Ras signaling and its effector RREB1 are required for the dissociation of MEE cells in palatogenesis

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Keywords: Ras, Rreb1, MEE cells, EMT, TGF-β3, Palatogenesis

Summary statement:
RREB1, a known transcriptional factor that acts downstream of Ras signaling, is expressed in the MEE region and required for the dissociation of MEE during palatal fusion.

Abstract
Cleft palate is one of the major congenital craniofacial birth defects. The etiology underlying the pathogenesis of cleft palate has largely remained unelucidated. Dissociation of the medial edge epithelium (MEE) at the contacting region of palatal shelves and subsequent migration or apoptosis of MEE cells is required for the proper MEE removal. Ras Responsive Element Binding Protein 1 (RREB1), a RAS transcriptional effector, has recently been shown to play a crucial role in developmental EMT, in which loss of epithelial characteristics is an initial step, during mid-gastrulation of embryonic development. Interestingly, the involvement of RREB1 in cleft palate has been indicated in humans. Here, we demonstrated that pan-Ras inhibitor prevents the dissociation of MEE during palatal fusion. Rreb1 is expressed in the palatal epithelium during palatal fusion, and knockdown of Rreb1 in palatal organ culture resulted in palatal fusion defects by inhibiting the dissociation of MEE cells. Our present findings provide evidence that RREB1-mediated Ras signaling is required during palatal fusion. Aberrant RREB1-mediated Ras signaling might be involved in the pathogenesis of cleft palate.
Introduction

Cleft palate is one of the major congenital craniofacial birth defects that result from failure of growth, elevation, and fusion of the palatal shelves during embryogenesis (Dixon, Marazita, Beaty, & Murray, 2011; Murray, 2002). This condition shows anatomical impairments combined with various defects involving the soft and hard palates, the nasal septum and the alveolar ridge. It is well accepted that cleft palate is a multifactorial disease caused by a combination of genes and environmental factors. While recent studies using genetically modified mice help to identify the genetic and environmental etiology for cleft palate, the etiology underlying the pathogenesis of cleft palate has largely remained unknown.

In mice, the palatal fusion begins at the midline of the palatal shelves following vertical palate elevation above the tongue and bilateral outgrowth of the palatal shelves toward each other. Following contact of the palatal shelves at the medial edge epithelium (MEE), the intervening epithelium between the palatal shelves merges to form the epithelium seam, which is subsequently removed to allow mesenchymal continuity across the fused palate (Bush & Jiang, 2012; Ferguson, 1988). Fusion of the palatal shelves is crucial for the correct formation of the palate, and its defect can lead to cleft palate.

Transforming growth factor-beta 3 (TGF-β3) has been established as a critical gene for causing cleft palate through regulating epithelial fusion in mice and humans (Lidral et al., 1998; Taya, O’Kane, & Ferguson, 1999; Zhu et al., 2010). While the roles of TGF-β3 have been widely studied in the dissociation of MEE during palatal fusion, the cross-talk between TGF-β3 and other signaling pathways in palatogenesis and
regulatory mechanism in that process remains unexplored (Nakajima, F Shuler, Gulka, & Hanai, 2018).

The RAS proteins are members of a large superfamily of low-molecular-weight GTP-binding proteins. RAS plays pivotal roles in numerous basic cellular functions, including regulation of proliferation, differentiation, and apoptosis (Downward, 2003). Physiological and oncogenic activation of RAS, encoded by one of three isoforms HRAS, KRAS, or NRAS, is coupled to a wide range of downstream signaling pathways, including RAF-MEK-ERK (MAPK), phosphoinositide 3-kinases (PI3Ks), and Rho GTPases family (Rajalingam, Schreck, Rapp, & Albert, 2007).

Ras Responsive Element Binding Protein 1 (RREB1) is a zinc finger transcription factor that binds to RAS-responsive elements (RREs) of gene promoters in response to Ras signaling activation (Mukhopadhyay et al., 2007). Recently, Su et al. (Su et al., 2020) reported that RREB1 plays a pivotal role in developmental EMTs during mid-gastrulation of embryonic development by integrating TGF-β and Ras signals. Importantly, developmental EMTs are regulated by tissue-specific mechanisms and occur in a temporal and spatial manner. Studying the context-dependent regulatory molecular mechanisms of these developmental EMTs is very important for understanding cell plasticity during embryonic development and for clarifying the pathogenesis of developmental diseases, including cleft palate, in which EMT-like loss of epithelial characteristic is at least partially involved.

