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Fate-mapping of the epithelial seam during palatal fusion rules out epithelial–mesenchymal transformation

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Abstract

During palatogenesis, fusion of the palatine shelves is a crucial event, the failure of which results in the birth defect, cleft palate. The fate of the midline epithelial seam (MES), which develops transiently upon contact of the two palatine shelves, is still strongly debated. Three major mechanisms underlying the regression of the MES upon palatal fusion have been proposed: (1) apoptosis has been evidenced by morphological and molecular criteria; (2) epithelial–mesenchymal transformation has been suggested based on ultrastructural and lipophilic dye cell labeling observations; and (3) migration of MES cells toward the oral and nasal areas has been proposed following lipophilic dye cell labeling. To verify whether epithelial–mesenchymal transformation of MES cells takes place during murine palatal fusion, we used the Cre/lox system to genetically mark *Sonic hedgehog*- and *Keratin-14*-expressing palatal epithelial cells and to identify their fate in vivo. Our analyses provide conclusive evidence that rules out the occurrence of epithelial–mesenchymal transformation of MES cells.

Keywords: Palate; Cleft palate; Epithelial–mesenchymal transformation; Fate-mapping; Sonic hedgehog

Introduction

The mammalian secondary palate develops through complex and critical events. Perturbation of palatogenesis by genetic or environmental factors results in cleft palate, a common birth defect (Ferguson, 1988; Murray and Schutte, 2004). In mammalians, secondary palate development is initiated through outgrowths of the internal surface of the maxillary processes of the first pharyngeal arch. In the mouse embryo, this process is initiated at 12 days post-coitum (dpc). The bilateral outgrowths form the palatal shelves, which initially grow downwards in a vertical position parallel to the lateral surfaces of the tongue. By 14 dpc, the palatal shelves elevate into a horizontal position above the tongue and make contact with each other, first in the region of the second rugae palatinae (middle third of the palate). Thereafter, fusion spreads from this point in posterior and anterior directions. Anteriorly, the palatal shelves also fuse with the primary palate and with the nasal septum (Ferguson, 1988). Upon contact between the opposing shelves, the medial edge epithelia (MEE) promote the formation of the midline seam, which becomes evident at 15–15.5 dpc in the mouse embryo. The MEE consists of basal columnar cells covered by flat peridermal cells.

The fate of the MEE, which forms the midline epithelial seam (MES), upon palatal shelf fusion is still unclear and this matter is subject to considerable disagreements. Previous studies have provided ultrastructural and molecular evidence for the occurrence of apoptosis in the disappearing MES cells (Glucksmann, 1951; Saunders, 1966; DeAngelis and Nalbandian, 1968; Smiley and Dixon, 1968; Shapiro and Sweney, 1969; Smiley and Koch, 1975; Mori et al.,

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1994; Tanigushi et al., 1995; Cuervo and Covarrubias, 2004; Cuervo et al., 2002). However, other mechanisms have been suggested, such as epithelial–mesenchymal transformation (EMT) of the MES, i.e., a transdifferentiation of MES cells into mesenchymal cells (Fitchett and Hay, 1989; Griffith and Hay, 1992; Hay, 1995, 2005; Shuler et al., 1991, 1992; Nawshad and Hay, 2003; Nawshad et al., 2004). Alternatively, MES cells have been suggested to disappear by migrating along the midline towards the nasal and oral epithelia (Carette and Ferguson, 1992). Others suggested that all events, including apoptosis, migration, and EMT of the MES cells, are likely to occur (Mori et al., 1994; Martínez-Álvarez et al., 2000).

During palatogenesis, a number of signaling molecules, transcription factors, and extracellular components are essential for palatal shelf growth, elevation, and fusion (Murray and Shutte, 2004 and references therein). Sonic hedgehog (Shh), a member of the Hedgehog family of signaling proteins, is broadly expressed in the maxillary epithelium before elongation of the palatal shelves in the 11 dpc mouse embryo (Rice et al., 2004). At 13.5 dpc, *Shh* expression becomes restricted to the MEE and to the developing rugae palatinae (Bitgood and McMahon, 1995; Zhang et al., 2002; Rice et al., 2004).

