# OCCLUDING JUNCTIONS AND CELL BEHAVIOR

# IN PRIMARY CULTURES OF NORMAL

# AND NEOPLASTIC MAMMARY GLAND CELLS

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ABSTRACT

Cells dissociated from normal prelactating mouse mammary glands or from spontaneous mammary adenocarcinomas, when grown at high density on an impermeable substrate, form nonproliferating, confluent, epithelial pavements in which turgid, blister-like domes appear as a result of fluid accumulation beneath the cell layer. To compare the structure of the fluid-segregating cell associations in normal and tumor cell cultures with that of lactating gland in vivo, we have examined such cultures alive and in thick and thin sections and freeze-fracture replicas. Pavement cells in all cases are polarized toward the bulk medium as a lumen equivalent, with microvilli and continuous, well-developed occluding junctions at this surface. Between the pavement and the substrate are other cells, of parenchymal or stromal origin, scattered or in loose piles; these sequestered cells are relatively unpolarized and never possess occluding junctions. Small gap junctions have been found in the pavement layer, and desmosomes may link epithelial cells in any location. Under the culture conditions used, development of the epithelial secretory apparatus is not demonstrable; normal and neoplastic cells do not differ consistently in any property examined. A dome's roof is merely a raised part of the epithelial pavement and does not differ from the latter in either cell or junction structure. We suggest that dome formation demonstrates the persistence of some transport functions and of the capacity to form effective occluding junctions. These basic epithelial properties can survive both neoplastic transformation and transition to culture.

In confluent, high-density cell culture, normal or neoplastic epithelial cells from several mammalian sources spontaneously form multicellular, blister-like, hollow domes (2, 9, 14, 19, 24, 32, 39, 40, 48–50, 58). Leighton et al. (32, 33) recorded by time-lapse photography of MDCK (canine kidney cell line) cultures the growth, rupture, and collapse of domes, "like gently boiling oatmeal." McGrath et al. (39, 41), using primary cultures of mouse mammary tumor cells, found that replication and release of mammary tumor virus (MTV) were highest under conditions that also promoted dome formation and that these conditions included, besides high cell density, insulin and hydrocortisone in the medium. The authors suggested for both the kidney and mammary cells that fluid pressure in the dome cavity was maintained by active secretion, presumably related to the process

occurring in the normal tissue of origin. Confirmation of fluid transport capabilities of cultured cells has recently been provided by Misfeldt et al. (43): confluent MDCK cultures on membrane filters clamped in Ussing chambers exhibited transepithelial potential difference, resistance, and polarized water flux comparable to values reported for intact epithelial organs.

Fluid secretion in lactating mammary gland (35) and release of MTV virions in infected glands and tumors (41) are polarized activities characteristic of relatively differentiated cells forming alveoli. Normal cells of alveoli and ducts are linked by occluding junctions (51), which provide the paracellular permeability barrier essential for effective polarized transport. To determine to what extent cultured cells resemble mammary epithelium in structural organization, we have examined dome-forming primary cultures of cells dissociated from normal mammary glands and mammary adenocarcinomas,[1] with particular attention to morphological polarity and cell junctions. We report here that occluding junctions and other surface features of a transporting epithelium, polarized toward the culture medium, are equally displayed in cultures from both sources by cells forming a confluent surface sheet; the many cells lying beneath this layer are minimally polarized and nonconfluent; and cells in domes are not different from neighboring nondome cells. Some of our observations have been presented briefly elsewhere (22).

The structure and function of membrane junc-

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[1] Mouse mammary adenocarcinoma is considered to be nonmalignant, as it rarely is observed to invade adjacent nonmammary tissue or to metastasize. Surgical or other "cure" of the primary tumor is not often attempted, however, and death from the primary lesion occurs soon enough that early metastatic foci could go undetected. This possibility gains some support from published data from experiments designed to detect metastases: in mice of various C3H sublines, reported incidences of spontaneous pulmonary metastasis from untreated primary tumors reach 28% (56) and 70% (57); from untreated first-generation implants, 80% (57%); and after surgical removal of first-generation implants, 31% (53) and 35% (57). Preliminary results of experiments in progress by one of us (D. R. Pitelka) suggest that both metastasis and invasion by mammary tumors occur more frequently in C3H/Crgl mice than has been recognized. Consideration of potential malignancy thus requires more data than are yet available; the present paper is concerned only with the neoplastic status of the mammary tumor cells.

tions (i.e. gap and tight junctions, intimate appositions involving modifications in structure of the joined membranes) have been frequently reviewed and will not be described here (see 4, 8, 51, 54, 55). In agreement with Bennett (4), we will use "occluding junction" for the continuous tight-junction bands that surround the apical surfaces of epithelial cells, joining cells in a single-layered mosaic, as opposed to focal or discontinuous tight junctions.

## MATERIALS AND METHODS
### Dissociation and Culturing Procedures

Spontaneous mammary tumors from BALB/cfC3H/ Crgl or from C3H/Crgl multiparous female mice were used singly or pooled (three to five tumors), minced, and dissociated in trypsin-Versene as described by McGrath (39). Normal mammary glands from 14- to 19-day pregnant females of the above strains or BALB/cCrgl were minced and dissociated by the collagenase method of Lasfargues (31). The cell suspension was washed, filtered through 150-$\mu$m mesh Nitex cloth (Tobler, Ernst and Traber, Inc., Elmsford, N. Y.) in all cases and, additionally, through 10-$\mu$m mesh Nitex for accurate cell counting, counted in a hemocytometer, and plated at high density ($\sim$5 × 10$^5$ cells/cm$^2$ of substrate) in 35-mm Falcon plastic Petri dishes in Dulbecco's modification of Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.), supplemented with 2 mM glutamine, 15% fetal calf serum, insulin 10 $\mu$g/ml, hydrocortisone 5 $\mu$g/ml, penicillin 100 U/ml, and streptomycin 100 $\mu$g/ ml. The medium was replaced every 2-3 days. Nonepithelial cultures were prepared from gland-free mammary fat pads (11) of 6-wk old virgin mice by the procedure used for normal gland. In addition to our cultures, we examined cultures from strains RIII/Crgl, A/Crgl, and GR/Crgl, prepared by others in this laboratory. No significant differences in cell or culture morphology were noted among the different mouse strains.

Tumorigenicity of cultured cells was tested by subcutaneous injection into isogenic mice of 10$^5$ cells from 14-day old primary normal and tumor cultures. Tumors developed from six of six tumor cell implants and from neither of two normal cell implants.

### Processing of Cells for Microscopy

Petri dish cultures were fixed in situ in modified (1% paraformaldehyde, 3% glutaraldehyde, 0.1 M Na cacodylate) Karnovsky's fluid (28) for 30 min at room temperature, postfixed 1 h in cacodylate-buffered osmium tetroxide, stained in 0.5% uranyl acetate in Veronal-acetate buffer for 30 min, dehydrated in ethanol, and embedded in a thin layer of Epon. The polymerized Epon disk was freed by breaking away the plastic dish. For some cultures, tannic acid (52) was introduced during fixation.

For freeze-fracturing, cultures fixed for 10-15 min in modified Karnovsky fixative were scraped from the dish with a rubber policeman. Clumped cell sheets were soaked in 20% glycerol, frozen in Freon at liquid $N_2$ temperature, and fractured and replicated at $-115°C$ (44) in a Balzer's freeze-fracture apparatus. In later experiments, small areas scraped from fixed, glycerine-soaked cultures were sandwiched between metal surfaces before freezing, as described elsewhere (22).

