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Original Article

Molecular and morphologic changes during the epithelial-mesenchymal transformation of palatal shelf medial edge epithelium *in vitro*

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ABSTRACT The fate of the medial edge epithelial (MEE) cells during palatal fusion has been proposed to be either programmed cell death or epithelial-mesenchymal transformation. Vital cell labeling techniques were used to mark the MEE and observe their fate during palatal fusion *in vitro*. Fetal mouse palatal shelves were labeled with DiI and allowed to proceed through fusion while maintained in an organ culture system. The tissues were examined at several stages of palatal fusion for the distribution of DiI, presence of specific antigens and ultrastructural appearance of the cells. The MEE labeled with DiI occupied a midline position at all stages of palatal fusion. Initially the cells had keratin intermediate filaments and were separated from the underlying mesenchyme by an intact basement membrane. During the process of fusion the basement membrane was degraded and the DiI-labeled MEE were in contact with the mesenchymal-derived extracellular matrix. In the late stages of fusion the DiI-labeled MEE altered their cellular morphology, had vimentin intermediate filaments, and were not associated with an identifiable basement membrane. DiI-labeled cells, without an epithelial phenotype, remained present in the midline of the completely fused palate. The data indicate that the MEE did not die but underwent a phenotypic transformation to viable mesenchymal cell types, which were retained in the palatal mesenchyme.

KEY WORDS: *medial edge epithelium, palatogenesis, cell lineage, epithelial-mesenchymal transformation, programmed cell death*

Introduction

Development of the mammalian secondary palate requires a series of precise changes in tissue morphology and cell differentiation (for reviews, Moscona and Monroy, 1984; Ferguson, 1988; Greene, 1989). The entire sequence of events occurs during a brief period of fetal development (Walker and Fraser, 1956; Pourtois, 1966, 1972). Interruption of the sequence of tissue- and cell-developmental events occurring during palatogenesis can result in a cleft palate birth defect (Ferguson, 1991).

The epithelial cells covering the medial edges of the opposing palatal shelves have a critical role in the final stages of palatal ontogeny (Pourtois, 1966; Smiley, 1970; Morgan, 1976; Pratt, *et al.*, 1980). Complete fusion of the secondary palate requires that the medial edge epithelium (MEE) disappear from the midline of the palate in order to permit mesenchymal confluence (Farbman, 1969; Mato *et al.*, 1972; Chaudhry and Shah, 1973; Morgan 1976). Both *in vivo* and *in vitro* examinations have noted the absence of MEE following complete fusion of the palate (Coleman, 1965; Smiley and Koch, 1971, 1972, 1975; Morgan, 1976).

Fusion of the secondary palate in organ culture requires tissues which have achieved the capacity for fusion *in vivo* (Moriarity *et al.*, 1963; Coleman, 1965; Pourtois, 1966, 1972). MEE transiently develops the state of cell differentiation that permits palatal fusion (Pourtois, 1966; Smiley and Koch, 1971, 1972, 1975). The morphologic changes in the MEE during palatal fusion *in vitro* (Smiley and Koch, 1971; Tyler and Koch, 1975; Ferguson *et al.*, 1984), the intracellular enzymatic changes (Idoyaga-Vargas *et al.*, 1972; Greene and Pratt, 1978; Greene and Garbarino, 1984), and the response of cultured palatal shelves to teratogenic agents

Abbreviations used in this paper: EMT, epithelial-mesenchymal transformation; DiI, 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; MEE, medial edge epithelium; PCD, programmed cell death