In order to verify whether EMT takes place during palatal shelf fusion, we took advantage of a genetic approach that allows irreversible cell marking to trace the fate of MES cells in vivo. We used transgenic mice carrying the *ShhGFPCre* allele (Harfe et al., 2004) as well as transgenics expressing *Cre* under the regulation of the keratin-14 promoter (*K14-Cre*) (Turksen et al., 1992; Dassule et al., 2000). K14 is widely expressed in ectodermally derived structures, including the oral epithelia and the skin and its appendages (Byrne et al., 1994). When the above transgenic strains are crossed with the *Rosa-loxP-stop-lacZ* reporter mice (*R26R*) (Soriano, 1999), Cre-recombination-mediated activation of the reporter will mark MES cells as well as their descendants. Consequently, if EMT did indeed occur, *lacZ*-positive mesenchymal cells would be visible in the fused palate.

Materials and methods

Male mice carrying the *ShhGFPCre* (Harfe et al., 2004) or the *K14-Cre* (Dassule et al., 2000) alleles were crossed with females carrying the *R26R* conditional reporter allele (Soriano, 1999) to generate *ShhGFPCre/+; R26R/+* as well as *K14-Cre/+; R26R/+* embryos. Alternatively, *ShhGFPCre/+; R26R/R26R* or *K14-Cre/+; R26R/R26R* males were also bred with Swiss–Webster females to produce embryos (50%) carrying both the transgene and the *R26R* allele. For embryo staging, noon of the day of patent vaginal plug was considered as 0.5 dpc. Embryo staging was confirmed by examination of sections of developing teeth and various external features. Mice were genotyped as described (Soriano, 1999; Dassule et al., 2000; Harfe et al., 2004).

Entire bodies, heads, or maxillae from embryos at 15, 15.5, 16, 16.5, 17.5, and 18.5 dpc were fixed overnight in 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4 containing 2 mM $MgCl_2$, 2.25 mM EGTA, pH 8 and 0.02% NP-40 (IGEPAL CA-630; Sigma, St. Louis, MO, USA). After washing in rinse buffer (PBS, pH 7.5, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% NP-40), the embryos were either stained as whole mounts in staining solution or allowed to sink in 30% sucrose in phosphate buffer/$MgCl_2$ and then embedded in OCT compound and frozen on dry ice to generate cryosections. Cryostat sections were fixed in 0.2% PFA in 0.1 M phosphate buffer, pH 7.4 for 5 min, rinsed in PBS and incubated in the β-galactosidase staining solution at 37°C from a few hours to overnight. The β-galactosidase staining solution consisted of 1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 20 mM Tris–HCl, pH 7.3 in phosphate buffer, pH 7.4. After staining, whole mounts were post-fixed in neutral-buffered formalin and processed for paraffin embedding. All serial sections (6 μm) along the entire rostral–caudal axis of the palates were counterstained with Nuclear Fast Red.

Immunohistochemistry was performed essentially as described earlier (Gritli-Linde et al., 2001). Mouse monoclonal anti-cytokeratin 14 (clone LL002) was from YLEM (Rome, Italy). Rat anti-E-cadherin and rabbit monoclonal anti-cleaved caspase-3 (Asp175) were from Zymed Laboratories (South San Francisco, CA, USA) and Cell Signaling Technology (Beverly, MA, USA), respectively. Immunostaining for cleaved caspase-3, a marker for apoptosis, was made subsequently to processing the specimens for β-galactosidase histochemical staining.

Results

At 15 dpc, the palatal shelves have made contact with each other and with the primary palate. In 15 dpc *ShhGFPCre/+; R26R/+* embryos, β-galactosidase activity was present throughout the oral surface of the palate, including in the rugae palatinae as shown by whole-mount staining (Fig. 1A). β-galactosidase staining was robust in the epithelium of the rugae palatinae (Fig. 1A), which express *Shh* up until 16.5 dpc (Bitgood and McMahon, 1995; and data not shown). β-galactosidase activity in the epithelial covering of the primary palate was expected, given the expression of *Shh* mRNA in the ectoderm of the frontonasal and maxillary processes at 11.5 dpc (data not shown), which give rise to the upper lip and primary palate. As expected, *lacZ*-positive cells covered the oral surface of the primary and secondary palates of 15 dpc *K14-Cre/+; R26R/+* embryos (data not shown). This staining pattern was maintained at later developmental stages, including 15.5, 16, 16.5, 17.5, and 18.5 dpc both in *ShhGFPCre/+;*

