Analysis of cell migration, transdifferentiation and apoptosis during mouse secondary palate fusion

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Malformations in secondary palate fusion will lead to cleft palate, a common human birth defect. Palate fusion involves the formation and subsequent degeneration of the medial edge epithelial seam. The cellular mechanisms underlying seam degeneration have been a major focus in the study of palatogenesis. Three mechanisms have been proposed for seam degeneration: lateral migration of medial edge epithelial cells; epithelial-mesenchymal trans-differentiation; and apoptosis of medial edge epithelial cells. However, there is still a great deal of controversy over these proposed mechanisms. In this study, we established a [Rosa26-C57BL/6] chimeric culture system, in which a Rosa26-originated ‘blue’ palatal shelf was paired with a C57BL/6-derived ‘white’ palatal shelf. Using this organ culture system, we observed the migration of medial edge epithelial cells to the nasal side, but not to the oral side. We also observed an anteroposterior migration of medial edge epithelial cells, which may play an important role in posterior palate fusion. To examine epithelial-mesenchymal transdifferentiation during palate fusion, we bred a cytokeratin 14-Cre transgenic line into the R26R background. In situ hybridization showed that the Cre transgene is expressed exclusively in the epithelium. However, β-galactosidase staining gave extensive signals in the palatal mesenchymal region during and after palate fusion, demonstrating the occurrence of an epithelial-mesenchymal transdifferentiation mechanism during palate fusion. Finally, we showed that Apaf1 mutant mouse embryos are able to complete palate fusion without DNA fragmentation-mediated programmed cell death, indicating that this is not essential for palate fusion in vivo.

KEY WORDS: Mouse secondary palate, Cell migration, Apoptosis, Apaf1 mutant mice, K14-Cre transgenic mice, Epithelial-mesenchymal transdifferentiation

INTRODUCTION

The development of the mammalian secondary palate involves multiple steps. During mouse development, the palate mesenchymal cells are specified within the bilateral maxillary processes at embryonic day (E) 11.5 (Ferguson, 1988; Murray and Schutte, 2004). From E12.5, the two palate shelves grow down vertically along the two sides of the tongue. Each palate shelf consists of a neural crest-derived mesenchymal core surrounded by epithelial cells that originate from the cranial ectoderm. The vertical growth of the palate shelves continues until E13.5 (Ferguson, 1988; Murray and Schutte, 2004).

At E14.5, the vertical palate shelves elevate above the tongue, grow horizontally towards each other, and contact each other at the medial edge epithelium (MEE) region along the facial midline. The contact initiates adhesion between the two MEE layers, and triggers a series of cellular and biochemical reactions that lead to the fusion of the palate shelves at E15.5 (Ferguson, 1988; Murray and Schutte, 2004). During fusion, the MEE layers from the two palate shelves first merge to form the MEE seam, which subsequently undergoes degeneration, giving rise to the formation of a continuous palate that separates the oral and nasal cavities (Ferguson, 1988).

The cellular mechanism underlying seam degeneration and the fate of the MEE seam cells have been a major focus of the field for more than a decade. However, controversies still remain on this issue. Three major models have been proposed for seam degeneration: epithelial-mesenchymal transdifferentiation (EMT) (Griffith and Hay, 1992; Nawshad et al., 2004; Shuler et al., 1992); MEE cell apoptosis (programmed cell death) (Cuervo and Covarrubias, 2004); and lateral migration of MEE cells (Carette and Ferguson, 1992).

The cell migration model was first proposed by Carette and Ferguson, who suggested that the MEE cells may migrate to the oral and nasal sides of the palatal shelf during fusion (Carette and Ferguson, 1992). To date, there have only been very limited studies to evaluate this model experimentally (Cuervo and Covarrubias, 2004).

