

# TGF $\beta$ 3 promotes transformation of chicken palate medial edge epithelium to mesenchyme in vitro

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## SUMMARY

Epithelial-mesenchymal transformation plays an important role in the disappearance of the midline line epithelial seam in rodent palate, leading to confluence of the palate. The aim of this study was to test the potential of the naturally cleft chicken palate to become confluent under the influence of growth factors, such as TGF $\beta$ 3, which are known to promote epithelial-mesenchymal transformation. After labeling medial edge epithelia with carboxyfluorescein, palatal shelves (E8-9) with or without beak were dissected and cultured on agar gels. TGF $\beta$ 1, TGF $\beta$ 2 or TGF $\beta$ 3 was added to the chemically defined medium. By 24 hours in culture, medial edge epithelia form adherent midline seams in all paired groups without intact beaks. After 72 hours, seams in the TGF $\beta$ 3 groups disappear and palates become confluent due to epithelial-mesenchymal transformation, while seams remain mainly epithelial in control, TGF $\beta$ 1 and TGF $\beta$ 2 groups. Epithelium-derived mesenchymal cells are identified by carboxyfluorescein fluorescence with confocal microscopy and by membrane-bound carboxyfluorescein isolation bodies with electron microscopy. Labeled fibroblasts

completely replace the labeled epithelia of origin in TGF $\beta$ 3-treated palates without beaks. Single palates are unable to undergo transformation, and paired palatal shelves with intact beaks do not adhere or undergo transformation, even when treated with TGF $\beta$ 3. Thus, physical contact of medial edge epithelia and formation of the midline seam are necessary for epithelial-mesenchymal transformation to be triggered. We conclude that there may be no fundamental difference in developmental potential of the medial edge epithelium for transformation to mesenchyme among reptiles, birds and mammals. The bird differs from other amniotes in having developed a beak and associated craniofacial structures that seemingly keep palatal processes separated in vivo. Even control medial edge epithelia partly transform to mesenchyme if placed in close contact. However, exogenous TGF $\beta$ 3 is required to achieve complete confluence of the chicken palate.

Key words: TGF $\beta$ , epithelial-mesenchymal transformation, medial edge epithelium, carboxyfluorescein, chicken palate development, craniofacial morphogenesis

## INTRODUCTION

Palatogenesis is a very important event in craniofacial development of the group of higher vertebrates known as amniotes. In alligator, rodent and human, the medial edge epithelia (MEE) of the paired palatal shelves that arise from the maxillary processes make contact to form a two-layered midline seam (Ferguson, 1988). Then, the epithelial seam disappears and the palate becomes confluent. In the avian embryo, palatal shelves form in similar fashion, but the MEE do not fuse and the palate does not become confluent. Later, the MEE stratify and keratinize to line the medial edge of the naturally cleft palate (Lillie, 1908).

The formation and disappearance of the MEE seam have been the focus of many studies of the easily accessible rodent palate. Early investigations suggested that programmed cell death is the mechanism for the disappearance of the seam (Glucksmann, 1951; Saunders, 1966). However, this conclusion has been

challenged by recent workers (Fitchett and Hay, 1989; Griffith and Hay, 1992; Shuler et al., 1992), who studied the rodent MEE in vivo and in vitro by transmission electron microscopy (TEM) and with lineage markers, such as carboxyfluorescein and DiI, to obtain definitive evidence that the MEE undergo epithelial-mesenchymal transformation (EMT) to produce fibroblasts that form the connective tissue that creates palatal shelf confluence (but see Carrette and Ferguson, 1992).

Earlier studies of chicken palatogenesis suggested that failure of apoptosis in the MEE plays the causal role in preventing avian palatal fusion. This conclusion implies that a fundamental difference exists in the developmental potential of avian MEE as compared to other amniotes (Shah and Crawford, 1980; Koch and Smiley, 1981; Greene et al., 1983; Shah and Cheng, 1988). However, no cell lineage studies have been done in chicken. Ferguson and Honig (1985) induced chicken palate fusion in vitro and in vivo by lacerating the MEE of each palatal shelf. Because this surgical procedure not only removed epithelia, but

also injured underlying connective tissue, we think connective tissue wound healing played the important role in achieving confluence in that experiment, which did not test the developmental potential of the intact MEE.

The demonstration of EMT by the rodent palate MEE (Fitchett and Hay, 1989; Griffith and Hay, 1992; Shuler et al., 1992) raises the possibility that the deficiency in the chicken palate may not be failure of apoptosis, but rather is due to failure of EMT. TGF $\beta$ s have been shown to promote EMT in chicken cardiac cushions (Potts et al., 1991; Runyan et al., 1992; Dickson et al., 1993). TGF $\beta$  type II receptors are expressed in early chicken embryos (Barnet et al., 1994), and antibodies to this receptor block EMT in the chicken AV canal (Brown et al., 1996). TGF $\beta$ s promote many other cellular events, such as differentiation, proliferation, extracellular matrix production and migration (Roberts and Sporn, 1990; Kingsley, 1994), by binding this same serine/threonine kinase receptor to activate signaling pathways (Massague, 1992; Wrana et al., 1994).

The possible roles of TGF $\beta$ s in palatogenesis, as well as other growth factors including EGF, FGF and PDGF, have been studied in the rodent (Abbott and Pratt, 1987; Abbott et al., 1988; Gawel-Thompson and Greene, 1989; D'Angelo and Greene, 1991; Gehris et al., 1991; Gehris and Greene, 1992; Dixon and Ferguson, 1992; Sharpe et al., 1992). The rodent palatal MEE is rich in TGF $\beta$ 3 mRNA (Fitzpatrick et al., 1990; Pelton et al., 1990), but contains little TGF $\beta$ 1 or  $\beta$ 2 message (Williams et al., 1991; Gehris et al., 1991). There is abundant TGF $\beta$ 3 protein associated with rodent MEE (Fitzpatrick et al., 1990; Pelton et al., 1990), while chicken palate contains almost none (this report). The predominant phenotype of TGF $\beta$ 3 knock-out mice is partial, but not complete, cleft palate (Proetzel et al., 1995; Kaartinen et al., 1995). TGF $\beta$ 3 (but not TGF $\beta$ 1 or  $\beta$ 2) antisense-oligonucleotides and antibodies block palate fusion in mouse (Brunet et al. 1995; Kaartinen et al., 1997). However, effects of TGF $\beta$ s on chicken palatogenesis have not been studied.

In this paper, we show that TGF $\beta$ 3, but not TGF $\beta$ 1 or TGF $\beta$ 2, promotes chicken palatal confluence. In order that the fate of the MEE could be followed, we labeled epithelia with carboxyfluorescein and then cultured palatal shelves in chemically defined medium for 24-72 hours with various TGF $\beta$ s. Carboxyfluorescein is a better lineage marker than DiI (Shuler et al., 1992; Carrette and Ferguson, 1992), because it can be identified not only by confocal microscopy, but also by TEM, since it withstands plastic embedding. Cells compartmentalize it into membrane-bound isolation bodies that are identified readily at the ultrastructural level (Griffith and Hay, 1992).

We demonstrate for the first time here that chicken palatal MEE will form a midline seam if placed in close contact after removal of the beak. This seam undergoes partial EMT even without growth factor addition, but TGF $\beta$ 3 promotes full confluence of chicken palates by EMT.

