Death is the major fate of medial edge epithelial cells and the cause of basal lamina degradation during palatogenesis

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Summary

During mammalian development, a pair of shelves fuses to form the secondary palate, a process that requires the adhesion of the medial edge epithelial tissue (MEE) of each shelf and the degeneration of the resulting medial epithelial seam (MES). It has been reported that epithelial-mesenchymal transformation (EMT) occurs during shelf fusion and is considered a fundamental process for MES degeneration. We recently found that cell death is a necessary process for shelf fusion. These findings uncovered the relevance of cell death in MES degeneration; however, they do not discard the participation of other processes. In the present work, we focus on the evaluation of the processes that could contribute to palate shelf fusion. We tested EMT by traditional labeling of MEE cells with a dye, by infection of MEE with an adenovirus carrying the *lacZ* gene, and by fusing wild-type shelves with the ones from *EGFP*-expressing mouse embryos. Fate of MEE labeled cells was followed by culturing whole palates, or by a novel slice culture system that allows individual cells to be followed during the fusion process. Very few labeled cells were found in the mesenchyme compartment, and almost all were undergoing cell death. Inhibition of metalloproteinases prevented basal lamina degradation without affecting MES degeneration and MEE cell death. Remarkably, independently of shelf fusion, activation of cell death promoted the degradation of the basal lamina underlying the MEE ('cataptosis'). Finally, by specific labeling of periderm cells (i.e. the superficial cells that cover the basal epithelium), we observed that epithelial triangles at oral and nasal ends of the epithelial seam do not appear to result from MEE cell migration but rather from periderm cell migration. Inhibition of migration or removal of these periderm cells suggests that they have a transient function controlling MEE cell adhesion and survival, and ultimately die within the epithelial triangles. We conclude that MES degeneration occurs almost uniquely by cell death, and for the first time we show that this process can activate basal lamina degradation during a developmental process.

Key words: Morphogenesis, Apoptosis, Cell migration, Mouse

Introduction

The secondary palate forms by fusion of a pair of shelves that originate on the inner side of the maxillary process (reviewed by Ferguson, 1988). Upon meeting at the midline of the oropharyngeal cavity, the shelves adhere to each other at their medial edge epithelia (MEE) giving rise to the medial epithelial seam (MES). The MES initially consists of a multilayered epithelium that later becomes a single epithelial layer. MES degeneration continues by fragmentation of the adhered region forming epithelial islands (called epithelial pearls) along the MES. These islands degenerate resulting in fused palate shelves.

The MEE is composed of a basal columnar cell layer covered by flat cells that constitute the periderm. During shelf growth, MEE is histologically undistinguishable from oral or nasal epithelium, but it acquires distinctive features just prior to fusion. At the molecular level, the MEE region appears defined by the expression of several genes such as *Tgfb3* (Fitzpatrick et al., 1990), *Egfr* (Brunet et al., 1993), *Tgfa* (Citterio and Gaillard, 1994) and *Fos* (Yano et al., 1996). It is believed that periderm cells shed before fusion to allow intimate contact between shelves (Fitchett and Hay, 1989).

Epithelial-mesenchymal transformation (EMT) is considered relevant for MES degeneration (Fitchett and Hay, 1989; Griffith and Hay, 1992; Shuler et al., 1991). EMT stands for the transdifferentiation of packed epithelial cells to more loose mesenchymal cells, a process that involves basal lamina degradation (and dramatic changes in the cytoskeleton), and cell-cell and cell-extracellular matrix interactions (Boyer et al., 1996). The migratory capacity of mesenchymal cells allows them to move far from their site of origin. Once the EMT process occurs, transdifferentiated cells can give rise to specific cell types, just as neural crest cells do (Duband et al., 1995), or can contribute to form structures such as the heart valves, which are derived from endocardial cells (Markwald et al., 1975). In the case of secondary palate, transdifferentiated mesenchymal cells would not have a specific function. Several lines of evidence suggest that EMT actually occurs during palate shelf fusion (Fitchett and Hay, 1989; Griffith and Hay, 1992; Martinez-Alvarez et al., 2000; Shuler et al., 1991; Shuler et al., 1992). However, because only few cells have been detected to

have transdifferentiated and because there is lack of quantitative analyses, other mechanisms for MES degeneration should be considered. Migration of MEE cells towards the nasal and oral regions has also been proposed to participate in shelf fusion (Carette and Ferguson, 1992). Cell death, which has been known for many years to occur in the developing palate (DeAngelis and Nalbandian, 1968; Farbman, 1968; Smiley and Dixon, 1968), was until only recently implicated in MES degeneration (Cuervo et al., 2002; Martinez-Alvarez et al., 2000; Mori et al., 1994; Taniguchi et al., 1995). MES degeneration could also result from a combination of cellular mechanisms such as those described above.

The aim of the present work was to evaluate the relevance of EMT, epithelial cell migration and cell death in palate shelf fusion. Our results revealed a fundamental role of cell death in MES degeneration, without a significant contribution from EMT or basal MEE cell migration. However, we show data indicating that the ordered migration of periderm cells out from the basal MEE is necessary for normal shelf fusion. Furthermore, in contrast to the activation of cell death by the degradation of basal lamina (i.e. anoikis), we identified the activation of basal lamina degradation as a consequence of MEE cell death (‘cataptosis’).

Materials and methods

Animal handling and palate dissection

CD-1 and EGFP (Hadjantonakis et al., 1998) mouse strains were used in this study. Pregnant females were sacrificed by cervical dislocation between 13.5 and 14.5 days post coitus (d.p.c.). The day of detection of vaginal plug was found at 0.5 d.p.c. Palate dissection was carried out as previously described (Cuervo et al., 2002).

Organ culture

Whole palates were cultured on filters floating on serum-free medium as previously described (Cuervo et al., 2002). We also developed a palate slice culture system based on that reported by Knight et al. (Knight et al., 1999). Initially shelves of whole palates were allowed to contact for 3 hours and were then embedded in 5% low-melting point agarose (SeaPlaque GTG, FMC Bioproducts, Rockland, ME) in McCoy medium (Microlab, México). Slices (200 μm) were obtained using a vibratome (Leica VT1000S, Wetzlar, Germany) and collected in cold PBS (5.4 mM potassium chloride, 138 mM sodium chloride, 22 mM glucose, 2 mM sodium-potassium phosphate, pH 7.2). Slices were placed at the bottom of a 35 mm petri dish and covered with a layer of 1% low-melting point agarose and 2 ml of McCoy medium. At the end of culture, live slices were washed with PBS, fixed with 4% paraformaldehyde and processed for TUNEL in wholemount (Conlon et al., 1995). Cytochalasin D (6 μM; Sigma, St Louis, MO), cycloheximide (20 μg/ml; Sigma, St Louis, MO), retinoic acid (20 μM; Sigma, St Louis, MO), staurosporin (20 μM Sigma, St Louis, MO), BB3103 MMP inhibitor (10 μM; British Biotech, Oxford, UK), or z-VAD (100 μM z-VAD; Biomol, Plymouth, PA) were added directly to the culture medium. z-VAD anti-apoptotic activity has also been tested in explant cultures of limbs undergoing interdigital regression, and of developing spinal cords undergoing motoneuron degeneration. Similarly, the inhibitory activity of BB3103 on metalloproteinases has been tested by zymography using gelatin as substrate, and by the ability to avoid the natural degradation of basal lamina in explant cultures containing müllerian and wolfian ducts. All reagents remained in the medium for the whole culture period.

