ORIGINAL ARTICLE

NL Brown
JR Sandy

Tails of the unexpected: palatal medial edge epithelium is no more specialized than other embryonic epithelium

Authors' affiliations:
NL Brown, JR Sandy, Division of Child Dental Health, University of Bristol Dental School, Bristol, UK

Correspondence to:
Jonathan Sandy
Division of Child Dental Health
University of Bristol Dental School
Lower Maudlin Street
Bristol BS1 2LY
UK
E-mail: jonathan.sandy@bristol.ac.uk

Structured Abstract

Authors – Brown NL, Sandy JR

Objective – To determine whether palatal medial edge epithelium (MEE) is specialized in its ability to disappear compared with other embryonic, non-palatal, epithelium.

Subjects – Embryonic tissues harvested from CD1 mice.

Methods – Organs were cultured in 2 ml of DMEM/F12 supplemented with 300 μg/ml L-glutamine and 1% penicillin/streptomycin. Organs were cultured under various conditions including opposing other organs and opposing an inert material for a period of 6 days. Tissues were then processed for histological examination.

Results – MEE of shelves opposing nothing persisted, whereas MEE of shelves contacting another shelf disappeared. When a tail was placed against a palatal shelf the MEE disappeared, as did the epithelium from the tail, resulting in fusion between the shelf and tail. Furthermore, when palatal shelves were placed against an inert material the MEE disappeared, suggesting pressure alone is a sufficient stimulus to initiate disappearance of the MEE, and that the interaction between the two palatal shelves is not a prerequisite for the disappearance of MEE. Moreover, when two embryonic tails were cultured in close apposition they fused, as did paired limbs. Non-palatal epithelia also disappeared after contact with inert materials. Epithelial disappearance began within 24 h of contact, but there was an age limit.

Conclusion – These findings suggest that embryonic epithelium from non-specific sites around the body has the ability to disappear with mechanical contact resulting in fusion of tissues. MEE may not be as specialized as once thought.

Key words: embryology; epithelium; fusion; palate

Introduction

Normal palate development is a crucial developmental process, if it fails a cleft results and neonatal feeding is difficult because of an inability to create an effective oral seal necessary for swallowing. Most forms of abnormal palate development result in lethality for all animals except humans, who have the ability to repair the defect. In man, cleft lip and palate occurs in about 1 in 700 live births (1). The aetiology is regarded as multifactorial and there are no recognized strategies to prevent palatal clefting.

Human and mouse secondary palate development is strikingly similar. Two mesenchymal swellings surrounded by a layer of epithelium derived

Dates:
Accepted 3 January 2007

To cite this article:
Brown NL, Sandy JR:
Tails of the unexpected: palatal medial edge epithelium is no more specialized than other embryonic epithelium
Orthod Craniofacial Res 10, 2007; 22–35

from the first branchial arch form and grow on either side of the tongue. In the mouse, lateral palatine processes (shelves) can be clearly seen at embryonic day (ED) 13.5. These palatal shelves then elevate above the tongue, the medial edge epithelium (MEE) of shelves contact, adhere (ED14.5), form a transitory midline epithelial seam (MES) and finally fuse after loss of the MES (ED15.5) (2, 3).

The MEE of each palatal shelf before shelf contact consists of two layers, the inner basal layer and superficial periderm (4–6). It has been proposed that periderm is lost either by peeling/sloughing or migration prior to shelf contact, or in the posterior palate may become trapped between the shelves after contact and undergo apoptosis within the MES (4, 7–10). It is generally accepted that cells of the basal layer form the MES and a number of mechanisms have been proposed for its disappearance; epithelial to mesenchymal transformation (EMT) (4, 11–14), apoptosis (15–17) and epithelial migration to either the oral or nasal epithelium (18, 19) or finally a combination of the mechanisms (20). The role of EMT has recently become a contentious issue. A number of studies have considered EMT as a mechanism during MES loss, yet others have reinforced it (14, 21, 22). Despite these studies, it is still not clear which mechanism or combination of mechanisms are responsible for MEE/MES disappearance, or even what initiates them. Furthermore, total epithelial loss at the medial edge can occur before palatal shelves even contact, resulting in direct apposition of mesenchyme (23). There are also regional differences in gene expression, fusion ability, cell death and teratogen susceptibility along the length of the shelf (17, 24, 25). However, during normal palatogenesis the final result is mesenchymal continuity and fusion in the midline of the palate.

Development of avian palates differs. The palatal shelves grow horizontally towards one another and contact but never fuse, and the persistence of the MEE results in a non-fused persistent seam between adhered shelves (26). Despite this lack of epithelial breakdown, contact between the palatal shelves is necessary for normal development, and a true avian cleft palate (rather than a physiological cleft) still results in mortality (27). Interestingly, if paired chicken palatal shelves are cultured in contact after removal of the beak the MEE will start to disappear without the need of any additional growth factors (28). Alligator palatogenesis is similar to that of humans with confluence in the midline, but the posterior one-fifth of the secondary palate grows along the side of the tongue and unites with the floor of the mouth (29). If the alligator palatal shelf is able to fuse with the floor of the mouth as well as the opposing palatal shelf, and chick shelves are capable of fusing in culture when placed in close apposition, does the MEE at the appropriate time of normal palate development break down simply because of mechanical contact?

Mutant mice with a genetic deletion in either Jagged 2 or Fgf10 are of interest. Mutants develop a cleft palate, but the palatal shelves fuse with the lateral borders of the tongue (30, 31). The epithelium of the tongue and palate unite, the MEE disappears (as does the epithelium of the tongue in the region of contact), and the mesenchyme of the palate becomes continuous with the tongue. Other mutant mice provide evidence for a mechanical trigger for MEE breakdown. Homozygous Msx-1 mice have cleft palate, yet in a similar manner to avian palates, when the palatal shelves are cultured in close proximity, they fuse (32). Col11a1 mutant mice also develop cleft palate, and as in the homozygous Msx-1 mice the 'cleft' palatal shelves fuse when cultured (33). Epidermal growth factor receptor-deficient ($Egfr^{-/-}$) mice have facial defects including a high incidence of cleft palate yet palatal shelf explants from the $Egfr^{-/-}$ mice fuse in culture (34). Furthermore, mice with transforming growth factor beta (TGF-β) receptor 2 conditional gene ablation have complete cleft secondary palate, but the midline epithelium of the mutant palatal shelf remains functionally competent to mediate palatal fusion once the palatal shelves are placed in close contact *in vitro* (35). This suggests the cause of cleft palate in these animals is failure of the palatal shelves to make contact and adhere in the first instance. Certain strains of mice have an increased incidence of palatal clefts, for example 15–40% of newborn CL/Fr strain mice are affected with spontaneous cleft lip and palate (36–41). Sixty per cent of the clefts are bilateral cleft lip which, in this strain, may be associated with poorly developed secondary palate and wide cleft (42). When palatal shelves are dissected from CL/Fr embryos with bilateral cleft lip and cultured so that the palatal shelves are in contact, the shelves fuse within 48 h (43). Moreover, when normal murine palatal shelves at ED13.5 are cultured, a critical distance of 0.48 mm is necessary for palatal clefting (44). This

inter-palatal separation of 0.48 mm or greater, consistently results in non-fusion.

Other studies of mice with cleft palate report non-fusion after culture of the mutant palatal shelves. TGF-β signalling is one of a number of molecular pathways that are crucial to normal palatogenesis (3, 45). TGF-β_3 signalling operates in the MEE and MES regression is mediated by the TGF-β type II and the TGF-β type I receptor (Alk5)/Smad pathway (22, 24, 46, 47). While inactivation of epithelial Alk5 or Tgfbr2 generates partial cleft palate (22, 24, 46), TGF-β_3 mutants display either a complete or partial secondary cleft (48, 49). Furthermore, when shelves of the TGF-β_3 knockout mouse or the TGF-β type I receptor gene *Alk5* mutant are cultured in close apposition the MES persists and shelves fail to fuse (15, 46, 50).

