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Stem cells in mammary gland differentiation and cancer

PHILIP S. RUDLAND AND ROGER BARRACLOUGH

Cancer and Polio Research Fund Laboratories, Department of Biochemistry, University of Liverpool, PO Box 147, Liverpool L69 3BX, UK

Summary

Evidence based on ultrastructure and immunocytochemical staining suggests that morphological gradations between epithelial and myoepithelial cells, and possibly between epithelial and alveolar-like cells, can occur in terminal ductal structures of rat and human mammary glands. In neoplastic disease the benign, carcinogen-induced rat and benign, human mammary tumours can contain epithelial, myoepithelial-like and alveolar-like cells, whereas their malignant counterparts mainly contain only epithelial-like cells. Clonal epithelial cell lines from normal rat mammary glands, from benign tumours and from SV40-transformed human mammary cultures can differentiate to either myoepithelial-like or alveolar-like cells. In those of the rat, the differentiation processes occur in steps, intermediate cells along the myoepithelial-like pathway resemble the morphological intermediates in the terminal ductal structures *in vivo*. Changes in specific polypeptides characterize each of the intermediate cells *in vitro*. One of the earliest increases observed in the myoepithelial-like pathway *in vitro* is that due to a novel protein p9Ka, whereas the major increases in Thy-1 antigen and the basement membrane proteins laminin and type IV collagen occur at later steps. The nucleotide sequence of the gene for p9Ka is related to that of the small, regulatory calcium-binding proteins, and antibodies raised to synthetic fragments of its predicted amino acid sequence react with only myoepithelial cells within the rat mammary parenchyma. Increases in the production of p9Ka and Thy-1 are largely due to increases in their messenger RNAs, possibly arising at the level of transcription of the DNA, whereas the increases in production of laminin and type IV collagen occur at a post-transcriptional level. The normal transcriptional promoter sequences of TATA or CAAT are not found adjacent to the genes for p9Ka or Thy-1. Cells and cell lines from malignant rat mammary tumours of increasing metastatic potential and from malignant areas of human ductal carcinomas largely fail to yield fully differentiated myoepithelial-like or alveolar-like cells in culture; however, weakly metastasizing rat cells yield variants which may retain a vestige of the myoepithelial phenotype. It is suggested that novel regulatory transcriptional element(s) may control the production of some of the proteins along the normal myoepithelial-like pathway, and that these elements may be relatively unique in their capacity to become inoperative in the malignant breast cancer cell.

Identification of cell types and development of the normal mammary gland

The mammary glands of both adult rats and humans consist of a system of branching ducts terminating in alveoli and embedded in a fatty stroma (Raynaud, 1961). The mammary ducts are composed of one or more layers of cuboidal, epithelial cells, some of which border a lumen that is continuous throughout the ductal system. The epithelial cells are surrounded by a layer of elongated, myoepithelial cells (Hollman, 1974; Vorherr, 1974). These two fully differentiated cell types have been distinguished in the past by their characteristic ultrastructural morphologies. The ductal epithelial cells possess apical microvilli and specialized junctional complexes

Key words: mammary gland, stem cells *in vivo* and *in vitro*, differentiation and cancer.

with associated desmosomes, whereas the myoepithelial cells possess smooth muscle-like myofilaments with pinocytotic vesicles and basement membranes on their basal surfaces. A third functionally differentiated cell type, the secretory cell, is found in the mammary alveoli. This cell type is characterized by its ultrastructure and, during lactation, by the synthesis and secretion of milk products (Ozzello, 1971; Radnor, 1971).

More recently, immunocytochemical stains have been used to distinguish between the different cell types (Table 1). In the rat, the epithelial cells are stainable by antisera to milk fat globule membrane (MFGM) (Warburton *et al.* 1982*a*) and keratin monoclonal antibody (MAB) LE61 (Taylor-Papadimitriou *et al.* 1983). The myoepithelial cells can be stained by antisera to vimentin, actin, myosin (Dulbecco, 1982; Warburton *et al.* 1982*a*), keratin MAB LP34 (Taylor-Papadimitriou *et al.* 1983; Warburton *et al.* 1987), and by the lectins *Griffonia simplicifolia*-1 (GS-1) and pokeweed mitogen (Hughes, 1988). The basement membrane, which is probably synthesized at least in part by the myoepithelial cells, stains with antisera to laminin, type IV collagen and Thy-1 antigen (Dulbecco, 1982; Rudland *et al.* 1982; Warburton *et al.* 1982*a*; Monaghan *et al.* 1983). In the human gland, antisera (Heyderman *et al.* 1979) and MABs (Foster *et al.* 1982; Taylor-Papadimitriou *et al.* 1983) to MFGM are primarily against a single glycoprotein termed epithelial membrane antigen (EMA) (Ormerod *et al.* 1984; McIlhinney *et al.* 1985), whilst the two lectins (Hughes, 1988) and the antiserum to human keratin (Gusterson *et al.* 1982) fail to show any discriminatory staining. MAB LICR-LON-23.10 which recognizes basal cells of the skin and blood vessels (Gusterson *et al.* 1985) and MABs to the common acute lymphoblastic leukaemia antigen (CALLA) (Gusterson *et al.* 1986) preferentially recognize the human myoepithelial cells. The secretory alveolar cells from both species are characterized by being stainable by peanut lectin (Newman *et al.* 1979) and, during lactation, by antisera to their respective caseins (Rudland *et al.* 1983*a*; Earl & McIlhinney, 1985) (Table 1).

Table 1. *Summary of the immunocytochemical staining patterns of rat and human mammary cells* in vivo

Reagent[a]	Epithelial cells	Myoepithelial cells	Alveolar cells
Anti-MFGM[b], EMA[c]; MABs to EMA	+	−	+
Peanut lectin alone	−	−	+
Anti-caseins[b,c]	−	−	+
Keratin MAB LE61	+	−	+
Anti-actin/myosin	−	+	−
Keratin MAB LP34	−[b](±)[c]	+	−
MAB LICR-LON-23.10	−	+[c]	−
MABs to CALLA	−	+	−
GS-1/PWM lectins	−	+[b]	−
Anti-laminin/type IV collagen/Thy-1[b,c]	−	+[d]	−

[a] Abbreviations as used in text; MAB, monoclonal antibody ±, weakly stained; [b] rat; [c] human; [d] basement membrane adjacent to myoepithelial cells.