Cell labeling

Complete MEE labeling was obtained by submerging whole palates in a 10 μM solution of 5-6-carboxy 2-7-dichlorofluorescein diacetate succinimidyl ester (CCFSE; Molecular Probes, Eugene, OR) in PBS. Samples were incubated at 37°C for 15 minutes in the dye solution and washed twice with PBS before culturing. Selective labeling of periderm cells with the same dye was attained by a short incubation (30 seconds) of the palate explants in the same dye solution at room temperature. Transfections with an adenovirus carrying the *lacZ* reporter gene (Ad-*lacZ*) were carried out at 37°C for 1.5 hours as previously described (Cuervo et al., 2002). Chimaeric palates were formed using one shelf from a CD1 embryo and the other from an EGFP embryo. Shelf fusion under this condition took about 36 hours. In all cases described above palates were cultured for different time periods, fixed with 4% paraformaldehyde, embedded in paraffin or agarose, and sliced for histology, immunohistochemistry or cell death detection.

Periderm cell removal

To remove superficial cells, palates were incubated in 0.25% trypsin solution in Versene (Invitrogen, Grand Island, NY) at 4°C for 5 minutes. After incubation, palate medial edges were extensively washed with the same solution until the thin periderm cell layer came off. Samples were washed with PBS and then incubated in DMEM containing 10% serum at room temperature for 10 minutes. Shelves were separated from the rest of the tissue and cultured on filters. The same treatment was given to control palates except that wash with 0.25% trypsin was not performed.

Histology, immunofluorescence and TUNEL procedures

Cell death detection by the TUNEL method was performed using commercial kits (Roche, Mannheim, Germany) or a whole-mount procedure as reported by Conlon et al. (Conlon et al., 1995). Immunohistochemistry for laminin (Rabbit anti-laminin, Sigma, St. Louis, MO) was performed according to standard protocols. Except for the Ad-*lacZ* labeling, labeled cells were detected by epifluorescence (Eclipse TE 300, Nikon, Japan). Double detections combined EGFP or fluorescein (CCFSE; in situ cell death detection kit, fluorescein, Roche) with rhodamin (in situ cell death detection kit, rhodamin, Roche) or Alexa-fluor 594 (goat anti-rabbit; Molecular Probes, Eugene, OR). Photographs were taken with a digital camera (CoolSnap, Roper Scientific Inc., Trenton, NJ).

Results

Fate of MEE cells after palate shelf fusion

To study the shelf fusion process that is required for secondary palate formation, we have cultured palate shelves integrated to the nasal region of the head (Cuervo et al., 2002). In this culture system, complete shelf fusion occurs within 24 hours (Fig. 1A, T/L) whereas, when isolated shelves are used, fusion occurs within periods of time of around 48 hours. Hence, any cellular process participating in fusion must occur within the 24 hours time window in our culture conditions. A frequently used protocol to determine the fate of MEE cells consists on the specific labeling of these cells with dyes that can not diffuse trough the basal lamina. One such dye is the CCFSE, which, once inside a cell, becomes modified and can not diffuse to other cells. After MEE labeling with CCFSE, we forced the contact between shelves and followed the fate of the labeled cells at different time points during the fusion process. Epithelial cell viability was not affected by CCFSE labeling (data not shown). We previously reported some differences in cell death activation within different MEE regions. Cell death in the anterior MEE region is activated shortly after shelf contact, whereas, cell death in the posterior MEE region is