However, fusion does occur when a wild-type palatal shelf is cultured with a TGF-β_3 knockout shelf. It has been hypothesized that TGF-β_3 synthesized in the wild-type shelf diffuses across and induces phenotypic changes in the knockout shelf in order for fusion to occur (6). A point of note with respect to this cleft model is that all TGF-β_3 knockouts exhibit cleft palate to some degree. The degree of severity of the cleft is dependent on the background of the knockout mouse (49). When TGF-β_3 knockout mice are kept on a mixed background (129 × CF-1) partial cleft palate is frequent and total cleft palate is rare. In those mice with partial clefts a few elongated islands of the MEE persist in the fused region (6). Regardless of these islands of epithelium, the area within the palate is defined as being fused. This suggests the majority of TGF-β_3 knockout shelves have retained an ability to fuse, albeit partially. Furthermore, in other murine knockouts that exhibit cleft palate, such as the TGF-β_2 (51), GAD67 (52) and Lhx8 models (53), not all homozygotes have cleft palate. It seems then that deletion of the gene alone in these animals is not sufficient to cause a cleft. Genetically identical siblings reinforce the concept that the cause of clefting is not due to genetics alone. In monozygotic twins one sibling may have a cleft with the other unaffected (54).

When all of these points are considered (with the exception of the paired TGF-β_3 knockout palatal shelf culture results), evidence seems to indicate that although the aetiology of cleft palate is multifactorial, ultimately it is due to a deficiency in palatal shelf growth/proliferation with absent or inadequate contact of opposing palatal shelves.

A number of methods for culture of palatal shelves have been described (50, 55–57), but some methods (such as roller culture) are not suitable if organs need to be static for the duration of culture. The Trowell procedure is more frequently used than others. The dissection technique involves removal of the palatal shelves with disruption of their natural relationships. The MEEs are artificially approximated, and this method lends itself well to the study of epithelial disappearance. We used this technique to test the hypothesis that any prolonged mechanical contact with palatal MEE (at the time of normal palatal shelf contact) is sufficient to initiate breakdown of the epithelium, regardless of the nature of the opposing tissue.

Materials and methods

Organ culture

CD1 mice were mated overnight and females were then isolated the next morning. The presence of a vaginal plug was taken to represent ED0.5, and if pregnant, the females were killed by cervical dislocation at the appropriate embryonic age. Embryos within each litter were divided so that control experiments were performed within that litter. Palatal shelves were dissected from ED13.5 embryos as described by Taya et al. (6). Tails and forelimbs were also removed from ED13.5 embryos with a simple incision to detach the tail or limb from the trunk of the embryo. Care was taken to minimally handle each specimen thus avoiding disruption of the epithelium. The embryonic tissues were cultured as described by Taya et al. (6) and Erfani et al. (58). Briefly, 2 ml of DMEM/F12 (Gibco, Paisley, UK) supplemented with 300 μg/ml L-glutamine (Sigma, Poole, Dorset, UK), 50 μg/ml glycine (Sigma), 100 μg/ml ascorbate (Sigma), and 1% penicillin/streptomycin (Sigma) was placed into each well of a 6-well companion plate (Becton Dickinson, Cowley, Oxford, UK). Dissected ED13.5 tissues (palatal shelves, limbs and tails) or whole embryos (ED13.5–16.5) were then placed into 0.45 μm pore size PET track-etched membrane cell culture inserts (Becton Dickinson). Individual palatal shelves were either cultured with the MEE contacting the other palatal shelf MEE from the same embryo, with the palatal MEE contacting wax, or with the palatal MEE contacting the tail of the same embryo. Unopposed palatal shelves and unopposed embryonic

tails were cultured as controls. Tails were also cultured in close contact with another tail, and limbs were cultured either unopposed or contacting other limbs. Whole embryos were cultured either alone or in contact with another embryo. A summary of all organ culture pairings can be seen in Fig. 1. Where contact was required, tissues were placed in close contact without distorting the tissues. Culture inserts containing the organs were placed inside the companion plate containing medium and cultured at 37°C in a humidified atmosphere. Culture medium was replaced after 3 days for tails, limbs and palates, and daily for whole embryo culture. After 6 days tissues were prepared for histological analysis.

Histology

All cultured embryonic material was fixed in formal saline (BDH, Poole, Dorset, UK) for 24 h and prepared for histology on a Shandon processor 2LE with graded industrial methylated spirits (IMS, BDH), xylene (BDH) and paraffin wax baths. Tissues were then embedded in paraffin wax, sectioned on a microtome (2 μm thick cross sections) and stained with haematoxylin and eosin for histological analysis. The epithelium at the point of contact was examined for its disappearance using standard light microscopy. The presence of epithelial disappearance with mesenchymal continuity was defined as fusion.

Immunohistochemistry

Wax-embedded tissue sections 2 μm thick were placed onto polylysine-coated slides and incubated overnight at 37°C. This was followed by three xylene rinses for 3 min each, a rinse in IMS, a distilled water rinse, and immersion in 3% hydrogen preroxide (BDH) for 15 min followed by another distilled water rinse. Then 200 μl of a 2% stock of trypsin (DAKO, Ely, Cambridgeshire, UK) plus 3.8 ml of 1% calcium chloride (BDH) was placed onto each slide and left at 37°C for 30 min, followed by a wash with phosphate-buffered saline (PBS, Sigma). Normal swine serum (controls; DAKO, Ely, Cambridgeshire, UK) or primary antibody (1:70 pan cytokeratin polyclonal; Zymed, San Francisco, CA, USA) was then added for 1 h at room temperature followed by a further PBS wash. Swine anti-rabbit secondary antibody (1:400, DAKO) was then placed on each slide for 30 min at room temperature followed once more by a thorough PBS wash. Streptavidin horseradish peroxidase (1:500, DAKO) was placed over sections and left for

