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*Short Contribution*

# Bulging medial edge epithelial cells and palatal fusion

CONCEPCIÓN MARTÍNEZ-ÁLVAREZ[1], ROSA BONELLI[1*], CONSUELO TUDELA[1*], ANGEL GATO[2], JESÚS MENA[1], SHARON O'KANE[3] and MARK W.J. FERGUSON[3]

*[1] Departamento de Ciencias Morfológicas I, Facultad de Medicina, Universidad Complutense de Madrid. Spain.*
*[2]Departamento de Anatomía, Facultad de Medicina, Universidad de Valladolid, Spain.*
*[3]Cells, Immunology and Development Division, School of Biological Sciences. The University of Manchester, Manchester, U.K.*

**ABSTRACT The surface of the medial edge epithelium of embryonic day 12, 13 and 14 mouse palatal shelves was observed utilising Environmental Scanning Electron Microscopy (ESEM). This technique offers the advantage of visualisation of biological samples after short fixation times in their natural hydrated state. Bulging epithelial cells were observed consistently on the medial edge epithelium prior to palatal shelf fusion. Additionally, we have used ESEM to compare the morphology and surface features of palatal shelves from embryonic day 13 to 16 mouse embryos that are homozygous null (TGF-$\beta_3$ -/-), heterozygous (TGF-$\beta_3$ +/-) or homozygous normal (TGF-$\beta_3$ +/+) for transforming growth factor beta-3 (TGF-$\beta_3$). At embryonic day 15 and 16 most TGF-$\beta_3$ +/- and +/+ embryos showed total palatal fusion, whilst all TGF-$\beta_3$ null mutants had cleft palate: the middle third of the palatal shelves had adhered, leaving an anterior and posterior cleft. From embryonic day 14 to 16 abundant cells were observed bulging on the medial edge epithelial surface of palates from the TGF-$\beta_3$ +/- and +/+ embryos. However, they were never seen in the TGF-$\beta_3$ null embryos, suggesting that these surface bulges might be important in palatal fusion and that their normal differentiation is induced by TGF-$\beta_3$. The expression pattern of E-Cadherin, β-catenin, chondroitin sulphate proteoglycan, β-Actin and vinculin as assayed by immunocytochemistry in these cells shows specific variations that suggest their importance in palatal shelf adhesion.**

KEY WORDS: *Environmental scanning electron microscopy, TGF-$\beta_3$, null mutant mice, E-cadherin, β-catenin, chondroitin sulfate, β-actin, vinculin*

In mammals, the definitive palate is formed by the fusion of the primary palate with the fusing secondary palatal shelves. The epithelial cells covering their tips (medial edge epithelial cells: MEE) contact in the midline at embryonic day 14.5 (E14.5) (Ferguson, 1988). They then adhere and undergo programmed cell death (Mori *et al.,* 1994; Taniguchi *et al.,* 1995; Martínez-Álvarez *et al.,* 2000), epithelial-mesenchymal transformation (Fittchet and Hay, 1989; Griffith and Hay, 1992; Shuler *et al.*, 1992; Martínez-Álvarez *et al.*, 2000) and migration to the oral and nasal sides of the palate (Carette and Ferguson, 1992). Some of these events have been reported to be dependent on transforming growth factor beta-3 (TGF-$\beta_3$) (Kaartinen *et al.,* 1997; Martínez-Álvarez *et al.*, 2000) and the disruption of the TGF-$\beta_3$ gene results in cleft palate, both in humans (Lidral *et al.,* 1998) and in mice (Proetzel *et al.,* 1995; Kaartinen *et al.,* 1995; Taya *et al.,* 1999). Scanning Electron Microscopic (SEM) studies showed the presence of small round cells, cellular debris, filamentous material, filopodia and lamellipodia over the MEE. These were suggested to be representative of degenerative changes of superficial cells of the MEE which facilitate subsequent adhesion (Schüpbach *et al.*, 1983). However, no one has previously investigated the morphological appearance of the palatal medial edge epithelia using Environmental Scanning Electron Microscopy (ESEM). ESEM allows the investigation of biological tissues fixed for only a very short time and without dehydration or gold coating, as in conventional SEM. This means that the surface features of the tissue are observed in as near a natural state as possible. So we have taken advantage of this technique to visualise the prefusion surface of E12 to E14 mouse palatal MEE, together with the morphology and surface features of E13 to E16 mouse palates from embryos which are homozygous null (TGF-$\beta_3$ -/-), heterozygous (TGF-$\beta_3$ +/-) or homozygous normal (TGF-$\beta_3$ +/+) for transforming growth factor beta-3. We have found numerous bulging MEE cells present on the

