressed TGF-β3 (Fig. 4 B, D, F, H, arrowhead). On the other hand, in non-canonical TGF-β signaling, Pp38 was rarely observed in the anterior palatal mesenchyme, but it was expressed in the posterior palate, with strong expression throughout the

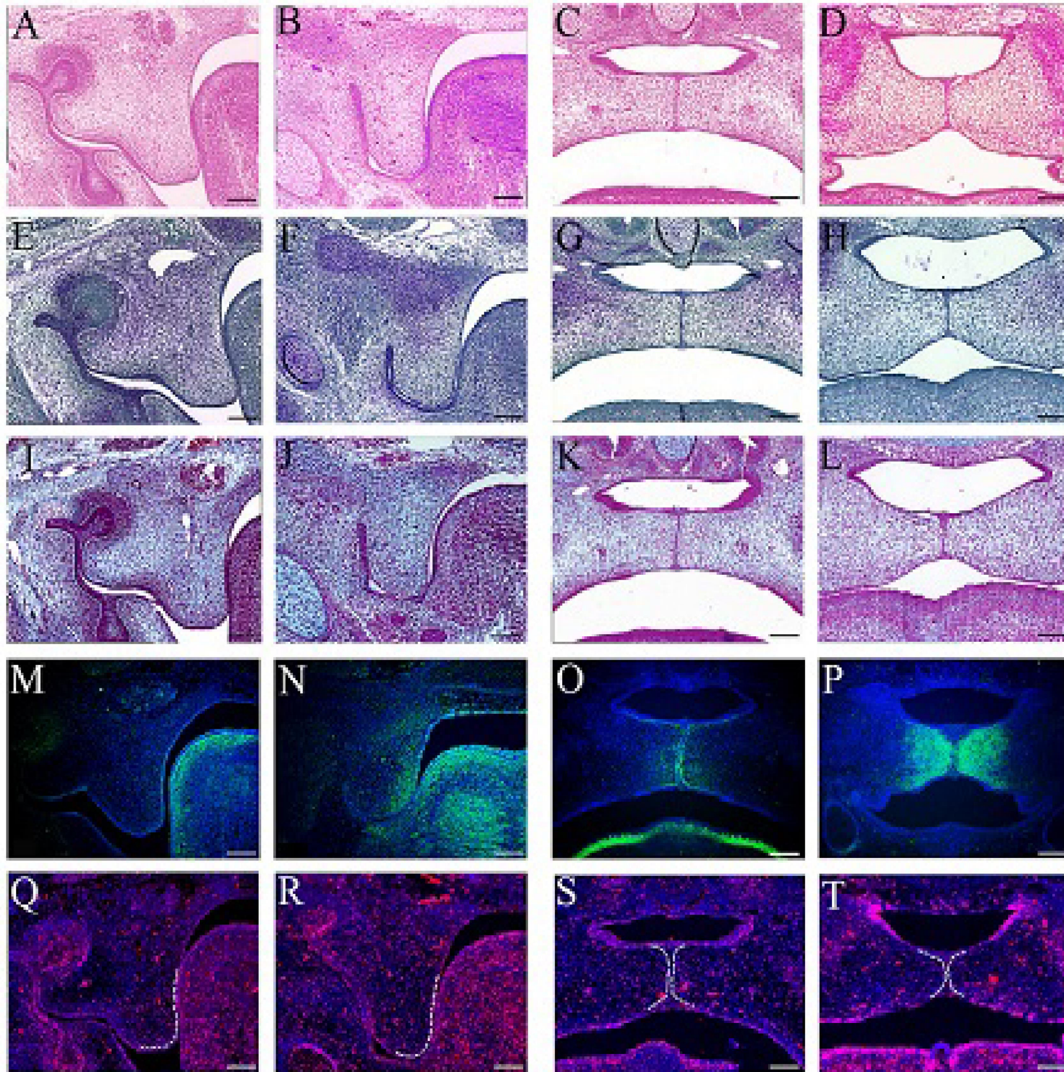

Figure 2. The histological and immunohistochemical appearance of the anterior and posterior palates. H&E, PAS, and Azan staining at E13.5 (A, B, E, F, I, J) and E14.5 (C, D, G, H, K, L). Immunohistochemical expressions of tenascin C and Ki67 in the anterior and posterior palates at E13.5 (M, N, Q, R) and E14.5 (O, P, S, T). Cell nuclei are stained with DAPI. White dotted line indicated MEE and MES. Scale bar: 100 µm.

