In vitro development of palatal tissues from embryonic mice

II. Tissue isolation and recombination studies

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SUMMARY

The epithelial and mesenchymal tissues of the secondary palate from 12-, 13-, and 14-day embryonic mice were enzymatically separated and cultured in isolation and in homochronic and heterochronic recombinations. In both homochronic and heterochronic recombinations, epithelial differentiation was similar to that in vivo. In heterochronic recombinations, epithelium differentiated according to a schedule appropriate for the age of the epithelium rather than for the age of the mesenchyme, suggesting that differentiation of palatal epithelium is temporally determined as early as 12 days of gestation. Palatal epithelium cultured in isolation was capable of limited differentiation. Potential for differentiation therefore exists within the isolated palatal epithelium at an early stage of palatal development, and epithelial-mesenchymal interactions are required during palatal development to support full epithelial differentiation.

INTRODUCTION

During development, the normal differentiation of complex organs involves interactions between the tissues of these organs. Isolation and growth of tissues in vitro and tissue recombination experiments have demonstrated that the extent to which the tissues of an organ are dependent upon reciprocal interactions varies among different types of tissues, and changes within a single organ during its development (see e.g. Kollar, 1972).

The embryonic mammalian secondary palate is an organ that is of potential interest for studies of tissue interactions. Though the mesenchyme does not have histologically distinct regional differences, the epithelium includes three well-defined regions, nasal, medial, and oral, each with a different developmental fate. The development of this organ therefore allows for comparative studies between different types of epithelia within a single organ system.

In the mouse, the secondary palate first becomes morphologically distinct

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on the 12th day of gestation, at which time it exists as two low bilateral processes extending from the paired maxillary processes. Between the 14th and 15th days of gestation, the palatal processes reorientate from a vertical position alongside the tongue to a horizontal position above the tongue and fuse with one another along their medial surfaces. This fusion brings about the separation of the oral and nasal cavities. The medial-epithelial lamina which is formed between apposing palatal processes disrupts during palatal fusion, and the mesenchymal tissues of the two processes become confluent. By 17 days of gestation, the dorsal epithelium of the palate, which constitutes the floor of the nasal cavity, has differentiated into a pseudostratified ciliated columnar epithelium, and the ventral epithelium, which constitutes the roof of the oral cavity, has differentiated into a stratified squamous epithelium (Vargas, Nasjleti & Azcurra, 1972; Tyler, 1975a; Tyler & Koch, 1975).

The results of both in vivo and in vitro studies have suggested that tissue interactions are important in the differentiation of the secondary palate. The occurrence of cell death within the medial-epithelial lamina between fusing palatal processes has been well documented (Farbman, 1968; Shapiro & Sweeney, 1969; Smiley & Koch, 1971, 1972; Mato, Smiley & Dixon, 1972; Holmstedt & Han, 1973), and certain in vitro recombination experiments have been interpreted as showing that this cell death is induced by the palatal mesenchyme immediately prior to the time of palatal fusion (Pourtois, 1972).

In the present study, epithelial-mesenchymal interactions in the embryonic palate were further investigated through tissue isolation and tissue recombination experiments. The palatal epithelium or palatal mesenchyme were cultured as isolated tissues under various conditions. Their differentiation was also studied by direct and transfilter recombination using homochronic and heterochronic tissue combinations. The results of these experiments support the concept that mesenchymal factors are important for the histogenesis of palatal epithelium and show that palatal epithelium has a potential for limited differentiation in isolation. Brief abstracts of certain aspects of this study have been published (Tyler & Koch, 1974; Tyler, 1975b).

**METHODS**

*Tissue preparation*

The tissues used in this study were taken from embryonic mice of strains C57BL and Brown Belt. The adult animals were mated between 9.00 and 11.00 a.m.; the day on which copulation plugs were observed was designated as day zero of gestation. Pregnant females were killed at 9.00 a.m. on the day that tissues were dissected. Fetuses were aseptically removed from the uterus and the organs to be used were immediately dissected in a mixture of Tyrode’s solution and horse serum (1:1, v/v). In the course of the study, the epithelium and mesenchyme from the secondary palate of 12-, 13-, and 14-day embryos, and
the epithelium from the tongue, foot-pad, and nasal cavity of 12- to 14-day embryos, were used.

