Int. J. Exp. Path. (1998), **79,** 193–206

Stem Cell Review

Stem cells in breast epithelia

PETER LI, ROGER BARRACLOUGH, DAVID G. FERNIG, JOHN A. SMITH AND PHILIP S. RUDLAND

Cancer and Polio Research Fund Laboratories, School of Biological Sciences, University of Liverpool, Liverpool, UK

Received for publication 1 December 1997
Accepted for publication 1June 1998

Summary. The rodent and human nonpregnant mammary glands contain epithelial, intermediate and myoepithelial cells which have all been isolated as cell lines *in vitro*. Transforming growth factor-α (TGFα) and basic fibroblast growth factor (bFGF) are produced by myoepithelial cells and can stimulate the growth of intermediate stem cells *in vitro*. Epithelial and intermediate cells behave like stem cells *in vitro*, since they can differentiate into alveolar-like and myoepithelial cells. The myoepithelial differentiation pathway is associated with the early expression of a calcium-binding regulatory protein called p9Ka and the protease, Cathepsin D. Myoepithelial cells are also present in benign lesions but not in malignant mammary carcinomas of rats or humans, whose resultant cell lines fail to differentiate completely along the myoepithelial cell pathway. Loss of the myoepithelial cell in some invasive carcinomas may be compensated, at least in part, by changes in malignant cells. Overexpression of TGFα and/or *erb*B receptors may reduce the requirement for TGFα, whilst ectopic production of bFGF and its receptors and p9Ka/Cathespin D may assist in tumorigenesis and in metastasis, respectively. Thus compensation for, or retention of, molecules potentially involved in the differentiation of mammary cells may be a mechanism by which malignancy progresses in some human invasive carcinomas.

Keywords: mammary development, breast cancer, stem cells

The existence of stem cells in the breast is required to account for the developmental behaviour of the mammary gland. In rodents and humans, the normal mammary gland consists of a branched system of ducts which, prior to puberty, terminate in end-buds and thereafter, in alveolar lobules and finally in alveoli during pregnancy and lactation (Figure 1) (Russo *et al*. 1982).

Correspondence: Dr Peter Li, Cancer and Polio Research Fund Laboratories, School of Biological Sciences, Life Sciences Building, University of Liverpool, Crown Street, Liverpool, L69 7ZB, UK.

These developmental changes in ductal structures necessitate the presence of a stem cell population in order to create the cellular components of each mammary duct. The formation of a lactating gland is repeated upon every successful reproductive cycle, and is also dependent upon stem cells to repopulate the resting mammary gland. Regeneration of a mammary ductal tree capable of secreting milk products can be achieved with any particular ductal segment of the mammary gland, regardless of age and stage of development, when placed within cleared fat pads in rodents (Ormerod

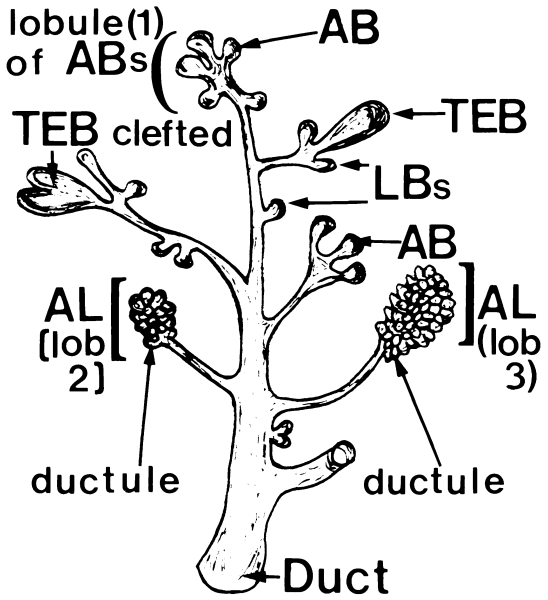

Figure 1. Schematic diagram of the ductular structures in the developing mammary gland. TEB, terminal end-bud; TEB clefted, terminal end bud partially divided by a cleavage furrow at its apex, further cleavage results in alveolar buds; LBs, lateral buds; ABs, alveolar bud; AL, alveolar lobule. Classification of lobules: type 1 (lob 1), synonymous with lobule of ABs; type 2 correspond to ALs containing smaller numbers of larger ductules (lob 2); type 3 are ALs containing larger numbers of smaller ductules (lob 3). Modified from Russo & Russo (1987) and Rudland (1993).