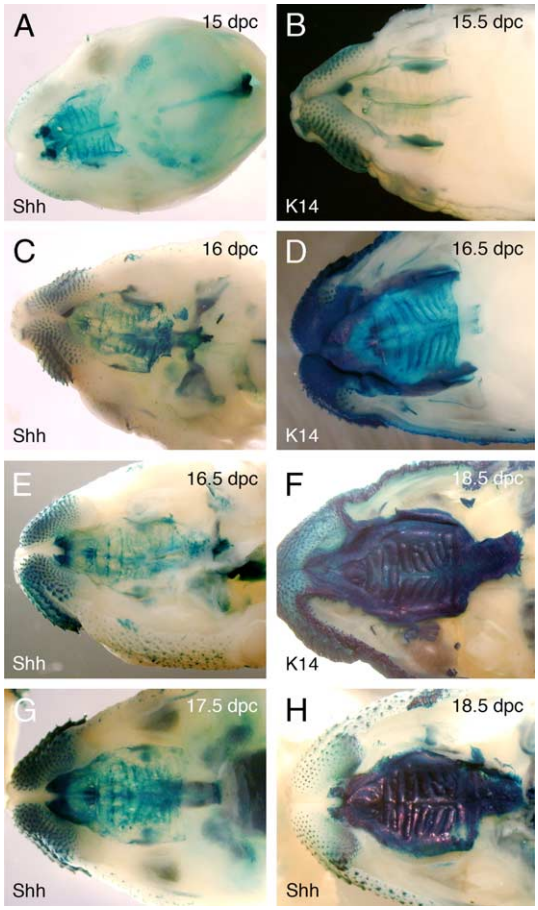

Fig. 1. Whole-mount β-galactosidase staining of palates from *ShhGFPCre/+; R26R/+* (A, C, E, G, H) and *K14-Cre/+; R26R/+* (B, D, F) embryos. Note the progressive increase of β-galactosidase activity in the oral epithelium with advancing development.

R26R/+ and *K14-Cre/+; R26R/+* embryos (Figs. 1B–H). Furthermore, the oral surface of the palates as well as the rest of the oral mucosa displayed a progressive increase in β-galactosidase activity as development proceeded (Figs. 1A–H). This was likely an effect of the progressive accumulation of β-galactosidase within individual cells, increased cell size, as well as increased cell number following stratification of the oral epithelium.

Sections from 15 dpc *ShhGFPCre/+; R26R/+* and from *K14-Cre/+; R26R/+* embryos stained for β-galactosidase revealed enzyme activity along the palatal MES as well as along the oral and nasal surfaces of the fusing palatal shelves (Figs. 2A–C and data not shown).

At later developmental stages (15.5–16, 16.5, 17.5, and 18.5 dpc), the palatal shelves displayed quasi- or total confluence. Examination of serial sections from *ShhGFPCre/+; R26R/+* and *K14-Cre/+; R26R/+* embryos at 15.5–17.5 dpc revealed the presence, in some sections, of well-demarcated epithelial islands consisting of β-galactosidase-positive cell clusters surrounded by *lacZ*-negative mesenchymal cells (Figs. 2D–I and data not shown). These were located at the palatal midline at different dorso-ventral and rostro-caudal levels as well as along the nasopalatine junction, the zone of fusion between the epithelial covering of the nasal septum and palatal epithelium. The latter were present only in the anterior portion of the secondary palate (Fig. 2D). Areas devoid of epithelial islands were found at different rostro-caudal levels at 15.5–17.5 dpc (data not shown). Palates at 18.5 dpc were virtually devoid of the epithelial remnants along the nasopalatine junction as well as at the palatal midline (Figs. 2J–L and data not shown).

In some specimens at 15–16 dpc, several embryonic sites, including the palate, tongue, and rugae palatinae, showed some background staining which was associated with mesenchymal components (blood vessels, cells, and extracellular matrix) that were located close to *lacZ*-positive epithelial cells (data not shown). This could be due to overstaining or to leakage of the stain during dehydration and processing in xylene. Nevertheless, discrimination between specific and non-specific staining was straightforward.

At none of the developmental stages examined was there any evidence of palatal mesenchymal cells displaying any specific β-galactosidase activity, thus providing strong evidence against the occurrence of EMT during palatal shelf fusion (Fig. 2 and data not shown). Putative epithelially derived mesenchymal cells should display strong β-galactosidase staining at late developmental stages (16–18.5 dpc) had they existed; however, this was not the case.

Immunostaining for activated caspase-3 showed that both the regressing MES and epithelial islands are fated to disappear by apoptosis (Figs. 3A–C). The epithelial identity of the islands was confirmed by immunostaining with antibodies anti-cytokeratin 14 (Fig. 3D) and anti-E-cadherin (Fig. 3E).