In humans, there is no direct genetic evidence for the involvement of RREB1 mutation in palatal fusion defects. However, the association of RREB1 with cleft palate has been indicated in some congenital disorders. Chromosome 6pter-p24 deletion syndrome (OMIM #612582) is a chromosomal disorder carrying a terminal deletion of the short
arm of chromosome 6 (6p), the region that includes the \textit{RREB1} gene. The 6pter-p24 deletion syndrome is characterized by developmental delay/mental retardation, reduced muscle tone, Dandy-Walker malformation, hearing loss, anomaly of eyes, cardiac abnormalities, and abnormal craniofacial features often associated with orofacial clefting (Davies et al., 2004; Topping, Harris, & Moss, 2002). \textit{RREB1} is also a candidate factor in the pathogenesis of DiGeorge syndrome, which displays craniofacial abnormalities, including cleft palate. The expression of \textit{RREB1} is dysregulated by an epigenetic mechanism in DiGeorge syndrome. These observations indicate the involvement of the \textit{RREB1} gene in the pathogenesis of cleft palate in humans. However, the role of \textit{RREB1} in the dissociation of MEE, a process with steps partially overlapping the biological process of developmental EMT, during palatogenesis has not been determined yet.

Here, we demonstrated the essential roles of Ras signaling in the dissociation of MEE during palatal fusion. In addition, the present study provides the first evidence that \textit{RREB1} is expressed at the fusing surface of the secondary palate and is required for the dissociation of MEE in palatogenesis.

\textbf{Results}

\textit{Pan-Ras inhibitor prevents palatal fusion in organ culture.}

Palatal organ culture is a well-established method to examine the fusion of palatal epithelium (Brunet et al., 1995). To examine the roles of Ras signaling in palatal fusion, we used Pan-Ras inhibitor (Pan-Ras-IN-1) in palatal organ cultures. Pan-Ras-IN-1 has been well characterized as an inhibitor of Ras signaling (Welsch et al., 2017). We also used a specific TGF-\(\beta\) RI kinase inhibitor, SB431542, which has been demonstrated to
inhibit palatal fusion in palatal organ cultures (Dudas, Nagy, Laping, Moustakas, & Kaartinen, 2004; Inman et al., 2002). After 48 hours of culture, control cultures showed complete fusion of the palatal shelves (Figure 1A). The group treated with SB431542 at 20 μM showed complete fusion defects of the palatal shelves covered with intact MEE cells. Similarly, treatment with pan-Ras-IN at 20 μM inhibits palatal fusion in palatal organ cultures. E-cadherin is the most commonly used marker specific for the epithelial cells covering the palatal shelves. EMT-like loss of epithelial characteristics is an essential process in which E-cadherin disappears prior to the removal of MEE during palatal fusion. P63 is one of the key transcriptional factors whose mutations result in cleft palate in humans and mice (Thomason, Dixon, & Dixon, 2008; van Bokhoven & Brunner, 2002). Down-regulation of P63 in MEE of the palatal shelves is required for palatal fusion by promoting periderm migration and reducing the proliferative potential of MEE (Richardson et al., 2017). The immuno-staining of E-cadherin and P63 demonstrated that E-cadherin and P63 double-positive MEE cells disappeared from the contact region of the palatal shelves after 2 days of culture in the control group (Figure 1B). In contrast, persistent expression of E-cadherin and P63 was observed in MEE despite forced contact between the palatal shelves in the SB431542 and Pan-Ras-IN-1-treated groups. Since MAPK/ERK pathway is one of the downstream pathways of Ras proteins, we examined the effects of pan-Ras-IN on the expression of pERK in the organ cultures. Pan-Ras-IN-1 treated cultures showed substantially lower expression of pERK at the remaining MEE seam, as shown by immunohistochemical staining (Figure 1C). Interestingly, Yamamoto et al. demonstrated that EGF treatment of palatal explants prevented palatal fusion and MEK inhibitor rescued the EGF-induced inhibition of palatal fusion (Yamamoto, Cui, & Shuler, 2003). These
observations indicate that appropriate activation of the MAPK/ERK pathway is required for the normal process of palatal fusion. Western blotting demonstrated decreased expression of pERK in Pan-Ras-IN-1-treated cultures and pSmad2/3 in SB431542-treated cultures, respectively (Figure 1D). Taken together, our results indicated that Ras signaling is required for the dissociation of MEE, which is an essential process for palatal fusion.