& Rudland 1986; Smith & Medina 1988; Rudland 1991). These transplantation studies suggest the presence of stem cells throughout the mammary gland during the life of an animal.

A relationship between the presence of stem cells and the predisposition to carcinogenesis exists in the mammary gland. The greatest concentration of stem cells are found in end-buds and terminal ducts and it is these ductal structures within the pubertal mammary gland which are more susceptible to tumorigenesis. This association can be demonstrated in rodents with chemicals such as 7, 12-dimethylbenz[a]anthracene and N-nitrosomethylurea (NMU) whose carcinogenic effectiveness decreases after 50 days (puberty), which correlates with the presence of terminal end-buds (TEBs) and terminal ducts (Huggins *et al.* 1961; Dao 1969; Gullino *et al.* 1975; Russo *et al.* 1977). These ductal structures are present in greater numbers in prepubertal and adolescent women (Dawson 1934) and it is this age-group of Japanese victims of the Hiroshima and Nagasaki atomic detonations that were most susceptible to carcinogenesis induced by resulting irradiation (McGregor *et al.* 1977).

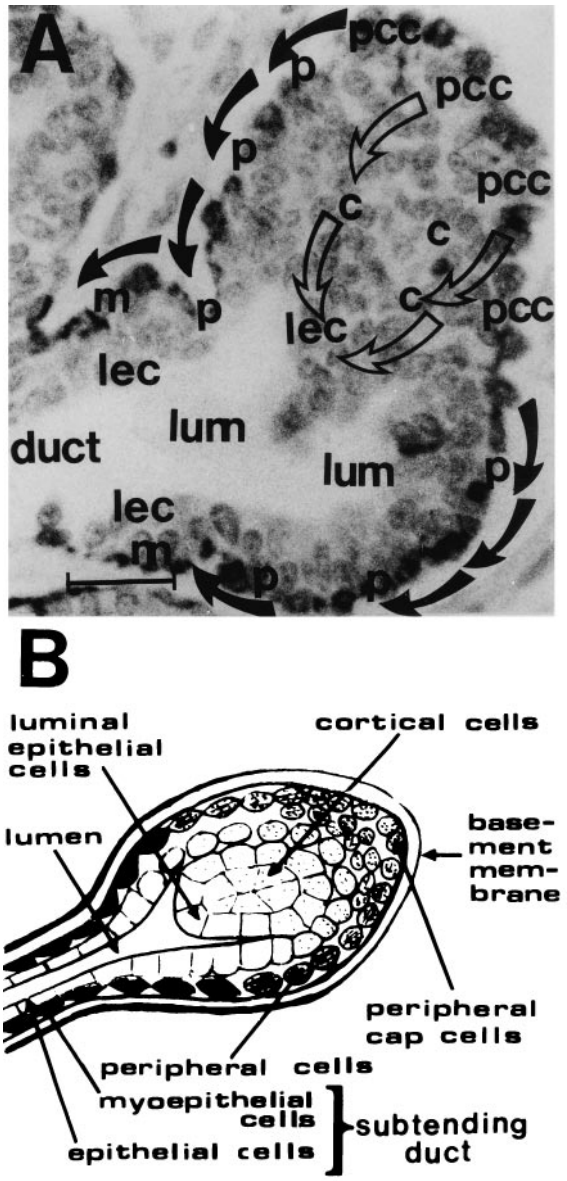

Figure 2. Staining of growing terminal budded structures with myoepithelial-specific markers. A. Section of human terminal end-bud incubated with MAb to smooth-muscle actin. Staining of the peripheral cap cells (pcc) gradually increases the further such cells are positioned from the apical tip (solid arrows). A decrease in staining intensity occurs from the peripheral cap cells (pcc) to the central cells (cc) and is absent in the luminal epithelial cells (lec) (outlined arrows) which line the lumen (lum). Magnification $\times 720$. Bar $= 20\,\mu$m. B. Schematic representation of staining patterns. Actin/myosin antisera moderately stain peripheral cap cells (dotted circles) and this staining increases in peripheral cells (p) which are closer to the subtending duct until they merge with myoepithelial cells (m) of the duct (black ovoids). Central cells are only weakly stained by antisera to actin/myosin (dotted squares). Differential staining for the basement membrane proteins, laminin and Type IV collagen, occurs in the TEB. A thin band of staining is present at the apical tip and becomes thicker around the TEB and the subtending duct. Modified from Rudland (1993).

Table 1. Summary of breast cell lines

Mammary tissue	Cell line	Identity	Differentiate
Normal rat	Rama 704	Epithelial	Yes
	Rama 401	Myoepithelial-like	No