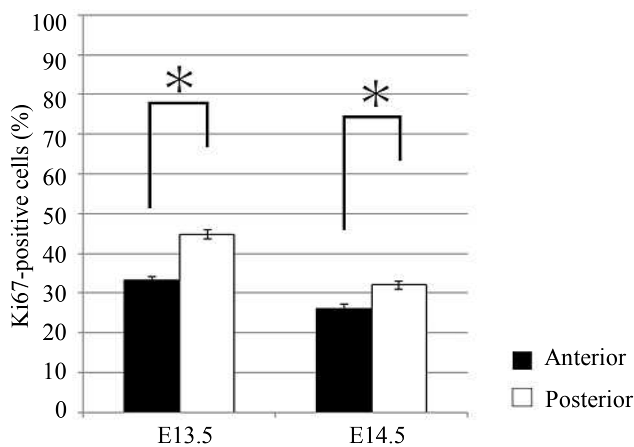

Figure 3.Quantification of the number of Ki67-labeled nuclei in the anterior (black bars, n=5) and posterior palates (white bars, n=5 at E13.5, n=7 at E14.5) at E13.5 and E14.5. Error bars represent the S.D. *P<0.01

entire palatal epithelium; its expression was not specific to MEE (Fig. 4 C, G). To compare each signal expression pattern in the anterior-posterior axis statistically, positive signal expression in nuclei of palatal mesenchyme was counted (Figure 5). From these immunohistochemical observations, at E13.5, the Smad3-dependent pathway was more activated in anterior than posterior palatal mesenchyme. On the other hand, Pp38 was more activated in posterior than in anterior palatal mesenchyme. In the palatal epithelium at E13.5, Pp38 expression was strongly observed in the entire palatal epithelium, but Smad3 was specifically expressed in MEE (Fig. 4 B, C, F, G, arrowhead).

The opposing expression patterns of Smad3 and Pp38 at E14.0

The immunohistochemical analysis at E13.5 showed that Smad3 and Pp38 had different expression patterns between the anterior and posterior palate regions. To compare the expression

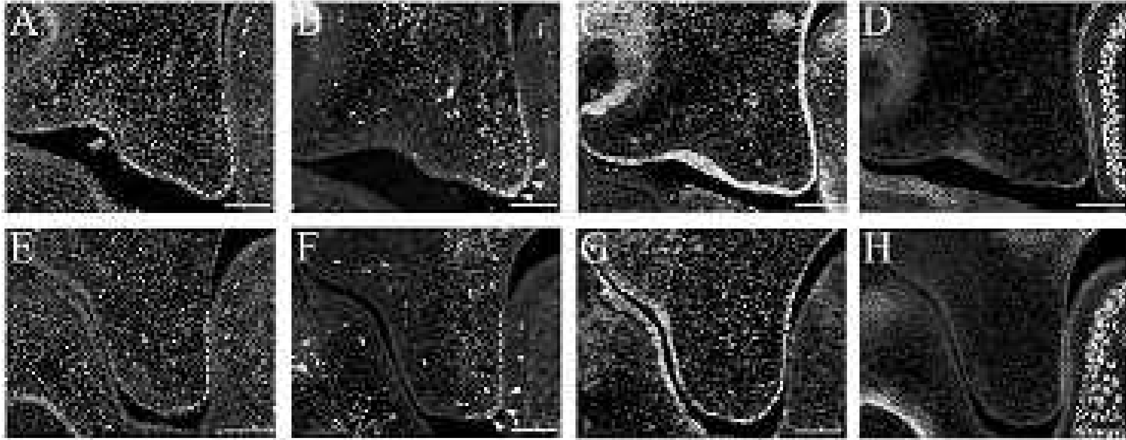

Figure 4. Smad2/3, Pp38, and TGFβ3 expressions at E13.5
Immunohistochemical expressions of Smad2/3, Pp38, and TGFβ3 in the anterior palate (A-D) and in the posterior palate (E-H) at E13.5. Cell nuclei are stained with DAPI. The dotted line indicates the MEE with TGFβ3 expression. Scale bars: 100 μm.

