Human Mammary Luminal Epithelial Cells Contain Progenitors to Myoepithelial Cells

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The origin of the epithelial and myoepithelial cells in the human breast has not been delineated. In this study we have addressed whether luminal epithelial cells and myoepithelial cells are vertically connected, i.e., whether one is the precursor for the other. We used a primary culture assay allowing preservation of basic phenotypic traits of luminal epithelial and myoepithelial cells in culture. The two cell types were then separated immunomagnetically using antibodies directed against lineage-specific cell surface antigens into at best 100% purity. The cellular identity was ascertained by cytochemistry, immunoblotting, and 2-D gel electrophoresis. Luminal epithelial cells were identified by strong expression of cytokeratins 18 and 19 while myoepithelial cells were recognized by expression of vimentin and a-smooth muscle actin. We used a previously devised culture medium (CDM4) that allows vigorous expansion of proliferative myoepithelial cells and also devised a medium (CDM6) that allowed sufficient expansion of differentiated luminal epithelial cells based on addition of hepatocyte growth factor/scatter factor. The two different culture media supported each lineage for at least five passages without signs of interconversion. We used parallel cultures where we switched culture media, thus testing the ability of each lineage to convert to the other. Whereas the myoepithelial lineage showed no signs of interconversion, a subset of luminal epithelial cells, gradually, but distinctly, converted to myoepithelial cells. We propose that in the mature human breast, it is the luminal epithelial cell compartment that gives rise to myoepithelial cells rather than the other way around.

Introduction

The resting mammary gland of a number of species such as mouse, rat, and human essentially consists of a branching ductal system lined by an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells. The question of a stem cell population in the mammary gland has remained intriguing for almost as long as the two participating epithelial cell types have been described (for review see Rønnov-Jessen *et al.,* 1996). The two cell types are believed to have a common ectodermal origin which possibly branches into separate lineages. Whereas the luminal epithelial cells differentiate in an entirely tissue specific manner, the myoepithelial cells differentiate toward a more general contractile phenotype as also seen in certain other exocrine glands (Rønnov-Jessen *et al.,* 1996). The question of how myoepithelial cells arise is important not only from a developmental perspective, but also from the point of view of the stability of the differentiated state. During the resting state, the cellular renewal is slow, but after gestation, lactation, and involution where myoepithelial cells almost disappear, the lineage equilibrium is somehow restored (Ferguson and Anderson, 1983; Umemura *et al.,* 1996). Additional interest arises from the perspective of tumor biology where the ratio between the two lineages change radically. For example, whereas in a number of frequent but unquestionably benign lesions such as sclerosing adenomatosis, the ratio shifts in favor of myoepithelial cells, the opposite is the case whenever premalignant lesions such as atypical hyperplasias and carcinomas *in situ* are apparent (Guelstein *et al.,* 1988; Gusterson *et al.,* 1982). Moreover, in overtly invasive breast cancer of almost every species including mouse, rat, feline, or human, the myoepithelial component is practically absent (Gusterson *et al.,* 1982). These observations have led to speculations that breast cancer arise in “stem cells” which then are pushed toward the luminal epithelial lineage bypassing the myoepithelial pathway (Rudland, 1993; Rudland *et al.,* 1995).

Studying the cellular lineages of the breast especially that of human has been hampered by lack of convincingly separated expandable cell populations with minimal phenotypic drifting in culture (Dairkee and Heid, 1993). We have previously devised culture conditions for preservation of defined phenotypic traits in primary culture (Petersen and van Deurs, 1987, 1988). Here we take advantage of these preserved traits to separate differentiated cells immunomagnetically based on their specific expression of lineage-related surface glycoproteins (Clarke *et al.,* 1994; Gomm *et al.,* 1995). With these tools in hand, we have addressed the question of which of the two lineages may serve as a cellular source of the other. We show that a subset of luminal epithelial cells can make the conversion to myoepithelial cells while the reverse does not occur.

Materials and Methods

Culture of Breast Organoids

Fresh mammary tissues were obtained from patients undergoing reduction mammoplasty for cosmetic reasons. The tissue was minced into small pieces in Dulbecco's modified Eagle's medium–Ham's F12 (DME-F12)2 supplemented with 50 mg/ml gentamycin (Garamycin, Schering, Kenilworth, NJ) and 2 mM glutamine (Sigma Chemical Co., St Louis, MO) as previously described (Petersen and van Deurs, 1987). The disaggregated tissue was further digested for 16 h at 37°C under gentle rotation in DME-F12 medium containing 900 IU/ml collagenase (CLS III; Worthington Biochemical Corp., Freehold, NJ). Blood vessels and fibroblasts were removed from the organoid suspension by centrifugation of the collagenase digest as previously described (Rønnov-Jessen and Petersen, 1993). The pellet was washed twice in DME-F12 medium. The epithelial organoids were further minced by pulling the suspension through a 0.4-mm-diameter (27 guage x ¾) needle twice. After washing two more times in DME-F12 the cleared epithelial organoids were resuspended in chemically defined medium, CDM3, and plated on T-25 flasks (Nunc, Roskilde, Denmark) coated with Vitrogen (Vitrogen 100, 8 μg/cm2, Collagen Corporation, Palo Alto, CA) as described (Petersen and van Deurs, 1987, 1988). To demonstrate the cell types in the original cell population, organoids were scraped off and sectioned for immunostaining (see below) before the cells had spread into monolayer.

Cell Separation

Cell types were separated from each other after organoids had spread out to monolayers in primary culture or had been passaged once. Cells were trypsinized and resuspended in *N*-(2-hydroxyethyl)piperazine-*N*9-2-ethanesulfonic acid (Hepes) buffer with 0.5% (w/v) bovine serum albumin (BSA, fraction V, A4919, Sigma) and filtered using a 100-mm nylon mesh (Millipore, Hedehusene, Denmark) to remove residual cell clumps. The cell suspension was incubated for 30 min at 4°C with the primary monoclonal antibody (mAb), 115D8, directed against sialomucin (kindly provided by Jo Hilgers, Amsterdam, The Netherlands) or J5 directed against common acute lymphoblastic leukemia antigen (CALLA) or CD10 antigen (Coulter Clone, Struers Kebo Lab., Albertslund, Denmark) diluted 1:100 and 1:10, respectively, in Hepes/BSA. The cells were then washed twice in Hepes/BSA and incubated for 15 min at 4°C with goat anti-mouse IgG microbeads (Miltenyi Biotec, U.S.A., purchased from AH Diagnostics, Århus, Denmark) diluted

1:5 in Hepes/BSA and washed twice in Hepes/BSA. Cell separation was carried out by use of the MiniMACS magnetic cell separation system according to the enclosed instructions (Miltenyi Biotec). To obtain 100% pure populations, cell suspensions were passed through the column in a free flow without needles attached and the retained cells were washed, eluted, and plated.