## MATERIALS AND METHODS

### Labeling and culturing of chicken palates in vitro

Fertile M145D white chicken eggs were purchased (Spafas) and incubated at 40°C in a moist atmosphere. Chicken embryos were harvested at Hamburger-Hamilton stage 35 (E8-9 days) and put into

warm Hanks Balanced Salt Solution (HBSS). The head without lower jaw was placed in a solution of 5-,6-carboxy 2-7-dichlorofluorescein diacetate succinimidyl ester (CCFSE; Molecular Probes, Inc.) in HBSS at 37°C for 1 hour. Whole heads were treated so that palate epithelia would be labeled, but not pre-existing palate mesenchyme as no cut surface is nearby and CCFSE does not cross intact epithelium (Griffith and Hay, 1992). CCFSE was prepared as a 10 mM stock solution in dimethyl sulphoxide (DSMO) diluted 1:200 (v:v) in HBSS for use. After CCFSE exposure, heads were rinsed well in fresh HBSS. Labeled palatal shelves were dissected with or without beaks and cultured as described below (Fig. 1A).

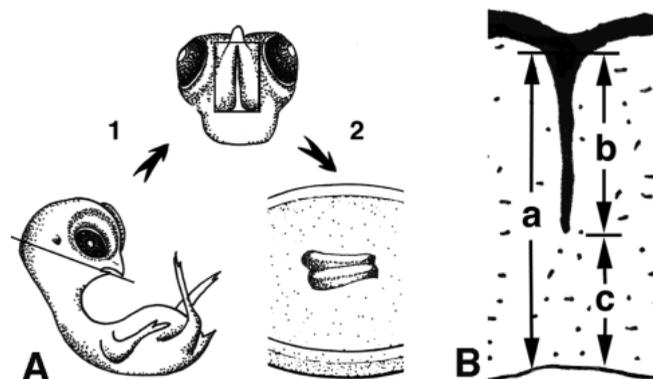
Palatal shelves were cultured nasal side down on 0.5% agar gels, either singly or in pairs with medial edges touching. However, contact was not close in palates left attached to their beaks. The 0.5% agar was made up in 1:1 DMEM:F12 medium (JRH Biosciences) at pH 7.4, supplemented with 1% penicillin-streptomycin (GIBCO). Controls were cultured in serum-free, chemically defined medium, which consisted of Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12 (1:1 DMEM:F12; JRH Biosciences) at pH 7.4, with 3.15 mg/ml glucose, 2.5 mM L-glutamine, 0.055 mg/ml sodium pyruvate, 0.25 mg/ml ascorbic acid and 1% penicillin-streptomycin (GIBCO). 0.1 ng/ml, 1 ng/ml and 10 ng/ml TGF $\beta$ 3, 10 ng/ml TGF $\beta$ 1 and 10 ng/ml TGF $\beta$ 2 (R&D) were added to serum-free, chemically defined medium for experimental groups. Cultures were maintained for 24 or 72 hours in humidified incubators at 37°C, in 5% CO<sub>2</sub>, 95% air, and the medium was changed every 24 hours. Total palates examined (345) are described in Table 1. 90% of these were processed for paraffin sectioning and the remaining 10% (all paired palates without beaks) for TEM.

### Confocal microscopy

Palates exposed to CCFSE were fixed after culture for 24 and 72 hours in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 2 hours. They were then rinsed in PBS and processed for paraffin embedding. Serial sections 7  $\mu$ m thick were deparaffinized and mounted unstained for fluorescence confocal microscopy (Zeiss 185). After removal of coverslips, slides were then stained with hematoxylin and eosin (H and E).

### Morphometric methods

Slides of palates cultured without beaks for 72 hours were examined systematically. Slides contained consecutive ribbons of serial cross



**Fig. 1.** (A) Diagram showing preparation of a pair of palatal shelves for CCFSE-labeling and organ culture on agar. 1, Lower jaw was removed and the rest of head was exposed to CCFSE; 2, palatal shelves were dissected and placed on agar. In some cases (not shown), palates were left attached to beaks. (B) Diagram showing measurements carried out on cross sections of fused palates. a, Total length of adherence; b, total length of the midline seam; c, length of confluence. Confluence (%) per section =  $c/a \times 100\%$ .

**Table 1. A summary of numbers of palates cultured**

Treatment		Number of palates cultured	Number of palates adherent (%)
Single palates (cultured 72 hours)	Control	15	0
	TGFβ1 (10 ng/ml)	15	0
	TGFβ2 (10 ng/ml)	15	0
	TGFβ3 (10 ng/ml)	15	0
Paired palates with intact beak (cultured 72 hours)	Control	25	0
	TGFβ3 (0.1 ng/ml)	15	0
	TGFβ3 (1 ng/ml)	15	0
	TGFβ3 (10 ng/ml)	15	0
Paired palates without beak (cultured 24 hours)	Control	15	13 (86.7%)
	TGFβ1 (10 ng/ml)	15	14 (93.3%)
	TGFβ2 (10 ng/ml)	15	13 (86.7%)
	TGFβ3 (10 ng/ml)	15	14 (93.3%)
Paired palates without beak (cultured 72 hours)	Control	30	25 (83.3%)
	TGFβ1 (10 ng/ml)	25	23 (92.0%)
	TGFβ2 (10 ng/ml)	25	22 (88.0%)
	TGFβ3 (0.1 ng/ml)	15	13 (86.7%)
	TGFβ3 (1 ng/ml)	30	26 (86.7%)
	TGFβ3 (10 ng/ml)	30	27 (90.0%)

sections, stained by H and E, oriented from anterior to posterior for each palate. Measurements were made on every tenth consecutive section of the middle 100 sections of each palate, using a measuring grid inserted in a 10× ocular lens (mesh unit=1 μm with 20× objective). Confluence (%) per section was calculated as the length of the confluent region (c, Fig. 1B) divided by the total length of adherence (a, Fig. 1B) including any remaining seam (b, Fig. 1B). The average confluence (%) of each group was calculated by adding the average confluence (%) in the sections of each palate and dividing by the total number of palates examined.

#### Electron microscopy

Palates were fixed at room temperature in 4% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 hour, then

rinsed in buffer, post-fixed in cold 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour, and stained en bloc for 30 minutes with saturated uranyl acetate in distilled H<sub>2</sub>O. They were dehydrated through graded ethanols and propylene oxide, and embedded in Epon/Araldite. Thin sections were examined for TEM after counterstaining with lead citrate as previously described (Fitchett and Hay, 1989).

