Reproductive Toxicology 50 (2014) 129–133

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Short communication

Strain-dependent effects of transforming growth factor-β1 and 2 during mouse secondary palate development

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ARTICLE INFO

Article history:
Received 9 September 2014
Received in revised form 15 October 2014
Accepted 23 October 2014
Available online 1 November 2014

Keywords:
Secondary palate
TGF-β1
TGF-β2
Cleft palate

ABSTRACT

Cleft palate is a common birth defect affecting 1 in 700 births. Transforming growth factor-βs (TGF-βs) are important signaling molecules, and their functions in murine palate development have received great attention. TGF-β3 is expressed exclusively in palatal epithelial cells and mediates epithelial fusion, whereas the importance of TGF-β1 and 2 in palate have not yet been demonstrated in vivo, since inactivation of *Tgf-β1* or *Tgf-β2* genes in mice did not reveal significant palate defects. We hypothesized that TGF-β1 and TGF-β2 can compensate each other during palate formation. To test this, we generated *Tgf-β1* and *Tgf-β2* compound mutant mice and found that approximately 40% of [$Tgf\text{-}\beta1^{+/-}$; $Tgf\text{-}\beta2^{-/-}$] compound mutant embryos display cleft palate on C57 background. In addition, 26% of $Tgf\text{-}\beta2^{-/-}$ embryos on 129 background, but not in C57 or Black Swiss, displayed cleft palate. TGF-β1 and 2 functions are required for murine palate development in strain-dependent manner.

1. Introduction

Cleft palate is a common birth defect with a frequency as high as 1:700 births [1]. During mammalian embryonic development, the formation of a complete and functional palate shelf is a multi-step process involving palatal shelf initiation, vertical growth, and elevation followed by horizontal growth and fusion [2–4].

In the past decades, studies on mouse secondary palate development have led to significant insights into the molecular mechanisms governing mammalian palatogenesis [4–6]. In mice, a group of cells within the bilateral maxillary processes are specified to be palatal mesenchymal cells and form the palatal outgrowths on embryonic day 11.5 (E11.5) [6]. By E12.5, the two palatal outgrowths further grow into the oral-nasal cavity to form two palatal shelves that contain a block of mesenchymal cells surrounded by multiple layers of epithelial cells [2,6]. From E12.5 to E13.5, the developing palatal shelves grow vertically along the two sides of the tongue followed by elevation above the tongue on E14.5 [2,4]. The two elevated shelves continue to grow horizontally toward each other until they meet along the facial midline at their medial edge epithelium (MEE) regions. This leads to the fusion of the two MEEs to form a single continuous secondary palate on E15.5 that separates the oral and nasal cavities [2,4]. Genetic studies with mouse embryos have disclosed that the aforementioned processes are controlled by a number of signaling and regulatory pathways including BMP, SHH, Wnt, PDGF, SHOX2, MSX1 and ZEB1 [7–17].

Transforming growth factor-β (TGF-β) 1, 2, and 3 are the three main founding members of TGF-β superfamily that signals through binding and activating type I and II receptors, and the signaling cascade is further transduced by activating transcription factors Smad2/3 and Smad4 [18–21]. The functional significance of TGF-β1, 2 and 3 during palate development has been a major focus for many years [22–25]. The role of TGF-β3 in mediating palate fusion has been well established [22,26,27]. On E14.5, *Tgf-β3* mRNA is expressed in the MEE region of the mouse palate [23,24] and disruption of the *Tfg-β3* gene in mice or in palate organ culture results in cleft palate due to a fusion defect [22,26–28]. Consistent with biochemical studies [18], the function of TGF-β3 in palate fusion is mediated by Smad2, since the overexpression of *Smad2* in *Tgf-β3* mutant palate epithelial cells rescues the fusion defect [29]. Interestingly, studies with palatal epithelium cells suggested that TGF-β3 induces cell cycle arrest and epithelial to mesenchymal trans-differentiation (EMT), which could be a mechanism underlying TGF-β3 in mediating palate fusion [30,31]. In addition, a recent study revealed that TGF-β3 may also promote MEE fusion by activating the p38 MAPK pathway [32]. It appears that the expression and function of TGF-β3 during palate development is restricted to the palatal epithelial cells only.

