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Functional role of TGF- β receptors during palatal fusion *in vitro*

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Abstract

Objective—Reported expression patterns for TGF- β receptors (T β R-I, -II, and -III) during palatogenesis suggest that they play essential roles in the mechanisms leading to palatal fusion. The purpose of this study was to compare the functions of the three T β Rs during palatal fusion.

Methods—Using organ culture of mouse palatal shelves, expression levels of T β R-I, -II, and -III were suppressed by transfecting the siRNAs siT β R-I, -II, and -III, respectively. Phosphorylation of SMAD2 was examined as an indicator of downstream signaling *via* each T β R. Linkage between TGF- β signaling and critical events in palatal fusion led to the use of, MMP-13 expression as an outcome measure for the function of the TGF- β receptors.

Results—The siRNA treatment decreased the expression level of each receptor by more than 85%. When treated with either siT β R-I or -II, palatal shelves at E13 + 72 h were not fused, with complete clefting in the anterior and posterior regions. The middle palatal region following treatment with either siT β R-I or -II had fusion from one-half or one-third of the palatal region. Treatment with siT β R-III resulted in a persistent midline seam of medial edge epithelium (MEE) in the anterior region with islands of persistent MEE in the middle and posterior regions of the

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midline. Treatment with all three siT β R_s altered the pattern of SMAD2 phosphorylation. Palatal shelf cultures treated with siT β R-I or -II, but not -III, showed altered MMP-13 expression levels.

Conclusion—The ability to identify and recover MEE and palatal mesenchymal cells during palatal fusion will aid in the evaluation of the different mechanistic events regulated by each T β R during palatogenesis.

Keywords

palatal development; TGF- β receptor; TGF- β signalling; SMAD2 phosphorylation; MMP-13 expression; siRNA

1. Introduction

Cleft palate birth defects occur with an incidence of 1/500–1/1000 birth in humans.^{1,2} Several mechanisms for these malformations have been hypothesized that affect palatal shelf adhesion and fusion including insufficient palatal size, altered elevation, and changes in the medial edge epithelium (MEE).^{3–5} In normal mouse palatal development, a multilayer epithelial seam is formed by the adhesion of two opposing MEE populations, which cover the tips of the palatal shelves at embryonic day 14 (E14).³ The MEE seam thins to a single-cell layer at E14.5, while MEE cells accumulate at the oral and nasal aspects forming epithelial triangles.³ The epithelial seam disappears by E15, such that only mesenchymal cells are observed in the palatal midline.³

The multiple molecular mechanisms regulating palatogenesis, each of which represents a potential target for the etiology of cleft palate, are only beginning to be understood. In particular, the role of the transforming growth factor (TGF)- β signaling pathway and the expression patterns of the three TGF- β receptor (T β R) types, T β R-I, -II, and -III, have been shown to be important during palatogenesis.^{6–23} Binding of TGF- β to complex of T β R-I and -II on the cell surface activates the serine/threonine kinase signaling pathway,^{24–26} initiating an intracellular signaling cascade to alter gene expression. Upon binding TGF- β , these T β R_s reorient to provide a catalytically favorable presentation for the cytoplasmic kinase domains.^{11,21,22,24} After binding TGF- β , T β R-II catalyzes the phosphorylation of T β R-I.^{24–37} In contrast, T β R-III lacks a cytoplasmic protein kinase domain and has been considered to be a nonsignaling receptor.^{25,26} T β R-III may facilitate binding of TGF- β ligands to T β R-II, which, in turn, recruits and phosphorylates T β R-I.^{27–36} Thus, T β R-III may bind TGF- β and present it to T β R-II, to enhance the effect of the ligand.^{33–36}