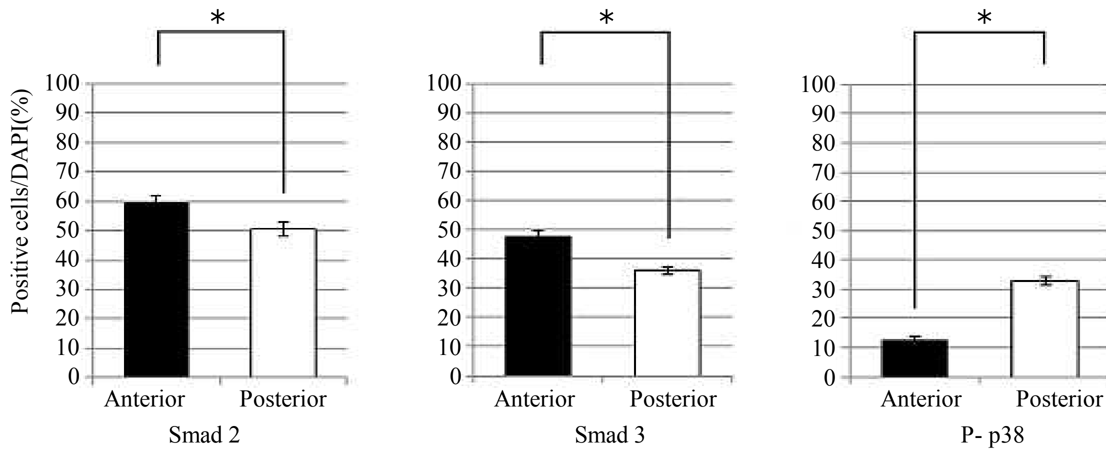

Figure 5. Quantification of the number of positive cells in nuclei out of the total nuclei in the anterior (black bars, n=5) and posterior palates (white bars, n=5) in palatal mesenchyme. Error bars represent the S.D. *P<0.01.

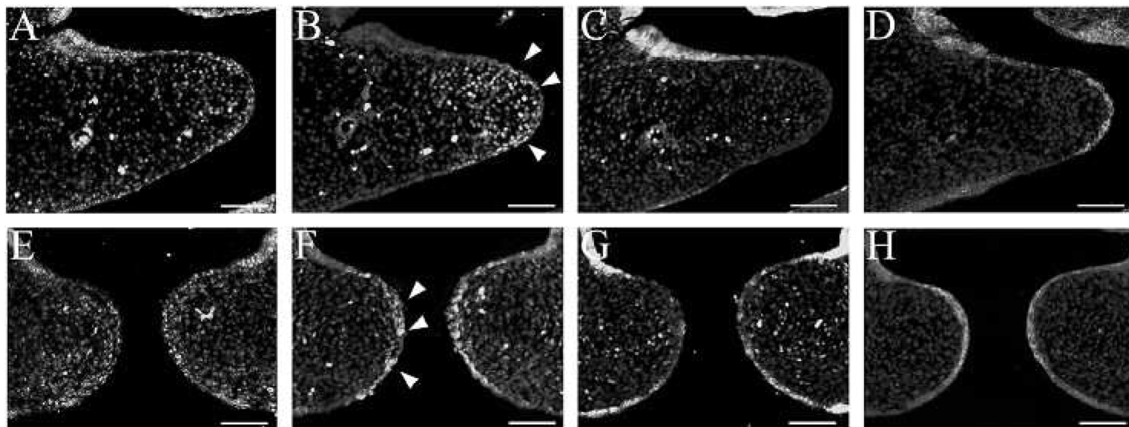

Figure 6. Smad2/3, Pp38, and TGFβ3 expressions at E14.0
Immunohistochemical expressions of Smad2/3, Pp38, and TGFβ3 in the anterior palate (A-D) and posterior palate (E-H). Cell nuclei are stained with DAPI. Scale bars: 100 μm.