Discussion

The fate of the MES during palatal development is still controversial (Martínez-Álvarez et al., 2000; Nawshad and Hay, 2003; Cuervo and Covarrubias, 2004; Nawshad et al., 2004; Takigawa and Shiota, 2004). In this study, we used an in vivo genetic marking to track cells of the MES during palatal shelf contact and confluency. We showed that MES cells disappeared totally from the confluent palate. Transient cell aggregations, visualized as *lacZ*-positive epithelial islands surrounded by *lac-Z*-negative mesenchymal cells, were detected at different levels along the rostro-caudal and dorsal–ventral axes of the palate. At none of the developmental stages examined were any *lacZ*-positive mesenchymal cells detected at any palatal rostro-caudal and dorsal–ventral level, thus ruling out the occurrence of EMT during palatal fusion.

The existence of EMT of the MES was first suggested based on transmission electron microscopy (TEM) examination and vimentin immunostaining (Fitchett and Hay, 1989) as well as on in vitro and in utero epithelial cell tracking with the lipophilic molecule carboxyfluorescein (Griffith and Hay,

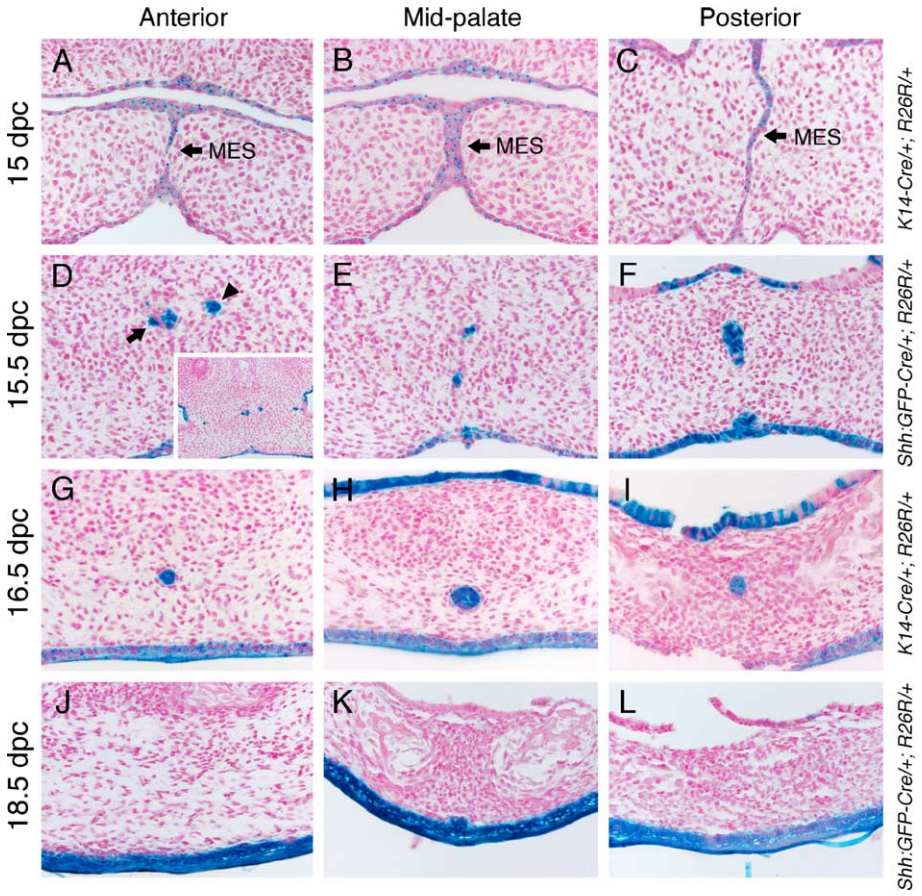

Fig. 2. Frontal sections from 15 dpc *K14-Cre/+; R26R/+* (A–C), 15.5 dpc *ShhGFPCre/+; R26R/+* (D–F), 16.5 *K14-Cre/+; R26R/+* (G–I), and 18.5 dpc *ShhGFPCre/+; R26R/+* (J–L) embryos at the anterior (A, D, G, J), middle (B, E, H, K), and posterior (C, F, I, L) segments of the palate stained for β-galactosidase. MES, midline epithelial seam (arrows in panels A–C). Sections through the nasopalatine junction show numerous *lacZ*-positive epithelial islands (arrowhead in panel D and inset) which represent remnants from the fusion between the nasal septum and the palatine shelves. Epithelial clusters at the palatal midline (D–I; arrow in panel D) along the dorsal–ventral axis of the palate, which represent remnants from the medial epithelial seam are also *lacZ*-positive. Inset in panel D is a low-magnification view of the micrograph. No arrows were added in panels E, F, and G–I to avoid masking of mesenchymal cells. At 18.5 dpc, the palate is virtually cleared from *lacZ*-positive epithelial islands (J–L). Mesenchymal cells are totally devoid of β-galactosidase activity. Note that in *ShhGFPCre/+; R26R/+* embryos, the nasal epithelium consists of areas displaying *lacZ*-positive squamous cells (F) as well as areas displaying *lacZ*-negative mucous cells (F, L). Negative areas may also be due to partial epithelial loss during processing of specimens (K).