## Microscopy of Cultured Cells

Living cells in Petri dishes were observed with phase contrast in a Zeiss inverted microscope; cells on glass cover slips placed in some of the dishes were examined and photographed in a Zeiss Photomicroscope II equipped with phase-contrast and Nomarski differential interference-contrast optics. Cultures embedded in Epon disks were examined in a stereomicroscope and the Photomicroscope. Selected areas of the disk were cut out with a razor blade, reembedded in Epon, and sectioned perpendicular to the cell layer. Thick sections, stained with toluidine blue, were examined and photographed in the Photomicroscope, and thin sections, stained with uranyl acetate and lead citrate, in a Siemens Elmiskop 1 or Elmiskop 102.

We have examined thick sections of one to several individual cultures from each of 31 different tumor dissociations and eight normal-gland dissociations, thin sections of one or more cultures from 19 of the tumor and five of the normal dissociations, and freeze-fracture replicas from eight of the tumor and five normal dissociations.

## RESULTS

### Growth Pattern and Morphology of Living Cultures

Mouse mammary cells plated at a density of $\sim 5 \times 10^5$ cells/cm$^2$ typically reach confluence within 1-2 days. Cell counts, measurements of DNA synthesis, and mitotic indices (9, 24) indicate that those cells of the mixed epithelial-stromal inoculum that settle out and adhere to the dish undergo at most one or two rounds of division before reaching a saturation density that is not appreciably higher than the plating density. Domes begin to appear shortly thereafter.

Confluent living cultures of either normal or tumor cells appear to be typical epithelial pavements of close-fitting polygonal cells (Fig. 1). Domes during the first 2 wk of culture usually are less than 2 mm in diameter, roughly hemispherical, turgid, with distinct margins: they collapse when ruptured with a fine microneedle. On the substrate beneath the dome, flattened cells may be present singly or in a discontinuous layer. Domes form on glass or plastic, but they do not form on the permeable substrate provided by a membrane filter (33, 43).

Irregularities that sometimes develop in older cultures include fusion of domes to form large, shallow blisters, the appearance of additional bulbous or tubular expansions from the roofs of domes, or the freeing of cell sheets that roll up and rearrange themselves as strings of spherical cysts.

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Figs. 1-3, 12, and 13 are light micrographs; all others are electron micrographs. All sections illustrated are cut perpendicular to the culture substrate. Figs. 14, 15, and 17-22 are freeze-fracture replicas. The circled arrow on these designates the shadowing direction.

FIGURE 1    Living tumor cells on a glass cover slip, 3 days in culture. The polygonal cells form a completely confluent pavement. Nomarski optics. $\times$ 220.

FIGURE 2    Thick section of a 4-day culture of normal cells. Under the continuous upper cell layer are other cells, single or loosely piled. Toluidine blue stain, bright-field optics. $\times$ 560.

FIGURE 3    Thick section of a 4-day culture of tumor cells. Two nucleated cell bodies and cytoplasmic areas of other cells lie under the continuous upper layer. Toluidine blue stain, Nomarski optics. $\times$ 1,000.

FIGURE 4    3-day tumor cell culture. Cell packing is relatively close; slender processes from the cell surfaces interdigitate in the intercellular space. One nucleated cell body lies directly beneath another, and extensions from neighboring cells penetrate between the two and between the lower cell body and the substrate. Microvilli are present but sparse on the luminal cell surface. Cytoplasmic differentiation in these cells is average for our primary cultures. A desmosome (d) links two cells at the upper left. Bar = 2.0 $\mu$m. $\times$ 8,000.

FIGURE 5    2-day tumor cell culture. A single-layered area of the culture is shown. Cells are almost columnar here, and intercellular spaces, of moderate width, are bridged by slender cell processes. Upper cell surfaces bear microvilli; basal surfaces are flattened against the substrate. Bar = 2.0 $\mu$m. $\times$ 5,400.
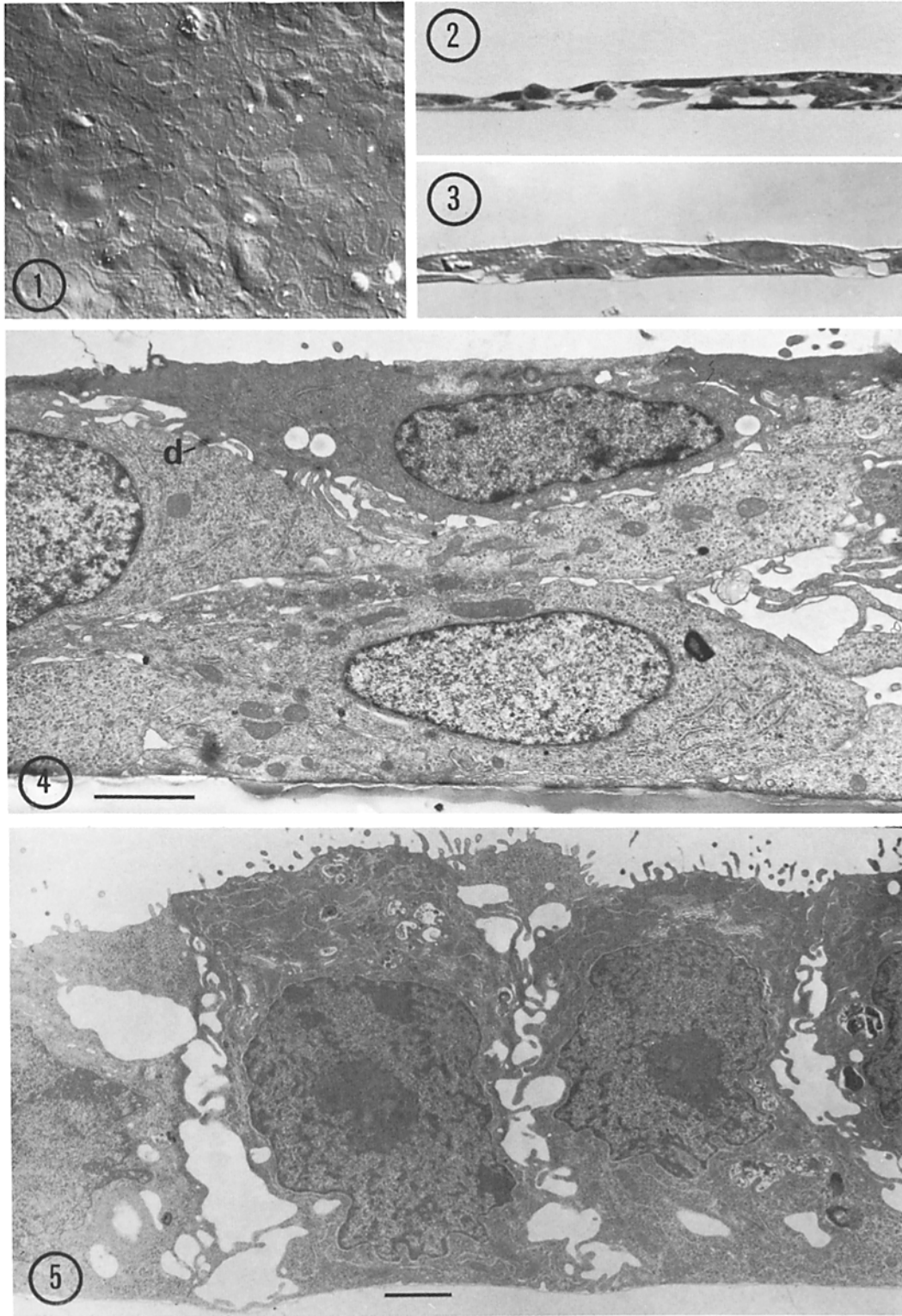
Fibroblasts, which usually comprise a small and easily controlled (48) proportion of cells from mammary adenocarcinomas, are more abundant in normal mammary preparations and may outgrow the epithelial cells after 1-2 wk. Most of our observations were made during the first 2 wk of culture, when the epithelial pavement covers most of the culture dish (but occasional bare spots appear in even the densest cultures). Since normal and tumor cell cultures do not differ detectably in other respects to be considered here, our descriptions apply to both.