TGF- β signaling through T β R-I (also known as activin receptor-like kinase 5, Alk5) has been shown to be required for upper lip and midline fusion of the hard and soft palates.^{12,27} During craniofacial development, T β R_s are distributed in cell types that are critical for fusion in the embryological process. T β R-I expression is distributed in the midline epithelial seam, as well as in the oral and nasal epithelia, but not in the underlying mesenchyme.¹⁷ T β R-II is expressed in the palatal epithelial and mesenchymal cells.³⁰ T β R-III shows an MEE-specific expression pattern at critical stages of palatal fusion.^{15–17} T β R-I conditional knockout mice (Tgfr1^{-/-}/Alk5^{-/-} mice)²⁸ and T β R-II knockout mice³¹ are lethal around E10.5, a developmental stage that precedes the formation of the palatal shelves and initiation

of palatal development. A T β R-III homozygous null mutant model has been developed, but the mice are lethal around E13.5 again prior to the developmental steps critical for palatal development and fusion.³³ Hence, it has not been possible to analyze these null mutants for their effects on palatal development. However, epithelial-specific deletion of the T β R-I gene (*Alk5^{fl/fl};K14-Cre*)¹² or the T β R-II gene (*Tgfb^{2fl/fl};K14-Cre*)^{10,13} causes clefts in the posterior soft palate and the anterior hard palate. In T β R-II conditional knockout mice, the middle region of the palate has persistence of MEE in the midline seam between E14.5 and E16.5.¹³

TGF- β transduces an intracellular signal through the T β R-I-mediated phosphorylation/activation of SMAD2 or SMAD3, which each can form a complex with the common-mediator Smad, SMAD4. The complex undergoes nuclear translocation and interacts with DNA in a cell-specific manner to effect transcription of TGF- β -responsive genes.^{25,37} In palatal development, SMAD2 and SMAD3 are present in the MEE; however, SMAD2 is predominantly phosphorylated during palatal fusion.^{38,39} Persistence of the MEE has been linked to a decreased level of phosphorylated SMAD2 (p-SMAD2), which, in turn, has been correlated with an increase in MEE cell proliferation.⁴⁰

Extracellular matrix (ECM) is an essential component of many biological processes involving cell migration, proliferation, and differentiation, as well as cell-cell interactions.⁴¹ ECM turnover is controlled by multiple enzymes. In particular, matrix metalloproteinases (MMPs) function primarily at the cell surface or in the extracellular space. Their proteolytic activity is controlled through zymogen activation and inhibition by endogenous proteinase inhibitors, known as the tissue inhibitors of metalloproteinases (TIMPs).^{41–45} During palatal fusion, MMP-13 expression is highly induced in the palatal MEE^{16,42} and responsive to TGF- β 3 stimulation in the degrading midline seam.⁴²

To investigate and confirm the role of T β Rs in palatogenesis, we directly examined the functions of the three T β Rs through suppression with siRNAs. MMP-13 expression was examined in organ cultures of mouse palatal shelves, in which the T β Rs were specifically knocked-down. The findings in this study provide additional information and confirmation of the functional role of each T β R during palatal fusion.

2. Experimental Procedures

2.1. Palatal organ culture

Timed-pregnant Swiss-Webster mice were used for these studies (Slc: ICR mouse; Sankyo laboratory, Tokyo, Japan). Female mice were mated for 2 h. Presence of a vaginal plug was used to determine E0. At E13, palatal shelves—which were in a horizontal position above the tongue, but not yet adhering to each other—were dissected and placed in pairs on Millipore filters, in the correct anterior-posterior orientation and with the medial edge epithelium in contact (Figure 1 A and B). Palatal shelves were cultured at 37 °C in 8% CO₂ for up to 72 h at the air-fluid interface in Grobstein organ culture dishes (Falcon, Japan) containing BGjb medium (Gibco, Japan).

Cultures were analyzed at the developmental stages determined by previous studies.^{15,16,40,45} Specifically, they were analyzed at three key time points considered to be equivalent to critical stages in palatogenesis *in vivo*: (1) 24 h (equivalent to E14), when the palatal shelves became adherent and the epithelial seam forms in the midline; (2) 48 h (equivalent to E14.5), when palatal fusion begins and the midline epithelial seam begins to break down; and (3) 72 h (equivalent to E15), when palatal fusion is complete and the MEE disappears resulting in continuity of the mesenchyme. All experiments that used this time schedule were completed in triplicate.