1992). Those authors suggested that the cells of the MES as well as the cells of the epithelial seam along the nasopalatine junction never die; rather, they undergo EMT to produce fibroblasts which form the connective tissue that creates palatal shelf confluence. Others used the same (Cuervo and Covarrubias, 2004) or alternative (Carette and Ferguson, 1992) cell labeling approaches but were unable to find evidence of EMT of the MES. Adding to the controversy and casting doubts on the use of lipophilic dyes in cell tracking, Takigawa and Shiota (2004) recently reported that healthy epithelial cells in palatal shelf cultures in suspension do not take up DiI and carboxyfluorecein dyes; in contrast, these dyes are excluded by healthy shelves.

It has been argued (Nawshad et al., 2004) that EMT of the MES upon palatal fusion constitutes the obvious candidate mechanism of choice, as it would prevent disruption of mesenchyme continuity and, thus, prevent the formation of a cleft palate. For those authors, a failure of the EMT step of palate development constitutes one cause of the birth defect, cleft palate (Nawshad and Hay, 2003; Hay, 2005). However, MES degeneration is a progressive process, though rapid, and previous TEM analysis has shown that concomitant with the regression of the MES, mesenchymal cells migrate to the palatal midline and fill up the space that was occupied by a portion of the MES (see Fig. 8 in Ferguson, 1988). We also observed such a scenario after examination of serial sections at 15.5 dpc. In other words, there is no evidence of any void, i.e., a cell-free area that develops as a result of MES regression. Thus, transdifferentiation of epithelial cells into mesenchymal cells would be both a costly and redundant mechanism.

In this study, given the progressive accumulation of β-galactosidase in the MES and other epithelia as embryogenesis proceeded, we would have expected to see strong, or at least some, β-galactosidase staining of some mesenchymal cells, had they been generated via EMT of MES cells. However, this was not the case; the palates were totally devoid of such *lacZ*-positive mesenchymal cells.