*Abbreviations used in this paper:* CSPG, chondroitin sulphate proteoglycan; E, embryonic day; ESEM, environmental scanning electron microscopy; MEE, medial edge epithelium; SEM, scanning electron microscopy; TGF-$\beta_3$, transforming growth factor-beta3; TGF-$\beta_3$ +/+, +/- or -/-, TGF-$\beta_3$ homozygous normal, heterozygous or homozygous null.

***Address for reprints:** Dr. Concepción Martínez-Álvarez. Dpto. Ciencias Morfológicas I. Facultad de Medicina. Universidad Complutense de Madrid. Ciudad Universitaria. Avda. Complutense s/n. 28040, Madrid. Spain. Tel.: 34 - 91 - 394 1380. Fax: 34 - 91 - 394 1374. e-mail: calvarez@eucmax.sim.ucm.es

* Both authors contributed equally to this work.

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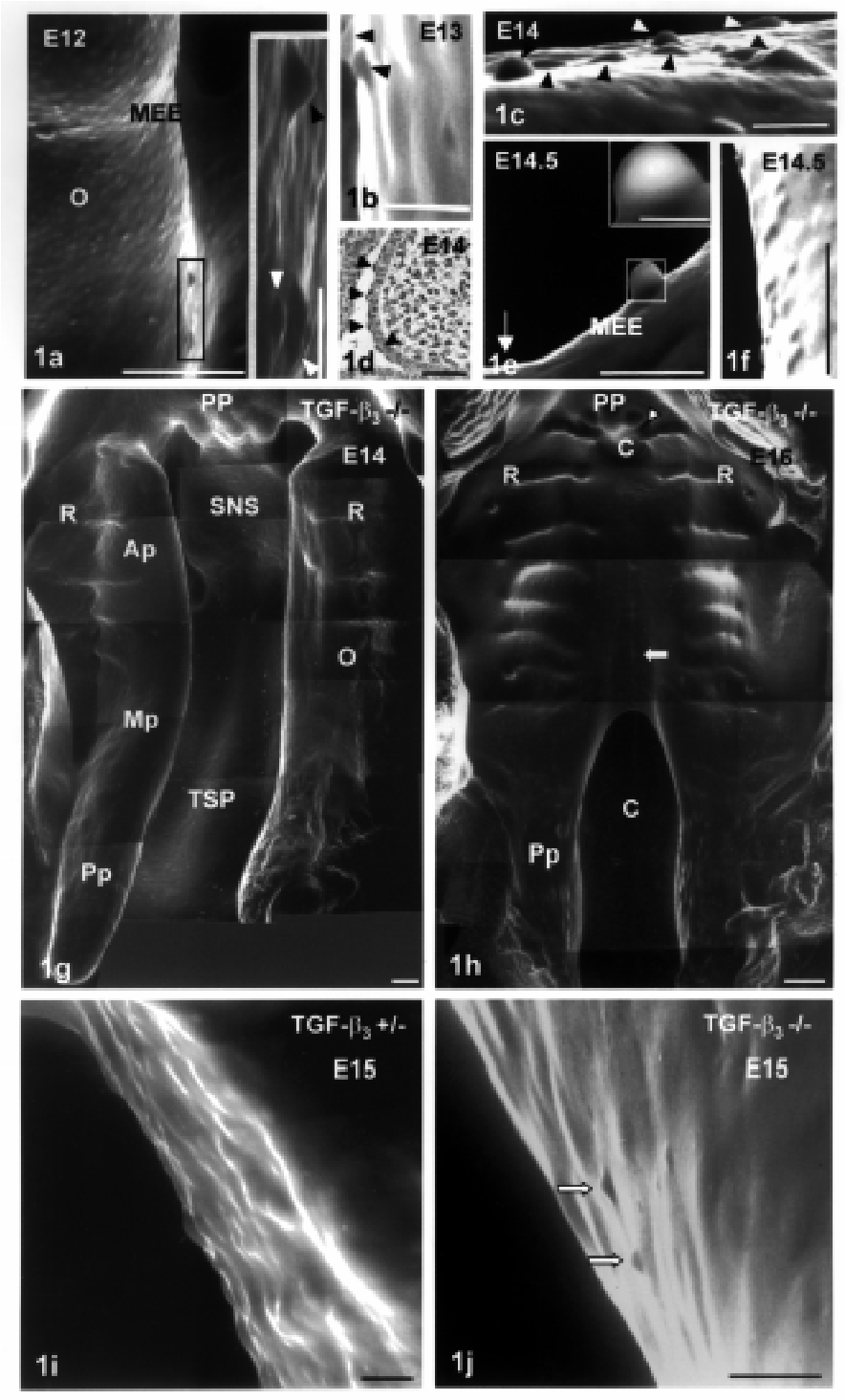