The purified cells were plated on T-25 Vitrogen-coated flasks. The myoepithelial cells were cultured in CDM4 as previously described (Petersen and van Deurs, 1988). The luminal epithelial cells were cultured in modified CDM3 and referred to as CDM6 by addition of 10 ng/ml human recombinant hepatocyte growth factor (H1404, Sigma) and reduction of epidermal growth factor to 20 ng/ml. The purified cells were maintained in these media up to seven passages without a change in intermediate filament profile. One flask from each separation was stained with immunoperoxidase to intermediate filaments and quantified to assess the quality of the separation (see below).

Immortalization of Myoepithelial Cells

Purified myoepithelial cells were immortalized with sterile filtered retrovirus supernatant from the PA317 LXSN 16E6E7 packaging cell line (CRL-2203, ATCC, Rockville, MD) in the presence of 8 µg/ml polybrene (Sigma). Infected cells were selected in the presence of 100 µg/ml G418 (Life Technologies).

Immunocytochemistry

The cultures were rinsed, fixed in methanol, dried, and stained for immunoperoxidase as previously described (Petersen and van Deurs, 1988). Frozen biopsies were sectioned at an 8-µm setting in a cryostat. The sections were dried for 15 min at room temperature and fixed as previously described (Petersen and van Deurs, 1988). The mAbs were anti-intermediate filaments against CK17 (M7046; Dako, Glostrup, Denmark), CK18 (F3006; Trichem ApS, Denmark), CK19 (BA17, Dako), and vimentin (VIM; MEDAC, GmbH). Other antibodies used were against α-sm actin (1A4; Sigma), sialomucin (115D8), β4-integrin (Chemicon International Inc., AH Diagnostics), and CALLA (J5). Rabbit anti-mouse immunoglobulins (Z259, Dako) were used as secondary antibodies and a peroxidase conjugated anti-peroxidase mouse mAb was used as tertiary antibody (P850, Dako). The cultures were counterstained with haematoxylin and mounted in Aquamount (Gurr®; BDH Chemicals Ltd, Poole, UK). The number of positive cells (cytoplasmic staining) were quantified as previously described (Rønnov-Jessen *et al.,* 1992). Ten randomly selected fields were counted by use of a 25X objective and a 10X ocular mounted with a grid.

Double labeling immunofluorescence was performed as previously described (Petersen *et al.,* 1992). Primary antibodies recognized CK18 (CAM 5.2, IgG2a; Becton Dickinson, Alberslund, Denmark) or CK17 (M7046, IgG2b) both combined with a mAb against vimentin (VIM, IgG1). The secondary antibodies were Texas redconjugated goat anti-mouse IgG2a (1080-07; Southern Biotechnology Associated Inc., Birmingham, AL) or IgG2b (1090-07; Southern Biotechnology) combined with FITC-labeled goat anti-mouse IgG1 (1007-02; Southern Biotechnology). The flasks or sections were mounted in 20-µl Fluoromont G (100, Southern Biotechnology) containing 2.5 mg/ml *n*-propylgallate (Sigma).

Confocal Microscopy

Double-labeling immunofluorescence was visualized using a Zeiss LSM 510 laser scanning microscope (Carl Zeiss, Jena GmbH). Sections were observed by use of a 63X objective and sliced in the Z-plane into 0.25-µm-thick focal planes and exposed to visualize FITC and Texas red.

Immunoblotting

The cells of a near-confluent T-25 flask were lysed for 30 min at 4°C with gentle agitation in Laemmli buffer, and samples were subjected to SDS–PAGE on 12% acrylamide gels and run overnight (Rønnov-Jessen *et al.,* 1992). The loading of lanes were equilibrated based on protein determinations by the Bio-Rad protein assay (Bio-Rad Laboratories, Copenhagen, Denmark). The proteins were blotted to an Immobilon-P transfer membrane (Millipore Co., Bedford, MA) and stained as previously described (Rønnov-Jessen *et al.,* 1992). Briefly, the lanes were washed for 15 min in phosphatebuffered saline (PBS)/0.05% Tween 20 (v/v) and blocked for 30 min in PBS/3% BSA (w/v) at room temperature. Between incubations of 30 min with primary, secondary, and tertiary antibodies, the lanes were washed three times for 10 min in PBS/Tween 20. The antibodies were against CK18 (1:100, CK5; Sigma), CK19 (1:100, BA17), vimentin (1:50, VIM), α-sm actin (1:1000, 1A4), and subsequently rabbit anti-mouse immunoglobulins (Z259) and a peroxidase-conjugated anti-peroxidase mouse mAb (P850). The lanes were stained in 12 mg 3,3,39,59-tetramethylbenzidine (Merck, Bie & Berntsen, Rødovre, Denmark) and 40 mg dioctylsodium sulfosuccinate (Merck) in 5 ml of ethanol in combination with 10 µl 30% H2O2 in 15 ml of citrate–phosphate buffer, and fixed for 1 min in 2.0 mg/ml dioctylsodium sulfosuccinate in 30% ethanol.

2-D Gel Electrophoresis

A subconfluent well of a 24-well dish was incubated for 1 h with 100 µCi [^{35}S]methionine (SJ 204, Amersham, Birkerød, Denmark) in methionine-free medium, rinsed six times in PBS, and lysed as previously described (Rønnov-Jessen and Petersen, 1993). Equal amounts of counts (approximately 10^6 cpm) were applied to all gels. First dimension was run overnight at 400 V in tubes with an inner diameter of 2 mm. Second dimension was run for approximately 18 h at 8–10 mA per gel under conditions described previously (Rønnov-Jessen and Petersen, 1993).

Results

Normal human breast epithelium expresses an array of markers that is indicative of only two epithelial lineages in the mature gland. The immature human breast gland and the rodent mammary gland in addition contain basal cells and cortical cells with distinct phenotypes (Anbazhagan *et al.,* 1998; Kordon and Smith, 1998; Smith, 1996). In the mature gland the luminal epithelial cells express CK18, CK19, and sialomucin while the myoepithelial cells express vimentin and α-sm actin, β4-integrin, and CALLA (Jones *et al.,* 1997; Petersen and van Deurs, 1988; Taylor-Papadimitriou *et al.,* 1989). In primary and secondary passaged cultures, luminal epithelial cells are generally identified as islets, and myoepithelial cells as the

surrounding streams, and the dual expression pattern is preserved in a complementary fashion (Petersen and van Deurs, 1988; Taylor-Papadimitriou *et al.,* 1989). Previous attempts to expand the mixed population in any of the existing culture media (CDM3-5) did not lead to sufficient purity of either of the cell types, and the cultures would senesce about 10–20 generations later (Mørk *et al.,* 1990; Petersen and van Deurs, 1987).

Isolation and Characterization of Luminal Epithelial and Myoepithelial Cells

We have tried a number of modifications to separate luminal and myoepithelial cells using antibodies to the exterior portion of the lineage specific cell surface markers sialomucin (MAM-6) and CALLA (J5) (Clarke *et al.,* 1994; Gomm *et al.,* 1995). The most reproducible and high efficient separation was achieved by use of two consecutive separations before passage 3. To purify luminal epithelial cells, we first removed myoepithelial cells using J5 against CALLA; the luminal epithelial cells were retained using MAM-6 against sialomucin. Myoepithelial cells were purified by rapid passage of cells through two MAM-6 columns.