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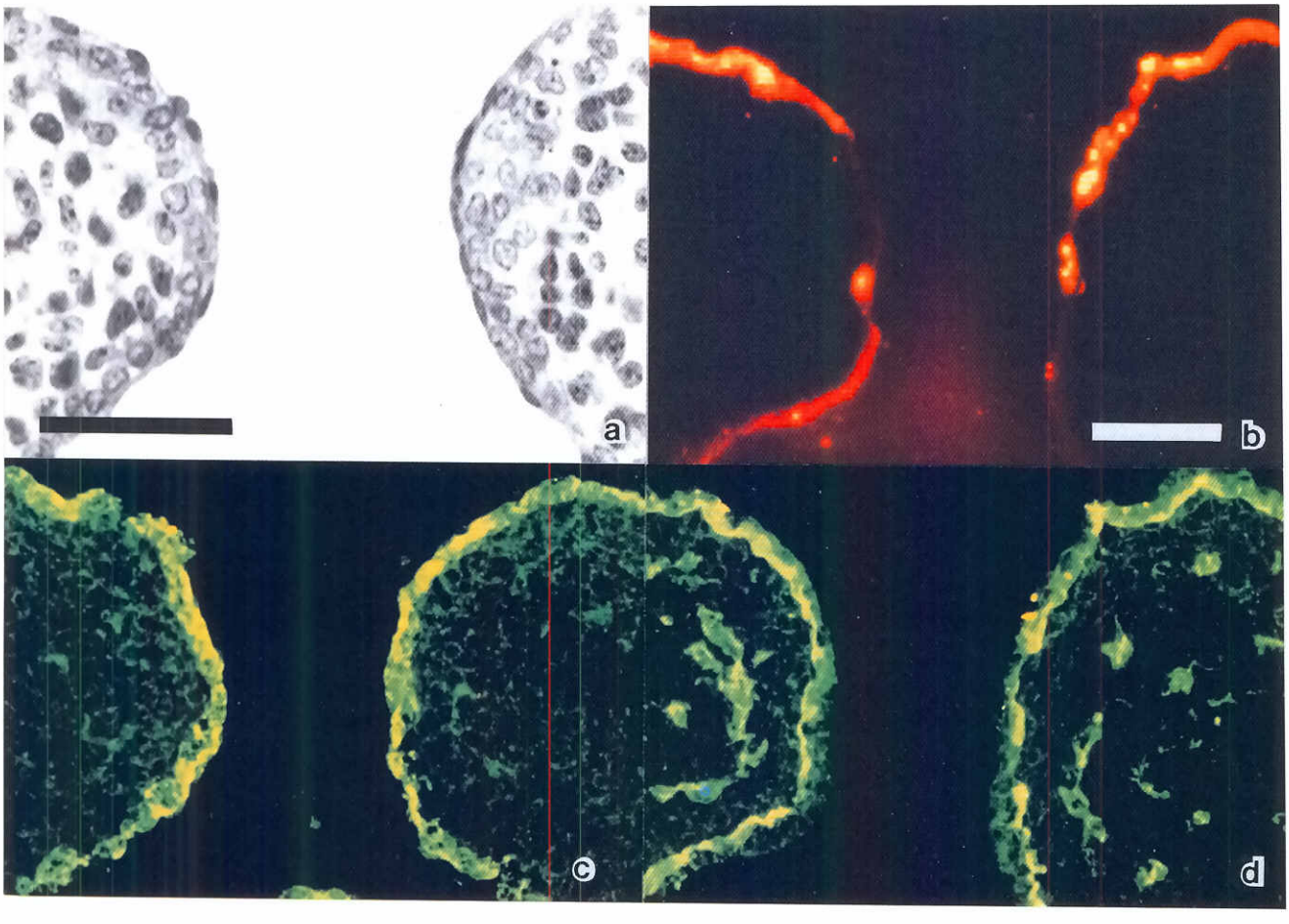
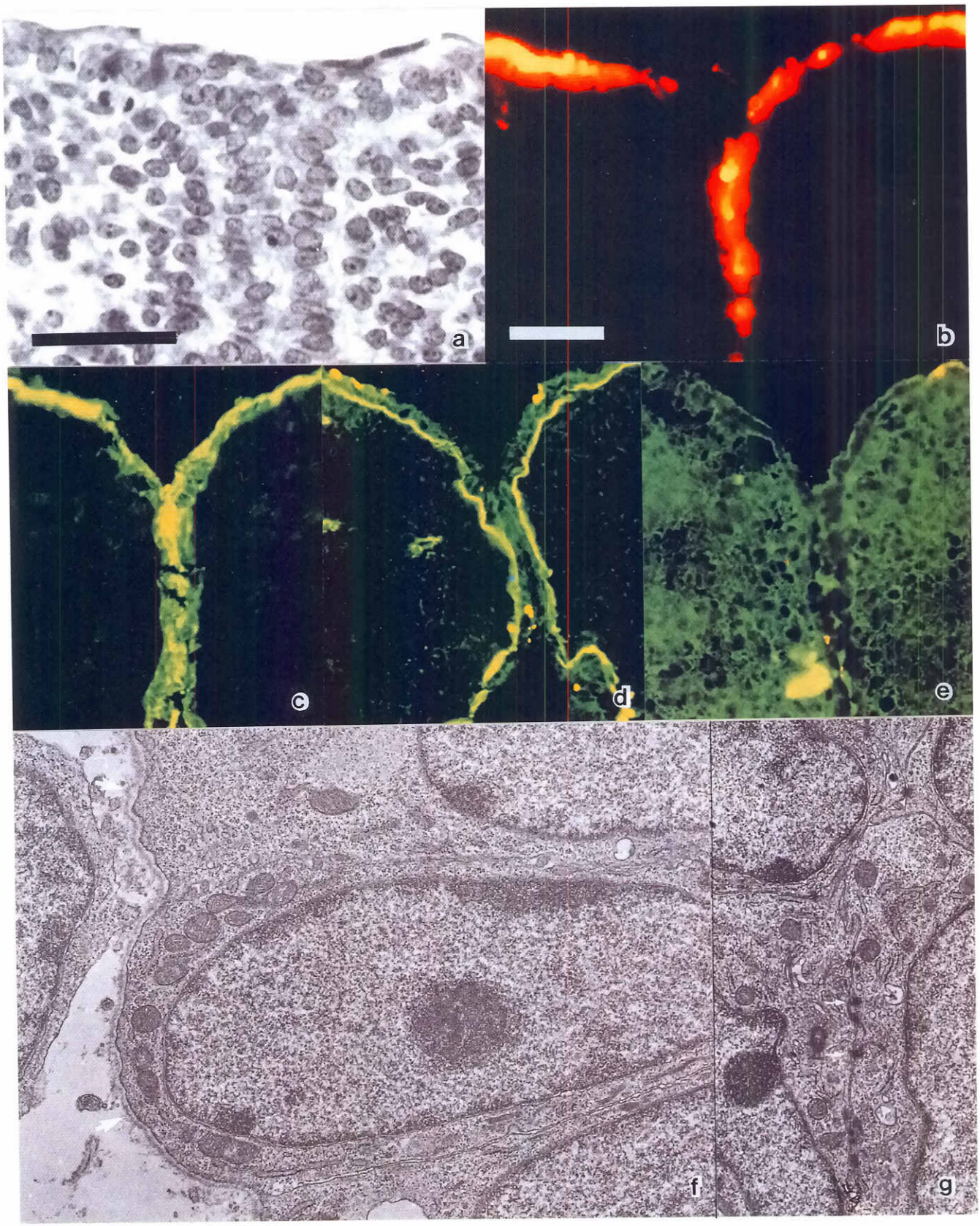