**Pan-Ras inhibitor attenuates MEE cells migration in unpaired palatal explants.**

We previously established an unpaired palatal explants model using epithelial-GFP labeled mice for tracing the behavior of MEE cells at the developing secondary palate (Aoyama et al., 2019). In this unpaired palatal explants culture system, MEE cells can disappear from the medial edge of the single palatal shelf independently from palatal shelf contact and midline seam formation. Furthermore, the distribution and timing of this epithelial removal are closely similar to those of the disappearance of the MEE in vivo reported in previous studies (Charoenchaikorn et al., 2009; Takigawa & Shiota, 2007). While the unpaired palatal explants culture system has some limitations, this culture system enables us to monitor the epithelial behavior, including epithelial cell migration on the surface of the secondary palatal shelf. Therefore, we used the unpaired palatal explants model to further examine the role of Ras signaling in the cellular behavior of MEE cells during palatal fusion. The results showed that the MEE cells migration and the subsequent mesenchymal exposure were strongly disturbed in both the 20 μM SB431542-treated and the 20 μM pan-Ras-IN-treated groups (Figure 2A). In addition, frontal section confirmed that palatal shelves were covered with the intact MEE cells and E-cadherin expression was retained at the MEE region in both the
SB431542-treated and pan-Ras-IN-treated groups (Figure 2B). These results suggest that Ras signaling is required for the loss of E-cadherin that is an essential process of the MEE dissociation in palatogenesis.

*Pan-Ras inhibitor reduces apoptosis of MEE cells in organ culture.*

Apoptosis is also an important cellular event during MEE removal from the fusing palatal shelf. Next, we examined the effects of Pan-Ras inhibition on the apoptosis of MEE cells in palatal organ culture. TUNEL assays revealed that the number of TUNEL-positive cells in the remaining MEE cells was markedly reduced in the pan-Ras-IN-treated and SB431542-treated groups compared to the control group (Figure 2C). Ki67 immunostaining demonstrated that the proliferation activity was maintained in the remaining MEE cells in the pan-Ras-IN-treated and SB431542-treated groups (Figure 2D). Together, these results demonstrated that Pan-Ras inhibitor-treated MEE cells continued to proliferate and did not undergo cell death, and these observations are consistent with the effects of TGF-b RI kinase inhibitor application.

*Synergistic effects of TGF-b RI kinase inhibitor and pan-Ras inhibitor during palatal fusion*

We also conducted simultaneous application of SB431542 and Pan-Ras-IN-1 to examine the synergistic effects of TGF-β3 signaling and Ras signaling on palatal fusion in palatal organ culture. A single application of SB431542 at lower than 1 μM or Pan-Ras-IN-1 at lower than 5 μM failed to inhibit the fusion of palatal shelves (Figure 3A, B). However, simultaneous application of SB431542 at 0.5 μM and Pan-Ras-IN-1 at 5 μM attenuated palatal fusion by blocking the removal of MEE in palatal organ
culture (Figure 3C). These synergistic effects of SB431542 and Pan-Ras-IN-1 suggest a cooperative role of TGF-β3 signaling and Ras signaling in the dissociation of MEE during palatal fusion.

**Rreb1 is expressed in the palatal epithelium during palatogenesis.**

RREB1 has recently been shown to play a crucial role in developmental EMT during mid-gastrulation of embryonic development (Su et al., 2020). We hypothesized that RREB1 might be involved in the dissociation of MEE during palatal fusion. However, the expression pattern and the contribution of Rreb1 in palatogenesis have not been examined yet. Toward this end, we examined the expression patterns of *Rreb1* mRNA in palatogenesis. In whole-mount preparations at E14.0 and E14.5, *Rreb1* mRNA was expressed at the MEE region of the secondary palate and palatal rugae (Figure 4A). The expression of Rreb1 was also prominent at the fusing surface of the secondary palate (Figure 4B). These observations suggest that Rreb1 may play an essential role in the epithelium, including MEE, rather than the mesenchyme, of palatal shelves in palatogenesis.

**Pan-Ras inhibitor downregulates the expression of Rreb1 in palatal organ culture.**

Next, we examined the effects of Ras signaling inhibition on the expression of *Rreb1* in the palatal shelf during palatal fusion. Pan-Ras-IN treatment dramatically decreased the expression of *Rreb1* after 6 hours of inhibitor application (Figure 4C). Even after 24 hours of inhibitor application, *Rreb1* expression was still downregulated compared to the level in the control palatal shelf (Figure 4C). Consistently, Pan-Ras-IN treatment for 24 hours decreased RREB1 protein expression in the palatal shelf (Figure 4D). These
results indicate that Ras signaling is essential for the expression of *Rreb1* in palatal shelves.