The apoptosis model has long been proposed for MEE seam degeneration. However, only recently have relevant experimental data been obtained regarding this hypothesis. Cuervo and colleagues examined apoptosis in the MEE seam during palatal fusion in vivo and in vitro using TdT-mediated dUTP nick end labeling (TUNEL) assay (Cuervo et al., 2002; Cuervo and Covarrubias, 2004). Activation of caspase 3 has been detected in the triangular area of the palate at E15.5 (Vaziri et al., 2005). As to the functional requirement of apoptosis in mediating seam degeneration, contradictory results were reported by different investigators. Cuervo and Covarrubias reported that the addition of cell death inhibitors, e.g. z-VAD, in palate organ culture prevented seam and basement membrane degeneration in vitro (Cuervo and Covarrubias, 2004). By contrast, Takahara and colleagues reported that palatal shelves treated with caspase inhibitors, YVAD-CHO and DEVD-CHO, still underwent normal fusion in vitro (Takahara et al., 2004). It is important to note that both reports are based on in vitro culture experiments involving the use of chemical inhibitors. Because cell death is highly sensitive to the environment and the condition of the cell, one has to be cautious in applying in vitro cell death data to the in vivo situation. In addition, specificity is often a concern with the use of chemical inhibitors.
In contrast to cell migration and cell death, epithelial-mesenchymal transdifferentiation has been extensively studied. However, the experimental data on this issue remains inconsistent and controversial. Carette and Ferguson labeled the mouse palate epithelial cells with Dil, traced the labeled cells during palatal fusion in vitro, and found no labeled cells in the mesenchymal region after fusion (Carette and Ferguson, 1992). Similar in vitro results were recently obtained by Cuervo and Covarrubias, using CCFSE as a labeling reagent (Cuervo and Covarrubias, 2004). By contrast, Schulle and colleagues approached this question in mouse embryos in vivo by intraperitoneal (IP) injection of Dil, and observed labeled cells in the mouse palatal mesenchymal area at E15.5 (Shuler et al., 1992). Sun and colleagues labeled the chicken palatal epithelium with CCFSE, cultured the palate pairs in vitro with Tgfβ3, and found that the treated chicken palatal pairs underwent fusion, with a high level of CCFSE signal present in the medial edge mesenchymal area (Sun et al., 1998).

Along with the physical labeling approach, a molecular biology based labeling approach has also been applied to address this issue. Martinez-Alvarez and colleagues infected the palate shelves with a retroviral vector that constitutively expressed the bacterial lacZ gene, and cultured the infected palatal pairs in vitro in complete medium with serum. Following in vitro palate fusion and β-galactosidase (β-gal) staining, the investigators observed blue cells in the mesenchymal region (Martinez-Alvarez et al., 2000). By contrast, Cuervo and Covarrubias infected the palatal shelves with a lacZ-expressing adenoviral vector and cultured them in serum-free medium, but no β-gal-positive cells were observed in the mesenchymal region after in vitro palatal fusion (Cuervo and Covarrubias, 2004). Very recently, a Cre-LoxP-based genetic labeling system has also been applied to study this issue. Vaziri and colleagues examined mice expressing Cre recombinase under the control of Shh and cytokertatin 14 regulatory elements in the R26R background, and observed no β-gal-positive cells in the mesenchymal region after palatal fusion in vivo (Vaziri et al., 2005). However, the labeling intensity in MEE cells prior to fusion was rather low in that study.

As to the molecular mechanism mediating MEE seam degeneration, Tgfβ3 has been shown to be essential for MEE degeneration by a genetic inactivation approach (Kaartinen et al., 1995; Proetzel et al., 1995). Tgfβ3 is a growth factor with multiple biological functions and activities (Massague, 1998). Several mechanisms have been proposed for Tgfβ3-mediated seam degeneration, including facilitating epithelial-mesenchymal transdifferentiation (Kaartinen et al., 1997; Nawshad and Hay, 2003; Sun et al., 1998), initiating cell adhesion (Gato et al., 2002; Sun et al., 1998), and promoting basement membrane and cell matrix degeneration (Blavier et al., 2001).