The goal of this study was to examine the summative effect of the knockdown of gene expression. The 72-h time point was the most appropriate for analyzing the impact of gene expression changes on the entire process of palatal fusion because, at this time point, the palatal shelves should be completely fused and none of the MEE cells should be in the midline position.

2.2. Transfection of siRNAs

RNA interference by siRNAs was performed as previously described.^{15,40,46} The siRNAs against T β R-I, -II, and -III, designated as siT β R-I, -II, and -III, respectively, were from Thermo Scientific (SMARTpool ON-TARGETplus; L-040617-05-8 -0010, L-040618-05-8 -0010, and L-046537-05-8 -0010, respectively). Experimental groups of palatal shelves were treated with siT β R-I, -II, or -III at final concentrations of 50, 100, and 300 nM in BGjb medium containing 0.2% Lipofectamine 2000 (Invitrogen).¹⁵ The control group was transfected with a scrambled duplex control siRNA (Stealth RNAiTM siRNA Negative Controls, Invitrogen). The culture media was changed 48 h after siRNA transfection.^{15,40} The palatal fusion process was analyzed by characterizing the midline region at E13 + 72 h.

2.3. Histological evaluation

Cultured palatal tissues were harvested after 72 h of organ culture and processed for examination with traditional frozen-section hematoxylin and eosin (H&E) light microscopy. Briefly, frozen, 10- μ m-thick sections were prepared from the cultured palates and processed for H&E staining.¹⁵ The examined region of the palate was restricted to between the most posterior end of the primary palate and a secondary palate position defined by the posterior boundary of the maxillary first molar. The regions of the secondary palate were defined as follows: anterior (primary palate to third ruga), middle (third to sixth ruga), and posterior (sixth ruga to end of the secondary palate). Every fifth section from each cultured palate was examined by light microscopy.⁴⁰ The control siRNA group ($n = 30$) and three siRNA-treated groups ($n = 30$ in each) were observed.

2.4. Immunofluorescence analysis of T β Rs in palatal organ culture

Frozen 10- μ m-thick sections were prepared from the cultured palatal shelves at E13 + 24 h for control palate to identify normal expressions, and at E13 + 72 h for treatment palate to analyze the effects of transfection of siRNA. Immunohistochemistry was performed with primary antibodies (Santa Cruz, CA) against T β R-I (sc-9048), -II (sc-400), and -III (sc-28975), in accordance with previously described procedures.^{15,16} Secondary fluorescein isothiocyanate (FITC)-labeled antibodies were used to localize primary antibody binding.

FITC fluorescence was identified with a Zeiss fluorescent microscope at excitation/emission wavelengths of 490/520 nm. Vectashield® Mounting Medium with DAPI (Vector Labs., Burlingame, CA) was used to prevent rapid loss of fluorescence during microscopic examination and for nuclear staining.

2.5. Western blotting

Protein expression levels of T β R-I, -II, -III, total SMAD2, p-SMAD2, and MMP-13 were quantified by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, following the standard methods for the Boehringer Mannheim Chemiluminescence Blotting Kit (Roche, Indianapolis, IN).^{15,16,40} For Western blotting, MEE cells at E13 + 24 h were carefully dissected under the microscope from the midline seam region of each pair of palatal shelves (Fig. 1A and B).^{15–17,38,40} The 24-h culture period was selected because early changes in MEE gene expression levels are critical for initiating the cascade of events required to complete palatal fusion.