Separation of the epithelial and mesenchymal tissues of an organ rudiment was achieved by treatment with a 3% trypsin-pancreatin solution (3:1; w/w in calcium- and magnesium-free Tyrode's solution). Organ rudiments were incubated in this enzyme solution at 4 °C; young rudiments were incubated for 30 min, older rudiments were incubated for as long as 60 min. After enzymatic treatment, the organ rudiments were transferred to the Tyrode's-serum mixture. Agitation with a small-bore pipette or gentle manipulation with cataract knives was then sufficient to separate the loosened epithelium from the mesenchyme.

**Culturing procedures**

Tissues were placed on Millipore filters (0.45 μm porosity and 25 ± μm thick, from the Millipore Filter Corp., Bedford, Massachusetts), supported by plexiglass rings (Grobstein, 1956), and incubated in contact with a complex culture medium (Eagle's basal medium supplemented with 1% glutamine, 3% 11-day chick-embryo extract, 10% horse serum, and 100 units/ml of penicillin and streptomycin). Cultures were maintained at 37.5 °C in a humidified incubator gassed with 5% CO₂ in air. The culture medium was replaced every 48-72 h. The living cultures were photographed at 24 h intervals.

**Culture of isolated tissues**

Isolated palatal epithelium was explanted onto various types of substrate and orientated with either its apical or basal surface against the substrate. Epithelium with its apical surface against a Millipore filter was cultured either with or without an overlying plasma clot. Epithelium with its basal surface against the substrate was cultured in contact with (1) a Millipore filter, (2) a collagen gel, (3) a plasma clot, or (4) nutrient agar. Isolated palatal mesenchyme was explanted onto Millipore filters.

The behavior in culture of isolated palatal epithelium was compared with that of non-palatal epithelia, i.e. epithelia from embryonic tongue, nasal cavity, and foot-pad, grown on Millipore filters either with or without an overlying plasma clot.

Collagen gels were made from acetic acid extracts of collagen, extracted from adult rat tail tendons (Ehrmann & Gey, 1956), and 'gelled' by exposure to ammonium vapors for 30 min (Simkovic, 1959). Plasma clots were made from unheparinized chicken plasma mixed immediately before use with a 30% solution of embryo extract in Tyrode's solution (1:1, v/v). Agar substrates were made from a 1% agar solution diluted with culture medium (2:1, v/v) immediately before use. Collagen, plasma and agar substrates were supported by the Millipore filters of culture assemblies.
Culture of recombinant tissues

Recombinations of palatal epithelium and palatal mesenchyme were made in both homochronic and heterochronic combinations. In homochronic combinations, palatal epithelium and mesenchyme of the same embryonic age were recombined either as direct recombinants (in which the epithelium was placed in direct contact with the mesenchyme) or as transfilter recombinants (in which the two tissues were separated by the Millipore filter of a culture assembly). In transfilter recombinations, the mesenchyme was positioned on the lower surface of the filter such that either its oral surface or its nasal surface was against the filter; the mesenchyme was held in place with a plasma clot and its orientation was recorded. Epithelium was positioned with its basal side against the upper surface of the filter directly opposite the mesenchyme.

Heterochronic recombinations were made between 12- and 14-day palatal tissues: 12-day palatal epithelium was cultured transfilter to 14-day palatal mesenchyme, and 14-day palatal epithelium was cultured transfilter to 12-day palatal mesenchyme.

Histological procedures

After 1–5 days of incubation, representative cultures were fixed in Bouin’s fluid. Fixed cultures were embedded in paraffin blocks, and the 4 μm serial sections cut from these blocks were stained with either hematoxylin, eosin and alcian blue (pH 2.7–3.0), or by the McManus method for the periodic acid–Schiff reaction (Pearse, 1960).