**Fig. 1. The developing palate as observed under ESEM.** *1a to 1f show the MEE of Albino Swiss mouse palates. 1g and 1h are montages made from pictures taken at high magnifications from overlapping fields of E14 (1g) and E16 (1h) TGF-$\beta_3$ null mutant palates. 1i and 1j are high magnifications of the E15 TGF-$\beta_3$ heterozygous (1i) and null mutant (1j) palatal MEE.* ***(1a)*** *Middle third of an E12 mouse right palatal shelf. The boxed area is shown at higher magnification on the right hand side. Some cell-like protrusions are indicated (arrowheads).* ***(1b)*** *Middle third of the medial edge epithelium of an E13 mouse palatal shelf. The arrowheads indicate some cell-like protrusions.* ***(1c)*** *Anterior third of the medial edge epithelium of an E14 mouse unfused palatal shelf. Note the increased number of cell-like protrusions (arrowheads).* ***(1d)*** *Histological section of the medial edge epithelium of a similar staged palate. Several protruding superficial cells are indicated by the arrowheads.* ***(1e)*** *Medial edge epithelium of an E14.5 mouse partially fused palate. The arrow points to the anterior extent of the fusion. A cell-like protrusion is boxed. A higher magnification of the bulge inside the boxed area can be seen in the top right corner.* ***(1f)*** *E14.5 mouse partially fused palate. Many cell-like protrusions can be seen over the medial edge epithelial surface.* ***(1g)*** *E14 TGF-$\beta_3$ null mutant palate. The anterior palate has elevated and five rugae are visible. The middle palate has remodelled. The primary palate shows three characteristic bulges and the secondary nasal septum displays two lateral prominences.* ***(1h)*** *Palate of an E16 TGF-$\beta_3$ null mutant mouse. The primary palate has not fused to the secondary palate (arrowhead). The palatal shelves have not fused between the first and the second rugae nor posterior to the eighth rugae, so displaying an anterior-posterior cleft (C). Several bulges are observed in the midline, indicating the midline seam (arrow).* ***(1i)*** *Numerous cells are bulging on the MEE surface of an E15 TGF-$\beta_3$ heterozygous palate.* ***(1j)*** *In an E15 TGF-$\beta_3$ null mutant palate, the MEE surface is flat throughout the length of the shelf and only very few protruding cells are observed (arrows). Ap: Anterior palate. MEE: Medial edge epithelium. Mp: Middle palate. O: Oral epithelium. PP: Primary palate. Pp: Posterior palate. R: Ruga. SNS: Secondary nasal septum. TSP: Tectal septal process. Scale bar in 1b: 10 $\mu$m; in 1c, 1e, 1j and in the inserts of 1a and 1e: 20 $\mu$m; in 1i: 50 $\mu$m; in 1a, 1d, 1f and 1g: 100 $\mu$m; in 1h: 200$\mu$m.*