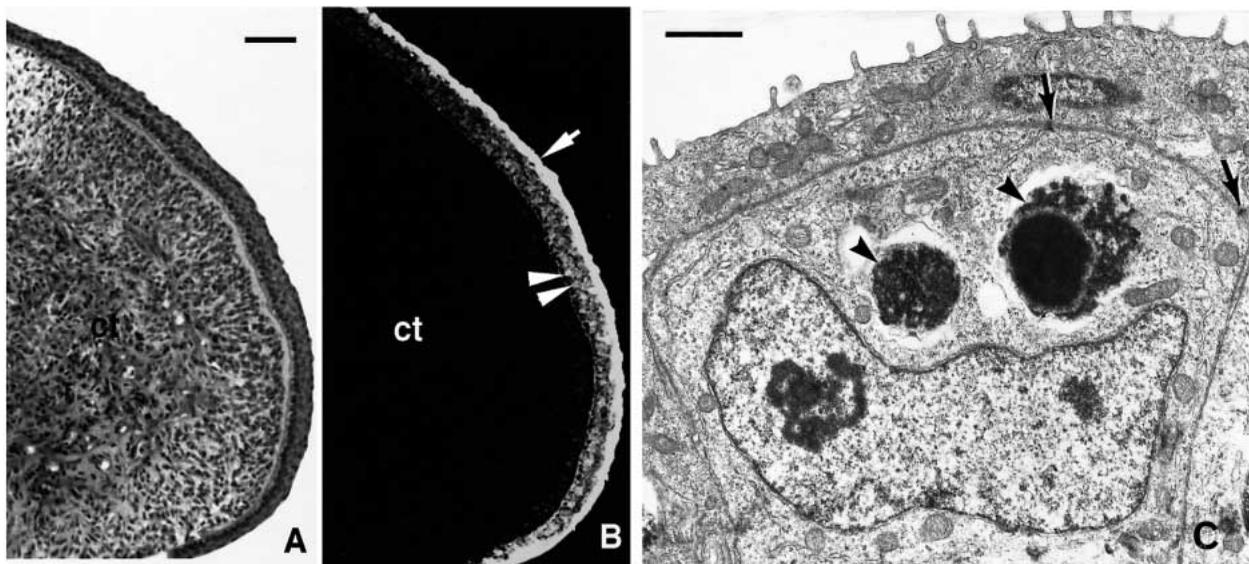
#### Immunohistochemistry

Palates were collected at stage 35 and after 24 hours in culture (chicken) and at E15 (rat) and fixed in 2% paraformaldehyde at room temperature for 1 hour. Paraffin sections (7 μm thick) were deparaffinized and rehydrated, washed in Tris-HCl-buffered saline (TBS), pH 7.4, and blocked with TBS containing 2% goat serum and 2% bovine serum albumin (BSA, Sigma). Primary antibodies (anti-TGF β1, β2 and β3, a gift from Dr Leslie Gold (NYU) and/or purchased from R and D) were added (2.5 μg/ml) and sections incubated at 4°C overnight. After washing in TBS, sections were incubated with secondary antibodies (1:500) (anti-rabbit IgG-FITC, Boehringer Mannheim) at 37°C for 1 hour and examined with a Zeiss 185 confocal microscope.

## RESULTS

### Organ culture of chicken secondary palate

Hamilton-Hamburger stage 35 (E8-9) chicken embryos were chosen for study, because the palatal shelves are well developed and moving medially. The embryonic MEE consists of two cell layers (periderm, basal cells), as in the rodent when the palatal shelves adhere. The morphology of palates remains fair until 72 hours in culture without serum, but tissue necrosis begins thereafter. The chemically defined culture medium is modified from an existing method that supports growth of vertebrate embryonic palates in vitro (Ferguson et al., 1984; Ferguson and Honig, 1985). It was necessary to omit serum to investigate the effects of growth factors.



**Fig. 2.** Single palate cultured for 72 hours after labeling with CCFSE for 1 hour. (A) H and E stain. (B) Confocal fluorescent view. Outer peridermal cells (arrow) are labeled best, but CCFSE passes through to the basal MEE cells (arrowheads). The connective tissue compartment is negative (ct), indicating that label does not penetrate past epithelium. Bar in A, 100 μm (also applies to B). (C) TEM showing CCFSE isolation bodies (arrowheads) in the epithelium of palate cultured for 72 hours. These bodies correspond to fluorescent bodies viewed by confocal microscopy (arrowheads, B). Periderm is connected to basal cells via desmosomes (arrows). Bar, 1 μm.

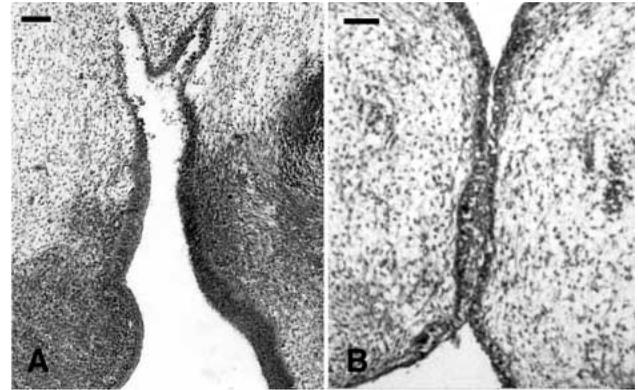
### CCFSE labeling

After 1 hour of exposure to CCFSE, palatal peridermal cells are intensely labeled (Fig. 2B, arrow), while the basal cells that receive the label indirectly through gap junctions from the peridermal cells are less well labeled. Inside the cell, esterases cleave off acetates to release the fluorophore as a water-soluble compound that cannot diffuse out of labeled cells across the plasmalemma (Griffith and Hay, 1992). Since CCFSE cannot cross from the plasmalemma of the basal cell through the basement membrane, the underlying connective tissue (ct, Fig. 2B) is not labeled.

The fluorescent bodies observed by confocal microscopy (Fig. 2B, arrowheads) can be resolved by TEM (Fig. 2C, arrowheads) as membrane-bound CCFSE-containing bodies into which the cell isolates the foreign material (Griffith and Hay, 1992). Griffith and Hay (1992) have demonstrated that these bodies are not lysosomes by co-staining palate sections with the Gomori stain for lysosomal acid phosphatase. Also, the labeled cells are healthy looking, with euchromatic nuclei and normal appearing organelles (Fig. 2C), whereas lysosomes usually characterize dying cells.

### Culturing of single palates

After single palates are cultured for 72 hours, the MEE of control and experimental groups stratify to about 3-4 cell layers thick (Fig. 2A). The CCFSE-labeled epithelia cannot form a seam, and the connective tissue in the control (Fig. 2B, ct) and TGF $\beta$ 1-3 groups is not labeled, indicating that no transformation of MEE to mesenchymal cells occurs in single palates in any of the groups.