MEE cells with the midline seam tissue were homogenized at 85 °C in lysis buffer (50 mM Tris-HCl, 2% SDS, 10 glycerol). Homogenates were boiled for 10 min, centrifuged, and the supernatants were used to determine the protein content with the DC Protein Assay Kit (Bio-Rad Labs, Hercules, CA). Each protein sample was resolved by 10–12% SDS-PAGE, and electroblotted onto polyvinylidene difluoride membranes (Roche). Specific polyclonal antibodies against T β R-I (sc-9048), -II (sc-400), and -III (sc-28975) were used (Santa Cruz, CA; 1:250). To identify downstream signaling, specific polyclonal antibodies for SMAD2 (3122) and p-SMAD2 (3104) were used (Cell Signaling Technology, Inc., MA; 1:1000). To investigate MMP-13 expression, specific polyclonal antibodies for MMP-13 (sc-30073) were used (Santa Cruz, CA; 1:250). The anti-GAPDH monoclonal antibody (MAB374) was used to identify a standard housekeeping gene as a control (Chemicon International, 1:1000). Blots were exposed to horseradish peroxidase-conjugated goat anti-rabbit IgG for 30 min at room temperature and reacted with substrate (Luminol/H₂O₂) according to the manufacturer's protocol (Roche). The signal intensities were quantitated by densitometric analysis with Scion Image (Scion Corp.).

2.6. Quantitative evaluation of MMP-13 in palates treated with siT β R-I, -II, and -III

To investigate MMP-13 expression after treatment of the palatal organ cultures with siRNA, real-time reverse transcription–polymerase chain reaction (RT-PCR) was performed to quantify the mRNA expression levels. MEE cells were isolated from the palatal shelf tissues by microscopic dissection at E13 + 24 h (Fig. 1A and B). Isolated MEE tissue was placed in a collecting tube containing RLT buffer (Qiagen, CA) with 10% β -mercaptoethanol and homogenized. The RNeasy Mini Kit (Qiagen, CA) was used for total RNA extraction and purification. Extracted RNA was purified and quantified by spectrophotometry.^{15,16,40}

Total mRNA samples from MEE cells were prepared and reverse-transcribed into cDNA. Aliquots containing equal amounts of mRNA were subjected to RT-PCR. First-strand cDNA synthesis was performed with 1 μ g of DNase-treated total RNA in 20 μ L containing first-strand buffer, 50 ng of random primers, 10 mM of a dNTP mixture, 1 mM Dithiothreito, and 0.5 U of reverse transcriptase at 42 °C for 60 min. The cDNA mixtures were diluted five-

fold in sterile distilled water, and 2- μ L aliquots were subjected to real-time RT-PCR with the SYBR Green I dye. Real-time RT-PCR was performed in 25 μ L containing 1 $\times$ real-time PCR buffer, 1.5 mM of a dNTP mixture, 1 $\times$ SYBR Green I, 15 mM MgCl₂, 0.25 U of ExTaq Polymerase (RT-PCR version; TaKaRa, Tokyo, Japan), and 20 mM of specific primers. The primers were designed with the Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA). The primer pairs were as follows: for MMP-13, 5'-GTCTTCCCCG TGTCCAAAAG A-3' and 5'-TGACCTGGGA TTTCCAAAAG A-3', and with a product size of 105 bp; and for GAPDH, 5'-CAATGACCCC TTCATTGACC-3' and 5'-GACAAGCTTC CCGTTCTCAG -3', with a product size of 106 bp.

PCR was carried out in a thermal cycler (Smart Cycler, Cepheid, Sunnyvale, CA). Data were analyzed with Smart Cycler software (ver. 1.2d). The PCR conditions were 95 °C for 3 s and 60 °C for 20 s for 35 cycles. Measurements were taken at the end of the annealing step at 60 °C in each cycle. All real-time RT-PCR reactions were performed in triplicate. The mRNA expression levels were calculated and normalized to the level of GAPDH mRNA.

2.7. Statistical analysis

Results from multiple groups were compared by analysis of variance (ANOVA) and Tukey's-HSD multiple comparison tests. The acceptable level of significance was set at $p < 0.05$. Data were analyzed with the SPSS software.

3. Results

3.1. Normal palatal development and expressions of three types of T β R in vitro in palatal shelf organ culture

The in vitro normal palate without siRNA treatment showed one or two layers of MEE cells in the midline seam of the palate in the anterior, middle, and posterior regions at E13 + 24 h (Fig. 2A, a–c). At E13 + 72 h, specimens of normal palates achieved complete palatal fusion (Fig. 2A, d–f).