wild type palatal shelves before fusion that are very uncommon on the MEE of TGF-$\beta_3$ null palates, suggesting that these surface bulging cells may be involved in palatal fusion. Recently, we have demonstrated that these bulging cells are not dead (Martínez-Álvarez *et al.*, 2000) and we now provide further evidence for their role in palatal shelf adhesion.

**Morphology of the developing palate as visualised by ESEM**

No specimens at any time point showed degenerative material consistently present over the MEE surfaces. Most of the superficial MEE cells at E12 (Fig.1a) and E13 (Fig. 1b) were flattened and their cellular boundaries were not clearly distinguishable. At E14, however, many superficial cells became prominent, especially in the more anterior regions of the palatal shelves (Fig. 1c). They were round in shape and similar to the adjacent cells in size, resembling round bulging epithelial cells. Histology confirmed this aspect (Fig. 1d). In partially fused palates, these bulges were also seen in the MEE of the still unfused parts of the shelves, less frequent or absent in the region anterior to fusion (Fig. 1e), but frequent in the region posterior to the fusion zone (Fig. 1f).

Palatal shelves in all TGF-$\beta_3$ +/+, +/- and -/- (Fig. 1g) E13 and E14 mice displayed a similar appearance (Table 1) and size. Likewise, there were no differences in the primary palate and secondary nasal septum between the TGF-$\beta_3$ -/-, +/+ and +/- embryos. The palatal shelves of all E15 and E16 mice studied had partially or totally fused (Table 1). Fusion was complete in the palates of 5 of 6 TGF-$\beta_3$ +/+ and 9 of 11 +/- E15 mice, as well as in the palates of 3 of 5 TGF-$\beta_3$ +/+ and 9 of 10 +/- E16 mice. 1 +/+ and 2 +/- E15 and 1 +/+ and 1 +/- E16 mice showed a very small unfused region in the soft palate (Table 1), which was considered a normal delay in the fusion process. One TGF-$\beta_3$ +/+ E16 and all E15 and E16 TGF-$\beta_3$ null mutant mice showed both anterior and posterior cleft palates, with only the middle third of the palate fused (Table 1) (Fig. 1h). None had isolated anterior or posterior clefts, nor associated cleft lip. In all these embryos, the anterior cleft occurred in the region between the first and the second rugae. The primary palate had an apparently normal morphology, but fusion with the anterior part of the secondary palate had failed. Posterior clefts affected in all cases the region placed between the eighth and ninth rugae and the whole posterior soft palate. Therefore, the fused region was located between the second and eighth rugae (Fig. 1h).

No differences were detected on the MEE surface between TGF-$\beta_3$ +/+ , +/- and TGF-$\beta_3$ -/- palates at E13 (Table 1). In all E13 specimens the MEE surface was flat, with very few protruding cells throughout the entire length of the palatal shelves. However, many protruding cells were observed on the MEE surface in all E14 to E16 TGF-$\beta_3$ +/+ and +/- mice (Fig. 1i), whilst the entire MEE surface was flat with few bulging cells in the TGF-$\beta_3$ -/- (Fig. 1j) (Table 1) embryos. The medial edge epithelium of the almost totally fused +/+ and +/- E15 and E16 embryos was thick and flat (Fig. 1i), however, the medial edge epithelium in the anterior-posterior clefted palates was thin and rounded (Fig. 1j) .