Fig. 1. Palatal shelf morphology and antigen localization in the medial edge epithelium prior to the onset of fusion. *Palatal shelf tissues were dissected from mouse fetuses of gestational age 14 d 3 h and labeled with DiI* in vitro. *The tissues were frozen, sectioned and reacted with specific antisera. The localization of the primary antibody was detected with FITC-labeled secondary antibodies. The distribution of DiI and FITC was examined by fluorescence microscopy.* **(a)** *Hematoxylin and eosin stain of the medial edges of the palatal shelves.* **(b)** *DiI localization.* **(c)** *Keratin immunolocalization.* **(d)** *Laminin immunolocalization. Bars 50 μm (a-d).*

(Hassell, 1975; Hassell and Pratt, 1977; Tyler and Pratt, 1980; Goldman, 1984; Pratt *et al.*, 1984), have all been shown to resemble the changes observed *in vivo*. Thus the process of palatal fusion in organ culture recapitulates the set of events that occur during palatogenesis *in vivo*.

The final fate of the MEE during palatal fusion has not been determined either *in vivo* or *in vitro*. Previous studies have proposed that the MEE undergo programmed cell death (PCD) during their disappearance from the midline of the palate (Shapiro and Sweney, 1969; Hudson and Shapiro, 1973; Pratt and Martin, 1975; Pratt and Greene, 1976; Pratt *et al.*, 1984). An alternative MEE fate was proposed by Fitchett and Hay (1989) who observed ultrastructural and molecular changes which indicated that the MEE may have undergone an epithelial-mesenchymal transformation (EMT) and remained as viable cells in the palate connective tissue. PCD and EMT are alternative ways to accomplish the fusion of the palate; however distinctly different molecular changes would be associated with their respective mechanisms. It may be expected that these

Fig. 2. Adherent stage of palatal fusion with a 2 cell layer thick midline epithelial seam, following 2 h of culture *in vitro*. *Palatal shelf tissues were dissected from mouse fetuses of gestational age 14 d 3 h and labeled with DiI* in vitro. *The palatal shelves were placed in organ culture with their medial edges touching. After 2 h of culture the tissues were frozen, sectioned and reacted with specific antisera or processed for ultrastructural examination. The localization of the primary antibody was detected with FITC-labeled secondary antibodies. The distribution of DiI and FITC was examined by fluorescence microscopy.* **(a)** *Hematoxylin and eosin stain of palatal shelf tissues.* **(b)** *DiI localization.* **(c)** *Keratin immunolocalization.* **(d)** *Laminin immunolocalization.* **(e)** *Vimentin immunolocalization.* **(f)** *Basement membrane separating MEE from mesenchyme (arrows) x15,000.* **(g)** *Desmosomal connections between opposing MEE (arrows) (x5,000). Bars 50 μm (a-e).*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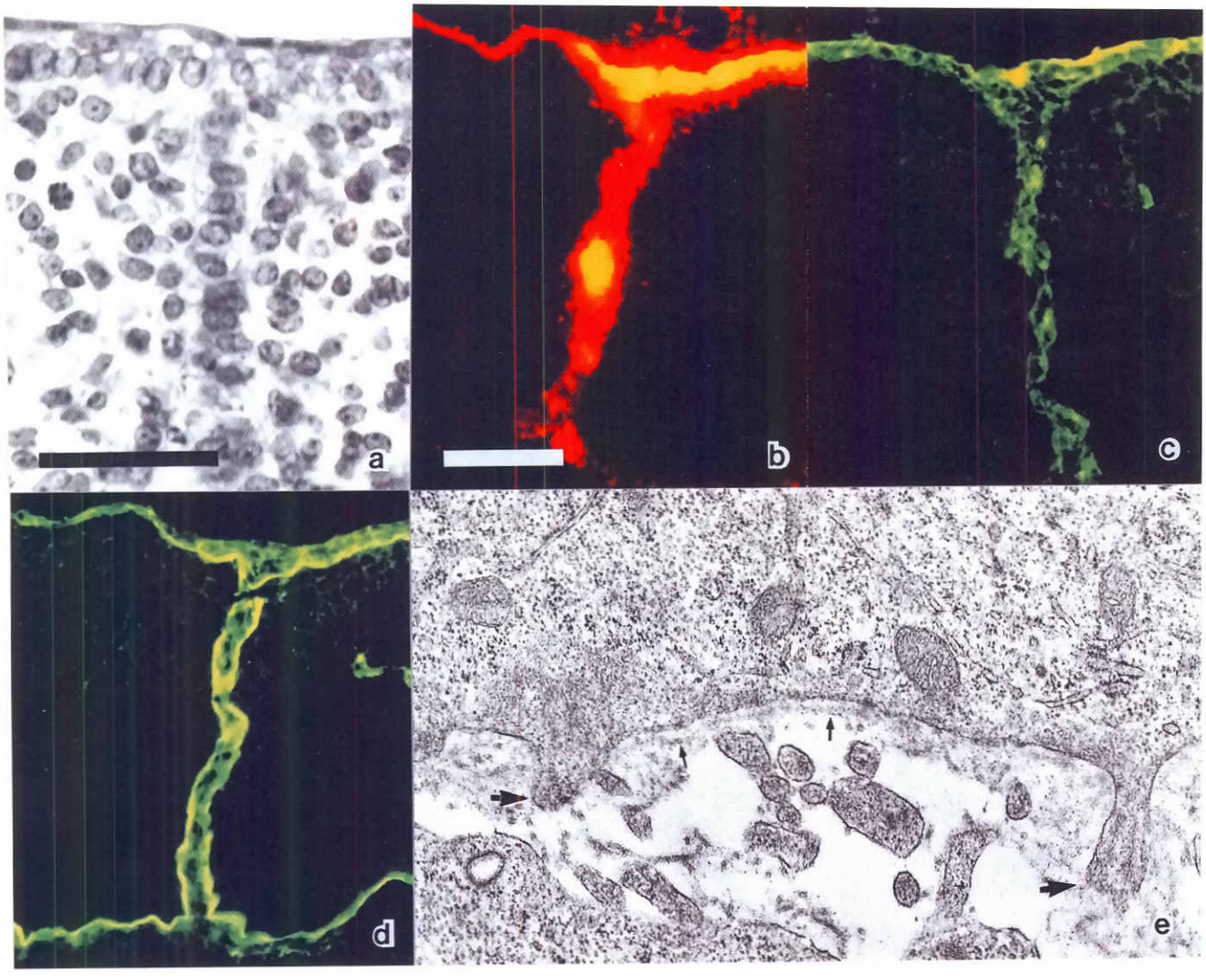


