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Invited Review

Epithelial stem cells and their possible role in the development of the normal and diseased human breast

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Summary. The developing breasts of peripubescent girls consist of ducts and budded structures, which can subdivide to alveolar buds/lobules with advancing maturity and finally to secretory alveoli during pregnancy and lactation. Immunochemical reagents have been used to visualize the three major cell types in histological sections of mature/pregnant breasts, the epithelial cells which line ducts/ductules, the smooth muscle-like myoepithelial cells and the casein-secretory alveolar cells. Ductal budded structures contain basal cells intermediate in immunocytochemical staining characteristics between epithelial and myoepithelial cells. Immortalization of primary epithelial cultures of normal breasts by simian virus 40 yields epithelial cell lines that can differentiate to myoepithelial-like and to secretory alveolar-like cells; similar cell types are identifiable in primary cultures. Immunocytochemical staining shows that both hyperplastic and neoplastic benign lesions contain myoepithelial-like cells, and, under suitable hormonal conditions, alveolar-like cells, but invasive carcinomas contain neither differentiated cell type. Primary cell cultures of benign hyperplastic and neoplastic lesions contain epithelial, myoepithelial-like and presumptive alveolar-like cells whilst malignant cell fractions of invasive carcinomas contain only epithelial cells. Spontaneously-immortalized epithelial cell lines from hyperplastic benign breast disease can generate myoepithelial-like and alveolar-like cells, whilst standard epithelial cell lines from pleural effusions and novel epithelial cell lines from primaries of invasive carcinomas fail to differentiate to either cell type. It is suggested that epithelial/intermediate stem cells exist in a basal position predominantly in terminal structures of growing breasts, and that they are the major cell type involved in benign hyperplastic, benign neoplastic and malignant breast diseases. The acquisition of the malignant phenotype is associated with the carcinoma cells having a greatly impaired ability to differentiate to myoepithelial and to alveolar cells.

Key words: Breast development, Immunocytochemical staining, Stem cell cultures, Epithelial differentiation, Failure in carcinomas

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Anatomical structures within the developing breast

The development of the female human breast occurs in three distinct phases that are largely controlled by the levels of circulating hormones. The first phase occurs during fetal development, initially under the influence of maternal hormones, and results in the formation of a rudimentary organ consisting of simple, branched ducts (Dawson, 1934; Salazar et al., 1975; Anbazhagan et al., 1991).

The second phase occurs with the approach of puberty between the ages of 10 and 12 years (Monaghan et al., 1990). This phase consists of a rapid extension of the ductal tree and generation of its branching pattern by lengthening of existing ducts, by dichotomous branching of the growing ductal tips and by monopodial branching from the sides of existing ducts (Dawson, 1934; Vorherr, 1974). During this period of rapid growth the ducts sometimes become enlarged or swollen of their termini and occasionally produce large bulbous terminal end buds (TEBs) up to 1 mm in length (Fig. 1) (Table 1) (Russo and Russo, 1987; Rudland, 1991). These terminal ducts/TEBs contain the most actively proliferating cells in the breast parenchyma (Russo and Russo, 1987) and are responsible for ductal elongation and probably for dichotomous branching by bifurcation (Rudland, 1991). Thereafter the number of terminal ducts/TEBs decreases (Dawson, 1934). Monopodial branching is produced by the growth of smaller lateral buds (LBs) (Table 1) at the sides of existing ducts (Dawson, 1934; Vorherr, 1974).

Formation of lobules of alveolar buds (ABs) (Table 1)

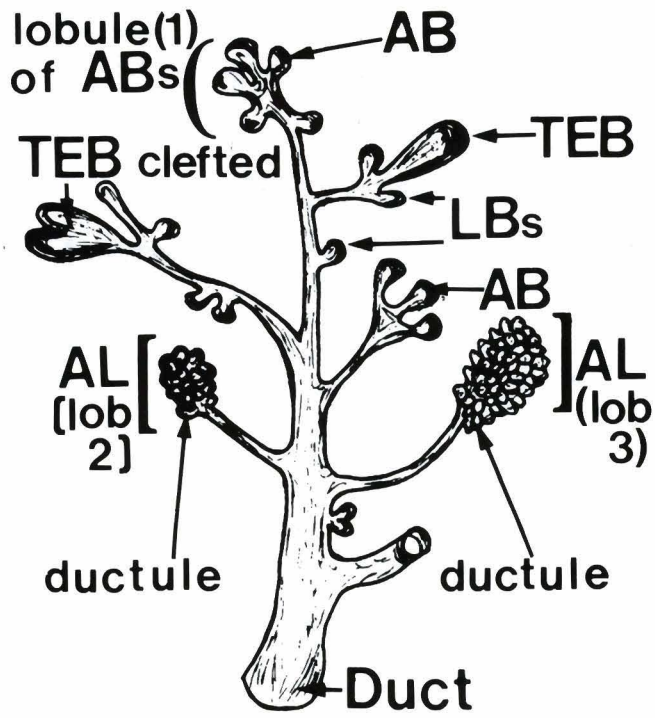

Fig. 1. Diagrammatic representation of the structures present in the developing breast. TEB, terminal end bud; TEB clefted, terminal end bud subdivided by a cleavage furrow at its tip, further cleavage results in alveolar buds; LB, lateral bud; AB, alveolar bud; AL, alveolar lobule. The lobules have been classified previously as type 1 (lob 1), synonymous with lobule of ABs, and types 2 and 3 which correspond to ALs containing smaller numbers of larger ductules (lob 2) and ALs containing larger numbers of smaller ductules (lob 3), respectively. Modified from Russo and Russo (1987).

occurs within 1 to 2 years after the onset of the first menstrual period. They consist of 3-5 lobes of a similar size to that of lateral buds and arise from terminal ducts and TEBs; smaller ABs can also arise from LBs (Russo et al., 1990; Rudland, 1991). The existence of TEBs, LBs and terminal ducts that can subdivide into ABs during the first and subsequent menstrual cycles in humans is similar to the much more detailed studies in rodents (Russo and Russo, 1978; Ormerod and Rudland, 1984). However, this transition in humans is not so sharp as in rodents since the occasional TEB has been

Table 1. Characteristics of different structures of the developing breast.

PARAMETER	STRUCTURE[a]			
	TEB	LB	Lobule of ABs	AL
Number of buds or ductules/structure	1	1	3-5	6-60
Area of total structure (mm^2±SD) x100[b]	1.9±0.8	0.53±0.17	4.4±0.9	10±4
Area of bud/ductule (mm^2±SD) x100[c]	-[f]	-[f]	0.42±0.10	0.17±0.08
Number of cells per cross-section of bud/ductule ±SD[d]	62±6	34±8	33±6	13±3
Number of cell layers in bud/ductule[e]	>4	2-3	2-3	1-2

[a]: abbreviations: AB, alveolar bud; AL, alveolar lobule containing individual ductules; LB, lateral bud; TEB, terminal end bud; ductule is synonymous with acinus. [b]: significant differences (n=20) between columns (Student's t-test, $p<0.01$). [c]: significant differences (n=20) for TEB, LB/AB and ductule (Student's t-test, $p<0.05$). [d]: significant differences (n=20) for TEB, LB/AB and ductule (Student's t-test, $p<0.01$). [e]: the cross-section includes any lumina present; ductules contain a discontinuous layer of basal cells. [f]: same as for total structure above.

observed in mature 18-year-old females (Russo and Russo, 1987). Thereafter, sprouting of new ABs can gradually occur over a period of years during puberty, and their further subdivision gives rise to alveolar lobules (ALs) consisting of many smaller ductules (Dawson, 1934; Vorherr, 1974). The number of individual ductules within the ALs increases somewhat with age reaching up to 60 or more in mature cycling 17- and 18-year-old females (Table 1). The terminal ductal structures that include ABs and ALs correspond to the terminal ductal lobular units (TDLUs) (Fig. 1) (Hamperl, 1970; Ozzello, 1971). There is, however, much variation in glandular structure both within the same breast and between the breasts for women of the same age (Dawson, 1934; Dabelow, 1957; Drife, 1986).

The third phase occurs during pregnancy and lactation. During the first few months of pregnancy there is a marked increase in large clusters of ALs, some of which can contain up to 200 individual ductules (Russo et al., 1988). They become distended and form secretory alveoli during the lactational phase (Salazar and Tobon, 1974; Russo and Russo, 1987). Even at this stage of

Fig. 2. Immunocytochemical staining for epithelial, myoepithelial and alveolar cells in mature normal breasts. **A.** Section incubated with MAbs to human MFGM showing staining of epithelial cells (e) surrounding the lumen but no staining of myoepithelial cells (m). **B.** Section incubated with MAb to cytokeratin 18 showing only some of the epithelial cells in the main duct staining (arrows), the myoepithelial cells are unstained (m), l = lumen. **C.** Section incubated with MAb to smooth-muscle actin showing strong staining of myoepithelial cells (m) in extralobular terminal (etd) and in intralobular terminal ducts (itd) and ductules (dtl), the epithelial cells (e) are unstained. **D.** Section incubated with MAb to cytokeratin 14 showing staining of the ductal myoepithelial cells (m) but no staining of the epithelial cells (e). **E.** Frozen section incubated with MAb to CALLA showing strong staining of all myoepithelial cells (m) in a main duct, the epithelial cells (e) and stroma are unstained. **F.** Section incubated with MAb to Type IV collagen showing a stained band of basement membrane around the outside of ductules (thin arrows); the epithelial cells (e) and fibroblasts are unstained, but blood vessels are stained strongly (thick arrow). **G.** Section of pregnant breast incubated with MAb to κ-casein showing extensive staining of epithelial cells (e) in alveolar lobules and in the intralobular terminal duct (itd) (arrow). **H.** Section of lactating breast incubated with MAb to κ-casein showing extensive staining of the secretory epithelial or alveolar cells (e), globules in the lumen (g) and luminal debris (arrow), the myoepithelial cells (m) are unstained. All Methacarn-fixed, paraffin-embedded material except E. Original magnification x 230, Bars = 75 μm.