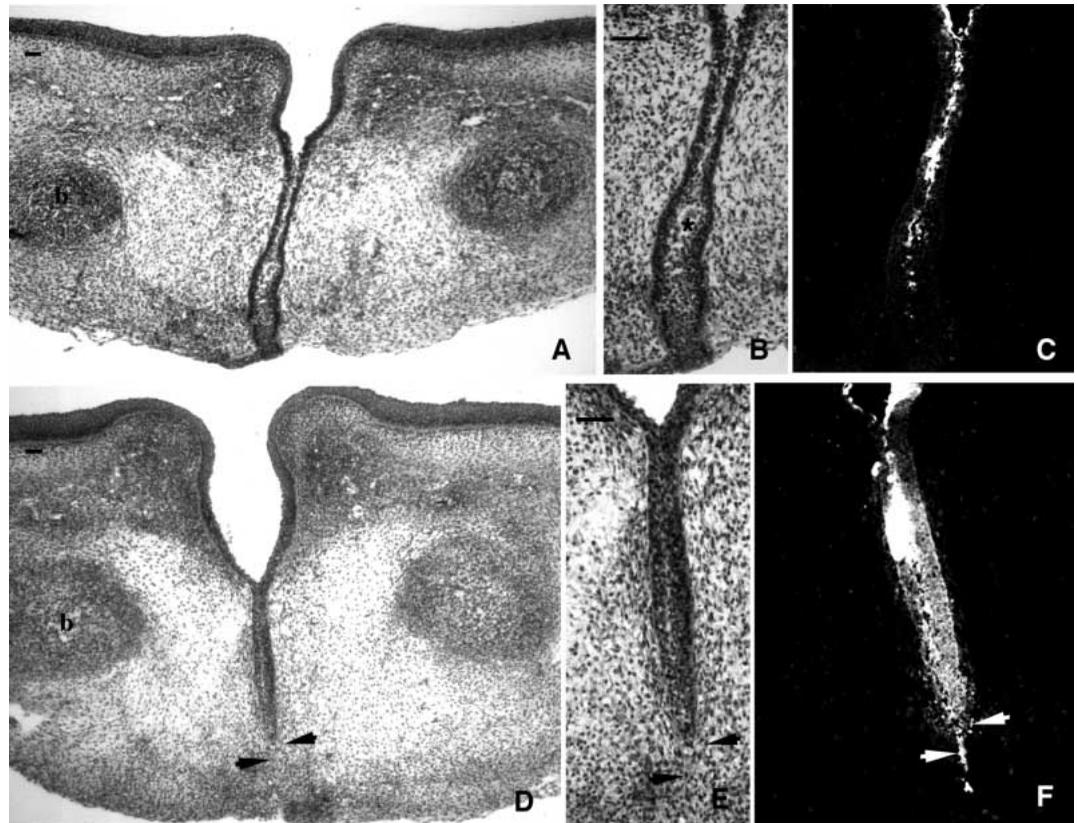


**Fig. 3.** Cross sections of cultured palates cultured with (A) or without (B) intact beaks. (A) Palatal shelves cultured with an intact beak do not fuse after 3 days in culture with (shown) or without TGF $\beta$ 3 treatment. (B) After the beak is removed, two palatal shelves are able to maintain contact and they form a midline seam within 24 hours under conditions that otherwise are the same as in (A). Bars, 100  $\mu$ m.

### Culturing of paired palates with beaks intact

Even after culture for 72 hours, the MEE of paired palates with intact beaks do not make contact or become adherent, even when treated with TGF $\beta$ 3 (Table 1, Fig. 3A). CCFSE labeling also shows that no EMT occurs. However, we found that after the beak is removed, the two palatal shelves are able to maintain contact with each other form a midline seam within 24 hours (Fig. 3B).

**Fig. 4.** Paired palates cultured for 24 hours after CCFSE-labeling. (A,B,D,E) are H and E stains and (C,F) are confocal fluorescent views of the same sections. Epithelia are better preserved on the oral side of the palate (top), which does not face the agar. Control palate (A,B,C) exhibits midline epithelial seam containing CCFSE, but the mesenchyme is not labeled. In TGF $\beta$ 3 (10 ng/ml) treated (D,E,F), paired MEE form a seam that has started to break into CCFSE-labeled mesenchymal cells (arrows, D,E,F). Asterisk, peridermal cells trapped in the seam; b, developing bone. Bars, 100  $\mu$ m.

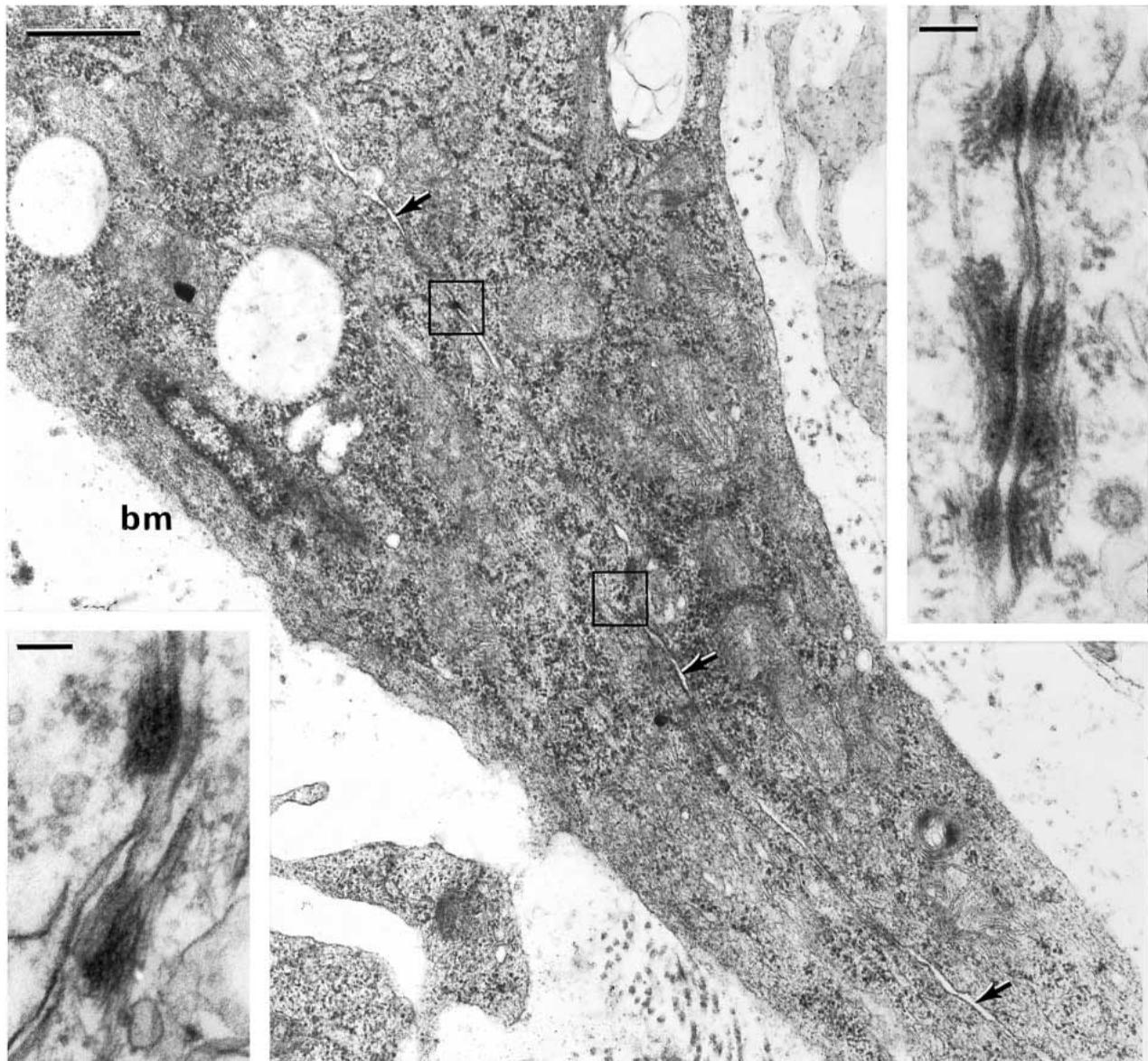


### Culturing of paired palates with beaks removed

When paired chicken palates without beaks are placed in close contact in vitro, 'fusion' occurs in all groups. Two distinct events occur during the 'fusion' we observed. The first is formation of midline epithelial seam, which we shall term 'adherence,' because true desmosomes form between basal layers of the opposing MEE when they make contact. The second is epithelial-mesenchymal transformation of the MEE, which we shall call 'confluence,' because the epithelial seam is replaced by a continuous layer of fibroblasts and extracellular matrix holding the palate together. Complete confluence is only observed in TGF $\beta$ 3-treated groups, whereas adherence occurs in all groups of paired palates without beaks.