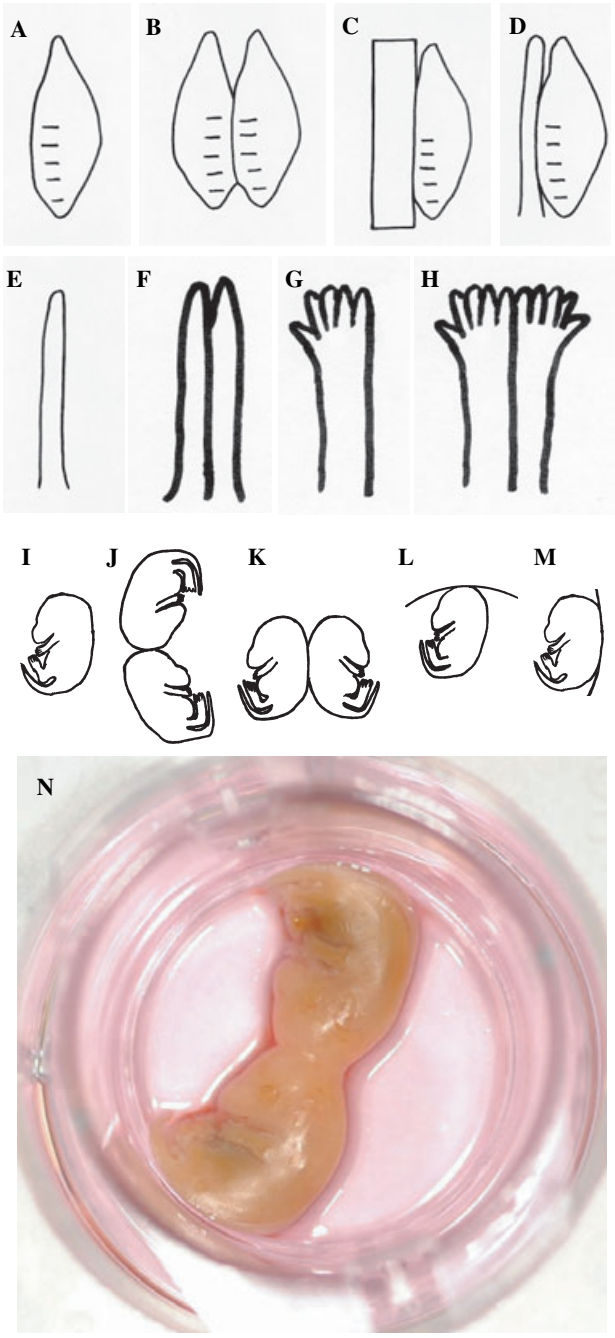

Fig. 1. Summary of the different organ culture pairings and controls used. A palatal shelf alone (A), two palatal shelves with medial edge epithelium in contact (B), a palatal shelf with medial edge epithelium contacting wax (C), a palatal shelf with medial edge epithelium contacting a tail (D), a tail cultured alone (E), two tails cultured in contact (F), a limb cultured alone (G), and two limbs cultured in contact (H). Whole embryos were cultured alone (I), with heads in contact (J), with backs in contact (K), with the embryo head contacting the side of the Perspex culture dish (L), and with its back contacting the side of the Perspex culture dish (M). Photograph of two embryos cultured with heads contacting (N).

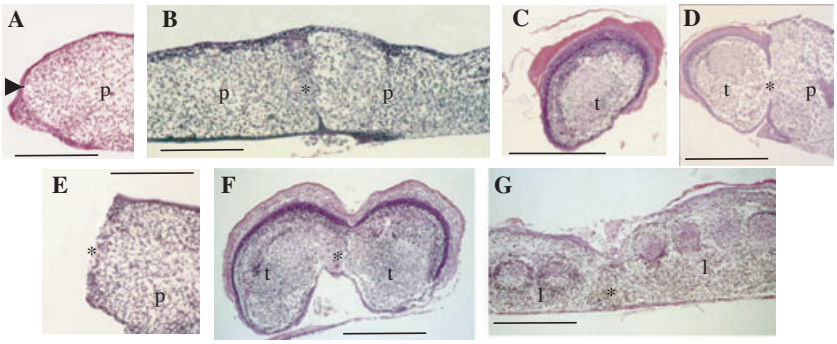

Fig. 2. Haematoxylin and eosin-stained sections of embryonic day 13.5 tissues after culture for 6 days with or without contact. The medial edge epithelium (arrowhead) of palatal shelves (p) cultured without contact remained intact (A), and when palatal shelves were cultured in contact, the epithelium disappeared (*), resulting in fusion (B). Epithelium of tails (t) cultured alone remained continuous (C), but when tails were cultured in contact with the palatal shelf medial edge epithelium, fusion between the palatal shelf and tail occurred with disappearance of epithelium from the tail and palate (D). Medial edge epithelium disappeared from palatal shelves cultured in contact with wax (E). Wax cannot be seen, as this is lost during histological preparation. Epithelium of the tail disappeared at the contact point when two tails were cultured in contact, resulting in fusion of the two tails (F). Limbs (l) cultured in contact also fused, with concomitant loss of epithelium (G). Scale bars represent 0.1 mm.

30 min, again followed by a thorough PBS wash. Diaminobenzidine (DAKO) was placed on each slide for 6 min. Slides were then rinsed and stained with haematoxylin for 40 s. After another water rinse and three rinses in IMS, slides were immersed in xylene for 5 min and then mounted with cover slips, allowed to dry and viewed under an Olympus light microscope.

Statistics

The Fishers exact test was applied to test the significance of the culture results.

Results

Contact from a non-palatal source can initiate palatal MEE disappearance

As a negative control, individual ED13.5 palatal shelves were cultured with the MEE free of any contact (Fig. 2A). In all shelves, after 6 days of culture the epithelium remained continuous at the medial edge. Positive controls comprised pairs of palatal shelves (30 individual shelves in total) cultured with their MEE in contact and 14 of 15 pairs fused (Fig. 2B), the mesenchyme was continuous and the palatal shelf MEE had disappeared. As a further control, 15 embryonic tails were cultured alone, and without exception, the epithelium remained continuous with no evidence of epithelial disappearance (Fig. 2C).

In the first of the experimental groups, 12 palatal shelves were cultured so that each shelf opposed the tail from the same embryo. The hypothesis tested was that the mechanical contact from the tail would be adequate to initiate MEE disappearance. In 10 of 12 shelves the epithelium disappeared at the medial edge (Fig. 2D). Furthermore, in these shelves the epithelium of the tail also disappeared, resulting in fusion between the palate and tail. The contact between tail and palate had initiated disappearance of the MEE. Results are summarized in Table 1.

Table 1. **Epithelial disappearance in murine embryonic day 13.5 organs cultured for 6 days following harvest**

Epithelial contact during culture	Epithelial disappearance	*p* (Fishers exact)
Palatal shelf MEE unopposed	0/15	
Palatal shelf MEE contacting palatal shelf MEE	28/30 (14/15 pairs)	<0.0001
Palatal shelf MEE contacting wax	10/13	<0.0001
Tail unopposed	0/15	
Tail contacting palatal shelf MEE (or vice versa)	10/12 (10/12 pairs)	<0.0001
Tail contacting tail	24/28 (12/14 pairs)	<0.0001
Limb unopposed	0/5	
Forelimb contacting forelimb	12/12 (6/6 pairs)	<0.001

MEE, medial edge epithelium.

Contact from a non-embryonic source can initiate palatal MEE disappearance

The second experimental group consisted of 13 individual ED13.5 shelves that were cultured with the MEE contacting wax. This was to test the hypothesis that non-embryonic contact from the wax would initiate MEE disappearance. In 10 of 13 shelves the MEE disappeared (Fig. 2E, Table 1). Wax was dissolved/melted during the histology process and therefore cannot be seen in Fig. 2E. This enabled the palatal tissue to be removed with minimal trauma prior to microtome sectioning. The mechanical contact of the wax against the MEE appeared to have initiated its breakdown and after the 6 days of culture no MEE could be identified.

Embryonic epithelium that does not normally disappear can be induced to do so by contact with another embryonic epithelial surface

Previously, when palatal shelves were cultured against epithelium from the tail, epithelial disappearance was seen in both the palate and tail (Fig. 2D). Tail epithelium does not fuse to any other structure during normal development, but could be induced to do so in culture by providing contact with the palatal MEE. Further experimental groups examined epithelium from structures other than that of the palate. During normal palatogenesis the MEE disappears and this could influence adjacent epithelium with signalling across the contact point. The hypothesis tested was that epithelium which does not normally disappear *in vivo* will not break down regardless of the contact. This was conducted by culturing tails contacting tails, limbs contacting limbs, and in whole embryo culture, heads contacting heads and backs contacting backs.

Twenty-eight tails were cultured in pairs closely opposing each other. Of the 14 pairs, 12 achieved fusion with the epithelium disappearing at the site of contact (Fig. 2F, Table 1). Six forelimbs were cultured alone, and none showed epithelial breakdown, yet when a further six pairs of limbs (12 individual limbs in total) were cultured in close apposition, all six achieved fusion with the epithelium disappearing at the site of contact (Fig. 2G, Table 1).