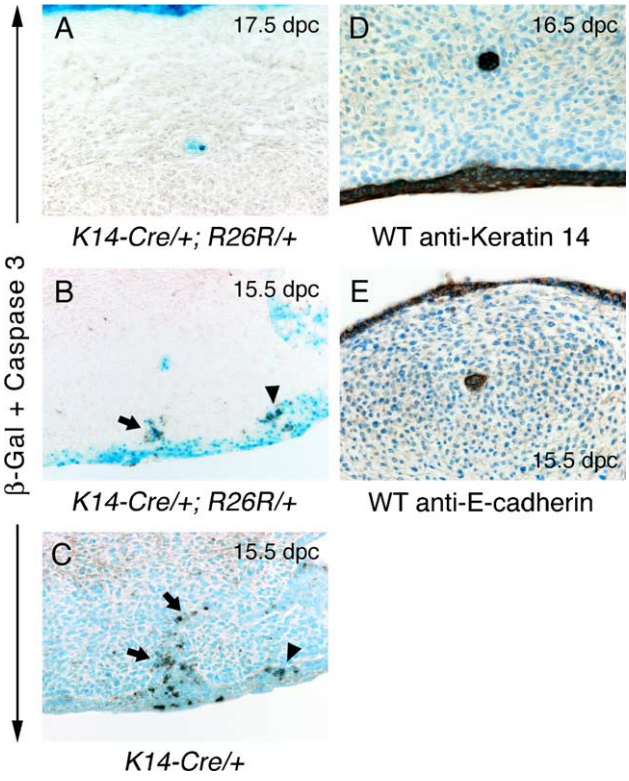

Fig. 3. Immunohistochemistry with anti-activated caspase-3 (A–C), anti-cytokeratin 14 (D), and anti-E-cadherin (E) antibodies. Section through the anterior segment of the palate from a 17.5 dpc *K14-Cre/+; R26R/+* embryo showing activated-caspase-3 immunostaining (brown color) in a *lacZ*-positive epithelial island (A). Section through the anterior palatal region from a 15.5 dpc *K14-Cre/+; R26R/+* embryo showing anti-activated caspase-3 immunostaining in the regressing *lacZ*-positive MES (arrow) as well as in the lateral epithelium, the latter representing remnants from the fusion between the maxillary and intermaxillary processes (arrowhead) (B). Section through the anterior palate from a 15.5 dpc *K14-Cre/+* embryo (devoid of an *R26R* allele) showing cells positive for activated caspase-3, indicating a prelude to apoptosis in both the regressing MES (arrow) and the epithelium at the junction of fusion between the maxillary and intermaxillary processes (arrowhead) (C). Sections through the middle palatal segment from 16.5 (D) and 15.5 (E) dpc embryos showing cytokeratin 14 (D) and E-cadherin (E) immunostaining of epithelial islands.

Furthermore, the use of two different transgenic systems to track MEE cells and their progeny during palatogenesis gives further strength to our findings. Recently, Cuervo and Covarrubias (2004) addressed the issue of the fate of the MES by an in vitro palatal culture system using carboxyfluorecein labeling, infection with an adenovirus carrying the *lacZ* gene, and fused chimeric palatal shelves from *Egfp* and wild-type mouse embryos. Those authors found no evidence of EMT of the MES, thus supporting previous findings with DiI labeling (Carette and Ferguson, 1992). However, those studies have been criticized for different reasons, including the use of a static culture system in vitro, the absence of cytology in the fluorescence images, distribution of the labels "in a hit or miss fashion", as well as trapping of dying peridermal cells in the seam during in vitro manipulation (Griffith and Hay, 1992; Nawshad et al., 2004; Takigawa and Shiota, 2004).

EMT during male Müllerian duct regression, a process originally proposed based on morphological criteria (Trelstad et al., 1982), has been evoked as an argument to support the occurrence of EMT during late mammalian embryogenesis (14 dpc in mice), thus providing support for the occurrence of such a mechanism during palatogenesis (Fitchett and Hay, 1989; Griffith and Hay, 1992; Martínez-Álvarez et al., 2000). However, accumulating molecular evidence suggests that apoptosis accounts for the ultimate fate of the Müllerian duct in the male embryo (Roberts et al., 1999, 2002), supporting earlier ultrastructural studies (Djehiche et al., 1994).

The concept of EMT during palatogenesis has been used extensively as a basis to assign biological roles to a number of factors, including TGFβ3 (Kaartinen et al., 1997; Sun et al., 1998; Nawshad and Hay, 2003), Snail (Martínez-Álvarez et al., 2004), Lef1 and/or Smads (Nawshad and Hay, 2003; Dudas et al., 2004; Nawshad et al., 2004; Hay, 2005). There is no reason to doubt that these factors play a crucial role during palatogenesis, as they display specific expression and activation patterns. In addition, mutations in *Tgfβ3* generate murine and human non-syndromic cleft palate (Kaartinen et al., 1995; Proetzel et al., 1995; Lidral et al., 1998). However, the present study indicates that they do not regulate EMT during palatal fusion.

Our immunohistochemical analysis showed that the *lacZ*-positive regressing MES as well as the epithelial islands displayed immunoreactivity for activated caspase-3. This indicates that these structures are fated to die by apoptosis, which thus lends further support to previous studies, from those dating from the early 1950s to the most recent ones using morphological and molecular criteria for apoptosis (Glucksmann, 1951; Saunders, 1966; DeAngelis and Nalbandian, 1968; Smiley and Dixon, 1968; Shapiro and Sweney, 1969; Smiley and Koch, 1975; Mori et al., 1994; Tanigushi et al., 1995; Cuervo et al., 2002; Cuervo and Covarrubias, 2004).

In conclusion, this in vivo genetic fate-mapping of the MEE rules out, with confidence, the occurrence of EMT as a mechanism underlying the regression of the MES during palatal fusion. This should, thus, close a more than decade-long chapter of divergent opinions.

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