Birefringent elements within various epithelia were detected using a Zeiss polarizing microscope. The sign of birefringence, (+) or (−), was determined with a first-order red-retardation plate (Schmitt, 1944); the surface of the epithelium was used as reference for the retardation colors observed, and positive birefringence was determined as being parallel to the epithelial surface.

The results are based upon 321 cultures of isolated epithelia, 26 cultures of isolated mesenchyme, 81 cultures of homochronic recombinations, and 35 cultures of heterochronic recombinations.

RESULTS

Isolated palatal epithelium

Both the age of the tissue and the organ culture conditions had an effect upon the growth of isolated palatal epithelium.

Millipore filter substrate

Palatal epithelium from 13- and 14-day embryos, positioned with its basal surface against a Millipore filter, adhered to the substrate and spread to a moderate extent during the culture period. Spreading was not uniform throughout the epithelial sheet. In over 90% of the cases, the nasal epithelium spread
to approximately four times its original size while the oral epithelium spread little or not at all (Fig. 1A–D). In several cases, the oral epithelium retracted into a dense nodule of tissue (Fig. 1D). The medial-epithelial region became constricted between 24 and 48 h of incubation, and, during subsequent incubation, often became so constricted that only a narrow neck of cells remained between the oral and nasal epithelia (Fig. 1D). Histological examination revealed this medial constriction to be a region of cellular debris (Fig. 1I). The nasal epithelium developed into cuboidal cells, many of which were ciliated (Fig. 1K). The oral epithelium developed into a sheet of squamous cells varying in thickness from 1 to 5 cell layers (Fig. 1L). Under polarized light, these squamous cell layers displayed positive birefringence.

Twelve-day palatal epithelium, grown with its basal surface against a Millipore filter, did not spread as a cell sheet. Instead, it retracted, and peripheral cells migrated outward as individual cells or as cellular aggregates. Histological examination showed that cells of the nasal epithelium became squamous to rounded ciliated cells, and cells of the oral epithelium organized into layers of squamous cells which displayed positive birefringence under polarized light (Figs. 1M, N).

Palatal epithelium, from all age groups tested, positioned with its apical surface against a Millipore filter, failed to adhere or to spread upon the substrate; instead, by 24 h of incubation, it curled into a rounded mass. A plasma clot placed over epithelium so positioned permitted extensive spreading of 13- and 14-day palatal epithelium (Fig. 1E–H). During the incubation period, an interruption occurred in the epithelial sheet in the medial region, and within 24 h of its appearance, extended the length of the medial region. The time of appearance of this discontinuity corresponded to the time of in vivo medial epithelial disruption. Histological examination of these cultures revealed that the region of discontinuity contained only cellular debris or cells with pycnotic nuclei (as in Fig. 1I). Oral and nasal epithelial differentiation was similar to that described for 13- and 14-day palatal epithelium cultured with its basal surface against a Millipore filter. The cilia of the nasal epithelial cells were located on the surface which corresponded to the initial apical surface of these cells, i.e. the surface facing the Millipore filter substrate.

The behavior of 12-day palatal epithelium differed from that described above for 13- and 14-day palatal epithelium when positioned with its apical surface against a filter and covered with a plasma clot. The epithelium did not spread as an epithelial sheet but dispersed into small cell clusters. Histological examination showed that a large percentage of the epithelial cells died under these culture conditions; cell death was generally not confined to the medial region. In a few cultures, however, cellular debris could be seen between an intact nasal and oral epithelium (Fig. 1J); in these cases, the differentiation of the nasal and oral regions was similar to that of 12-day palatal epithelium cultured with its basal surface against a Millipore filter.
Fig. 1. For legend see facing page
Collagen-gel substrate

In cultures of palatal epithelium on a collagen gel, the opacity of the substrate interfered with observations of the living cultures. Fixed and stained whole-mount preparations, however, showed that the oral and nasal palatal epithelium of all age groups tested spread extensively, and the medial region thinned (Fig. 2A). Histological sections showed that the nasal epithelium varied from a ciliated cuboidal to a ciliated columnar epithelium (Fig. 2C). The oral epithelium was organized as a stratified epithelium of squamous cells (4–12 cell layers thick) (Fig. 2D) in which only the outer squamous cell layers were birefringent. Birefringence of the oral epithelium occurred no earlier in vitro than in vivo (Fig. 2E, F).