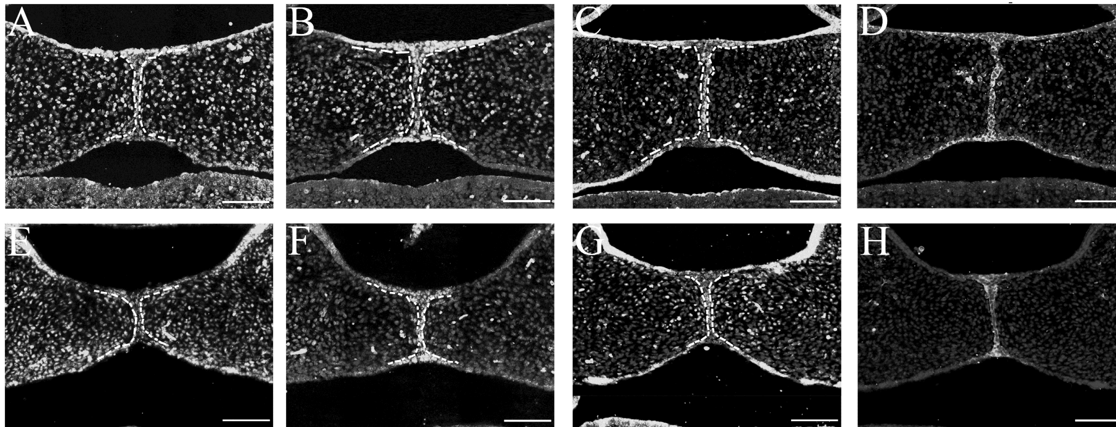

Figure 7. Smad2/3, Pp38, and TGFβ3 expressions at E14.5
Immunohistochemical expressions of Smad2/3, Pp38, and TGFβ3 in the anterior palate (A-D) and posterior palate (E-H) at E14.5. Cell nuclei are stained with DAPI. The dotted line indicates the MES with TGFβ3 expression. Scale bars: 100 μm.

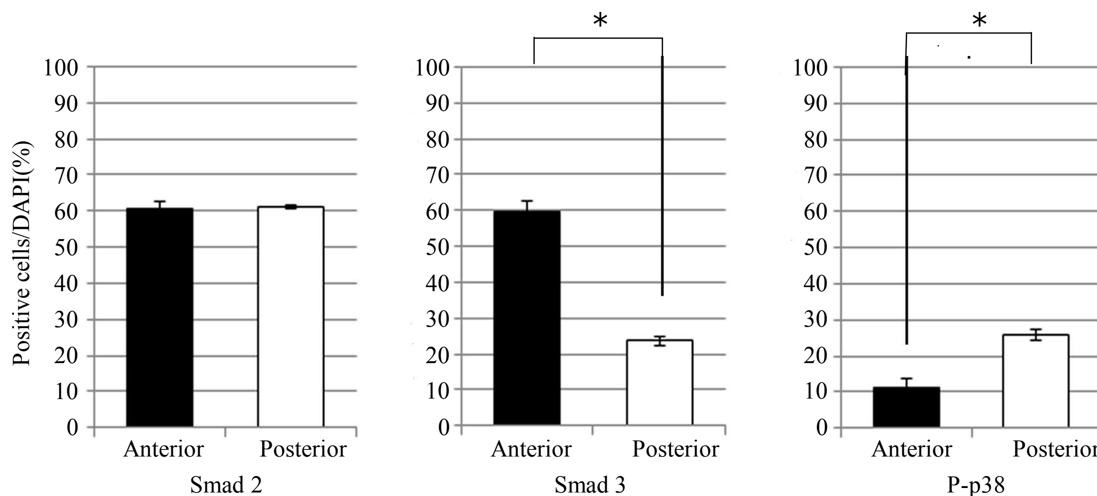

Figure 8.Quantification of the number of positive cell nuclei out of the total nuclei in the anterior (black bars, n=5) and posterior palates (white bars, n=7) in palatal mesenchyme. Error bars represent the S.D. *P<0.01