To characterize the cells and as a further measure of purity, the cells were analyzed for expression of additional lineagespecific markers: CK18 and CK19 for luminal epithelial cells and vimentin and a-sm actin for myoepithelial cells. Whereas uncultured and primary cultured cells expressed a mixture of these markers, separation of cells into luminal and myoepithelial cells resulted in the total mutual exclusion of the markers characterizing each cell type as assessed by immunoblotting (Fig. 1). The expressed markers were not carry over from the phenotype *in vivo,* but rather continued to be evolved in a lineage-specific pattern as evaluated by 2-D gel electrophoresis and fluorography of labeled cells (Fig. 2). The success rate in obtaining cultures that were better than 98% pure was one in three biopsies. The purity, as evaluated by immunocytochemical staining for CK18 and vimentin, was in the range of 98.7–100% for luminal cells and 99.3–99.7% for myoepithelial cells (*n* 5 10; Fig. 3).

Expansion of Cells

Once purified, we expanded the myoepithelial and luminal epithelial cells for up to seven passages using CDM4 and CDM6 media, respectively. During the course of expansion, the phenotypes were continuously monitored by immunocytochemistry and 2-D gel electrophoresis. Since balance of different expressed genes is a hallmark of homeostasis, and since the nature of the cytoskeleton is a prominent means of distinguishing epithelial and myoepithelial cells, we asked whether the ratios of different keratins may represent a lineage. Whereas some of the cytoskeletal proteins such as α-sm actin in myoepithelial cells changed with time in culture (see below), the ratio between CK8 and CK14 as revealed by PD-QUEST image analysis remained stable: 4:1 in luminal cells and 1:10 in myoepithelial cells independent of passage number. We therefore used these ratios as a stable marker of the two cell types in higher passages. Thus defined, luminal epithelial cells retained a strong expression of CK18 and CK19, and they behaved in a lineage-specific manner inside a reconstituted basement membrane in that they formed typical acinus-like spheres with a central lumen (not shown; Petersen *et al.,* 1992). In contrast, myoepithelial cells did not display any luminal characteristics, and there was no expression of CK19, filamentous CK18, or surface sialomucin. These cells down-regulated α-sm actin and CALLA, but retained their myoepithelial characteristics as demonstrated by the total absence of CK18 and CK19, and the homogenous expression of vimentin as well as the

strong and uniform surface expression of another myoepithelial marker, the β4-integrin (Jones *et al.,* 1997). Moreover, they regained their lineage-specific α-sm actin expression at confluency in the presence of serum, and in the reconstituted basement membrane they formed tight balls without a central lumen as did primary myoepithelial cells (not shown).

Conversion of Luminal Cells to Myoepithelial Cells

To assay for bipotency of the cells, we switched the culture media of the two lineages. In CDM6, the myoepithelial cells remained unchanged for up to 2 weeks before they stopped growing and eventually apoptosed. There were no signs of luminal epithelial differentiation as revealed by morphology or staining for CK19. Since the myoepithelial cells eventually died in the CDM6 medium, we repeated this experiment with myoepithelial cells immortalized by retroviral infection with HPV16E6E7. Even with these cells, which readily survived the shift in medium for more than 10 days, we did not see a conversion in terms of CK19 expression to a luminal epithelial cell phenotype (data not shown).

Purified luminal epithelial cells, on the other hand, once placed in CDM4, showed a distinct focal conversion to myoepithelial cells as judged by the gradual morphological reappearance of cellular "streams" between the luminal epithelial islets, by the gradual loss of CK18 and CK19, and by gain of vimentin-, CALLA-, and serum-inducible α-sm actin (Fig. 4). Cellular sorting of the converted cells based on β4-integrin expression followed by 2-D gel electrophoresis additionally confirmed the myoepithelial protein pattern (Fig. 5). Whenever converting foci were apparent they showed cells that stained for both myoepithelial and luminal epithelial markers at the interphase (Fig. 4). Such dual staining was not observed in the original myoepithelial population. To obtain an estimate of the frequency of luminal epithelial cells with the capacity to convert, we quantified the number of converted cells immediately after morphological conversion was evident, that is, 4 days after switching the medium to CDM4. The frequency of cells having lost the luminal epithelial marker CK18 was 4.5 6 3.2%, and of cells having gained the myoepithelial marker β4-integrin was 2.2 ± 1.2%. This indicates that β4-integrin does not appear as readily as CK18 disappears. Thus, there is a hierarchy of gain of markers for myoepithelial cells. In this regard, a-smooth muscle actin appeared last (not shown). The frequency of conversion in a control propagated in CDM6 was 0%.

Having identified conversion among luminal epithelial cells in culture we looked for cellular conversion *in vivo.* This was done by use of two double-staining immunofluorescence protocols and confocal microscopy. In the first protocol we double stained for the two myoepithelial markers vimentin and a-sm actin where double staining was taken to reflect the ultimate level of myoepithelial differentiation. We next looked for vimentin-positive cells that were not costained for α-sm actin as a reflection of the maturation pathway seen in culture. Such cells were in fact apparent in sections of breast tissue as located suprabasally in occasional ducts and acini (Fig. 6). The other protocol allowed us to test whether the vimentin-positive suprabasal cells occasionally showed additional expression of the luminal epithelial marker CK18. As seen in Fig. 7, we were able to demonstrate the existence of double-stained suprabasal cells. While these were rare, typically only one or two cells per an entire section of tissue containing 3–10 lobules (Fig. 7), they nevertheless are the exceptions that prove the rule. Similar cell types could be demonstrated in the original, crude cellular populations from which our cultures were derived as demonstrated

in sections of organoids from primary cultures (not shown). Thus, our observations in culture, and the conclusions drawn from them are supported by the stainings of the tissue and cells of origin.

