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Epithelial-mesenchymal transformation during palatal fusion: carboxyfluorescein traces cells at light and electron microscopic levels

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Summary

During the fusion of rodent embryo palatal shelves, the cells of the outer epithelial layer slough off, allowing the cells of the medial edge basal layer to form a midline seam that undergoes epithelial-mesenchymal transformation, as judged by electron microscopy and immunohistochemistry. In this study, we analyze the fate of the transformed cells using a lipid soluble dye to label the medial edge epithelium in situ. Prefusion E14 mouse palates were exposed in vitro or in vivo to a fluoresceinated lipid soluble marker, carboxydichlorofluorescein diacetate succinimidyl ester (CCFSE), which localizes in epithelia as a lipid insoluble compound that does not pass into the connective tissue compartment. The midline seam that formed after 24 hours contained labelled epithelial cells that were replaced by individually labelled mesenchymal cells where the seam transformed. By light microscopy, the labelled cells were seen to contain intensely fluorescent bodies that do not react for acid phosphatase. We were able for the first time to identify these structures by electron microscopy as CCFSE isolation bodies. The cells with isolation bodies are clearly healthy and able to participate in subsequent development of the palate. At 4 days after labelling, individual CCFSE containing cells present in the palate mesenchyme occupy both midline and lateral areas and can clearly be classified as fibroblasts by electron microscopy. CCFSE is a far more useful marker than another lipid soluble marker, DiI, for following cells, because the cells can be fixed and identified both at the light and electron microscope levels. Interestingly, if labelled palatal shelves are not allowed to fuse in vitro, the basal epithelial cells do not form mesenchyme after sloughing, indicating that formation of the epithelial midline seam is necessary to trigger its epithelial-mesenchymal transformation.

Key words: epithelial-mesenchymal transformation, carboxyfluorescein, electron microscopy, cell tracer, palate mesenchyme.

Introduction

Tissue phenotypic transformations, from epithelial to mesenchymal cells and vice versa have been well documented during embryonic development. The initial transformation, from epithelial to mesenchymal, occurs in higher vertebrates during the invagination of epiblast derived cells through the primitive streak to form mesoderm (Hay, 1968). Other well-studied examples of epithelial-mesenchymal transformations include neural crest (LeDouarin, 1982), sclerotome (Solursh et al., 1979) and cardiac cushion mesenchyme (Markwald et al., 1984). Mesenchymal-epithelial transformations occur in somite formation (Hay, 1968), kidney development (Ekblom, 1984) and formation of the caudal or secondary neural tube (Griffith et al., 1992). Recently, it has been shown that epithelial-mesenchymal transformations also occur during embryonic remodelling. In 1982, Trelstad et al. presented TEM (transmission electron microscope) evidence that disappearance of the Mullerian duct in male rat embryos occurs by conversion of epithelial cells to mesenchyme, rather than cell death. Subsequently, similar TEM evidence indicated that disappearance of the cervical sinus in chick embryos involves epithelial-mesenchymal transformation (Chatrain and Hay, 1985). A careful reexamination of palatal fusion in the rat embryo by Fitchett and Hay (1989) produced definitive TEM evidence for phenotypic transformation of the MEE (midline epithelial seam) into mesenchymal cells to achieve mesenchymal confluence across the palate. In addition, the seam was shown to turn on vimentin, generally a mesenchymal intermediate filament (Zuk et al., 1989; Hay, 1990), prior to the transformation and to lose the epithelial determinant, syndecan (Fitchett et al., 1990).

The objectives of the present study were to extend the ultrastructural and immunohistochemical study of Fitchett and Hay (1989) by following the fate of the transformed rodent epithelial MEE mesenchymal cells, using a nonharmful long-lived cell lineage tracer. Various lipid soluble dyes have been used in the past as cell lineage tracers, including the fluorescent dyes, DiI (1,1 dioctadecyl 3,3,3′,3′ tetramethyllindocarbocyanine perchlorate; Honig and Hume, 1986, 1989) and carboxyfluorescein diacetate suc-

cinimidyl ester (CFSE; Bronner-Fraser, 1985). These dyes have the enormous advantage of labelling external epithelia specifically, without the necessity of transplanting the labelled cells. DiI remains hydrophobic, presumably within plasmalemma, and does not stand up to fixation. Its presence can only be detected in frozen sections. CFSE diffuses across the membrane, where intracellular esterases cleave off the acetates to release the fluorophore as a water-soluble compound that cannot diffuse out of the labelled cells.

In this study, we used a dichloro-substituted derivative of CFSE, carboxydichlorofluorescein diacetate succinimidyl ester (CCFSE) as a tracer for transforming MEE cells in vitro and in vivo, because we found that its fluorescence resists bleaching better than CFSE. The advantages of the cytoplasmic CCFSE product are that it is a stable cytoplasmic marker and it stands up to formaldehyde fixation and paraffin embedding. We show here for the first time that the CCFSE product is taken up into phagosome-like isolation bodies that can be identified in plastic sections by light as well as electron microscopy. The CCFSE isolation bodies are well fixed by routine TEM procedures. TEM allowed us to identify the descendants of the basal MEE cells as fibroblasts whose distribution is restricted to the connective tissue of the palate and nasal palatal junction.

In the interim since we first reported our results (Griffith and Hay, 1991), Schuler et al. (1991) published a report of the fusion of DiI-labelled mouse palates in vitro. They confirmed the report of Fitchett and Hay (1989) that keratin is present only in the epithelial seam (not in the mesenchyme) and showed that the midline seam contained DiI. Although they conclude that DiI-labelled connective tissue does indeed appear, cytology of the frozen sections does not permit definitive characterization of cell type. Our study provides this important cytological evidence at the electron microscopic level. More recently, Carette and Ferguson (1992) published a DiI study of mouse palate development in which they conclude that the DiI-labelled midline seam gives rise to cells that migrate into and become part of the epithelium lining the dorsal and ventral side of the palate. We disagree with their conclusions and present our reasons for this opinion in detail in the Discussion.

Methods and materials

In vitro labelling and culture of palates

Timed pregnant CD 1 females were purchased from the Charles River Breeding Laboratories. While palatal fusion is known to occur at different developmental ages in different strains of mice (Walker and Fraser, 1956), we found that in CD 1 embryos, apposition and contact occurs between E14 and E15. At 14 or 14½ days of gestation (day of vaginal plug = day 1), the dams were killed and the embryos dissected out of the uterus into a dish of warm Hanks' balanced salt solution (HBSS). Each embryo was killed by decapitation. The mandible and tongue were removed from each head and discarded. The rest of the head was placed in a solution of 5 (and 6) carboxy 2′,7′ dichlorofluorescein diacetate succinimidyl ester (CCFSE; Molecular Probes, Inc.) in HBSS for 1 hour at 37˚C. The CCFSE was prepared as a 10 mM stock solution in dimethyl sulphoxide (DMSO). This stock solution was diluted 1:500 in HBSS and prewarmed at 37˚C prior to use. After CCFSE exposure, the heads were rinsed in fresh HBSS and the entire palatal shelf nasal septum ensemble or precontact palatal shelves connected by their anterior end were dissected out (Fig. 1). Single shelves were also dissected out, labelled in CCFSE and cultured. Control palates were exposed to HBSS containing the same amount of DMSO as the experimental for 1 hour at 37˚C, then cultured as below.

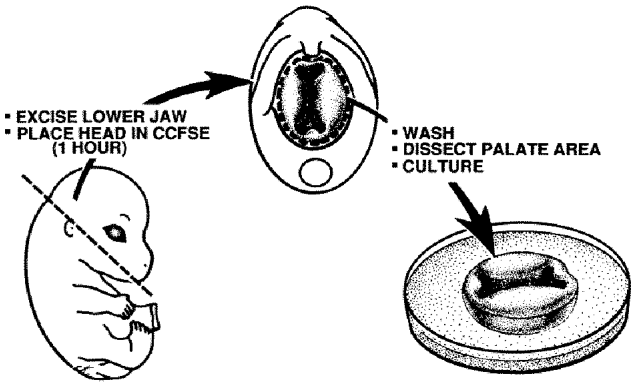

Fig. 1. Diagram showing preparation of a palatal assembly for in vitro CCFSE labelling and organ culture.

The palates were placed nasal surfaces down on an agar gel in a Falcon 3037 organ culture dish, overlaid with a thin film of medium to provide a liquid-air interphase. Two different gels were tried. The first comprised Medium 199 (GIBCO) containing 10% foetal calf serum with penicillin and streptomycin in 0.5% agar. The second gel comprised BGJ_b medium (GIBCO) supplemented with 2.8 mg/ml L-glutamine, 6 mg/ml BSA and 1% insulin transferrin selenium (ITS+^{TM}Premix; GIBCO) in 0.5% agar with penicillin and streptomycin, a modification of a protocol provided by Dr Barbara Abbott (Abbott and Buckalew, 1992). The liquid medium lacked the agar. For cultures up to 24 hours, palates were maintained in a humidified incubator at 37˚C, under 5% CO_2. Palates in long-term cultures (up to 4 days) were exposed to a constant stream of 50% O_2, 5% CO_2 and 45% N_2.

We have found that the BGJ_b protocol provided the healthiest cultures and consequently, all Figs in this report illustrate palates cultured by the second protocol. It seems likely, from our comparison of palates cultured in different media, that reports of numerous phagosomes in developing palates (see Carette and Ferguson, 1992) can be attributed to poor culture conditions. In addition, the abnormally large pieces of cartilage reported in palatal cultures by other investigators (Ferguson et al., 1984) did not occur in palates cultured on BGJ_b agar, in an atmosphere of 50% oxygen.