### Cellular adhesion in the MEE

In order to investigate whether the bulging medial edge epithelial cells are involved in palatal shelf adhesion, we labelled the E14.5 mouse partially fused palate with monoclonal antibodies against E-Cadherin, β-catenin, chondroitin sulphate proteoglycan (CSPG), β-actin and vinculin. The presence of E-Cadherin and CSPG was investigated because they are usually involved in epithelial adhesion during development (Nagafuchi *et al.*, 1994). β-catenin, which normally links α-catenin to the cadherin cytoplasmic domain, was investigated because its presence does not always correlate with E-cadherin expression (Nagafuchi *et al.,* 1994) and it might even diminish adhesion in response to growth factors and cell transformation (Kinch *et al.,* 1995). We also investigated potential differences in the organisation of the actin cytoskeleton and vinculin expression between the bulging and other MEE cells. The expression patterns for E-cadherin and β-catenin were similar, with most bulging cells showing a basolateral localisation, leaving the apical surfaces negative (Figs. 2a and b). Basal and suprabasal MEE cells were E-cadherin and β-catenin positive all around the cellular surface. By contrast, CSPG labelling was negative for all MEE cells except for the apical surfaces of the surface cells, with the staining being intensively positive on the surface of the cellular bulges (Fig. 2c). Staining the just apposed palatal shelves with the anti-β-actin monoclonal antibody demonstrated actin filaments orientated perpendicular to the apical surface of the bulging cells. By contrast, actin was organised in a circumferential pattern in all other MEE cells (Fig. 2d). Vinculin labelling was intense in both those bulging surface cells establishing contact in the midline and in the bulging but not yet touching cells (Fig. 2e). Vinculin expression was much reduced in the other non-bulging MEE cells (Fig. 2e).

Our results suggest an important role for the MEE cellular bulges during palatal fusion. They increase on the MEE surface following an anterior-posterior gradient, as does palatal fusion, and decrease significantly in the clefted TGF-$\beta_3$ null mutant mouse palates. Cells were reported to protrude over the palatal epithelial surfaces and were hypothesised to be shed in order to allow fusion of the basal epithelial layers of the adjoining palatal shelves (Fitchett and Hay, 1989). Others, however, did not find sloughing cells and reported all the MEE layers as forming the fusion seam (Farbman, 1969). By TUNEL labelling of E14.5 mouse palates, we have recently demonstrated that most protruding MEE cells are not dead (Martínez-Álvarez *et al.*, 2000). Our present findings suggest that they are actively involved in palatal shelf adhesion. These cells are the first elements establishing contact when both palatal shelves meet, and the only showing oriented actin filaments and vinculin expression under their apical surface. This is in accordance with a dynamic role for them in cellular adhesion, as vinculin is involved in the linking of actin filaments to integrins that attach cells to each other and the extracellular matrix (Burridge *et al.*, 1988). These bulging cells strongly express CSPG. CSPG is important for epithelial adhesion during neural tube closure (Trasler and Morris-Kay, 1991; Alonso *et al.*, 1998). Greene and Kochhar (1974) demonstrated the presence of a coat of glucosaminoglycans covering the MEE surface prior to palatal shelf contact, suggesting that glucosaminoglycans have a role in palatal shelf adhesion. However, we are the first to demonstrate that the bulging MEE cells are specifically involved in the initial palatal adhesion. The pattern of expression of β-Actin and CSPG in these cells could correspond to the presence of syndecan, whose extracellular domain is formed by chondroitin and heparin sulphate proteoglycans, whilst