Discussion

Luminal Cells Give Rise to Myoepithelial Cells in the Adult Gland

We demonstrate here that sorted epithelial cells from the adult resting human breast can give rise to myoepithelial cells while the opposite does not occur. This observation is of interest because the current perception of renewal of breast cells rely on the existence of a population of subluminal epithelial cells referred to as “clear cells,” “null cells,” or “basal cells.” These are supposed to be equivalent to “cap cells” which are believed to be the putative stem cells of the developing prepubertal human breast and of the virgin rodent gland (Ferguson, 1985; Rudland, 1987, 1991; Sonnenberg *et al.,* 1986; Williams and Daniel, 1983; for recent reviews see Petersen *et al.,* 1997; Rønnov-Jessen, 1996). Cap cells are highly proliferative and are characterized by a very low level of mammary-specific differentiation. They may migrate into the luminal body cells and change their phenotype or migrate up along the subtending ducts to mature into fully differentiated myoepithelial cells (Dulbecco *et al.,* 1982; Williams and Daniel, 1983). The concept of subluminal, basal cells have been taken to support the notion that cap-like cells in the form of basal cells are present in the entire mammary gland and that these would be responsible for the cellular renewal for both luminal and myoepithelial cells. Such cells have been identified ultrastructurally as clear cells in both human and mouse, and evidence for their stem cell properties has been obtained by reimplanting different parts of the mouse mammary gland into cleared fat pads with the result that entire new glands were generated (Chepko and Smith, 1997; Smith and Medina, 1988). While this is plausible from a developmental point of view, it is reasonable to question whether what is true during development is also true for the stability of the differentiated state, i.e., for cellular renewal to take place in the adult organism. In particular, it is important to consider that the human breast, in contrast to the gland from virgin mouse, does not need to undergo further lobular development once postpubertal (for review see Rønnov-Jessen *et al.,* 1996). In fact, some confusion might be avoided if for the moment, the two processes of development and maintenance of the differentiated state were considered as separate processes, possibly involving different cellular compartments. Our findings reported here that we do not detect undifferentiated, subluminal cells after subtraction of the bona fide luminal cells from myoepithelial cells, and that mature myoepithelial cells in our hands do not give rise to luminal epithelial cells, leave little option other than the conclusion that a subset of luminal epithelial cells is key in cellular renewal in the mature gland. An epithelial to myoepithelial conversion was proposed previously as an explanation for the cellular transitions seen in a rat mammary epithelial cell line, although the lineage affiliations were only assessed morphologically (Rudland, 1987). In this case, however, the epithelial progenitor cell was considered to be a less differentiated putative stem cell. If such cells exist in the human breast, we must conclude that we somehow lost them during isolation. What the results indicate is the existence of progenitor cells rather than true stem cells. This would be consistent with the existence of secondary progenitor cells as described in mouse and rat (Chepko and Smith, 1997).

Luminal Epithelial Cells May Acquire Stem Cell-Like Function Postmaturation

Evidence that overtly differentiated luminal cells may indeed take over from cap (or basal) cells once the primitive ductal state is passed comes from a number of additional observations: First, while cap cells proliferate in early terminal end buds, proliferation occurs only in the luminal epithelial cells in the mature breast (Ferguson, 1985). This then could explain the almost exclusive occurrence of tumors (carcinomas) with luminal epithelial phenotype of the breast. Second, the expression of *bcl-2* is believed to reflect cellular longevity and stem cell properties (Nathan *et al.,* 1994). In the 16-week-old breast bud during embryogenesis, *bcl-2* is expressed in the basal cells (equivalent to cap cells in rodents) whereas already in the infant breast, expression switches to that of the luminal epithelial compartment (Nathan *et al.,* 1994). In glands that resemble the mammary gland, such as the submandibular gland, the *bcl-2*-expressing cells are identical to the intercalated duct putative progenitor cells (Pammer *et al.,* 1995). As is the case for the human breast, the cells responsible for cell renewal and also for cancer in the salivary gland are luminal epithelial and not basal cells (Ballagh *et al.,* 1994). If for the moment our hypothesis that the "stem" cell population may not necessarily be identical to the renewing cell population is accepted, then the question arises as where the stem cells could be located in the breast? It is again useful to draw a parallel to another skin derivative, namely, the hair follicles. Cellular turnover in this structure is confined to the basal bulge cells. However, when cellular longevity in culture was used as a measure of "stem" cell properties, the microdissected isthmus cells stood out as the candidate (Yang *et al.,* 1993). To return to the human breast, it is interesting that the basal cells in the isthmus area of lacteferous ducts and in the areola epidermis also stand out from the rest of the epidermis. These "basal" cells—analogous to mature epidermal cells—express CK19 (Bartek *et al.,* 1990). This keratin is found exclusively in the simple luminal epithelial cells of breast and not in the myoepithelial compartment. Again there is a gradual shift in the large ducts from basal expression to that of a luminal.

Transitional Cell Types Coexpress Luminal Epithelial Cytokeratins and Vimentin

As mentioned previously the earlier data from cultured rat mammary epithelial cell lines had also indicated that luminal epithelial cells were converted to myoepithelial cells only (Rudland, 1987). The conversion described in our study, similar to that of rat mammary epithelial cells operates with the transient coexpression of vimentin and luminal epithelial keratins in intermediate cells. *In situ* equivalents of such cells have not been described in the breast until now, probably due to the transient nature of the event and the fact that it would occur only in a small subset of cells. However, a look at another skin derivative, namely, the eccrine sweat gland from which the breast is derived, may help in the search for such intermediate stages. When sweat glands are fully formed at week 22, and the cap cells of the end buds have matured into complete myoepithelial cells, further growth relies on cells other than the cap cells. At this stage luminal secretory cells are believed to serve as precursor to myoepithelial cells because they retain the residual feature of immaturity up to adulthood, i.e., the coexpression of vimentin along with simple keratins (Moll and Moll, 1992).

Epithelial Progenitor Cells Are Linearly Related

In conclusion, we have provided evidence that luminal cells are precursors to myoepithelial cells rather than the other way around and that they may in fact be related in a unidirectional manner rather than both being derived directly from a common stem cell. This finding may be viewed within a broader context of decision making during diversification of cells not only *in vivo* but also in culture. The fact that an extrinsic factor may trigger a subset of cells to differentiate in a different lineage supports the hypothesis put forward by Brown *et al.* (1988), where the progenitor cells were postulated to be related in a linear manner, rather than having diversified along two distinct pathways. If the exterior signal is not present or the cells lose their ability to respond to the microenvironment appropriately, the progenitor continues to expand along the luminal epithelial lineage. Thus one additional aspect of malignant transformation in the breast may be the inability to convert to myoepithelial phenotype, a postulate that could explain why more than 90% of human breast cancers are luminal in their phenotype.

Finally, three sets of independent experiments performed recently lend strong support to the interpretation of our data. Using restriction length polymorphism, Noguchi *et al.* (1995) clearly showed that certain benign tumors which retain the normal dual luminal epithelial and myoepithelial composition are monoclonal in origin. Second, it has been shown that "normal" tissue close to invasive cancer indeed contain loss of heterozygocity (LOH) especially at chromosome 3p22-25 (Deng *et al.,* 1996). In the process of separating luminal epithelial and myoepithelial cells in phenotypically normal lesions, Lahkani *et al.* found that the LOH is contained in both the luminal epithelial and the myoepithelial compartment, again suggesting monoclonality (M. J. O'Hare, Ludwig Institute, London, personal communication).

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References

Anbazhagan, R., Osin, P. P., Bartkova, J., Nathan, B., Lane, E. B., and Gusterson, B. A. (1998). The development of epithelial phenotypes in the human fetal and infant breast. *J. Pathol.* **184,** 197–206.

Ballagh, R. H., Kudryk, K. G., Lampe, H. B., Moriarty, B., Mackay, A., Burford-Mason, A. P., and Dardick, I. (1994). The pathobiology of salivary gland. III. PCNA-localization of cycling cells induced in rat submandibular gland by low-dose x-radiation. *Oral. Surg. Oral Med. Oral Pathol.* **77,** 27–35.