**Knockdown of *Rreb1* prevents palate fusion in organ culture**

RNA interference has been utilized within a palate organ culture model to specifically and efficiently knock down the target gene with low toxicity. To examine the potential roles of *Rreb1* in MEE during palatal fusion, we used siRNA to knock down *Rreb1* in palatal shelf organ culture experiments. *Rreb1* knockdown cultures showed incomplete fusion of the palatal shelves (Figure 5A). The expression of E-cadherin and P63 was retained in the MEE seam of *Rreb1* knockdown cultures, as detected by immunohistochemical staining (Figure 5B). TUNEL staining demonstrated that the number of TUNEL-positive cells in the remaining MEE cells was substantially decreased in *Rreb1*-knockdown cultures compared to the control cultures. Ki67 immunostaining revealed that the proliferative activity was retained in the remaining MEE cells in *Rreb1* knockout cultures (Figure 5E, F). These effects of *Rreb1* knockdown in palate organ cultures were consistent with the effects observed in pan-Ras inhibitor-treated palate organ cultures.

The decreased expression of *Rreb1* mRNA and RREB1 protein in *Rreb1* knockdown palatal shelves was confirmed by qPCR or western blotting, respectively (Figure 5C, D). The expression of phosphorylated ERK was slightly decreased in *Rreb1* knockdown cultures (Figure 5D).
Discussion

For the cellular mechanisms underlying MEE removal in palatal fusion, MEE seam degradation and subsequent MEE migration has been proposed as the major model. TGF-β has been extensively examined as the central signaling molecule in cancer and developmental EMT. However, it has been shown that the activation of TGF-β signaling is insufficient for the complete EMT (Safina et al., 2009). Ras signaling is also well known to modulate the EMT process, particularly in cancer (Rajalingam et al., 2007). Interestingly, the cooperative roles of Ras and TGF-β-Smad signaling in EMT have been reported previously in human cancer cells and human keratinocyte HaCaT cells. p63 is a critical regulator of epithelial cell proliferation and differentiation and acts downstream of TGFβ-Smad and Ras signaling. It was reported that the ΔNp63 isoform, having the opposite function to p63, is necessary for the activation of the Ras signaling-dependent EMT gene program induced by TGF-β in multiple breast cancer cells (Sundqvist et al., 2020). While the role of EMT in palatal fusion remains controversial, the dissociation of MEE at least partly shares morphological and molecular mechanisms with EMT. In palatogenesis, TGF-β3-induced down-regulation of p63 in the MEE is required for palatal fusion by promoting MEE migration and reducing the proliferative potential of the MEE seam (Richardson et al., 2017). Note that down-regulation of p63 in Tgfb3−/− mice can restore MEE cells’ fate. In the present study, we demonstrated that Ras signaling via its effector RREB1 regulates the dissociation of MEE and the subsequent migration of MEE cells during palatogenesis (Figure 6). In this context, the recent indication of the TGF-β signaling and Ras signaling cross-talk through the interaction between Smad and RREB1 in the process of palatal fusion is of particular interest.
Ras signaling mediates receptor tyrosine kinase (RTK) signaling, including FGF, PDGF, and EGF signaling. FGFR1 and FGFR2 knockout mice models demonstrated the crucial of FGF signaling for cell proliferation in the growth of palatal shelves (Hosokawa et al., 2009; Kawai et al., 2019). PDGFR-a mutant mice developed cleft palate due to a defect of the palatal shelf growth associated with the reduced extracellular matrix production. Unlike FGFR and PDGFR signaling, EGFR signaling is primarily crucial in the palatal epithelium during palatal fusion. While the penetration of the cleft palate phenotype is not high, EGFR knockout mice show cleft palate due to defects of the MEE seam disruption (Miettinen et al., 1999). In addition, the involvement of EGFR signaling in EMT has been reported in cancer progression and metastasis (Lo et al., 2007). Thus, EGFR might be a major candidate receptor involved in the activation process of Ras-Rreb1 signaling during palatal fusion.