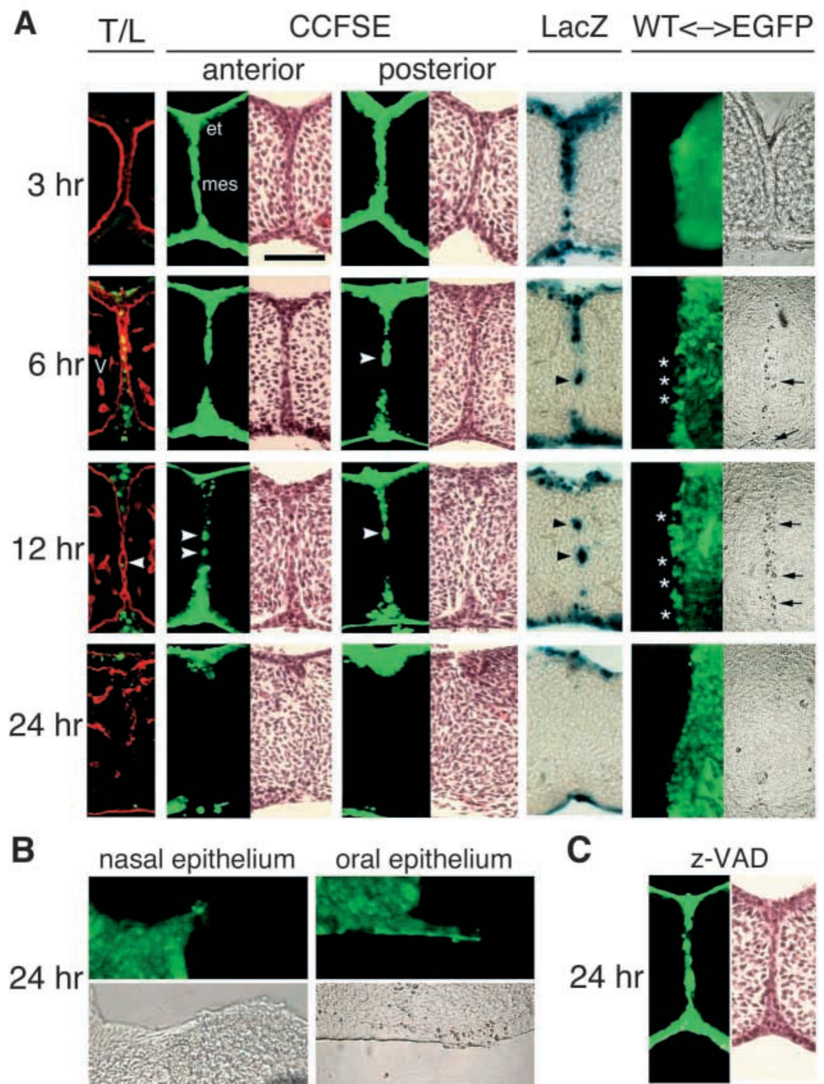

Fig. 1. Analysis of MEE cell fate using different approaches. (A) Left lane (T/L) shows the cell death and basal lamina degradation patterns during palate shelf fusion in vitro (0-24 hours). Cell death detection by TUNEL (green) and laminin immunohistochemistry (red) were performed on the same slice. Laminin-specific immunohistochemistry detected basal lamina as well as blood vessels (V). Three hours after shelf contact, MES and basal lamina were unaltered. By 6 hours, concomitant cell death and basal lamina fragmentation indicate that MES degradation had begun. By 12 hours, MES degradation was very advanced, showing many dying cells within the epithelial triangles and few dying in the MES within the epithelial pearls (arrowhead) as indicated by the surrounding basal lamina. No MEE cells (alive or dead) and laminin were detected in the region surrounding the MES at the end of culture (24 hours). MEE cells of palates of equivalent stages were labeled with CCFSE or Ad-*lacZ* (LacZ in figure) before contact and their fate was analyzed after 3, 6, 12 and 24 hours in anterior and posterior palatal regions. Epithelial pearls were evident at 6 and 12 hours after contact (arrowheads) using both labeling protocols. At the end of culture (24 hours) no labeled cells were detected in the MES. At no time was the presence of labeled cells evident in the mesenchyme compartment. By joining one wild-type shelf with one from the EGFP mouse strain, we produced chimeric palates (WT↔EGFP). At no time were EGFP-positive cells detected in the wild-type mesenchyme compartment of these chimeric palates. Interestingly, chimeric palates showed intercalation of MEE cells (asterisks), at the time abundant apoptotic bodies were detected (arrows). After 24 hours of culture, fusion was complete and no mesenchymal cell migration was detected between halves. et, epithelial triangles; mes, medial epithelial seam. (B) Although no EGFP-positive cells were detected in the wild-type mesenchyme compartment of chimaeric palates, EGFP-positive cells (green) were detected in the wild-type oral and nasal epithelia. (C) CCFSE-labeled cells are not detected in the mesenchyme compartment when cell death is inhibited by z-VAD. Scale bar: 100 µm.

observed prior to shelf contact. It is therefore possible that different mechanism control fusion in the anterior and posterior MEE regions; thus, the fate of MEE cells was studied in both regions. After 3 hours of contact, adhesion between shelves was very strong and there were no signs of MEE cell death, basal lamina degradation, migration or EMT (Fig. 1A, 3 hours T/L and CCFSE). Three hours later, the MES started to disrupt and many dying cells could be observed (Fig. 1A, 6 hours T/L and CCFSE). Epithelial triangles were clearly identified at this time, and stained cells were not detected outside the MES. Twelve hours after contact, many dying cells and remnants of basal lamina were still present within the MES (Fig. 1A, 12 hours T/L and CCFSE), but by 24 hours, all dead cells were absent and the basal lamina was completely degraded (Fig. 1A, 24 hours T/L and CCFSE). Very few stained cells were detected outside the MES after this period of time, and among those, most of them were detected as dying cells (Table 1) probably inside phagocytes.

An alternative strategy to follow the fate of developing cells is by using viral vectors that carry a reporter gene. A recent report shows that labeling of cells with a retroviral vector carrying the *lacZ* gene allows the detection of many labeled cells within the mesenchyme compartment after fusion (Martinez-Alvarez et al., 2000). However, infection with a retroviral vector requires induction of cell proliferation by

Table 1. Quantification of MEE labeled cells in the mesenchyme compartment after fusion

Labeling method (culture condition)	Number of slices analyzed (width)	Number of labeled cells in mesenchyme	Number of TUNEL-positive cells
CCFSE	94 (12 µm)	12	12
CCFSE (+z-VAD)	100 (12 µm)	0	0
Ad-*lacZ*	60 (50 µm)	3	n.d.

MEE cells of palates were labeled with CCFSE or Ad-*lacZ* before contact and cultured for 24 hours. CCFSE labeled palates were also cultured in the presence of z-VAD. Slices from those palates (three for each condition) were produced and labeled cells in the mesenchyme compartment were searched along anterior and posterior palatal regions. Cell death was also determined in the same samples by the TUNEL technique. n.d., not determined.

serum. As serum could cause artifacts during fusion, we preferred to use an adenovirus-based vector, also carrying the *lacZ* gene, which does not require serum for infection. Cell labeling with the adenoviral vector used here was more extensive than that reported using the retroviral one. As with the dye, most of the MEE infected cells were dying and none was detected outside the MES (Fig. 1A, LacZ column; Table 1).

We also followed the fate of MEE cells by forming chimaeric palates between a wild-type shelf and a shelf from a mouse embryo that expresses constitutively the GFP protein (WT↔EGFP). In this situation, the cells that undergo EMT would be detected only if they cross the MES. That is, transdifferentiated cells that migrated within the same shelf would not be detected. Of course, in principle, mesenchymal cells present prior to shelf contact could also migrate between shelves during or after fusion. No cells were detected to cross the MES (Fig. 1A, WT↔EGFP), supporting the low frequency of EMT occurrence and also the limited migratory ability of mesenchymal cells from one shelf to another. Regardless of the behavior of MEE and mesenchymal cells, nasal and oral epithelial cells were observed to migrate between shelves (Fig. 1B). From these experiments, it was also interesting to observe that as the double adhered epithelial layer turned into a single epithelial layer, intercalation of epithelial cells from both shelves (i.e. EGFP-positive and EGFP-negative cells) became obvious (Fig. 1A, 6-12 hours/WT↔EGFP).

Fate of MEE cells in a palate slice culture system

In order to follow the fate of MEE cells continuously during MES degeneration, we established a slice culture system in which cell migration can be studied in further detail. MEE cells were labeled with CCFSE and shelves were put in contact as described above. Three hours later, 200 μm slices were obtained. At this time, MES appeared intact without signs of degeneration (Fig. 2; 3 hours). Next, slices were cultured as described in the Materials and methods. In slices cultured for 3 hours (i.e. 6 hours after contact), the MES thinned; 6 hours later it seemed fragmented (Fig. 2; 6 hours and 12 hours, respectively). At this later time, no labeled cells were found outside the MES. Twenty-four hours after contact, the MES could not be visualized by phase-contrast microscopy, but fluorescent cells were still detected within the fusion region (Fig. 2, 24 hours). The great majority of remaining cells at this time was of dying cells (Fig. 2, TUNEL). Therefore, in this culture system, MEE fusion takes place in the absence of detectable EMT contribution.