Figure 1

(A–D) A series of photographs showing in vitro development of isolated 14-day palatal epithelium cultured with its basal surface against a Millipore filter. The photographs show the living culture at 0, 24, 48, and 72 h of incubation. At 0 h (Fig. A), the medial region is indicated by an anterior and posterior notch; the oral epithelium (oe) is distinguished by the presence of rugae; the nasal epithelium is indicated by ne. At 48 h (Fig. C), the medial region is becoming constricted. At 72 h (Fig. D), the constriction within the medial region is extreme; the oral epithelium is a dense nodule of tissue, and the nasal epithelium is a flattened sheet. × 25.

(E–H) A series of photographs showing in vitro development of isolated 14-day palatal epithelium cultured with its apical surface against a Millipore filter and covered with a plasma clot. The photographs show the living culture at 0, 24, 48, and 72 h of incubation. The medial region is indicated by two hairs that were positioned anteriorly and posteriorly at the beginning of the culture period. At 0 h (Fig. E), the oral epithelium (oe) is distinguishable by the presence of rugae; the nasal epithelium is indicated by ne. At 48 h (Fig. G), a discontinuity is present in the medial region of the epithelial sheet. By 72 h (Fig. H), the discontinuity extends the length of the medial region. × 25.

(I) Photomicrograph of a transverse section through isolated 14-day palatal epithelium positioned with its basal surface against a Millipore filter and cultured for 48 h. Within the medial region is cellular debris. The nasal epithelial cells are rounded and sparsely ciliated. (Hematoxylin, eosin and alcian blue.) × 480.

(J) Photomicrograph of a transverse section through isolated 12-day palatal epithelium positioned with its apical surface against a Millipore filter, covered with a plasma clot, and cultured for 120 h, showing in one of the cultures that was viable that cellular debris is present within the medial region. (Hematoxylin, eosin and alcian blue.) × 300.

(K–L) Photomicrographs of a section through the nasal region (Fig. K) and the oral region (Fig. L) of isolated 14-day palatal epithelium positioned with its basal surface against a Millipore filter and cultured for 72 h. (Hematoxylin, eosin and alcian blue.) × 1200.

(M–N) Photomicrographs of a section through the nasal region (Fig. M) and the oral region (Fig. N) of isolated 12-day palatal epithelium positioned with its basal surface against a Millipore filter and cultured for 120 h. Epithelial differentiation is less advanced than that of similarly cultured 14-day palatal epithelium shown in Fig. 1 K–L. (Hematoxylin, eosin and alcian blue.) × 1200.
Fig. 2. For legend see facing page
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Plasma-clot substrate

Palatal epithelium of all age groups tested spread extensively when grown with its basal surface against a plasma clot. The medial epithelium became opaque and uneven in appearance at a time which corresponded to the time of in vivo medial epithelial disruption (Fig. 2B); in histological preparations, squamous cells and cellular debris were noted in this region. The nasal epithelium differentiated into a ciliated cuboidal to columnar cell sheet, and the oral epithelium became layers of squamous cells (6-12 cell layers thick) which displayed positive birefringence under polarized light. Thus epithelial differentiation was similar to that of palatal epithelium cultured on a collagen gel.

Agar substrate

Isolated palatal epithelium, explanted with its basal surface against a nutrient agar substrate, did not survive.

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Figure 2

(A) Photograph of a fixed and stained culture of isolated 14-day palatal epithelium positioned with its basal surface against a collagen gel and incubated for 72 h. The oral epithelium (oe) and nasal epithelium (ne) have spread extensively. The medial region is thinned and transparent. (Eosin.) × 30.

(B) Photomicrograph of living isolated 12-day palatal epithelium cultured with its basal surface against a plasma clot for 72 h showing that the medial region (arrow) has become darkened, a modification which was shown in histological sections to be caused by the presence of cellular debris within this region. × 90.