## Overlapping and Multilayering in Culture

Light microscope examination of thick sections perpendicular to the substrate reveals that the cultures are not conventional monolayers. Single-layered areas are present, but, under much of the confluent epithelial pavement layer, other cells appear in varying numbers and arrangements; examples are shown in Figs. 2 and 3. Low magnification electron micrographs confirm that cells of the surface layer, in both monolayer and multilayer areas, invariably are in close mutual contact immediately adjacent to the bulk medium (Figs. 4-6). Below this contact zone, remaining surfaces of pavement cells and the cells that appear sequestered beneath the pavement make intermittent contact or close approximation with each other or with the substrate, the volume of intercellular space varying widely.

Some pavement cells rest squarely on the substrate (Fig. 5). Others overlap extensively, and it sometimes is evident that a pavement cell can reach the substrate micrometers away from the area integrated in the pavement. As a rule, however, the nucleus is in that part of the cell facing the bulk medium, so that little nuclear overlap occurs among pavement cells. The frequency of nucleated cell bodies seen in both thick and thin sec-

tions to lie well below the surface layer or under other nuclei (Figs. 2-4, 6) therefore indicates that there is a considerable population of wholly sequestered cells and that many pavement cells do not touch the substrate at all. We have not examined serial thin sections through entire cells, but areas such as those pictured are so typical of our cultures that the possibility that every cell is somewhere integrated in the pavement layer is remote. Sequestered cells tend to become flattened parallel to the substrate; they may overlap extensively and often pile up in loose mounds.

The pavement layer itself thus is equivalent to an epithelial monolayer, but in large areas of these primary cultures it rests on top of other epithelial cells, stromal cells, or debris and does not make regular contact with a solid substrate. In one experiment, cultures were fixed at hourly intervals; multilayering in epithelial islands was observed by 8-10 h.

Cultures of nonepithelial cells dissociated from gland-free fat pads lack a continuous surface layer. Multilocular fat cells, nucleated blood cells, and many fibroblastic cells overlap freely and make frequent, discontinuous contacts. They often form loose mounds like the piles of sequestered cells in predominantly epithelial cultures.

## Cell Polarity and Ultrastructure

The medium-facing surface of the pavement cells is structurally equivalent to the luminal surface of intact mammary or other single-layered transporting epithelia: it typically bears sparse to abundant microvilli, and occluding junctions abutting this surface provide continuous intercellular adhesion. We will refer to it as the luminal surface. Like MTV-infected mammary cells in vivo, the pavement cells release B particles usually by budding from this surface, directly or from microvilli (Fig. 6, inset). Below the occluding zone,

FIGURE 6 Another area of same tumor cell culture as Fig. 5. Cells tend to be rounded, and intercellular spaces are wide. Long processes from all cells extend into the spaces, often making near contact with processes from other cells. Microvilli are abundant on the cell surfaces facing the bulk medium (at left); in and among the microvilli are round, dark bodies that represent budding and mature MTV particles. In this field, only the binucleate cell at the center appears to reach from pavement to substrate. Bar = 2.0 $\mu$m. × 7,200. The *inset* shows budding MTV particles. One particle, sectioned tangentially, is emerging from a hump on the cell surface. The second, at the tip of a microvillus whose base is out of the section plane, has a nearly complete internal dense shell, enveloped by a membrane whose unit structure resembles that of the cell surface. Both particles bear a fringe of short surface projections characteristic of MTV. Bar = 0.2 $\mu$m. × 90,000.
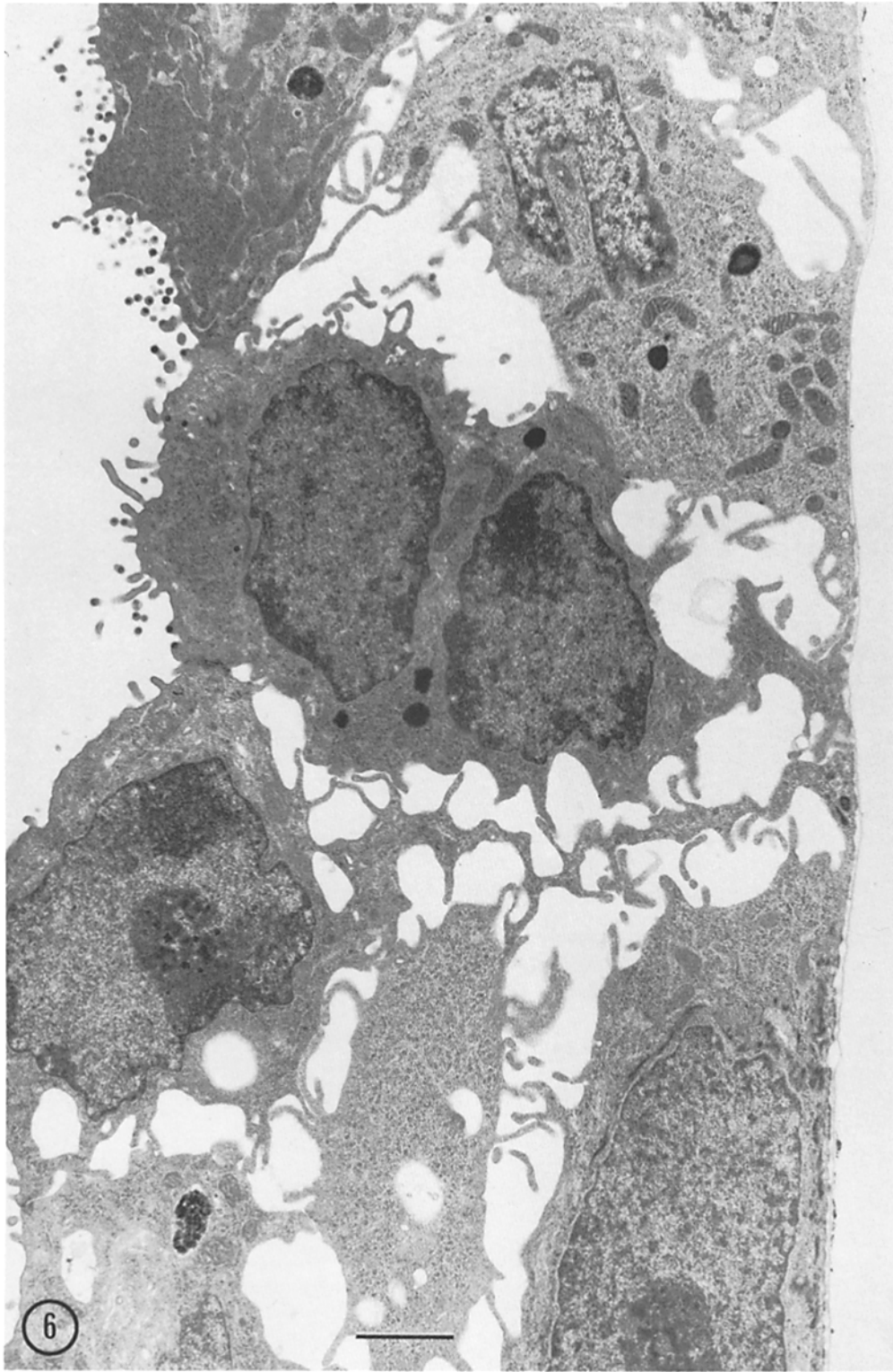
polarization is slight; pavement and sequestered cells bear many irregular, long, cell processes, lamellar or filiform, projecting into intercellular spaces or inserted between cells and the substrate (Figs. 4–7). Budding of MTV from both pavement and sequestered cells into these spaces in tumor cell cultures is more common than into laterobasal intercellular spaces in vivo. Flattening of basal cell surfaces against available substrate is common but not consistent.