Relationship between cell death and basal lamina degradation

Despite the failure to detect EMT during MEE fusion in our in vitro system, it is still possible that it takes place but it is immediately followed by cell death. Under normal conditions, cell death would preclude the detection of EMT as the cause of MES degeneration. To address this issue, we blocked cell death with an inhibitor of caspases, z-VAD, and searched for labeled cells in the mesenchyme compartment. We previously showed that this inhibitor is very effective for blocking cell death in rugae and MES (Cuervo et al., 2002) (see also Fig. 4). No such labeled cells were found in treated palates (Fig. 1C; Table 1). These results indicate first, that failure to detect MEE

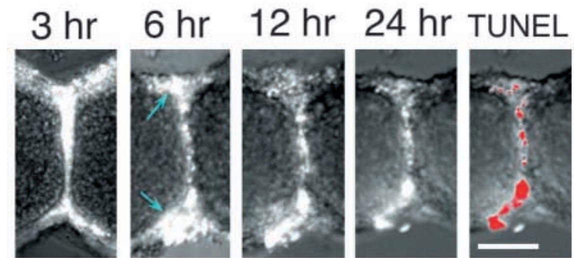

Fig. 2. Time-course analysis of MES degeneration in a live palate slice. MEE cells of 14.5 dpc palate shelves were labeled with CCFSE (bright signal) before contact. Three hours after contact, 200 μm transverse slices were produced. Selected individual slices were cultured, and micrographs of same slices were taken at 3, 6, 12 and 24 hours. At the end, cultured slices were processed for cell death detection (TUNEL positive, red). Note the accumulation of labeled cells in epithelial triangles at 3 and 6 hours of culture (blue arrows). At 6 and 12 hours of culture, the fragmented MES was obvious. At the end of culture (24 hours), the remaining labeled cells were detected as dying cells and none was clearly detected in the mesenchyme. These experiments were repeated more than three times at least in triplicate for each condition. Scale bar: 100 μm.

transdifferentiated cells was not because they died soon after differentiation, and second, that EMT can not compensate for MES degeneration in the absence of cell death.

In the previous experiments, we also assessed the integrity of the basal lamina. To allow EMT, the basal lamina needs to degrade by a mechanism involving metalloproteinases (MMPs). It has actually been proposed that basal lamina degradation is one of the earliest events of EMT (Lochter et al., 1997; Song et al., 2000). In agreement with the rare occurrence of EMT during shelf fusion, we did not detect basal lamina degradation when cell death was blocked (Fig. 3, z-VAD). Interestingly, MMP inhibition prevented basal lamina degradation and blocked fusion without affecting the occurrence of cell death (Fig. 3, MMI). Toxic effects, or other different than preventing basal lamina degradation, by MMP inhibitors were not observed in these experiments, given that palate shelves looked histologically unaltered and cell death was not detected in ectopic regions (Fig. 3 and data not shown). Therefore, it appears that cell death is not induced by the basal lamina degradation that should accompany EMT, but rather basal lamina degradation appears to be a secondary event activated by the dying cells (‘cataptosis’; see Discussion).

To obtain supporting evidence for the occurrence of cataptosis, we treated untouched palate shelves with RA, a very strong MEE cell death inducer. Under these conditions, RA-induced cell death was also accompanied by basal lamina degradation (Fig. 4, mee/RA). Basal lamina degradation was not an independent event regulated by RA, as cell death inhibition under this condition also inhibited basal lamina degradation (Fig. 4, mee/z-VAD/RA). Interestingly, cataptosis was restricted to the MEE region, as cell death in rugae epithelium (natural or RA-induced) had no effect in the integrity of basal lamina (Fig. 4, control and rugae/RA). Finally, a general activation of cell death by staurosporin also induced basal lamina degradation specifically in the MEE region and preferentially in regions underlying apoptotic cells, whereas basal lamina remained intact in the surrounding tissue (Fig. 4/staurosporine). Therefore, these data suggest that as a

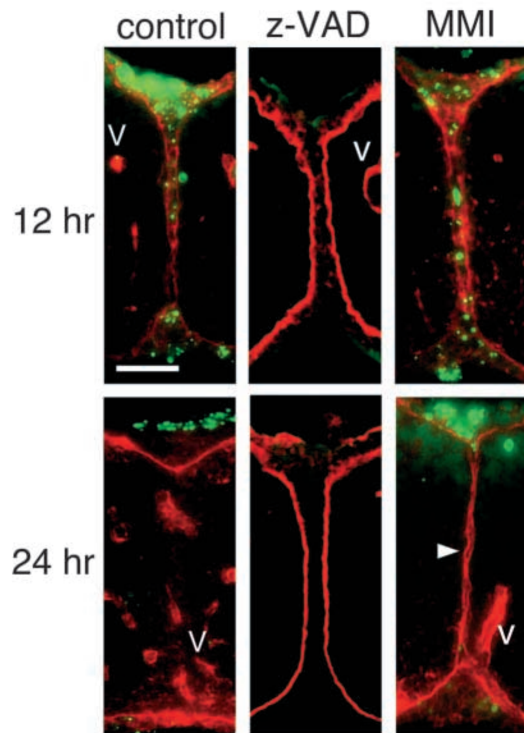

Fig. 3. Relationship between cell death and basal lamina degradation. Shelves of developing palates were put in contact and cultured in the presence of either a caspase inhibitor (z-VAD) or a metalloproteinase inhibitor (MMI). At the end of culture (24 hours), cell death (green) and laminin (red) were detected in the same palate slice by TUNEL and specific immunohistochemistry, respectively. After 12 hours in culture, control palates show an advanced MEE cell death and basal lamina degradation. At the end of culture, MEE and basal lamina completely disappeared. However, z-VAD treatment inhibited cell death but basal lamina remained intact. Application of 10 µM BB3103 (MMI) did not alter the apoptotic fate of MEE cells but, as expected, inhibited basal lamina degradation (arrowhead). These experiments were repeated more than three times with at least a triplicate for each condition. v, blood vessel. Scale bar: 50 µm.