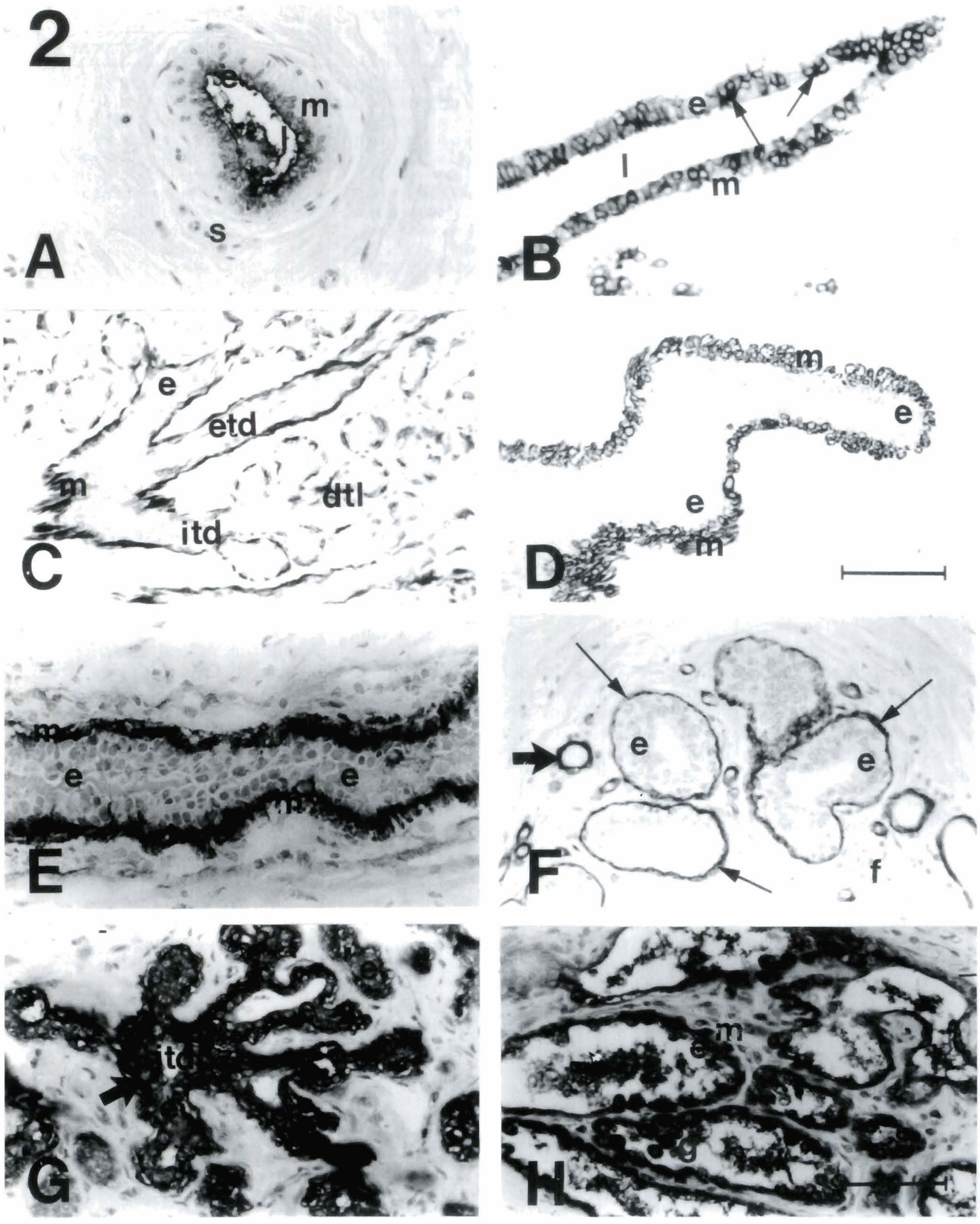
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Table 2. Immunocytochemical reagents: their reactive determinants and cellular staining patterns.

REAGENT[a]	REACTIVE DETERMINANTS[b]	REFERENCE/SOURCE	BREAST STAINING[d]
Anti-actin	Smooth muscle actin	Bussolati et al., 1980/Miles Labs	Myoepithelial, b/v
Anti-EMA	EMA (PAS-O) of MFGM	Ormerod et al., 1985/Sera Labs	Epithelial
Anti-laminin	All species laminin	Warburton et al., 1982	Basement membranes
Anti-myosin	Smooth muscle myosin	Warburton et al., 1982	Myoepithelial, b/v
Anti Type IV-collagen	All species Type IV collagen	Warburton et al., 1982	Basement membranes
Anti-vimentin	Vimentin	Hynes and Destree, 1978	Myoepithelial, fibroblasts, b/v
MAb 24.128	Human Type IV collagen	Hancock and Atkins, 1984/Dako Labs	Basement membranes
MAb A12[c]	CALLA gp100	Carrel et al., 1983/Sera Labs	Myoepithelial
MAb CKB1	Keratin 14	Dairkee et al., 1985/Sigma	Myoepithelial
MAb LE61[c]	Keratin 18	Lane, 1982	Epithelial
MAb LICR-LON-14.1	Human κ-casein	Earl and McIlhinney, 1985	Alveolar
MAb LICR-LON-23.10[c]	135 Kd glycoprotein	Gusterson et al., 1985	Myoepithelial, b/v
MAb LICR-LON-32.2	Human ß-casein	Earl and McIlhinney, 1985	Alveolar
MAb LICR-LON-M3	Sugar residue of MFGM	McIlhinney et al., 1985	Epithelial, trace of myoepithelial
MAb LICR-LON-M8	EMA (PAS-O)	Ormerod et al., 1984, 1985	Epithelial
MAb LICR-LON-M18	Gal (1-4)GlcNAcß(1-6)	Gooi et al., 1983	Epithelial
MAb LICR-LON-M24	Sugar residue of MFGM	McIlhinney et al., 1985	Epithelial
MAb LP34	Keratins 4, 5, 6, 10, 18	Taylor-Papadimitriou et al., 1983/Dako Labs	Myoepithelial, weaker epithelial
MAb MA-931	Smooth and skeletal muscle actin	Enzo Biochemicals, New York	Myoepithelial, b/v
MAb MA-933	Smooth muscle actin	Enzo Biochemicals, New York	Myoepithelial, b/v
MAb PHM6[C]	CALLA gp100	Pilkington et al., 1984	Myoepithelial
MAb PKK2	Keratins 7, 16, 17, 19	Holthofer et al., 1984/Lab Systems	Myoepithelial, weaker epithelial
MAb PKK3	Keratin 18	Holthofer et al., 1984/Lab Systems	Epithelial
MAb V9	Vimentin	Osborn et al., 1984/Dako Labs	Myoepithelial, fibroblasts, b/v
Peanut lectin	Galß(1-3)GalNAc	Newman et al., 1979/EY Labs	Epithelial

[a]: anti-polyclonal antiserum raised in rabbits; MAb, monoclonal antiserum raised in mice. [b]: abbreviations as in text and Rudland and Hughes (1989). [c]: frozen tissues only; tissues for the remainder were normally fixed in Methacarn and embedded in paraffin (Warburton et al., 1982). [d]: predominantly from Rudland and Hughes (1989), except MAb CKB1 to cytokeratin 14 from Aung et al. (1993); b/v, blood vessel.

development more primitive stages of budding which have lagged behind the general degree of development can be found up to the time of birth. They have been regarded either as virginal lobules refractory to endocrine stimuli (Dabelow, 1957) or a reserve to replace degenerating or exhausted areas of the gland (Dawson, 1935).

Identification of different cell types in mature breasts

The ducts and ductules usually consist of two cell types, an inner lining of epithelial cells and an outer, sometimes discontinuous lining of myoepithelial cells (Fig. 2). Both structures are separated from the stroma by a basement membrane (Ozzello, 1971; Salazar and Tobon, 1974). A third cell type that lines large distended ductules or alveoli during pregnancy and lactation, the alveolar cell, is responsible for production and eventual secretion of milk (Vorherr, 1974). Within the ducts, ductules and alveoli, epithelial cells, myoepithelial cells and alveolar cells can normally be distinguished in histological sections by their position and their characteristic ultrastructural features (Vorherr, 1974). Thus epithelial cells possess microvilli and typical intercellular junctions including desmosomes. The myoepithelial cells usually possess an irregular nucleus with peripheral heterochromatin, myofilaments, pinocytotic vesicles which line the inner plasma membrane and basement membrane, often connected by hemidesmosomes to the outside of the plasma membrane. The alveolar cell has a typical secretory apparatus (Ozzello, 1971).

More recently, histochemical and immunocytochemical reagents have been used to distinguish epithelial cells, myoepithelial cells and alveolar cells (Table 2). Peanut lectin (Newman et al., 1979), antiserum to epithelial membrane antigen (EMA) (Sloane and Ormerod, 1981), monoclonal antibodies (MAbs) to human milk fat globule membranes (MFGM) (Foster et al., 1982a; Taylor-Papadimitriou et al., 1983). and MAbs to cytokeratin 18 stain the epithelial cells throughout the parenchymal structures; the myoepithelial cells are largely unstained (Table 2). Antisera and monoclonal antibodies to MFGM stain the luminal membrane of all epithelial cells, irrespective of their position in the breast (Fig. 2A), whereas peanut lectin and MAbs to keratin 18 (Fig. 2B) stain relatively few epithelial cells in main ducts but more epithelial cells in terminal lobular structures (Rudland and Hughes, 1989). Removing terminal sialic acid residues from histological sections with neuraminidase enables many more luminal epithelial cells to react with peanut lectin (Rudland and Hughes, 1989). None of the above reagents stains the stromal cells.

In contrast, antisera to the cytoskeletal components of smooth muscle, actin (Fig. 2C) (Bussolati et al., 1980), myosin (Gusterson et al., 1982), MAbs to cytokeratins 5 and 14 (Fig. 2D) (Dairkee et al., 1985; Nagle et al.,

1986), to the intermediate filamental protein vimentin (Rudland and Hughes, 1989) and to cell surface determinants including the common acute lymphoblastic leukemia antigen (CALLA) (Fig. 2E) (Gusterson et al., 1986) and a 135 kd glycoprotein (Gusterson et al., 1985) stain the myoepithelial but not the epithelial cells (Table 2). In addition antisera to laminin and Type IV collagen (Fig. 2F) stain the basement membrane which is usually associated with myoepithelial cells (Table 2) (Barsky et al., 1982; Gusterson et al., 1982). However, MAbs to vimentin also stain stromal fibroblasts, and these antibodies, together with those of the smooth muscle-specific actin/myosin, of the 135 kd surface glycoprotein and of laminin and Type IV collagen also stain stromal blood vessels (Rudland and Hughes, 1989), thus necessitating a combination of suitable antibodies to identify unambiguously myoepithelial cells (Table 2). This is particularly true when the myoepithelial cells occur in TDLUs and show reduced staining with the above antibodies (Rudland and Hughes, 1989), suggesting the presence of a less mature form of myoepithelial cell. Finally, MAbs to the human caseins stain only the third cell type, the alveolar cell in pregnant (Fig. 2G) and lactating (Fig. 2H) women (Table 2) (Earl and McIlhinney, 1985; Rudland and Hughes, 1989).