In vivo labelling of palates

Dams at days 13½ to 14 of pregnancy were anaesthetized by an intraperitoneal injection of Avertin (an aqueous solution of 2,2,2 tribromoethanol in 2 methyl 2 butanol) of 0.1 ml per 5 g body weight. A midline incision was made through the abdominal wall under sterile conditions. The uterine horns were pulled out and injections of CCFSE were administered to the embryos through the uterine walls. In each dam, embryos in one uterine horn were given intra-amniotic injections of the dye, while the embryos in the other horn served as controls for viability and specificity of labelling. Each embryo received 0.5 μl of CCFSE stock, after which the uterine horns were eased back into the abdomen. The abdominal cavity was then flushed with sterile saline and sutured. The pregnancy was then allowed to continue for another 3 to 4 days, until the embryos reached E17 or E18, when osteogenesis

within the palate was well underway. The dams were then killed and the embryonic palates dissected out and processed for microscopy. A total of 17 CCFSE-treated embryos from 4 litters were recovered.

Microscopy

Palates were fixed in 4% paraformaldehyde in 0.1 M Tris-buffered saline (TBS), pH 8.0, with 4% (w/v) polyvinylpyrollidone (PVP, M_r 40 000). They were then rinsed in TBS and processed for routined paraffin wax embedding. Serial sections of 6 μm thicknesses were deparaffinized and quenched for autofluorescence in TBS containing 50 mM ammonium chloride, pH 8.0 for ½ hour before mounting to look for CCFSE-labelled cells by epifluorescence using conventional or confocal light microscopy.

Palates were also fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 to 7.4 and then postfixed in 1% osmium tetroxide in the 0.1 M cacodylate buffer for transmission electron microscopy. They were en bloc stained with a saturated aqueous uranyl acetate solution, dehydrated through a graded ethanol series, infiltrated with propylene oxide and Spurr's resin and then embedded in Spurr's. 1 μm sections were taken of CCFSE-treated and control palates. Photographs of these sections showing disintegrating seams were used for semi-quantitative analysis. Each section was divided into midline seam and mesenchymal cells in the midline region and measurements of the surface area of each region were taken. The number of isolation bodies per unit surface area was scored for each region. A one-way Anova was performed to determine statistical significance. A score of $P<0.05$ was considered statistically significant.

Thin sections from the same blocks were examined by transmission electron microscopy, both unstained and counterstained with a lead citrate solution (Venable and Coggeshall, 1965).

The normal development of murine palates was also examined by fixing the palatal assemblies of embryos from E14 to E18, postnatal day 1 and adults in Bouin's fluid and processing for routine paraffin embedding. Serial 6 to 8 μm sections of palates at each developmental stage were stained with haematoxylin and eosin (H and E) for light microscopy.

Gomori stain for lysosomal acid phosphatase

In order to show that CCFSE isolation bodies are not merely lysosomes in dying epithelia, we fixed and stained palates after 24 hours in organ culture for acid phosphatase, a lysosomal enzyme that serves as a marker for dead or dying cells. Palates were fixed overnight in cold calcium formalin, after which they were rinsed in 0.1 M sodium acetate buffer, pH 5.2, containing 7.5% sucrose. They were then reacted with a preheated (60˚C for 1 hour) mixture of 0.12% lead nitrate and 0.3% β-glycerophosphate in the sodium acetate buffer at 37˚C for 1 hour. The palates were rinsed with buffer and acid-phosphatase-positive cells were visualised by reaction with a 1% ammonium sulphide solution. The palates were then briefly fixed in formalin again and routinely processed for paraffin sectioning. Controls were reacted in a mixture that lacked the β-glycerophosphate. 6 μm serial sections were photographed under Nomarski optics to visualise acid-phosphatase-positive cells and through a FITC filter for visualising CCFSE-labelled cells.

Results

Mouse palatogenesis

Palatogenesis in the mouse occurs in a similar fashion to that previously described for rats (Hughes et al., 1967; Hayward, 1969; Fitchett and Hay, 1989). The palatal shelves move from vertical to horizontal orientation between 13 and 14 days and the medial edges of the two shelves then approach each other (arrows, Fig. 2A). During the time of apposition and contact at late E14, the medial edge epithelia (MEE) comprise two layers: basal cells and superficial periderm cells. Prior to medial edge contact, the cells of the MEE peridermal layer die and slough off (p, Fig. 2A),

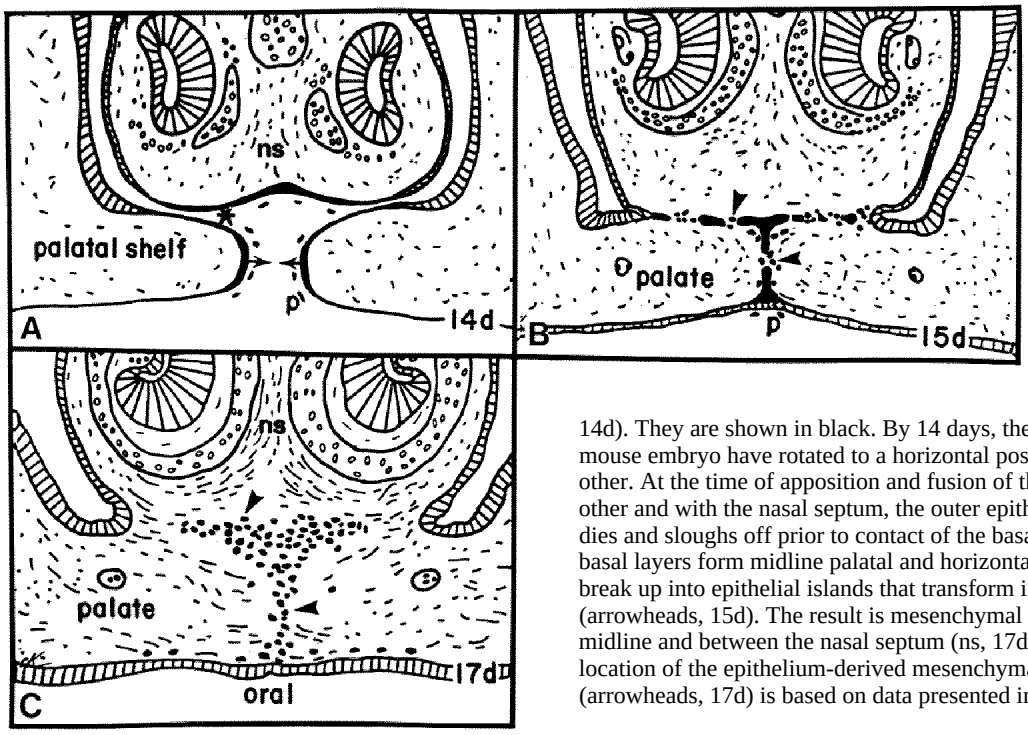

Fig. 2. Camera-lucida drawings showing the successive stages in the fusion of the secondary palatal shelves in the mouse. Based on our previous observations (Fitchett and Hay, 1989), the epithelia contributing mesenchymal cells during palatal fusions are the dorsal palatal and ventral nasal epithelia (*,14d) and the medial edge epithelia (arrows, 14d). They are shown in black. By 14 days, the palatal shelves of the CD 1 mouse embryo have rotated to a horizontal position and are approaching each other. At the time of apposition and fusion of the palatal shelves with each other and with the nasal septum, the outer epithelial layer, the periderm (p), dies and sloughs off prior to contact of the basal epithelial cells. The fused basal layers form midline palatal and horizontal palate nasal seams, which break up into epithelial islands that transform into mesenchymal cells (arrowheads, 15d). The result is mesenchymal confluence across the palate midline and between the nasal septum (ns, 17d) and dorsal palate. The location of the epithelium-derived mesenchymal cells shown in black (arrowheads, 17d) is based on data presented in this paper.

allowing the epithelial cells of the basal layers to come into close contact and develop junctional contacts, such as desmosomes, with cells of the opposite side (Fitchett and Hay, 1989). The two-cell-wide midline seam that results thus forms a strong connection between the two palatal shelves. Once the seam is established, the component cells, by undergoing epithelial-mesenchymal transformation, can disappear without disrupting palatal continuity. In its anterior portion, the palate becomes fused to the nasal septum (ns, Fig. 2A) by a similar process. The basal epithelial cells form epithelial seams that break up into epithelial islands. These islands subsequently transform into mesenchymal cells (arrowheads, Fig. 2B). By E16, there is mesenchymal confluence across the palate. The mesenchymal cells deriving from palate and nasal epithelium are prominent in the midline (black cells, arrowheads, Fig. 2C) and also migrate laterally, especially in areas where newly forming bone is peripheral in its location.

In our organ cultures, palates developed in a very similar manner to those in vivo.

Fate of the labelled cells

After 1 hour in CCFSE solution, the periderm is well-labelled (arrowheads, Fig. 3), having been exposed directly to the dye. All of the sloughing peridermal cells are labelled (p, Fig. 3C). Labelling within the basal layer, which received dye through gap junctional contacts, is more diffuse (arrow, Fig. 3A). The amount of label in the basal cells and its distribution from one part of the epithelium to another, varies. Some basal cells are intensely labelled (*, Fig. 3C). The dye is rendered lipid insoluble once it has entered the epithelium. Therefore, it does not cross the basal plasmalemma and the underlying connective tissue cells are unlabelled (fibroblasts, Table 1A).