In cultured palatal shelves without siRNA treatment, T β R-I expression was detected in palatal epithelial cells including medial edge of epithelial (MEE) (Fig. 2B, a).¹⁷ T β R-II expression was observed in both the palatal epithelium and mesenchyme (Fig. 2B, b),³⁰ and T β R-III expression was identified in the MEE cells (Fig. 2B, c).¹⁷ These results were consistent with findings in previous studies.^{17, 30}

3.1. Effects of knocking-down T β R-I, -II, and -III in palatal tissue using siRNA

Western blot analysis of cultured palatal shelf tissue at E13 + 24 h was performed to determine whether siT β R-I, -II, and -III were effective at knocking-down expression of the T β Rs (Fig. 3A–C). Expression levels of T β R-I, -II, and -III were calculated relative to GAPDH (Fig. 3D), with the signal intensities quantified by densitometric analysis. The siRNAs had a dose-dependent effect, with the greatest reduction in expression (>85% decrease vs. control siRNA) being obtained with 300 nM siT β R-I, -II, or -III (Fig. 3D). GAPDH expression was not affected in the experimental or control siRNA treatment group (Fig. 3A–C).

3.2. Immunohistochemistry results of T β R expression in siT β R-treated palates

To confirm the effects of siRNA transfection, we observed the expression levels of T β R-I, -II, and -III by immunohistochemistry. Palatal shelf organ cultures were transfected with 300 nM siT β R-I, -II, or -III. Cultures were subjected to immunohistochemistry at E13 + 72 h to determine whether transfection with each siRNA was effective in altering the developmental program for the fate of MEE and the palatal shelves (Fig. 4A, E, and I). In cultured palatal shelves treated with siT β R-I, T β R-I expression was not detected in either the palatal epithelial or mesenchymal cells (Fig. 4A), T β R-II expression was observed in both the palatal epithelium and mesenchyme (Fig. 4B),³⁰ and T β R-III expression was identified in the MEE cells (Fig. 4C). Similarly, treatment of cultured palatal shelves with siT β R-II resulted in an absence of T β R-II expression (Fig. 4E), whereas the T β R-I and -III expressions were not affected (Fig. 4D and F). The siT β R-III treatment demonstrated the same specificity of effect on only T β R-III expression (Fig. 4G–I).

3.3. Phenotype of palatal fusion after treatment with siT β R-I, -II, and -III in in vitro organ culture

The process and completeness of palatal fusion were analyzed by using the classification of fusion shown in Fig. 5, which is a slight modification from a previous study.²⁰ Palatal fusion phenotypes of siT β R-treated organ cultures were further classified into the following five patterns (Fig. 5):

Type A: Palatal shelves remained apart or only contacted.

Type B: Palatal MEE remained present, with either one or two layers.

Type C: Small epithelial islands of MEE cells remained, but comprised less than one-third of the palatal midline.

Type D: MEE was present in triangles at the oral and nasal epithelial aspects.

Type F: Palatal shelves achieved complete fusion.

The phenotype of representative control siRNA-treated palatal shelf organ cultures at E13 + 24 h was a double layer of MEE cells in the anterior region (Fig. 6A, a), with a single-cell layer of MEE in the middle and posterior regions^{17,30} (Fig. 6A, b and c). These phenotypes did not differ from those observed in normal palatal development without control siRNA treatment (Fig. 2 A, a–c). In organ cultures treated with siT β R-I, the anterior, middle, and posterior regions of the palate were not fused (Fig. 6A, d–f). In cultures treated with siT β R-II, the anterior and posterior regions were not fused, but the opposing MEE areas were in contact in the middle region (Fig. 6A, h). In cultures treated with siT β R-III, the anterior and posterior regions were not fused, and the middle region was in contact (Fig. 6A, j–l). We compared the control and treated samples at E13 + 24 h according to the palatal classification scheme shown in Fig. 5. Control palates were 70–80% (20–23/30 palates) type B (Fig. 6B, a). Cultures treated with siT β R-I or -II were 90% (26–28/30 palates) type A (Fig. 6B, b and c). Specimens treated with siT β R-III were type A or type B (Fig. 6B, d).