Desmosomes (Fig. 7) link epithelial cells at various locations in some cultures but are rare or absent in others. Away from the occluding zone, apparent fusion of the outer leaflets of two cell membranes in a focal tight junction is sometimes seen (Figs. 9, 10). Membranes of adjacent cells often approach within 10 nm of each other, with filamentous material in the intervening space that may be merely the fused and compacted surface coats of the two cells (Fig. 8). Small dense plaques in the subjacent cytoplasm of confronting cell processes are seen occasionally; they resemble small intermediate junctions (29) or very early contacts between cultured fibroblasts (23). Sites of closest contact between basal cell surfaces and the thin line visible in sections that represents material coating the substrate are often marked by cytoplasmic densities and extracellular plaques (Fig. 11).

Cells in these primary cultures vary considerably in degree of development of cytoplasmic organelles but never approach the differentiated state of secreting mammary epithelium. Internal polarization is not evident, and neither endoplasmic reticulum nor Golgi apparatus appears highly developed. Lysosomes of various kinds are frequent in both pavement and sequestered cells.

One aspect of culture adaptation by both epithelial and other cell types is an increased abundance of cytoplasmic filaments. Bundles of 85–100-Å filaments appear at various locations, and 50–70 Å microfilaments often form tracts or lattices along surfaces facing the medium or the substrate and in cell processes (Figs. 7, 11). Microtubules are numerous, but not notably more so than in intact tissue. Myoepithelial cells, distinguished in the intact gland by their abundant parallel microfilaments, are not identifiable in our cultures.

Accommodation to crowded culture conditions thus appears to reduce the several cell types from the mammary gland to roughly similar states of cytoplasmic development, so that internal distinguishing features tend to disappear. Epithelial cells

may be identified with certainty if they display occluding junctions, well-developed desmosomes, or either cytoplasmic A or budding B virus particles (neither particle has been seen in nonepithelial mammary cells in our mice). Sequestered cells without any of these features generally cannot be classified. Macrophages and lymphocytes on top of the epithelial pavement, where they may be seen in living cultures, do retain their identifying features. Where the epithelial pavement is actually interrupted by strands or mounds of fibroblastic cells, the surface layer is discontinuous, as in nonepithelial cultures; occluding junctions and desmosomes are absent.

## Domes

The roof of a dome is continuous with the surrounding epithelial pavement (Fig. 12) and is similarly polarized toward the bulk medium as a lumen equivalent. As in the pavement, cells may vary in shape from almost cuboidal to squamous. Underlying cells, when present, may adhere either to the roof of the dome or to the substrate beneath it; often they aggregate in a cellular annulus at its periphery (Fig. 13).

Other three-dimensional structures formed from the epithelial pavement, such as strings of balloon-like spheres developing from detached sheets, also are polarized toward the bulk medium; the cavity of the balloon, like that of the dome, is basal and not luminal. The only exceptions seen were rare small lumina, complete with tight junctions and microvilli, within large clusters of cells in young cultures established from cells not filtered through fine-meshed Nitex; we assume that these were undissociated alveolar remnants, trapped within cell aggregates.

## Ultrastructure and Distribution of Membrane Junctions

The continuous close fit of cell borders in the epithelial pavement is evident in freeze-fracture replicas of luminal surfaces (Figs. 14, 15). Wherever an overlapping cell is broken away along the junction line, the ridge and groove pattern of an occluding junction is exposed (Figs. 17–20). In some replicas, prepared by the sandwich technique (22), such breaks have exposed stretches of continuous lateral-to-apical membrane fracture faces with uninterrupted occluding-junction belts up to 18 μm long. Thin sections cut perpendicular to the junctional membranes show the conventional se-
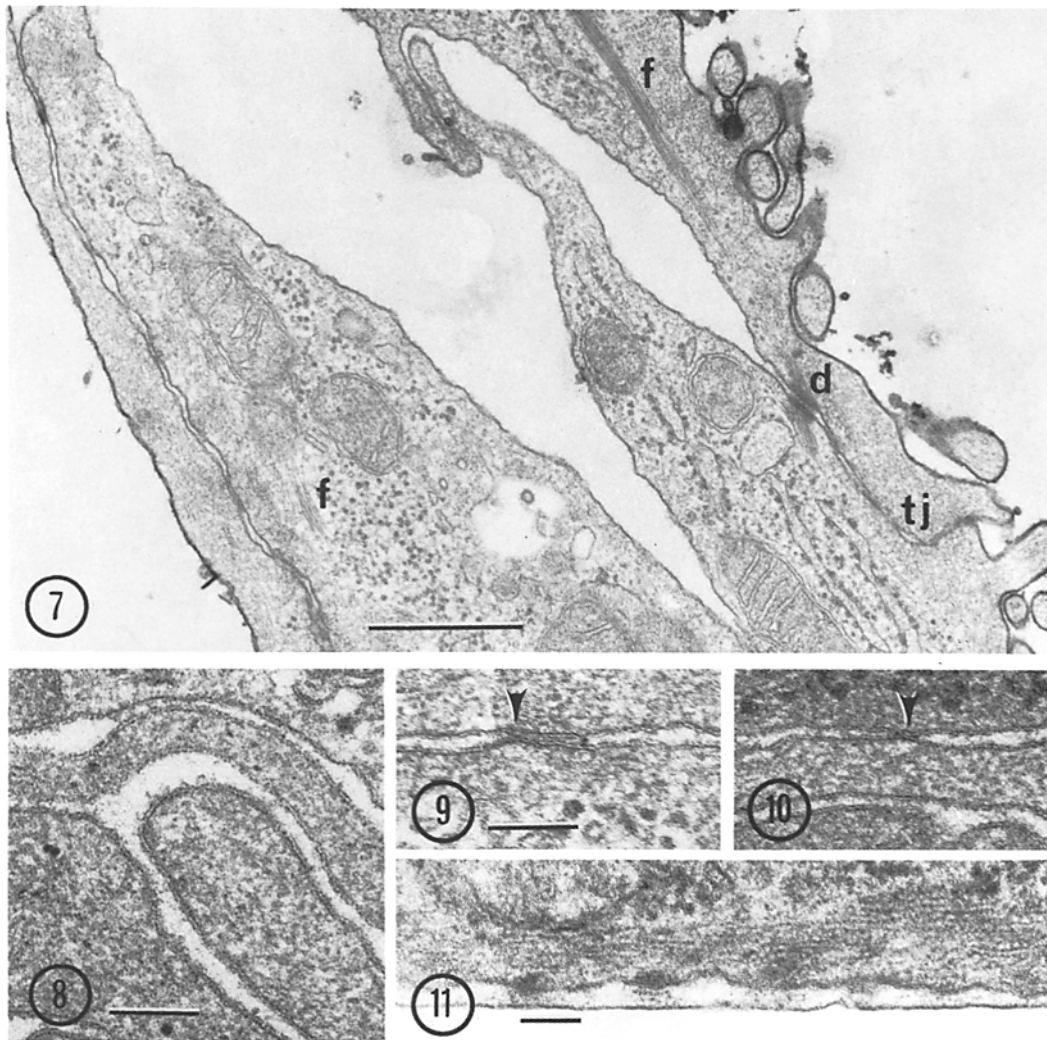
FIGURE 7   8-day tumor cell culture fixed in aldehyde solution containing tannic acid, which has stained the entire upper surface of the cells. A tight junction (*tj*) and a desmosome (*d*) link the cells at their luminal border. A meshwork of microfilaments occupies the layers of cytoplasm adjacent to the bulk medium and to the substrate in these cells; bundles of coarser filaments (*f*) are seen elsewhere. Bar = 0.5 μm. × 40,000.