Benign DMBA rat tumour	Rama 25	Epithelial	Yes
	Rama 25-I1, I2, I4	Intermediate	Yes
	Rama 29	Myoepithelial-like	No
	Rama 37	Epithelial	Yes
Weakly metastasizing rat tumour T*R*2 CL	Rama 600	Epithelial	Very incompletely
Moderately metastasizing TMT-081 rat tumour	Rama 800	Anaplastic epithelial	No
Strongly metastasizing rat SMT-2 A	Rama 900	Anaplastic epithelial	No
Normal human immortalized by SV40	SVE3, Huma 7	Epithelial	Yes
	Huma 25 and 62	Myoepithelial-like	No
Benign breast disease, parent HMT-3522	Huma 121, 123	Epithelial	Yes
	Huma 101, 109	Myoepithelial-like	No
Human ductal carcinoma	Ca2–83	Malignant epithelial	No
Metastatic pleural effusion	MCF-7		
	ZR-75	Malignant epithelial	No

Reviewed in detail previously (Rudland 1987, 1993).

Thus stem cells provide a population of cells in the breast with the ability to create a secretory parenchyma during normal mammary development. In addition, neoplastic development of the mammary gland appears to be associated with the greater susceptibility of stem cells to carcinogenesis. This review focuses on the work performed in our laboratories on the biology of stem cells and how control of their growth is linked to the normal and neoplastic development of the mammary gland.

Cellular composition of normal and neoplastic mammary glands

The normal mammary gland is composed of two compartments separated by a basement membrane (Rudland 1993). The branching system of ducts constitutes the mammary parenchyma which is embedded within a mesenchyme containing clusters of adipocytes, permeated by fibroblasts, nerves and blood vessels. During the development of malignant tumours, the density of the blood vessels increases and the basement membrane and the fatty environment are reduced or lost completely (Folkman *et al*. 1989; Aung *et al*. 1993).

The normal mammary parenchyma consists of three main cell types. Epithelial cells form the lining of ducts, and alveoli are lined by the alveolar cells which are responsible for the secretion of milk products, including casein, during lactation. Both cell types are surrounded by a sometimes discontinuous layer of myoepithelial cells situated adjacent to the basement membrane (Ozzello 1971). Epithelial, myoepithelial and, if the animal or patient is pregnant, alveolar cells are present in benign hyperplastic and neoplastic lesions in the mammary gland. However, only epithelial cells are present in malignant carcinomas of rodents and humans; no myoepithelial or secretory alveolar cells are present even if the subject is pregnant (Rudland 1987, 1993).