Bartek, J., Bartkova, J., and Taylor-Papadimitriou, J. (1990). Keratin 19 expression in the adult and developing human mammary gland. *Histochem. J.* **22,** 537–544.

Brown, G., Bunce, C. M., Lord, J. M., and McConnell, F. M. (1988). The development of cell lineages: A sequential model. *Differentiation* **39,** 83–89.

Chepko, G., and Smith, G. H. (1997). Three division-competent, structurally-distinct cell populations contribute to murine mammary epithelial renewal. *Tissue Cell* **29,** 239–253.

Clarke, C., Titley, J., Davies, S., and O'Hare, M. J. (1994). An immunomagnetic separation method using superparamagnetic (MACS) beads for large-scale purification of human mammary luminal and myoepithelial cells. *Epith. Cell Biol.* **3,** 38–46.

Dairkee, S., and Heid, H. W. (1993). Cytokeratin profile of immunomagnetically separated epithelial subsets of the human mammary gland. *In Vitro Cell. Dev. Biol.* **29A,** 427–432.

Deng, G., Lu, Y., Zlotnikov, G., Thor, A. D., and Smith, H. S. (1996). Loss of heterozygosity in normal tissue adjacent to breast carcinomas. *Science* **274,** 2057–2059.

Dulbecco, R., Henahan, M., and Armstrong, B. (1982). Cell types and morphogenesis in the mammary gland. *Proc. Natl. Acad. Sci. USA* **79,** 7346–7350.

Ferguson, D. J. P. (1985). Ultrastructural characterisation of the proliferative (stem ?) cells within the parenchyma of the normal "resting" breast. *Virchows Arch. [Pathol. Anat.]* **407,** 379–385.

Ferguson, D. J. P., and Anderson, T. J. (1983). A morphological study of the changes which occur during pregnancy in the human breast. *Virchows Arch. [Pathol. Anat.]* **401,** 163–175.

Gomm, J. J., Browne, P. J., Coope, R. C., Liu, Q. Y., Buluwela, L., and Coombes, R. C. (1995). Isolation of pure populations of epithelial and myoepithelial cells from the normal human mammary gland using immunomagnetic separation with dynabeads. *Anal. Biochem.* **226,** 91–99.

Guelstein, V. I., Tchypysheva, T. A., Ermilova, V. D., Litvinova, L. V., Troyanovsky, S. M., and Bannikov, G. A. (1988). Monoclonal antibody mapping of keratins 8 and 17 and of vimentin in normal human mammary gland, benign tumors, dysplasias and breast cancer. *Int. J. Cancer* **42,** 147–153.

Gusterson, B. A., Warburton, M. J., Mitchell, D., Ellison, M., Neville, A. M., and Rudland, P. S. (1982). Distribution of myoepithelial cells and basement membrane proteins in the normal breast and in benign and malignant breast diseases. *Cancer Res.* **42,** 4763–4770.

Jones, J. L., Royall, J. E., Critchley, D. R., and Walker, R. A. (1997). Modulation of myoepithelial-associated a6b4 integrin in a breast cancer cell line alters invasive potential. *Exp. Cell Res.* **235,** 325–333.

Kordon, E. C., and Smith, G. H. (1998). An entire functional mammary gland may comprise the progeny from a single cell. *Development* **125,** 1921–1930.

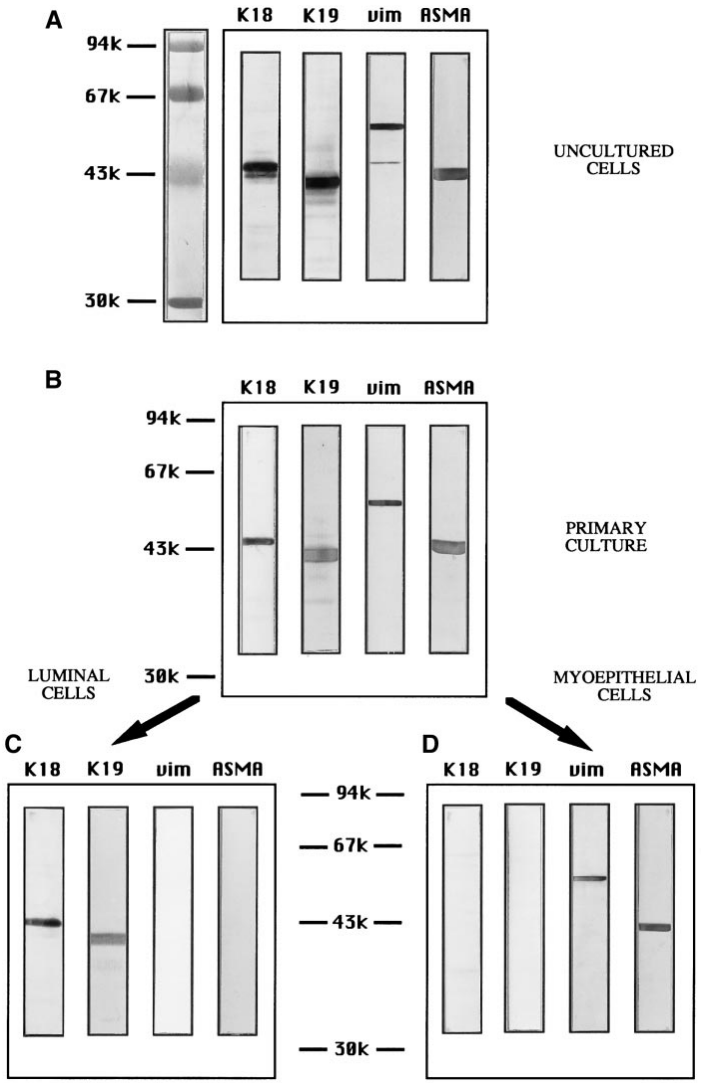
Moll, I., and Moll, R. (1992). Changes of expression of intermediate filament proteins during ontogenesis of eccrine sweat glands. *J. Invest. Dermatol.* **98,** 777–785.