The development of the mammary parenchyma takes place predominantly after birth, but prior to puberty (Myers, 1919; Dawson, 1934), by the lengthening and branching of primative ducts within the mammary fat pad. During this period of growth, the ducts terminate in globular structures called terminal end buds in rats (TEBs), which contain most of the dividing parenchymal cells (Dawson, 1934; Russo *et al.* 1982). The number of globular structures reaches a maximum in rats of about 20 days old (Russo *et al.* 1982), and in humans of about 13 years old (Dawson, 1934) (Fig. 1A). Thereafter the number rapidly declines as the globular structures differentiate to terminal ducts and alveolar buds in rats (Russo & Russo, 1978) or to terminal ductal alveolar units (TDLUs) in humans (Dawson, 1934) (Fig. 1B). The alveolar buds and TDLUs are the direct precursors of the secretory alveoli. In rats the TEBs and to a lesser extent the alveolar buds consist of a heterogeneous collection of cells which show a gradation in ultrastructural and immunocytochemical-staining characteristics towards the epithelial cells on the one hand, and to the myoepithelial cells of the subtending duct on the other hand (Williams & Daniel, 1983; Ormerod & Rudland, 1984). Some of the terminal structures in humans also show some evidence for similar morphological gradations (Stirling & Chandler, 1976; Smith *et al.* 1984; Rudland& Hughes, 1989). Thus some evidence exists *in vivo* relating epithelial cells

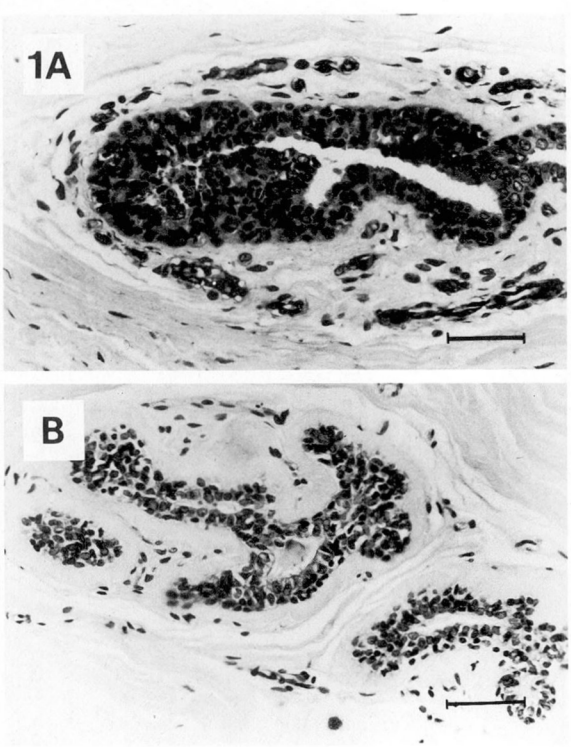

Fig. 1. Histological sections of human terminal ductal structures. Mammary gland from: A, 13 year old girl showing a terminal end-bud in longitudinal section; B, 14 year old girl showing a terminal ductal-lobuloalveolar unit. Bars, 50 μm.

to myoepithelial cells on the one hand and to alveolar cells on the other hand, although this evidence is stronger in the rat than in the human at present.

Development and cellular structure of mammary tumours

The susceptibility of the rat mammary gland to chemical carcinogenesis correlates with the presence in the gland of TEBs and terminal ducts (Russo *et al.* 1977). The tumours induced by dimethyl-benzanthracene (DMBA) (Huggins *et al.* 1961) or nitrosomethyl urea (NMU) (Gullino *et al.* 1975) are predominantly cytologically benign in the authors' experience (Williams *et al.* 1981). These relatively benign tumours contain areas of epithelial and elongated, myoepithelial-like cells in duct-like arrangements (Murad & von Haam, 1972). However, many of the elongated, myoepithelial-like cells possess a more undifferentiated appearance than the myoepithelial cells of mature mammary ducts (Dunnington *et al.* 1984*a*). Hormonal stimulation of the host leads to production of a small proportion of alveolar-like cells which can synthesize casein (Supowit & Rosen, 1982). However, the amount of casein and casein mRNA produced by these cells is only 1–5 % of that produced by the alveolar cells of the lactating mammary glands in normal rats, when animals bearing the tumours are subsequently mated (Herbert *et al.* 1978; Supowit & Rosen, 1982; Rudland *et al.* 1983*a*). Chemical induction in partially immune-deficient rats that are then subjected to nonspecific immunostimulation can produce metastatic tumours (Table 2) which disseminate widely, some like the human disease (Kim, 1979). However, no cells with any myoepithelial characteristics are seen in such malignant carcinomas (Dunnington *et al.* 1984*a*).

The primary carcinogens which induce mammary tumours in humans are

Table 2. *Origins of the mammary cell lines discussed*

Mammary tissue	Cell line	Identity
Normal rat	Rama 704	epithelial
	Rama 704E	myoepithelial-like
	Rama 401	myoepithelial-like
Benign DMBA rat tumour	Rama 25	epithelial
	Rama 25-I	epithelial/myoepithelial intermediates
	Rama 29	myoepithelial-like
Benign DMBA syngeneic rat tumour	Rama 37	epithelial
	Rama 37E5	myoepithelial-like
Weakly metastasizing rat tumour, TR2CL	Rama 600	epithelial
Moderately metastasizing rat tumour, TMT-081	Rama 800	anaplastic epithelial
Strongly metastasizing rat tumour, SMT-2A	Rama 900	anaplastic epithelial
Normal human transformed by SV40	SVE3	epithelial
	Huma 7	epithelial
	Huma 25	myoepithelial-like
	Huma 62	myoepithelial-like
Human ductal carcinoma	Ca2-83	epithelial