Histochemical and immunocytochemical reagents can identify the main cell types in normal and neoplastic mammary tissue (Rudland 1993). Furthermore, these reagents have immunolocalized subsets of epithelial and myoepithelial cells, and cells intermediate between these cell types which occur particularly in growing end-bud structures (Figure 2) (Rudland 1991). Cap cells line the apex of the end-bud and are stained by both epithelial- and myoepithelial- specific markers. Cytochemical staining with these markers diverge from cap cells towards cortical cells for epithelial-specific markers and towards peripheral cells for myoepithelial-specific markers. This staining pattern suggests that cap cells in the end-bud contains stem cells which can differentiate into epithelial or myoepithelial cells. Similar properties are found in single-cell-cloned epithelial cell lines, such as Rama 25, 37 and 704, derived from normal and benign lesions of rat and human glands which differentiate, in a stepwise manner, to myoepithelial-like and secretory alveolar-like cells in culture. Cell lines of the intermediate cells along the myoepithelial pathway have also been isolated (Rudland 1987, 1993). In contrast, rat and

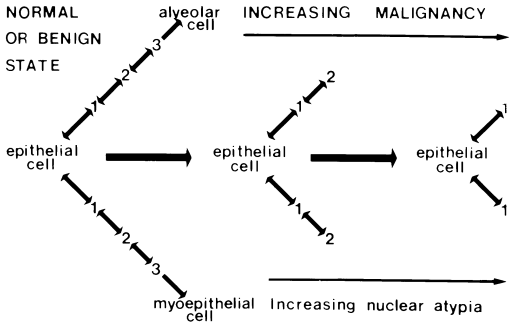

Figure 3. A cellular model for the normal and neoplastic development of the mammary gland. Normal epithelial cells in terminal ductal structures can differentiate into myoepithelial cells and secretory alveolar cells via a series of cellular intermediates represented by numerals (Rudland 1987). While retaining the ability to differentiate into myoepithelial and alveolar cells, such epithelial and closely-related intermediate cells can give rise to benign hyperplastic and neoplastic lesions. Further injury to epithelial/intermediate cells leads to a decreasing ability to differentiate into other cell types resulting in cellular malignancy (Rudland 1993).

human breast carcinomas fail to produce single-cell-cloned epithelial cell lines that differentiate to a myoepithelial or alveolar cell phenotype in culture (Figure 3), and this result correlates with the disappearance of the myoepithelium and the inability to give rise to alveolar cells in breast carcinomas. The absence of the myoepithelial cell layer represents the loss of the growth-stimulatory environment produced by these cells which is normally additional to that provided by the basement membrane and fatty stroma. The intercellular signals regulating growth of the three main parenchymal types are now considered using the cell lines shown in Table 1.

Hormonal agents and micronutrients

The growth of the mammary gland is regulated by the systemic levels of mammatrophic hormones. Estrogen promotes growth of ducts whereas progesterone stimulates lobuloalveolar development, and prolactin induces the differentiation of alveoli (Lyons 1958; Nandi 1958). Mammary development *in vivo* also requires hydrocortisone and insulin which are thought to synergise with the three primary mammatrophic hormones (Figure 4) (Topper & Freeman 1980). In addition, the stromal compartment of the mammary gland produces local trophic factors which are involved in the regulation of mammary development, since rodent parenchymal tissues and cells will grow when transplanted into the mammary fat pad, but not when transplanted subcutaneously (DeOme *et al.* 1959).

The malignancy of tumours in the mammary gland is inversely related to their requirement of systemic hormones for growth. Benign tumours, e.g. the carcinogen-induced tumours of rats (Huggins *et al.* 1961) and the spontaneously arising benign tumours of humans (Hinton *et al.* 1986), are structurally similar to the normal mammary gland and require mammatrophic hormones to grow, as do some (low-grade) malignant tumours of rats (Williams *et al.* 1985) and humans. These mammary cancers are classified as hormone-dependent tumours and comprise 25–30% of all human carcinomas (Vorherr 1980). The remaining 70–75% of human malignant tumours no longer require mammatrophic hormones for growth and are therefore known as hormone-independent. Furthermore, the fat pad is required for growth of hyperplastic rodent lesions but not for neoplastic growth of lesions (DeOme *et al.* 1959), which suggests that changes in both local and systemic growth control occur in the development of breast carcinoma.