After 24 hours in culture, CCFSE labelling is present in the cells of the midline epithelial seam, in the epithelial islands (Fig. 4A) and in mesenchymal cells that at this time are located in the midline (Fig. 4C). In palates with breaking seams, all 28 cultured contained labelled mesenchymal cells (Table 1B). Brightly labelled spots, which TEM identifies as isolation bodies (see below), can now be distinguished within the cytoplasm of CCFSE-labelled cells (arrowheads, Fig. 4A). Sloughed periderm can still be seen (arrow, Fig. 4A). Mesodermal confluence (Fig. 4D) is achieved by transformation of the epithelial seam to mesenchyme (Fig. 4C).

TEM observations showed that the large inclusions in CCFSE-labelled epithelium consist of fibrogranular material encased by cell membranes. They resemble autophagic vacuoles (Holtzman, 1976), but they do not represent parts of the cell entering lysosomes. They do not contain acid phosphatase (see below), nor is the captured product a digestible protein. We decided to use the term *isolation bodies* introduced by Locke and Collins (1965) to describe them, because their function appears to be to isolate CCFSE from the cytosol. The epithelial cells that contain these isolation bodies (arrowheads, Fig. 5) are otherwise healthy in appearance, i.e., the cells have euchromatic nuclei, intact organelles, keratin (k, Fig. 5), desmosomes (inset, Fig. 6) and ribosome rich cytoplasm (r, Fig. 5). Fig. 5 is from the

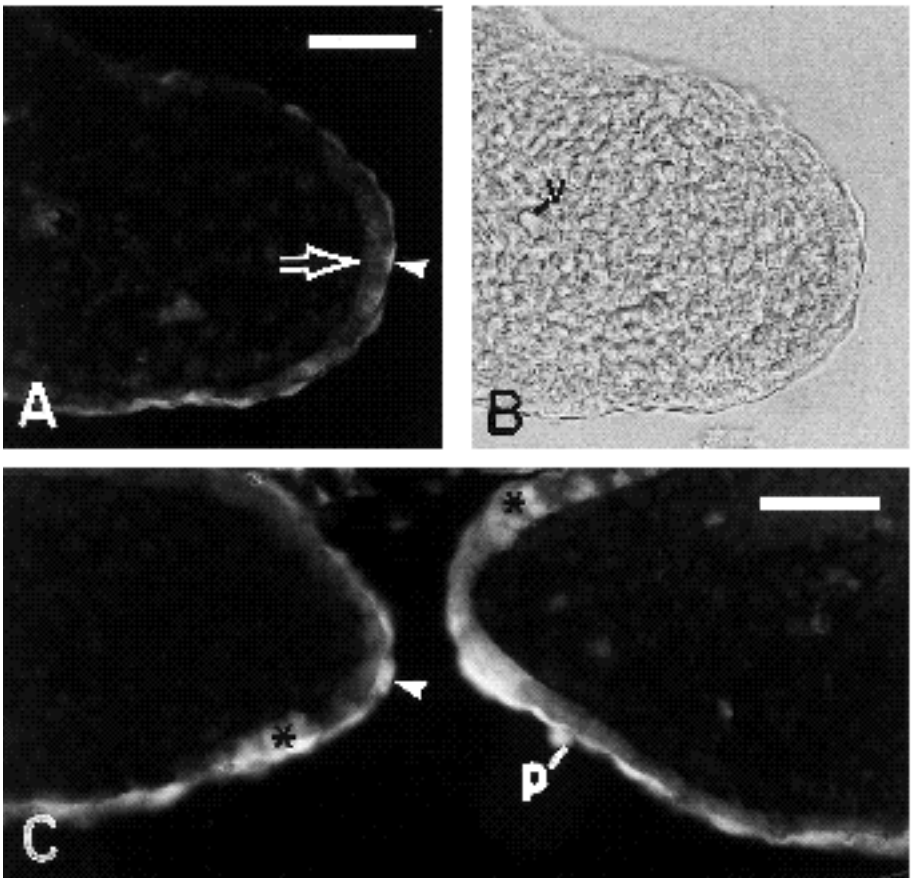

Fig. 3. E14 palatal shelves after 1 hour labelling in CCFSE. The nasal side is at the top in all Figs. Figs 3, 4, 9 and 10 are paraffin sections. (A,B) Fluorescent and Nomarski views of a single palate labelled in vitro. (C) A pair of palatal shelves labelled in vitro. The epithelium is diffusely labelled (arrow, A) and, although occasional clumps (*, C) occur, TEM reveals no isolation bodies 1 hour after labelling. The periderm is well-labelled (arrowheads). There is variable label in the basal epithelial layer, as CCFSE has diffused in from the periderm via gap junctions. Note that different areas of the epithelium in A and C label better than others in a random fashion. As a result, the number and location of mesenchymal cells deriving from these epithelia that can be identified by CCFSE label will vary. A sloughed periderm cell is labelled (p). v, blood vessel. Bar, 50 μm.

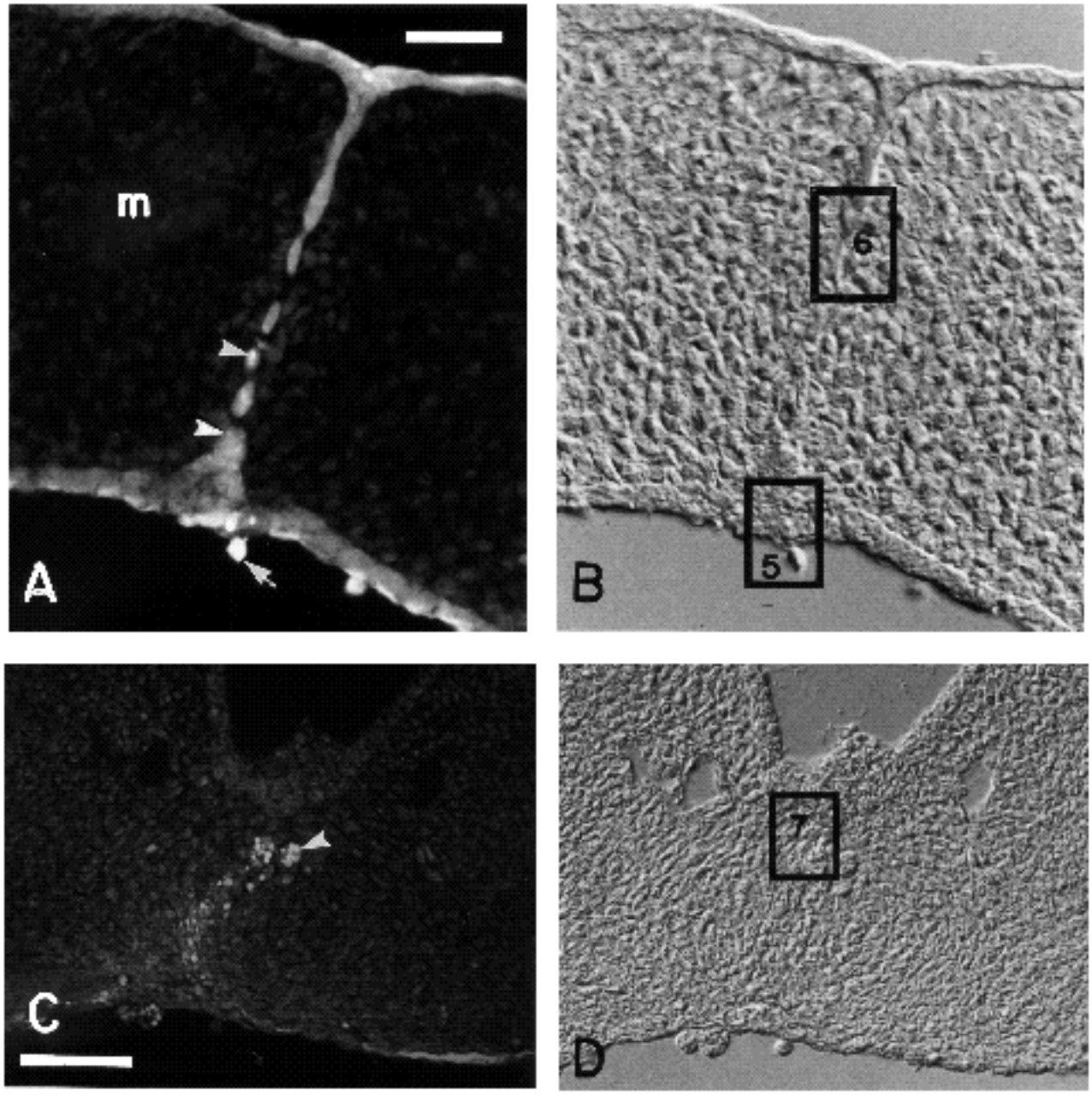

Fig. 4. One day after labelling in vitro, CCFSE labelling is present in the cells of the midline seam (A,B) and in the mesenchyme-like cells deriving from epithelium in the region of the seam (C,D). These labelled mesenchyme-like cells are indistinguishable from others in the mesoderm after attainment of palatal confluence, as shown in D (photographed with Nomarski optics). The location of electron micrographs in Figs 5 and 6 are shown in B (also Nomarski optics) and of Fig. 7 in D. The CCFSE, originally diffuse in distribution, is condensing into one or more fluorescent spots per cell (arrowheads, A and C), which represent dye packaged within isolation bodies. Mesenchymal cells situated outside the midline seam (m, A) have no isolation bodies showing that the CCFSE absorbed through the periderm was confined to the epithelial cells and did not pass through the basal plasmalemma into the mesenchyme. Periderm staining is brighter than that of the basal epithelial cells. A sloughed periderm cell is labelled (arrow, A). Palate C was cultured in a medium (Abbott) that promoted faster development and it already has sloughed most of the CCFSE-containing surface epithelial cells. In the region shown here, the nasal septum epithelium does not fuse with the palate. Bars, 25 μm (A) and 50 μm (C).

region labelled 5 in Fig. 4B. It can be seen that the sloughed periderm cell (p) contains isolation bodies. It would seem that the cells respond to the foreign material (CCFSE) in their cytoplasm by sequestering it in membrane-bound compartments.