In this study, we first employed an in vitro [Rosa26-C57BL/6] chimeric culture system to examine epithelial cell migration in cultured palate fusion in vitro. We also used a Cre-LoxP-based genetic labeling system, a similar approach to Vaziri and colleagues (Vaziri et al., 2005), to label and follow the palate epithelium during palate fusion in vivo. We bred a cytokertatin 14-Cre (K14-Cre) transgenic mouse line (Vasioukhin et al., 2001) into the R26R background (Soriano, 1999). In this combination, the expression of Cre recombinase is driven by a cytokertatin 14 (K14) promoter and enhancer, a well-characterized, epithelium-specific regulatory element (Vasioukhin et al., 2001). Thus, the R26R reporter locus will be specifically activated and irreversibly labeled in the epithelium. Finally, to find out whether apoptosis is functionally required for seam degeneration in vivo, we examined MEE cell apoptosis and palatal fusion in Apaf-1 deficient mice, in which caspase 3, a key effector caspase, is not activated in embryonic cells (Honarpour et al., 2000).

**RESULTS**

**Migration of MEE seam cells during palatal fusion in culture**

To investigate the migration of MEE seam cells during palatal fusion, we performed a [Rosa26-C57BL/6] chimeric culture in which a palatal shelf dissected from Rosa26 mice (Zambrowicz et al., 1997) was paired with another palatal shelf dissected from C57BL/6 mice. We assumed that, if lateral migration of MEE seam cells occurs during palatal fusion in vitro, the cells that emerge from the seam will split randomly to either side of the pair, and that the Rosa26-originated epithelial cells may be present in the oral or nasal side of the C57BL/6 half of the pair and can be detected by β-gal staining. In other words, if β-gal-positive epithelial cells can be detected in the C57BL/6 palate, it is reasonable to believe that the lateral migration of MEE seam cells occurs during palatal fusion, at least in vitro. As shown in Fig. 1B, β-gal-positive cells are indeed present in the C57BL/6 palatal nasal epithelium in a [Rosa26-C57BL/6] chimeric pair cultured oral side up, indicating a migration of cells to the nasal side of the palate during fusion in vitro.
Interestingly, we observed that MEE seam cells can migrate, at least to the nasal side of the C57BL/6 palate (data not shown). We therefore conclude that MEE seam cells can migrate, at least to the nasal side during in vitro palatal fusion, whereas migration to the oral side is still an unsolved question. Notably, all the [Rosa26×C57BL/6] chimeric explants examined so far (n>15) have shown emerging cells on only the nasal side of the C57BL/6 palate, not on the oral side. To exclude the possibility of a positional effect, we cultured the palate explants nasal side up; however, we still found β-gal-positive cells present only in the nasal sides of the C57BL/6 palates (data not shown). We therefore conclude that MEE seam cells can migrate, at least to the nasal side during in vitro palatal fusion, whereas migration to the oral side is still an unsolved question. Interestingly, we observed β-gal-positive cells in the C57BL/6 palate in posterior regions in which the two shelves are still separated (Fig. 1C). This observation prompted us to hypothesize that the MEE seam cells may also migrate along the anteroposterior (AP) axis. To confirm this possibility, we sectioned the paired explants in the manner indicated in Fig. 1D. We found that β-gal-positive cells did indeed extend posteriorly (Fig. 1E). Because the blue staining in the C57BL/6 palate is continuous rather than dispersed (Fig. 1C,E), it is unlikely that the blue signals in the C57BL/6 side are the cell attachments from the Rosa26 palate. Based on these observations, it is likely that during in vitro palatal fusion, MEE cells commit to migration in two directions: migrating nasally along the oral-nasal axis; and migrating posteriorly along the AP axis (see Discussion for details).