Histochemical organisation and cellular composition of ductal buds in developing human breasts

In addition to the discrete cell types observed in mature breasts, subsets of epithelial cells (Hagueneau and Arnoult, 1959) and myoepithelial cells (Emerman and Vogel, 1986; Hamperl, 1970; Smith et al., 1984) and/or cells intermediate between these two types (Ozzello, 1971; Stirling and Chandler, 1976) have been reported at the ultrastructural level, the latter particularly in basal positions in growing terminal ductal structures of young women. Immunocytochemical staining of such

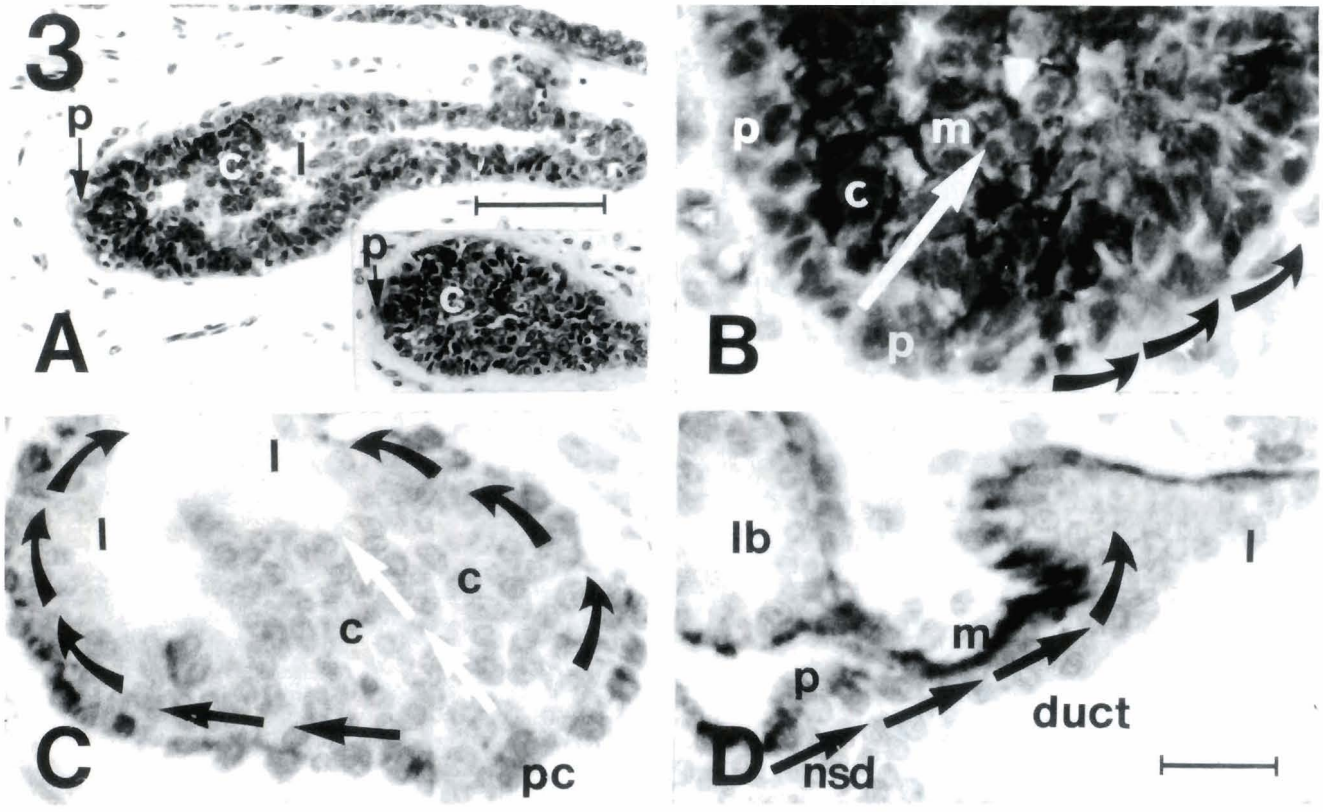


Fig. 3. Histochemical and immunocytochemical staining of terminal end bud structures in prepubescent developing breasts. **A.** Longitudinal section of terminal end bud (TEB) stained by haematoxylin and eosin with central lumen (l). Inset. TEB without central lumen. Peripheral cells are seen at the distal tip (p) and within the centre (c) of the cellular mass. **B.** Section of TEB incubated with MAbs to MFGM showing the distal tip at higher magnification with weak staining of the loosely-packed, peripheral cells (p), which is gradually reduced the nearer the cells become to the subtending duct (curved arrows). Staining of the cells increases towards the centre (white arrow), first cytoplasmically (c) and then membranously (m). **C.** Section of TEB incubated with MAb to smooth muscle actin showing the distal tip at higher magnification with the loosely-packed peripheral cells (pc) stained moderately; this staining increases gradually the further such cells are positioned from the distal tip (black arrows). The staining decreases for the close-packed cells (c) in the middle of the TEB (white arrows), and is lost completely from the luminal epithelial cells (l). **D.** Section of TEB incubated with MAb to smooth-muscle actin showing its proximal region at higher magnification with the now more-closely-packed peripheral cells (p) increasing their staining to form a continuum with the myoepithelial cells (m) of the neck region of the subtending duct (nsd). The luminal epithelial cells are unstained and a lateral bud (lb) is also shown. Magnification A, x 230. B, C, D x 730; Bars = 75 μm for A and 20 μm for B, C, D.

structures confirms this opinion (Rudland, 1991).

TEBs, LBs, terminal ducts and ABs of pre- and peripubescent 12 to 13-year-old girls contain more than two cellular layers and TEBs in particular often contain a solid mass of cells (Fig. 3A), in contrast to most of the ALs of mature cycling women (Russo and Russo, 1987; Rudland, 1991). Larger, more bulbous terminal ducts are also encountered in growing infant breasts (Anbazhagan et al., 1991). Immunocytochemical staining of pre- and peripubescent breasts with antisera, MAbs and peanut lectin to EMA/MFGM delineates the luminal surfaces of the epithelial cells that line buds/ductules in ABs and ALs, as well as staining both cytoplasmically and peripherally epithelial-like cells in nonluminal regions, including central cells of the TEBs (Fig. 3B). This staining occurs in a graded manner with peripheral, more loosely connected cells showing weak staining which gradually increases the more centrally positioned the cells become (Figs. 3B, 4A). Similar staining patterns are obtained with epithelial-specific MAbs to keratin 18 in TEBs, although many more epithelial-like cells are stained in the growing terminal budded structures and lobules than in the mature main ducts (Rudland and Hughes, 1989; Rudland, 1991).

Immunocytochemical staining for markers associated with myoepithelial cells gives the reciprocal result to that above for the growing budded structures in such young girls (Fig. 4B). In particular, antibodies to smooth muscle actin (Rudland, 1991), myosin (unpublished results) and vimentin (Rudland, 1991) stain the peripheral cells at the distal tips of TEBs, some terminal ducts and small LBs in an intermediate manner. The staining intensity of peripheral cells gradually increases from the tip of the TEBs (Fig. 3C) to reach virtually the same intensity of staining as the myoepithelial cells of the subtending duct (Fig. 3D). The majority of the central cells are stained weakly, if at all (Figs. 3C, 4B). Peripheral cells at the distal regions of ABs show a more intense staining than equivalent cells in TEBs (Fig. 3D), but this still increases in intensity to that of the myoepithelial cells of the subtending duct (Rudland, 1991). MAbs to the myoepithelial-specific cytokeratin 14 show enhanced staining of the epithelial-like cells over the peripheral cells in some growing TEBs, terminal ducts, LBs and ABs. Antibodies to the basement membrane components Type IV collagen and laminin stain the majority of the surfaces of the peripheral/basal cells in terminal budded structures. The thickness of the stained band of basement membrane proteins is reduced round the distal tips of the TEBs and is increased round the necks of the TEBs, and some peripheral cells around the necks and tips show

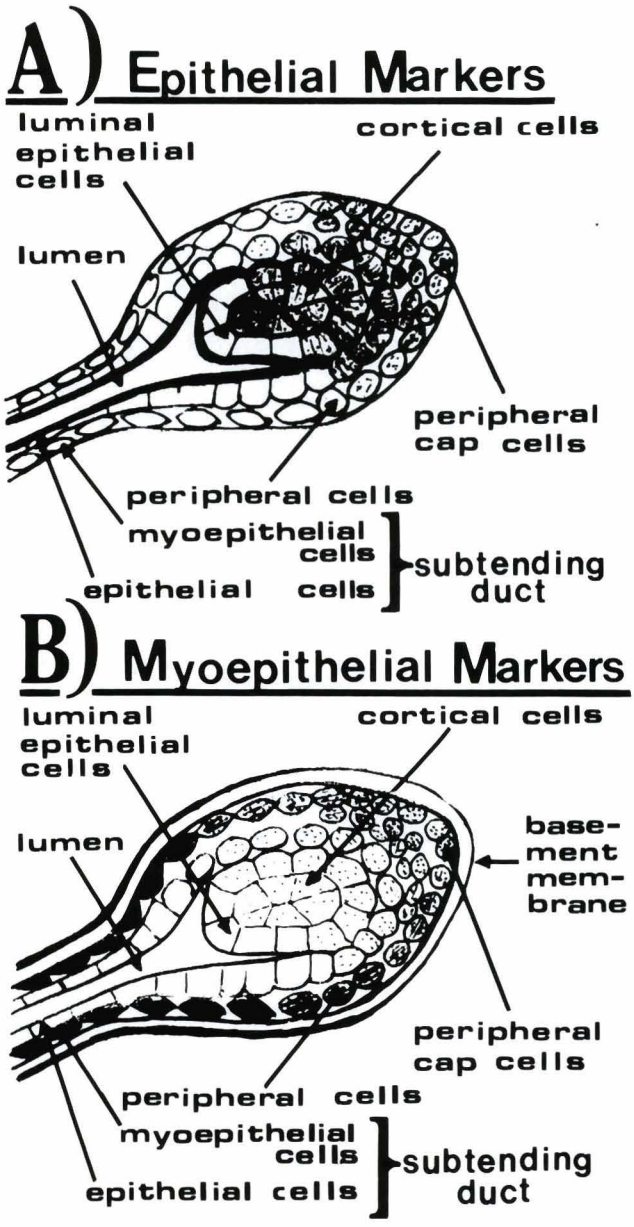


Fig. 4. Schematic representation of the staining patterns for epithelial and myoepithelial markers in TEBs. **A.** Epithelial markers: histochemical staining with MAbs to MFGM, anti-EMA and PNL after treatment of the sections with neuraminidase. The loosely-packed peripheral cells are stained weakly (dotted circles). This staining becomes stronger in the central epithelial cells where it is predominantly cytoplasmic (black squares), and becomes intense along the apical surfaces of the epithelial cells which line the lumina in both the duct and TEB (semi-black squares). Gradations in morphology and intensity of staining are observed. The proximal peripheral cells and the myoepithelial cells of the subtending duct are unstained. **B.** Myoepithelial markers: histochemical staining for smooth muscle actin and basement membrane proteins. The peripheral cells are stained moderately for actin (dotted circles). The elongated morphology and intensity of staining increases for the peripheral cells closer to the subtending duct, and they merge eventually with those of the myoepithelial cells of the duct (black ovoids). The central epithelial cells show usually only a weaker staining for actin (dotted squares) and this staining is usually not apparent in the luminal epithelial cells of the TEB and duct. Staining for the basement membrane proteins laminin and Type IV collagen encircles completely the TEB, producing a thin band at its distal tip and gradually increasing in thickness in the neck regions of the TEB, where it merges with that of the subtending duct.

cytoplasmic staining for the basement membrane proteins (Rudland, 1991) suggesting the site of synthesis (Fig. 4B).