After chicken palatal shelves are cultured in close contact for 24 hours, the MEE in the TGF $\beta$ 1-, TGF $\beta$ 2-, TGF $\beta$ 3-treated and control groups adhere and form a midline epithelial seam (Fig. 4A,D) similar to that observed in rodent palatogenesis. Both in control and TGF $\beta$ -treated groups, peridermal cells are trapped in some areas of the seams (Fig. 4B, \*), an artifact which also occurs in rodent palates if the shelves are juxtaposed before the periderm is able to slough (Waterman and Meller, 1974; Fitchett and Hay, 1989).

The major difference between the TGF $\beta$ 3-treated and other groups at 24 hours is that the midline seams have started to break up into mesenchymal-like cells in the TGF $\beta$ 3 groups (Fig. 4D-F, arrows). Confocal microscopy demonstrates that the labeled seams are replaced by CCFSE-labeled



**Fig. 5.** Electron microscopy showing the plasmalemmal apposition area (arrows) of two basal MEE layers in a control seam after 24 hours in culture. Desmosomes are differentiating here (squares). Examples of such desmosomes are shown at higher magnification in the insets. The left-hand inset depicts two half desmosomes that will probably induce matching halves in opposed cells. In the right-hand inset, four well-developed desmosomes are aligned on opposed midline plasmalemmas of a seam. Remnants of the basement membrane are disappearing (bm). Bar, 0.5  $\mu$ m; insets, 0.1  $\mu$ m.

mesenchymal cells, which leave the seam to create connective tissue and/or migrate into nearby stroma (Fig. 4F, arrows).

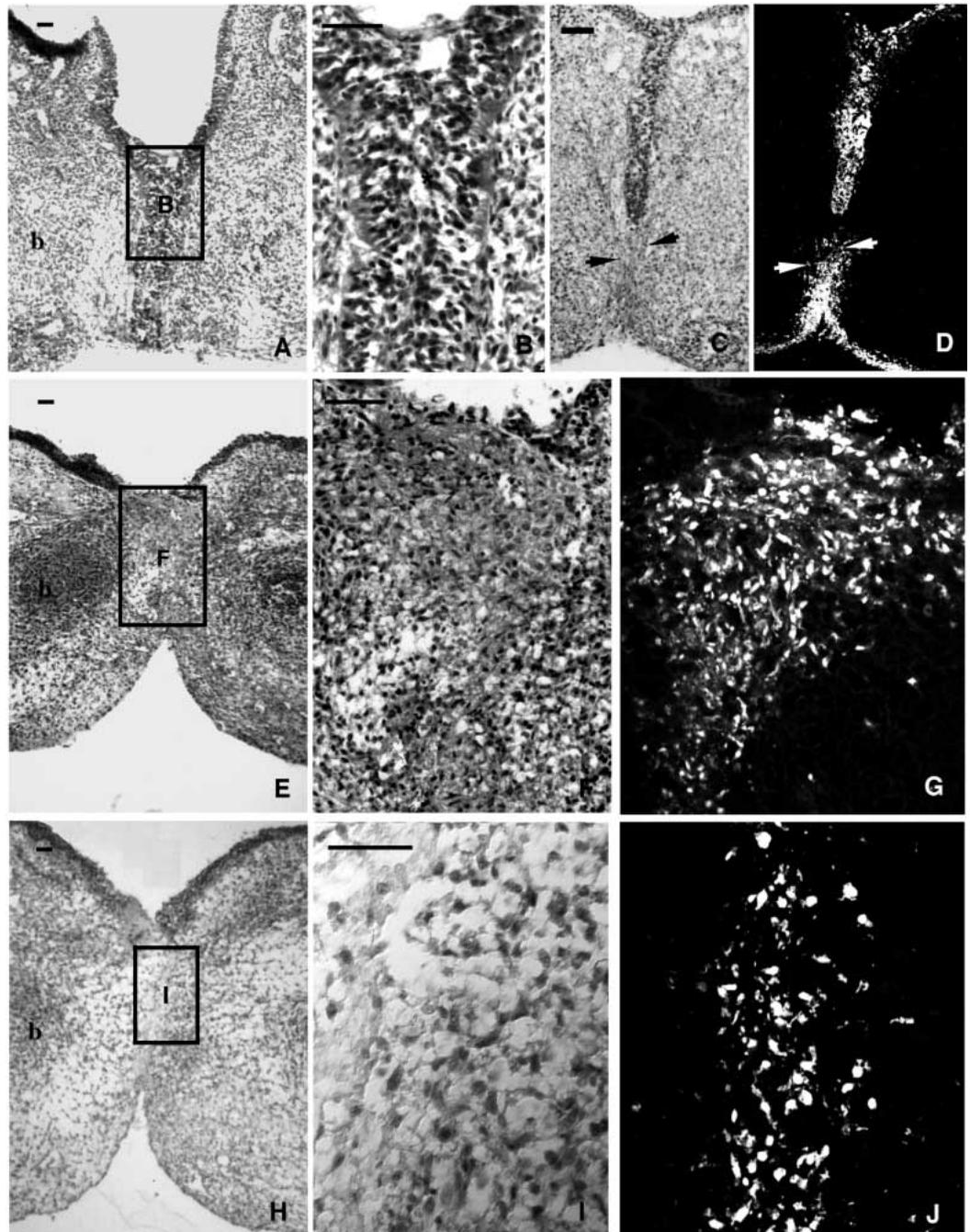
In areas where peridermal cells are not present, basal cell layers form numerous new desmosomes between them (Fig. 5, squares and insets). The trapped peridermal cells apparently die and are removed by phagocytosis, as two-layered seams soon predominate (Fig. 5). Both half desmosomes and well-developed desmosomes occur between the two basal layers comprising the seam (Fig. 5, insets). A similar pattern is observed in rodent palatogenesis (see Discussion).

After culture for 72 hours, most midline seams in the control, TGF $\beta$ 1 and TGF $\beta$ 2 groups are still intact and the MEE retain epithelial characteristics (Fig. 6A,B). However, part of the midline seam is now replaced by confluent mesenchyme in

some control palates (Fig. 6C,D, arrows). In areas where the CCFSE-labeled seam has disappeared, CCFSE-labeled mesenchymal cells are found in the connective tissue compartment (Fig. 6D, arrows), indicating that the MEE in the control groups can undergo EMT, resulting in partial confluence of the palate.

In contrast, midline seams in the TGF $\beta$ 3-treated group disappear completely and palates become totally confluent (Fig. 6E-J). The MEE of the seam undergo EMT to become fibroblasts. CCFSE-labeled cells present within the connective tissue compartment (Fig. 6G,J) correspond to areas of confluent fibroblasts, as seen by H and E staining (Fig. 6F,I).