While cleft palate phenotype is not a major clinical feature in RASopathies, there are some indications regarding the association of Ras signaling with the pathogenesis of cleft palate in humans. Couser et al. reported that some cases exhibited cleft lip and/or palate phenotype in Noonan-like syndrome. High and narrow palate with submucous clefting is a typical craniofacial feature in Cardiofaciocutaneous syndrome, which is caused by mutations in KRAS, BRAF, MAP2K1, and MAP2K2 (Chrzanowska, Fryns, & Van den Berghe, 1989). In addition, microdeletions within chromosome 22q11.2 exhibit haplo-insufficient ERK2 expression and reportedly cause cleft palate (Ben-Shachar et al., 2008).

A limitation of the present study is our lack of use of a genetically engineered mouse with Rreb1 defect for the analysis of palatal fusion. The conventional knockout mice of Rreb1 exhibit lethality at early embryonic development (Morgani, Su, Nichols,
Massagué, & Hadjantonakis, 2021; Su et al., 2020). Thus, we cannot examine the cleft palate phenotype in Rreb1 knockout mice using the conventional knockout mouse model. While conditional knockout mice targeting Rreb1 using the Cre/loxP system have not been generated yet, we may be able to test the phenotype of epithelial-specific knockout of Rreb1 in the future. While our approaches using small molecular inhibitor and RNAi in palate organ cultures cannot completely rule out non-specific off-target effects, these approaches have been accepted as an alternative method when a genetic mouse model is not available. Another limitation of the study is the use of MEE cells for in vitro cell biological experiments. To the best of our knowledge, there are no available MEE cells or normal oral epithelial cells for analyzing the molecular mechanism of MEE dissociation in palatogenesis. Establishing MEE cells that can mimic MEE dissociation in palatal fusion would be an excellent approach to examine the molecular interactions and signaling cross-talk in palatogenesis.

In conclusion, the present study demonstrated that Ras signaling is essential for dissociation of MEE during palatogenesis. RREB1, a known transcriptional factor that acts downstream of Ras signaling, is expressed in the MEE region and required for the dissociation of MEE during palatal fusion. With these findings, we provide essential information on the requirement of the Ras/RREB1 signaling axis in the dissociation of MEE in palatogenesis. However, the upstream activators of Ras signaling and the downstream molecules which activate RREB1 during palatal fusion remains to be seen.
Materials and methods

Animals

We used transgenic mice in which GFP was expressed under the control of the Cytokeratin-14 promoter (K14-GFP) (Vaezi, Bauer, Vasioukhin, & Fuchs, 2002). Mice expressing the transgene were identified by the green fluorescent glow of the skin surface. Mature female mice of C57BL/6J (CLEA, Tokyo, Japan) were mated overnight with a K14-GFP male mouse, and the day on which a vaginal plug was found was designated as day 0 of pregnancy. Time course observation of palatal shelf development was performed by dissecting out K14-GFP mice maxilla from E14.0–E15.5.

Dissection and organ culture

On E14, wild-type C57BL/6J mouse embryos were quickly immersed in BGJb medium (Gibco). The palatal shelves were removed using forceps under a dissecting microscope. Isolated palatal shelves were placed in pairs on 0.4 μm porosity Millipore filters (Millipore Corporation, USA), nasal epithelium down, media edges in contact, on 35 mm-tissue culture dishes (FALCON, France). The culture medium was composed of BGJb medium with or without Pan-ras-IN-1 and/or SB431542. Pan-ras-IN-1 was purchased from MedChemExpress (MCE, USA). SB431542 and/or 0.5, 1.0, 2.0, 5.0, 10 or 20 μM SB431542(Sigma, USA). Samples were pretreated for 6h prior to the organ culture. Palatal cultures were performed at 37 °C with 5% CO2. All the medium and treatment solutions were replaced every 24 h.
Time-Lapse Imaging and Quantitative Analysis

Time-lapse images of explant cultures were captured using an all-in-one fluorescence microscope (BZ-X700, Keyence, Osaka, Japan), equipped with filters for GFP (excitation: 475 nm, emission: 525 nm) and DAPI (excitation: 360 nm, emission: 460 nm) channels. The instrument was controlled by the BZ Viewer version 1.0 software of the microscope (Keyence, Osaka, Japan).

Study approval

All of the animal experiments were performed in strict accordance with the guidelines of the Animal Care and Use Committee of the Osaka University Graduate School of Dentistry, Osaka, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of Osaka University Graduate School of Dentistry. Mice were housed in the animal facility at the Department of Dentistry, Osaka University. Welfare guidelines and procedures were performed with the approval of the Osaka University Graduate School of Dentistry Animal Committee.