completely unknown, apart from two exceptions where women had been exposed to high doses of radiation after atomic bomb explosions at Hiroshima and Nagasaki (McGregor *et al.* 1977). As in rats, the most susceptible developmental stage for these radiation-induced breast cancers is probably in prepubertal/adolescent females (McGregor *et al.* 1977). The specific phenotypic feature which best correlates with increased risk of neoplastic disease in humans is the presence of atypical epithelial cell proliferations in terminal ductal structures (Wellings *et al.* 1975). These atypical structures probably represent a spectrum from benign lesions to carcinoma-in-situ (Wellings & Yang, 1983), the direct precursor to mammary carcinoma, although there are contrary views (Azzopardi, 1979). Ultrastructural (Ahmed, 1978; Azzopardi, 1979; Macartney *et al.* 1979; Gould *et al.* 1980) and immunocytochemical techniques (Albrechstein *et al.* 1981; Barsky *et al.* 1982; Bussolati *et al.* 1980; Macartney *et al.* 1979; Gusterson *et al.* 1982; 1985; 1986) have shown that some myoepithelial cells are always present in the major categories of benign breast disease (epitheliosis, adenosis and fibroadenoma), but they are almost entirely lost in infiltrating ductal carcinomas. Similarly, in the few cases examined with well-characterized reagents, pregnant/lactating women bearing benign tumours can produce neoplastic cells that secrete casein, whereas none were seen in malignant carcinomas (Earl & McIlhinney, 1985; Earl, 1987). Thus the broad pattern of malignant cell types in human breast neoplasms is similar, to some extent, to that found in the corresponding rat mammary tumours; both the myoepithelial cell and the putative alveolar cells are lost in malignant compared with nonmalignant tumours.

Differentiation of cultured stem cell lines isolated from normal and benign neoplastic mammary glands

To determine whether one cell type can give rise to another cell type directly, it is frequently necessary to obtain immortalized cell lines cloned from a single cell, and to observe the different cell types that such a system will generate.

In the rat, limited digestion of mammary glands or carcinogen-induced mammary tumours yields 'organoids' that can subsequently adhere to the surface of a tissue culture vessel and produce growing cultures of epithelial cells (Hallowes *et al.* 1977*b*; Rudland *et al.* 1977). After a few passages most of these cells die out, but the occasional spontaneously transformed, immortalized epithelial cell is generated which can eventually be cloned (Bennett *et al.* 1978). In this way single-cell-cloned epithelial cell lines have been obtained from the normal mammary glands of 7-day-old inbred, Furth-Wistar rats (Ormerod & Rudland, 1985), from DMBA-induced benign tumours of out-bred, Sprague-Dawley rats (Bennett *et al.* 1978), or inbred, Furth-Wistar rats (Dunnington *et al.* 1983) and from an NMU-induced rat mammary tumour (Dulbecco *et al.* 1981) (summarized in Table 2). Cultures of mammoplasty specimens from otherwise normal human breasts that have been obtained in virtually the same way as those from the rat mammary glands, however, fail to undergo spontaneous transformation events, and eventually die out after

several passages in culture (Hallowes *et al.* 1977*a*; Stampfer *et al.* 1980; Easty *et al.* 1980; Rudland *et al.* 1989*a*). To obviate this problem, human epithelial cells have been immortalized by transforming them with simian virus 40 (SV40) (Fig. 2) (Chang *et al.* 1983; Rudland *et al.* 1989*b*). All the rat and human epithelial cell lines discussed above behave in a similar manner (Table 2), and thus the results of one, rat mammary 25 (Rama 25) from a benign rat mammary tumour, is described in detail below.

The epithelial cell lines, both rat and human, are conveniently cultured on a plastic substratum where they grow with a cuboidal morphology (Fig. 2A). When such cultures become densely packed, small, dark, polygonal cells are formed (Fig. 2B) which can contain vacuoles or 'droplets' at their peripheries (droplet cells) (Fig. 2F) (Bennett *et al.* 1978). These droplet cells form hemispherical blisters or domes (Fig. 2F) that arise from the unidirectional pumping action of the ouabain-sensitive sodium/potassium ATPase (Paterson *et al.* 1985*a*). The overall process can be accelerated with the erythroleukaemic differentiating agent, dimethyl sulphoxide (Friend *et al.* 1971; Bennett *et al.* 1978), or retinoic acid (Rudland *et al.* 1983*b*) in the presence of the mammotrophic hormones, prolactin, estrogen, hydrocortisone and insulin. Such cultures of Rama 25 produce authenticated rat β-casein, although the small amounts (50–100× less than in lactating rat mammary glands) may reflect the neoplastic origins of this particular cell line (Warburton *et al.* 1983). The discrete morphological stages observed in the formation of the casein-secreting, doming cultures *in vitro* (Paterson *et al.* 1985*b*) are paralleled by changes in a small number of specific polypeptides (Paterson & Rudland, 1985*a*). The final casein-secreting stage resembles alveolar-like cells, particularly those found in the benign rat mammary tumours (Rudland *et al.* 1983*a*). In addition, the human mammary epithelial cell lines can also keratinize in culture (Fig. 2E), whereas this is observed infrequently in the corresponding rodent cell lines (Rudland *et al.* 1989*b*).

Although the epithelial cell lines have been single-cell cloned at least once, confluent cultures at high passage-number yield ridges of elongated cells and subconfluent cultures yield from 0·1 % (human cell lines: Rudland *et al.* 1989*b*) up to 3 % (rat cell lines: Warburton *et al.* 1982*b*; Ormerod & Rudland, 1985) of clones of cells with an elongated morphology (Fig. 2C). Similar morphological forms occur in epithelial cell lines of mouse mammary tumours (Sanford *et al.* 1961; Dexter *et al.* 1978; Hager *et al.* 1981). From a comparison of the ultrastructure and immunocytochemical-staining characteristics of histological sections of rat (Ormerod & Rudland, 1982; Warburton *et al.* 1982*b*) and human mammary glands (Gusterson *et al.* 1982, 1985, 1986) and of their primary cultures (Warburton *et al.* 1985; Rudland *et al.* 1989*a*,*b*), the elongated cells derived from such cultures are thought to be related to myoepithelial cells rather than to fibroblasts (Fig. 2D). However, the final phenotype of these elongated cells can vary. In general, cells of a more mature myoepithelial phenotype have been derived from the epithelial cells of normal mammary glands, e.g. the rat cell lines Rama 704E (Ormerod & Rudland, 1985) and Rama 401 (Warburton *et al.* 1981*b*), than from the epithelial cells of mammary tumours, e.g. the rat cell lines Rama 29 (Bennett *et al.* 1978) and Rama 37E5