patterns of Smad2, Smad3, and Pp38, sections were selected before palatal shelves attached at around E14.0, and immunohistochemistry was performed. Smad2 expression in palatal mesenchyme was almost the same along the anterior-posterior axis, and its expression in palatal epithelium was not dependent on the MEE region (Fig. 6 A, E). Smad3 expression was higher in anterior than in posterior palatal mesenchyme (Fig. 6 B, F). In the posterior palate, Smad3 expression was diffuse in the posterior mesenchyme, but clearly seen in the MEE (Fig. 6 B, D, F, H, arrowhead). Conversely, Pp38 expression in palatal mesenchyme at E14.0 was downregulated only in the anterior but not in the posterior palatal mesenchyme (Fig. 6 C, G). Interestingly, Pp38 expression in the palatal epithelium was clearly inhibited only in the MEE region (Fig. 6 C, G).

Smad2/3 and Pp38 MAPK kinase activities during palatogenesis at E14.5

At E14.5, each palatal shelve was attached in the midline, and the MEE started to disappear for formation of the palatal shelf. In regular palatal development, cell differentiation was stimulated from E14.5, and bone and skeletal muscle formation started in the anterior and posterior palates, respectively. Smad2 expression was ubiquitously observed in the mesenchyme of both the anterior and posterior palates (Fig. 7 A, E), but Smad3 expression was statistically decreased in the posterior palate (Fig. 7 B, F, and Figure 8). Pp38 expression was clearly increased in posterior palatal mesenchyme (Fig. 7 C, G, and Figure 8). In palatal epithelium, TGF-β3 was especially expressed in the MEE (Fig. 7 D, H). Smad3 was clearly expressed in the MEE region with overlap of TGF-β3 expression in both anterior and posterior palates (Fig. 7 B, F). On the other hand, Pp38 expression was inhibited only in the MEE region (Fig. 7 C, G).

Discussion

The tissue in the palatal region can be divided into the bony hard and the muscular soft palates, each having a specialized function, such as occlusion, speech, or swallowing. In the initiation stage of palatogenesis, palatal mesenchyme is mostly composed of neural crest-derived cells in the anterior-posterior axis, and after the palatal shelves are fused, paraxial mesoderm-derived cells migrate into the posterior palate and contribute to palatal muscles with neural crest-derived cells[22-24]. Therefore, neural crest-derived cells mostly contribute to palatal mesenchyme in both the anterior and posterior parts until E14.5. According to the histological analysis of H&E, PAS, and Azan staining, palatal mesenchyme did not show specific differences in the anterior-posterior axis. However, different results were seen for cell proliferation and tenascin C expression, which clearly suggested that the palatal mesenchymal cells are already characterized in the anterior-posterior axis from E13.5, the initiation stage of palatal fusion.

Analyses of some gene expressions in mice have already elucidated the specific regional gene expression patterns in the anterior-posterior axis during palatogenesis[3, 25]. The expressions of transcription factors, such as Msx1, Shox2, or Tbx22, showed specific patterns in the anterior or posterior palate and suggested tissue-specific regulation in the anterior-posterior palatal mesenchyme[26]. However, specific regulation of growth factor in the anterior-posterior axis has not been well reported.

TGF-β signaling is crucial in regulating organogenesis during embryonic development, and it has been known as a key regulator for many developmental events, such as migration, cell proliferation, differentiation, ECM deposition, and the epithelial-mesenchymal transition in craniofacial development[23,27-29]. The cleft palate phenotypes of gene-mutated mice with ligands and receptors of TGF-β signaling provide strong evidence that TGF-β signaling plays an important role in both palatal epithelium and mesenchyme[4]. Human linkage studies have also shown that TGF-β signaling is important for palatogenesis. A rare variant of TGFB3 gene was found in Caucasian cleft palate patients[30]. Loeys-Dietz syndrome with mutations in TGFβR1 or TGFβR2 exhibits a partial cleft palate or bifid uvulae[31].