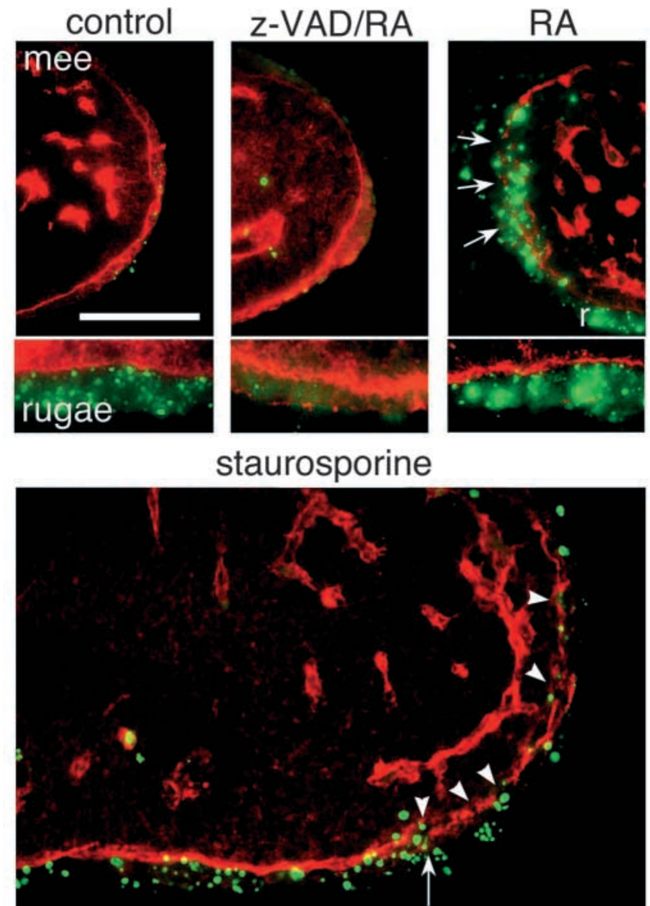

Fig. 4. Activation of basal lamina degradation by MEE cell death stimuli. Individual palate shelves were cultured without contact in the presence of retinoic acid (RA; a MEE cell death activator) or staurosporine (a broad-spectrum cell death activator). At the end of culture (10 hours), cell death (green) and laminin (red) were detected in the same palate slice by TUNEL and specific immunohistochemistry, respectively. RA induced extensive cell death in the MEE and rugae (r) of isolated shelves, but basal lamina degraded only in the MEE apoptotic region (arrows). Treatment with z-VAD blocked RA-induced cell death and basal lamina remained intact. Generalized induction of epithelial cell death with staurosporine activated the degradation of the basal lamina underlying the dying MEE cells (arrowheads), whereas basal lamina underlying the MEE adjacent epithelium (left from the arrow) was unaffected. These experiments were repeated more than three times at least in triplicate for each condition. Scale bar: 100 µm.

consequence of cell death activation in the MES, the MMPs responsible for basal lamina degradation gets activated.

Migration of the periderm cells associated to the MEE

As previously shown, most MEE cells die during fusion. However, another component associated to the MEE is the periderm that covers most of the shelf surface that first comes into contact with the opposite shelf. In order to label periderm cells preferentially, we stained palate shelves for a very short period of time (i.e. 30 seconds). The selectivity of this staining procedure can be clearly seen in slices of these preparations (see, for example, Fig. 5, CCFSE/2 hours). To determine the fate of periderm cells, they were followed during the fusion process. In contrast to MEE cells that appeared to die in situ, periderm cells migrated to the oral and nasal ends of the MES, contributing to the formation of the epithelial triangles, where most of them died (Fig. 5, 2-8 hours).

To gain insights on the role of periderm cells during fusion, we inhibited cell migration with cytochalasin D, which blocks actin polymerization. As expected, periderm cells did not migrate, epithelial triangles did not form and complete adhesion did not occur (Fig. 6A). Interestingly, cell death in both basal MEE and the overlying periderm was not triggered in the presence of cytochalasin D (Fig. 6B). Blockade of actin polimerization-depolimerization does not appear to interfere in general with the cell death execution process, as the drug did not modify cell death in rugae (Fig. 6C). Cycloheximide also inhibited migration and the epithelial triangles did not form; however, in this case, cell death in rugae was affected as well (data not shown). These data together suggest that migration of periderm cells out of the MEE area is essential to initiate the fusion process.

In the previous experiments, it is possible that cell death was

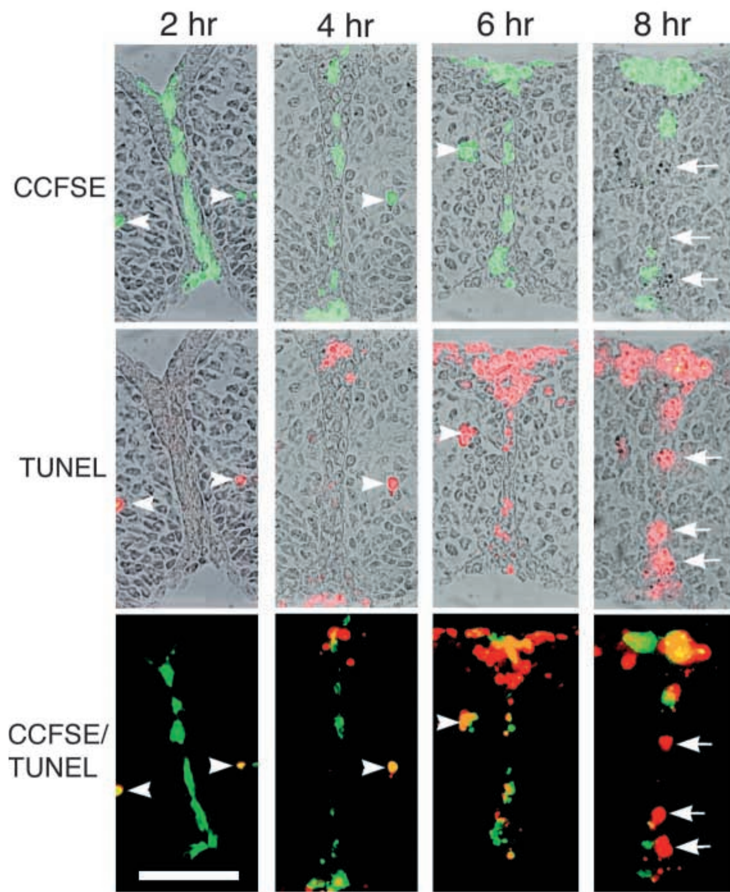

Fig. 5. Analysis of periderm cell fate. Periderm cells were labeled with CCFSE as described in the Materials and methods, and palates were cultured for 8 hours. Samples were analyzed every 2 hours. At the beginning of culture (2 hours), periderm cells were confined to the MES middle line between the two basal MEE; cell death was not detected at this time. As fusion proceeded (4, 6 and 8 hours), accumulation of labeled cells occurred at the apex of the MES, constituting a large proportion of epithelial triangle cells. Most labeled cells died within the epithelial triangles (yellow cells; see also Fig. 5). As noted here, basal MEE cells appeared to die in situ within the epithelial pearls (arrows at 8 hours; compare with Fig. 1). Arrowheads indicate autofluorescent erythrocytes. These experiments were repeated more than three times at least in triplicate for each condition. Scale bar: 100 μm.

not activated in the MEE because periderm cells act as a barrier for intimate contact between shelves, a conceivable requirement for cell death activation and fusion (Cuervo et al., 2002). To test this possibility, we removed this cell layer by controlled trypsin treatment (see Materials and methods). Palate shelves lacking periderm cells adhered, activated cell death and fused. However, epithelial triangles did not form, resulting in a thinner secondary palate (Fig. 7A). Interestingly, viability of MEE cells in isolated shelves markedly decreased when periderm cells were removed (Fig. 7B). In conclusion, periderm cells do not appear to be necessary for the fusion process itself, but they need to migrate out of the MES to allow contact and cell death activation.