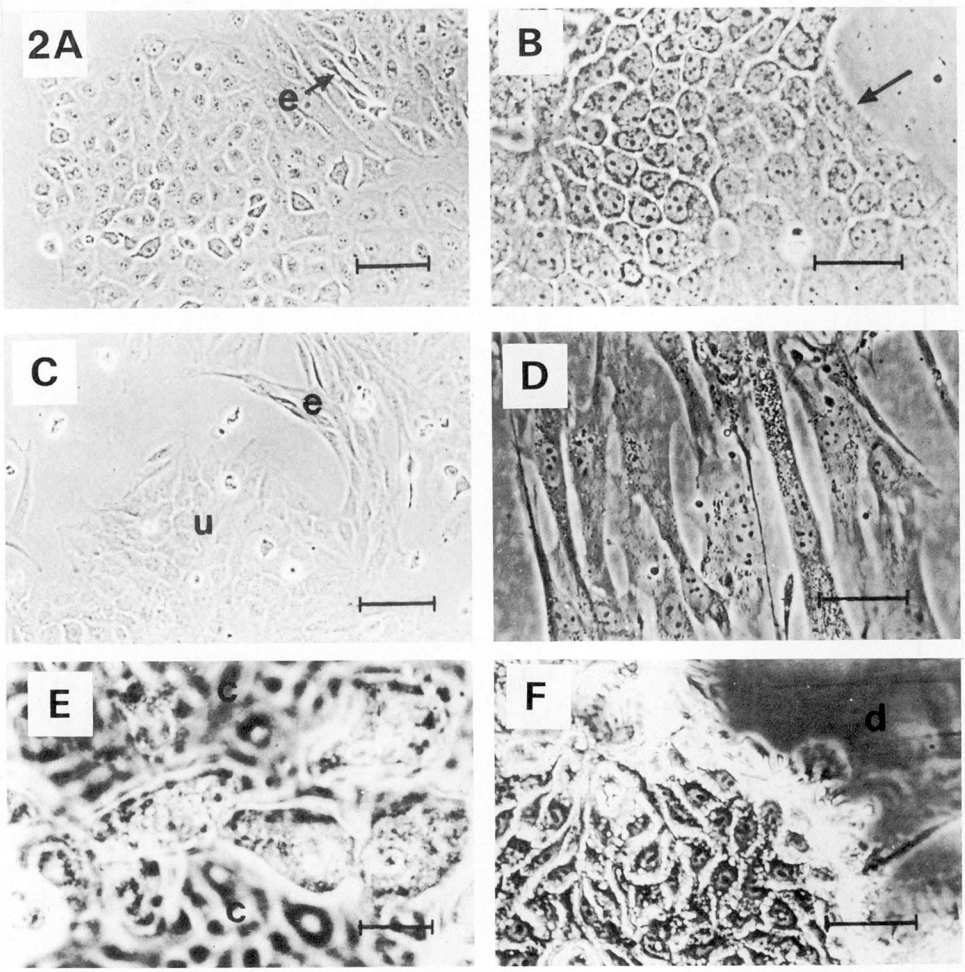

Fig. 2. Phase-contrast morphology of SV40-transformed normal human mammary cells in culture. The morphology of the corresponding rat cells is identical unless otherwise specified. A. Colony of cuboidal epithelial cells from the SV40-transformed cell line SVE3 showing the very occasional elongated cell (e). B. Cuboidal epithelial cells of a subclone of SVE3, human mammary (Huma) 7 showing more compact gray cells (<——) and dark cells. C. Elongated, myoepithelial-like cells derived from SVE3, Huma 25 which, when sparse are very elongated in appearance (e), but when more confluent appear pseudocuboidal (u) due to over-and-underlapping of cellular processes. D. Mammary fibroblastic cells. E. SVE3 cuboidal epithelial cells (c) undergoing desquamation by shedding thin, enucleated cellular residues into the medium; this rarely occurs in the corresponding rat cells. F. Small, dark, 'droplet cells' with associated hemispherical blister or dome (d) in the sheet of SVE3 cells (Rudland *et al.* 1989*b*). Bars: A,C, 100 μm; B,D,F, 50 μm; E, 20 μm.

(Dunnington *et al.* 1983) (Table 2). This result is consistent with the finding in the previous section that the better differentiated myoepithelial cells occur in normal rat mammary glands rather than in their tumours. Thus, based on the above results, the

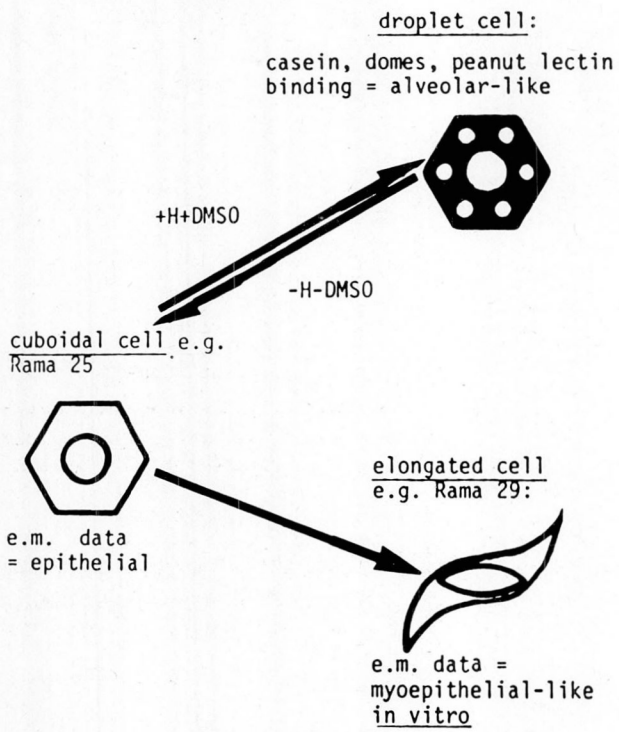

Fig. 3. Diagram of the intercellular conversions of Rama 25 epithelial cells. Rama 25 cuboidal, epithelial cells derived from a benign rat mammary tumour can differentiate to droplet cell/doming-alveolar-like cells with mammatrophic hormones prolactin, estrogen, hydrocortisone, insulin and dimethyl sulphoxide (DMSO) or to elongated, myoepithelial-like cells (e.g. the cell line Rama 29). e.m., electron microscopic.

majority of the more-elongated cells *in vitro* are classified as myoepithelial-like rather than as mature myoepithelial cells (Rudland *et al.* 1980, 1989*b*).