Assessment of palatal fusion and histological analysis

The palatal phenotypes were first evaluated with a dissecting microscope. For histology, dissected samples were fixed in 4% paraformaldehyde, equilibrated in graded sucrose, and embedded in Tissue-Tek (OCT compound, Sakura). The tissue samples were sectioned into 15 μm slices.
**Immunohistochemistry and TUNEL staining**

Immunostaining of frozen sections was performed as previously described (Inubushi, Nozawa, Matsumoto, Irie, & Yamaguchi, 2017). The following antibodies were used in this study: polyclonal rabbit anti-E Cadherin (1:50, ab15148, Abcam), monoclonal rabbit anti-Ki67 (1:50, ab16667, Abcam), monoclonal mouse anti-P63 (1:50, #B1320, Santa Cruz Biotechnology), Alexa 488-labeled goat anti-rabbit IgG (#A11034, 1:500), Alexa 555-labeled goat anti-mouse IgG (#A21422, 1:500), and Alexa 555-labeled goat anti-rabbit IgG (#A21428, 1:500) from Invitrogen. Apoptotic cells were identified by using an *in situ* cell death detection kit (Roche, catalog #11684795910) according to the manufacturer’s instructions.

**Immunoblotting**

Protocols for immunoblotting were described previously (Inubushi, Lemire, Irie, & Yamaguchi, 2018). Briefly, cells were lysed in ice-cold RIPA buffer containing 50 mmol/L Tris-HCl Buffer (pH 7.6), 150 mmol/L NaCl, 1% Nonidet P40 Substitute, 0.5% sodium deoxycholate and 0.1% SDS. Protease inhibitor cocktail was purchased from Promega (Germany). Following a 30 min lysis period on ice, lysis samples were centrifuged at 15,000 rpm for 20 min at 4°C to prepare cell lysates. Ten μg of lysate was subjected to SDS-PAGE on an 8–16% Tris-glycine gel (Invitrogen), followed by electroblotting onto an Immobilon PVDF membrane (EMD Millipore). The ECL Western Blotting Substrate (Nacalai Tesque, #07880) was used to detect signals. The following antibodies were used in this study: monoclonal mouse anti-RREB1 (1:200, B-7, Santa Cruz Biotechnology), monoclonal rabbit anti-pSmad2/3 (1:1000, #8685, Cell Signaling Technology), monoclonal rabbit anti-Smad2/3 (1:1000, #8685, Cell Signaling
Technology), monoclonal rabbit anti-pERK (1:1000, #4370, Cell Signaling Technology), polyclonal rabbit anti-ERK (1:1000, #9102, Cell Signaling Technology), α-tubulin (1:1000, T6074, Sigma), horseradish peroxidase-conjugated goat anti-rabbit IgG (#1706565, 1:500) and goat anti-mouse IgG (#1706516, 1:500) from Bio-Rad.

**siRNA Transfection in Palatal Culture System**

E13.5 palatal shelves were pre-cultured in serum and antibiotic-free medium for 4 hours. The medium was then replaced with siRNA-containing medium according to the manufacturer’s instructions. Transfection Reagent (Lipofectamine RNAiMax, Invitrogen) was mixed with siRNA targeting mouse RREB1 or scrambled control (Silencer Select siRNAs, Thermofisher). We used two different siRNAs for mouse RREB1. Cultured palatal tissues were harvested after 48 hours for organ culture, immunoblotting or real-time PCR procedures, respectively.

**RNA extraction and qPCR analysis**

The E14.0 palatal shelves were incubated with or without RAS inhibitor for 6 hours and 24 hours. After the cultures, we dissected only the MEE region as much as possible using forceps under a dissecting microscope. Protocols for RNA extraction and qPCR analysis were described previously (Inubushi et al., 2012). Total RNA was extracted from the dissected tissues using IsogenII (Nippon Gene) according to the manufacturer's protocol, then reverse transcribed to cDNA using oligo (dT) with reverse transcriptase (Takara). For real-time PCR, aliquots of total cDNA were amplified with the TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Data acquisition and analysis were performed with a Step One Real-Time PCR System using
Step One Software, Version 2.1 (Applied Biosystems). The PCR products were quantified using *gapdh* as the reference gene. The primers and TaqMan probes were purchased from Applied Biosystems.