Discussion

Our studies indicate that the primary and major fate of MEE cells of secondary palate shelves is death, a requirement for MES degeneration and fusion. We did not find evidence of EMT, and migration could be detected only for the periderm cells that overlie the MEE. Furthermore, we found, for the first time, that basal lamina degrades as a consequence of cell death, emphasizing the relevance of this latter process in shelf fusion.

Several years ago, Fitchett and Hay (Fitchett and Hay, 1989) presented the first evidence to suggest that EMT causes MES degradation. Subsequently, other authors presented additional evidences supporting this role of EMT (Kaartinen et al., 1997; Martinez-Alvarez et al., 2000; Shuler et al., 1991; Shuler et al., 1992). Currently, this idea prevails, in some cases neglecting the participation of cell death (Young et al., 2000). In some of our experiments, we used experimental strategies similar to those used in previous reports, but using an improved palate culture system in which shelf fusion occurs within a similar time window as observed in vivo. Under these conditions, we were unable to detect any obvious participation of EMT. The few labeled cells found around the MES at the end of culture were undergoing cell death (Table 1). Furthermore, when cell death was prevented, EMT was still not detected, indicating that EMT does not compensate for the inability to eliminate MEE by cell death. Because it is possible that individual transdifferentiated cells escaped to our detection methods, we analyzed each MEE cell within a 200 μm MES region using a novel slice culture system. Again, we failed to detect any evidence of EMT and found, instead, that most MEE cells were dying.

Why the discrepancy between our data and those previous reports? With some exceptions (Sun et al., 1998), very few MEE cells have been reported to undergo EMT, and in most reports quantitative analyses are lacking. Moreover, in none of these reports, has it been determined whether the assumed transdifferentiated cells are the dying cells or whether they are phagocytes containing dying cells instead. These drawbacks make it difficult to estimate the contribution of EMT to MES degeneration. It is also possible that palate shelf fusion does not occur equally along the rostrocaudal axis, which might explain the discrepancies if the studies were performed at various points along this axis. We considered this possibility and, thus, studied MES degeneration along the complete length of the rostrocaudal axis. Another possible explanation for the contrasting results obtained in our experiments is the use of an improved culture system. Dissected shelves are usually put together and then cultured in the presence of serum to allow fusion. We have found that our culture system allows a more precise contact between shelves and an efficient fusion in the absence of serum. Shuler et al. (Shuler et al., 1992) also labeled MEE in vivo with DiI and showed that clumps of labeled cells remain around the fusion line. It is possible that artifactual staining occurred in those experiments (DiI can easily precipitate), as individual cells could not be visualized and DiI membrane incorporation would not ensure the transfer of the dye to other more internal cells. Despite these apparently conflicting results, we propose that cell death is the major contributor to MES degeneration, even considering a low occurrence of EMT.

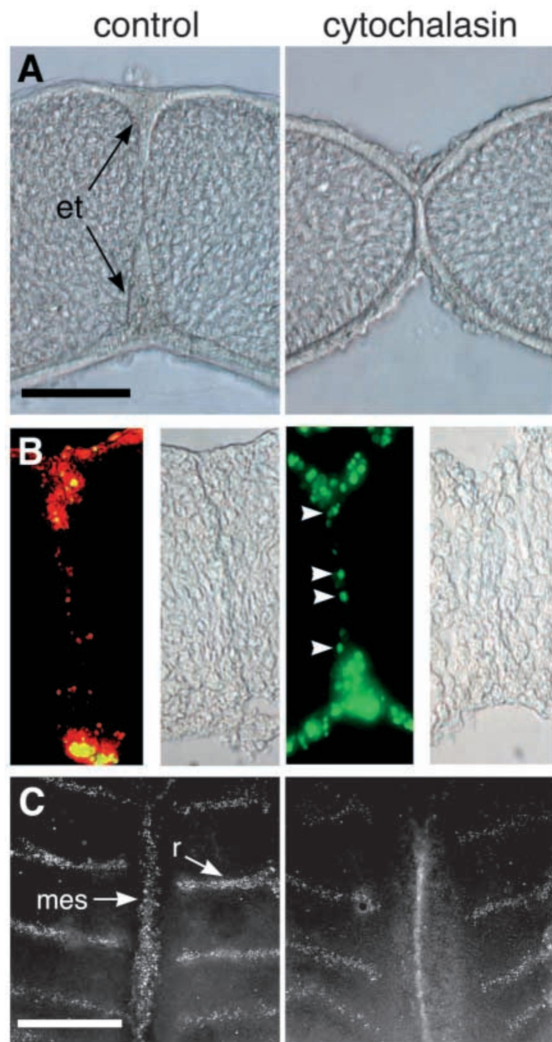

Fig. 6. Effect of inhibition of periderm cell migration on cell death and fusion. After periderm cell labeling (green), palate shelves were put in contact and cultured in the presence or absence of 6 μM cytochalasin D for 10 hours. (A) When cytochalasin D was included in the medium, palate morphology showed the lack of epithelial triangles (et) and weak shelf adhesion. To detect cell death, palates were either stained in whole-mount with Acridine Orange (C; bright spots) or slices processed for the TUNEL technique (B; red). (B) Periderm cells of control palates died within epithelial triangles (yellow; see also Fig. 3), whereas those from cytochalasin D-treated palates did not reach the oral and nasal closures and did not die (green cells; arrowheads). (C) Specific reduction in cell death was observed in the mes of cytochalasin D-treated palates with a minimum effect in rugae (r). These experiments were repeated more than three times at least in triplicate for each condition. Scale bars: in A, 100 μm for A; in C, 500 μm for C.