(C-D) Photomicrographs of a section through the nasal region (Fig. C) and oral region (Fig. D) of isolated 14-day palatal epithelium positioned with its basal surface against a collagen gel and cultured for 27 h. Epithelial differentiation is more advanced than that of isolated palatal epithelium cultured on a Millipore filter (as shown in Fig. 1 K-N). (Hematoxylin, eosin and alcian blue.) × 1200.

(E-F) Polarization photomicrographs of sections through the oral epithelium of isolated 12-day palatal epithelia positioned with their basal surfaces against collagen gel and cultured for 48 and 96 h respectively. At 48 h (Fig. E), the oral epithelium consists of layers of squamous cells but exhibits no birefringence under polarized light. The oral epithelium first displays birefringence at 96 h of incubation (Fig. F). (Hematoxylin, eosin and alcian blue.) × 480.

(G-J) A series of photographs showing in vitro development of isolated 13-day palatal mesenchyme at 0, 24, 48, and 72 h of incubation, respectively. The differentiation of membrane bone (b) and cartilage (c) is distinguishable. The anterior end of the mesenchyme is to the left. × 26.

(K-L) Photomicrographs of transverse sections through isolated 14-day palatal mesenchyme cultured for 72 h showing the eosin-staining bony trabecula that forms anteriorly (Fig. K), and the alcian blue-staining cartilage (Fig. L) that forms posteriorly from a chondrogenic region that was included in the explant. (Hematoxylin, eosin and alcian blue.) × 300.
Isolated non-palatal epithelia

In cultures of non-palatal epithelia (tongue, nasal cavity, and foot-pad) grown either with their basal surfaces against a Millipore filter or with their apical surfaces against a Millipore filter and held with a plasma clot, no constriction or disruption of the central region occurred; instead, spreading was uniform.

Isolated palatal mesenchyme

Palatal mesenchyme grown in isolation on Millipore filters progressively spread and flattened during the culture period (Fig. 2G–J). In histological sections, mitotic figures were seen throughout the mesenchyme.

Membrane bone and cartilage formed in the cultured isolated mesenchyme of all age groups tested. The timing of bone formation was approximately 24 h in advance of that which occurs in vivo. Cartilage formation, not normally seen in the palate in vivo, occurred 24 h prior to bone formation and was restricted to a region posterior to the region of bone formation (Fig. 2K, L).

Homochronic direct recombinations of palatal tissues

In homochronic direct recombinations, the epithelium spread on the surface of the mesenchyme, but its edges often curled to form epithelial vesicles. In all cases, epithelial and mesenchymal differentiation was similar to that of cultured intact palatal processes (see Tyler & Koch, 1975). After incubation periods which brought the tissues to an equivalent of 17 days of gestation (72 h for recombinations of 14-day tissues, 96 h for recombinations of 13-day tissues, and 120 h for recombinations of 12-day tissues), the nasal epithelium had become a pseudostratified ciliated columnar epithelium, similar in height and ciliation to the nasal epithelium of intact palatal processes cultured for similar times; the oral epithelium had become a stratified squamous epithelium similar in thickness and histology to that of the corresponding cultured intact palatal processes, and in the mesenchymal tissue, bone and cartilage formed in a pattern similar to that of cultured intact palatal processes.

Homochronic transfilter recombinations

Differentiation was similar among homochronic transfilter recombinations of 12-, 13-, and 14-day palatal tissues. In addition, regardless of the age of the tissues, palatal epithelium cultured transfilter to the oral surface of palatal mesenchyme (Fig. 3A) differentiated in a pattern identical to that of palatal epithelium cultured transfilter to the nasal surface of palatal mesenchyme (Fig. 3B, C). In each case, the nasal epithelium differentiated into a pseudostratified ciliated columnar epithelium and the oral epithelium differentiated into a birefringent (+) stratified squamous epithelium (Fig. 3D) similar to that of cultured intact palatal processes. Within the medial epithelial region there was an area of cellular debris; the nasal and oral epithelia overlapped one
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another in this region (as in Fig. 3A). Within the mesenchyme, bone and cartilage formed in a pattern similar to that of isolated palatal mesenchyme. The pattern was independent of the orientation of the mesenchyme.