FIGURE 8   Interdigitating cell processes in a 7-day culture of tumor cells. The intercellular gap becomes as narrow as 5 nm at the top of Fig. 8; the material within the gap resembles cell coat material visible elsewhere on these membranes. Bar = 0.1 μm. × 120,000.

FIGURES 9 and 10   7-day tumor cell cultures. Arrowheads indicate points of apparent fusion of outer leaflets of adjacent cell membranes at sites remote from the occluding junction. Bar = 0.1 μm. × 120,000.

FIGURE 11   Lower surface of a cell resting on the substrate in a normal cell culture, showing parallel filaments in the cytoplasm and a series of substrate contacts marked by extracellular and cytoplasmic dense plaques. Bar = 0.1 μm. × 80,000.

ries of fusions of outer membrane leaflets (Fig. 16). Within the epithelial pavement in primary cultures grown under dome-promoting conditions, we have never, either in sections or in replicas, found an interrupted junctional belt or a lateral-to-apical membrane face lacking an occluding junction. We have occasionally found isolated tight-junction ridges in replicas of lateral membrane faces remote
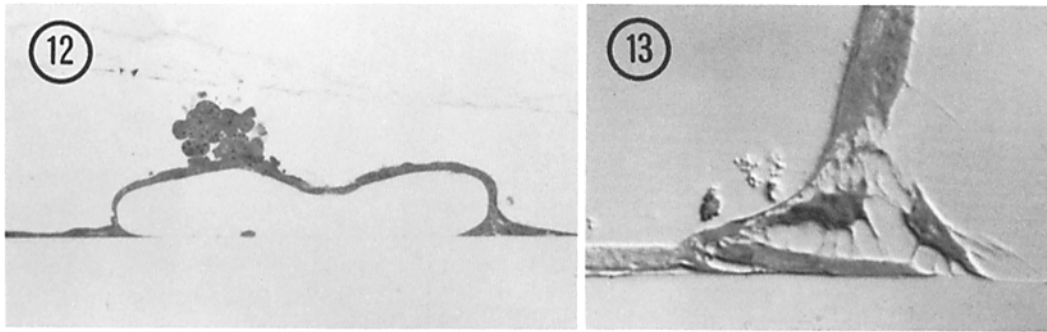
FIGURE 12  Vertical section through a dome from a 14-day tumor cell culture. The roof of the dome has partly collapsed during processing, as hydrostatic pressure within the cavity is not maintained after fixation. The cluster of cells on top of the dome is a development occasionally seen in older cultures. Note the thickened cellular annulus on the substrate at the periphery of the dome. Toluidine blue stain, bright-field optics. × 190.

FIGURE 13  Higher magnification of a vertical section through the annulus of a dome in a 5-day tumor cell culture. At least three sequestered cells comprise the annulus in this section. Dome cavity to the right. Nomarski optics. × 1,200.

from any occluding zone, in agreement with evidence from thin sections (Figs. 9, 10).

In the intact mammary gland, the occluding-junctional network is highly irregular in pattern in all nonlactating states, but in alveolar epithelium at parturition, coinciding with a change in permeability (36), it promptly becomes compact, with ridges oriented predominantly parallel to the luminal surface (51). Cultured cells resemble the nonlactating tissues in displaying no consistent network pattern, but variations in mesh size and shape are somewhat less extreme. A continuous border ridge is present on the luminal side of the junction, but extended loops and loose ends are common on the abluminal side (Figs. 17-20). The number of ridges in any one transect of the belt varies from two to 12 or more and its depth from 30 to 600 nm. In occasional areas, ridges are predominantly oriented perpendicular to the luminal surface (Fig. 19), a variation we have not seen in the original tissue.

Gap junctions (Figs. 21, 22) appear in or near the occluding zone in both normal and tumor cultures, often enough to suggest that cell coupling by these structures is at least not unusual. Most of them are small, around 0.1-0.3 μm in diameter, and are composed of particle aggregates without a uniform packing order. We have occasionally seen gap junctions elsewhere in both replicas and sections, but the convolution of membranes below the occluding zone makes recognition difficult, and they may be more common than we can demonstrate.

DISCUSSION

Domes

These studies have shown that mammary epithelial cells in high-density primary culture readily form a confluent sheet, within which continuous belts of tight junctions maintain close cell-to-cell adhesion at the surface facing the bulk medium.

FIGURE 14  Freeze-fracture of the A faces (cytoplasmic leaflets exposed by cleavage of the plasma membrane) of the luminal membranes and the lines of contact between parts of four cells (numbered 1-4) in a 6-day normal cell culture. All of the surfaces seen are studded with the fractured bases of microvilli; where several microvilli arise from a common hillock on the cell surface, the fracture profile is elongate or irregular. Bar = 2.0 μm. × 9,000.