The general lack of growth-stimulatory effects by mammatrophic hormones *in vitro* led to the identification of agents which stimulate DNA synthesis and division directly in mammary cells (Sirbasku 1978). A number of growth-promoting agents have been discovered in culture studies with defined media which may be classified as essential nutrients. One of the most important cellular micronutrients is iron and the iron-binding protein, transferrin, is essential for the growth of many cells in defined culture medium including our mammary cell lines (Rudland *et al.* 1977). In addition to transferrin, haemoglobin and ferritin promote growth of the Rama 37 mammary epithelial cell line (Wilkinson *et al.* 1998) which suggests that the requirement of iron may be an artefact of the culture system. However, transferrin constitutes 2% of the total cytosolic protein in pregnant and lactating mammary tissue (Keon & Keenan 1993) which also contains proliferating cells that possess a high number of transferrin receptors (Walker & Day 1986). Transferrin and its mRNA are also present within the developing rat mammary gland. These reports suggest that transferrin can act like a growth factor, either in an autocrine or paracrine manner if the protein is produced locally, and/or in an endocrine manner if levels within the circulation vary considerably.

MCF-7 breast carcinoma cells secrete transferrin in culture and its secretion is stimulated by oestrogen and reduced by tamoxifen (Vandewalle *et al.* 1989). Cell lines from malignant breast tumours contain a higher number of transferrin-receptors per cell compared to those which are derived from nonmalignant sources (Table 2). Medium containing oestrogen results in higher numbers