Heterochronic recombinations of palatal tissues

Necrotic cells and cellular debris were present within the medial epithelial region of 12-day palatal epithelium grown transfilter to 14-day palatal mesenchyme by 72 h of incubation (Fig. 3E). This corresponds to the time that medial epithelial disruption occurs in cultures of intact 12-day palatal processes. The nasal and oral epithelia at this time were undifferentiated, and the oral epithelium exhibited no birefringence. By 120 h of incubation, the oral epithelium was a stratified squamous epithelium (Fig. 3F) with positive birefringence in its outer cell layers, but was slightly thinner than that of intact 12-day palatal processes, cultured for a similar time, because of fewer squamous cell layers. The nasal epithelium had differentiated into a pseudostratified ciliated columnar epithelium (Fig. 3G) similar in height and ciliation to that of intact 12-day palatal processes cultured for a similar time. The oral and nasal epithelia overlapped within the medial region.

In the converse heterochronic recombination, the timing of epithelial differentiation again reflected the original age of the epithelium rather than that of the mesenchyme. Differentiation of 14-day palatal epithelium cultured transfilter to 12-day palatal mesenchyme was similar both in its histology and timing to that of cultured intact 14-day palatal processes. As in homochronic transfilter recombinations, the oral and nasal epithelia overlapped one another within the medial region (Fig. 3H).

DISCUSSION

The results suggest that isolated palatal epithelium exhibits a capacity for limited differentiation and that complete epithelial differentiation is fostered by the presence of mesenchyme. The potential for histological differentiation proved to be inherent within the palatal epithelium by at least 12 days of gestation. (Younger palatal epithelium was not tested due to size limitations and to a significant decline of epithelial viability in culture.) Furthermore, it appears that regression of the medial palatal epithelium is a ‘programmed’ event as had been previously suggested (Shapiro, 1968; Shapiro & Sweney, 1969). Since cell death in vitro may be the result of adverse environmental conditions, it is important to confirm that this particular cell death is in fact a result of intrinsic rather than extrinsic factors. Several observations are relevant:

(1) In viable cultures, cellular debris and cells with pyknotic nuclei were restricted to the medial epithelial region while the cells of the nasal and oral epithelia appeared healthy.

(2) The time of cell death within the medial region was not correlated with
the length of the culture period but corresponded to the time of *in vivo* medial epithelial disruption.

(3) In cultures in which unsuitable culture conditions existed, as for example on agar, the cells of the medial region displayed no greater sensitivity to the adverse culture conditions than did the cells of the oral and nasal regions. Instead, under such conditions, cell death occurred first at the periphery of the cell sheet, and was directly correlated to the length of the culture period.

(4) Cultured epithelia from sources other than the palate (tongue, nasal cavity and foot-pad) did not display the pattern of cell death observed in cultured palatal epithelium.

Cell death as a mechanism of morphogenesis is not unusual, for example in eliminating various transient tissues and organs during development (see e.g. Glücksmann, 1951; Saunders, 1966). In the case of the palate, it appears that programmed cell death assists in the removal of the medial palatal epithelium.

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**Figure 3**

(A) Photomicrograph of a transverse section through a transfilter recombination between 14-day palatal epithelium and 14-day palatal mesenchyme cultured for 72 h showing that within the medial region there is cellular debris (arrow). The nasal epithelium, a pseudostratified ciliated columnar epithelium, and the oral epithelium, a stratified squamous epithelium, overlap within the medial region. The oral surface of the mesenchyme is against the filter. (Periodic acid–Schiff reaction.) × 480.

(B–C) Photomicrographs of a transverse section through a transfilter recombination between 14-day palatal epithelium and 14-day palatal mesenchyme in which the nasal surface of the mesenchyme is against the filter. The culture was incubated for 72 h, and the differentiation of the nasal epithelium (Fig. B) and the oral epithelium (Fig. C) is similar to that of 14-day palatal epithelium cultured transfilter to the oral surface of palatal mesenchyme (shown in Fig. 3 A). (Hematoxylin, eosin and alcian blue.) × 480.