Fig. 3. Early stages of palatal fusion with a single cell layer thick midline epithelial seam continuous with other surface epithelia. *Palatal shelf tissues were dissected from mouse fetuses of gestational age 14 d 3 h and labeled with DiI* in vitro. *The palatal shelves were placed in organ culture with their medial edges touching. After 8 h of culture the tissues were either frozen, sectioned and reacted with specific antisera or processed for ultrastructural examination. The localization of the primary antibody was detected with FITC-labeled secondary antibodies. The distribution of DiI and FITC was examined by fluorescence microscopy.* **(a)** *Hematoxylin and eosin stain of palatal shelf tissues.* **(b)** *DiI localization.* **(c)** *Keratin immunolocalization.* **(d)** *Laminin immunolocalization.* **(e)** *Projections (large arrows) of MEE through the discontinuous basement membrane (small arrows) (x25,000). Bars 50 μm (a-d).*

two mechanisms would be differently affected by either drugs or environmental agents known to be associated with the induction of cleft palate birth defects. Thus a determination of the fate of the MEE is necessary to ascertain the mechanism of action of known teratogens.

In order to definitively characterize the developmental fate of the MEE, the present study labeled individual MEE cells with a vital dye prior to the onset of palatal fusion. The exogenous marker, DiI (1,1-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) was incorporated into the plasma membranes of MEE and used to trace their cell lineage (Serbedzija *et al.*, 1989, 1990; Bronner-Fraser, 1990). Incorporation of DiI in MEE prior to palatal fusion provided a marker to identify this specific population of cells during the subsequent stages of palatogenesis *in vitro* and determine their fate. The results of the present study indicated that murine MEE could be labeled *in vitro* with DiI, that the incorporation of DiI did not alter the morphologic events of palatal development *in vitro*, and that the DiI-labeled MEE cells could be detected at all stages of palatal fusion *in vitro*.

Results

Palatal development

The process of palatal fusion was compared between DiI-labeled palatal shelves and unlabeled control palatal shelves. The exposure of the palatal shelves to DiI was not associated with obvious adverse effects on the sequence of morphologic events during palatal shelf fusion *in vitro* (Figs. 1-5).