**Whole-mount in situ hybridization**

Whole-mount *in situ* hybridization was performed using fixed E14.0, E14.5 and E15.0 palates. The digoxigenin-labeled RNA probes were prepared using a DIG RNA labeling kit according to the manufacturer's protocol (Roche) employing each cDNA clone as the template. The probes were synthesized from fragments of *Rreb1* (Allen Institute for Brain Science; https://alleninstitute.org/) and amplified with T7 and SP6 adaptor primers through PCR, as described previously (Sarper et al., 2018). After hybridization, the signals were visualized according to their immunoreactivity with anti-digoxigenin alkaline phosphatase-conjugated Fab fragments (Roche).

**Statistics**

Statistical methods were not used to predetermine sample size. Statistical analyses were performed with GraphPad Prism 8. Student’s two-sided t-test and two-way ANOVA were used under the assumption of normal distribution and observance of similar variance. A p-value of <0.05 was considered significant. Bonferroni post hoc analysis was performed where applicable. Values are expressed as mean ± SD. For all of these experiments, variances between groups were similar and data were symmetrically distributed. Data shown are representative images; each analysis was performed on at least three mice per genotype. Immunostaining was performed at least in triplicate. For
other experiments, the numbers of biological replicates, animals, or cells are indicated in the text.

**Acknowledgements**

We thank Ms Yuki Okamoto and Akiko Okura for valuable assistance in the histological, molecular and protein work.

**Funding**

This work was supported by grants-in-aid for scientific research programs from the Japan Society for the Promotion of Science (#19KK0232, #20H03896 to T.I. and #21K17159 to G.A.).

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Figure 1. *Pan-Ras inhibitor prevents the palate fusion in organ culture*

(A) Palatal shelf organ cultures were performed for 48 hours using a pair of the dissected shelves from wild-type mice at E14.5. Dissected shelves were pretreated with SB431542 (20uM), PAN-RAS-IN-1 (20uM) or DMSO for 6h prior to the organ cultures.
Histological sections of horizontal palatal shelves showed the complete fusion defects of the palatal shelves, and the remaining MEE in SB431542 treated and PAN-RAS-IN-1 treated groups. Arrowheads indicate the contact region of the paired shelves. The lower panel displays the incidence of palatal fusion in each treatment group. (B) The persistent expression of E-cadherin and P63 was observed in the MEE region despite forced contact between the palatal shelves in SB431542 and Pan-Ras-IN-1 treated groups. (C) The overall expression of phosphorylated ERK (pERK) is decreased in Pan-Ras-IN-1 treated group. Note that the positive expression was observed at the oral epithelial triangle region in the control culture (arrowhead). In contrast, the Pan-Ras-IN-1 treated cultures show substantially lower expression of pERK at the remaining MEE seam. (D) Western blotting confirmed the decreased expression of pERK in Pan-Ras-IN-1 treated cultures. The expression of pSmad2 is decreased by SB431542 treatment in cultured palatal shelves. pp, primary palate; sp, secondary palate. Scale bar: 200 μm. The experiments in B, C, and D were performed at least 3 times with similar results.
Figure 2. Pan-Ras inhibitor attenuates MEE cells migration and apoptosis in unpaired palatal explants.

Epithelial behavior in unpaired palatal explant model. (A) Fluorescence microscopic images showing the view of medial edges of the unpaired palatal explant model after 0, 6, and 18 hours of culture. White dotted lines indicate the area of mesenchymal exposure. SB431542 (20uM) and pan-Ras-IN (20uM) treatment completely inhibited the migration of MEE cells and subsequent mesenchymal exposure. (B) Frontal section of the palatal shelf of K14-GFP mice after 18 hours of unpaired culture. E-cadherin
expression is retained at the MEE region in both SB431542 treated and Pan-Ras-IN treated groups. (C) Analysis of cell death after 24 hours of palatal shelf organ cultures. The number of TUNEL-positive cells in remaining MEE cells (arrowheads) is markedly reduced in pan-Ras-IN treated and SB431542 treated groups. (D) Analysis of cell proliferation after 24 hours of palatal shelf organ cultures. The proliferation activity is maintained in the remaining MEE cells (arrowheads) in pan-Ras-IN treated and SB431542 treated groups. A, anterior; P, posterior. Scale bars: 200 μm in A, B; 100 μm in C, D. These experiments were performed at least 3 times with similar results.
Figure 3. Synergistic effects of TGF-β RI kinase inhibitor and pan-Ras inhibitor during palatal fusion