Mørk, C., van Deurs, B., and Petersen, O. W. (1990). Regulation of vimentin expression in cultured human mammary epithelial cells. *Differentiation* **43,** 146–156.
Nathan, B., Anbazhagan, R., Clarkson, P., Bartkova, J., and Gusterson, B. (1994). Expression of BCL-2 in the developing human fetal and infant breast. *Histopathology* **24,** 73–76.
Noguchi, S., Aihara, T., Koyama, H., Motomura, K., Inaji, H., and Imaoka, S. (1995). Clonal analysis of benign and malignant human breast tumors by means of polymerase chain reaction. *Cancer Lett.* **90,** 57–63.
Pammer, J., Horvat, R., Weninger, W., and Ulrich, W. (1995). Expression of bcl-2 in salivary glands and salivary gland adenomas: A contribution to the reserve cell theory. *Pathol. Res. Pract.* **191,** 35–41.
Petersen, O. W., Rønnov-Jessen, L., Howlett, A. R., and Bissell, M. J. (1992). Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. *Proc. Natl. Acad. Sci. USA* **89,** 9064–9068.
Petersen, O. W., Rønnov-Jessen, L., Weaver, V. M., and Bissell, M. J. (1997). Differentiation and cancer in the mammary gland: Shedding new light on an old dichotomy. *Adv. Cancer Res.* **75,** 135–161.
Petersen, O. W., and van Deurs, B. (1987). Preservation of defined phenotypic traits in short-time cultured human breast carcinoma derived epithelial cells. *Cancer Res.* **47,** 856–866.
Petersen, O. W., and van Deurs, B. (1988). Growth factor control of myoepithelial-cell differentiation in cultures of human mammary gland. *Differentiation* **39,** 197–215.
Rudland, P. S. (1987). Stem cells and the development of mammary cancers in experimental rats and humans. *Cancer Metast. Rev.* **6,** 55–83.
Rudland, P. S. (1991). Histochemical organization and cellular composition of ductal buds in developing human breast: Evidence of cytochemical intermediates between epithelial and myoepithelial cells. *J. Histochem. Cytochem.* **39,** 1471–1484.
Rudland, P. S. (1993). Epithelial stem cells and their possible role in the development of the normal and diseased human breast. *Histol. Histopathol.* **8,** 385–404.
Rudland, P. S., Fernig, D. G., and Smith, J. A. (1995). Growth factors and their receptors in neoplastic mammary glands. *Biomed. Pharmacother.* **49,** 389–399.
Rønnov-Jessen, L. (1996). Stromal reaction to invasive cancer: The cellular origin of the myofibroblast and implications for tumor development. *Breast J.* **2,** 320–339.
Rønnov-Jessen, L., Celis, J. E., van Deurs, B., and Petersen, O. W. (1992). A fibroblast-associated antigen: Characterization in fibroblasts and immunoreactivity in smooth muscle differentiated stromal cells. *J. Histochem. Cytochem.* **40,** 475–486.
Rønnov-Jessen, L., and Petersen, O. W. (1993). Induction of a-smooth muscle actin by transforming growth factor-b1 in quiescent human breast gland fibroblasts: Implications for myofibroblast generation in breast neoplasia. *Lab. Invest.* **68,** 696–707.
Rønnov-Jessen, L., Petersen, O. W., and Bissell, M. J. (1996). Cellular changes involved in conversion of normal to malignant breast: The importance of the stromal reaction. *Physiol. Rev.* **76,** 69–125.
Smith, G. H. (1996). Experimental mammary epithelial morphogenesis in an *in vivo* model: Evidence for distinct cellular progenitors of the ductal and lobular phenotype. *Breast Cancer Res. Treat.* **39,** 21–31.
Smith, G. H., and Medina, D. (1988). A morphologically distinct candidate for an epithelial stem cell in mouse mammary gland. *J. Cell Sci.* **89,** 173–183.

Sonnenberg, A., Daams, H., van der Valk, M. A., Hilkens, J., and Hilgers, J. (1986). Development of mouse mammary gland: Identificationof stages in differentiation of luminal and myoepithelial cells using monoclonal antibodies and polyvalent antiserum against keratin. *J. Histochem. Cytochem.* **34,** 1037–1046.
Taylor-Papadimitriou, J., Stampfer, M., Bartek, J., Lewis, A., Boshell, M., Lane, E. B., and Leigh, I. M. (1989). Keratin expression in human mammary epithelial cells cultured from normal and malignant tissue: Relation to in vivo phenotypes ad influence of medium. *J. Cell Sci.* **94,** 403–413.
Umemura, S., Osamura, R. Y., and Tsutsumi, Y. (1996). Cell renewal and functional morphology of human lactating breast: Presentation of a new mode of cell death ("magentosis") characterized by nuclear periodic acid-Schiff reactivity. *Pathology Int.* **46,** 105–121.
Williams, J. M., and Daniel, C. W. (1983). Mammary ductal elongation: Differentiation of myoepithelium and basal lamina during branching morphogenesis. *Dev. Biol.* **97,** 274–290.
Yang, J. S., Lavker, R. M., and Sun, T. T. (1993). Upper human hair follicle contains a subpopulation of keratinocytes with superior in vitro proliferative potential. *J. Invest. Dermatol.* **101,** 652–659.