In the palatal epithelium, it has been thought that the Smad2-dependent pathway works mainly under TGF-β signaling in MEE, because transgenic overexpression of Smad2 could lead to partial recurrence of palatal fusion of *Tgfb3*-knockout mice[16]. In the present study, Smad2 expression did not show any characteristic expression pattern differences in the MEE region of the anterior-posterior axis. Interestingly, Smad3 expression was clearly activated in the MEE and MES during palatogenesis. It was suggested that activation of Smad3, in addition to the Smad2-dependent pathway, is required for the disappearance of the MES under TGF-β3 regulation. Interestingly, the present data showed that Pp38 expression was clearly inhibited in the MEE/MES of palatal epithelium. It has been shown that Smad4 and p38 MAPK function redundantly in mediating TGF-β signaling to regulate the disappearance of the MES during palatal fusion[21]. Smad4 is a component of the heterotrimeric complex with Smad2 or Smad3 for nuclear translocation and transcriptional activation of the downstream target genes under TGF-β signaling. With conditional deletion of Smad4 in palatal epithelium, the *K14-cre*;*Smad4$^{fl/fl}$* mice did not have disrupted palatal fusion, since Pp38 expression was activated in palatal epithelium in *K14-cre*;*Smad4$^{fl/fl}$* mice. Taken together, TGF-β3 regulates the disappearance of the MES during palatal fusion mainly through the Smad2/Smad3-dependent pathway, but the p38 MAPK pathway can be activated in the presence of a defect of canonical Smad-dependent TGF-β signaling. In the present examination, Smad2/Smad3 and Pp38 expression patterns in palatal epithelium did not show differences between the anterior and posterior regions. This suggests that intra-cellular signaling in the palatal epithelium is common along the entire anterior-posterior axis for the disappearance of the MES under TGF-β3 regulation.

In palatal mesenchyme, Smad2 expression was widely observed and did not show a specific pattern in the anterior-posterior axis. However, Smad3 showed stronger expression in the anterior region from E13.5 and was clearly inhibited in posterior mesenchyme. The Smad3-dependent pathway may have a specific role in anterior palatal mesenchyme, such as maxillary and palatine bone formation. On the other hand, Pp38 expression was clearly higher in posterior than in anterior palatal mesenchyme during palatogenesis. It leads us to consider that the activation of non-canonical TGF-β signaling has an exclusive and original role in the posterior palate. *Tgfb3* mutant mice showed a partial cleft of the posterior palate depending on the mouse strain[8, 9]. Furthermore, an isoform-specific role for *Tgfb3* in the palatal epithelium during posterior palate development was examined, which cannot be fully substituted by *Tgfb1*[32]. In addition, *K14-cre*;*Tgfbr2$^{fl/fl}$* mice exhibit partial or submucous cleft palate with MES remaining, and these mice have dramatic deformation of the palatine muscles[15]. Given these characteristic defects of the posterior palate in these *Tgfb3* and *K14-cre*;*Tgfbr2$^{fl/fl}$* mice, it was suggested that TGF-β signaling in palatal epithelium also affects molecular signaling in posterior palatal mesenchyme. The non-canonical Pp38-dependent pathway in posterior palatal mesenchyme may have the specific function of forming the soft palate with palatal muscle development under TGF-β control. The p38 MAPK kinase pathway is not regulated solely by TGF-β signaling; other growth factors also activate downstream target genes through Pp38, such as BMP or FGF. To discuss the redundancy of the non-Smad pathway of TGF-β signaling in the posterior palatal region, further investigations of the role of other growth factors are needed.

In the present study, we showed the different Smad-dependent/independent signaling expression patterns along the anterior-posterior axis. TGF-β signaling might regulate cell proliferation

and differentiation of palatal mesenchyme and the disappearance of the MEE through different intracellular pathways. These redundant functions of canonical and non-canonical signaling pathways of TGF-β might be important for the composition and characteristics of the hard and soft palates.

Acknowledgements

This work was supported in part by grants from the Japan Society for the Promotion of Science (JSPS) KAKENHI [KIBAN C; No. 24593116] and the MEXT-Supported Program for the Strategic Foundation at Private Universities to K.O.

Conflict of Interest

The authors have declared that no COI exists.

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