(D) Polarization photomicrograph of the oral epithelium shown in Fig. 3B. The outer squamous cell layers of the epithelium are birefringent under polarized light. × 660.

(E) Photomicrograph of a transverse section through a heterochronic transfilter recombination between 12-day palatal epithelium and 14-day palatal mesenchyme cultured for 72 h. The medial epithelial region is disrupted and the oral and nasal epithelia are undifferentiated. (Hematoxylin, eosin and alcian blue.) × 300.

(F–G) Photomicrographs of a transverse section through a transfilter recombination between 12-day palatal epithelium and 14-day palatal mesenchyme cultured for 120 h. The oral epithelium (Fig. F) is a thin stratified squamous epithelium (slightly thinner than that shown in Fig. 3 B). The nasal epithelium (Fig. G) is a pseudostratified ciliated columnar epithelium (similar to that shown in Fig. 3 C). (Hematoxylin, eosin and alcian blue.) × 480.

(H) Photomicrograph of a transverse section through a heterochronic transfilter recombination between 14-day palatal epithelium and 12-day palatal mesenchyme cultured for 72 h showing the medial region where there is overlapping of the nasal and oral epithelia. The differentiation of the nasal and oral epithelia is similar to that of homochronic transfilter recombinations shown in Fig. 3 A–C. (Hematoxylin, eosin and alcian blue.) × 660.
The results of this study indicate that this program is determined in the medial epithelium of the embryonic mouse palate by 12 days of gestation and is not dependent thereafter upon the presence of mesenchyme for its expression.

These results conflict with an earlier report (Pourtois, 1972) in which it was concluded that the medial palatal epithelial death is induced by palatal mesenchyme immediately prior to the time of the disruption. Pourtois (1972) reported that epithelial disruption occurred in heterochronic recombinations of palatal tissues only if the palatal mesenchyme had been excised at the time of *in vivo* palatal fusion; palatal epithelium recombined with young palatal mesenchyme was said to remain intact. Unfortunately, detailed methods of that study have not been reported; the region of palatal mesenchyme thought to induce epithelial disruption was not defined, nor is it possible to confirm the specific region of palatal epithelium that was tested. The culture conditions used differed from those of the present study, and a recent report has shown that the culture conditions under which palatal processes are grown can affect both the behavior and the ontogeny of the explant (Smiley and Koch, 1975).

Isolated nasal and oral palatal epithelium exhibited a potential for differentiation *in vitro* even when isolated as early as 12 days of gestation. The nasal epithelium differentiated into a ciliated sheet of usually cuboidal, but sometimes columnar, cells. The oral epithelium became organized into layers of squamous cells with positive birefringence.

Birefringence, though not in itself a definitive criterion for differentiation, is typically used as a diagnostic criterion for keratinization (Rothman, 1954; Matoltsy, 1958; Wolman, 1975). It is difficult, however, to distinguish between intrinsic birefringence, which is due to the ordered arrangement of molecular moieties, and form birefringence, which is due to the ordered arrangement of objects within a medium of a different refractive index (Wolman, 1975). Several observations indicate that the oral region of isolated palatal epithelium displayed intrinsic rather than form birefringence:

1. The birefringence of the isolated oral epithelium was orientated parallel to the epithelial surface as it is in the fully differentiated oral epithelium.
2. Birefringence in the isolated epithelium became discernible at the same time as in the oral epithelium of the *in vivo* palate.
3. The appearance of birefringence in isolated oral epithelium was not coincident with a change in cell shape. In isolation, the oral epithelium became layers of squamous cells at least 24 h prior to the first appearance of birefringence.