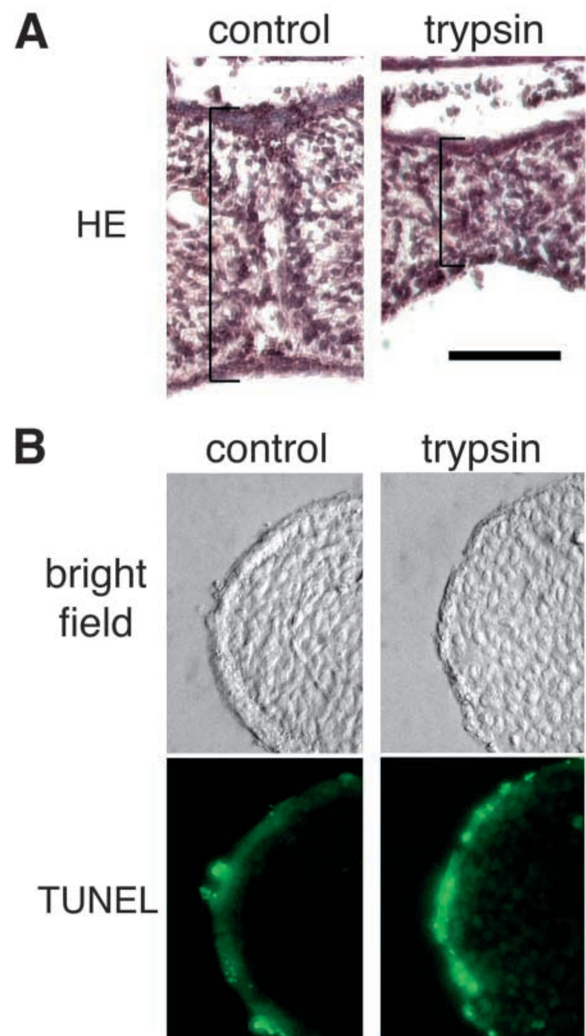

Fig. 7. Effect of periderm cell removal on basal MEE cell viability and shelf fusion. Periderm cells were removed by washing the MEE region after controlled trypsin digestion performed on 14.5 dpc palate shelves before contact. Control palates were also treated with trypsin but washing was not performed. (A) Treated shelves were put in contact and fusion was analyzed 24 hours later by standard Hematoxylin-Eosin staining (HE). Although MES degenerated, proper fusion between 'denuded' palate shelves did not occur. Note the absence of epithelial triangles and marked reduction in MES thickness when compared with a control sample (brackets). (B) Isolated halves were cultured for 10 hours and cell death analyzed with the TUNEL technique. More dying MEE cells were detected in 'denuded' palates (i.e. without periderm cells). These experiments were repeated more than three times at least in triplicate for each condition. Scale bar: 100 μm.

MEE cell migration has also been proposed as a mechanism for MES degeneration (Carette and Ferguson, 1992). However, those studies did not take into account the migration of the periderm cells that overlay the MEE. In our study, we stained periderm cells preferentially and demonstrated that soon after contact they migrate toward the oral and nasal cavities and form the epithelial triangles. In keeping with this observation, when migration was blocked or periderm cells were selectively eliminated, the epithelial triangles did not form. Shelf fusion did take place in the absence of periderm cells, but it resulted in a thinner palate. It has been considered that periderm cells shed before contact (Fitchett and Hay, 1989); our data indicate, however, that epithelial triangles result from periderm cell migration, a process that appears to be necessary for proper fusion. Furthermore, a relevant finding of the present work was that periderm cell migration plays role in activation of cell death of both periderm and basal MEE cells. Periderm cells are

likely to produce the filopodia and to be the source of the proteoglycans required for shelf adhesion (Gato et al., 2002; Taya et al., 1999). We propose that periderm cell migration is relevant for the efficient shelf fusion regulating adhesion and cell death activation.

Anoikis is a term given to the process of cell death induced by the lack of contact with the extracellular matrix. The basal lamina has been considered to be an essential survival factor for epithelial cells in vitro and in vivo (Coucouvanis and Martin, 1995; Ruoslahti and Reed, 1994). For example, it has been shown that during mammary gland involution or Müller duct regression, disrupting the underlying extracellular matrix induces epithelial cell death (Pullan et al., 1996; Roberts et al., 2002). This prompted us to assess whether the trigger for MEE cell death activation was basal lamina degradation. Blocking basal lamina degradation by inhibiting MMP activity, however, had no effect on cell death. These results contrast with two recent reports (Blavier et al., 2001; Brown et al., 2002) showing partial or no MEE degeneration in the presence of the same MMP inhibitor used here. In our experiments, we demonstrated [with high reproducibility even at the low inhibitor dose (10 μM)] that the basal lamina was intact. In the aforementioned reports, the integrity of basal lamina was not demonstrated, and hence, their results can be interpreted as incomplete MEE degeneration. Our observations in the presence of the MMP inhibitor do not imply that basal lamina has no survival activity on MEE cells, but suggests that MEE cell death is not triggered by basal lamina degradation. On the contrary, we found that cell death activates basal lamina degradation (see Fig. 4). To our knowledge, this is an unprecedented finding that gives a new function to the process of cell death. We propose the term 'cataptosis' (a Greek word meaning downfall) to describe this phenomenon. Cataptosis may occur in different developmental process involving tissue regression, in order to coordinate cell degeneration with extracellular matrix degradation. In the palate, the basal lamina degradation activity is restricted to the dying MEE cells, suggesting that specific factors give them this property (see below). This conclusion is also in opposition to the participation of EMT, as inhibition of basal lamina degradation can block EMT (Song et al., 2000), and MMPs can directly induce EMT (Lochter et al., 1997).

Collagen IV and laminin, the most abundant components of basal lamina, are likely to be the major MMPs substrates during basal lamina degradation. MMPs are found extracellularly and also bound to the plasma membrane (MT-MMPs) (Birkedal-Hansen, 1995). The MMP activity could be regulated at different levels. MMP gene expression is characteristic during tissue remodeling but postranslational regulation is crucial for enzymatic activity. With the exception of MT-MMPs, MMPs are synthesized as inactive proenzymes that need to be processed by other enzymes, such as plasmin or other MMPs, to become active (Nagase, 1997). Furthermore, MMP activity can be negatively regulated by direct binding of proteins such as members of the tissue inhibitor of metalloproteinases (TIMP) family (Gomez et al., 1997). Among the several MMPs and their inhibitors described to date, *MT1-Mmp* (*Mmp14* – Mouse Genome Informatics), *Mmp2*, *Mmp3*, *Mmp9*, *Mmp13*, *Timp1* and *Timp2* are expressed in the developing palate (Blavier et al., 2001; Morris-Wiman et al., 2000). *MT1-Mmp*, *Mmp13* and *Timp2* are specifically expressed in the MEE at the time fusion occurs (Blavier et al., 2001). These genes could be the ones that provide the MEE with the distinct ability to activate cataptosis. MMP2 and MMP13 can digest collagen IV (Knäuper et al., 1997), suggesting a role for these enzymes in basal lamina degradation, although their role in vivo has not been demonstrated. TIMP2 could prevent extracellular matrix degradation; it has been shown, however, that it is also relevant for the efficient MMP2 activation in vivo (Wang et al., 2000). MT1-MMP, conversely, could be the initiator of a cascade of MMPs involved in cataptosis. Recently, it was reported that MT1-MMP and MMP2 translocate from the cytoplasm to the plasma membrane in endothelial cells upon stimulation of apoptosis (Levkau et al., 2002). Furthermore, MT1-MMP can activate proMMP13 (Knäuper et al., 2002). However, as *Mt1-Mmp* null mutants do not have an obvious palate phenotype (Holmbeck et al., 1999), the latter hypothesis would imply that redundant mechanisms are activated during MES cataptosis.