FIGURE 15  Replica of a similar fracture of the luminal membranes of five cells in a 6-day tumor cell culture. The continuous close fit of contacting cell borders is evident. Microvilli are more abundant along cell borders than elsewhere. Bar = 2.0 μm. × 9,000.
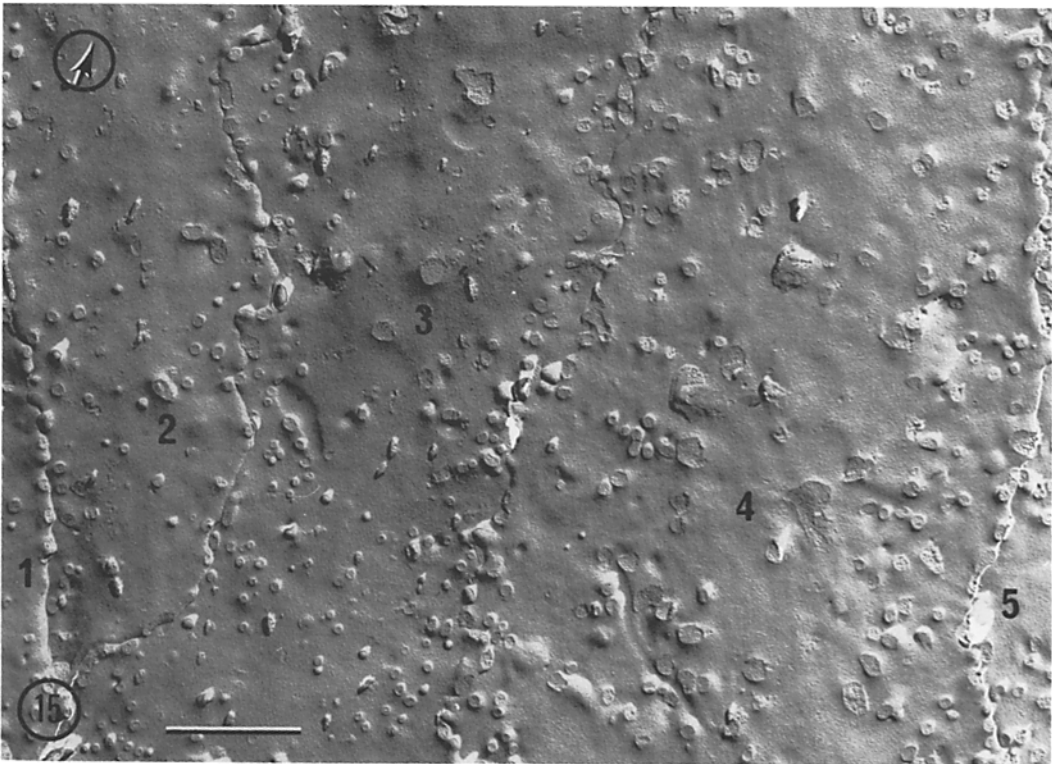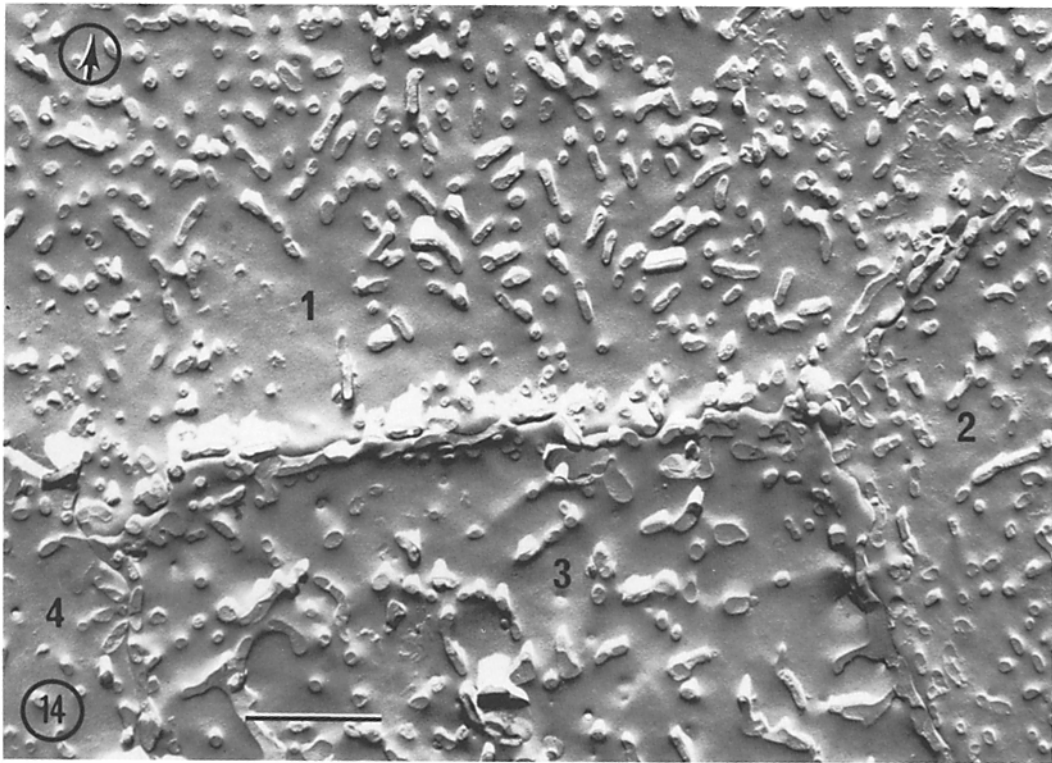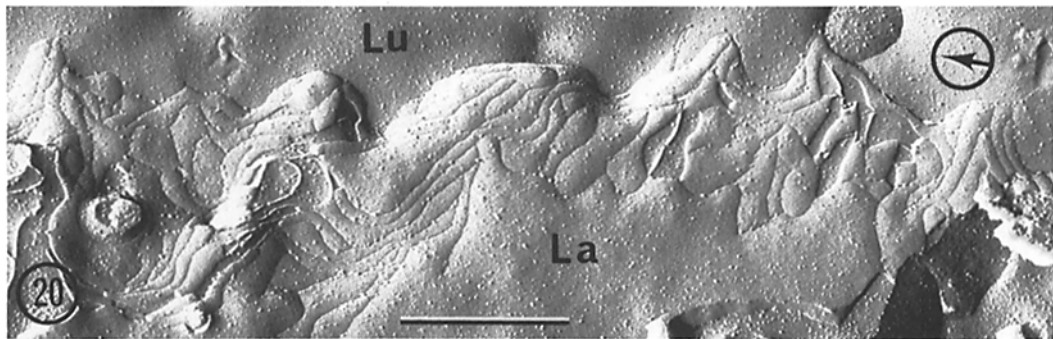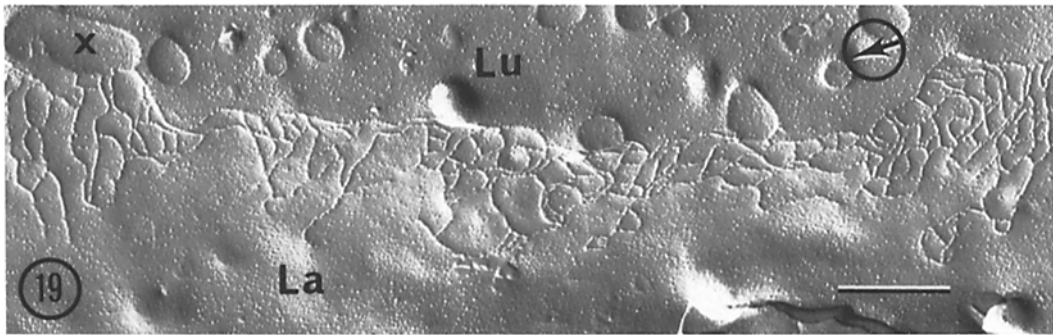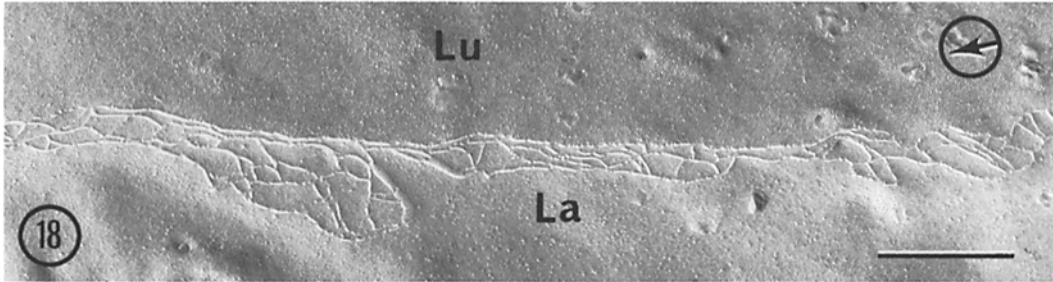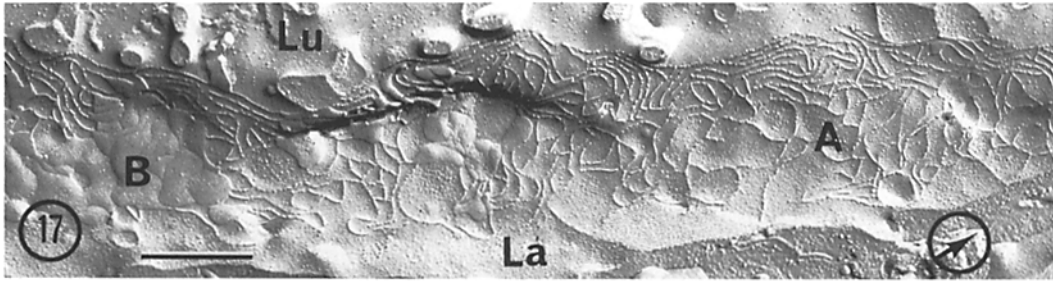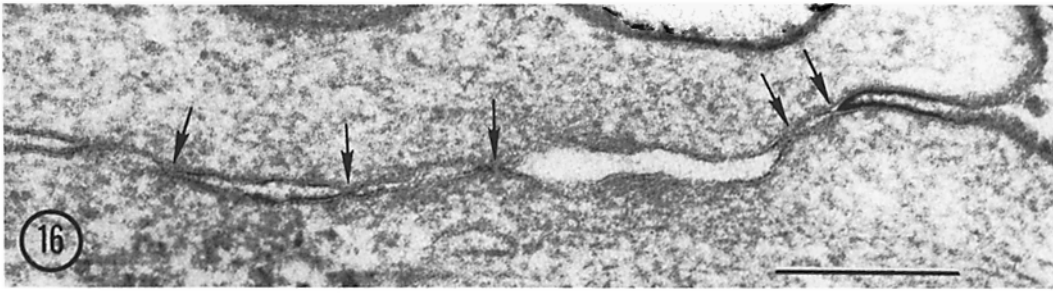
That such junctions are truly occluding, that is, that they can provide an effective permeability barrier, is indicated by the demonstration of polarized water transport in filter-grown cultures of morphologically similar MDCK cells (43) and by measurement of spontaneous transepithelial potential difference and resistance in filter-grown mammary tumor cell cultures (D. S. Misfeldt, unpublished observations), as well as by dome formation. It appears that, in cultures on an impermeable substrate, a dome forms when transported fluid accumulating under the confluent pavement exerts enough pressure to lift the layer locally. The presence of domes, visible to the naked eye, constitutes acceptable evidence that transport is occurring and that the mosaic of occluding junctions is complete and effective at least in and around each dome. It further is evidence that the cells involved are epithelial, since other cell types are not known to form occluding junctions.