Epithelial-mesenchymal transdifferentiation (EMT) during palatal fusion in vivo

To examine EMT during palatal fusion in vivo, it is necessary to label the palatal epithelium specifically prior to palatal fusion. For that purpose, we bred a cytokeratin 14-Cre (K14-Cre) transgenic mouse line (Vasioukhin et al., 2001) in the R26R background (Soriano, 1999) to generate [K14-Cre; R26R] embryos. Because the Cre transgene is under the control of an epithelial-specific K14 regulatory element (Vasioukhin et al., 2001), the R26R reporter locus will be activated only in epithelial cells and will lead to irreversible β-gal labeling of those cells. Therefore, the β-gal-positive cells in [K14-Cre; R26R] embryos are epithelial in origin, and the presence of β-gal-positive cells in palatal mesenchymal region after fusion will be an indication of epithelial-mesenchymal transdifferentiation.

We first carried out in situ hybridization with the Cre anti-sense RNA probe in [K14-Cre; R26R] embryos to validate that the expression of the Cre transgene in those embryos is indeed epithelium specific. We found that the Cre transgene is highly expressed in the entire palatal epithelium at E13.5 (Fig. 2B). At E14.5, the expression becomes heterogeneous along the AP axis of palate. In the anterior and posterior region, the Cre transgene is expressed roughly in the entire palatal epithelium (Fig. 2C,E), whereas expression in the middle region is restricted to the oral side of palate epithelium and the triangular area (Fig. 2D). The expression of the Cre transgene becomes undetectable in the palate at E15.5 (data not shown). No expression was observed in mesenchymal cells throughout palate development. Therefore, the Cre transgene in this K14-Cre line confers an epithelium-specific expression, at least in the development of the secondary palate.

We then examined the β-gal staining pattern in palate development in the [K14-Cre; R26R] embryos. We found that the β-gal labeling intensity in the palatal epithelium varies among the [K14-Cre; R26R] embryos, ranging from high density to almost no labeling (data not shown). Moreover, the labeling efficiency also varies with respect to the region of palatal epithelium, as some [K14-Cre; R26R] embryos are strongly labeled in the oral and nasal palatal epithelium, but are weakly labeled in the MEE seam, whereas others may be strongly labeled in the MEE seam region, but poorly labeled in oral and nasal sides of palate. This phenomenon is frequently observed in various Cre mice. Therefore, the labeling level in the oral or nasal palatal epithelium does not necessarily reflect the labeling situation in the seam. In order to trace MEE fate during...
fusion, it is essential that the \([\text{K14-Cre}; \text{R26R}]\) embryos selected for the analysis are strongly labeled with \(\beta\)-gal in their MEE seams. For that purpose, we focused on the stages between E14.5 and early E15.5, in which the seam had not completely vanished and the remaining seam can be used as an internal control for the \(\beta\)-gal-labelling situation in the seam cells. Fig. 3A shows the \(\beta\)-gal staining in the anterior palate of a \([\text{K14-Cre}; \text{R26R}]\) embryo at early E14.5, in which the two palate shelves had just made contact. The MEE region of this embryo is strongly labeled with \(\beta\)-gal, but no \(\beta\)-gal signal was detected in the mesenchymal cells, particularly the medial edge mesenchyme region, indicating that no EMT had occurred before fusion (Fig. 3A). In the initial stage of seam degeneration, some \(\beta\)-gal-positive cells are located off the midline within the mesenchymal region (Fig. 3B). Around late E14.5 and
E15.0, seam degeneration is more advanced and more β-gal-positive cells were found away from the midline; in addition, a large portion of medial edge mesenchymal cells are β-gal-positive (Fig. 3C,D), indicating that epithelial-mesenchymal transdifferentiation occurs during seam degeneration. At E15.5, the seam degeneration is complete, and a large patch of β-gal-positive cells with typical mesenchymal cell morphology is present in the medial edge region (Fig. 3E). The high intensity of β-gal staining in the degenerating seam indicated that the MEE seams of the examined embryos are strongly labeled with β-gal (Fig. 3B-D). In this experiment, eight out of 18 embryos at E14.5, and eight out of 16 embryos at E15.5, gave high-level labeling in the degenerating or residual seam cells, and all of them showed good signals in the mesenchymal region. These results demonstrate that EMT does occur during palate fusion in vivo.