At E13 + 48 h, control cultures had only islands of MEE cells persisting in the midline in the anterior and posterior regions^{17,30} (Fig. 7A, a and c), while the middle region was beginning

to fuse with one-third of the remaining MEE, primarily at the oral aspect (Fig. 7A, b). Treatment with siTβR-I altered the pattern of palatal fusion compared to the controls. In siTβR-I-treated cultures, the anterior and posterior regions were not fused (Fig. 7A, d and f), and the middle region was in contact in less than one-third of the midline (Fig. 7A, e). Treatment with siTβR-II resulted in a similar phenotype to siTβR-I (Fig. 7A, g–i). Treatment with siTβR-III resulted in a persistent single layer of MEE cells in all regions (Fig. 7A, j–l). The proportions of the palatal phenotypes at E13 + 48 h with each treatment are presented in Fig. 7B.

The phenotypes of palatal fusion at E13 + 72 h were compared between the controls and specimens treated with siTβR-I, -II, or -III. Control palatal shelf organ cultures were completely fused in the anterior, middle, and posterior regions^{17,30} (Fig. 8A, a–c), with patterns identical to normal palatal development (Fig. 2A, d–f). However, the palatal shelves in the anterior and posterior regions remained unfused when the organ cultures were treated with siTβR-I (Fig. 8A, d and f) or siTβR-II (Fig. 8A, g and i). With both siTβR-I and -II, the middle regions were in contact but not fused (Fig. 8A, e and h). Organ cultures treated with siTβR-III had a single layer of MEE cells remaining in the anterior region (Fig. 8A, j), as well as persistent MEE islands in the middle and posterior regions (Fig. 8A, k and l). At E13 + 72 h, the control cultures in all regions were over 90% (28–29/30 palates) type F (Fig. 8B, a–c). Cultures treated with siTβR-I and -II were 80% (23–24/30 palates) type A in the anterior and posterior regions (Fig. 8B, b and c) and 80% (24/30 palates) type B in the middle region. The siTβR-III-treated palatal cultures were approximately 80% (24/30 palates) type C or D (Fig. 8B, d).

3.4. SMAD2 phosphorylation levels in MEE cells after treatment with siTβR-I, -II, and -III

To identify downstream TGF-β signaling after treatment with siTβR-I, -II, or -III during palatal fusion, the total SMAD2 and p-SMAD2 expression levels were determined by Western blot analysis (Fig. 9A, a), and the relative amount of p-SMAD2 (p-SMAD2/total SMAD2) was calculated (Fig. 9A, b). All of the palatal shelf cultures treated with siTβRs demonstrated decreased p-SMAD2 expression levels compared to cultures treated with control siRNA ($p < 0.05$, $n = 5$). The lowest level of p-SMAD2 was observed in the cultures treated with siTβR-I or -II ($n = 5$) (Fig. 9A, a and b). There was no significant difference between the outcomes with siTβR-I and -II. The p-SMAD2 expression of was significantly higher in siTβR-III-treated specimens than in the cultures treated with the other siRNAs.

3.5. MMP-13 expression after treatment with siTβR-I, -II, and -III

The MMP-13 expression was directly compared between organ cultures treated with siTβR-I, -II, or -III. The amount of MMP-13 protein present in palatal organ cultures treated with siTβR-I or -II was decreased compared to the levels observed in the control palatal tissue (Fig. 9B, a). MMP-13 gene expression was quantified in MEE tissues from palatal organ cultures treated with siTβR-I, -II, or -III. The MMP-13 gene expression was reduced compared to control specimens after treatment with siTβR-I or -II ($p < 0.05$; Fig. 9B, b), but not after treatment with siTβR-III.