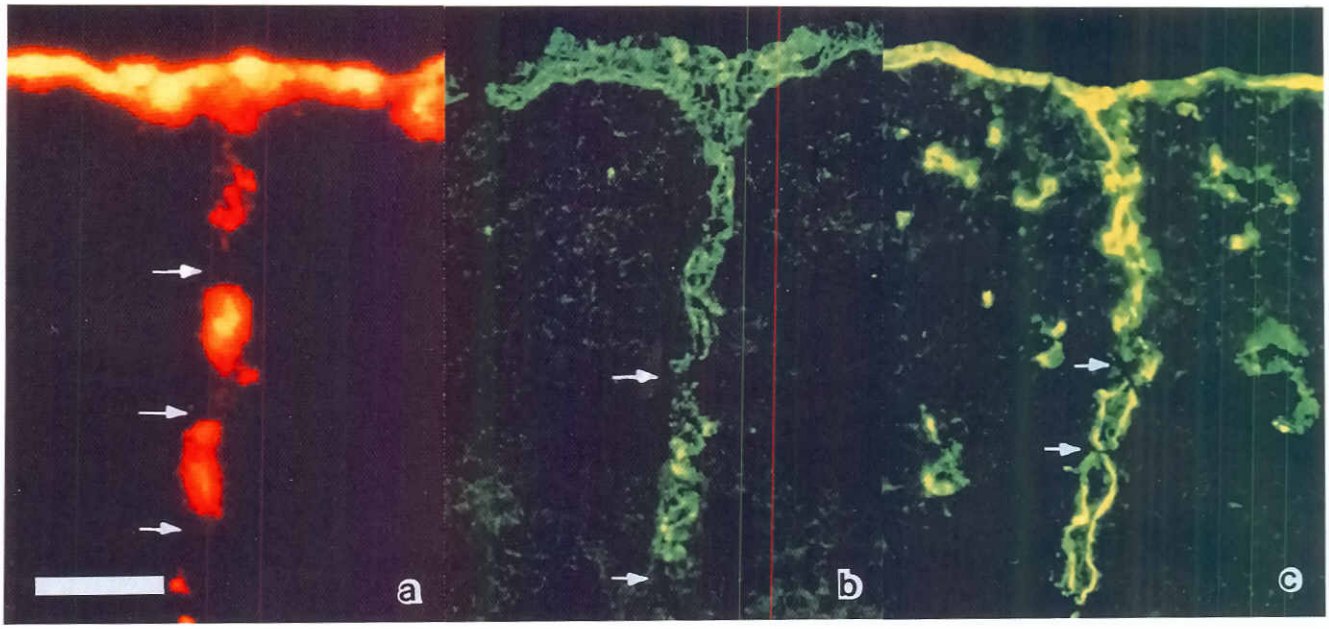


Fig. 4. Late stages of palatal fusion with a discontinuous midline epithelial seam. *Palatal shelf tissues were dissected from mouse fetuses of gestational age 14 d 3 h and labeled with DiI* in vitro. *The palatal shelves were placed in organ culture with their medial edges touching. After 20 h of culture the tissues were frozen, sectioned and reacted with specific antisera The localization of the primary antibody was detected with FITC-labeled secondary antibodies. The distribution of DiI and FITC was examined by fluorescence microscopy.* **(a)** *DiI localization.* **(b)** *Keratin immunolocalization.* **(c)** *Laminin immunolocalization. Bar 50 μm (a-c). Arrows indicate breaks in the continuity of the MEE.*

MEE prior to adherence

The DiI localization was examined in the palatal shelf tissues prior to the initial adherence of the MEE (0 h of organ culture). The palatal shelves were covered by a layer of epithelium, recognizable by light microscopic criteria (Fig. 1a). The epithelium covering the palatal shelves was labeled with DiI (Fig. 1b). All the epithelial cells contained the epithelial-specific intermediate filament keratin (Fig. 1c) and were separated from the underlying mesenchyme by a continuous basement membrane (Fig. 1d). The underlying mesenchymal cells were devoid of DiI-associated fluorescence (Fig. 1b), which indicated that DiI had not penetrated the epithelium during the labeling process.

MEE appearance following adherence

The palatal shelves were adherent by 2 h in organ culture (Fig. 2a). A two cell layer thick seam of epithelial cells was present in the midline of the palate (Fig. 2a). The DiI-associated fluorescence was restricted to epithelial cells covering the palatal shelves and in the midline seam (Fig. 2b). DiI-associated fluorescence was not observed in the palatal mesenchyme (Fig. 2b). Keratin proteins were immunolocalized only in the epithelial cells which had DiI-associated fluorescence (Fig. 2c). The MEE was continuous with the oral epithelium and both epithelia were separated from the mesenchyme by a continuous basement membrane (Fig. 2d). Vimentin was immunolocalized in the mesenchymal cells and was absent from the MEE and surface epithelia (Fig. 2e). The ultrastructural appearance of the MEE was columnar cells containing mitochondria, rough endoplasmic reticulum, free ribosomes and an intact nucleus (Fig. 2f). The basal lamina separating the MEE from the mesenchyme was well-defined and continuous (Fig. 2f). Desmosomal junctions had been formed between the MEE of the opposing palatal shelves (Fig. 2g). The cells with DiI-associated fluorescence had features consistent with an epithelial phenotype based on standard morphologic criteria, immunolocalization of specific antigens and ultrastructural characterization.

MEE during thinning of the midline seam

The progression of palatal fusion was associated with the reduction of the midline MEE seam to a single layer of cells (*e.g.*, after 8-16 h of organ culture) (Fig. 3a). The epithelial cells in the midline were continuous with the oral epithelium (Fig. 3a). Cells with DiI-associated fluorescence were present only in the epithelium on the oral surface of the palatal shelves and in the midline seam (Fig. 3b). Keratin proteins were immunolocalized only in the cells with an epithelial morphology and with DiI-associated fluorescence (Fig. 3c). The epithelial cells on the oral surface and in the midline seam were separated from the underlying mesenchyme by an apparently continuous layer of basement membrane laminin (Fig. 3d). The ultrastructural appearance of the MEE cells was more cuboidal and the cells contained organelles and nuclei which appeared not to be degraded. The integrity of the basement membrane was altered and at numerous sites filopodial processes from MEE were protruding through the basal lamina and in contact with the mesenchymal extracellular matrix (Fig. 3e). The pattern of DiI localization compared to the immunodetection of phenotype-specific antigens was similar to that observed at earlier stages of palatal shelf fusion (Figs. 1 and 2). Discontinuities in the basement membrane, which were detected by electron microscopy, represented the first change in the relationship of the epithelial cells to the mesenchyme (Fig. 3e). Epithelial filopodia projecting through these breaks in the

basement membrane permitted contact between the epithelial cells and the mesenchymally-derived extracellular matrix.

MEE during breakup of the midline seam

The MEE midline seam became fragmented and lost its continuity with the surface epithelium between 20 and 28 h of organ culture. The distribution of both DiI fluorescent cells and phenotype-specific antigens changed at this stage of palatal fusion. The oral surface epithelium contained DiI with a prominent concentration in the stratum corneum (Fig. 4a). Individual and groups of DiI-labeled cells could be identified in the midline of the palate, in positions previously occupied by the MEE (Fig. 4a). The DiI-associated fluorescence was identified in cells with either an epithelial or mesenchymal morphology by light microscopic criteria. The distribution of both keratin-containing cells in the midline and basement membrane laminin in the midline had discontinuities not present at earlier stages of development (Figs. 4b,c). At the electron microscopic level the MEE cells were irregularly organized, with a more spindle-shaped morphology. At this stage of palatal fusion, the DiI and antigen distribution patterns did not have the continuity present at earlier developmental points.