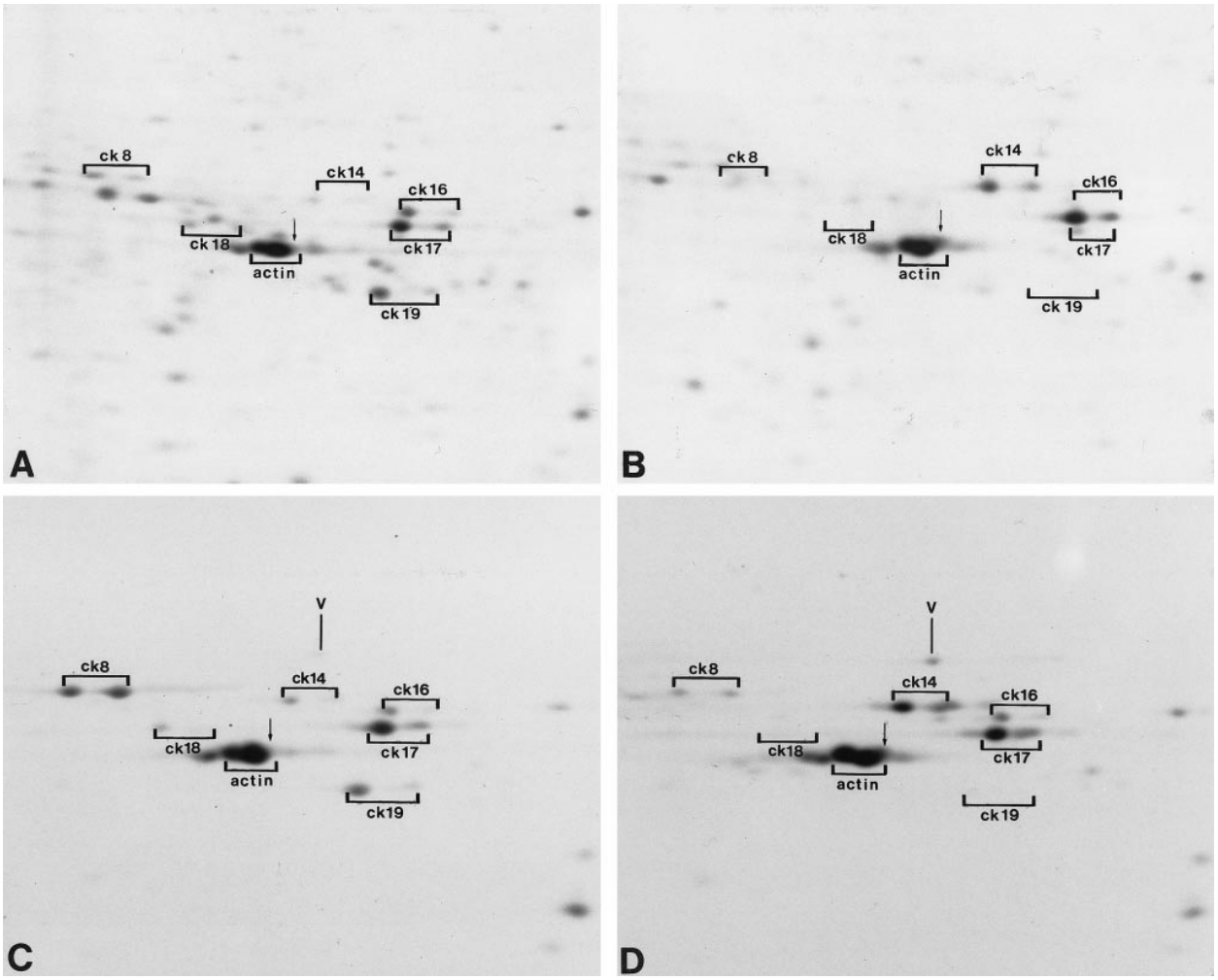
Figures

FIGURE 1



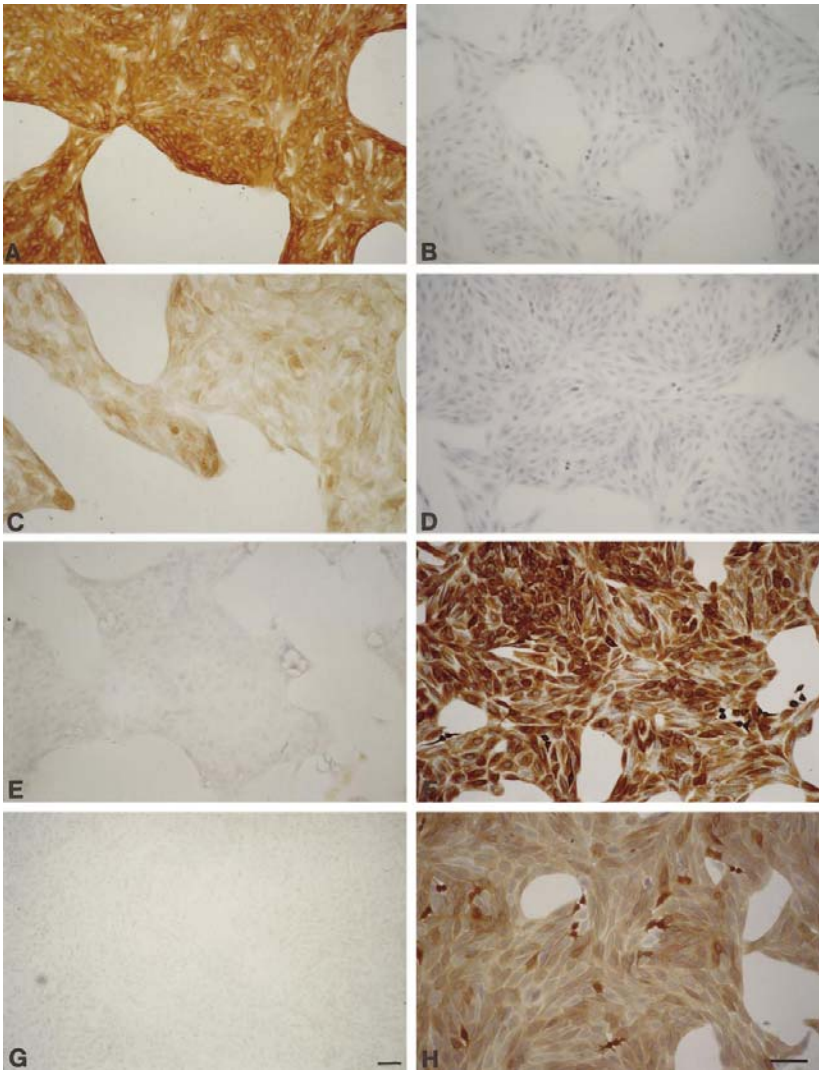
Characterization of human mammary epithelial cells. Uncultured cells (A), primary culture (B), purified luminal epithelial cells (C), and purified myoepithelial cells (D). (A, B) Homogeneous expression of the cytokeratins 18 and 19 (K18–K19), vimentin (vim) and α-sm actin (ASMA) in uncultured cells (A) and in primary culture (B). (C, D) The luminal epithelial cells (C) expressed CK18 and CK19 only whereas myoepithelial cells (D) expressed vimentin and α-sm actin.

FIGURE 2



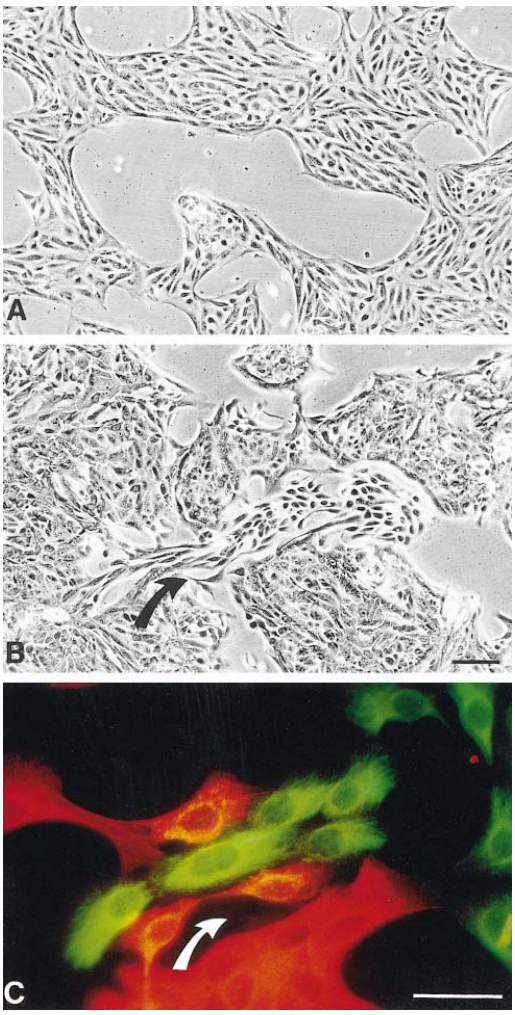
2-D gel electrophoresis of methionine labelled proteins of purified human breast epithelial cells in culture. Total lysate (A, B) and enriched cytoskeletal preparations (C, D). (A, C) Protein pattern of the purified luminal epithelial cells indicating a high ratio of CK18 and CK19 to CK14. (B, D) The opposite pattern is observed for myoepithelial cells.