The above results suggest that morphological intermediate cell types can exist in a basal position within growing terminal ducts and budded structures, particularly in TEBs. Moreover, the intermediate cells at the distal tips can show morphological and immunocytochemical transitions to the central epithelial-like cells on the one hand and to the myoepithelial cells of the subtending duct on the other hand (Fig. 4). In addition there is increased staining for the epithelial-specific keratin 18 and myoepithelial-specific keratin 14 of similar cells in the budded structures. These results suggest that staining with both keratin antibodies is not reciprocally related in such structures and may relate more to the different physiological state of cells in mature ducts and in growing budded structures.

Isolation of immortalized cell lines from normal breasts of a potential stem cell nature

To investigate the relationship between the three individual cell types within the breast, human mammoplasty specimens have been maintained in short term-culture (Easty et al., 1980; Stampfer et al., 1980) and immortalized with simian virus 40 (SV40) (Chang et al., 1983) to obtain single-cell-cloned epithelial-like cell lines, e.g. SVE3 and human mammary (Huma) 7 (Rudland et al., 1989a). The single-cell-cloned epithelial cell lines repeatedly give rise to more-elongated cells at a frequency of about 0.1%, and these cells can be cloned; representative clones are Huma 25 from SVE3 and Huma 62 from Huma 7, and these are completely stable (Fig. 5). In addition, two further morphological cell types are observed within the cultures of the epithelial-like cell lines, large flat cells (Edwards et al., 1984) and dark droplet cells associated with domes (Fig. 6) (Rudland et al., 1989a). In addition to being grown on flat plastic dishes, these cell lines can also be grown on floating gels of Type I collagen to mimic, to a certain degree, a stromal matrix (Rudland et al., 1991), and as small tumour nodules in nude mice. The later regress after about 10 days (Rudland et al., 1989a). All three types of growth environment have been used for subsequent analysis.

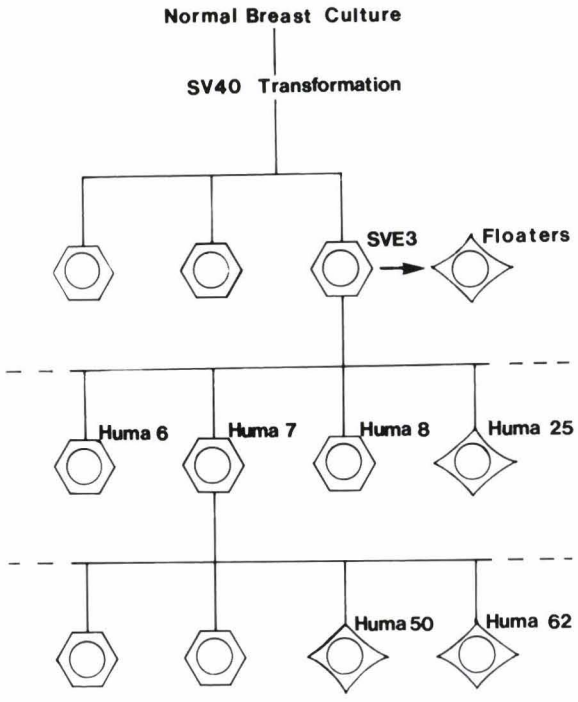


Fig. 5. Scheme of isolation of cell lines from simian virus 40-transformed normal breast epithelium. The cell lines are shown as epithelial (hexagonal cells) and more-elongated, myoepithelial-like (star-shaped cells); those cell lines isolated by single-cell cloning are connected by continuous lines, and those cell strains floating in the medium or floaters and isolated as such are shown by an arrow.

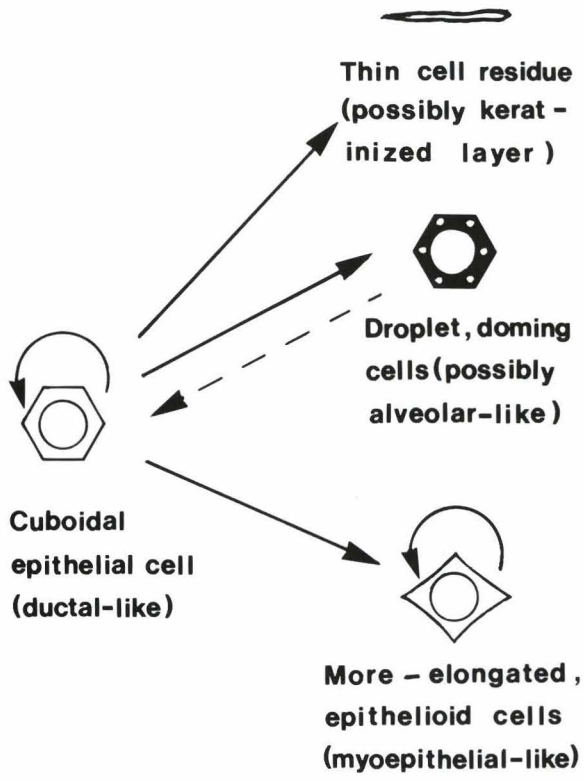


Fig. 6. Summary of the intercellular conversions of normal and benign hyperplastic cell lines. The cuboidal epithelial cell lines from SV40-immortalized normal breast epithelial cells and from a spontaneously immortalized epithelial cell line HMT-3522 from hyperplastic benign breast disease can either replicate (curved arrow) or give rise to different cell types (straight arrow). Conversion to thin cellular residues of squamous-like cells or to more-elongated myoepithelial-like cells is irreversible, but that to droplet/doming, presumptive alveolar-like cells can be reversed (discontinuous lines).

Table 3. Summary of histochemical staining of different cell types in culture.

ANTIBODY OR REAGENT[a]	CELL TYPE[b]		
	Epithelial like	Large, flat	More-elongated
Epithelial-related			
Anti-EMA, peanut lectin, MAbs to MFGM	+	++	-
MAbs to keratin 18	+	±	-
Epithelial and myoepithelial-related			
MAbs to complex keratins, PKK2 and LP34	±	++	+
Myoepithelial-related			
Anti-smooth muscle actin, myosin[c]	-	-	+
MAb to keratin 14[d]			
Anti-vimentins[c, e]			
MAbs to CALLA, 135 kd glycoprotein[c]	±	±	+
Anti-Type IV collagen, laminin[c]	-	-	+
CONCLUSIONS	Epithelial	Squamous-like	Myoepithelial-like

[a]: abbreviations as in the text and Table 1; [b]: identical results for primary breast cell cultures (Rudland et al., 1989b); SV40-immortalized primary epithelial cells (Rudland et al., 1989a), primary cultures of benign hyperplastic (benign breast disease) and neoplastic (fibroadenoma) disease (Rudland et al., 1985), and spontaneously immortalized cell lines from benign breast disease (Rudland et al., in preparation). Key: ±, weak and diffuse staining; +, most cells stain; ++, extremely strong staining for both immunofluorescent and immunocytochemical reagents; [c]: also stain blood vessels in stroma; [d]: staining with MAb to cytokeratin 14 has only been performed on the benign hyperplastic cell lines as yet (Rudland et al., in preparation); [e]: also stains stromal fibroblasts.

Immunofluorescent and immunocytochemical staining with reagents against MFGM/EMA stain the epithelial-like cells in a peripheral manner and the larger flat cells very intensely confirming both cells' epithelial characteristics (Table 3). The epithelial-like cells also stain with epithelial-specific MAbs to keratin 18, but the large flat cells fail to do so appreciably (Table 3). The flat cells, however, stain intensely with MAbs to more complex keratins, suggesting a squamous form of the epithelial cells (Table 3) (Rudland et al., 1989a, 1991). Similar immunostaining with antibodies to smooth muscle actin, myosin, vimentin, CALLA and the 135 kd protein predominantly stains the more-elongated cells, albeit in a rather heterogeneous manner, suggesting a mesenchymal origin (Table 3). However, the fact that the more-elongated cells produce basement membrane proteins laminin and Type IV collagen, and, shortly after conversion from epithelial cells, keratin intermediate filamental proteins, which may or may not be retained on long-term passaging, suggests that they are not simply fibroblastoid cells (Boyer et al., 1989), but are more closely related to myoepithelial cells (Table 3) (Rudland et al., 1989a, 1991). Ultrastructural analysis confirms that some of the more-elongated cells possess a phenotype consistent with that of immature myoepithelial cells, although dense networks of myofilaments are encountered only rarely in such growing cultures (Rudland et al., 1989a, 1991), as observed in an immortal myoepithelial cell line from a human salivary gland adenoma (Shirasuna et al., 1986). The epithelial-like, squamous-like and myoepithelial-like cell types have also been observed in primary cultures of human breasts (Table 3) (Rudland et al., 1989b). The most strongly anti-EMA/MFGM-staining and anti-CALLA-staining cell types have also been separated by fluorescence-activated cell sorting (O'Hare et al., 1991). The former cell type possibly corresponds more to the squamous epithelial-like cells and the latter more to myoepithelial-like cells. However, the peripherally, more-moderately anti-EMA-stained epithelial cells which proliferate in early cultures (Rudland et al., 1989b) and which correspond to the virally-immortalized epithelial cell lines (Rudland et al., 1989a) appear to have been missed by this technique.