MEE cells following disappearance of the midline seam

The palatal shelves were completely fused following 40 h of organ culture and cells with an epithelial morphology could not be identified in the midline region by light microscopy (Fig. 5a). DiI-labeled cells were present in the midline region of the palate, an area previously associated strictly with fusion of the MEE (Fig. 5b). The DiI-labeled cells did not contain keratin intermediate filaments (Fig. 5c) and were no longer associated with a laminin-containing basement membrane (Fig. 5d). The DiI fluorescence had a plasma membrane distribution in the midline palatal cells (Fig. 5e) and these cells contained vimentin (Fig. 5f). The cells in the midline could not be distinguished ultrastructurally from the surrounding mesenchymal cells (Fig. 5g). The DiI-containing cells, originally present as MEE, were retained in the palatal mesenchyme as cells with a mesenchymal phenotype.

Discussion

This study was designed to observe directly the medial edge epithelial cells throughout the series of developmental events associated with fusion of the secondary palate *in vitro*. Labeling of the MEE *in vitro* permitted precise identification of the cells at specific stages of palatal fusion as well as characterization of phenotype-specific antigens. The use of MEE-specific markers ensured reproducible interpretations of whether the disappearance of the MEE was the result of either programmed cell death (PCD) or epithelial-mesenchymal phenotypic transformation (EMT). This was accomplished by the correlation of DiI label with the expression of phenotype-specific markers in the same cells. The present data support the interpretation of an epithelial-mesenchymal transformation fate for the medial edge epithelium during palatal fusion *in vitro*.

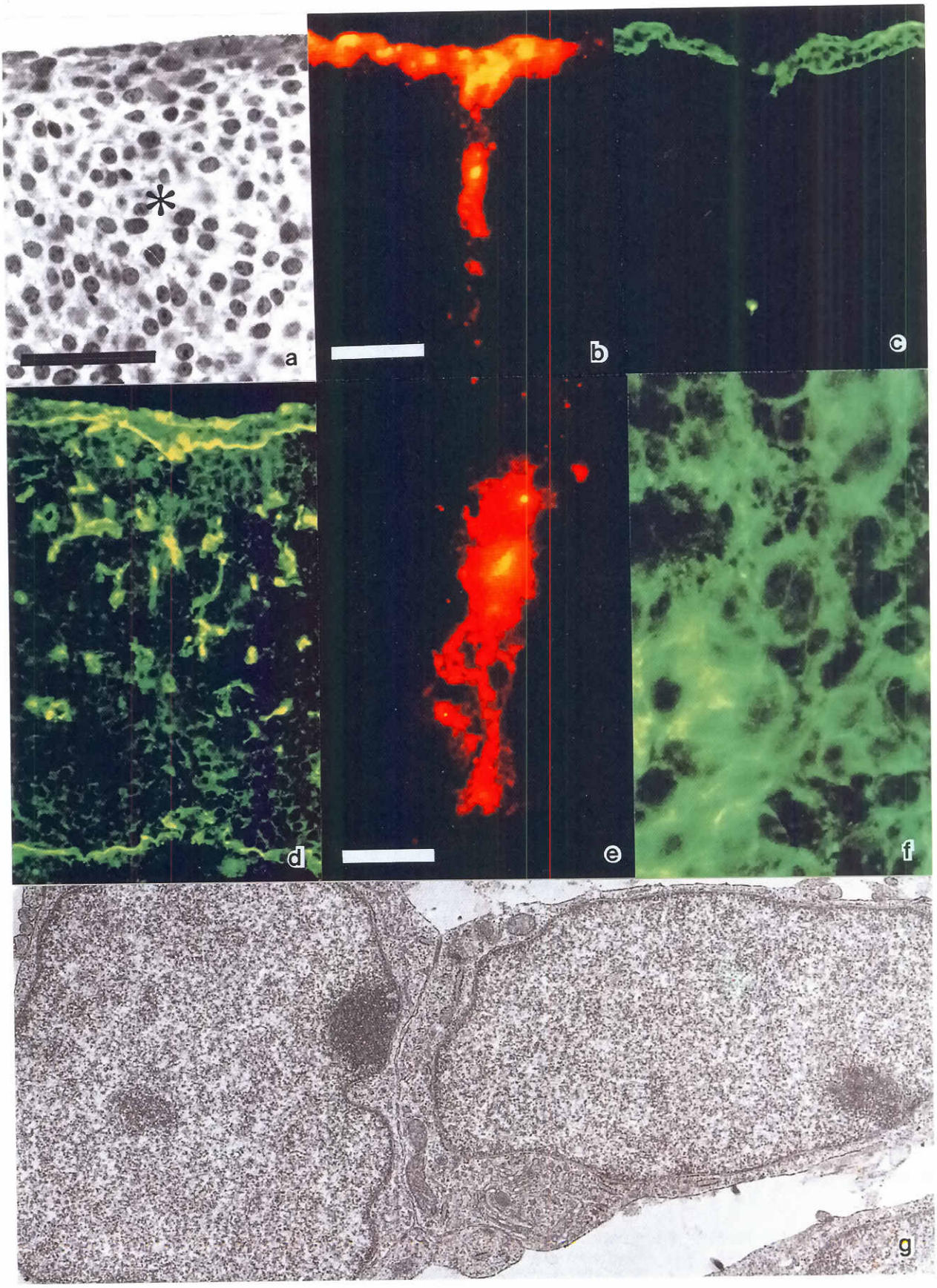
EMT has been observed at several anatomic locations *in vivo* (Markwald *et al.*, 1977; Trelstad *et al.*, 1982). For example, both the Müllerian duct and the cardiac cushion have been shown to contain groups of epithelial cells which undergo phenotypic transformation (Trelstad *et al.*, 1982; Potts and Runyan, 1989; Potts *et al.*, 1991). The sequence of events observed at other anatomic locations during EMT *in vivo* is similar to the pattern of cellular changes observed during palatal fusion *in vitro*. EMT is under both genetic and epigenetic control and represents an alternative fate to programmed cell death for epithelial cells during embryonic development (Markwald *et al.*, 1978; Runyan and Markwald, 1983; Runyan *et al.*, 1990; Valles *et al.*, 1990; Potts *et al.*, 1991). Characterization of possible molecular events associated with EMT has been accomplished in *in vitro* models (Greenburg and Hay, 1982, 1986, 1988; Boyer *et al.*, 1989; Valles *et al.*, 1990). The phenotypic transformation has been proposed to be a response to either contact with an extracellular matrix element or exposure to a growth factor.

