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Overexpression of Smad2 in Tgf-β3-null mutant mice rescues cleft palate

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Abstract

Transforming growth factor (TGF)-β3 is an important contributor to the regulation of medial edge epithelium (MEE) disappearance during palatal fusion. SMAD2 phosphorylation in the MEE has been shown to be directly regulated by TGF-β3. No phospho-SMAD2 was identified in the MEE in Tgf-β3-null mutant mice (Tgf-β3$^{-/-}$), which was correlated with the persistence of the MEE and failure of palatal fusion. In the present study, the cleft palate phenotype in Tgf-β3$^{-/-}$ mice was rescued by overexpression of a Smad2 transgene in Keratin 14-synthesizing MEE cells following mating Tgf-β3 heterozygous mice with Keratin 14 promoter directed Smad2 transgenic mice (K14-Smad2). Success of the rescue could be attributed to the elevated phospho-SMAD2 level in the MEE, demonstrated by two indirect evidences. The rescued palatal fusion in Tgf-β3$^{-/-}$/K14-Smad2 mice, however, never proceeded to the junction of primary and secondary palates and the most posterior border of the soft palate, despite phospho-SMAD2 expression in these regions at the same level as in the middle portion of the secondary palate. The K14-Smad2 transgene was unable to restore all the functional outcomes of TGF-β3. This may indicate an anterior–posterior patterning in the palatal shelves with respect to TGF-β3 signaling and the mechanism of secondary palatal fusion.

Keywords: TGF-β3; Smad2; Keratin 14; Overexpression; Mouse; Cleft palate; Palatal fusion; Medial edge epithelium; Epithelial–mesenchymal transformation; Rescue

Introduction

Transforming growth factor (TGF)-β3 has been shown to play an important role in the events regulating the disappearance of the medial edge epithelium (MEE) during palatal fusion. Tgf-β3-null mutant (Tgf-β3$^{-/-}$) mice have a cleft palate that is the principal developmental defect visualized at birth in these mice (Kaartinen et al., 1995; Proetzel et al., 1995). Previous studies suggested that all Tgf-β3$^{-/-}$ mice exhibited cleft palate, however, the severity of the cleft varied between different mouse genetic backgrounds (Kaartinen et al., 1995; Koo et al., 2001; Proetzel et al., 1995). The cleft palate phenotype was most severe on the C57BL/6 background, in which about 50% of Tgf-β3$^{-/-}$ mice had a complete cleft while the remainder had partial clefts. Thus, Tgf-β3 knockout mice with a C57BL/6 background have been most often used in the analysis of secondary palate development (Blavier et al., 2001; Cui et al., 2003; Gato et al., 2002; Kaartinen et al., 1997; Martínez-Álvarez et al., 2000a,b; Taya et al., 1999).

The TGF-β family initiates signaling by triggering a phosphorylation cascade initiated by TGF-β receptors (TβRs) and members of the SMAD family. SMAD2/3 are phosphorylated by the TβR complex and then bind to SMAD4. The SMAD2/3–SMAD4 complex is translocated into the nucleus to participate in an alteration of gene expression (Heldin et al., 1997; Massagué, 1998). Previously, we examined the distribution pattern of TGF-β3, TβRs, and SMADs in the developing palate to understand the restriction of TGF-β3 signaling to the MEE in a

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developmental stage specific manner (Cui and Shuler, 2000; Cui et al., 1998, 2003). We found that the expression pattern of TGF-β3, receptors TβR-I and-II, and SMAD2 were consistent. They were localized predominantly to the epithelium covering the palatal shelves, which included the palatal oral epithelium, palatal nasal epithelium, and MEE before and during palatal shelf contact in the midline (Cui and Shuler, 2000; Cui et al., 1998, 2003). Expression of TβR-III (betaglycan) and SMAD2 phosphorylation, however, was shown to be spatially restricted to the MEE and temporally correlated with the disappearance of the MEE during palatal fusion. Both TβR-III and phospho-SMAD2 were present in the MEE immediately prior to palatal shelf contact in the midline, and both retained an exclusive distribution in the MEE as fusion progressed (Cui and Shuler, 2000; Cui et al., 2003). The results suggested that TGF-β3 exerted its developmental role in palatal fusion in an autocrine manner through TβR-I and-II binding. The role of TβR-III was hypothesized to localize and mediate TGF-β3's effect on MEE by specific presentation in the MEE. As a consequence, SMAD2 phosphorylation was spatially restricted to the MEE. In Tgf-$\beta3^{-/-}$ mice, the palate had a typical distribution of TGF-β1 and-β2 ligands and receptors (Taya et al., 1999), however, no phosphorylated SMAD2 was identified in the MEE (Cui et al., 2003). The absence of phospho-SMAD2 was correlated with the persistence of the MEE and failure of palatal fusion in Tgf-$\beta3^{-/-}$ mice. The results suggested that TGF-β3 was required for initiating and maintaining SMAD2 phosphorylation in the MEE that was required for alterations in gene expression necessary to complete palatal fusion. The endogenous TGF-β1 and-β2 alone were unable to trigger the SMAD2 phosphorylation to rescue the MEE fate in vivo (Cui et al., 2003).