The epithelial cell lines can thus give rise to both alveolar-like cells and myoepithelial-like cells (Fig. 3). They are therefore possible candidates for stem cells for the mammary gland, since they can undergo *in vitro* the morphological transitions observed in terminal ductal structures *in vivo* (pp. 97–98) and in primary cultures *in vitro* (Rudland, 1987). Moreover, when grown on floating collagen gels which mimic the stromal matrix of the mammary gland, such epithelial cell lines form branched, duct-like structures reminiscent of the immature ducts found in neonatal mammary glands (Bennett, 1980; Ormerod & Rudland, 1982, 1985, 1988), further confirming their possible stem-cell properties.

Identification and regulation of discrete differentiation stages to myoepithelial-like cells *in vitro*

Cloned cell lines that are intermediate (I) in morphology and known marker content between Rama 25 epithelial cells and elongated, myoepithelial-like cells have been

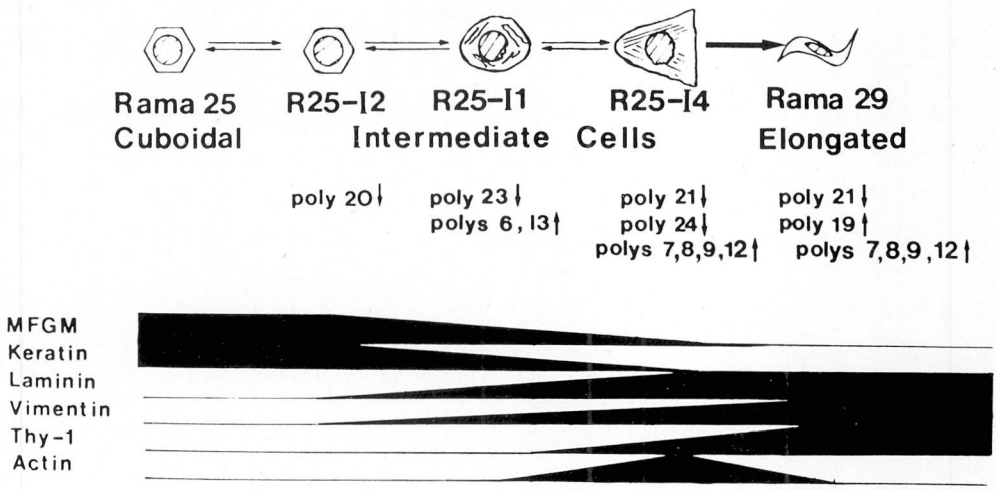

Fig. 4. Diagram of the differentiation of Rama 25 epithelial cells along a myoepithelial-like pathway. The cell lines intermediate in morphology between Rama 25 cuboidal epithelial and elongated, myoepithelial-like cells (e.g. Rama 29) are designated by R25-I etc. (Rudland *et al.* 1986). They are also thought to resemble similar cells in the direct conversion of Rama 25 cuboidal cells to elongated cells. Only the last stage is irreversible. The polypeptide changes associated with each stage are also shown; the numbers correspond to those reported previously (Paterson & Rudland, 1985*b*); polypeptide 13 is p9Ka (Barraclough *et al.* 1982).

isolated, and they form a series in the order: Rama 25 cuboidal cells, Rama 25-I2, Rama 25-I1, Rama 25-I4, and elongated cells, e.g. Rama 29 (Table 2; Fig. 4: Rudland *et al.* 1986). When grown on floating collagen gels, the intermediate cell line Rama 25-I2 forms more-mature, duct-like structures than the parental Rama 25, and ultrastructural and immunocytochemical analysis suggests that Rama 25-I1 and Rama 25-I4 resemble the intermediate cells of the terminal ductal structures *in vivo* (Rudland *et al.* 1986). The intermediate cell lines *in vitro* thus may represent the heterogeneous cells observed earlier (pp. 97–98) in budded structures *in vivo*. The above cellular order is maintained for increasing abundance of 7 polypeptides which are characteristic of elongated, myoepithelial-like cells and decreasing abundance of 4 polypeptides which are characteristic of cuboidal epithelial cells (Fig. 4) (Paterson & Rudland, 1985*b*; Rudland *et al.* 1986). The majority of these new proteins do not correspond to the known proteinaceous markers of the myoepithelial cells since the two dimensional-gel systems used to identify them cannot easily detect the lower levels of most of the known marker proteins of the myoepithelial cell.

One of the earliest detectable increases in a protein along the myoepithelial-like differentiation pathway is that due to polypeptide 13 (Fig. 4) (Rudland *et al.* 1986), a novel protein of 9000 mol.wt. termed p9Ka by Barraclough *et al.* (1982, 1984*a*). The nucleotide sequence of its gene (Fig. 5) suggests that it may be related to a class of small, regulatory, calcium-binding proteins (Fig. 6) (Barraclough *et al.* 1987*b*). The p9Ka protein may therefore serve to trigger the changes in the cytoskeleton (Paterson & Rudland, 1985*b*) which have been observed along this pathway to myoepithelial-

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       10         20         30         40         50         60         70         80         90
GGATCCAGATGAGAGATTCTGGTACGGAGGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTATTGCACAAGA

      100        110        120        130        140        150        160        170        180
ATGAAAACTGAAAAACAAGCAGTATATAAATGGCTCCCGGAGATTCTGAGATGCTGAGGCTTGCTTGTATGTTGCTATAGTGTATGTTGG

      190        200        210        220        230        240        250        260        270
TGCTTGGGAGCCACTGTCATGGATAGGTATGTTGCTGGGTCATCCAAGCCAGTGTGTGGACACTCAGGTACAGGAAGCAAAGTGAAGGCA

      280        290        300        310        320        330        340        350        360
TCAGCAGGCATTTTTGTTTTACGATGTTTAAATTACACTTATTTTATTTGTGTGTACGAGTGTATGGGTGGGGATGGGGCAAATGCCAAG