When grown on floating collagen gels or as tumour nodules in nude mice, the epithelial cell lines form rod or cord-like structures, a few of which, particularly those grown on gels are hollow and superficially resemble ducts, a few others have more bulbous, solid ends like TEBs, whilst a further few consist of grape-like structures some of which superficially resemble alveolar lobules. A few such duct-like structures and many sheets of cells on gels consist of two or more cellular layers, the outer/basal layer possessing less epithelial and more myoepithelial properties and the inner/upper layer of epithelial cells consisting of cells correctly polarised with respect to the lumen/medium (Rudland et al., 1991). Lactating nude mice bearing tumour nodules induced by the epithelial cell lines can form hollow sac or grape-like clusters that secrete human-specific forms of casein (Rudland et al., 1989a). These casein-secretory cells may then bear some relationship to alveolar cells and it is tempting to speculate that they may also be related to the droplet/doming cells observed in primary cultures (Rudland et al., 1989b) and in epithelial cell lines (Rudland et al., 1989a), as shown for the equivalent rat cells (Rudland, 1987a,b). Thus the epithelial cell lines can give rise to myoepithelial-like and to alveolar-like cells (Fig. 6), although the full expression of the differentiated phenotype is difficult to achieve in either case, as well as to cells more intermediate in marker characteristics between epithelial and myoepithelial cells. These intermediate cells superficially resemble morphologically intermediate cells found particularly in basal positions in growing terminal ductal structures (Stirling and Chandler, 1976; Rudland, 1991). Thus the results for cellular interconversions in culture are consistent with the phenotypic gradations seen *in vivo*, and therefore the intermediate/epithelial cells may represent stem cells from the breast, in line with the much more detailed observations in rodents (Rudland, 1987a,b).

Since the same epithelial cell lines can also give rise, at least superficially, to many of the structures of the

Table 4. Summary of breast lesions analysed immunocytochemically.

BREAST LESION	NUMBER OF PATIENTS	(PREGNANT)	CELL TYPES[a]
Benign diseases			
Fibrous changes	31		epithelial, myoepithelial-like (alveolar-like)[f]
Fibrocystic disease	242[c]	(1)	
Lobular hyperplasia	5		
Papilloma	6		
Fibroadenoma	76	(1)[e]	
Phyllodes tumour	8		
Carcinoma-in-situ			
Ductal	8	(2)	epithelial
Lobular	4		
Invasive carcinoma			
Tubular	4		epithelial
Ductal[b]	137[d]		
Medullary	3		
Colloid	7	(2)	
Lobular	11		

[a]: parenchymal tissue only, carcinoma-in-situ also possess host myoepithelial cells surrounding the lesions; [b]: not-otherwise-specified; [c]: this includes cystic changes (22), duct ectasia (9), apocrine metaplasia (16), adenosis (133), blunt duct adenosis (15), sclerosing adenosis (25), epitheliosis (49); the last four contain areas associated with some of the other types of benign breast disease; [d]: this includes 6 with ductal carcinoma-in-situ with comedo (3) or cribriform (3) pattern and 6 with scirrhous reaction; [e]: lactating adenoma; [f]: secretory alveolar-like cells detected only in appreciable quantities in lesions in pregnant women, otherwise 5-10% of the lesions in nonpregnant women contained small numbers (<5%) of secretory cells.

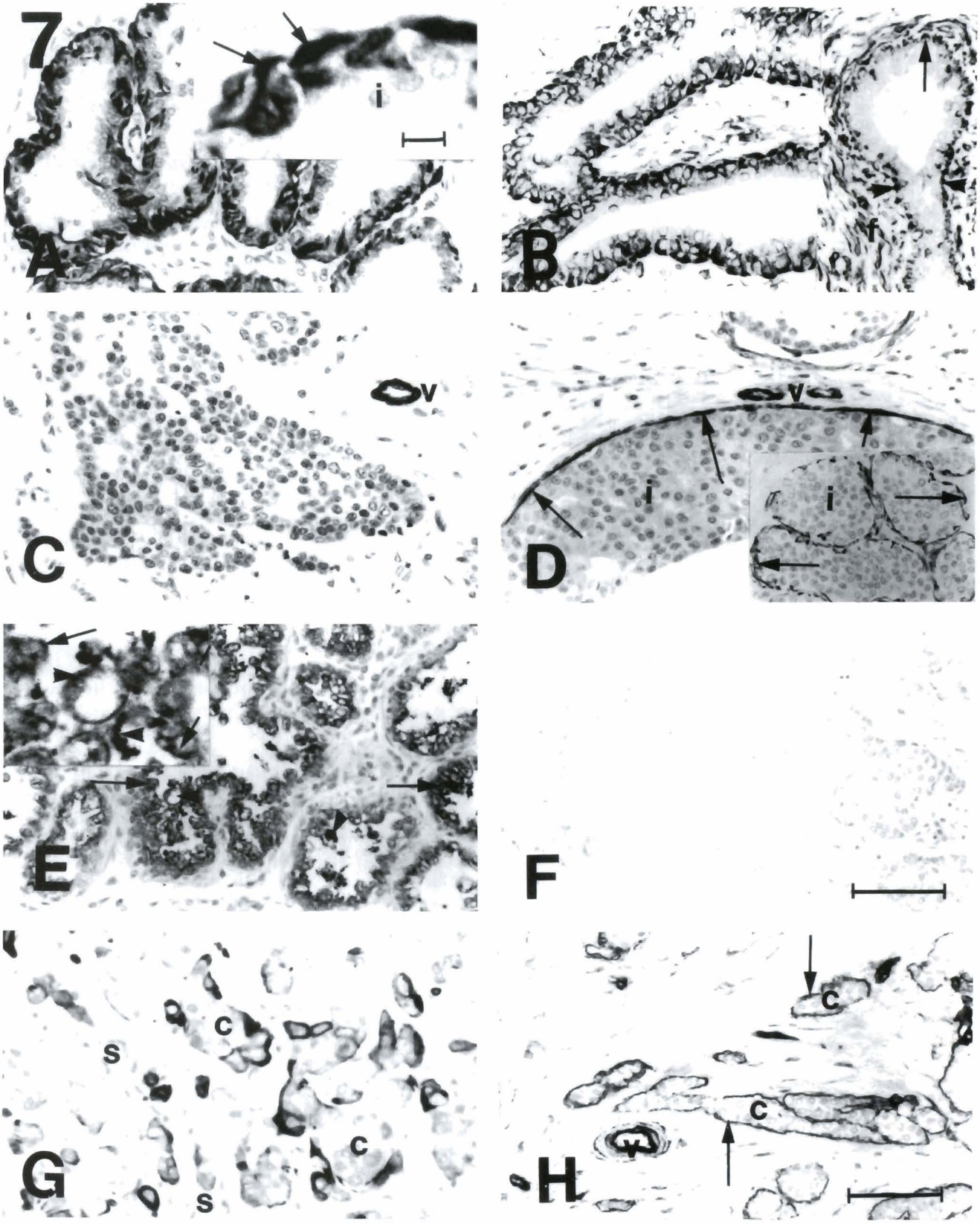
parenchyma of the breast, they may indeed represent a stem cell population (Rudland et al., 1991). However, these epithelial cell lines also produce cells and structures not normally encountered in the breast, the most notable being the flat, squamous-like cells (Fig. 6) and corresponding sheets and whorls of keratinizing epithelium on collagen gels (Rudland et al., 1991) and in regressing tumour-nodules in nude mice (Rudland et al., 1989a). However, it is unlikely that viral transformation has resulted in this extra phenotype, since much larger numbers of squamous-like cells are produced in primary cultures (Edwards et al., 1986; Rudland et al., 1989b), and their production is therefore probably due to a reaction to the nonphysiological conditions of tissue culture with their emphasis on cell growth. The reason for senescence of the primary cultures is predominantly due to the rapid production of such near-terminal squamous-like cells from the growing epithelial cells (Rudland et al., 1989b), and transformation with SV40 yields epithelial cells with a considerably reduced ability to produce the squamous-like cells (Rudland and Barraclough, 1990), thereby probably effecting immortalization.

Identification of different cell types in benign and malignant breast diseases

As discussed in earlier sections, the terminal structures of the breast are the sites of most breast growth (Russo and Russo, 1987), and they are also the most likely initial sites for much of the hyperplastic benign breast disease and many of the benign and malignant breast neoplasias (Wellings et al., 1975; Ishige et al., 1991). Breast carcinomas are thought to arise mainly from carcinomas-in-situ (Page et al., 1987), and they in turn are thought to arise predominantly from epithelial hyperplasia (or epitheliosis: Page et al., 1987) that are similar in many respects to those encountered in hyperplastic benign breast disease, particularly that associated with atypia (Simpson et al., 1982; Wellings and Misdrop, 1985; Norris et al., 1988). The major benign neoplasms of the breast, the fibroadenomas and adenomas, however, appear to arise by proliferation of the entire ductal/ductular unit and rarely develop into carcinomas (Fechner, 1987). The detailed cellular origins of breast tumours, however, are still unclear.

Morphological criteria and topographical relationships alone are inadequate when attempting to relate the organisation and differentiation of the normal breast with cellular structures observed in its tumours. For example the presence (Murad, 1971) and absence (Azzopardi, 1979) of myoepithelial cells in invasive carcinomas of the breast have been reported using histological identification at the light and electron microscopic levels. This discrepancy in invasive carcinomas of the breast is probably due to confusion of tonofilaments in epithelial cells with myofilaments in myoepithelial cells (Rudland, 1987b). Immunocytochemical staining for cytoskeletal markers of the myoepithelial cell, smooth-muscle actin (Bussolati et al., 1980), myosin (Gusterson et al., 1982), intermediate-filamental-proteins vimentin (Rudland et al., 1993),