One strategy hypothesized to rescue the cleft palate phenotype in Tgf-β3-deficient mice was to achieve MEE-specific overexpression of Smad2 in the Tgf-$\beta3^{-/-}$ MEE. The Keratin 14 promoter driving Smad2 expression (K14-Smad2) in the MEE appeared to be capable of providing overexpression of Smad2 in the MEE. It has been shown that in K14-Smad2 transgenic mice there was overexpression of Smad2 mRNA and translation of the Smad2 mRNA into protein in Keratin 14-synthesizing epidermal cells (Ito et al., 2001). The overexpressed SMAD2 was phosphorylated and translocated into the nucleus, regulated TGF-β target gene expression, and triggered a positive feedback on TGF-β signaling in a pathway-specific manner (Ito et al., 2001). Keratin 14 has been shown to have high levels of expression in the MEE in embryonic palatal epithelial sheet cultured in vitro by immunohistochemistry (Carette et al., 1991). We thus hypothesized that overexpression of K14-Smad2 in the MEE of Tgf-β3-null mutants could rescue the cleft palate phenotype via a positive induction of the TGF-β signaling pathway. This approach would bypass an initial requirement for TGF-β3 ligand binding to the TGF-β receptors to initiate SMAD2 phosphorylation in the MEE.

The goal of this study was to attempt rescue of the cleft palate phenotype in the Tgf-$\beta3^{-/-}$ mice by overexpression of a Smad2 transgene in the MEE following mating Tgf-β3 heterozygous mice (Tgf-$\beta3^{tm1Doe}$) with mice carrying the Smad2 transgene-driven by the K14 promoter (K14-Smad2).

Materials and methods

Animals and genotyping

Tgf-$\beta3^{tm1Doe}$ heterozygous mice (Tgf-$\beta3^{+/-}$) had been backcrossed for at least 12 generations onto the C57BL/6J background (Jackson Laboratories, Bar Harbor, ME). Tgf-$\beta3^{+/-}$ mice were bred to generate Tgf-$\beta3^{-/-}$ newborns to determine the palatal phenotype in highly backcrossed C57BL/6J genetic background of the Tgf-β3 knockout mice.

K14-Smad2 transgenic mice were generously provided by Dr. Yang Chai (Ito et al., 2001). Tgf-$\beta3^{tm1Doe}$ heterozygous mice were mated with K14-Smad2 mice to generate a Tgf-$\beta3^{+/-}$/K14-Smad2 line, which was then crossmated to produce Tgf-$\beta3^{-/-}$/K14-Smad2 newborns to determine whether MEE-directed overexpression of a Smad2 transgene could rescue the cleft palate phenotype in Tgf-$\beta3^{-/-}$ mice.

The genotype of the mice was determined by PCR using genomic DNA extracted from tail biopsies of either adults or newborns. Primers used to detect the Tgf-β3 knockout were located in intron 5 and intron 6 of Tgf-β3 (Taya et al., 1999). Primers used to detect the K14-Smad2 transgene were designed to be specific for the K14 region (Ito et al., 2001).

Fetuses obtained from timed pregnant C57BL/6J mice were used to immunolocalize phospho-SMAD2 in the developing palate. Fetuses were obtained between embryonic (E) days 14.5 and 15.5 as previously described (Cui et al., 1998).

Assessment of palatal fusion

Newborn mice were collected immediately post partum. Neonatal heads were immediately dissected. The jaw and tongue were removed to evaluate both hard and soft palate by direct observation with a dissecting microscope. Palatal fusion was scored on a 0 to 13 point scale. The criteria for this scale are as follows. (i) The nine rugae were used to establish landmarks to locate specific fusion positions on the palatal shelves (Sakamoto et al., 1991). The starting and ending fusion points were documented by the position of ruga. The total number of rugae fused was scored. For example, if partial fusion starts at the 3rd ruga and ends at the 5th ruga, the score is 3. There are a total of 9 points equaling fusion at all 9 rugae that can be assigned for fusion of the secondary hard palate. (ii) The pterygoid