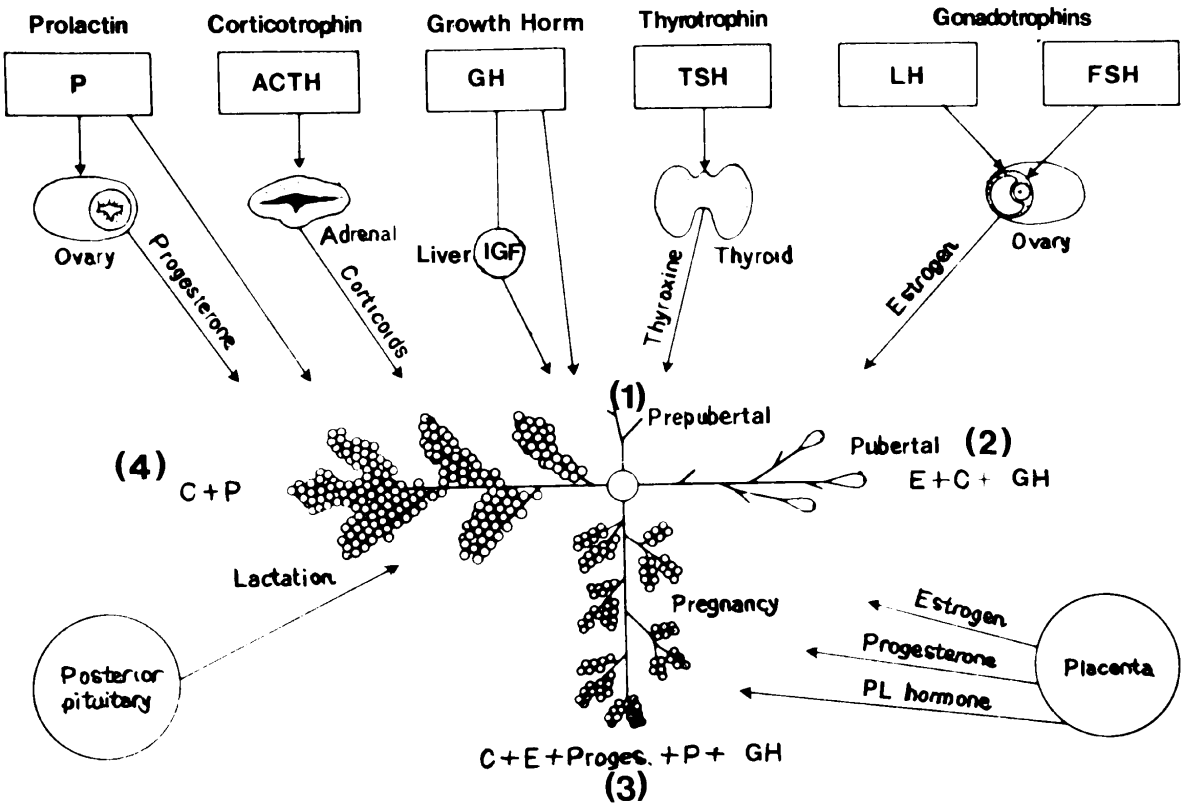

Figure 4. Involvement of hormones at different stages of mammary development. 1. Prepubertal gland. 2. Pubertal gland. 3. Pregnant gland. 4. Lactation. Modified from Lyons (1958).

of transferrin-receptors for the MCF-7 breast cancer cell line, but not when they are cultured in medium antagonistic to oestrogen action. Furthermore, 71% of invasive breast carcinomas contain cells which overexpress transferrin-receptors. Thus, the growth of breast carcinomas may, at least in part, be enhanced by a hormonally controlled autocrine loop involving the overexpression of transferrin and its receptor.

Table 2. Binding of transferrin to breast cell lines

Cell line	Kd (nM)	No. of receptors per cell
SVE3 normal epithelial	44±28	41±14 k
MCF-7 malignant epithelial, oestrogen-free	120±70	350±120 k
MCF-7 malignant epithelial, plus oestrogen	200±120	810±160 k

The Kd and number of receptors were calculated from the data pooled from at least two independent binding experiments and presented as the mean±standard error. The binding of [^{125}I] diferric transferrin was performed and analysed as described previously (Fernig *et al.* 1990, 1992).

Mammary growth-stimulating activity in pituitary glands

Prolactin, like oestrogen and progesterone, has little effect on the growth of cultured mammary cells. However, pituitary extracts are mitogenic for many cells (Sirbasku *et al.* 1982) including the Rama cell lines. Pituitary-derived mammary growth factor (PMGF) stimulates growth of Rama 25 epithelial but not the Rama 29 myoepithelial-like cells. Although it has only been partially purified, PMGF is distinguishable from bovine prolactin, growth hormone and other known growth factors which occur in the pituitary gland (Smith *et al.* 1984; Wilkinson *et al.* 1998). The activity of PMGF is higher from the pituitary glands of lactating or perphenazine-treated rats than in untreated virgin females which suggests that the growth factor has a role to play in mammary development *in vivo* (Smith *et al.* 1984). Despite both originating from the pituitary gland and probably under similar hypothalamic control, prolactin stimulates the production of casein in alveolar-like cells as observed in confluent epithelial cultures (Warburton *et al.* 1983). Thus, in respect to function, prolactin induces