In whole embryo culture, epithelium did not disappear on either the heads or the backs of control embryos cultured without contact (Fig. 3A, Table 2). Those embryos cultured with heads together or backs together resulted in epithelial disappearance (10 of 12 pairs and 9 of 12 pairs respectively, Fig. 3B,C, Table 2). Collectively these results disprove our original hypothesis and demonstrate embryonic epithelium that does not normally disappear can be induced to do so if placed in contact with another embryonic epithelial surface that also does not normally disappear.

Embryonic epithelium that does not normally disappear can be induced to do so with contact from a non-embryonic source

To investigate whether the contact applied to embryonic epithelium that does not normally disappear needed to be of embryonic origin, embryos were cultured contacting a non-embryonic surface, in this case the perspex side of the culture insert. Twelve whole embryos were cultured with their heads contacting perspex, and after the 6-day culture period, in 11 of 12 the epithelium had disappeared (Fig. 3D, Table 2). This demonstrated that embryonic epithelium which does not normally disappear could be induced to do so with contact from a non-embryonic source.

In culture, embryonic epithelium starts to disappear within 2 days and fully disappears within 5 days of the initial contact, resulting in fusion

A time course to investigate the length of contact time needed to initiate breakdown of the epithelium was conducted using whole ED13.5 embryos. Following contact between palatal shelves *in vivo* the MEE disappears within a short space of time (24–48 h); in culture this process can take longer (up to 5–6 days). Based on these observations our hypothesis was that a similar length of contact time would be necessary in our model with epithelium that does not disappear *in vivo*. The results are summarized in Table 3. Control embryos not cultured at all showed no epithelial disappearance. The control embryos cultured for 6 days behaved as expected: no epithelial disappearance was seen in embryos without contact and in embryos cultured for 6 days epithelium disappeared in all but one pair of heads. The embryos separated after 1 day of culture had no evidence of epithelial disappearance. Those fixed immediately after the 1-day culture did have epithelial disruption but this was not evident in

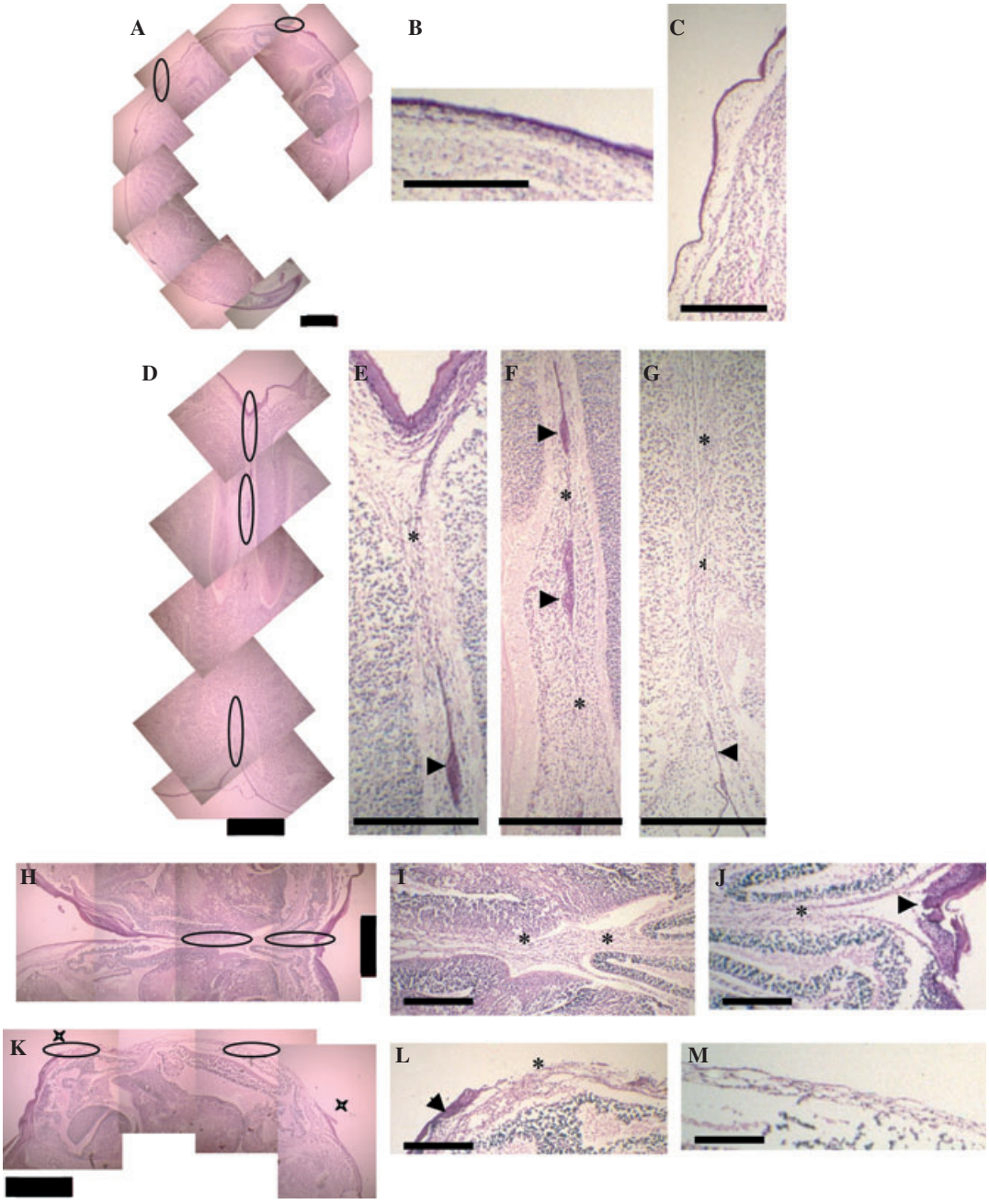

Fig. 3. Haematoxylin and eosin-stained sections of embryonic day 13.5 whole embryos after culture for 6 days with or without contact. There was no loss of epithelium in control embryos (A). The circled areas are magnified and demonstrate continuous epithelium on the head (B) and back (C). Pairs of embryos cultured with their backs contacting had loss of epithelium at the contact site (D). The circled areas are magnified (E–G), and demonstrate epithelial loss with resultant fusion between embryos (*) with a few remaining islands of epithelium (arrowhead). Epithelium also disappeared when embryos were cultured with heads in contact (H). The circled areas are magnified (I, J). When heads of embryos were cultured in contact with the side of the culture dish (K), the epithelium in contact with the Perspex (between the two star symbols) disappeared. The circled areas are magnified (L, M), and show that the area of contact with the Perspex resulted in loss of epithelium. Scale bar represents 1 mm for A, D, H and K, 0.1 mm for B, C, E–G, I, J, L and M.

embryos cultured for a further 5 days. When embryos were separated after 2 days of contact, epithelium had been lost, but only in a few embryo pairings. After 4 days of contact, epithelium had disappeared in the majority of embryo pairings, and after 5 days six of the pairings could not be separated and histological analysis revealed epithelial merging between embryos at the periphery of the contact point. Of those that could be separated, epithelial loss had occurred in all but one pair of embryos.