4. Discussion

The present results indicate that there are regional differences in the process of secondary palatal fusion when palatal organ cultures are treated with siRNAs specific for the three T β R. The anterior and posterior regions of the cultured palates were particularly susceptible to the inhibition of fusion when the cultures were treated with siT β R-I or -II. Inhibition of T β R-III with siT β R-III delayed the fusion process compared to the controls. The results provide evidence that T β R-I, -II, and -III are all necessary to mediate the effects of TGF- β during normal palatal development.^{10,12,15,17,28,30}

Inhibiting T β Rs by siRNA treatment resulted in a demonstrable effect on the downstream signaling by altering the levels of p-SMAD2. The treatment of the palatal organ cultures with the siRNAs specific for the Type I and Type II receptors significantly decreased the levels of p-SMAD2. Since the p-SMAD2 is critical to the changes required in the cells the reduction in p-SMAD2 will secondarily alter the patterns of expression of TGF- β responsive genes.^{24,30,38–40} In the present study, the MEE was persistent and complete fusion did not occur in the cases of gene knockdown, indicating a significant effect on palatal fusion. Thus, knocking-down T β Rs could affect the downstream signaling pathway of TGF- β and, ultimately, the process of palatal fusion.

MMP-13 offered a further example of altered gene expression linked to T β R knockdown. The MMP-13 expression was significantly reduced by treatments with siT β R-I and -II; however, knockdown of the nonsignaling T β R-III did not alter MMP-13 expression. This new information may provide a means to classify the inhibition of palatal fusion in ways linked to the function of T β Rs. The MMP family of proteins is involved in ECM breakdown in normal physiological processes, including embryonic development.^{16,41–43} MMP-13 is intensely expressed in MEE and adjacent mesenchymal cells in the midline seam.^{41,42} A previous study revealed that MMP-13 expression is decreased in TGF- β 3^{-/-} palates at E14.5 compared to wild-type controls.⁴² The previous study suggested that both TGF- β 1 and TGF- β 3 have a high affinity for TGF β 2, however, TGF- β 2 has a low affinity for T β RII and may require T β R-III as an accessory receptor for high-affinity interaction with the heteromeric-signaling complex.²¹ In our previous data, MMP-13 expression in MEE cells was highest at E13 + 24 h, and MMP-13 mRNA was present at the site of contact between the palatal shelves.¹⁶ In the present study, MMP-13 expression decreased after treatment with siT β R-I or -II, but not -III. Thus, proteolytic degradation of the ECM by MMP-13 is linked to TGF- β signaling, which may be mainly associated with TGF- β 1 and/or TGF- β 3. Furthermore, T β R-III may function as an accessory receptor to directly affect downstream signaling but may not affect indirect signaling such as ECM expression.

In summary, the ability to identify and recover MEE and palatal mesenchymal cells during palatal fusion will provide the opportunity for closely evaluating the different mechanistic events regulated by each T β R during palatogenesis. The present study provides additional insight into the TGF- β -related signaling pathway that is critical during palatal development.

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Research highlight

- When treated with either siT β R-I or -II, palatal shelves at E13+72h were not fused.
- Treatment with siT β R-III resulted in a persistent midline seam of MEE at E13+72h.
- Treatment with all siT β Rs altered the pattern of SMAD2 phosphorylation.
- Treatment with siT β R-I or -II, but not -III, showed altered MMP-13 expressions.

Fig. 1. Sampling of MEE cells for protein and mRNA extraction

MEE cells were carefully dissected from the midline seam region of each pair of palatal tissue. Tissues were collected from the midline region, defined by the square area in A (coronal view) and B (oral side view). This specific tissue was dissected and used to extract the proteins and mRNA.

Fig. 2. Control palatal development and normal expression of TβRs in vitro in palatal organ culture

At E13 + 24 h, specimens without siRNA treatment showed one or two layers of MEE cells in the midline seam of the palate in the anterior (a), middle (b), and posterior regions (c). At E13 + 72 h, specimens of normal palate without siRNA treatment achieved complete palatal fusion in the anterior (d), middle (e), and posterior regions (f). TβR expression was detected in the cultured palatal shelves, TβR-I expression was detected in only palatal epithelium (Fig. 3A). TβR-II expression was observed in both the palatal epithelium and mesenchyme (Fig. 3B), and TβR-III expression was identified in the MEE cells (Fig. 3C).