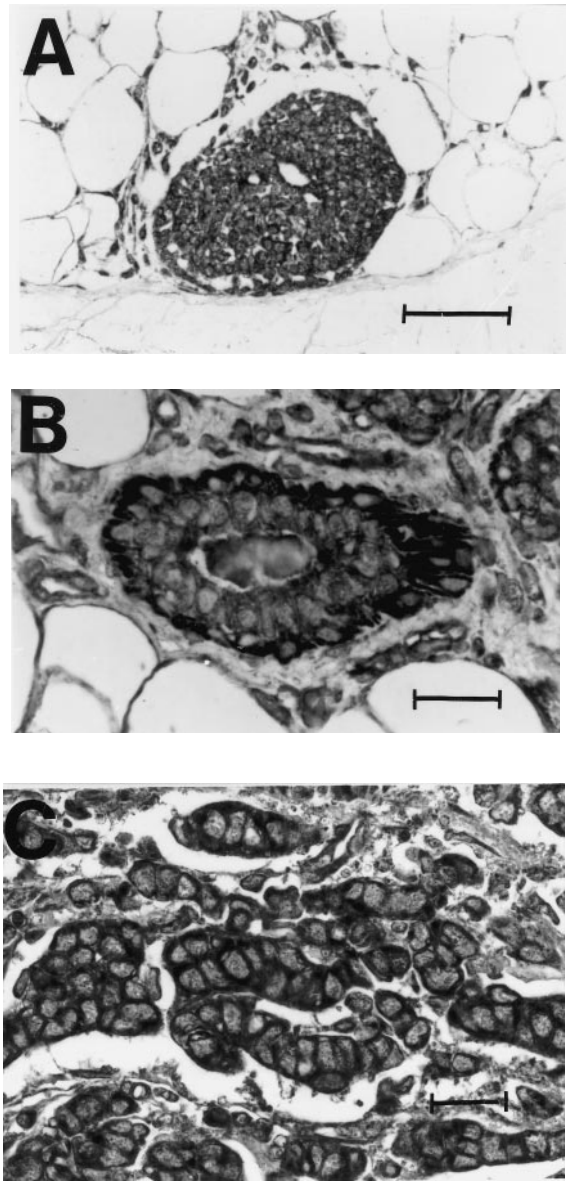

Figure 5. Immunocytochemical staining of mammary tissues by antisera to TGFα. A. Cross-section of a terminal end-bud from a 50-day old rat showing moderate to strong staining of parenchymal cells Magnification $\times$ 210. Bar = 75 μm. B. Strong staining of outer myoepithelial cells but little or no staining of the inner epithelial cells of a duct from a 50-day old rat. Magnification $\times$ 410. Bar = 25 μm. C. Human invasive ductal carcinoma showing intensive staining of the carcinoma cells. Magnification $\times$ 410. Bar = 25 μm.

differentiation of epithelial cells rather than proliferation which is stimulated by PMGF.

A growth response to PMGF is stimulated in epithelial cells in culture derived from benign tumours which regress *in vivo* after administration of antipituitary agents (Segaloff 1978; Hinton *et al*. 1986). However, hypophysectomy of rats or humans does not cause the regression of advanced tumours (Kim 1979; Vorherr 1980) nor does PMGF promote growth of rat (Rama 800, Rama 900) or human (Ca2–83) breast carcinoma cell lines which are highly malignant in nature. PMGF can still stimulate weakly metastasizing rat cell lines (Rama 600) (Smith *et al*. 1984) and hormone (including pituitary-ablative) therapy can aid about a quarter of all human breast cancers. The loss of differentiative ability and failure to respond to pituitary-derived growth factors are steps in the progression of the normal cell towards malignancy.

Growth factors of the epidermal growth factor family and their receptors

Transforming Growth Factor-α (TGFα) is the most abundant growth factor of the epidermal growth factor (EGF) family in the mammary gland. Myoepithelial-like (Rama 29) and, to a lesser extent, epithelial (Rama 25) cells in culture secrete mature, bioactive TGFα which has a molecular weight of 5616 D and is also found in the rat mammary gland *in vivo* (Figure 5A,B) (Smith *et al*. 1989). This is in agreement with immunofluorescent studies using antisera raised to TGFα which stained Rama 29 myoepithelial-like cells more intensely than Rama 25 epithelial cells. Cytochemical staining of immunoreactive (ir)-TGFα was also strongly associated with the myoepithelium but only weakly in the epithelial cells in the developing rat mammary gland. The intermediate cells within growing end-buds are stained at an intermediate level (Figure 5A,B). Alveolar secretions are also stained strongly for TGFα in lactating glands. However, the effects of TGFα (Smith *et al*. 1989), like those of EGF (Smith *et al*. 1984) are not restricted to a specific mammary cell type since TGFα stimulates the growth of fibroblastic, myoepithelial-like and epithelial cell lines from normal rat mammary glands and benign tumours. A paracrine or an autocrine role for TGFα in the mammary gland is suggested by the observation that the levels of the growth factor secreted by Rama 29 myoepithelial-like cells are sufficient to stimulate growth of the myoepithelial-like cells themselves, or of epithelial or stromal cell lines (McAndrew *et al*. 1994a).