TABLE 1

**PALATE DEVELOPMENT IN OFFSPRING FROM TGF-$\beta_3$ HETEROZYGOUS INTERCROSSES**

| | No. Embryos | T.U.P. | T.F.P. | S.P.C. | A.P.C. | M.E.E.-C.B. |
|---|---|---|---|---|---|---|
| **E13** | | | | | | |
| **Total** | 15 | 15 | 0 | 0 | 0 | |
| +/+ | 3 (20%) | 3 | 0 | 0 | 0 | + |
| +/- | 9 (60%) | 9 | 0 | 0 | 0 | + |
| -/- | 3 (20%) | 3 | 0 | 0 | 0 | + |
| **E14** | | | | | | |
| **Total** | 12 | 12 | 0 | 0 | 0 | |
| +/+ | 2 (16.6%) | 2 | 0 | 0 | 0 | +++++ |
| +/- | 6 (50%) | 6 | 0 | 0 | 0 | +++++ |
| -/- | 4 (33.3%) | 4 | 0 | 0 | 0 | + |
| **E15** | | | | | | |
| **Total** | 20 | 0 | 14 | 3 | 3 | |
| +/+ | 6 (30%) | 0 | 5 | 1 | 0 | +++++ |
| +/- | 11 (55%) | 0 | 9 | 2 | 0 | +++++ |
| -/- | 3 (15%) | 0 | 0 | 0 | 3 | + |
| **E16** | | | | | | |
| **Total** | 19 | 0 | 12 | 2 | 5 | |
| +/+ | 5 (26.5%) | 0 | 3 | 1 | 1 | +++++/+* |
| +/- | 10 (52.6%) | 0 | 9 | 1 | 0 | +++++ |
| -/- | 4 (21%) | 0 | 0 | 0 | 4 | + |

T.U.P.: Totally unfused palate; T.F.P.: Totally fused palate; S.P.C.: Only small posterior cleft; A.P.C.: Anterior and posterior cleft; M.E.E.-C.B.: Presence of cellular bulges on the surface of the medial edge epithelium; *: In only one spontaneous clefted palate