Table 2. **Epithelial disappearance in murine embryonic day 13.5 whole embryos cultured for 6 days following harvest**

Epithelial contact during culture	Epithelial disappearance	*p* (Fishers exact)
Embryo unopposed	0/12	
Embryos with heads contacting	20/24 (10/12 pairs)	<0.0001
Embryos with backs contacting	18/24 (9/12 pairs)	<0.0001
Embryos with head contacting perspex	11/12	<0.0001

Embryonic epithelium has a site-dependent age after which it will no longer disappear with contact

We believed that there would be a time point after which epithelium would no longer disappear. We tested this using organs and whole embryos of different ages (ED13.5, 14.5, 15.5 and 16.5) cultured for 6 days with or without contact. Different contact sites were also examined, heads and backs in whole embryos and tails and limbs in organ culture. The results are summarized in Table 4. Embryos or organs of any age cultured without contact did not lose epithelium. Epithelia on the heads of ED13.5, 14.5 and 15.5 embryos cultured with their heads contacting another head readily disappeared (20/24, 22/24 and 18/24 respectively), but epithelia on the heads of ED16.5 embryos did not (0/8). Similar results were obtained for heads in contact with perspex, but the epithelium of the back differed. When embryos were cultured with their backs in contact, epithelium was much less likely to disappear after ED13.5. In organ culture, paired tails and paired limbs demonstrated epithelial disappearance in ED13.5 and 14.5 organs, but by ED15.5 this was substantially reduced. This demonstrated that epithelium has altered susceptibility to disappearance depending on its location on the embryo.

Pan-cytokeratin immunostaining of paired ED13.5 or 16.5 embryos cultured with their backs in contact suggested that there was a greater amount of keratin on the backs of non-fused ED16.5 embryos than ED13.5 embryos, which did fuse (Fig. 4). The back epithelium also appeared thicker in ED16.5 embryos. This suggests that loss of epithelia is less likely and the higher the keratin levels and the thicker the epithelium.

Discussion

The observational data presented in this report are important in improving our understanding of epithelial loss during palatogenesis. The palate is one of a number of developing structures that rely on the coming together and joining of two distinct structures to form

Table 3. **Time course for disappearance of murine cranial epithelium in embryonic day 13.5 whole embryos cultured with or without contact**

Whole embryo–head epithelium contact	Culture length (days)	Epithelial disappearance	*p* (fishers exact)
Head unopposed	0	0/10	1
	6	0/10	1
Head contacting another head for the first day	1	0/10	1
	6	0/10	1
Head contacting another head for the first two days	2	2/10	0.2
	6	1/10	0.5
Heads contacting another head for the first four days	4	9/10	<0.0001
	6	8/10	<0.001
Heads contacting another head for the first 5 days	5	13/14*	<0.0001
	6	5/6	<0.005
Heads contacting another head for all six days	6	18/20 (9/10 pairs)	<0.0001

*Six pairs were inseparable.

Table 4. **Epithelial disappearance in murine whole embryo culture and organ culture of various aged embryonic tissue cultured for 6 days following harvest**

	Epithelial disappearance (embryonic day)			
Epithelial contact during culture	13	14	15	16
Whole embryo unopposed	0/12	0/12	0/12	0/4
Whole embryos with heads contacting heads	20/24 (10/12 pairs)*	22/24 (11/12 pairs)*	18/24 (9/12 pairs)*	0/8 (0/4 pairs)
Whole embryos with backs contacting backs	18/24 (9/12 pairs)*	8/24 (4/12 pairs)***	2/24 (1/12 pairs)	0/8 (0/4 pairs)
Whole embryos with heads contacting perspex	11/12*	12/12*	11/12*	2/4
Tails unopposed	0/15	0/12	0/11	ND
Tails contacting another tail	24/28 (10/12 pairs)*	10/12 (5/6 pairs)*	2/8 (1/4 pairs)	ND
Limbs unopposed	0/5	0/4	0/4	ND
Forelimbs contacting forelimbs	12/12 (6/6 pairs)**	6/8 (3/4 pairs)***	2/8 (1/4 pairs)	ND

*$p < 0.0001$, **$p < 0.005$, ***$p < 0.05$. ND, not done.

one, relying on merging or epithelial disappearance and subsequent fusion. Other facial structures include the cheeks, upper lip, mandible and nose, but similarities can be seen in structures also derived from surface ectoderm at more distant sites and at different stages of the developing embryo.

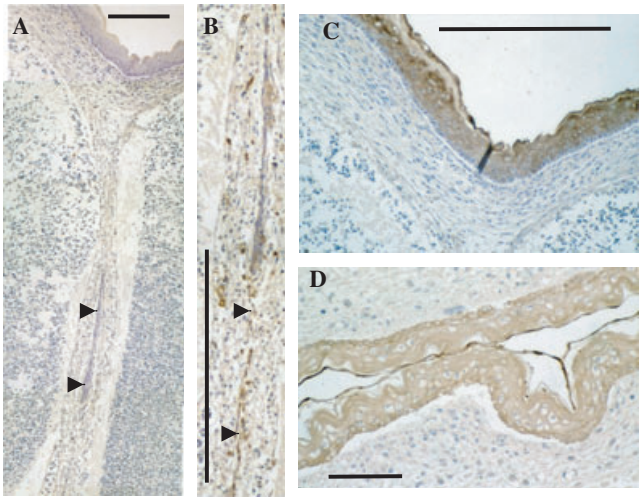

Fig. 4. Immunohistochemistry for pan-cytokeratin in embryos cultured with backs in contact. In the control section (A), the site of contact between the backs of two ED13.5 embryos show only a few epithelial remnants (arrowheads), but at the periphery of the contact point the epithelium of the two embryos had become thick and continuous with no distinguishable boundary between embryos. At the site of fusion, staining for keratin was only present in the few remaining epithelial islands (B). The epithelium at the periphery of the contact point stained heavily for keratin (C). Epithelium at the site of contact between the backs of two ED16.5 embryos remained thick and there was no epithelial loss (D). Keratin was present throughout the site of contact and a distinct boundary between the epithelium of each embryo remained. Scale bar represents 0.1 mm.

Normal development of the pharyngeal apparatus includes growth of pharyngeal arch II over pharyngeal arches III, IV and V to come into contact with arch VI. The surface ectoderm forming the contact point between these two arches disappears, resulting in fusion of arch II and VI. Fusion is also seen during primary neurulation and development of the external genitalia (59). Fusion of the urogenital swellings in the male genitalia gives rise to the penile urethra (60), and in the female forms the labia minora. In males failure of fusion between the urogenital swellings causes hypospadia. The two labioscrotal swellings meet in a similar manner, forming the scrotum in males which develops through merging and not by fusion (60).

The molecular and cellular events involved with closure of the neural groove during neurulation are poorly understood, but there are notable similarities to palatogenesis. Approximation of the edges of the neural folds in the dorsal midline, adherence, epithelial breakdown and fusion at several nucleation sites results in the formation of two separate epithelial layers, epidermal ectoderm and neuroepithelium, with intervening mesenchymal cells of the neural crest (61). Neural tube development occurs at an earlier stage of development than palatogenesis, but development of the upper lip, mandible and nose occur at a similar time to palatogenesis. As with palate development, all

rely on contact between facial processes with loss of intervening epithelium. Furthermore, development of the upper lip also relies upon fusion of the maxillary prominences, but differs by fusing with the medial nasal prominence rather than each other. Sun et al. (62) studied lip development in the chicken, and found major similarities with palate development. The bilateral two-layered embryonic epithelium that forms from contact between the primordia begins to slough prior to fusion, and brings the basal epithelial cells into close contact. Basal epithelial cells that contact each other produce numerous desmosomes to form the midline seam, and which begins to disappear through EMT. Numerous filopodia extend from the basal surfaces of epithelial cells, the spaces between them enlarge, and the seam breaks apart, leaving mesenchymal cells (62). What is interesting in the chicken model is that facial processes forming the lip fuse, as in human or mouse lip formation, but the MEE of palatal shelves persists. As it is known that chicken palatal shelves do have the ability to fuse if placed in close apposition (28), this physiological cleft is almost certainly caused by a lack of contact between shelves *in vivo* rather than an intrinsic failing of appropriate palatal shelf signals. Indeed, Sun et al. (28) suggested there might be no fundamental difference in developmental potential of the MEE for transformation to mesenchyme among reptiles, birds and mammals. The bird differs from other amniotes in having developed a beak and associated cranial structures that seemingly keep palatal processes separated *in vivo* (28).