Contact with Type I collagen was one mechanism proposed to initiate the series of intracellular changes that result in phenotypic transformation (Greenburg and Hay, 1988). Ordinarily epithelial cells would not be in contact with this extracellular matrix molecule, however breakdown of the basement membrane during palatal fusion permits contact between the MEE and Type I collagen in the mesenchyme. This sequence of events is similar to those observed in the phenotypic transformation of Müllerian duct cells (Trelstad *et al.*, 1982). For the temporal sequence of events observed in this study, the DiI-labeled MEE retained both an epithelial morphology and antigen characteristics, while the basement membrane was intact. At later stages of development, the DiI-labeled cells were not constrained by the basement membrane but were in intimate contact with the extracellular matrix and no longer had either an epithelial morphology or phenotype-specific antigens.

Transforming growth factor beta and acidic fibroblast growth factor have been shown to have an epigenetic regulatory role in the mechanism for EMT induction *in vitro* (Potts and Runyan, 1989; Valles *et al.*, 1990; Potts *et al.*, 1991). Recent reports have shown that the medial edge epithelial cells express transforming growth factor beta 3 (TGF B3) just prior to the onset of palatal fusion (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990; Gehris *et al.*, 1991). The expression of TGF B3 by MEE may represent an autocrine pathway regulating their phenotypic conversion.

The disappearance of cells with a recognizable epithelial phenotype from the midline seam of the palate is essential for complete fusion. The concept of a PCD fate for the MEE was based on three independent experimental observations: 1) MEE cells ceased DNA

Fig. 5. Complete fusion of the palate and mesenchymal confluence following 40 hours of culture *in vitro*. *Palatal shelf tissues were dissected from mouse fetuses of gestational age 14 d 3 h and labeled with DiI* in vitro. *The palatal shelves were placed in organ culture with their medial edges touching. After 40 h of culture the tissues were either frozen, sectioned and reacted with specific antisera or processed for ultrastructural examination. The localization of the primary antibody was detected with FITC-labeled secondary antibodies. The distribution of DiI and FITC was examined by fluorescence microscopy.* **(a)** *Hematoxylin and eosin stain of palatal shelf tissues.* **(b)** *DiI localization.* **(c)** *Keratin immunolocalization.* **(d)** *Laminin immunolocalization.* **(e)** *DiI localization.* **(f)** *Vimentin immunolocalization in the midline DiI positive region.* **(g)** *Ultrastructure of cells in the midline region following palatal fusion (x12,500). Bars 50 μm (a-d) and 20 μm (e,f).*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synthesis prior to fusion (Hudson and Shapiro, 1973; Pratt and Martin, 1975; Greene and Pratt, 1976); 2) MEE cells developed ultrastructural changes that were consistent with cell death (Farbman, 1968; Shapiro and Sweney, 1969; Chaudhry and Shah, 1973); 3) MEE lysosomal enzyme levels increased during palatal fusion (Hayward, 1969; Smiley, 1970; Pratt and Greene, 1976). These observations were used to develop further data, although subsequent studies used the absence of epithelial cells in the midline as the endpoint for the study of the mechanism of MEE cell death. A much different conclusion may be reached from those studies if the MEE disappear due to phenotypic transformation.

Of the three sets of data used to support PCD for the MEE, only ultrastructural evidence definitively demonstrates MEE cell death. However the presence of dead cells alone is not conclusive proof of PCD, even for proponents of PCD (Saunders, 1966). In those previous studies, only a subset of cells in the midline region were observed to have features consistent with cell death (Farbman, 1968; Shapiro and Sweney, 1969; Chaudhry and Shah, 1973). Moreover, it has been shown that peridermal cells caught in the midline will always degenerate (Waterman *et al.*, 1973; Waterman and Meller, 1975; Meller, 1980). and it was not always determined whether dying peridermal cells were the source of the cells with ultrastructural evidence of cell death. Since the peridermal cells are a distinctly different group of cells from the MEE involved in palatal fusion, their degeneration is not related to the fate of the MEE. In the present study the MEE did not exhibit features associated with cell death and contained intracellular organelles associated with a metabolically active cell.

Similarly, the cessation of DNA synthesis does not necessarily mean that a cell is destined to die. Many epithelial tissues and organs contain cells which are not actively replicating; however these cells retain the ability to reinitiate DNA synthesis and cell division following an appropriate stimulus, (*i.e.*, wounding). The cessation of DNA synthesis in the MEE may be related to the induction of a new pattern of gene transcription which is required for the phenotype transformation.