Palatal shelf organ cultures were performed for 48 hours using a pair of the dissected shelves from wild-type mice at E14.5 with specific inhibitor or DMSO as described below. (A) Dissected shelves were pretreated with SB431542 at 5 μM, 2 μM, 1 μM, 0.5
uM or DMSO for 6h prior to the organ cultures. (B) Pretreated with Pan-Ras-IN-1 at 10 uM, 5 uM or DMSO was performed 6h prior to the organ cultures. (C) Simultaneous treatment with SB431542 and Pan-Ras-IN-1 at the indicated concentration were performed 6h prior to the organ cultures. Histological sections of horizontal palatal shelves were stained with Hematoxylin and eosin (HE). Arrowheads indicate the contact region of the paired shelves in fused palatal shelves. The lower panel displays the incidence of palatal fusion in each treatment group. Scale bar: 200 μm.
Figure 4. *Rreb1 express in the palatal epithelium during palatogenesis*

(A) Whole-mount *in situ* hybridization of *Rreb1* in the developing palate of the wild type at E14.0 and E14.5. *Rreb1* was widely distributed in the MEE region of the secondary palate along the AP axis and palatal rugae at E14.0. The expression of *Rreb1* was also prominent in the midline region of the fusing secondary palate. Arrowheads indicate the MEE region of the secondary palate. Box denotes the MEE region of the
fusing palatal shelves. (B) Frontal section of the anterior and posterior region of palatal shelves in wild-type. \textit{Rreb1} was specifically expressed in oral epithelium, including fusing MEE at the anterior and posterior region. ns, nasal septum; pp, primary palate; sp, secondary palate. Scale bars: 500 μm. (C) The expression of \textit{Rreb1} in palatal shelf organ cultures with or without Pan-Ras-IN-1 at 20 μM. \textit{Rreb1} expression was significantly decreased after 6 and 24 hours of Pan-Ras-IN-1 application. The expression of \textit{Rreb1} was evaluated by qPCR. Gapdh was used as an internal control for normalization. Means ± SD (\textit{n} = 3) are shown as horizontal bars. \textit{P} values were determined by two-way ANOVA. *\textit{P} < 0.01. (D) Palatal shelves from organ cultures with or without Pan-Ras-IN-1 at 20 μM for 24 hours were lysed in RIPA buffer. Cell lysates were immunoblotted with antibodies to RREB1 and α-tubulin, respectively.
Figure 5. Knockdown of Rreb1 prevent the palatal fusion in organ culture

(A) Knockdown of Rreb1 by siRNA was performed using dissected shelves 48 hours prior to the paired palatal shelf organ culture. Then, the paired palatal shelf was cultured for 24 hours. Histological sections of horizontal palatal shelves showed the fusion defects of the palatal shelves and the remaining of the MEE in the Rreb1 knockdown group. Histological sections of horizontal palatal shelves were stained with...
Hematoxylin and eosin (HE). Arrowheads indicate the contact region of the paired shelves. The incidence of fusion of palatal shelves in each treatment group was shown at the lower panel. (B) The persistent expression of E-cadherin and P63 was observed at the contacting palatal shelves in the Rreb1 knockdown group. (C) Knockdown of Rreb1 by siRNA was performed using dissected shelves 48 hours. RNA was extracted from palatal shelves in each group. The expression of Rreb1 was evaluated by qPCR. Gapdh was used as an internal control for normalization. Means ± SD (n = 3) are shown as horizontal bars. P values were determined by two-way ANOVA. *P < 0.01. (D) Palatal shelves from control culture and Rreb1 knockdown culture were lysed in RIPA buffer. Cell lysates were immunoblotted with antibodies to RREB1, p63, phosphorylated ERK (pERK), ERK and α-tubulin, respectively. This experiment was performed 3 times with similar results. (E, F) Analysis of cell death and cell proliferation in palatal shelf organ cultures. Control and Rreb1 knockdown palatal shelves were cultures for 24 hours. (E) The number of TUNEL-positive cells in remaining MEE cells (arrowheads) is markedly reduced in Rreb1 knockdown cultures. (F) The proliferation activity is maintained in the remaining MEE cells (arrowheads) in Rreb1 knockdown cultures. Scale bar: 200 μm in A, B; 100 μm in E, F.
Figure 6. Schematic of the key findings. Ras signaling and its effector RREB1 are required for the dissociation of MEE seam in palatogenesis.