Fig. 3. Western blot analysis to assess the knockdown effects of siTβR-I, -II, and -III *in vitro* Experimental groups of palatal shelves were treated with siTβR-I, -II, and -III at final concentrations of 50, 100, and 300 nM. Control cultures were transfected with the scrambled duplex control siRNA. Protein expression levels of TβR-I (A), TβR-II (B), and TβR-III (C) were quantified by SDS-PAGE and Western blotting. (D) Comparison of all siRNA doses and controls. Data are presented as the mean $\pm$ SD, $*p < 0.05$.

Fig. 4. Immunofluorescence analysis of T β R in siRNA-treated palatal tissues
 Green indicates T β R staining. Blue (DAPI) indicates nuclear staining. Specimens were treated with siT β R-I (A–C), siT β R-II (D–F), and siT β R-III (G–I), followed by specific antisera to examine T β R-I (A, D, and G), T β R-II (B, E, and H), and T β R-III (C, F, and I).

Fig. 5. Classification of palatal phenotype

Palatal fusion was classified as described in the text.

Fig. 6. Phenotype of palatal fusion at E13 + 24 h after treatment with siTβR-I, -II, or -III
 (A) Representative phenotypes at the anterior, middle, and posterior regions, respectively, of palatal shelf organ cultures treated with control siRNA (a–c), siTβR-I (d–f), siTβR-II (g–i), and siTβR-III (j–l) at E13 + 24 h. (B) Ratios of the palatal classification, based on the treatment of the palatal shelf organ culture (n=30) (a: Control, b: siTβR-I, c: siTβR-II, d: siTβR-III).

Fig. 7. Phenotype of palatal fusion at E13 + 48 h after treatment with siTβR-I, -II, or -III
 (A) Representative phenotypes in the anterior, midline, and posterior regions, respectively, of palatal shelf organ cultures treated with control siRNA (a–c), siTβR-I (d–f), siTβR-II (g–i), and siTβR-III (j–l) at E13 + 48 h. (B) Ratios of the palatal classification, based on the treatment (a–d) of the palatal shelf organ culture (n=30) (a: Control, b: siTβR-I, c: siTβR-II, d: siTβR-III).

Fig. 8. Phenotype of palatal fusion at E13 + 72 h after treatment with siTβR-I, -II, or -III
 (A) Representative phenotypes in the anterior, midline, and posterior regions, respectively, of palatal shelf organ cultures treated with control siRNA (a–c), siTβR-I (d–f), siTβR-II (g–i), and siTβR-III (j–l) at E13 + 72 h. (B) Ratios of the palatal classification, based on the treatment (a–d) of the palatal shelf organ culture (n=30) (a: Control, b: siTβR-I, c: siTβR-II, d: siTβR-III).

Fig. 9. SMAD2 and MMP-13 expression levels after transfection with siTβR-I, -II, or -III
 (A) Total SMAD2 and p-SMAD2 expression levels were determined by Western blot analysis (a), and the relative amount of p-SMAD2 (p-SMAD2/total SMAD2) was calculated (b) (* $p < 0.05$; control vs siTβR groups, # $p < 0.05$; siTβR-I vs siTβR-III, \$ $p < 0.05$; siTβR-II vs siTβR-III) ($n = 5$ independent experiments). (B) MMP-13 protein expression levels in palatal organ cultures treated with control siRNA, siTβR-I, siTβR-II and siTβR-III (a) were determined by western blot analysis. And MMP-13 gene expression levels in palatal organ cultures treated with control siRNA, siTβR-I, siTβR-II and siTβR-III (b) were determined by real-time RT-PCR analysis (* $p < 0.05$; control vs siTβR treatment groups) ($n = 5$ independent experiments).