Fluid in the lactating mammary alveolus is transported from basal extracellular space to lumen; water and selected tissue-fluid constituents are passed into the lumen along with specific secretory products of the cells. Accumulation of fluid on the basal side of the epithelial sheet in culture therefore appears to be the reverse of the normal process. In the probable absence, however, of significant production of lactose, the major osmotically active milk component (34), other, less mammary-specific, transport functions may be disclosed. One that could lead to basally directed water flux is the action of a postulated sodium pump on the laterobasal cell membranes that is presumed responsible for the maintenance of low intracellular Na levels in the lactating mammary gland (34, 35).

Why domes form when and where they do is unexplained. Local variations in transcellular transport could be involved, but this would require pulses of activity shifting all over the pavement as domes come and go. Our evidence does not suggest that junctional belts are interrupted in nondome areas; thus it seems unlikely that dome sites are determined solely by the local absence of such discontinuities, although more subtle modulations in junction leakiness could play a role. We consider it more probable that transport and permeability are relatively constant and that domes form where

FIGURE 16   Section of an occluding junction in an 8-day tumor cell culture fixed in aldehyde with tannic acid. Points of fusion of the outer leaflets of the junctional membranes are indicated by arrows. Bar = 0.2 $\mu$m. × 125,000.

FIGURE 17   Replica of an occluding junction in a 6-day normal cell culture. The overlapping lip of one cell has been almost entirely broken away, exposing the ridges of the occluding junction on the A face ($A$) of the companion cell's membrane; this face is continuous from the lateral membrane ($La$) through the junction to the luminal membrane ($Lu$). In some areas ($B$), patches of the B face (outer leaflet) of the overlapping cell's membrane remain in place, showing grooves corresponding to the A-face ridges. The network of ridges in this sample is compact near its apical edge, looser and irregular near the lower edge, with several free-ended ridges. Bar = 0.5 $\mu$m. × 30,000.

FIGURE 18   Replica of an occluding junction in a 16-h tumor cell culture. As in Fig. 17, the A face is exposed continuously from the lateral cell membrane ($La$) through the junction to the luminal membrane ($Lu$). This junction is variable in width but its network is relatively simple, with continuous ridges at both luminal and abluminal edges. Bar = 0.5 $\mu$m. × 36,000.

FIGURE 19   Replica of a lateral-to-luminal membrane A face from a 10-day tumor cell culture. The network looks ragged, with many free ends on the abluminal edge and some areas where ridges are predominantly perpendicular to the luminal border. Nonetheless, there is no point where fewer than two ridges are present between lateral ($La$) and luminal ($Lu$) membranes. At $x$, the fracture plane passes into the cytoplasm of a hillock and the membrane bearing the luminal border ridge is broken away. Bar = 0.5 $\mu$m. × 30,000.

FIGURE 20   Replica of a lateral-to-luminal membrane B face from a 6-day tumor cell culture. The junctional network is a pattern of grooves (except at far left and other spots where the fracture plane shifts to the A face of the companion cell's membrane). The luminal border ridge ($Lu$) is continuous but strongly undulating, reflecting the curving line of contact often seen in luminal membrane fractures such as those shown in Figs. 14 and 15. $La$, lateral membrane. Bar = 0.5 $\mu$m. × 45,000.
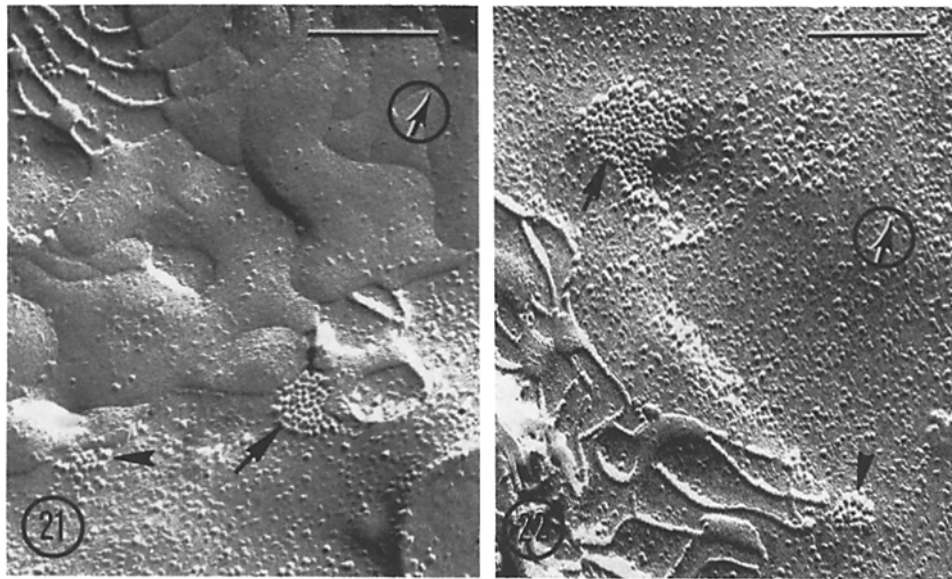
FIGURE 21 Part of the replica of a normal culture seen in Fig. 17, enlarged to show a small, round gap junction (arrow). The junctional particles are compactly arranged on the membrane A face; the cleavage plane then shifts to reveal the grooves of the adjacent occluding junction on the companion cell's membrane B face. A smaller cluster of particles at the arrowhead may indicate another gap junction. Bar = 0.2 μm. × 88,000.