**Palate fusion in vivo occurs without DNA fragmentation-mediated apoptosis**

Previous studies have reported that MEE cells undergo apoptosis (Cuervo et al., 2002; Cuervo and Covarrubias, 2004), and a recent study suggested that the apoptosis in palatal MEE cells is mediated by caspase 3 (Vaziri et al., 2005). To determine whether apoptosis is essential for palate fusion and seam degeneration during mouse development in vivo, we examined palate fusion in **Apaf1** mutant mouse embryos. The mouse **Apaf1** gene is the mouse homolog of the C. elegans CED-4 gene, which encodes a crucial component in the caspase-3-mediated apoptosis pathway (Green and Reed, 1998). **Apaf1** mutant mouse embryos are deficient in caspase 3 activation and display enormous apoptotic defects during embryonic development (Green and Reed, 1998; Honarpour et al., 2000). Surprisingly, we found that complete palate fusion still occurs in the **Apaf1** mutant embryos. Histological analysis showed that the **Apaf1** mutant palate forms a normal MEE seam at E14.5 (Fig. 4D), which undergoes degeneration at E15.5 (n=14; Fig. 4E). At E16.5 (n=10), the mutant embryos form a continuous palate with no residual seam cells (Fig. 4F). Interestingly, the mutant palates at E15.5 often present an enlarged triangular area that presents with more compacted cell density than wild-type palates (Fig. 5A,B). To rule out the possibility that caspase 3-independent apoptosis occurs in the **Apaf1** mutant palate, we carried out the TUNEL assay in both wild-type and **Apaf1** mutant embryos. We found few TUNEL-positive cells in the palate epithelium prior to E15.5 in wild-type embryos (data not shown), and apoptotic palate MEE cells become detectable as late as E15.5 (Fig. 5C). The majority of apoptotic cells are in the triangular area (Fig. 5C). This data is consistent with the previously reported caspase 3 activation in the triangular area of the palate at E15.5 (Vaziri et al., 2005). The TUNEL-positive cells are completely abolished in the **Apaf1** mutant palate at E15.5 (Fig. 5D). Therefore, the **Apaf1** mutant palate cells do not commit to DNA fragmentation-mediated programmed cell death. On the basis of these results, we conclude that apoptosis is not essential for palate fusion in vivo.

**DISCUSSION**

**Migration of MEE seam cells during palate fusion in vitro**

As illustrated in Fig. 6, our analysis with cultured [Rosa26×C57BL/6] chimeric palate pairs uncovered a three-dimensional movement of MEE seam cells during palate fusion: lateral migration to the nasal side of the palate and AP migration from the middle to the posterior. Interestingly, lateral migration to the oral side of the palate was not observed in this analysis. It is not clear whether lateral migration to the oral side of palate is blocked by an intrinsic mechanism or whether the lack of migration is simply due to the in vitro culture condition. Cuervo and Covarrubias reported lateral migration to both the nasal and oral side of the palate in culture using an eGFP-labeling system (Cuervo and Covarrubias, 2004). However, the cellular resolution was rather low in that reported study.

Lateral migration was first proposed by Carette and Ferguson (Carette and Ferguson, 1992), whereas the AP migration has not been reported previously. We speculate here that the AP migration may serve the dual functions of promoting seam degeneration and facilitating posterior palate fusion. Owing to palate topology, the two

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**Fig. 4. Hematoxylin and eosin staining reveals complete palate fusion and seam degeneration in **Apaf1** mutant embryos.** (A,D) The MEE seam (arrowhead) forms normally in both wild-type and **Apaf1** mutant embryos at E14.5. (B,E) The MEE seam undergoes degeneration in both wild-type and **Apaf1** mutant embryos at E15.5 to establish the mesenchyme confluence cross the midline (arrowheads). (C,F) At E16.5, both wild-type and **Apaf1** mutant embryos form a continuous palate with no sign of seam cells in the midline area (arrowhead).
palate shelves at E14.5 make close contact only in the middle region, whereas the posterior regions are widely separated, as illustrated in Fig. 2A. How the posterior palatal shelves achieve tight contact at E15.5 is still an unanswered question in palate development. The AP migration of MEE seam cells may serve as a ‘zipper’ to bring the two parts together.