Fig. 7. Immunocytochemical staining of benign and malignant breast diseases. **A.** Benign breast disease incubated with MAb to smooth-muscle actin showing staining of the myoepithelial cells, the inner epithelial cells (i) are unstained. Inset at higher magnification shows this more clearly. **B.** Fibroadenoma incubated with MAb to vimentin showing a strongly stained peripheral layer of myoepithelial cells; the inner cells are unstained. Inset shows a bud-like structure with weak staining, peripheral cells at the distal tip (arrow) and strong staining, peripheral cells adjacent to the subtending duct-like structure (arrowheads). The fibrous cells (f) are also well stained. **C.** Invasive ductal carcinoma incubated with MAb to smooth-muscle actin showing no staining of inner or peripheral cells, but blood vessels (v) are well stained. **D.** Carcinoma-in-situ incubated with MAb to smooth-muscle actin showing stained attenuated host myoepithelial cells (arrows) both in ductal and lobular carcinoma-in-situ (inset). The inner epithelial cells are unstained (i), but blood vessels are well stained (v). **E.** Adenomatous area of a pregnant women incubated with MAb to ß-casein showing strong staining of luminal cells (arrows) and secretions (arrowheads), the peripheral myoepithelial cells are unstained. Inset at higher power shows this more clearly. **F.** Invasive colloid carcinoma of the same pregnant women as in E incubated with MAb to ß-casein showing no staining of the malignant cells. **G.** Invasive ductal carcinoma incubated with MAb to cytokeratin 14 showing moderate staining of the majority of the carcinoma cells (c), the stromal cells (s) are unstained. **H.** Invasive ductal carcinoma incubated with MAb to laminin showing incomplete basement membrane (arrows) encircling the cords of infiltrating carcinoma cells (c). Original magnification x 230. Bar = 75 µm. Inset in A, original magnification x 730. Bar = 10 µm.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specific cytokeratins 5 and 14 (Nagle et al., 1986; Dairkee et al., 1988) and the cell-surface glycoproteins CALLA (Gusterson et al., 1986) and of 135 kd (Gusterson et al., 1985) (Table 2) have now firmly established the presence of such myoepithelial cells in benign breast disease (Fig. 7A) and in benign neoplastic tumours (Fig. 7B) (Table 4). Indeed cells intermediate in ultrastructural (Ozzello, 1971) and immunocytochemical-staining (Rudland et al., 1993) characteristics between epithelial and myoepithelial cells are observed in areas of epitheliosis and fibroadenomas, and such cells bear at least a superficial resemblance to intermediate cells growing in ductal terminal structures in normal glands (Fig. 7B). Fully differentiated myoepithelial cells possessing both smooth muscle actin/myosin and cytokeratins are absent from invasive carcinomas (Fig. 7C), but are retained as part of the normal host tissue surrounding carcinomas-in-situ (Fig. 7D) (Table 4) (Bussolati et al., 1980; Gusterson et al., 1982; Nagle et al., 1986; Dairkee et al., 1988; Rudland et al., 1993). The identification of cytokeratins may be required in certain circumstances to eliminate the possibility of smooth-muscle containing myofibroblasts (Ohtani and Sasano, 1980) being confused with myoepithelial cells (Gusterson et al., 1982). The epithelial cell markers for EMA/MFGM are invariably retained in all the observed benign and malignant conditions (Table 4) (e.g. Sloane and Ormerod, 1981; Foster et al., 1982b; Taylor-Papadimitriou et al., 1983). The loss of the myoepithelial cell has since been used as a diagnostic factor for separating apparently morphologically similar benign and malignant lesions, e.g. epitheliosis and ductal carcinoma-in-situ (Raju et al., 1990), microglandular adenosis and tubular carcinoma (Díaz et al., 1991) and benign and malignant papillary neoplasias (Raju et al., 1989).

As with the myoepithelial cell, the presence or absence of the secretory alveolar cell in invasive breast carcinomas has been disputed in the past using polyclonal antisera to detect casein and α-lactalbumin (Bussolati et al., 1973; Laurence, 1978; Walker, 1978; Fortt et al., 1979). However, highly-immunogenic EMA contaminants of the milk proteins are probably responsible for apparent serological detection of casein and α-lactalbumin in most, if not all such early studies (Ormerod et al., 1982). The use of highly specific MAbs to human ß and κ-caseins now shows the complete absence of secretory alveolar cells in malignant breast disease including tubular carcinoma, and only the relatively rare and isolated occurrence of such proteins in benign hyperplastic and neoplastic lesions (Table 4) (Bartkova et al., 1987; Earl et al., 1989; Rudland et al., 1993). However, these caseins are detected immunocytochemically only in the normal breasts of women in late pregnancy and during lactation (Bartkova et al., 1987; Earl et al., 1989; Rudland and Hughes, 1989), and hence may be anticipated to occur only in breast lesions in women in a similar hormonal state. In a hormonal state conducive to lactation in 16-18 wk pregnant females, two patients, one with benign breast disease and one with an adenoma, possess lesions in which the majority of epithelial cells are capable of staining for ß and κ-caseins, the one in areas of adenosis and the other in lactating adenomatous regions (Fig. 7E), respectively (Table 4). Staining for these caseins is not observed in areas of cystic expansion, apocrine metaplasia and sclerosing adenosis in such patients. However, in a further two patients who were 15-19 wk pregnant, no staining with antibodies to either of these caseins is observed in invasive carcinoma (Fig. 7F) or carcinoma-in-situ (Table 4), although ß- and κ-caseins are readily detected in noninvolved breast tissue and nonmalignant areas of the same patients (Rudland et al., 1993). Thus definitive markers for fully differentiated myoepithelial cells and, uder suitable hormonal conditions, for secretory alveolar cells are found in benign hyperplastic and neoplastic disease but are absent in carcinoma-in-situ and invasive carcinomas (Rudland et al., 1993).

The basement membrane components laminin and Type IV collagen are produced predominantly by the myoepithelial cells in the normal breast (Rudland et al., 1989b, 1991) and in hyperplastic and neoplastic benign breast lesions (Rudland et al., 1993) as well as in carcinomas-in-situ, resulting in complete encirclement of the parenchymal units (Gusterson et al., 1982; Barsky et al., 1983; Ekblom et al., 1984; Bosman et al., 1985; Willebrand et al., 1986). However, despite the disappearance of mature myoepithelial cells in invasive carcinomas, 11-15% of such carcinomas (Albrechstein et al., 1981; Nielson et al., 1983; Aung et al., 1993) produce a fragmentary but distinct basement membrane (Fig. 7H), whereas others completely lack it (Gusterson et al., 1982; Barsky et al., 1983; Ekblom et al., 1984; Willebrand et al., 1986; Charpin et al., 1986). Indeed there is an inverse relationship between the appearance of basement membrane proteins and histological grade, particularly between high and low grades in some series of breast carcinomas (Gusterson et al., 1982; Nielson et al., 1983; Aung et al., 1993). The significant reduction in basement membrane material with increased grade may be due to decreased deposition and/or increased destruction by collagenolytic enzymes (Nakajima et al., 1989; Montiagndo et al., 1990). The organisation of the fragmentary basement membrane appears to be more consistent with the former rather than the latter change (Gusterson et al., 1982; Rudland et al., 1993). Moreover cytokeratin 14, a specific marker for myoepithelial cells in normal breasts, is also found in 9-14% of cases of invasive ductal carcinoma (Dairkee et al., 1988; Wetzel et al., 1989), where it now resides within the malignant epithelial cells (Fig. 7G) (Aung et al., 1993). An even higher proportion of 50% of invasive carcinomas stain strongly with a MAb that also stains myoepithelial cells in mature, normal ducts (Skilton et al., 1990). These results suggest that although fully differentiated myoepithelial cells are absent in invasive carcinomas, some marker proteins of the myoepithelial cell are

Table 5. Adherence of cultured digests of different breast tissues to collagen gel.

BREAST TISSUE[a]	TOTAL NO. OF PATIENTS	NO. OF PATIENTS WITH DIFFERENT TYPES OF EPITHELIUM IN CULTURES		IDENTIFIABLE CELL TYPES
		Fast-adherent[b]	Loosely-adherent[c]	
Normal (reduction mammoplasty)	40	40	0	Epithelial, myoepithelial-like
Benign hyperplastic (benign breast disease)	20	20	0	Epithelial, myoepithelial-like
Benign neoplastic (fibroadenoma)	10	10	0	Epithelial, myoepithelial-like
Primary carcinoma (invasive ductal)	30[d]	25	27	Epithelial, (myoepithelial-like)[e]
Secondary carcinoma in lymph node	10[d]	0	9	Epithelial
Secondary carcinoma as pleural effusion	2	0	2	Epithelial

[a]: breast tissue has been digested with collagenase, except pleural effusions, and grown on collagen gel-coated dishes; [b]: fast-adherent colonies stick within 24 to 48 hr, they always contain epithelial and myoepithelial-like cells as determined by the markers described in the text; [c]: loosely-adherent colonies take greater than 72 hr to stick, if they do at all, and then are easily washed off; they contain only epithelial and not myoepithelial-like cells; [d]: a few samples contain no visible epithelium after digestion; [e]: myoepithelial-like cells only present in the fast-adherent and not in the loosely-adherent fraction.

retained by 9-50% or more of invasive carcinomas. These myoepithelial-related proteins are expressed to varying degrees by the malignant epithelial cells, and, at least for basement membranes, there is sometimes an inverse correlation between histological grade and loss of such ectopically expressed proteins.

Isolation of immortalized cell lines from benign and malignant breast lesions

Collagenase digests of benign and malignant breast lesions behaved differently on collagen gels. Digests of benign breast disease and benign tumours rapidly adhere to the collagen gel forming islands of nonmalignant epithelial-like cells surrounded by more-elongated cells similar to digests of normal breast (Hallowes et al., 1977; Smith et al., 1981). The former are confirmed as epithelial cells and the latter as myoepithelial-like cells using the markers described already (Table 5) (Rudland et al., 1985; Peterson and van Deurs, 1988; Yang et al., 1987). The primary tumours of invasive ductal carcinomas yield a virtually identical fast-adherent nonmalignant cellular fraction to that of the benign lesions (Fig. 8A) and relatively nonadherent cellular clusters (Fig. 8B) (Table 5). The latter eventually attach loosely to plastic petri dishes and form colonies of slow-growing malignant epithelial cells without any characteristic myoepithelial-like cells (Fig. 8C) (Rudland et al., 1985; Peterson and van Deurs, 1987; Peterson et al., 1990). Only the loosely-adherent, slow-growing cellular fraction of epithelial cells can be isolated from cultured lymph node metastases and pleural effusions (Fig. 8D) (Table 5) (Rudland et al., 1985), so confirming that these cells do indeed represent the malignant cell fraction in the primary invasive ductal carcinomas (Hallowes et al., 1983) rather than those cells of the fast-sticking cellular fraction (Smith et al., 1981, 1985; Woolman et al., 1985). Thus primary cultures of benign hyperplastic and neoplastic lesions yield both epithelial and myoepithelial cells, as well as indications of cells morphologically similar to alveolar cells under suitable hormonal conditions (Hallowes et al., 1977; Rudland et al., 1985). Malignant epithelial cell clusters and colonies, on the other hand, fail to demonstrate these differentiated cell types in culture (Table 5).