By TEM, we confirmed that connective tissue cells labeled

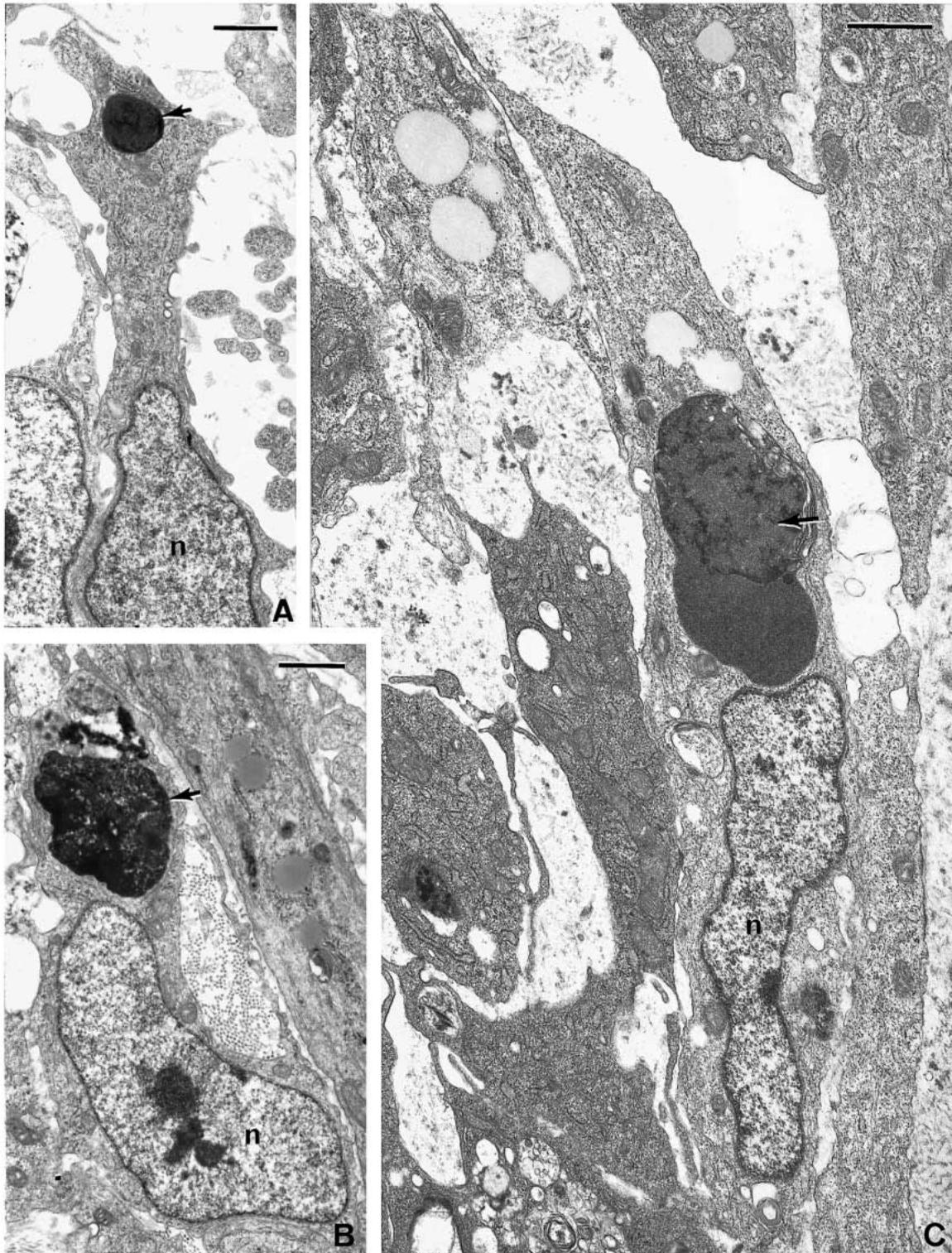


**Fig. 6.** Paired CCFSE-labeled palates cultured for 72 hours. (A,B,C,E,F,H,I) are H and E stained and (D,G,J) are confocal fluorescent views of the same sections. B, C, F and I are enlargements of the areas indicated in A, E and H, respectively. Some palates in the control group still have intact seams (A) with trapped peridermal cells (B, \*). In other control palates at 72 hours, partial confluence occurs (C,D, arrows). MEE-derived fibroblasts in the connective tissue are labeled by CCFSE (C,D, arrows). In contrast, the seams in the TGF $\beta$ 3-treated group have disappeared by 72 hours and the palates are completely confluent (E,H). The presence of labeled mesenchymal cells (G,J) identified as fibroblasts by H and E staining (F,I) indicates that EMT has occurred. TEM of such areas confirms this conclusion (see Fig. 7). E-G and H-J are from two different palates treated with TGF $\beta$ , demonstrating similar results. b, developing bone. Bars, 100  $\mu$ m; bars in C, F and I also apply to D, G and H, respectively.

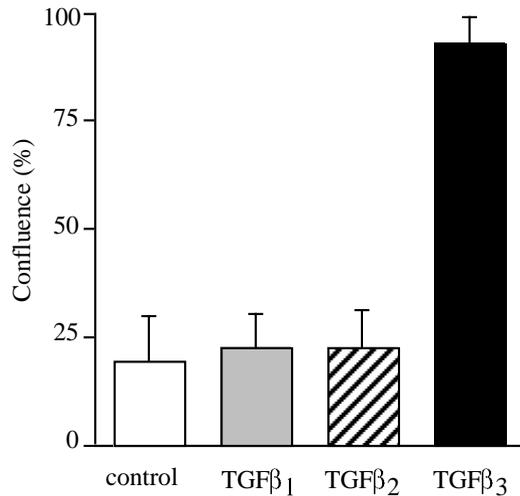
by CCFSE are fibroblasts, from labeled areas similar to those illustrated in Fig. 6G,J. Fibroblasts containing CCFSE isolation bodies (Fig. 7A,B,C, arrows) can be identified by their elongated shape, typical rough endoplasmic reticulum, and

relative lack of contact with adjacent cells. The CCFSE isolation bodies in the cytoplasm (Fig. 7, arrows) marks them as derived from CCFSE-labeled epithelium.

To determine the different effects of isoforms of TGFβ1-3



**Fig. 7.** TEM showing sections of three different transformed mesenchymal cells in an area similar to that in Fig. 6G,J. Typical isolation bodies are seen within the cytoplasm, identifying the cells as CCFSE-labeled (A,B,C, arrows). The elongated shape and prominent RER are typical of fibroblasts. Cells are healthy-looking, with euchromatic nuclei (n) and normal-appearing organelles. Bars, 1  $\mu$ m.



**Fig. 8.** Graph showing percentage of cross-sectional area exhibiting confluence in 85 palates cultured for 72 hours with different treatments. The average (%) confluence (see Fig. 1) in the control group, and in the TGFβ<sub>1</sub>, TGFβ<sub>2</sub> and TGFβ<sub>3</sub> groups (all at a dosage of 10 ng/ml), respectively, is 19.3±12.4% (n = 20), 21.9±9.4% (n = 21), 22.5±12% (n = 21) and 92.3±8.6% (n = 23). There is a large difference between the TGFβ<sub>3</sub> group and the other groups, but there is little or no difference between TGFβ<sub>1</sub>, TGFβ<sub>2</sub> and the control groups.