In areas where the midline seam is intact, TEM demonstrates isolation bodies within the seam cells (arrowheads, Figs 4A, 6), but not within the surrounding mesenchymal cells, confirming that the carboxyfluorescein has indeed been confined to the epithelial cells and has not passed

Table 1. *A summary of CCFSE localization during the various stages of palatal development*

Stage palate examined	No. palates examined	No. palates with label in the			
		nasal/oral epithel.	MEE	fibroblasts	bone
(A) Shelves before fusion (labelled and fixed on E14)	12	12 (100%)	12 (100%)	0	N.A.
(B) Fusing palatal shelves (fixed 24 hour post culture)					
(i) palates with intact seam	8	8 (100%)	8 (100%)	0	N.A.
(ii) palates with breaking seam	28	28 (100%)	28 (100%)	28 (100%)	N.A.
C. After palatal confluence					
(i) fixed after 3 to 4 days in culture	15	15 (100%)	N.A.	9 (60%)*	0
(ii)labelled in vivo until E17 to 18	17†	4 (100%)	N.A.	4 (100%)	0

*The 6 palates that did not contain labelled mesenchyme also showed poor labelling of oral and nasal epithelia.

†Only 4 of the embryos receiving CCFSE in the amniotic cavity contained any label. This speaks to the difficulty of injecting into a relevant part of the amniotic cavity in utero. The 4 palates labelled by CCFSE all contained positive fibroblasts.

through the basal plasmalemma into the mesenchymal cells (Figs 4A, 6). In the disappearing seam, isolation bodies are present in epithelial islands (arrowheads, Fig. 6). In the mouse (inset, Fig. 6), as in the rat (Fitchett and Hay, 1989), desmosomes and newly forming half desmosomes are present in the seam and the islands. The newly transformed mesenchymal cells of the midline (arrowhead, Fig. 4C; rectangle 7, Fig. 4D) contain isolation bodies (arrowheads, Fig. 7). Classification of the dye-containing cells as mesenchymal is based on several established criteria (Hay, 1990): (1) bipolar or stellate shape, (2) presence of pseudopodia and/or filopodia and (3) lack of epithelial cell junctions, such as desmosomes.

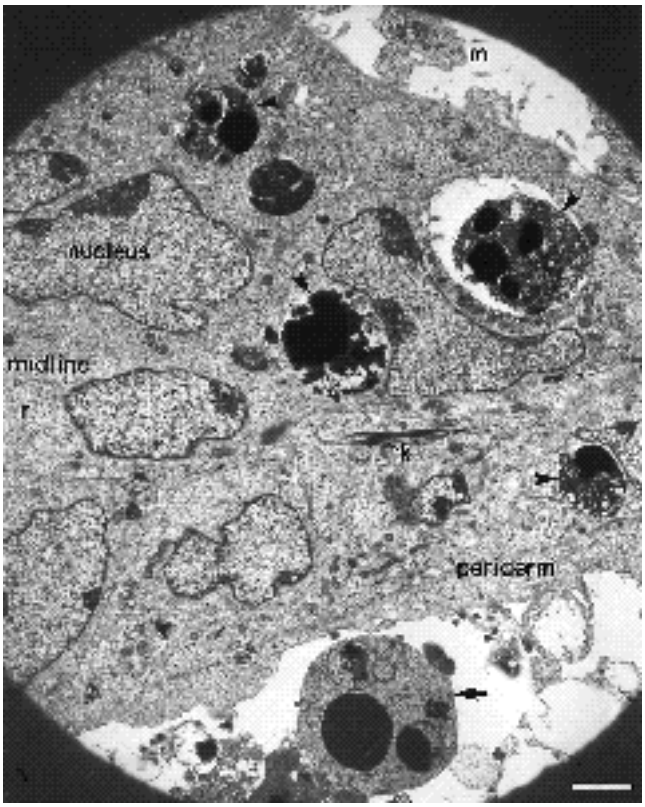

Fig. 5. TEM showing fluorescent isolation bodies in the CCFSE-exposed epithelium on the oral side of the midline seam (see square labelled 5 in Fig. 4B). A sloughed periderm cell (arrow) is present on the oral surface. Apart from the CCFSE isolation bodies (arrowheads), the epithelial cells are similar to those in the control and are healthy looking, that is, the nuclei are euchromatic, the cytoplasm is ribosome rich (r) and organelles are healthy. k, keratin filaments. Bar, 2 μm.

Comparisons between 1 μm sections through fusing control and CCFSE-labelled palatal shelves showed that the bright spots (isolation bodies) in the midline seam of dye-labelled palates correspond to phase-dense bodies identifiable in thin plastic sections (Fig. 8). They are 0.2 to 4.0 μm in diameter and are present in significant numbers only in the experimental palates. The difference between number of cytoplasmic phase dense bodies present in the midline, midline seam and midline mesenchymal cells of CCFSE palates as compared to control palates is statistically different ($P<0.05$, Table 2). In healthy cultures such as these, controls rarely contain lysosomes (see discussion by Fitchett and Hay, 1989).

We demonstrated that the isolation bodies are not lysosomes within a dying midline seam by staining palates with the Gomori stain for acid phosphatase, a major lysosomal enzyme marker. We found no correlation between the presence of CCFSE label and the presence of acid phosphatase (Fig. 9). Further evidence that the isolation bodies are not phagosomes was provided by TEM observations that the isolation bodies in the epithelia of labelled palates do not appear until about 12 hours after exposure to the dye. The epithelium, although clearly fluorescent after 1 hour of labelling (Fig. 3), does not contain isolation bodies as

Table 2. *A comparison between the number of isolation bodies found in the midline region of fusing palatal shelves after 24 hours in culture*

	No. isolation bodies per unit area (mean)	Std. deviation
EPITHELIAL SEAM		
control (n=6)	0.0143	0.012
CCFSE-labelled (n=6)	0.0437*	0.028
MIDLINE MESENCHYME		
control (n=6)	0.0006	0.001
CCFSE-labelled (n=6)	0.0028*	0.002

*significant difference from control ($P<0.05$).

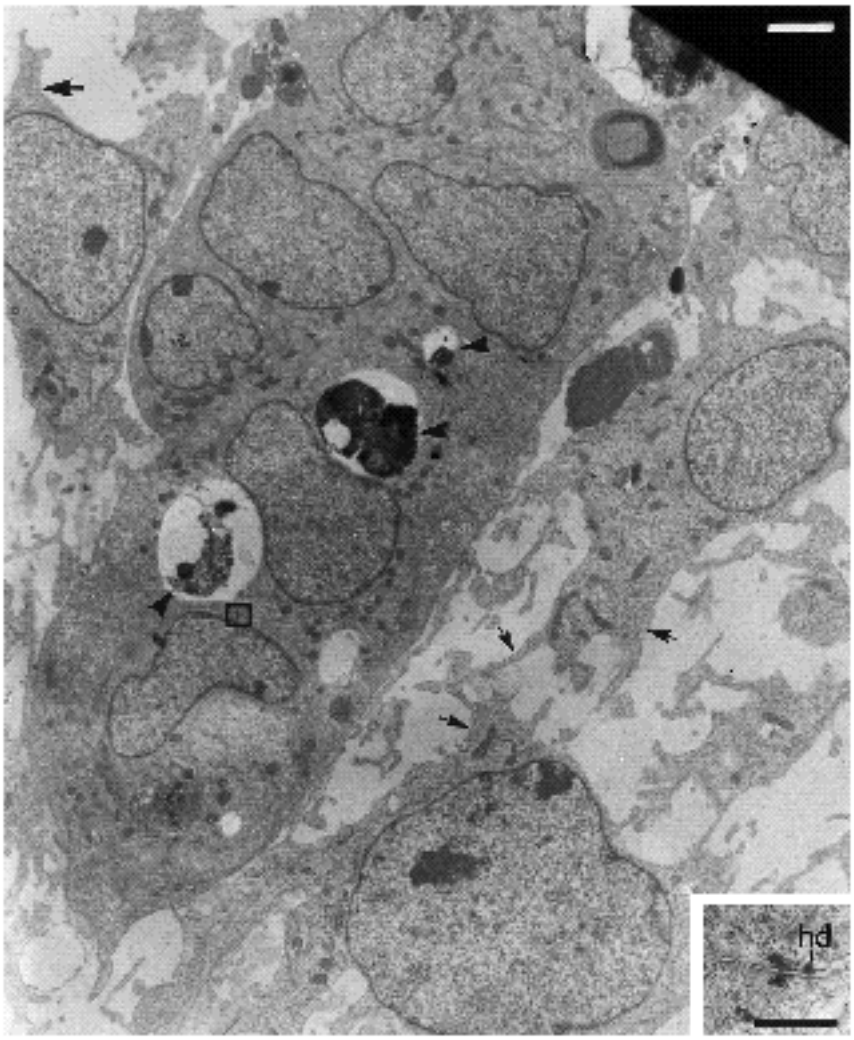

Fig. 6. This epithelial island is a remnant of the disappearing seam (as at the square labelled 6 in Fig. 4B). Isolation bodies are present (arrowheads). Mesenchymal cells are distinguished morphologically by their shape and well-developed pseudopodia and filopodia (arrows). The epithelial cells in the island are joined by desmosomes, one of which (square) is enlarged in the inset. Also shown in the inset is a half desmosome (hd) that probably is part of a newly forming junction, as described by Fitchett and Hay (1989) for rat. Bar, 2 μm; inset, 0.5 μm.

judged by TEM (data not shown). Thus, the CCFSE does not enter the cells by phagocytosis.