To establish whether epithelial cells from a benign breast lesion can give rise directly to other differentiated cell types, the spontaneously-immortalized diploid epithelial cell line established from hyperplastic regions of benign breast disease, HMT-3522 (Briand et al., 1987), has been subjected to conditions in culture that enhance production of more-elongated, myoepithelial-like cells in the SV40-immortalized normal breast epithelial cell system (Rudland et al., 1989a). At comparable frequencies to the SV40-immortalized system, a single-cell-cloned epithelial-like cell line of HMT-3522 and two further epithelial-like single-cell subclones, Huma 121 and Huma 123 have produced more-elongated cell lines, representatives of which are Huma 101 and Huma 109 from HMT-3522 and Huma 131 from Huma 123 (Fig. 9). The more-elongated cell lines are stable and do not revert. In addition, two further morphological cell types are observed within the cultures of the epithelial-like cell lines, the large flat cells and dark, droplet cells associated with domes; the latter cell type at a much lower frequency than in the SV40-immortalized normal breast cell lines (Table 6) (Rudland et al., in preparation). These cell lines have been grown on plastic, on collagen gels and as small tumour nodules in nude mice and analysed in exactly the same way as for the SV40-immortalized cell lines using immunofluorescent, immunocytochemical and ultra-

Fig. 8. Phase-contrast micrographs of collagenase-digested cultured invasive ductal carcinoma. **A.** Primary carcinoma, fast-adherent fraction to collagen gel showing some epithelial (e) but many more-elongated, presumptive myoepithelial cells (m). **B.** Primary carcinoma, loosely-adherent fraction 72 hr after plating on collagen-coated dishes showing no attached cells. **C.** Primary carcinoma, loosely-adherent fraction now plated on plastic dishes showing large epithelial-like cells (e), but no more-elongated myoepithelial-like cells. **D.** Secondary carcinoma now plated on plastic dishes showing large pleomorphic epithelial-like cells (e), but no more-elongated myoepithelial-like cells. Original magnifications x 160 for A, C; x 250 for B; x 400 for D. Bars = 75 μm.

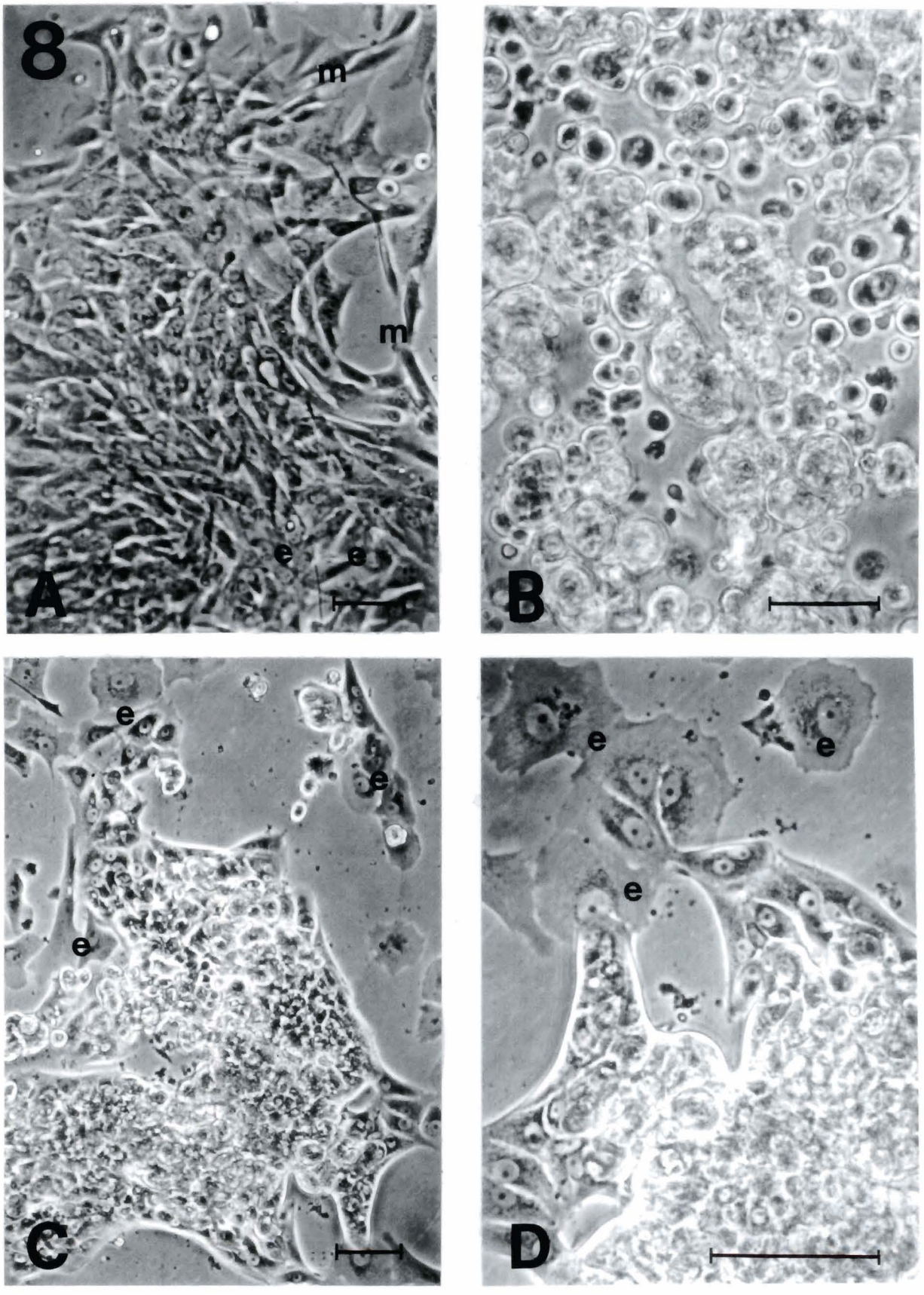
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Table 6. Summary of epithelial cell lines isolated and their differentiation characteristics.

BREAST TISSUE	IMMORTALIZATION	EPITHELIAL CELL LINES[a]	DIFFERENTIATED CELL TYPES
Normal (mammoplasty)	SV40	SVE3, Huma 7	Myoepithelial-like, alveolar-like, squamous-like
Benign hyperplastic (from HMT-3522)	Spontaneous	Huma 121, Huma 123	Myoepithelial-like, alveolar-like, squamous-like
Malignant[b] (invasive ductal carcinoma)	Spontaneous	$Ca_2$83, KM1	Squamous-like

[a]: references to isolated cell lines: SVE3, Huma 7 (Rudland et al., 1989a); Huma 121, 123 (Briand et al., 1987; Rudland et al, in preparation); $Ca_2$83 (Rudland et al., 1985); KM1 (K. McCarthy and P.S. Rudland, unpublished); [b]: in addition, the spontaneously immortalized cell lines from metastatic pleural effusions MCF-7 (Soule et al., 1973), ZR-75-1 (Engel et al., 1978) and T47D (Keydar et al., 1979) have been analysed but these too show no myoepithelial-like or alveolar-like cells.

structural techniques (Rudland et al., 1989b, 1991), with very similar results (Table 6) (Rudland et al., in preparation). Thus the epithelial nature of the epithelial-like cell lines has been confirmed and the more-elongated, large flat cells, and droplet/doming cells are related to myoepithelial-like, squamous-like and possibly alveolar-like cells, respectively (Table 6). Thus this epithelial cell line from hyperplastic benign breast disease can differentiate to myoepithelial-like and possibly alveolar-like cells in culture and *in vivo*, in a similar manner to the SV40-immortalized human breast epithelium, although the frequency of differentiation, particularly to the potential alveolar-like cell is considerably reduced (Fig. 6). Cell lines intermediate in marker characteristics between epithelial and myoepithelial-like cells have also been isolated from the cloned epithelial cell lines (Fig. 9) (Rudland et al., in preparation), and these are similar superficially to the intermediate cells described in benign hyperplasia (epitheliosis) in the previous section. Another spontaneously immortalized epithelial cell line isolated from benign breast disease, MCF-10 (Soule et al., 1990), has not yet been subjected to clonal analysis, although some of its cells express myoepithelial-related cytokeratin 14 (Tait et al., 1990), suggesting the possibility of its conversion to a myoepithelial-related phenotype.

In contrast, spontaneously immortalized cell lines from plural effusions MCF-7, ZR-75, T47D (Soule et al., 1973; Engel et al., 1978; Keydar et al., 1979) and from loosely-adherent cellular fractions of primary tumours $Ca_2$83 (Rudland et al., 1985) have been subjected to clonal analysis, but no myoepithelial-like or alveolar-like cells have been detected using the above immunohistochemical and ultrastructural criteria at frequencies where one cell in a million would have been capable of detection (Table 6). Moreover, other malignant epithelial cell lines developed from primary invasive carcinomas

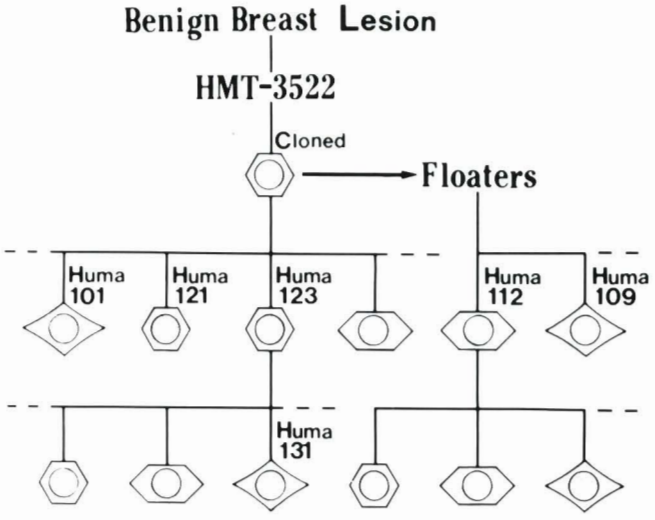

Fig. 9. Scheme of isolation of cell lines from the benign breast disease cell line HMT-3522. The cell lines are shown as epithelial (hexagonal cells), more-elongated, myoepithelial-like (star-shaped cells), and intermediate between epithelial and myoepithelial-like (elongated hexagonal cells).