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http://dx.doi.org/10.1016/j.reprotox.2014.10.018
0890-6238/

In contrast to TGF-β3, the function of TGF-β1 and 2 during palate development is still not clear, because *Tgf-β1* and *Tgf-β2* mutant mice do not reveal any significant palate defects, except that small percentage of *Tgf-β2* mutants displayed cleft palate on a mixed 129/Black Swiss background [33,34]. However, several lines of indirect evidence suggested that TGF-β1 and 2 play important roles in palate mesenchymal cells [25,35]. *Wnt1-Cre* mediated neural crest-specific deletion of the TGF-β type II receptor gene (*Tgfβr2*) lead to a complete cleft palate [35]. Because palatal mesenchymal cells, but not epithelial cells, originate from cranial neural crest (CNC) cells, this study demonstrated that TGF-β receptor functions in palate mesenchymal cells are essential for palate development. Considering that the expression and function of TGF-β3 are restricted to palate epithelial cells, the TGF-β ligands that function in the palatal mesenchymal cells are likely to be TGF-β1 and 2. Recently, all the three isoforms of TGF-β have been shown to be able to promote human embryonic palatal mesenchymal cell growth by activating *c-Myc* expression [36]. In addition, TGF-β1 and 2 have been suggested to be involved in facial endochondral ossification and suture formation [37,38]. In vitro studies also showed that TGF-β1and 2 interact with the Wnt signaling pathway in palate mesenchymal cells [39] and Wnt signaling has been demonstrated to be important for palate formation [12,13]. These reports suggested the functional involvements of TGF-β1 and 2 during palate formation. We reasoned that the lack of a phenotype in *Tgf-β1* and *Tgf-β2* mutants could be due to functional redundancy between TGF-β1 and 2 as they are both expressed in palate mesenchymal cells [23,24]. In this study, we tested this hypothesis by generating compound *Tgf-β1* and *Tgf-β2* mutants, and analyzed the effects on palate development.

2. Materials and methods

2.1. Mice

The *Tgf-β1* mutant line on a C57BL/6J background and the *Tgf-β2* mutant line on a Black Swiss background were obtained from The Jackson Laboratory (Maine, USA) (stock number 002220 and 003102, respectively.). These two lines were originally generated by Dr. Thomas Doetschman then at the University of Cincinnati. The mice were genotyped by PCR according to published reports [33,34], and The Jackson Laboratory data sheet. Briefly, a *Tgf-β1* mutant band around 800 bp is determined by PCR using (forward primer, 5′-gctt ta cgg tat cgc cgc tc-3′) and (reverse primer, 5′-tgc gac cca cgt agt aga cg-3′), and a 648 bp wild type band is detected with (forward primer, 5′- gga agg acc tgg gtt gga agt-3′) and (reverse primer, 5′-agc gcc cgg gtt gtg ttg gt-3′). For *Tgf-β2* genotyping, we used a set of three primers: (5′-aat gtg cag gat aat tgc tgc-3′), (5′-aac tcc ata gat atg ggg atg c-3′) and (5′-gca ggt agc cgg atc aag cgt a-3′). This primer set gives a 800 bp mutant band and a wild type band of 132 bp.

2.2. Phenotype analysis

Whole-mount views of embryonic heads were visualized and photographed according to Sandell's published method [40]. Briefly, tissues were fixed with 4% paraformaldehyde overnight followed by incubation with 10 μg/ml DAPI in PBS overnight or longer. The stained embryos were visualized and photographed under fluorescent dissecting microscope.

For histological analysis, embryonic heads were fixed in 4% paraformaldehyde overnight before they were processed for paraffin embedding. 10 μm sections were prepared and routine H & E staining was carried out.