      370        380        390        400        410        420        430        440        450
GGGCACTTCTTGTGAGAGTCAATCTGTTCCTTCTAGCATGTGGGCTCTGGAGATCAAACTCAGGTCATTGAGCTTGGTGGCAAGCACCTC

      460        470        480        490        500        510        520        530        540
TACCTACTGAGCCACCTGTTCAACACCCACCTGTAGGCATTTGTGTTCATAGTAGTTCATAGCCCTATGAACATATAGCACCTAGGCCAA

      550        560        570        580        590        600        610        620        630
GAGAGCCTGGCTTCCCCACCCCCTCCCCTTGTACCCCAACCTCTGCCACTTCATCTCACTCCTACTAGGCAGCTGGGTTTTTTCCCTCAC

      640        650        660        670        680        690        700        710        720
TGTAGGCCCCTGGGCAGGCAGCCAGCAGCCGCGCCCAACGCTGGGAGGGAGAAGAATGGGTCAGAGGCTGGAGCTTGTGGTTGAGTTGGG

      730        740        750        760        770        780        790        800        810
GAGTGAGTAAGCTGAGTGAGGGATGGAAAACTGCTGTTGTTGAGGCCAGGCCTGGGGGGGAGGCACAGAAGGCTGCTGGCATGAATTTCT

      820        830        840        850        860        870        880        890        900
AGAGTTTGAGTGGTAAGTTTTGCAAGTTTCAGAGCTTGAAGCACATATGAGCTTCTTGCCATCAGTGGGTACCACTCCTCTGATCTCCCT
                  ↑          ↑          ↑          ↑                    ↑          ↑
      910        920        930        940        950        960        970        980        990
GGGAGTGAGGTCGGTCTCTGGAAGTGCTCTTAGAGAGTAGGTTGGAGTAGAGCACTAAAAACGGGGACAGACTGAGTGTGACTTGAGTGA

     1000       1010       1020       1030       1040       1050       1060       1070       1080
TGCCTAGCAACATATATCCAGCTCTCAACACACTGTTGGTGTGGGTTGGAGAAGGCTACTTTTGTGTCTCCTGCCCCTAGGTCTCAACGG

     1090       1100       1110       1120       1130       1140
TCACCATG GCG AGA CCC TTG GAG GAG GCC CTG GAT GTA ATA GTG TCC ACC TTC CAC AAA TAC TCA GGC
    Met Ala Arg Pro Leu Glu Glu Ala Leu Asp Val Ile Val Ser Thr Phe His Lys Tyr Ser Gly

          1160       1170       1180       1190       1200       1210
AAC GAG GGT GAC AAG TTC AAG CTG AAC AAG ACA GAG CTC AAG GAG CTA CTG ACC AGG GAG CTG CCT AGC
Asn Glu Gly Asp Lys Phe Lys Leu Asn Lys Thr Glu Leu Lys Glu Leu Leu Thr Arg Glu Leu Pro Ser

          1230       1240       1250       1260       1270       1280       1290       1300
TTC CTG GGG gtgagtggatcctgtctgtgtattgcatatgtgatgcatccccaggaggaggctggggctggagatatctatctatct
Phe Leu Gly

  1310       1320       1330       1340       1350       1360       1370       1380       1390
atctatctatctatctatctatctatctatctatctatctatctatctatctatctatctatctatctatctatctatctatctatcta

  1400       1410       1420       1430       1440       1450       1460       1470       1480
catatatatatatatatatatatatatatatatatatatatatatatatatatctctccctactcctggcgcttggtatggaaccacaatga

  1490       1500       1510       1520       1530       1540       1550       1560       1570
accatctacttcacaccagccccccgttgagacaaggcttagaatgaagttaactgaagtggcacaggaaaaccacattaggtagtcagt

  1580       1590       1600       1610       1620       1630       1640       1650       1660
gtctgaaagcacagcctagatcaggacagtctttccggtgatgtgcaacagaaatcgagtttctgcttgtgaagacatgattgtggaggc

  1670       1680       1690       1700       1710       1720       1730       1740       1750
acacaaatgcctgcagatcttcccctcaatgacaccttatcttagttaacacctccttgtcatgacagttacctatagacatagttaaaa

  1760       1770       1780       1790       1800       1810       1820       1830       1840
caagcgtggggaagatgtggtcacatcctttcccagctagcccatgtgctcatctcacagttgagccctgaggctagcacggtgtctgca

  1850       1860       1870       1880       1890       1900       1910       1920
agccttcctgagctcctggctggaggtggcgtctaactgtacctcttctacctccag AGA AGG ACA GAC GAA GCT GCA TTC
                                                            Arg Arg Thr Asp Glu Ala Ala Phe
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  1930          1940          1950          1960          1970          1980          1990
CAG AAG CTG ATG AAC AAC TTG GAC AGC AAC AGG GAC AAT GAA GTT GAC TTC CAG GAG TAC TGT GTC TTC
Gln Lys Leu Met Asn Asn Leu Asp Ser Asn Arg Asp Asn Glu Val Asp Phe Gln Glu Tyr Cys Val Phe

  2000          2010          2020          2030          2040          2050          2060
CTG TCC TGC ATT GCC ATG ATG TGC AAT GAA TTC TTT GAG GGC TGC CCA GAT AAG GAG CCC CGG AAG AAG
Leu Ser Cys Ile Ala Met Met Cys Asn Glu Phe Phe Glu Gly Cys Pro Asp Lys Glu Pro Arg Lys Lys

    2070          2080      2090      2100      2110      2120      2130      2140
TGA AGA CTC CTC AGA TGAAGTGTTGGGCCAGTGGGGGAATCTTCCATGTTGGCTGTGAGCATAGTGCCTTACTCTGGCTTCTTCA
End               End

        2160      2170      2180      2190      2200      2210      2220      2230
TACATGTGCACAGTGCTGAGCAAGTTTAATAAAGAGTTTTGAAACTatgtctgagagactggagattgtgggtgggtgggtgggctgagg

        2250      2260      2270      2280      2290      2300      2310      2320
gagggtggtggcagagatggctggaagttgacctggagctttgtggggccaactagaaaaggttggggaggggtgagtgactatctgagt