3 to 4 days after the exposure to CCFSE, the nasal and oral epithelia have differentiated into pseudostratified ciliated columnar and stratified squamous epithelia, respectively. Only 4 of the 17 embryos that received an injection of CCFSE into the amnion showed any label in the palate (Table 1C). However, in all 4 cases, labelled cells were clearly present within the connective tissue compartment. In the anterior portion of the palate, where there is fusion of the nasal septum to the secondary palate, labelled cells are present in the connective tissue of the midline palate and junction of palate and nasal septum (Fig. 10). There was considerable variability in the location of the labelled fibroblasts (cf. Fig. 10A,C,E). This is due in part to the fact that some levels (as at A, B) contain prominent bone, which may block lateral migration of the midline fibroblasts. Interestingly, no CCFSE-labelled cells differentiate into bone (Fig. 10B, b). There is variability in epithelial uptake of label among palates (Fig. 3), and this also contributes to the variability in location and numbers of labelled fibroblasts. Similar results to those in vivo (Fig. 10) were obtained when we labelled palatal assemblies in vitro and cultured them for 4 days. 9 of the 15 palates that were cultured for 3 to 4 days contained labelled fibroblasts (Table 1C). Absence of fibroblasts in the other 6 palates does not appear to be the result of fibroblasts reentering (Carette and Ferguson, 1992) the epithelia, because neither oral nor nasal epithelium was labelled very well in these 6 cases. Rather, the result suggests that not enough labelled MEE cells were present to produce a significant number of fibroblasts.

By TEM, we confirmed that the labelled connective tissue cells are fibroblasts and that the palates, after culture, are still healthy. In the midline region of the palate identified as 11 in Fig. 10A, the mesenchymal cells are now clearly fibroblasts with the typical endoplasmic reticulum (RER, Fig. 11) and polarized Golgi apparatus (GA, Fig. 11) of fiber-producing connective tissue cells. They are elongate in shape and possess euchromatic nuclei. The fibroblast containing the isolation body (arrowhead, Fig. 11) derived from CCFSE-labelled epithelium. Adjacent bone, (b, Figs 10, 11) is not fluorescent (12, Fig. 10A) and does not contain isolation bodies (Fig. 12). The osteoblasts of this young membrane bone are round and are beginning to secrete bone matrix (m, Fig. 12). The morphology of the connective tissue cells derived from the fusion of the nasal palatal epithelium with the upper (nasal) palate surface (Fig. 2, 15d) is the same as that of the fibroblasts derived from the

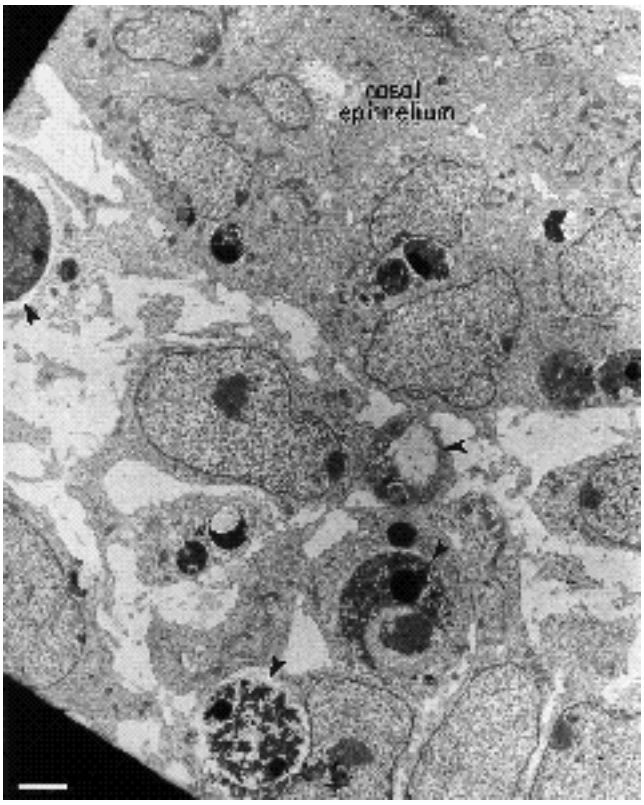

Fig. 7. TEM showing an area under the nasal epithelium after complete disappearance of the epithelial seam beneath it. Many midline-located mesenchymal cells have prominent isolation bodies (arrowheads). At this early point in culture, 24 hours after exposure to CCFSE, the basal epithelial cells in the surface epithelium still contain label. Bar, 2 μm.

fused medial palate epithelia. The elongate cells still contain isolation bodies 4 days after labelling (arrowhead, Fig. 13). Undoubtedly, some of the unlabelled fibroblasts derive from epithelia that were not well-labelled (Fig. 3A) or from proliferating fibroblasts that lost the original CCFSE isolation bodies during cell division. Even though the labelled connective tissue cells may be widely distributed (Fig. 10E), TEM reveals that all of the labelled mesenchymal cells are fibroblasts at this time. Unlabelled controls cultured for 4 days do not contain CCFSE isolation bodies (Fig. 14).

Single palatal shelves do not show epithelial-mesenchymal transformation

When an isolated palate shelf is grown in organ culture, the periderm of the MEE sloughs off by 24 hours. However, no CCFSE-labelled cells enter the mesenchyme. Instead, flattened but clearly delineated labelled epithelial cells remain on the surface of the MEE. At 96 hours, the medial edges are covered with nonkeratinized stratified squamous epithelium. The regenerated MEE contains CCFSE label, suggesting that the original basal cells regenerated the epithelium. Alternatively, the original oral or nasal epithelium could migrate into the medial edge to replace the sloughed cells. These results suggest that it is necessary for two palatal shelves to fuse for epithelial-mesenchymal transformation to be triggered.

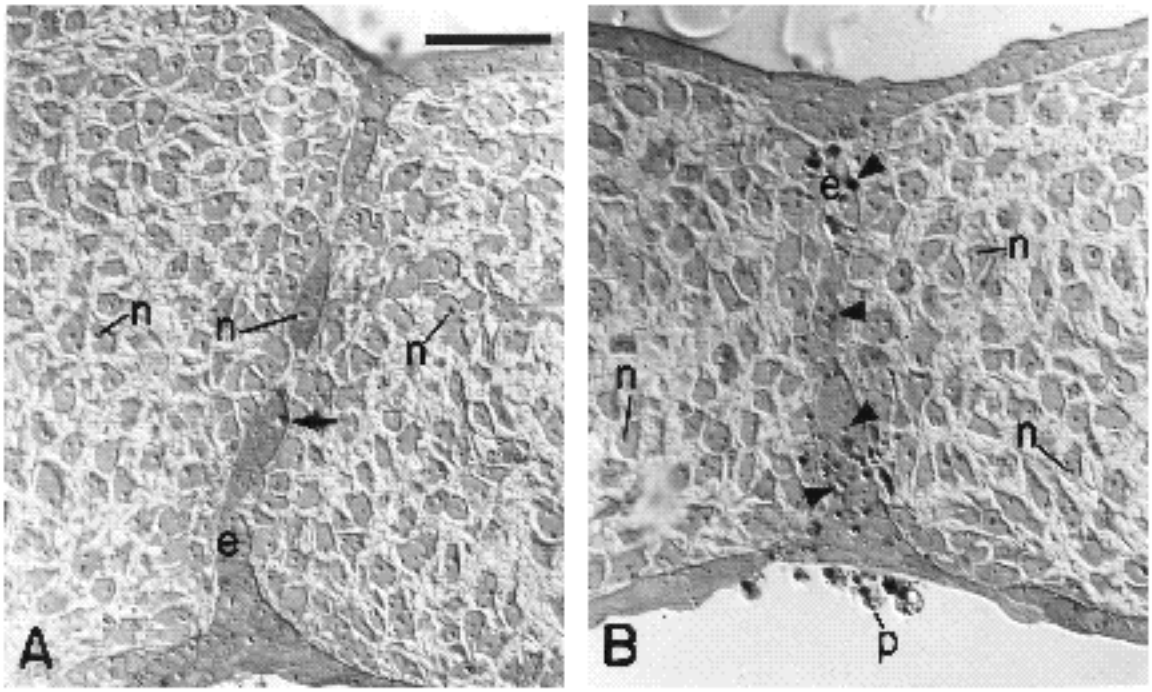

Fig. 8. 1 μm plastic sections through the transforming epithelial seams (e) of control (A) and carboxyfluorescein-labelled (B) palatal shelves showing that the isolation bodies (arrowheads, B) present in the dye-labelled palate can be identified at the light microscope level by Nomarski optics. The control does not contain a significant number of phagosome like bodies (arrow, A). Table 1 is a quantitative analysis of sections like these from 12 palates fixed after 24 hours in culture. p, sloughed periderm. n, nucleolus. Bar, 50 μm.