Table 1
Loss of TGF-β2 function affects mouse secondary palate formation in strain dependent manner. The *Tgf-β2* mutant line (The Jackson Laboratory) on a Black Swiss stain background was crossed back to C57 and 129 for 5 generations. The resulting *Tgf-β2* mutant lines, C57N10, and 129N5, were considered C57 and 129 backgrounds here, respectively.

Strains	*Tgf-β2*$^{-/-}$ Embryos	Cleft Palates	Penetration
129N5	34	9	26.5%
C57N10	29	0	0
Black Swiss	31	0	0

3. Results

3.1. Strain-dependent effects of TGF-β2 on mouse secondary palate formation

The original report on the *Tgf-β2* mutant line by Dr. Thomas Doetschman's group observed that 23% of *Tgf-β2*$^{-/-}$ mutant embryos displayed cleft palate due to the failure in shelf re-orientation [34]. We decided to follow up this observation and obtained the same *Tgf-β2* mutant line from the Jackson Laboratory. However, upon obtaining this line, we did not observe any cleft palate defects in *Tgf-β2*$^{-/-}$ mutant embryos. We suspected that the discrepancy was caused by strain background. The original report was based on the background of a mixture of 129 and Black Swiss [34], and the same line was maintained on a Black Swiss background by The Jackson Laboratory. We therefore decided to examine the *Tgf-β2* mutant embryos in different strain backgrounds by crossing the Jackson Laboratory *Tgf-β2* line back to C57BL/6J and 129SvEv for 10 and 5 generations. The resulting lines were designated C57N10 and 129N5, respectively.

We examined the formation of the palate on E15.5 and E16.5 *Tgf-β2*$^{-/-}$ mutant embryos on Black Swiss, C57N10 and 129N5 backgrounds. As shown in Table 1, *Tgf-β2* mutant embryos on C57N10 and Black Swiss backgrounds did not display any cleft palate (n = 29 and 31, respectively). However, 26% of *Tgf-β2*$^{-/-}$ mutant embryos on the 129N5 background gave rise to a complete anterior to posterior cleft (9 out of 34 mutant embryos), similar to the 23% reported previously on the 129 and Black Swiss mixed background [34]. Histological analyses revealed that the palate shelves in all the affected *Tgf-β2* mutant embryos failed to reorient (Fig. 1), consistent with the previous report [34].

Therefore, TGF-β2 function contributes to mouse secondary palate formation in a strain dependent manner.

3.2. Functional compensation between TGF-β1 and TGF-β2 during mouse palate formation in C57 background

Since both TGF-β1 and TGF-β2 are expressed in mouse palate mesenchymal cells, the lack of phenotype in *Tgf-β1* or *Tgf-β2* single mutant embryos could be due to the possible functional compensation between these two genes. To test this hypothesis, we obtained the *Tgf-β1* mutant on C57BL/6J background from The Jackson Laboratory and crossed it with our *Tgf-β2* mutant in C57N10, which can be considered as C57 background. Our first step was to make sure that there was no fetal lethality occurring in [*Tgf-β1*$^{+/-}$; *Tgf-β2*$^{+/-}$] mice. Among 71 progeny from the mating between *Tgf-β1*$^{+/-}$ and *Tgf-β2*$^{+/-}$ mice, 18 (25.3%) were [*Tgf-β1*$^{+/-}$; *Tgf-β2*$^{+/-}$], which met the standard Mendelian ratio of 25%. This indicated that all [*Tgf-β1*$^{+/-}$; *Tgf-β2*$^{+/-}$] mice survived to birth. The resulting [*Tgf-β1*$^{+/-}$; *Tgf-β2*$^{+/-}$] mice are normal and viable. As mentioned above, *Tgf-β2* mutant embryos on a C57 background never display any cleft palate defects, and no cleft palates have been reported in *Tgf-β1* mutant embryos since it was first generated in 1992 [33]. To study the