It is important to point out that this is an in vitro result and that the behavior in vivo requires further studies; for example, to trace the MEE fate by breeding a MEE-specific Cre line, when it is available, into the R26R background.

Epithelial-mesenchymal transdifferentiation during palate fusion in vivo

Our β-gal staining with [K14-Cre; R26R] embryos revealed that a significant number of mesenchymal cells are β-gal positive during and after palate fusion. Because our in situ data showed that the expression of the Cre transgene in the palate is exclusively restricted to the palatal epithelium, the β-gal-positive mesenchymal cells are epithelial in origin. We therefore provide strong evidence to support the occurrence of the long debated epithelial-mesenchymal transdifferentiation during palate fusion. Our results are different from a recent report by Vaziri and colleagues (Vaziri et al., 2005), who used a similar approach with a different K14-Cre transgenic line. The discrepancy may reside in the different Cre transgenic lines used in the studies. The β-gal-labeling level in MEE prior to fusion was rather low in that reported study, which could have meant that the signal was not sufficient to be detected. In addition, the embryos selected for analysis in that study demonstrated good palate fusion with almost no residual seam left. Because the labeling level in the oral and nasal palate epithelium does not necessarily correlate with the labeling situation in MEE, it is not clear how well the MEE seam cells were labeled in the embryos analyzed in the reported study.

The role of apoptosis in palate fusion and seam degeneration

Recent in vitro studies using caspase inhibitors gave different results with respect to the functional requirement of apoptosis in palate fusion in vitro. One report claimed that the addition of a pan-caspase inhibitor, z-VAD, prevents seam and basement membrane degeneration in vitro (Cuervo and Covarrubias, 2004), whereas another study reported that palate fusion occurs normally in vitro in the presence of the caspase inhibitors YVAD-CHO and DEVD-CHO (Takahara et al., 2004). In our current study, we took a genetic approach to address this issue in vivo. The mouse Apaf1 gene is the mouse homolog of the C. elegans CED-4 gene that encodes a protein factor essential for capase activation (Green and Reed, 1998). Targeted disruption of Apaf1 in mice leads to the failure of caspase 3 activation and numerous cell death-related defects (Cecconi et al., 1998; Honarpour et al., 2000). In the present study, we found that DNA fragmentation-mediated apoptosis was completely abolished in the palates of Apaf1 mutant embryos, as judged by the TUNEL assay (Fig. 5D). However, palate fusion and seam degeneration did occur in the Apaf1 mutant embryo (Fig. 4D-F). It is worth mentioning that a previous study with a different line of Apaf1 mutant mice described the persistence of the palate seam in the absence of the Apaf1 gene (Cecconi et al., 1998). However, the analysis in that study was only until E14.5, at which time the seam in a wild-type embryo is still evident. Cuervo and Covarrubias reported a much higher number of apoptotic cells in the MEE seam than in a wild-type embryo is still evident. Cuervo and Covarrubias reported a much higher number of apoptotic cells in the MEE seam than in a wild-type embryo (Cuervo and Covarrubias, 2004). Apaf1 mutation may be due to some kind of artificial increase in an in vitro system.

Our analysis with Apaf1 mutant embryos strongly demonstrates that the function of the observed apoptosis is not essential for palate fusion and seam degeneration. However, this does not mean that apoptosis does not contribute to palate fusion and seam degeneration during normal palate development, as it is obvious that MEE cell death can at least accelerate the removal of the MEE seam during palate fusion. In fact, Apaf1 mutant palates at E15.5 display abnormal-looking triangular areas that are larger and more compacted (Fig. 5B). This suggests that programmed cell death may play a role in the clearance of the
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triangle cells. It is also possible that more MEE cells commit to cell migration to the triangular areas when the cell death pathway is impaired.

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References