Another process involved with normal facial development is fusion of the mandibular prominences along the midline. This occurs in a similar manner to fusion of the palatal shelves, and medial epithelial cells disappear from the contacting edges. It is interesting that TGF-β_3 knockout mice have cleft palate despite shelf contact, the cleft being due to impaired fusion and elimination of the MES (49). In addition, TGF-β_3 mutants do not have cleft mandibles or other facial clefts, or indeed any other developmental defects except delayed lung maturation. One explanation for this phenotype is that palatal MEE disappearance and fusion may not be dependent on the same mechanisms that are present in the facial processes or at other sites such as neural crest. Mandibular fusion relies on epithelium migrating away from the site of fusion and into the surface and oral epithelia (63). This latter mechanism has also been considered to be important in MEE disappearance (18, 19). Another mechanism proposed for MEE disappearance is apoptosis (5, 10, 15–17). This is an important developmental mechanism that also occurs during normal formation of the limbs and digits, where cell death occurs in well-defined domains to sculpt the shape of the limb by eliminating cells between differentiating cartilages. The original paddle-shaped hand is modified with massive cell death by apoptosis within interdigital zones to form individual digits (64). Disruption of programmed cell death during limb formation causes syndactyly (digit fusions). This occurs at a similar time as palatogenesis, yet TGF-β_3 mutants do not have defects of the limbs or digits. The final mechanism proposed for palatal MEE disappearance is EMT (4, 11, 13). As with apoptosis, EMT is considered an important event and the earliest example of epithelio-mesenchymal transformation during embryonic development is the generation of the mesoderm from the primitive ectoderm marking the beginning of gastrulation (65). EMT also occurs during morphogenesis of a variety of organs including the lip (62), and the heart (66). Despite a direct implication of TGF-β_3 in mechanisms of epithelial–mesenchymal interaction from the TGF-β_3 knockout (48), structures such as the heart are seemingly unaffected in the TGF-β_3 mutant, but failure in signalling via the TGF-β type I receptor ALK5 leads to severe cardiovascular and pharyngeal defects (67). Despite the implication from TGF-β_3 mutants that MEE is a specialized tissue that has different mechanisms of disappearance to other epithelium, the results presented here challenge current beliefs and imply that palatal MEE may not be too dissimilar to surface epithelium elsewhere in the embryo during the initial stages of palatogenesis. In our experiments, contact with the epithelial surface, regardless of its site, was able to induce disappearance up to a certain age. This is in contrast to a palatal chimera study performed in the 1980s where palatal shelves from the chick, mouse and alligator were cultured in heterologous combinations, without being able to induce MEE loss (68). However, other workers could induce cytolysis of the MEE when hamster and chick palatal shelves were placed in contact with each other *in vitro* (69). It has been proposed that there is cross talk between palatal shelves (6). This seems unlikely as our results clearly demonstrate that not only MEE, but also embryonic epithelium that does not

normally break down can be induced to do so by contact even with inanimate objects or structures such as perspex or wax. MEE has always been regarded as being different to normal epithelium and has even been considered to be pre-programmed to disappear. In A/J mice that have spontaneous cleft lip and palate, palatal shelves do not contact yet MEE has been seen to disrupt thereby exposing the underlying mesenchyme. The epithelial discontinuity lasts just 1 day, but this suggests MEE is pre-programmed to disappear (23). Furthermore, 24–36 h prior to shelf contact in mammals, shelves seem to acquire pre-fusion characteristics such as cessation of DNA synthesis in the MEE, elevation of cyclic AMP and MEE cell death (69). Epithelium may even disappear completely at the shelf tips before contact is made between shelves (23). These and other *in vitro* studies imply contact is not a prerequisite for epithelial degeneration (70), but other research suggest contact is a necessity (17, 28, 71). Morphological changes prior to fusion including bulging MEE have also been reported (20, 72) which probably aids the initiation of contact and application of pressure between palatal shelf MEE. Other studies indicate morphological and functional differences at different regions of the MEE, including multiple MEE-specific expression patterns of an assortment of unrelated genes, MEE-specific apoptosis distribution, and sloughing cells occurring in the MEE but not in adjacent palatal surfaces (17, 24, 32).

The results presented in this study indicate that there is nothing special about the potential for MEE disappearance compared with epithelium for other sites of the same embryological age. Rather it is physical contact which initiates epithelial adherence and disappearance. Under organ culture conditions, palatal MEE of shelves cultured individually can selectively undergo EMT and apoptosis resulting in selective removal of the MEE (70). Oral and nasal epithelia that previously migrated away from the medial edge may subsequently migrate to cover the 'wound' created by removal of the MEE. The experimental design utilized in the present study, examining MEE after 6 days in culture, would indeed present an intact medial edge, but the epithelium at this stage may not be MEE, but could represent oral and nasal epithelia. It would therefore appear that if growth is delayed or disrupted then the relatively advanced stage of epithelial keratinization probably creates a physical barrier that is responsible for the prevention of palatal shelf fusion. The mature palate exhibits orthokeratinization on its oral surface and during normal murine development between ED14.5 and 15.5 there is a large increase in the mRNA levels of keratin and other markers for keratinization (73). Even the cleft Col11a1 mutant palates retain full potential for MEE adherence and EMT up to ED17.5/18.5, when epithelia keratinize and prevent the adherence of both normal and homozygous palatal shelves (33). As suggested by Sun et al. (62) keratinization of the epithelia prior to MEE contact and breakdown is the likely reason for the physiological avian cleft, and this is also a feasible explanation for the formation of cleft palate in humans. Delayed elevation or inadequate growth of one or both of the secondary palatal shelves combined with increased thickness and keratinization of the epithelium would more than likely result in formation of a cleft. Furthermore, if the palate was only partly fused at the time of keratinization, a partial cleft would result.

An explanation for the frequency of cleft lip alongside cleft palate in humans is that defective fusion between the maxillary process and frontonasal process (probably because of inadequate growth) results in primary palate distortion and subsequent separation between primary and secondary shelves resulting in an increased distance to bridge. Perhaps murine mutants with cleft palate as the only craniofacial defect are not ideal models for the study of human oro-facial clefting – for a number of reasons: rodents have the upper lip physiologically non-fused in the middle (cleft). Furthermore, the vast majority of mutants with oral clefts have cleft palate alone without defective lip formation (in contrast to humans), and most mutants have other developmental defects. Although syndromic clefting does occur in humans, isolated oral clefting is a better model to study. Finally, clefting in transgenic animals is not spontaneous. One example of an animal model that may be more appropriate than any other for the study of non-syndromic cleft lip and palate is the CL/Fr strain of mice, because, as in humans, this strain has spontaneous cleft lip with or without cleft palate.

Besides the MEE, other findings presented here may pose further questions in the aetiology of conjoined twins. The fusion in embryos between heads and also backs could be compared to the union that is seen in conjoined twins, although it is likely that fusion between twins occurs at a much earlier stage than

examined here. Furthermore, no fusion was evident between organs, and it is not even clear whether 'fusion or fission' is responsible for this disorder (74, 75), but the assumption that intact skin will not fuse to intact skin, including the ectoderm of the embryo (76), should be reviewed. We also believe that MEE loss does not require complex gene–environment interactions, rather it may arise from a functional mechanical contact which then triggers the loss of the MEE.

