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Rgs19 regulates mouse palatal fusion by modulating cell proliferation and apoptosis in the MEE

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ABSTRACT

Palatal development is one of the critical events in craniofacial morphogenesis. During fusion of the palatal shelves, removal of the midline epithelial seam (MES) is a fundamental process for achieving proper morphogenesis of the palate. The reported mechanisms for removing the MES are the processes of apoptosis, migration or general epithelial-to-mesenchymal transition (EMT) through modulations of various signaling molecules including Wnt signaling. RGS19, a regulator of the G protein signaling (RGS) family, interacts selectively with the specific α subunits of the G proteins (Gαi, Gαq) and enhances their GTPase activity. Rgs19 was reported to be a modulator of the Wnt signaling pathway. In mouse palatogenesis, the restricted epithelial expression pattern of Rgs19 was examined in the palatal shelves, where expression of Wnt11 was observed. Based on these specific expression patterns of Rgs19 in the palatal shelves, the present study examined the detailed developmental function of Rgs19 using AS-ODN treatments during *in vitro* palate organ cultivations as a loss-of-function study. After the knockdown of Rgs19, the morphological changes in the palatal shelves was examined carefully using a computer-aided three dimensional reconstruction method and the altered expression patterns of related signaling molecules were evaluated using genome wide screening methods. RT-qPCR and *in situ* hybridization methods were also used to confirm these array results. These morphological and molecular examinations suggested that Rgs19 plays important roles in palatal fusion through the degradation of MES via activation of the palatal fusion related and apoptotic related genes. Overall, inhibition of the proliferation related and Wnt responsive genes by Rgs19 are required for proper palatal fusion.

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Abbreviations: MEE, medial edge epithelium; MES, midline epithelial seam; EMT, epithelial-to-mesenchymal transition.

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1. Introduction

The palatal shelves originate bilaterally from the internal surface of the maxillary prominence. They are positioned and grow vertically on each side of the tongue. After growth, the subsequent palatal shelves elevate and fuse to a horizontal position above the tongue. They are fused with one another from the middle region to the adjacent regions, and the medial edge epithelia (MEE) then transforms to the midline epithelial seam (MES) (Chou et al., 2004; Martinez-Alvarez et al., 2000). The failure of these harmonized procedures can result in birth defects, such as a cleft lip and palate (Gritli-Linde, 2007; Lee et al., 2008; Liu et al., 2005; Taniguchi et al., 1995; Yu et al., 2009).

A cleft palate is a defect of craniofacial development and results from a dysfunction of palatal growth, shelf elevation and a MEE disappearance between the two secondary palatal shelves (Kang and Svoboda, 2002). The fate of the MEE, which forms the MES after palatal shelf fusion, is unclear. The three main mechanisms that account for the loss of MEE would be apoptosis (Cuervo and Covarrubias, 2004), epithelial migration (Jin and Ding, 2006) and an epithelial-to-mesenchymal transition (EMT) (Nawshad, 2008; Nawshad and Hay, 2003). Previously, the interactions between Wnt11 and Fgfr1b, which are key factors in convergent extension, modulate palatal cell proliferation for growth and apoptosis to fuse the palatal shelves (Kemler et al., 2004; Lee et al., 2008; Logan et al., 1999 In addition to these epithelial-mesenchymal interactions, several significant signaling pathways including TGFβ, BMP, FGF and Notch would involve in palatogenesis (Gritli-Linde, 2007; Lee et al., 2008; Liu et al., 2005; Taniguchi et al., 1995; Yu et al., 2009). In particular, during palate fusion, TGFβ3, Snail and Lef1 were established as essential growth and transcription factors inducing the removal of MES during palatal fusion (Nawshad, 2008; Nawshad and Hay, 2003). The Wnt family of secreted cytokines have been reported to be functionally important signaling molecules during palatogenesis (Chiquet et al., 2008; He et al., 2008; Lee et al., 2008; Warner et al., 2009). In many developmental processes, such as gastrulation and ectodermal formation of blastocysts, EMT is induced by the Wnt/β-catenin signaling pathways (Kemler et al., 2004; Logan et al., 1999).