        2340
catacagtgtat
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Fig. 5. The nucleotide sequence of the p9Ka gene. The sequence of the strand of DNA corresponding to the mRNA of p9Ka is shown. The arrows represent the multiple 5′ start sites of this mRNA, and the single intron and region beyond the 3′ terminus of the non-poly(A)-portion of the mRNA are shown in lower case letters. The potential p9Ka coding region is also shown with the corresponding amino acid residues beneath. The sequence corresponding to the particular complementary DNA to the mRNA for p9Ka that was used to isolate the genomic fragments is underlined. The numbers above the nucleotide sequence refer to the nucleotide immediately below the last digit (Barraclough *et al.* 1987*b*).

like cells (Warburton *et al.* 1981*a*,*b*). Antibodies to purified p9Ka and to a synthetic peptide corresponding to a short stretch of its deduced amino acid sequence (Barraclough *et al.* 1987*b*) bind only to myoepithelial cells in the rat mammary parenchyma, confirming the myoepithelial origins of p9Ka (Haynes, 1988). This protein and its messenger RNA coordinately increase initially in the intermediate Rama 25-I1 cells (Barraclough *et al.* 1984*a*,*b*; Rudland *et al.* 1986; B. R. Barraclough, unpublished results). Similarly Thy-1 protein and mRNA coordinately increase, but this increase occurs mainly in the Rama 25-I4 and elongated cells (Rudland *et al.* 1986; Barraclough *et al.* 1987*a*). These results suggest that asynchronous or stepwise regulation of the production of marker proteins for the myoepithelial-like cell is controlled mainly at the level of transcription of the DNA. This is not always the case. Thus the increase in laminin and type IV collagen which occurs mainly in the Rama 25-I1 and Rama 25-I4 cells (Rudland *et al.* 1986) is not due to major changes in their levels of mRNA (Warburton *et al.* 1986; Barraclough *et al.* 1987*a*) but, in the case of type IV collagen, to decreases in its rate of intracellular degradation (Warburton *et al.* 1986).

In the case of p9Ka, preliminary evidence suggests that at least part of the increase in its accumulation in the myoepithelial-like cells *in vitro* arises from an increased rate of transcription of its mRNA (B.R. Barraclough, unpublished observation). In many genes, regions of DNA which are important in controlling the synthesis of their mRNAs are often located immediately adjacent to those sequences that correspond to the 5′ end of their mRNAs. However, these regions, such as the TATA (Breathnach

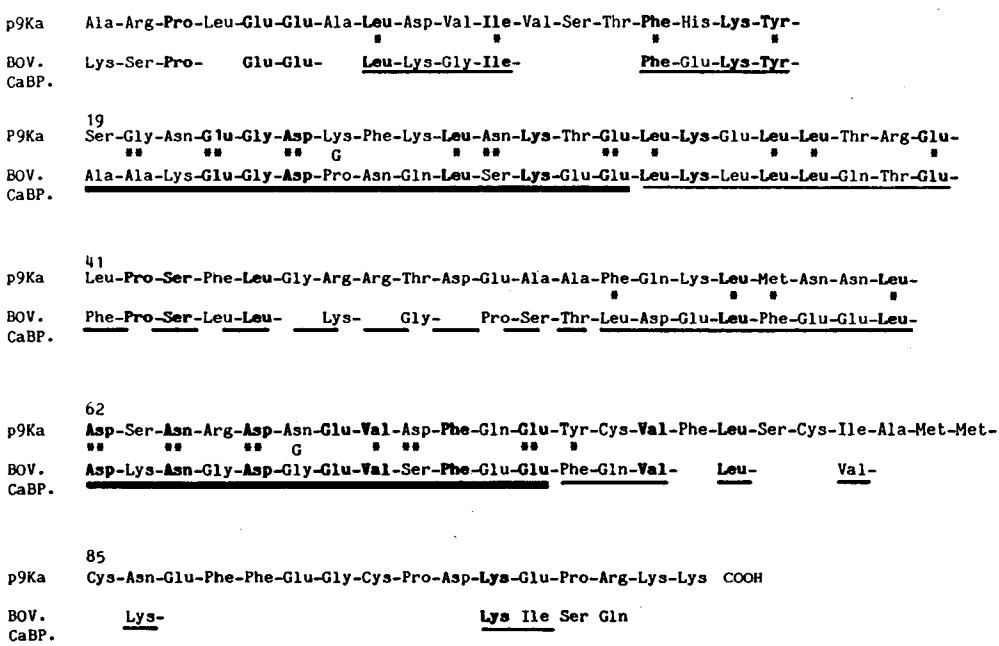

Fig. 6. The potential amino acid sequence of p9Ka and its relationship to the class of small, regulatory calcium-binding proteins. Amino acids are shown using the three letter code and are arranged to maximize the homology with the bovine intestinal calcium-binding protein (BOV CaBP), common residues are shown in bold type. The stars between the sequences show the preferred types of amino acid side chains in the general structure of the calcium-binding region of the small regulatory calcium-binding proteins: *, hydrophobic residue; **, oxygen-containing residue; and G, glycine. The helix, loop, helix arrangements of the calcium-binding sites are indicated by underscoring: helix, thin line; calcium-binding loop, thick line; and linker sequence, broken line (residues 41–53) (Barraclough & Rudland, 1988).

& Chambon, 1981) and CAAT (Benoist *et al.* 1980) consensus sequences are not found close to the p9Ka gene corresponding to p9Ka mRNA. In at least two other such cases, the murine Thy-1 gene (Giguere *et al.* 1985; Ingraham & Evans, 1986) and the 3-hydroxy-3-methylglutaryl coenzyme A reductase gene (Reynolds *et al.* 1984), multiple initiation sites for the transcription of the mRNAs have been reported, and this is also the case for the mRNA for p9Ka (Barraclough *et al.* 1987*b*). Thus it is possible that the genes for p9Ka and Thy-1 may contain a common or closely related novel promoter which regulates their expression between epithelial and myoepithelial-like cells.