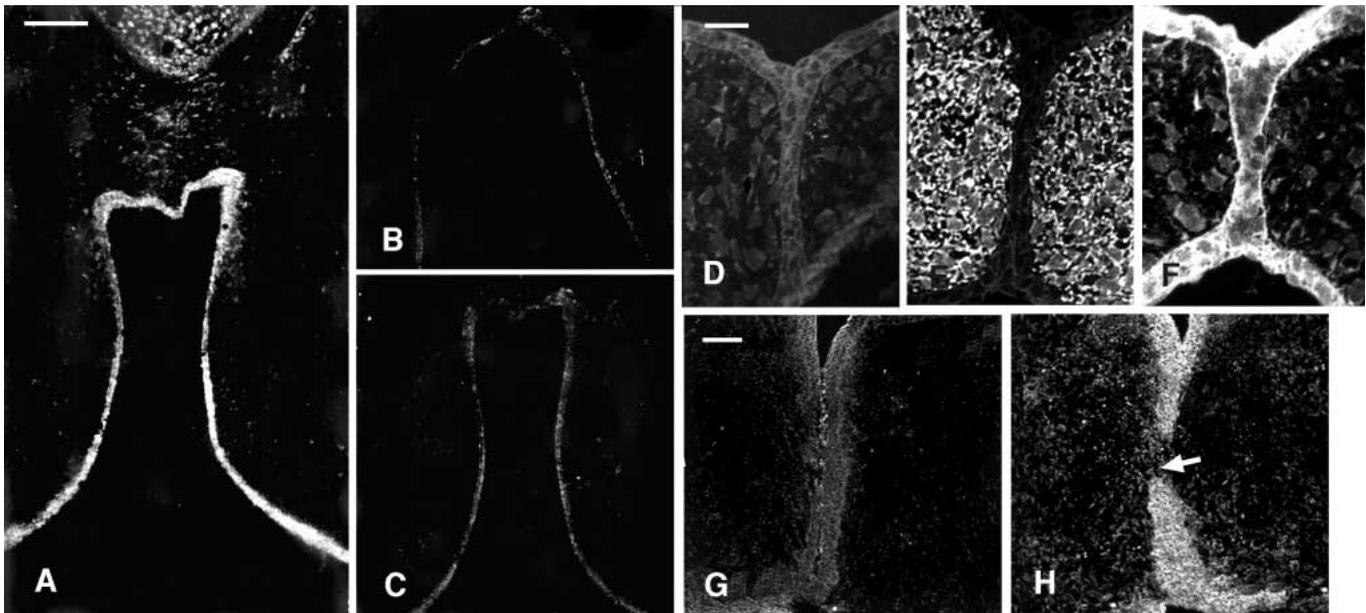
on confluence of the palates, we examined sections of palates cultured for 72 hours with a morphometric method (Fig. 8; see Fig. 1 and Materials and methods). The average confluences

(%) in control, TGFβ<sub>1</sub>, TGFβ<sub>2</sub> and TGFβ<sub>3</sub> groups (all at a dosage of 10 ng/ml), respectively, are 19.3±12.4%, 21.9±9.4%, 22.5±12% and 92.3±8.6% (Fig. 8). Thus, in control and TGFβ<sub>1</sub> and TGFβ<sub>2</sub> groups, the average cross-section area transformed is about 20%. Therefore, the partial confluence observed is not likely to be due to TGFβ<sub>1</sub> or -β<sub>2</sub>. There is a large difference, however, between these groups and the TGFβ<sub>3</sub> group (92% confluent; Fig. 8).

#### Immunohistochemical studies of TGFβs expression in vivo and in vitro

This study clearly demonstrates that it is TGFβ<sub>3</sub>, not TGFβ<sub>1</sub> or TGFβ<sub>2</sub>, that promotes complete confluence of chicken palates in vitro (Fig. 8). Since previous studies have shown that mouse MEE is rich in TGFβ<sub>3</sub>, we examined chicken MEE for endogenous TGFβ<sub>3</sub>. Our immunohistochemical studies demonstrate that E9 chicken MEE express TGFβ<sub>1</sub> (Fig. 9A), but very little TGFβ<sub>2</sub> (Fig. 9B) or TGFβ<sub>3</sub> (Fig. 9C). In contrast, rat palatal MEE express little TGFβ<sub>1</sub> (Fig. 9D) or TGFβ<sub>2</sub> (Fig. 9E), but a high level of TGFβ<sub>3</sub> (Fig. 9F) during the process of fusion. Rat palatal mesenchyme is rich in TGFβ<sub>2</sub>, however, (Fig. 9E). These new results from the rat differ from previous studies of developing mouse palates (see Introduction) in that they show localization of the abundant endogenous TGFβ<sub>3</sub> to basal sides of MEE (Fig. 9F).

In culture, TGFβ<sub>3</sub> expression in the chicken MEE is still very low (Fig. 9G). However, after treatment with TGFβ<sub>3</sub>, the midline seam accumulates exogenous TGFβ<sub>3</sub> diffusely in its cytoplasm (Fig. 9H). In areas where the seam has transformed to mesenchymal cells, TGFβ<sub>3</sub> is low (arrow, Fig. 9H).



**Fig. 9.** Immunohistochemistry showing the expression of TGFβs in vivo and in vitro. (A,B,C) Endogenous TGFβs in chicken palate in vivo. (D,E,F) Endogenous TGFβs in rat palate. (G,H) show the location of TGFβ<sub>3</sub> in chicken palates in vitro treated (H) or not treated (G) with exogenous TGFβ<sub>3</sub>. E9 chicken palatal MEE express TGFβ<sub>1</sub> (A), but very little TGFβ<sub>2</sub> (B) or TGFβ<sub>3</sub> (C). E15 rat palatal MEE express little or no TGFβ<sub>1</sub> (D) and TGFβ<sub>2</sub> (E), but large amounts of TGFβ<sub>3</sub> (F). After 24 hours in culture, control chicken MEE express little or no TGFβ<sub>3</sub> (G). After treatment with exogenous TGFβ<sub>3</sub> for 24 hours, however, TGFβ<sub>3</sub> can be detected in the midline seam (H) and adjacent oral epithelia, but not in the mesenchyme (arrow, H). The large mass at the top of A is beak cartilage. Bars, 100 μm (A, also applies to B and C; G, also applies to H), 50 μm (D, also applies to E and F).

## DISCUSSION

In this paper, we report the effects of TGF $\beta$ 3 on development of the secondary chicken palate *in vitro*, and more specifically on morphogenesis of the MEE, which *in vivo* do not become adherent or transform to mesenchyme. E8-9 (stage 35) chicken embryonic palatal shelves were labeled with CCFSE, and cultured in chemically defined media, lacking serum and with or without growth factors, for up to 72 hours. MEE of single shelves or paired shelves with beak attached do not adhere or undergo EMT. All paired palates with beak removed form adherent midline epithelial seams by 24 hours. In the TGF $\beta$ 3-treated group, the seam begins to transform into fibroblast-like cells by 24 hours, and by 72 hours the palate is completely confluent due to EMT of MEE, as judged by replacement of CCFSE-labeled epithelium by CCFSE-labeled fibroblasts. In the control, only 20% of the seam becomes confluent by 72 hours, and this is not promoted further by TGF $\beta$ 1 or TGF $\beta$ 2. Thus, two distinct events occur in beakless chicken palatal shelves cultured in close contact with each other, namely, formation of a midline seam (adherence) and EMT (confluence). Exogenous TGF $\beta$ 3 is required for the full developmental potential of the MEE to undergo EMT to be expressed in adherent chicken palates.