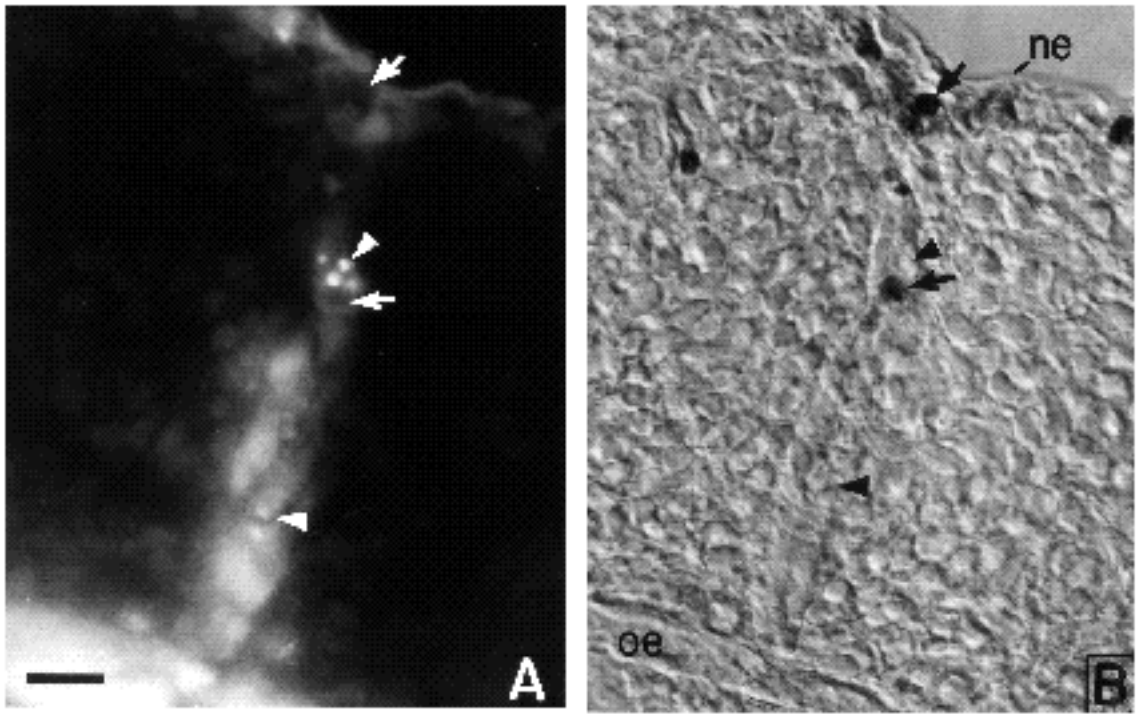

Fig. 9. Light micrographs of the same section viewed for fluorescence (A) and for Gomori stain (B). There is no correlation between the presence of acid phosphatase (arrows) and CCFSE (arrowheads). This palate was cultured for 24 hours after labelling with CCFSE; the nasal side faced the agar. This surface was not adequately oxygenated and the unhealthy nasal epithelium (ne) developed acid-phosphatase-rich autophagosomes not present in the oral epithelium (oe) growing at the air-medium interface. Bar, 25 μm.

Discussion

In this study, we examined the fate of the basal layers of the medial edge epithelia of mouse palatal shelves after they fuse to form the midline epithelial seam. Labelling of the living epithelium of the prefusion palate was accomplished by exposure, both in vitro and in vivo, to CCFSE, a lipid-soluble dye that diffuses into periderm, becomes lipid insoluble and enters basal cells via gap junctions. We followed labelled palates for 4 days, by which time they were fully differentiated and, in the case of the in vivo study, the fetuses were ready for delivery. To grow the palates for 4 days in vitro, it was necessary to experiment with several culture conditions. It was important to compare the in vitro developmental process with the in vivo, because most studies of palatal development have used in vitro models; they did so for the same reason that we did, namely accessibility. We have found that development of explanted palates up to 4 days in vitro using the BGJb protocol with 50% O_2 (see **Materials** and **methods**) is essentially the same as in vivo as indicated by successful fusion and osteogenesis.

Unlike DiI, which does not stand up to fixation, CCFSE-labelled tissue could be handled with ease. They could be fixed in formaldehyde and embedded in paraffin or plastic. In addition, the dye proved a heretofore unexpected marker for following osmium-fixed transforming cells at the electron microscopic level. Once within cells, CCFSE becomes packaged into intracellular parcels that we have called isolation bodies (see **Results** for terminology). This packaging has the obvious advantage to the cells of compartmentalizing materials with possible toxic effects and the unexpected advantage for us of providing an identifiable TEM marker. The presence of membrane-bound CCFSE did not harm the cells, as judged by their very healthy, euchromatic nuclei and participation in palate development.

We have confirmed by electron microscopy that the dye diffuses into the epithelial cells, and is not taken up by the cells by phagocytosis, by the lack of phagosomes in the fluorescent tissues up to 12 hours after labelling. The isolation bodies that appear at 24 hours might be called autophagosomes except that they lack acid phosphatase (see **Results**). We are confident that the dye does not leave the cells again after passing from the periderm to basal epithelial layer. This was confirmed by TEM observations that CCFSE isolation bodies are only seen within the cells of the midline epithelial seam and newly transformed mesenchyme. Preexisting mesenchymal cells in the palate do not contain isolation bodies.

In this study, we were able to use ultrastructural criteria to define the connective tissue cell to which the labelled epithelia cells give rise. 24 hours after labelling, the epithelial seam is transforming into individual stellate cells located mainly in the midline connective tissue. At 4 days, the labelled connective tissue cells could clearly be identified as fibroblasts by their elongate cell shape, abundant RER running parallel to the axis of the cell, and prominent pseudopodia and filopodia. They are not connected by epithelial junctions and they do not show other epithelial characteristics, such as apical basal polarity. At 4 days,

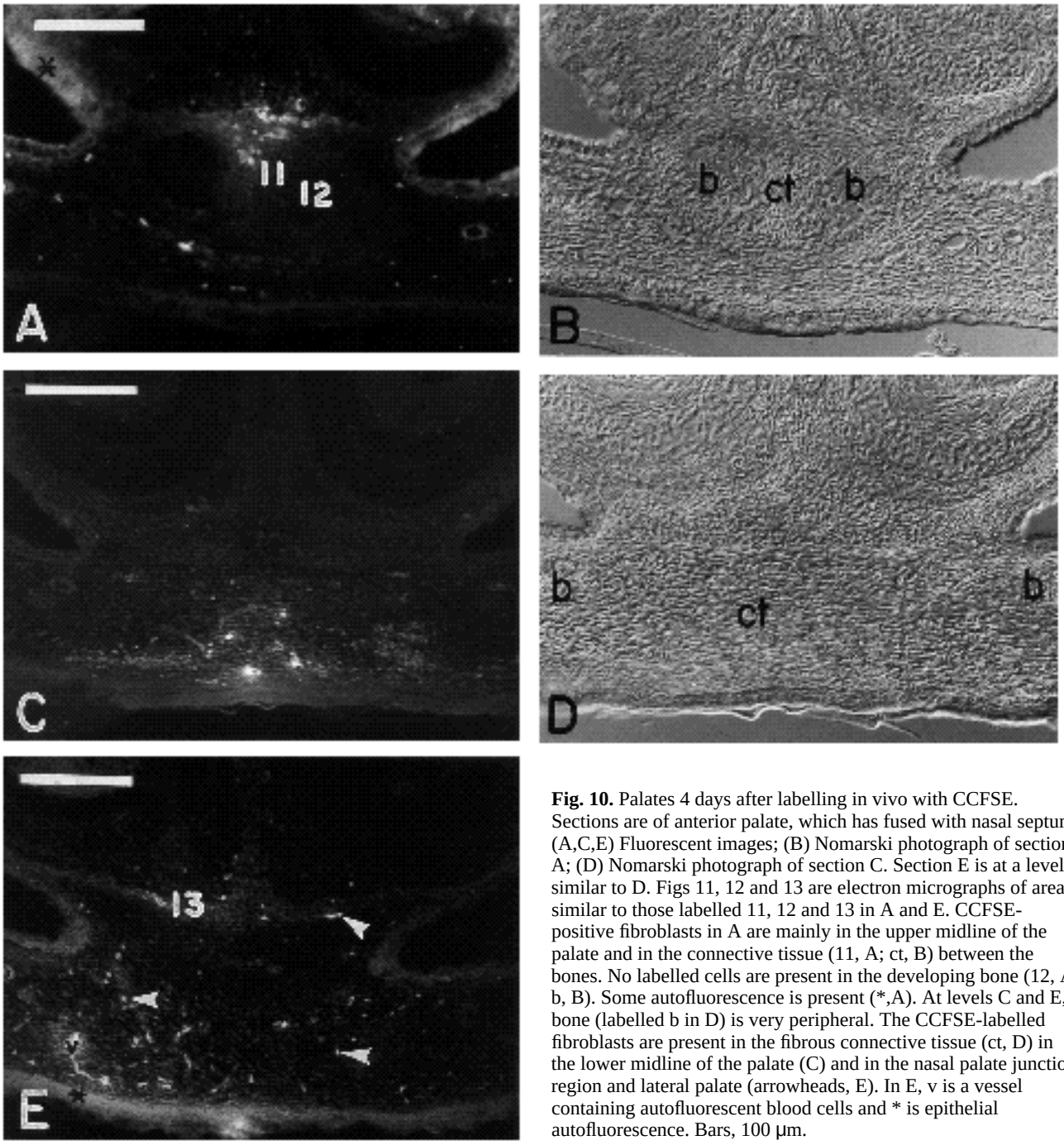

Fig. 10. Palates 4 days after labelling in vivo with CCFSE. Sections are of anterior palate, which has fused with nasal septum. (A,C,E) Fluorescent images; (B) Nomarski photograph of section A; (D) Nomarski photograph of section C. Section E is at a level similar to D. Figs 11, 12 and 13 are electron micrographs of areas similar to those labelled 11, 12 and 13 in A and E. CCFSE-positive fibroblasts in A are mainly in the upper midline of the palate and in the connective tissue (11, A; ct, B) between the bones. No labelled cells are present in the developing bone (12, A; b, B). Some autofluorescence is present (*,A). At levels C and E, bone (labelled b in D) is very peripheral. The CCFSE-labelled fibroblasts are present in the fibrous connective tissue (ct, D) in the lower midline of the palate (C) and in the nasal palate junction region and lateral palate (arrowheads, E). In E, v is a vessel containing autofluorescent blood cells and * is epithelial autofluorescence. Bars, 100 μm.