Carcinoma cells are characterized by their failure to differentiate to myoepithelial cells

In the rat, a variety of epithelial cell lines have been obtained from different transplantable tumours of the mammary gland, and these show similar metastatic potentials to those of their parental tumours (Table 2). These rat cell lines have been

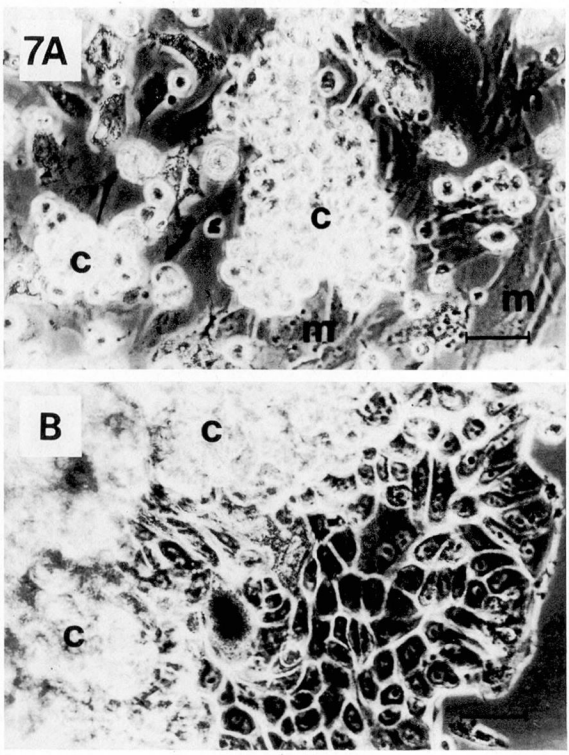

Fig. 7. Phase-contrast morphology of malignant mammary cell lines in culture. A. Clumps of cells of the cell line Rama 900 (c), isolated from the highly metastatic SMT-2A transplantable rat mammary tumour and growing with mesothelial-like feeder cells (m). B. Clumps of cells of the cell line Ca2-83 (c), isolated from a malignant human breast cancer, showing limited attachment and spreading of the epithelial cells on the substratum. Bars, 50 μm.

obtained with difficulty and grow as loosely-adherent colonies (Fig. 7A) much more slowly than cells from normal glands or benign tumours (Rudland, 1987). In contrast to cultured cells from normal and benign rat mammary glands, those from our metastasizing rat mammary carcinomas, as well as any cell lines developed from them, yield no myoepithelial cells nor any casein-producing, alveolar-like cells under the requisite hormonal conditions (Dunnington *et al.* 1984*b*; Williams *et al.* 1985; Rudland *et al.* unpublished). The weakly metastasizing Rama 600 cell line (Table 2) does contain a more elongated cellular component, but if this represents differentiation to a myoepithelial cell it is of a partial and incomplete nature, and probably reflects only a vestige of the complete pathway (Williams *et al.* 1985). Similarly, Rama 600 cells also appear to retain only a vestige of the differentiation pathway to alveolar cells (Rudland, 1988). Perhaps neoplastic transformation of the epithelial/intermediate cells results in a truncation of both differentiation pathways, and this truncation occurs earlier with increasing metastatic potential (Rudland, 1987).

Like most of the metastasizing rat mammary tumours, the culture of human

mammary carcinomas has been extremely difficult (Hallowes *et al.* 1977*a*; Kirkland *et al.* 1979). Routine digestion of over 100 primary infiltrating ductal carcinomas with collagenase, by slight modifications of the methods used for the benign rat mammary tumours (pp. 99–102), yields loosely adherent, malignant-looking cell clusters (Fig. 7B) and fast-adherent, less malignant-looking epithelium on collagen gels (Hallowes *et al.* 1983; Rudland *et al.* 1985). Metastases in axillary lymph nodes and pleural effusions yield only the loosely adherent clusters, whilst normal mammary glands and benign fibroadenomas yield only fast-adherent colonies (Hallowes *et al.* 1983; Rudland *et al.* 1985). These results suggest that the fast-growing adherent sheets of epithelium from primary ductal carcinomas (Smith *et al.* 1981) do not usually represent the most-metastasizing cell populations but, as in the rat above, the latter are best represented by the slow-growing, loosely adherent aggregates (Rudland, 1987). Continued passage of one preparation of loosely adherent cell clusters has yielded a continuously growing cell strain, Ca2–83 (Table 2; Fig. 7B), which has not yet undergone a period of crisis (Rudland *et al.* 1985), unlike most other cell lines established from malignant breast cancer cells (Semen *et al.* 1976; Lasfargues *et al.* 1978; Engel *et al.* 1978). Since the fast-adherent sheets of epithelium from cultures of different human mammary tissues always contain elongated, myoepithelial-like cells, but the loosely adherent clusters do not, myoepithelial-like cells are usually found in cultures of fibroadenomas and uninvolved peritumoral tissue adjacent to carcinoma (Rudland *et al.* 1985). However, they are almost invariably missing from cultures of metastases, from the cultures of the malignant cell strain Ca2-83, and from cultures of the loosely adherent aggregates of malignant cells of ductal carcinomas (Rudland *et al.* 1985). Moreover, Ca2-83 cells fail to produce casein and alveolar-like cells under the requisite hormonal conditions (Rudland, 1987).

The retention of the differentiating ability of the benign neoplastic cells from human breasts and its loss in human carcinoma cells in culture are facts which are consistent with both the pathology of neoplastic breast disease in humans (pp. 98–99) and the above findings from culturing the equivalent rat mammary tumours. The presence of abnormal organoidal structures of epithelial and myoepithelial-like cells in some of the primary ductal carcinomas and their absence in metastatic tumours (Rudland *et al.* 1985) probably reflects progression of the primary tumour from a less-malignant to a more-malignant phase. As in the rat, these findings in humans are also more likely to be consistent with a mutational event occurring in an epithelial stem cell with gradual truncation of its differentiation pathways during the progressive phase of the disease (Rudland, 1987) than with mutational events occurring simultaneously in the epithelial stem cell and an adjacent nondifferentiating epithelial cell that ultimately gives rise to the malignancy (Taylor-Papadimitriou *et al.* 1983). The loss of differentiating ability of epithelial stem cells to myoepithelial cells in the normal breast seems to be one of the few consistent changes wrought in the malignant breast cancer cell (Rudland, 1987). Thus the novel regulatory elements postulated in the previous section for transcriptional control of some of the events in the process of differentiation to myoepithelial cells may also be relatively

unique in their capacity to become inactivated in the malignant breast cancer cell.

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