The intracellular increase in lysosomal enzymes was also observed in a subset of cells in the midline of the palate during fusion (Hayward, 1969; Smiley, 1970; Pratt and Greene, 1976). The presence of degradative enzymes has been observed in both migratory and invasive cells (Liotta *et al.*, 1986; Goldfarb and Liotta, 1986; Tryggvason *et al.*, 1987), and has been associated with the ability of the cell to degrade extracellular matrix molecules and move through the tissue. During palatal fusion the basement membrane underlying the MEE is degraded and the MEE cells do migrate in the mesenchyme. Thus the presence of degradative enzymes can also be associated with EMT.

The cell lineage of the MEE was characterized in the present study by DiI labeling and demonstrated that the MEE, rather than undergoing PCD, underwent a process of EMT with the expression of a mesenchymal phenotype. The ability to definitively identify MEE cells at different stages of palatal fusion will permit characterization of the molecular changes which occur during phenotypic transformation and following exposure to teratogens.

Materials and Methods

Animals

Timed-pregnant Swiss-Webster mice were used for these studies. The females were mated for 3 h, the presence of a vaginal plug established day 0 and hour 0. The animals were maintained under standard conditions. The pregnant females were sacrificed by cervical dislocation at 14 d 3 h and the fetuses removed from the amniotic sacs aseptically and the stage of palatal development determined. Only fetuses with palatal shelves elevated above the tongue were used in these studies.

DiI labeling and organ culture

The epithelium of the palatal shelves was labeled with DiI (Molecular Probes) for cell lineage analysis. The mandibles were removed and the palatal region of the heads was submerged in 0.025% DiI (in normal saline with 1% ethanol) for 30 min at room temperature. The tissues were thereafter rinsed in culture medium to remove unincorporated DiI. Palatal shelves were dissected from 14 d 3 h fetal murine heads. The palatal shelves were placed in pairs on Millipore filters with their medial edges in contact. The palatal shelves were cultured at the air-medium interface in Grobstein organ culture dishes in BGJb medium (Gibco) at 37°C and a 5% CO_2/air atmosphere. The organ cultures were maintained for up to 72 h.

Sampling schedule

The explanted palatal shelves were examined by standard histologic techniques in order to determine the time required for *in vitro* recapitulation of the *in vivo* palatal fusion events. *In vitro*-maintained palatal shelves required 40 h to complete mesenchymal fusion; therefore sampling times were identified in the first 40 h of organ culture, which provided palatal shelf tissue representative of critical stages in the process of palatal fusion. The stages of palatal fusion selected were: adhesion of the medial edges, which occurred within 2 h of the initiation of organ culture; reduction of the MEE to a single layer of cells, which occurred by 8 h *in vitro;* discontinuity and breakdown of the MEE into clumps of cells, which occurred by 20 h *in vitro;* and palatal fusion, which was complete by 40 h *in vitro*. The cultured palatal shelves were either quick frozen for use in DiI/immunohistochemical analyses or fixed for use in transmission electron microscopic examinations.

DiI fluorescent techniques

Frozen sections, 8 μm thick, were prepared from the cultured palatal shelves. The sections were mounted on glass slides and immediately observed for DiI fluorescence with a Zeiss fluorescent microscope at an excitation wavelength of 546 nm and an emission wavelength of 563 nm. The DiI fluorescence was recorded photographically on Ektachrome 400 film. The frozen sections were either used immediately for immunohistochemical analyses or stored frozen for subsequent analysis.

Immunofluorescent techniques

The tissue sections, which had been analyzed for the pattern of DiI distribution, were reacted with one antiserum that was specific for either cell type-specific cytoskeletal elements or components of the epithelial basement membrane. Antisera (Sigma) to the intermediate filament proteins keratin (pancytokeratin) and vimentin and to basement membrane laminin were used to localize the corresponding protein epitopes. The binding of the primary antiserum was detected using FITC-conjugated secondary antisera specific for the immunoglobulin type of the primary antibody. The sections were mounted in aqueous medium, coverslipped and immediately observed for the FITC fluorescence with a Zeiss fluorescent microscope at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The pattern of FITC fluorescence was recorded photographically on Ektachrome 400 film. The sections were thereafter stained with hematoxylin and eosin for histologic examination of the tissue and characterization of the cell phenotype by standard morphologic indices. The presence of cytokeratin intermediate filaments and separation from the mesenchyme by a continuous basement membrane were used as criteria to identify the epithelial histogenesis of specific DiI populations. The presence of vimentin was used to identify the mesenchymal phenotype.

Electron microscopy technique

Palatal shelf tissues were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), for 2 h at room temperature and postfixed in 1% (w/v) osmium tetroxide for 1 h at 4°C. The tissues were dehydrated in graded alcohols, transferred to propylene oxide and embedded in Spurr resin. One-

micron-thick sections were cut to locate the MEE fusion area and subsequently trimmed for thin sectioning. Thin sections were cut, stained with uranyl acetate and lead citrate, and observed with a JEOL 1200 EX electron microscope at 80 kV.

Data analysis

The pattern of DiI fluorescence was compared with: 1) the immunohistochemical localization of the selected phenotype-specific antigens; 2) the light microscopic appearance of the palatal shelves; and 3) the ultrastructural features of the palatal midline cells. The use of identical tissue sections throughout the separate DiI, immunohistochemical and histologic techniques permitted precise characterization of the medial edge epithelial cells. The location of the MEE cells in the tissue at specific stages of development, the molecular features of the MEE cells and their relationship to the underlying mesenchyme could be compared to the temporal sequence of events during palatal fusion *in vivo*.

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