The retention of developmental potential of the chicken MEE for adherence and EMT is one of the most important discoveries of the present study. In the mammal, the MEE of opposing palatal shelves make close contact and form a midline seam, which then undergoes EMT, resulting in a confluent palate (Fitchett and Hay, 1989, 1992; Shuler et al., 1992). The reptile also forms a confluent palate (Ferguson, 1988). In birds, however, the MEE approximate but do not make close contact or fuse. Earlier studies stressed cytological differences between rodent and avian palatal MEE (Shah and Crawford, 1980; Koch and Smiley, 1981; Greene et al., 1983; Shah and Cheng, 1988). For example, compared to that of rodent, the avian MEE secretes less complex carbohydrates on the cell surface. However, these differences are not important to palate fusion, for we demonstrate that MEE of beakless chicken palates in close contact adhere and form a midline seam that undergoes some EMT even without TGF $\beta$ 3. Our study of the chicken palate indicates that for EMT to be induced, what is absolutely essential is for opposing MEE to adhere. This is in agreement with the demonstration by Griffith and Hay (1992) that rodent MEE does not undergo EMT if the palatal shelves are grown separately; instead epithelia stratify, but do not die as previously reported (Smiley and Koch, 1972). Moreover, MEE of single chicken palatal shelves and palates with intact beaks do not transform to mesenchyme even when treated with the same amount of TGF $\beta$ 3 as the paired groups.

What kind of communication could be occurring between the adherent epithelial cells of the newly formed MEE seam that promotes EMT? As in rodent, peridermal cells appear to slough or reabsorb and basal cells of the opposing MEE adhere, forming numerous desmosomes and half desmosomes (Ferguson, 1988; Fitchett and Hay, 1989; Griffith and Hay, 1992). Such half desmosomes in the developing avian cornea are believed to induce partners on opposing cells in order to become full desmosomes (Hay and Revel, 1969). We demonstrate similar desmosome morphology in the adherent avian MEE seam. It has become clear recently that the

functions of desmosomes go beyond simple mechanical linkage; desmosomes and other adherens junctions have newly recognized roles in signal transduction pathways (Garrod, 1993; Cowin and Burke, 1996). Tyrosine-phosphorylated protein is densely distributed in desmosomes in corneal epithelium (Amino et al., 1996). Plakoglobin, one of the major desmosomal proteins, is found in the nucleus, where it might have a gene target (Karnovsky and Klymkowsky, 1995). To identify the roles of newly formed desmosomes in the MEE seam is a major goal in future research; it is possible that desmosomes not only link epithelia together, but also mediate cell-cell communication in adherent palates.

What keeps the secondary chicken palatal shelves apart in the normal animal? We believe that the beak and associated craniofacial structures play an important physical role in preventing paired palatal shelves from contacting each other *in vivo* (Fig. 9A) as well as *in vitro* (Fig. 3A). The beak is a unique feature to avian craniofacial development, as it is not present in reptiles or mammals. When we removed the beaks *in vitro*, the majority of the palates were able to contact each other, and adhered to form midline seams. However, we show that beakless palates are still not able to reach full confluence *in vitro*, but require TGF $\beta$ 3 to undergo complete EMT. Since all palates in close contact form seams with desmosomes, TGF $\beta$ 3 may act downstream to desmosome-mediated contact to promote EMT.

The positive effects of TGF $\beta$ 3 on mesenchymal transformation are widely recognized. Potts and Runyan (1989) and Potts et al. (1991) used an *in vitro* bioassay of cell invasion into collagen gels to show that antibodies to TGF $\beta$  isoforms and antisense oligonucleotides to TGF $\beta$ 3 inhibit EMT in atrioventricular (AV) canal endothelia. EMT in the AV cushion consists of two distinct phases, endothelial activation (cell separation) followed by mesenchymal cell invasion, and the antisense TGF $\beta$ 3 oligonucleotides inhibit the latter (Potts et al., 1991). In palates, a distinct cell separation phase is not observed; basal lamina gradually disappears, filopodia appear along basal cell surfaces, and MEE transforms directly into fibroblasts (Fitchett and Hay, 1989; Griffith and Hay, 1992). TGF $\beta$ 3 appears to promote this entire process in the adherent chicken MEE.

In the AV cushion, TGF $\beta$ 3 mRNA levels correlate with cushion EMT induction (Potts et al., 1992), but high levels of TGF $\beta$ 2 appear in cushions after the activation phase, suggesting that other TGF $\beta$  isoforms are also involved (Dickson et al., 1993). Antibodies to the type II TGF $\beta$  receptor block cell activation and migration in the AV canal (Brown et al., 1996). The three isoforms use this same receptor, but may have different affinities for it. In addition, each isoform has unique upstream regulatory sequence, promoter and precursor (Roberts and Sporn, 1990; Kingsley, 1994). It is possible that TGF $\beta$ 1, - $\beta$ 2 and - $\beta$ 3 may have isoform-specific functions in developing organs (Schmid et al., 1991; Millan et al., 1991), but in cardiac EMT, TGF $\beta$ 1, - $\beta$ 2 and - $\beta$ 3 may all play a role.

We find that TGF $\beta$ 1 and TGF $\beta$ 2 do not promote palatal EMT beyond that observed in the control. There is previous evidence indicating that it is mainly TGF $\beta$ 3 that plays a role in palatogenesis. TGF $\beta$ 3 is abundantly and specifically expressed in pre-fusion mouse (Fitzpatrick et al., 1990; Pelton et al., 1990; Gehris et al., 1994) and rat (this report) MEE, where it colocalizes with *c-fos* (Yano et al., 1996); little or no

TGF $\beta$ 1 or - $\beta$ 2 is present (Williams et al., 1991; Gehris et al., 1991). The predominant phenotype of the TGF $\beta$ 3 knock-out mouse is a partial cleft palate defect (Proetzel et al., 1995; Kaartinen et al., 1995), whereas  $\beta$ 1-null mice undergo excessive inflammatory responses and early death (Kulkarni et al., 1993). TGF $\beta$ 3, but not TGF $\beta$ 1 and - $\beta$ 2, antisense oligonucleotides and antibodies have inhibitory effects on rodent palate fusion (see Introduction). We show here that there is little or no TGF $\beta$ 3 present in chicken palatal MEE and that exogenous TGF $\beta$ 3 must be added to stimulate EMT of the entire MEE. However, we have demonstrated clearly that TGF $\beta$ 3 is not necessary for the adherence stage of palatogenesis, contrary to the theory of Proetzel et al. (1995). Kaartinen et al. (1997) have recently come to the same conclusion. They showed that palatal shelves of TGF $\beta$ 3 null mice form a midline seam in vitro, but this seam does not undergo EMT unless treated with TGF $\beta$ 3.

In summary, we show here that the chicken secondary palatal shelves without beak will adhere and then form a midline seam in vitro, if put in close contact. This suggests that the developmental potential of chicken MEE is similar to that of other amniotes, even though the bird has a naturally cleft palate. Therefore, something must have happened to the avian palate in the process of the evolution of birds from reptiles that did not occur in mammals. These events seem to be (1) creation of the beak, which keeps the two palatal shelves apart, and (2) loss of growth factors, such as TGF $\beta$ 3, in the palate that are needed to bring about its full confluence by promoting EMT. Although the mechanisms of cleft palate formation in mammals, such as human, that normally form confluent palates remain to be fully understood, our data strongly suggest that lack of palatal shelf contact early on is one possible cause. The MEE may have the potential for EMT if the nonfusing embryonic palatal shelves can be put in close contact early on surgically and confluence optimized with TGF $\beta$ 3 treatment.

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