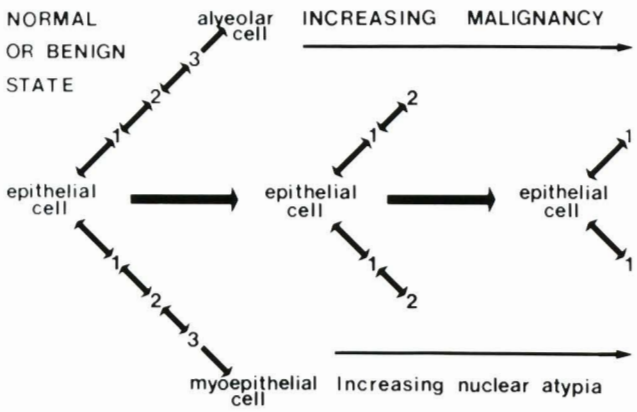

Fig. 10. Possible model for the development of malignant carcinoma cells of the breast. Normal epithelial cells in terminal ductal structures can differentiate into myoepithelial cells and into secretory alveolar cells by a series of cellular intermediates here represented by numerals. Details of such steps have only been worked out at present in rodents (Rudland, 1987a, b). It is suggested that many benign hyperplastic and neoplastic lesions arise directly from such epithelial/closely-related intermediate cells which then still retain much of their differentiating abilities to myoepithelial and to alveolar cells. Insults leading to malignant changes produce epithelial/related intermediate cells with a decreasing ability to differentiate into either cell type, and result at best in a retention of only limited expression of markers characteristic of myoepithelial and of alveolar cells in invasive carcinomas. The fact that more vestiges of myoepithelial cell rather than alveolar cell markers are retained in invasive ductal carcinomas, even under hormonal environments conducive to alveolar cell differentiation (Rudland et al., 1993), suggests that the malignant insult occurs/is expressed in epithelial/intermediate cells along the myoepithelial rather than the alveolar cell pathway.

fail to show any definitive features of myoepithelial cells (Lasfargues and Ozzello, 1971; Nordquist et al., 1975; Hackett et al., 1977; Minafra et al., 1989; Peterson et al., 1990), including KM1 (McCarthy and Rudland, unpublished results) (Table 6). All malignant epithelial cell lines show evidence of squamous-like cellular formations but to varying degrees. Thus, in agreement with the earlier pathological analyses (Section 5), benign hyperplastic and probably benign neoplastic lesions contain epithelial stem cells capable of differentiating to myoepithelial-like and to alveolar-like cells, whilst malignant epithelial cells fail to differentiate to these two cell types in culture.

Cellular model for the development of breast cancer

Previous sections have shown that epithelial stem cells exist in normal breasts and in benign hyperplastic and benign neoplastic lesions. These epithelial stem cells can probably give rise to the other major differentiated cell type, the myoepithelial-like cell, whereas the malignant epithelial cells fail to do so. There are two simple explanations for this phenomenon. (1) There are two types of epithelial cell in normal breasts; the one which can differentiate gives rise to benign lesions and the one which cannot gives rise to malignant lesions. (2) The ability of the epithelial stem cells to differentiate is impaired when they become malignant (Fig. 10).

Immunocytochemical staining patterns for cytokeratins, particularly cytokeratin 19, has suggested that two populations of epithelial cells exist within the normal breast; epithelial cells in benign tumours usually show a reduced expression whilst those in malignant carcinomas show an increased expression of this cytokeratin (Taylor-Papadimitriou et al., 1983; Taylor-Papadimitriou and Lane, 1987), in support of different cellular origins for benign tumours and carcinomas. However, detailed studies on markers of the normal myoepithelial cell, cytokeratin 14 (Wetzel et al., 1989; Aung et al., 1993), antigenic determinant(s) on the MCF-7 breast carcinoma cell line (Skilton et al., 1990), and the basement membrane proteins laminin and Type IV collagen (Albrechstein et al., 1981; Gusterson et al., 1982; Nielson et al., 1983) show that 9-50% of breast carcinomas retain a marker of the myoepithelial cell, and that such markers are now expressed by the malignant

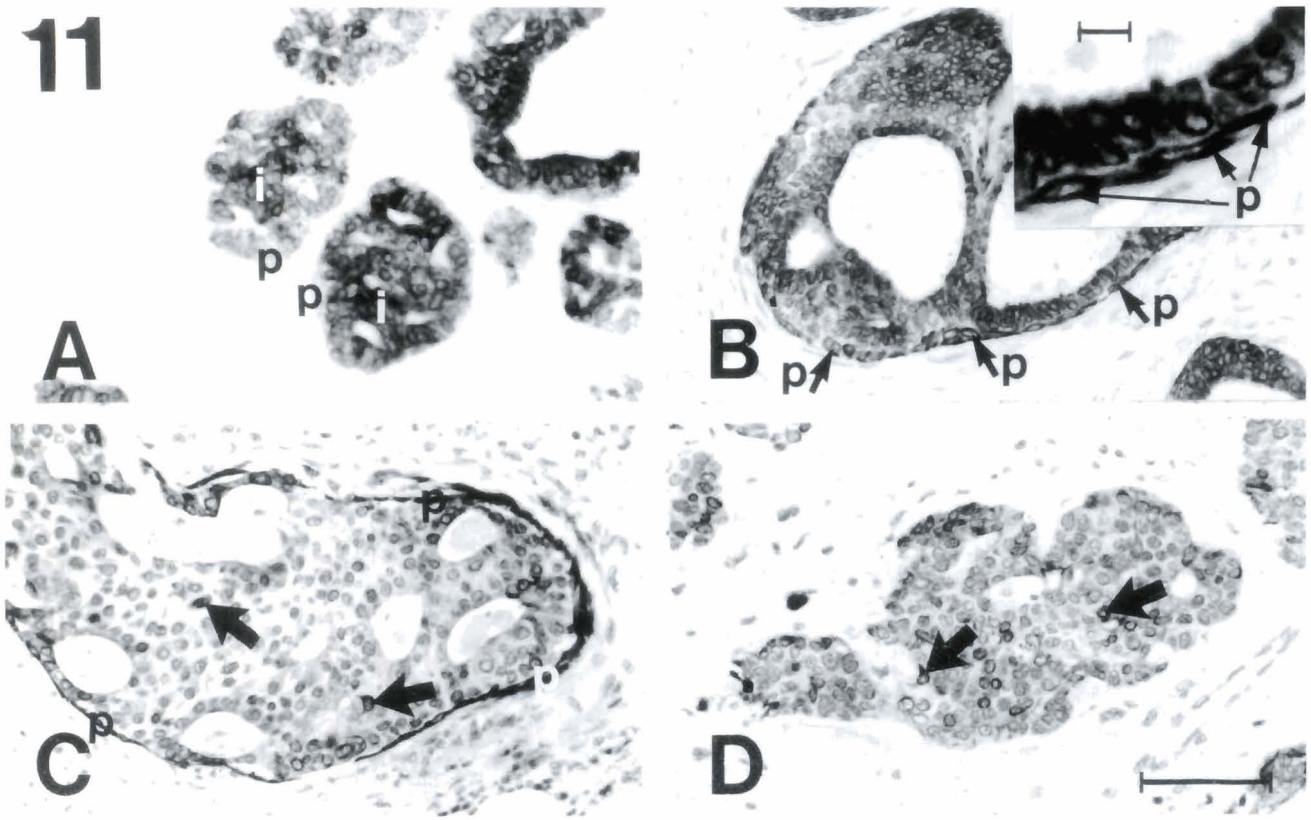

Fig. 11. Immunocytochemical staining of invasive ductal carcinoma by keratin MAb PKK2. **A.** Area of ductal hyperplasia (epitheliosis) showing an end bud-like structure with intensely staining inner cells (i) and only weak staining of the peripheral cells (p). **B.** Area of atypical hyperplasia with multiple layering of variably stained inner cells and a stained layer of attenuated, peripheral myoepithelial-like cells (p). Inset shows this more clearly. **C.** Ductal carcinoma-in-situ with cribriform pattern showing some inner neoplastic cells that are stained (arrows) and the strongly-stained peripheral myoepithelial cells (p). **D.** Invasive ductal carcinoma showing the very occasionally-stained neoplastic cell (arrows), the remainder are stained only weakly or not at all. Original magnification x 230. Bar = 75 μm. Inset, original magnification x 730. Bar = 10 μm.

epithelial-like cells. Indeed, there may be an inverse correlation between the presence of the basement membrane proteins and malignancy, as estimated by histological grade (Gusterson et al., 1982; Nielson et al., 1983; Aung et al., 1993). It has been suggested in humans from the identification of cells morphologically intermediate between epithelial and myoepithelial cells in growing ductal structures, benign breast hyperplasia and in cultured cell lines, and proven directly in the corresponding rat systems *in vivo* and *in vitro* (Rudland, 1987a,b) that differentiation of epithelial cells to myoepithelial-like cells proceeds in morphologically discrete steps. It is possible then that variable truncations of this differentiation pathway may accompany the malignant process, giving rise to a retention of vestiges of this pathway by the malignant epithelial-like cells, and thereby allowing ectopic expression of a limited number of myoepithelial-related markers (Fig. 10).

In order to help discriminate between the above two types of model, an antibody to complex keratins, PKK2, which preferentially recognises myoepithelial and not epithelial cells in normal mature ducts (Rudland and Hughes, 1989), but which stains strongly epithelial cells in benign hyperplastic and neoplastic lesions, has been used to identify stained cells in serial histological sections in several invasive ductal carcinomas. Usually PKK2-stained cells can be observed in areas of ductal hyperplasia (Fig. 11A), atypical hyperplasia (Fig. 11B), ductal carcinoma-in-situ (Fig. 11C) and close by in areas of invasive carcinoma, whereas they are rare or often absent from the remaining carcinomatous areas (Fig. 11D). Similar results have also been obtained with an antibody to epithelial-specific cytokeratin 18; it stains only relatively few epithelial cells in normal ducts, stains benign epithelial cells and malignant epithelial cells in ductal carcinoma-in-situ, but less often malignant epithelial cells in invasive carcinomas (Rudland et al., 1993). These results suggest that the loss of expression of specific keratins in invasive carcinomas is more likely to be due to selection against these keratins in the initial stages of malignant-cell proliferation than to a specific population of nonstaining, normal epithelial cells.

In normal breasts, keratin 18 is observed only in 5 to 10% of epithelial cells in main ducts, whilst keratin antibody PKK2 stains predominantly the myoepithelial cells in such ducts (Rudland and Hughes, 1989). However, the vast majority of epithelial cells in growing terminal ducts, end buds (Rudland, 1991) and alveolar lobules (Rudland and Hughes, 1989) in prepubertal and pregnant females stain with both anti-keratin 18 and PKK2 sera; but the staining pattern returns to that of the main ducts in nongrowing but lactating alveoli (Rudland and Hughes, 1989). The preferential staining of epithelial cells in benign hyperplastic and neoplastic lesions by antibodies to the above cytokeratins as well as staining of atypical hyperplasia, ductal carcinoma-in-situ, and adjacent invasive carcinoma suggests that all of these diseases of the breast have a common origin in the growing ductal buds and lobules; a suggestion consistent with conclusions drawn from the much more extensive examination of their subgross anatomies (Wellings et al., 1975). The most likely cellular candidates in these growing buds and lobules are those basal cells intermediate betwen epithelial and myoepithelial cells. Whether malignant transformation preserves preferentially the intermediate types closer to the epithelial end of the epithelial-myoepithelial developmental pathway or malignant transformation truncates the epithelial stem cells' ability to differentiate along this developmental pathway is unclear at present. However, the ectopic expression of a limited number of markers related to the myoepithelial cell by a good proportion of invasive carcinomas, particularly close to and including carcinoma-in-situ suggests that the insulted cell originally bears some similarities to the epithelial precursor or stem cells described for normal developmental processes of the breast.

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