Differences in the manner in which isolated palatal epithelium responded to various substrates demonstrated that the substrate can influence both the behavior and the differentiation of the epithelium. An agar substrate was found to be unsuitable for maintaining palatal epithelium. Similar results have been reported for epidermis (Dodson, 1967) and for corneal epithelium (Meier & Hay, 1974). The substrates which readily supported palatal epithelial differ-
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entiation were collagen gels and plasma clots. Both of these represent to a certain degree naturally occurring substrates: collagen is a major component of the basal lamina (Kahl & Pearson, 1967), and plasma clots serve as epidermal substrates during wound healing. The present study therefore lends support to recent investigations that have implicated collagen as playing a major role in epithelial behavior and differentiation (Grobstein & Cohen, 1965; Wessells & Cohen, 1966, 1968; Bernfield & Wessells, 1970; Hay, 1973; Meier & Hay, 1974).

Isolation studies suggested that palatal mesenchyme is capable of forming both bone and cartilage in the absence of continuous epithelial influences. The sum of the isolation and recombination studies indicates that the presence of epithelium does not influence the pattern or timing of the bone and cartilage formation for the age groups tested. In these respects, this system differs from that of the urodele limb-bud (Wilde, 1948) and human fetal long bones (Zaaijer, 1958), in which it has been shown that an epithelium is necessary for in vitro cartilage formation, and differs from that of the fetal mouse pubic joint in which it was reported that the epithelial covering inhibits in vitro cartilage formation (Crelin & Koch, 1965).

Since cartilage does not normally form in the in vivo palate, its presence in the mesenchyme of cultured palatal processes, noted by several investigators (Smiley & Koch, 1975, Fig. 8; Pratt, personal communication), bears explanation. Histological examination of a series of embryonic mouse heads has revealed that there is a region dorso-lateral to the posterior palate, probably one of the condensation centers of the basi-sphenoid (Hall, personal communication), in which cartilage develops at a time which corresponds to the time of cartilage formation in cultured palatal rudiments (Tyler, 1975a). It is therefore probable that at the time of palatal extirpation, a portion of this chondrogenic region is included in the explant, and that it is from this region that cartilage formation in cultured palatal rudiments is initiated.

The results from homochronic direct recombinations indicated that the separation procedures did not cause irreparable damage to the tissues or disrupt the timing of differentiation, since the tissues, when recombined, showed normal differentiation according to the in vivo schedule. Transfilter recombinations permitted precise positioning of the mesenchyme with respect to the epithelium; whether the nasal or the oral surface was in association with the epithelium, the mesenchyme supported full differentiation of 12- to 14-day palatal epithelium. These results indicate that in the mouse, as early as 12 days of gestation, the three epithelial regions of the palate do not have a highly specific requirement for their own particular type of mesenchyme in order to differentiate normally. Heterotypic recombinations between palatal epithelium and non-palatal mesenchyme support this statement (Tyler & Koch, 1976). In this respect, palatal epithelium is similar to epithelia such as that of the pancreas (Golosow & Grobstein, 1962) and thymus (Auerbach, 1960) which, when challenged with different types of mesenchyme, differentiate according to their originally determined pathways.
The mesenchymal factors necessary to support full differentiation of palatal epithelium were provided under both direct and transfilter recombination conditions. Whether or not this indicates that the epithelium does not require cellular contact with the mesenchyme in order to differentiate is still an unresolved question. Though it has been concluded from previous transfilter studies (Grobstein, 1956, 1957; Grobstein & Dalton, 1957; Koch & Grobstein, 1963; Meier & Hay, 1974) that transfilter tissue interactions are the result of the transfer of diffusible materials across the filter membrane, this conclusion has recently been contested (Nordling, Miettinen, Wartiovaara & Saxén, 1971; Wartiovaara, Nordling, Lehtonen & Saxén, 1974; Lehtonen, Wartiovaara, Nordling & Saxén, 1975), since cellular contacts occur in vivo between interacting tissues (e.g. Lehtonen, 1975).

Heterochronic recombinations tested the temporal stability of epithelial differentiation in the palate. The results showed that epithelial differentiation occurred on a schedule appropriate for the age of the epithelium rather than for the age of the mesenchyme, indicating that, within the parameters tested, the schedule of epithelial differentiation in the embryonic mouse palate is independent of mesenchymal influences by 12 days of gestation.

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