many of the labelled fibroblasts are located in the upper or lower midline of the palate and/or connective tissue at the palate nasal septum junction. Some, however, have moved throughout the palate to its lateral compartments, where at the very least (judging by their abundant RER and Golgi complexes) they help to manufacture the fibrous connective tissue matrix.

During initial contact of the palatal shelves, in the mouse, as in the rat (Fitchett and Hay, 1989), numerous desmosomes form to hold the two labelled basal epithelial layers together into a midline seam. This structural role that we proposed for the midline seam in attachment of the two shelves is gaining acceptance (Carette and Ferguson, 1992). We suggest still another role for the midline seam based on data in the present paper, namely, that formation of the midline seam is a prerequisite for the transformation of cells into mesenchyme to be triggered. Basal epithelial cells of CCFSE-labelled palatal shelves cultured singly do not transform, even though the periderm sloughs as previously noted Smiley and Koch (1972). Therefore, epithelial palatal fusion is necessary for achievement of palatal confluence by epithelial-mesenchymal transformation. Whether the

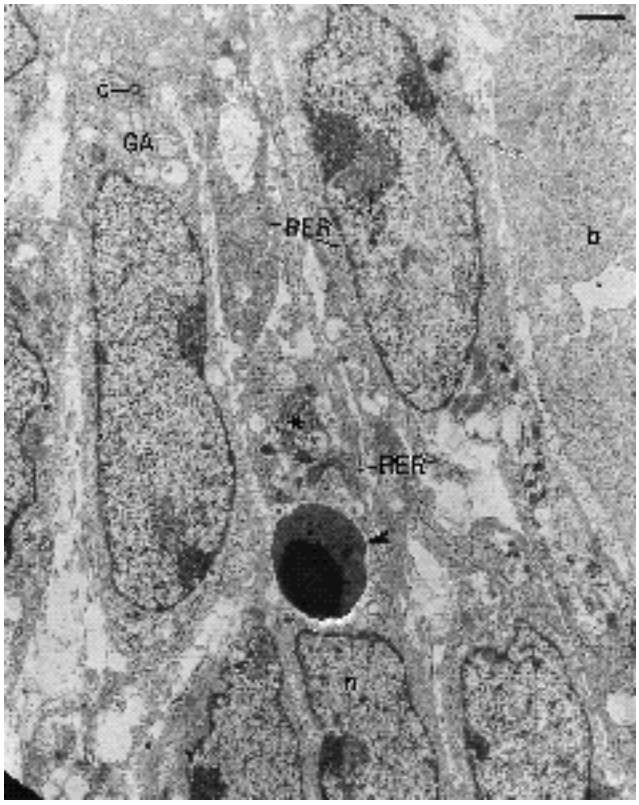

Fig. 11. TEM of a labelled fibroblast in an area of the palate similar to that marked 11 in Fig. 10A. A fibroblast in the center of the field contains a large isolation body (arrowhead). These mesenchymal cells are identified as fibroblasts by their elongate shapes and prominent RER running parallel to the long axis of the cell (RER). The debris (*) in this cell probably is a disintegrating isolation body. Its nucleus (n) and organelles appear healthy. Adjacent bone (b) is illustrated to better advantage in Fig. 12. c, centriole; GA, Golgi apparatus. Bar, 1 μm.

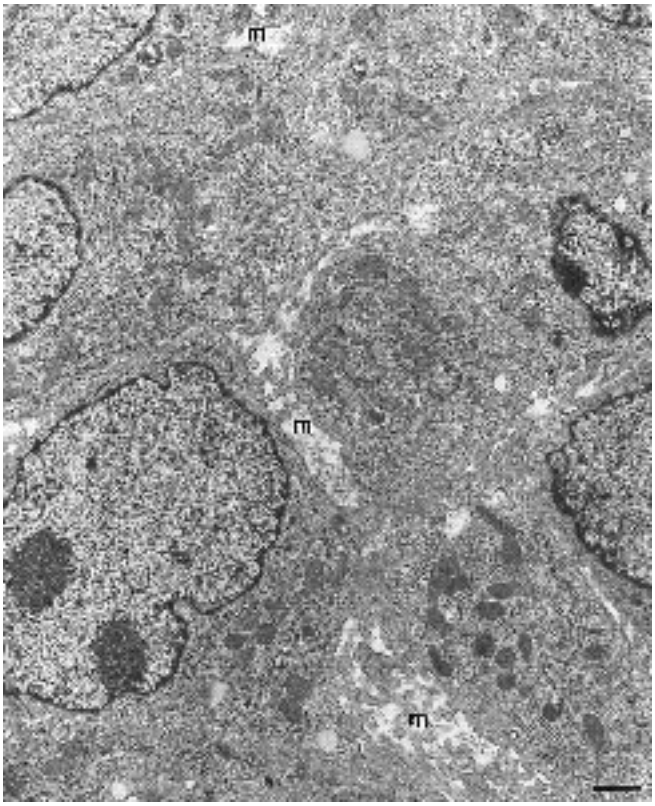

Fig. 12. TEM of early membrane bone in the area marked 12 (Fig. 10A) of a palate 4 days after exposure to CCFSE in vitro. No isolation bodies or fluorescence are seen in association with membrane bone. Bone matrix (m) has just begun to accumulate in the spaces between the osteoblasts. Bar, 1 μm.

resulting fibroblasts have developmental functions in addition to ECM synthesis as proposed above remains to be demonstrated.

Of some interest to the broad question of developmental potentials of mesenchymal cells, is the fact that the CCFSE-labelled mesenchymal cells did not make any contribution to the developing intramembranous bone within the palate, although they all become fibroblasts. This is consistent with existing evidence that osteoblast precursors acquire osteogenic potential through specific cell-cell interactions early in development. They appear to be transported into zones of ossification in the walls of blood vessels (Resnick et al., 1988). While little is known about the origin of the cells that give rise de novo to cranial membrane bone, the earliest blood vessels in the developing palate are in the region where bone forms later.

Carette and Ferguson (1992) reported that the disappearance of the midline seam in palatal fusion in vitro was due to MEE cells becoming transiently motile and migrating into the nasal and oral epithelia of the palate, where they become morphologically indistinguishable from the surrounding cells in 'epithelial triangles'. We have, however, demonstrated ultrastructurally here that the CCFSE-labelled epithelial seam cells of the MEE undergo epithelial-mesenchymal transformation. CCFSE-containing cells in the midline area after palatal confluence are clearly fibroblastic in morphology. The interpretation that these fibroblasts subsequently reenter epithelium at their final destination (Carette and Ferguson, 1992) is not supported by our data.

Our major criticism of the paper by Carette and Ferguson (1992) is that no cytology is presented, making the fluorescent images impossible to interpret. Several palates are exposed to DiI and frozen sections are examined at 24 and 48 hours with no fixation. The label is distributed in very hit or miss fashion, some in the seam and some in the oral epithelium, and it is not clear that enough label is present at 0 time and 24 hours to follow at 48 hours. Confocal images are then presented showing single living palates at 24, 48 and 120 hours, photographed as whole mounts at different levels into the tissue. It would not be possible from these images to say whether or not label is present in mesenchymal cells. Thus, the conclusion that labelled cells from the seam migrate back into the surface epithelium is not supported by the data presented by Carette and Ferguson (1992). Moreover, the so-called triangles of epithelium near the midline seam, which they reported accumulate such cells, are not a reproducible feature in our cultures. In the

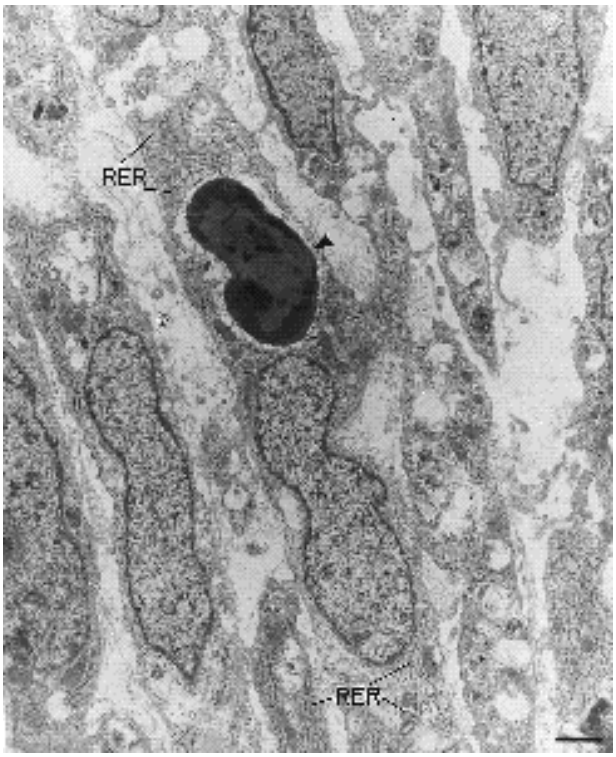

Fig. 13. TEM of a labelled fibroblast in an area of the palate similar to that marked 13 in Fig. 10E. The prominent RER (RER) and typical elongate cell shapes identify these cells unequivocally as fibroblasts. Arrowhead, CCFSE isolation body. Bar, 1 μm.