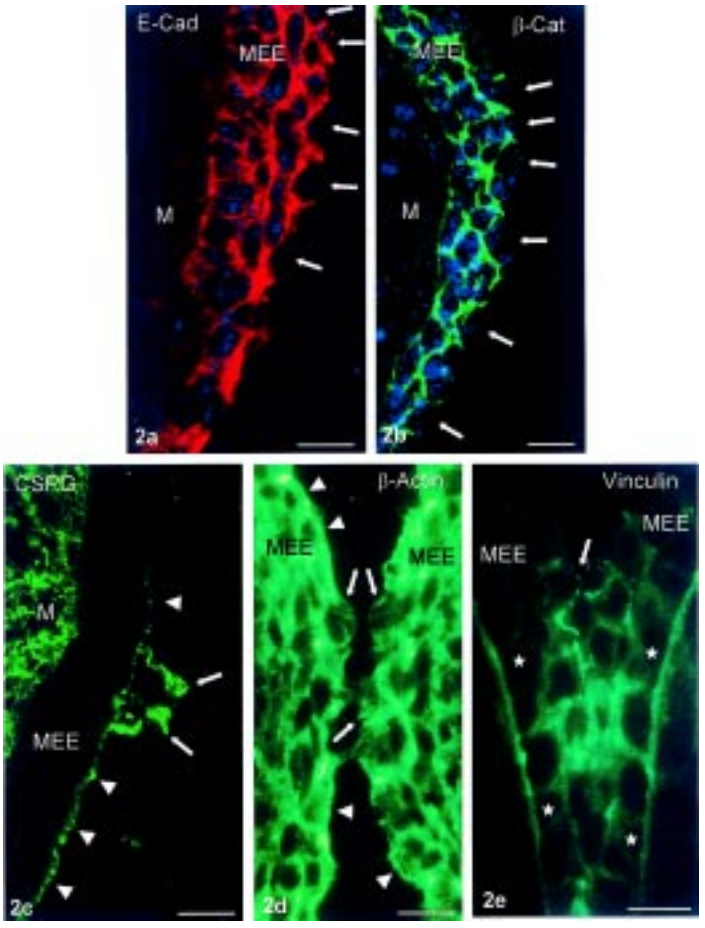


**Fig. 2. Immunolocalization of E-cadherin (2a), β-catenin (2b), chondroitin sulphate proteoglycan (2c), β-actin (2d) and vinculin (2e) in an E14.5 mouse palate.** *2a, 2b and 2c are confocal images, whilst 2d and 2e are fluorescence microscopy images.* ***(2a)*** *E-cadherin is localized to the circumference of most medial edge epithelial cells. Bulging cells only show positive staining on their basolateral surface, leaving unlabelled the apical surface (arrows).* ***(2b)*** *β-catenin immunostaining is observed underlying most medial edge epithelial cells. As for E-cadherin, the bulging cells are only β-catenin positive on their basolateral surfaces (arrows).* ***(2c)*** *Medial edge epithelial cells are CSPG negative except for the apical surface of the surface cells (arrowheads). Note the intense staining for CSPG on the surface of a bulging cell (arrows). The mesenchyme is also CSPG positive.* ***(2d)*** *Opposing bulging cells establishing contact. β-actin filaments are perpendicularly orientated under the apical cell surface (arrows), whilst they are circumferentially aligned in the non-protruding surface (arrowheads) and all other MEE cells.* ***(2e)*** *Prior to and during the initial contact of palatal shelves, the surface MEE cells stain positively for vinculin on their apical and lateral surfaces. Note the almost complete absence of vinculin in the basal epithelial cells (stars). The arrow indicates a vinculin positive bulging cell. GSPG: chondroitin sulphate proteoglycan; MEE: Medial edge epithelium; M: Mesenchyme; Scale bar: 10 μm.*

its intracellular domain interacts with the actin cytoskeleton. Syndecan-1 has been reported to be present in the fully formed midline epithelial seam of mouse palates (Sun *et al.*, 1998), but there is no evidence of its presence earlier in palate development. E-cadherin and β-catenin were absent from the MEE surface, indicating that initial palatal shelf adhesion is not likely mediated by this complex, although E-Cadherin is present around all MEE cells when the epithelial seam is fully formed (Sun *et al.*, 1998).

These cellular bulges observed here under ESEM or in routine histological sections (Fitchett and Hay, 1989) have never been reported in previous SEM studies. On the other hand, filopodia, lamellipodia and cellular debris, reported covering the prefusion areas of palatal processes by SEM (Schüpbach *et al.*, 1983; Abbott and Pratt, 1987, Taya *et al.*, 1999) are not observed by ESEM. Perhaps all these structures are different expressions of the same cellular events, depending on the technique used for visualisation. Dehydration used in SEM procedures could collapse the cellular bulges observed under ESEM, so that the underlying cytoskeleton (e.g. the perpendicular actin bundles) could be the only protruding structure.

The numerous cellular bulges observed on the MEE surface just prior to fusion seem to be a prerequisite for palatal fusion. TGF-$\beta_3$ could primarily modify superficial MEE cell morphology perhaps by altering the cytoskeleton, e.g. altered organisation/composition/binding elements of actin or cytokeratins. The non-bulging prefusion phenotype would decrease the area normally covered by adhesive proteoglycans and thus, decrease cell-cell adhesion so causing cleft palate. TGF-$\beta_3$ could also induce the synthesis of CSPG by the bulging MEE cells, as has been observed experimentally (Locci *et al.,* 1999) and thus directly stimulate initial palatal adhesion. Failure of this induction by TGF-$\beta_3$ would also result in failure of palatal fusion and cleft palate. Likely TGF-$\beta_3$ normally has multiple synergistic effects on the morphology and adhesive functions of the MEE cells so facilitating palatal fusion.

## Experimental Procedures

### Environmental Scanning Electron Microscopy

MF1 (Manchester strain) or TGF-$\beta_3$ +/- mice (from the Manchester colony) were timed mated and the day of finding a plug designated day zero. Thirteen E12, twenty E13 and forty one E14 MF1 mouse embryos were investigated. The numbers of TGF-$\beta_3$ +/+, +/- and -/- embryos recovered at E13, E14, E15 and E16 and utilised in this study are shown in Table 1. Pregnant mice were killed by an overdose of chloroform and the embryos removed by cesarian section. They were placed in cold Hank's balanced salt solution individually, for no more than thirty minutes, and decapitated. Care was taken to perform most of the dissection cranial to the plane of the palatal shelves to minimize the risk of cellular and tissue debris arising from cut surfaces settling over the palates when the sample was placed in its final orientation. The jaw and tongue of embryos older than E14 were removed, to allow better fixation of the palatal shelves. A portion of the tail of the TGF-$\beta_3$ embryos was used for PCR genotyping as described in Proetzel *et al.* (1995).