References

1. Slavkin HC. Incidence of cleft lips, palates rising. *J Am Dent Assoc* 1992;123:61–5.
2. Dudas M, Li WY, Kim J, Yang A, Kaartinen V. Palatal fusion – Where do the midline cells go? A review on cleft palate, a major human birth defect. *Acta Histochem* 2006 [doi:10.1016/j.acthis.2006.05.009].
3. Gritli-Linde A. Molecular control of secondary palate development. *Dev Biol* 2007;301:309–26.
4. Fitchett JE, Hay ED. Medial edge epithelium transforms to mesenchyme after embryonic palatal shelves fuse. *Dev Biol* 1989;131:455–74.
5. Holtgrave EA, Stoltenburg-Didinger G. Apoptotic epithelial cell death: a prerequisite for palatal fusion. An *in vivo* study in rabbits. *J Craniomaxillofac Surg* 2002;30:329–36.
6. Taya Y, O'Kane S, Ferguson MW. Pathogenesis of cleft palate in TGF-beta3 knockout mice. *Development* 1999;126:3869–79.
7. Waterman RE, Ross LM, Meller SM. Alterations in the epithelial surface of A-Jax mouse palatal shelves prior to and during palatal fusion: a scanning electron microscopic study. *Anat Rec* 1973;176:361–75.
8. Waterman RE, Meller SM. Alterations in the epithelial surface of human palatal shelves prior to and during fusion: a scanning electron microscopic study. *Anat Rec* 1974;180:111–35.
9. Nawshad A, LaGamba D, Hay ED. Transforming growth factor beta (TGFbeta) signalling in palatal growth, apoptosis and epithelial mesenchymal transformation (EMT). *Arch Oral Biol* 2004;49:675–89.
10. Cuervo R, Covarrubias L. Death is the major fate of medial edge epithelial cells and the cause of basal lamina degradation during palatogenesis. *Development* 2004;131:15–24.
11. Griffith CM, Hay ED. Epithelial-mesenchymal transformation during palatal fusion: carboxyfluorescein traces cells at light and electron microscopic levels. *Development* 1992;116:1087–99.
12. Shuler CF, Guo Y, Majumder A, Luo RY. Molecular and morphologic changes during the epithelial-mesenchymal transformation of palatal shelf medial edge epithelium *in vitro*. *Int J Dev Biol* 1991;35:463–72.
13. Shuler CF, Halpern DE, Guo Y, Sank AC. Medial edge epithelium fate traced by cell lineage analysis during epithelial-mesenchymal transformation *in vivo*. *Dev Biol* 1992;154:318–30.
14. Jin JZ, Ding J. Analysis of cell migration, transdifferentiation and apoptosis during mouse secondary palate fusion. *Development* 2006;133:3341–3347.
15. Glucksmann A. Cell death in normal vertebrate ontogeny. *Biol Rev* 1951;26:59–86.
16. Saunders JW. Death in embryonic systems. *Science* 1966;154:604–12.
17. Cuervo R, Valencia C, Chandraratna RA, Covarrubias L. Programmed cell death is required for palate shelf fusion and is regulated by retinoic acid. *Dev Biol* 2002;245:145–56.
18. Carette MJ, Ferguson MW. The fate of medial edge epithelial cells during palatal fusion in vitro: an analysis by DiI labelling and confocal microscopy. *Development* 1992;114:379–88.
19. Bittencourt MA, Bolognese AM. Epithelial alterations of secondary palate formation. *Braz Dent J* 2000;11:117–26.
20. Martinez-Alvarez C, Tudela C, Perez-Miguelsanz J, O'Kane S, Puerta J, Ferguson MW. Medial edge epithelial cell fate during palatal fusion. *Dev Biol* 2000;220:343–57.
21. Vaziri Sani F, Hallberg K, Harfe BD, McMahon AP, Linde A, Gritli-Linde A. Fate-mapping of the epithelial seam during palatal fusion rules out epithelial–mesenchymal transformation. *Dev Biol* 2005;285:490–5.
22. Xu X, Han J, Yoshihiro I, Bringas P Jr, Urata MM, Chai Y. Cell autonomous requirement for Tgfbr2 in the disappearence of medial edge epithelium during palatal fusion. *Dev Biol* 2006;133:3341–7.
23. Tsai HM, Verrusio AC. Epithelial breakdown in the palatal processes of mouse fetuses with spontaneous cleft lip and palate. *Teratology* 1977;15:121–3.
24. Dudas M, Nagy A, Laping NJ, Moustakas A, Kaartinen V. Tgf-beta3-induced palatal fusion is mediated by Alk-5/Smad pathway. *Dev Biol* 2004;266:96–108.
25. Hilliard SA, Yu L, Gu S, Zhang Z, Chen YP. Regional regulation of palatal growth and patterning along the anterior–posterior axis in mice. *J Anat* 2005;207:655–67.
26. Lillie FR. *The Development of the Chick: an Introduction to Embryology*. New York: Henry Holt and Co.; 1908.
27. Juriloff DM, Roberts CW. Genetics of cleft palate in chickens and the relationship between the occurrence of the trait and maternal riboflavin deficiency. *Poult Sci* 1975;54:334–46.
28. Sun D, Vanderburg CR, Odierna GS, Hay ED. TGFbeta3 promotes transformation of chicken palate medial edge epithelium to mesenchyme in vitro. *Development* 1998;125:95–105.
29. Shah RM, Ferguson MW. Histological evidence of fusion between the posterior palatal shelves and the floor of the mouth in Alligator mississippiensis. *Arch Oral Biol* 1988;33:769–71.
30. Jiang R, Lan Y, Chapman HD, Shawber C, Norton CR, Serreze DV et al. Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes Dev* 1998;12:1046–57.
31. Alappat SR, Zhang Z, Suzuki K, Zhang X, Liu H, Jiang R et al. The cellular and molecular etiology of the cleft secondary palate in Fgf10 mutant mice. *Dev Biol* 2005;277:102–13.
32. Zhang Z, Song Y, Zhao X, Zhang X, Fermin C, Chen Y. Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. *Development* 2002;129:4135–46.
33. Lavrin IO, McLean W, Seegmiller RE, Olsen BR, Hay ED. The mechanism of palatal clefting in the Col11a1 mutant mouse. *Arch Oral Biol* 2001;46:865–69.
34. Miettinen PJ, Chin JR, Shum L, Slavkin HC, Shuler CF, Derynck R et al. Epidermal growth factor receptor function is necessary for normal craniofacial development and palate closure. *Nat Genet* 1999;22:69–73.