FIGURE 3



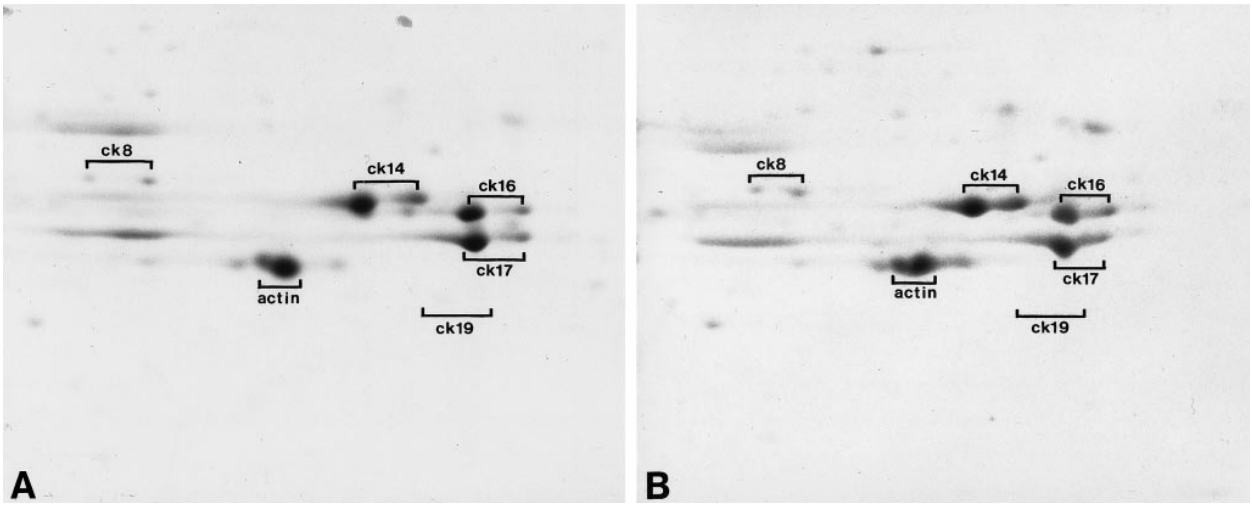
Immunoperoxidase staining of sorted, human breast epithelial cells. (Left) The luminal epithelial fraction. (Right) The myoepithelial cells. (A, B) Staining for CK18; (C, D) staining for CK19; (E, F) vimentin staining; (G, H) staining for α-sm actin. A–F and H, x83; G, x50; bar, 100 µm.

FIGURE 4



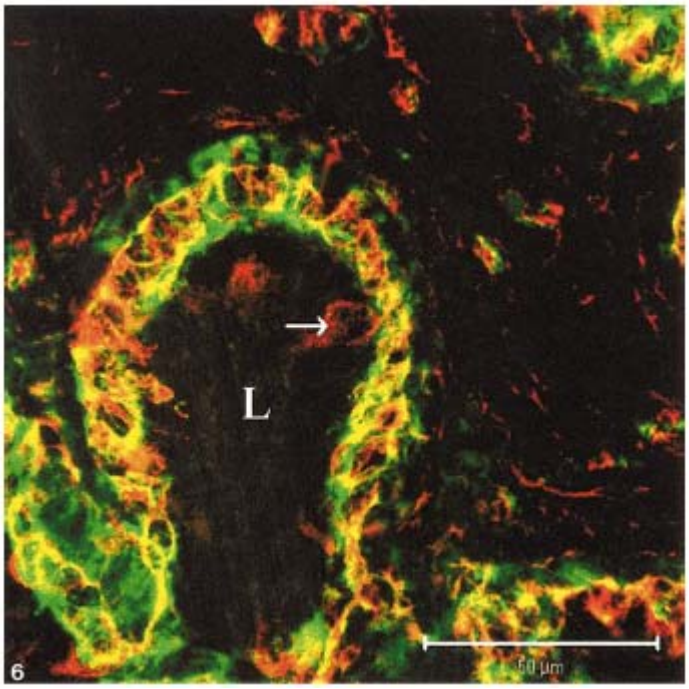
Conversion of luminal epithelial cells. (A) Phase contrast micrograph of luminal epithelial cells grown on CDM6 medium. Note the presence of only one morphologically distinct cell type. (B) Phase contrast micrograph of luminal epithelial cells grown in CDM6 medium followed by a switch to CDM4 medium. Note the gradual occurrence of a second cell type in addition to the luminal epithelial cells (arrow). (C) Double-labeling immunofluorescence of cells in the conversion zone of a culture treated as in (B). The Texas red staining represents CK18 and the FITC staining represents vimentin. Note the yellow-stained cells in the center of the conversion zone (arrow) expressing both markers. A, B, x83; C, x330; bar, 50 µm.

FIGURE 5



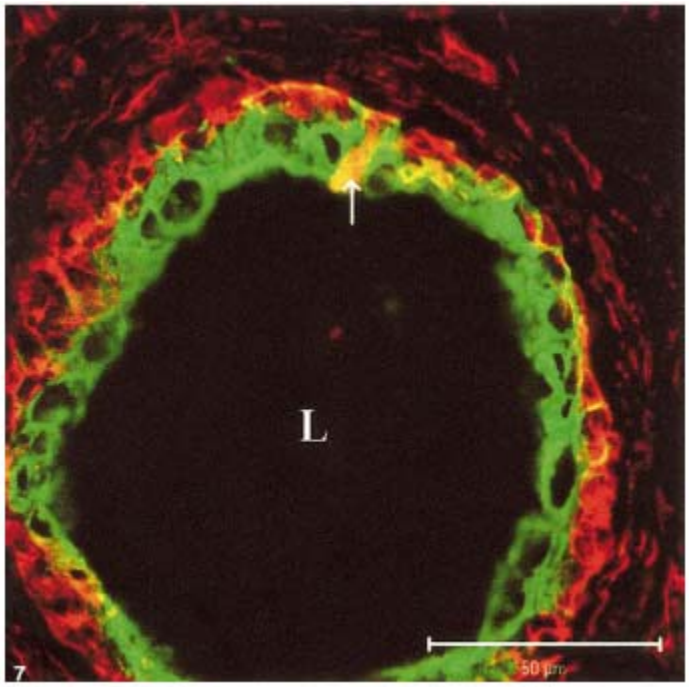
2-D gel electrophoresis protein patterns of converted luminal epithelial cells. (A) Control myoepithelial cells in CDM4 medium. (B) Converted luminal epithelial cells cultured in CDM4 medium. Note the near-identical protein expression patterns characteristic of myoepithelial cells in both fluorograms: CK18 and CK19 are absent, but there is strong expression of CK14.

FIGURE 6



Confocal image of a breast section double stained for α-sm actin (green) and vimentin (red) showing a large acinus/duct with a central lumen (L). The double-stained, mostly yellow cells represent the outer myoepithelial cell layer. The arrow indicates a suprabasal cell which stains for one myoepithelial marker only, that is, vimentin.

FIGURE 7



Confocal image of a breast section double stained with the luminal marker cytokeratin K18 (green) and the myoepithelial marker vimentin (red) showing a large acinus/duct with a central lumen (L). The outer layer of myoepithelial cells and the inner layer of luminal epithelial cells generally show no overlap in the staining pattern. The arrow indicates the occasional presence of a double-stained suprabasal cell.