Each head was fixed in 0.1 M sodium cacodylate buffered 1% glutaraldehyde, pH 7.3, for between one and forty eight hours at 4ºC. Samples not immediately visualised in the ESEM were stored in 0.1 M sodium cacodylate buffer, pH 7.3, at 4 ºC. Immediately prior to insertion into the ESEM, samples were rinsed in distilled water in order to eliminate from their surfaces any components of the previously used solutions, which could form crystals when the water vapour pressure was altered inside the ESEM chamber during investigation. The jaw and tongue of the E12 and E13 specimens were carefully removed at this time. Scanning electron microscopy was performed using an Environmental Scanning Electron Microscope ESEM (Electro Scan). Water vapour inside the specimen chamber had a pressure of 6.4 torr and a temperature of 8ºC to maintain 100% humidity on the surface of the sample. Photographs were taken with a Nikon P30T camera connected to the ESEM. Optimal visualisation of hydrated biological specimens in the ESEM occurs in the mid magnification range. It is therefore impossible to investigate and photograph the entire

palate in one picture, necessitating the construction of montages made from pictures taken at higher magnifications with overlapping fields.

**Immunohistochemistry**

Ten E14.5 Albino Swiss mouse heads were used for immunochemistry. For E-cadherin, β-catenin, β-actin and vinculin immunostaining, heads were fixed in 10 % buffered formaldehyde (pH = 7), whilst for anti-CSPG labelling Carnoy's fixative was used. Samples were embedded in paraffin and 5 μm thick sections were cut. E-cadherin, β-catenin, β-actin and vinculin epitopes were unmasked by treating the sections with microwave preheated 1mM EDTA (Sigma). Sections were incubated either with the rat IgG anti-mouse E-cadherin (10 μg/ml) (Takara Biomedicals), mouse IgG anti-human β-catenin (5 μg/ml) (Alexis), mouse IgG CS 56 anti-chondroitin sulphate (Sigma), mouse IgG1 anti-human β-actin FITC conjugate (dilution: 1:250) (Sigma) or mouse IgG1 anti-chicken vinculin (Sigma) monoclonal antibodies for two hours. Incubation was followed by the addition of the Cy™-3-conjugated rabbit anti-rat IgG secondary antibody (dilution: 1:125) (Jackson ImmunoResearch) for E-Cadherin immunostaining; the fluorescein conjugated horse anti-mouse IgG secondary antibody (5 μg/ml) (Vector) for β-catenin and vinculin immunostaining; and fluorescein conjugated goat anti-mouse IgM (Vector) for CSPG immunostaining, following the manufacturer's instructions. Fluorescence in the sections was visualized with a confocal microscope MRC-1024 (Bio-Rad, Hempstead, UK), using a helium-neon laser tuned to 543 nm with a BP 580/23 nm emission filter for E-cadherin staining, and an argon laser tuned to 488 nm with a BP 515/15 nm emission filter for β-catenin and CSPG staining. The software acquisition was Lasergraphics 3.2 (Bio-Rad). β-actin and vinculin staining was visualised using a Fluorescence Nikon Labophot microscope and photographed using Kodak Ektachrome film.

**Histological preparation**

Three E14 mouse heads were fixed in 4 % paraformaldehyde and embedded in paraffin. 6 μm thick sections were cut and hematoxylin and eosin staining was performed following standard procedures. Sections were studied using a Nikon Optiphot light microscope and photographed with a Nikon FX 35A camera.

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