The regulator of the G protein signaling (RGS) family consist of more than 20 members (De Vries et al., 2000). The RGS proteins play the role of GTPase and interact with the GTP-Gα subunits to limit their lifespan and terminate the downstream signaling pathways (Hollinger and Hepler, 2002; Ross and Wilkie, 2000). RGS19 is one of the first RGS members discovered and interacts selectively with the specific α subunits of the G proteins (Gαi, Gαq) and enhances their GTPase activity (De Vries et al., 1995). In addition, Rgs19 was examined as a regulator that attenuates Wnt signaling via inactivation of the Gαo subunit (Feigin and Malbon, 2007). In addition, a recent report showed that Rgs19 affects cardiac development and has negative effects on the heart function (Ji et al., 2010). Restricted Rgs19 expression was detected in the MEE of the palatal shelves in which Tgfβ3 and Wnt11 were expressed (Yang et al., 2008; Lee et al., 2008).

After knocking down of Rgs19, the morphological changes in palatal fusion and the altered expression patterns of the signaling molecules were examined using a genome wide screening method. The expression patterns of the candidate genes were then confirmed by RT-qPCR and *in situ* hybridization. In addition, the altered cellular events, such as cell proliferation and apoptosis, were evaluated to define the presumptive roles and signaling pathways of Rgs19 in palatogenesis.

2. Materials and methods

All experiments were performed according to the guidelines of the Kyungpook National University, School of Dentistry, Intramural Animal Use and Care Committee.

2.1. Animals

Adult ICR mice were housed in a temperature-controlled room (22 °C) under artificial illumination (lights on from 05:00 to 17:00), at 55% relative humidity, with free access to food and water. Mouse embryos were obtained from time-mated pregnant mice. The day on which a vaginal plug was confirmed then designated as embryonic day 0 (E0). We collected the time-designated embryos based on the morphological criteria, provided from the "the mouse atlas project" (http://www.emouseatlas.org).

2.2. In vitro organ culture

Procedures for *in vitro* organ culture were carried out at E13 for 1 or 2 days as described previously (Sohn et al., 2011). After the removal of brain forming tissues, dissected maxilla region including palatal shelves were cultivated in a 8-ml of BGJb medium containing 1% penicillin–streptomycin for 1 or 2 days in a 50-ml penicillin bottle. Three or four explants were put into one bottle, and the bottle was sealed airtightly using a rubber stopper and a metal clamp. The bottles were flushed for approximately 2 min with a gas mixture of 50% O_2, 45% N_2, and 5% CO_2. The bottles were incubated at 37 °C on a roller device (20 rpm) for 1 or 2 days. The culture bottles were flushed every 24 h with the same gas mixture. For Trowell's modified cultivations, palatal shelves were isolated from E13 mouse maxillae and cultured in medium without fetal bovine serum at 37 °C and 5% CO_2 for 1 or 2 days (Lee et al., 2008). The culture medium (DMEM/F12, Gibco) was supplemented with 20 μg/ml ascorbic acid (Sigma) and 1% penicillin/streptomycin and was renewed every 24 h.

2.3. Antisense-oligodeoxynucleotide (AS-ODN) treatments

AS-ODNs for Rgs19 were designed and treated during *in vitro* rolling organ culture (Hoffman et al., 2002; Kim et al., 2005; Mailleux et al., 2001). ODNs were designed as AS-ODN Rgs19 5′-TGTTTCTCAGCCTCATGT-3′, Sense (S)-ODN 5′-ACATGAGGCTGAGAAACA-3′. FITC-tagged Rgs19 AS-ODNs were used to detect the penetration level of AS-ODNs into the palate tissue. These ODNs were purchased from GENOTECH