FIGURE 22 Replica of part of an occluding junction and a small gap junction (arrow) from a tumor cell culture. A smaller cluster of particles at the arrowhead may indicate another gap junction. Bar = 0.2 μm. × 80,000.

direct or indirect (via sequestered cells) adhesion between pavement cells and the substrate is weak enough to be overcome by the pressure of accumulating fluid. Whatever its mechanism, dome formation requires that adhesions immediately around the periphery of the dome be firm enough to prevent these cells from lifting and frequent enough to restrict lateral flow of fluid under the pavement. If cell surfaces below the occluding junctions engage in slow movement, and if their structurally simple contacts are unstable, the dome will ultimately collapse as trapped fluid escapes laterally.

Domes are rare or absent in dense primary cultures grown without specific hormone stimulation (25); they do not form in favorable medium if cell density is too low, even when the pavement layer is fully confluent (9, 24). It may be significant that the density of lactating alveolar epithelial cells, calculated from measurements of average cell dimensions in scanning electron micrographs (51) and in thick sections of lactating tissue, lies within the range of 4–6 × 10⁵ cells/cm² of basal lamina surface, or approximately the same as in

our high-density cultures. We are currently investigating the control of dome formation by examining the structure, distribution, and effectiveness of cell-to-cell and cell-to-substrate contacts in confluent cultures under various conditions known to influence fluid transport or the occurrence of domes (9).

## Multilayering

Multilayering occurs in initial outgrowths from some explanted epithelial tissues (e.g. 17, 18, 21), but has not been commonly reported in primary cultures of dissociated normal epithelium. Since we find it in cultures established from single-cell suspensions as well as those containing many clusters, it is not attributable only to carry-over of cell associations from the original tissue. Since we find it in both young and old cultures and in medium- and low- as well as high-density cultures, it is not altogether a consequence of excessive crowding.

The rapid formation of the mammary epithelial pavement over whatever lies beneath it suggests that, when isolated epithelial cells retain a strong

capacity for forming tight junctions, they do so in preference to any other kind of attachment and to the exclusion of all cells (stromal or epithelial) not competent to share them. Formation of occluding belts appears always to be accompanied by structural differentiation of luminal vs. laterobasal cell surfaces, with the same polarity in all of the joined cells. In the forming epithelial sheet, that side facing the bulk medium becomes the luminal surface, regardless of whether the basal surfaces are resting on other cells or a solid support.

Several authors have reported (e.g. 1, 12, 13, 42) that fibroblasts are unable to attach and spread on the surface of a confluent epithelial sheet, but that individual cells on the substrate may spread under unattached edges of the sheet, where they may even move and divide. Something of this nature clearly occurs on a large scale during the development of our cultures. It may be significant that, whereas epithelial and fibroblastic cells in the experiments cited above were taken from different sources, those in our cultures were close neighbors in the mammary gland or tumor. Perhaps pavement cells in this case are more tolerant of the presence of other cells beneath them, and the sequestered cells may be more likely to thrive. Such multilayering may be more common in primary cultures of naturally mixed cell populations than is generally recognized.

### Comparison with Other Epithelial Cell Cultures

Many vertebrate epithelia have been grown successfully in primary or long-term culture, usually for studies of genetics, transformation, or control of synthetic function. Ultrastructural observations have been concerned mainly with cytoplasmic differentiation, and membrane junctions have received only passing notice. Since clear identification of a tight junction requires special techniques, micrographs published for other purposes usually are inconclusive. Use of thick sections cut perpendicular to the culture plane, which we have found useful for revealing variations in three-dimensional cell relationships, is rare.

Published electron micrographs suggest the presence of occluding junctions at the medium-facing surface in several cell types that also possess them in vivo, such as pigmented retinal epithelium (42), liver (21, 45), thyroid (15), and pancreas (47). Intercellular spaces resembling bile canaliculi are evident in thin sections of synthetically active liver cell cultures (7, 21, 30). Organoid rearrangement of cells in hormone-stimulated thyroid cell cultures produces follicle-like spaces described by Fayet et al. (15) as three-dimensional closed cavities surrounded by well-polarized cells with microvilli and tight junctions that exclude ruthenium red.

Established cell lines that are tumorigenic in vivo and form confluent epithelial pavements in vitro with occluding junctions and domes include MDCK (32, 43), one of two human cervical squamous carcinoma cell lines extensively analyzed by Auersperg (2), and two of three mammary tumor cell lines established by Yagi (58). The normal tissues of origin of the MDCK and mammary cell lines are transporting epithelia with characteristic occluding junctions. Normal stratified cervical epithelium is not known to have these junctions, but adjacent columnar mucosa and associated glands presumably do.

### Membrane Junctions in Neoplastic Cells in Culture

Mounting evidence that gap junctions play a role in cell-to-cell communication (see 4) has suggested to many workers that the failure of neoplastic cells in vivo and transformed cells in vitro to respond to normal growth controls may be related to deficient or defective junction formation. It appears now that, whereas some neoplastic or transformed cells fail to exhibit ionic coupling or detectable gap junctions, one or both of these properties have been demonstrated in others (e.g. 3, 6, 16, 26, 27, 37, 38, 46). Search for tight junctions in neoplastic or transformed cells of epithelial origin has been minimal, has not utilized freeze-fracture methods, and usually has not yielded enough information to permit distinction of focal tight from gap or other close junctions.

We have found both true occluding junctions and gap junctions in primary cultures of mammary tumor cells. They do not differ, in any way we can identify, from the membrane junctions in normal cell cultures. Gap junctions appear to be erratically distributed in both cell types, as they are in intact mammary gland (51), and we have not attempted to make quantitative comparisons.

Occluding junctions are strongly adhesive (5, 20). Although they may be dismantled by the organism during normal differentiation processes (10) and can accommodate to normal abrupt changes in cell shape (51), their role in maintaining

the transepithelial permeability barrier requires that they be relatively stable. It seems inevitable that establishment of such firm and continuous bonds should affect the behavior in culture of the cells involved, whether normal or neoplastic, and the close similarity of our normal and neoplastic cultures may be in part a consequence of this. The coherent behavior of epithelial cell sheets in culture has been noted in other contexts by many authors (e.g. 1, 12, 13, 42).

One might expect that tumor cells with the propensity to form tight junctions would be less likely than cells lacking this ability to invade adjacent tissues in vivo or to break away from the parent tumor and establish metastatic foci. We are currently using the occasional invasive or meta-static tumors found in our mouse colony to explore this problem.

It appears from the available data that at least those transporting epithelia that form occluding junctions in vivo retain the capacity to form them when the tissue is dissociated and grown under suitable conditions in cell culture, and this capacity may also be retained by cells from carcinomas originating in the tissues. An additional epithelial function that may survive both transformation and the transition to culture is polarized fluid trans-port. Cells that do preserve these basic functions should be subjects of choice for physiological experiments requiring the controlled conditions of the culture environment. Primary culture appears also to provide a system for testing whether loss of specific epithelial functions has a predictable re-lationship to degree of malignancy of breast and other epithelial tumors.

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