In summary, secondary palate shelf fusion process can be described as follows (Fig. 8). Initially, shelves approach each other until contact is made probably between filopodia from

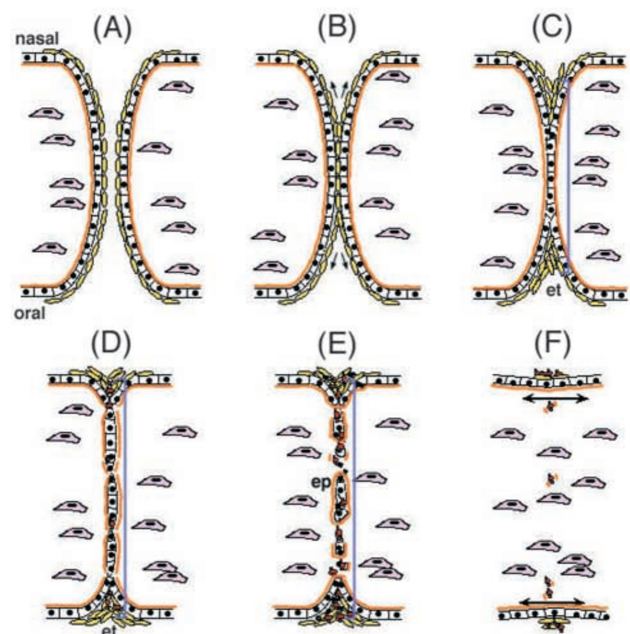

Fig. 8. Schematic representation of palate shelf fusion. (A) Initially, shelves approach each other at the time the periderm cells (yellow cells) overlying the basal MEE cells (white cells) emit filopodia. (B) First contact and adhesion occurs between periderm cells; proteoglycans appear to be important at this stage. Adhesion becomes stronger as periderm cells move up and down (arrows) the MES (bracket) forming the epithelial triangles (et). (C) Basal MEE cells of each shelf intercalate (convergent extension) resulting in a single epithelial layer. (D) MES breaks up and epithelial pearls (ep) form; periderm and MEE cells start to die within epithelial triangles and epithelial pearls, respectively (red cells). (E) MES, which is composed of periderm and basal MEE cells, essentially degenerates by cell death; dying cells activate basal lamina degradation (cataptosis; broken orange line). (F) Fusion is complete without a major mesenchymal cell movement across the midline; some oral and nasal epithelial cells do move across the middle line (double-headed arrows). Pink cells represent mesenchymal cells. Orange lines represent basal lamina.

periderm cells and with the help of proteoglycans. More intimate MEE contact proceeds through a process that is accompanied by the migration of periderm cells to the oral and nasal ends. Progressive adhesion appears to be controlled by periderm cell migration. If this periderm cell migration is not in place, a thinner palate would form. Periderm cells in the epithelial triangles could also be important for sealing the ends of the MES. Chimaeric palates CD1↔EGFP clearly reveal the intercalation between MEE cells from each shelf as recently reported (Tudela et al., 2002). This process, which results in a single epithelial sheath, represent a classical convergent extension phenomenon (Wallingford et al., 2002), and cause MES growth in both anteroposterior and oronasal axes. Strong adhesion between shelves probably originates from this MEE cell intercalation, and we propose that it is up to this stage that cell death is activated. Finally, dying cells actively promote the activation of MMPs such as MT1-MMP and MMP13 that cause basal lamina degradation. Basal lamina initially breaks down in fragments generating the epithelial pearls that later degenerate to produce the fused palate.

Two key molecules have been identified to be relevant in the fusion process. Retinoic acid appears to be essential for the control of cell death (Cuervo et al., 2002), and consequently for basal lamina degradation. TGFβ3, however, has been proposed to be involved in the control of EMT (Kaartinen et al., 1997; Sun et al., 1998). In the heart, strong evidence supports a role of TGFβ3 in EMT (Ramsdell and Markwald, 1997). *Tgfb3* knock-out mice display cleft palate, nevertheless, the specific TGFβ3 function that is responsible for this phenotype remains to be elucidated. *Tgfb3*$^{-/-}$ shelves lack the characteristic MEE associated filopodia normally observed prior to shelf contact, and also show a significant decrease in proteoglycans on the MEE surface (Gato et al., 2002; Taya et al., 1999). These data suggest that TGFβ3 is crucial for the initial contact and during adhesion between shelves. TGFβ3 could also play a role in the control of periderm cell migration. It is interesting to note that palates treated with cytochalasin D are a phenocopy of those from *Tgfb3*$^{-/-}$ embryos (see Fig. 6). Palates in both conditions show poor shelf adhesion, reduced cell death and lack of epithelial triangles (Martinez-Alvarez et al., 2000; Taya et al., 1999). Inhibition of actin polymerization would block cell motility, resulting in absence of filopodia and cell migration. In our experiments, it is unlikely that defective contact and adhesion, owing to lack of filopodia, is causing the unfused palate phenotype, because shelf contact was forced. Therefore, TGFβ3 might have a migration-promoting activity on periderm cells, as it appears to occur on endocardial cells transformed to mesenchyme (Ramsdell and Markwald, 1997). Despite the proapoptotic activity that has been demonstrated for TGFβ3 (Nguyen and Pollard, 2000; Opperman et al., 2000; Dunker et al., 2002), reduced MEE cell death in palates from *Tgfb3*$^{-/-}$ mice (Martinez-Alvarez et al., 2000) could result from defective adhesion or periderm cell migration. Additionally, TGFβ3 could influence the MMP activity initiated by cell death, because it is known that it regulates *Mmp13* expression in the palate (Blavier et al., 2001). Regulation in the opposite direction, however, could also occur: MMP13 could process the TGFβ3 precursor and in this way regulate its activity (Sternlicht and Werb, 2001; Yu and Stamenkovic, 2000). Further experiments are needed to determine the significance of this positive regulatory loop in basal lamina degradation.

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