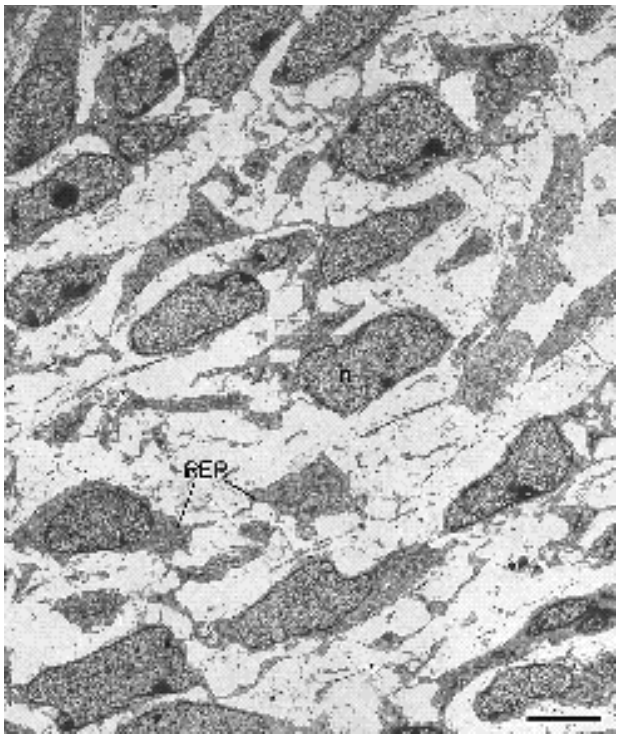

Fig. 14. TEM of a control palate treated as for Figs 11 to 12, but CCFSE omitted. After culture for 4 days, no isolation bodies are present in the midline connective tissue (above), or elsewhere in the control, and phagosomes are rare. n, nucleus; RER, rough endoplasmic reticulum. Bar, 5 μm.

present study, we traced labelled mesenchymal cells originating in the midline seam through their differentiation into fibroblasts, as judged by their ultrastructure. These fibroblasts are still present in abundance at just prior to birth (E18), while the label in the epithelium is greatly diminished.

In conclusion, we show here that CCFSE is superior to DiI for cytology; the label can be fixed for, and identified by, both light and electron microscopic observation. We have therefore been able to demonstrate with high resolution that epithelial to mesenchymal transformation of cells is a method for removal of epithelia during palatogenesis. The presence of living epithelial cells joined by desmosomes is paramount for the formation of a seam that holds the two fusing palatal shelves together. Once the connection is established, the epithelial cells are 'removed' by epithelial-mesenchymal transformation and integrated into the mesenchymal compartment of the palate, where they remain and can be identified later in palatal development, functioning as fibroblasts and playing as yet to be identified roles in morphogenesis. Our ability to identify CCFSE-labelled cells electron microscopically by their CCFSE-containing isolation bodies is a major step forward in extending the use of cell lineage markers to the ultrastructural level.

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References

Abbott, B. D. and Buckalew, A. R. (1992). Embryonic palatal responses to teratogens in serum free organ culture. *Teratology* **45,** 369-382.

Bronner-Fraser, M. (1985). Alterations in neural crest migration by a monoclonal antibody that affects cell adhesion. *J. Cell Biol.* **101,** 610-617.

Carette, M. J. M. and Ferguson, M. J. W. (1992). The fate of medial edge epithelial cells during palatal fusion in vitro: analysis by DiI labelling and confocal microscopy. *Development* **114,** 379-388.

Chartrain, I. M. H. and Hay, E. D. (1985). Fate of the avian cervical sinus: A new example of epithelial mesenchymal transformation during embryonic remodeling. *Anat. Rec.* **211,** 37a.

Ekblom, P. (1984). Basement membrane proteins and growth factors in kidney differentiation. In *The Role of Extracellular Matrix in Development*. (ed. R. L. Trelstad), pp. 173-206. New York: Alan R. Liss.

Ferguson, M. J. W., Honig, L. S. and Slavkin, H. C. (1984). Differentiation of cultured palatal shelves from alligator, chick and mouse embryos. *Anat. Rec.* **209,** 231-249.

Fitchett, J. E. and Hay, E. D. (1989). Medial edge epithelium transforms to mesenchyme after embryonic palatal shelves fuse. *Dev. Biol.* **131,** 455-474.

Fitchett, J. E., McAlmon, K. R., Hay, E. D. and Bernfield, M. (1990). Epithelial cells lose syndecan prior to epithelial mesenchymal transformation in the developing rat palate. *J. Cell Biol.* **111,** 145a.

Griffith, C. M. and Hay, E. D. (1991). Palatal fusion and epithelial mesenchymal transformation. *J. Cell Biol.* **115,** 145a.

Griffith, C. M., Wiley, M. J. and Sanders, E. J. (1992). The vertebrate tail bud: three germ layers from one tissue. *Anat. Embryol.* **185,** 101-113.

Hay, E. D. (1968). Organization and fine structure of epithelium and

mesenchyme in the developing chick embryo. In *Epithelial-Mesenchymal Interactions* (eds. R. Fleischmajer and R. E. Billingham), pp. 31-55. Baltimore: Williams and Wilkins.

Hay, E. D. (1990). Epithelial mesenchymal transitions. *Seminars in Dev. Biol.* **1,** 347-356.

Hayward, A. F. (1969). Ultrastructural changes in the epithelium during fusion of the palatal processes in rats. *Archs Oral Biol.* **14,** 661-678.

Holtzman, E. (1976). *Lysosomes: A Survey.* New York: Springer-Verlag.

Honig, M. G. and Hume, R. I. (1986). Fluorescent carbocyanine dyes allow living neurons of identified origin to be studied in long term cultures. *J. Cell Biol.* **103,** 171-187.

Honig, M. G. and Hume, R. I. (1989). DiI and DiO: versatile fluorescent dyes for neuronal labelling and pathway tracing. *TINS* **12,** 333-341.

Hughes, L. V., Furstman, L. and Bernick, S. (1967). Prenatal development of the rat palate. *J. Dent. Res.* **46,** 373-379.

LeDouarin, N. (1982). *The Neural Crest*. Cambridge: Cambridge University Press.

Locke, M. and Collins, J. V. (1965). The structure and formation of protein granules in the fat body of an insect. *J. Cell Biol.* **26,** 857-884.

Markwald, R. R., Runyan, R. B., Kitten, G. T., Funderburg, F. M., Bernanke, D. H. and Brauer, P. R. (1984). Use of collagen gel culture to study heart development: proteoglycan and glycoprotein interactions during the formation of endocardial cushion tissue. In *The Role of Extracellular Matrix in Development* (ed. R. L. Trelstad), pp. 323-350. New York: Alan R. Liss.

Resnick, D., Manolagas, S. C. and Niwayama, G. (1988). Histogenesis, anatomy and physiology of bone. In *Diagnosis of Bone and Joint Disorders* (eds. D. Resnick and Niwayama, G), pp. 1941-1974. Philadelphia: W. B. Saunders.

Schuler, C. F., Guo, Y., Majumder, A. and Luo, R. (1991). Molecular and morphologic changes during the epithelial mesenchymal transformation of palatal shelf medial edge epithelium in vitro. *Int. J. Dev. Biol.* **35,** 463-472.

Smiley, G. R. and Koch, W. E. (1972). An in vitro and in vivo study of single palatal processes. *Anat. Rec.* **173,** 405-416.

Solursh, M., Fisher, M., Meier, S. and Singley, C. T. (1979). The role of extracellular matrix in the formation of sclerotome. *J. Embryol. Exp. Morph.* **54,** 75-98.

Trelstad, R. L., Hayashi, A., Hayashi, K. and Donahoe, P. K. (1982). The epithelial mesenchymal interface of the male rat Mullerian duct: Loss of basement membrane integrity and ductal regression. *Dev. Biol.* **92,** 27-40.

Venable, J. H. and Coggeshall, R. (1965). A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25,** 407-408.

Walker, B. E. and Fraser, F. C. (1956). Closure of the secondary palate in three strains of mice. *J. Embryol. Exp. Morph.* **4,** 176-189.

Zuk, A., Matlin, K. S. and Hay, E. D. (1989). Type I collagen gel induces Madin-Darby canine kidney cells to become fusiform in shape and lose apical basal polarity. *J. Cell Biol.* **108,** 903-919.

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