35. Ito Y, Yeo JY, Chytil A, Han J, Bringas P, Jr, Nakajima A et al. Conditional inactivation of Tgfbr2 in cranial neural crest causes cleft palate and calvaria defects. *Development* 2003;130:5269–80.
36. Trasler DG, Machado M. Newborn and adult face shapes related to mouse cleft lip predisposition. *Teratology* 1979;19:197–206.
37. Millicovsky G, Ambrose LJ, Johnston MC. Developmental alterations associated with spontaneous cleft lip and palate in CL/Fr mice. *Am J Anat* 1982;164:29–44.
38. Trasler DG, Trasler TA. Left cleft lip predominance and genetic similarities of L line and CL/Fr strain mice. *Teratology* 1984;30:423–7.
39. Bronsky PT, Johnston MC, Sulik KK. Morphogenesis of hypoxia-induced cleft lip in CL/Fr mice. *J Craniofac Genet Dev Biol* 1986;2:113–28.
40. Wang KY, Juriloff DM, Diewert VM. Deficient and delayed primary palatal fusion and mesenchymal bridge formation in cleft lip-liable strains of mice. *J Craniofac Genet Dev Biol* 1995;15:99–116.
41. Nagata M, Amin N, Kannari Y, Hayatsu M, Ohashi Y, Oguro A. Isolated maxillary bending in CL/FR strain mice: observation of craniofacial deformity and inheritance pattern. *Cleft Palate Craniofac J* 1997;34:101–5.
42. Kadowaki S, Sakamoto M, Kamiishi H, Tanimura T. Embryologic features of term fetuses and newborns in CL/Fr mice with special reference to cyanosis. *Cleft Palate Craniofac J* 1997;34:211–7.
43. Hamachi T, Sasaki Y, Hidaka K, Nakata M. Association between palatal morphogenesis and Pax9 expression pattern in CL/Fr embryos with clefting during palatal development. *Arch Oral Biol* 2003;48:581–7.
44. Erfani S, Maldonado TS, Crisera CA, Warren SM, Lee S, Longaker MT. An in vitro mouse model of cleft palate: defining a critical intershelf distance necessary for palatal clefting. *Plast Reconstr Surg* 2001;108:403–10.
45. Dudas M, Kaartinen V. Tgf-beta superfamily and mouse craniofacial development: interplay of morphogenetic proteins and receptor signaling controls normal formation of the face. *Curr Top Dev Biol* 2005;66:65–133.
46. Dudas M, Kim J, Li WY, Nagy A, Larsson J, Karlsson S et al. Epithelial and ectomesenchymal role of the type I TGF-beta receptor ALK5 during facial morphogenesis and palatal fusion. *Dev Biol* 2006;296:298–314.
47. Cui XM, Shiomi N, Chen J, Saito T, Yamamoto T, Ito Y et al. Overexpression of Smad2 in Tgfβ3-null mutant mice rescues cleft palate. *Dev Biol* 2005;278:193–202.
48. Kaartinen V, Voncken JW, Shuler C, Warburton D, Bu D, Heisterkamp N et al. Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nat Genet* 1995;11:415–21.
49. Proetzel G, Pawlowski SA, Wiles MV, Yin M, Boivin GP, Howles PN et al. Transforming growth factor-beta 3 is required for secondary palate fusion. *Nat Genet* 1995;11:409–14.
50. Brinkley L, Basehoar G, Branch A, Avery J. A new in vitro system for studying secondary palate development. *J Embryol Exp Morphol* 1975;34:485–95.
51. Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola, H, Friedman R, Boivin GP et al. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* 1997;124:2659–70.
52. Asada H, Kawamura Y, Maruyama K, Kume H, Ding RG, Kanbara N et al. Cleft palate and decreased brain gamma-aminobutyric acid in mice lacking the 67-kDa isoform of glutamic acid decarboxylase. *Proc Natl Acad Sci U S A* 1997;94:6496–9.
53. Zhao Y, Guo YJ, Tomac AC, Taylor NR, Grinberg A, Lee EJ et al. Isolated cleft palate in mice with a targeted mutation of the LIM homeobox gene lhx8. *Proc Natl Acad Sci U S A* 1999;96:15002–6.
54. Christensen K, Fogh-Andersen P. Cleft lip (+/- cleft palate) in Danish twins, 1970–1990. *Am J Med Genet* 1993;47:910–16.
55. Lewis CA, Thibault L, Pratt RM, Brinkley LL. An improved culture system for secondary palatal elevation. *In Vitro* 1980;16:453–60.
56. Shiota K, Kosazuma T, Klug S, Neubert D. Development of the fetal mouse palate in suspension organ culture. *Acta Anat (Basel)* 1990;137:59–64.
57. Al-Obaidi N, Kastner U, Merker HJ, Klug, S. Development of a suspension organ culture of the fetal rat palate. *Arch Toxicol* 1995;69:472–9.
58. Erfani S, Maldonado TS, Crisera CA, Warren SM, Peled ZM, Longaker MT. Rescue of an in vitro palate nonfusion model using interposed embryonic mesenchyme. *Plast Reconstr Surg* 2002;109:2363–72.
59. Martin P, Wood W. Epithelial fusions in the embryo. *Curr Opin Cell Biol* 2002;14:569–74.
60. Van der Werff JF, Nievelstein RA, Brands E, Luijsterburg AJ, Vermeij-Keers C. Normal development of the male anterior urethra. *Teratology* 2000;61:172–83.
61. Colas JF, Schoenwolf GC. Towards a cellular and molecular understanding of neurulation. *Dev Dyn* 2001;221:117–45.
62. Sun D, Baur S, Hay ED. Epithelial-mesenchymal transformation is the mechanism for fusion of the craniofacial primordia involved in morphogenesis of the chicken lip. *Dev Biol* 2000;228:337–49.
63. Chai Y, Sasano Y, Bringas P, Jr, Mayo M, Kaartinen V, Heisterkamp N et al. Characterization of the fate of midline epithelial cells during the fusion of mandibular prominences *in vivo*. *Dev Dyn* 1997;208:526–35.
64. Garcia-Martinez V, Macias D, Ganan Y, Garcia-Lobo JM, Francia MV, Fernandez-Teran MA et al. Internucleosomal DNA fragmentation and programmed cell death (apoptosis) in the interdigital tissue of the embryonic chick leg bud. *J Cell Sci* 1993;106:201–8.
65. Viebahn C. Epithelio-mesenchymal transformation during formation of the mesoderm in the mammalian embryo. *Acta Anat (Basel)* 1995;154:79–97.
66. Markwald R, Eisenberg C, Eisenberg L, Trusk T, Sugi Y. Epithelial-mesenchymal transformations in early avian heart development. *Acta Anat (Basel)* 1996;156:173–86.
67. Wang J, Nagy A, Larsson J, Dudas M, Sucov HM, Kaartinen V. Defective ALK5 signaling in the neural crest leads to increased postmigratory neural crest cell apoptosis and severe outflow tract defects. *BMC Dev Biol* 2006;6:51.
68. Ferguson MW, Honig LS, Slavkin HC. Differentiation of cultured palatal shelves from alligator, chick, and mouse embryos. *Anat Rec* 1984;209:231–49.
69. Shah RM, Crawford BJ, Greene RM, Suen RS, Burdett D, King KO et al. In vitro development of the hamster and chick secondary palate. *J Craniofac Genet Dev Biol* 1985;5:299–314.
70. Takigawa T, Shiota K. Terminal differentiation of palatal medial edge epithelial cells in vitro is not necessarily dependent on palatal shelf contact and midline epithelial seam formation. *Int J Dev Biol* 2004;48:307–17.
71. Goss AN, Avery JK. Epithelial-mesenchymal interaction in palatal shelf fusion. An *in vitro* study. *Aust Dent J* 1975;20:152–6.

72. Martinez-Alvarez C, Bonelli R, Tudela C, Gato A, Mena J, O'Kane S et al. Bulging medial edge epithelial cells and palatal fusion. *Int J Dev Biol* 2000;44:331–5.
73. Brown NL, Knott L, Halligan E, Yarram SJ, Mansell JP, Sandy JR. Microarray analysis of murine palatogenesis: temporal expression of genes during normal palate development. *Dev Growth Differ* 2003;45:153–65.
74. Spencer R. Theoretical and analytical embryology of conjoined twins: Part I: Embryogenesis. *Clin Anat* 2000;13:36–53.
75. Spencer R. Theoretical and analytical embryology of conjoined twins: Part II: Adjustments to union. *Clin Anat* 2000;13:97–120.
76. Spencer R. Conjoined twins: theoretical embryologic basis. *Teratology* 1992;45:591–602.