Western blotting of ir-TGFα in extracts of rat mammary tissue and cell lines reveals that it is a protein with a molecular weight of 50 kD whose levels are higher than would be expected from the amount of bioactive TGFα detected in the same extracts. TGFα mRNA is found in the Rama 27 mammary fibroblast cells which are also stained by TGFα antiserum as are rat mammary

fibroblasts *in vivo* (McAndrew *et al*. 1994b). However, bioactive TGFα is not secreted by Rama 27 cells. These results indicate that growth control of the mammary gland by TGFα is complex in nature.

TGFα binds to the same receptor as EGF. Our rat mammary epithelial, myoepithelial-like and fibroblastic cell lines possess high-affinity EGF receptors (EGFR) with dissociation constants ranging from 0.4 nM to 1.3 mM for TGFα (Smith *et al*. 1989; Fernig *et al*. 1990). No alteration in EGFR numbers occurs when epithelial cells differentiate along the myoepithelial-like pathway *in vitro* (Fernig *et al*. 1990). This concurs with the view that locally produced TGFα may promote the proliferation of epithelial, intermediate and myoepithelial cell types *in vivo*.

Overexpression of TGFα and its receptor, EGFR, may be involved in the abnormal growth of malignant breast cells. This notion is supported by the detection of ir-TGFα in 30–70% of human breast carcinomas (Figure 5C) (Lundy *et al*. 1991; Umekita *et al*. 1992; McAndrew *et al*. 1994a) and that transgenic mice overexpressing TGFα are more prone to mammary cancers (Sandren *et al*. 1995). In a similar fashion to its counterpart in normal mammary cells, ir-TGFα in breast carcinomas appears as a 50 kD protein and its relationship to the mature, bioactive TGFα is unknown (McAndrew *et al*. 1994a). However, a rapidly growing tumour, due to an uncontrolled TGFα/EGFR autocrine loop, such as that found in squamous carcinomas of the head and neck (Cowley *et al*. 1984), is not found in breast carcinomas. These breast tumours are relatively slow growing and still under a degree of hormonal control during the early stages of malignancy. These observations suggest that the progression to an oestrogen-refractory state is more gradual in the development of a breast carcinoma in which changes in the TGFα/EGFR axis may play a vital contribution. The c-*erb*B-2 protooncogene encodes a 185 kD tyrosine kinase which is structurally related to the EGFR. The c-*erb*B-2 receptor is expressed in 20–30% of invasive breast carcinomas (Slamon *et al*. 1987; McCann *et al*. 1989; Winstanley *et al*. 1991) of which there is an inverse correlation between patient survival and c-*erb*B-2 expression, particularly in patients with no involved lymph nodes (Slamon *et al*. 1987; Winstanley *et al*. 1991). However, c-*erb*B-2 receptors have been immunolocalized on epithelial and alveolar cells during caprine mammary development and these have been suggested to be a potential population of stem cells (Li *et al*. 1998), whilst transfection of the rat equivalent of the c-*erb*B-2 gene, *neu*, enabled normal rodent mammary epithelial cells to secrete casein the presence of prolactin (Marte *et al*. 1995). Such reports suggest that c-*erb*B-2 receptor may play a role in the development and differentiation of the mammary gland as well as in breast neoplasia.

Fibroblast growth factors in normal mammary development

The Fibroblast Growth Factors (FGFs) form a family of growth factors whose effects are mediated by two types of receptors on mammary cells. High-affinity tyrosine kinases are ultimately responsible for generating the primary intracellular signals, whilst low-affinity heparan sulphate proteoglycans (HSPGs) are a type of glycosaminoglycan which can store and activate FGFs to enable binding to the high-affinity receptor (Fernig & Gallagher 1994). In our cell lines, bFGF and the high- (Kd 30–280 pM; approximately 2×10^4 per cell) and low-affinity (Kd 1–20 nM; approximately 3×10^6 per cell) receptors for the growth factor are produced by the myoepithelial-like cells and, to a lesser extent, the intermediate cells. Epithelial cells do not synthesize either type of receptor nor the growth factor itself (Fernig *et al*. 1990, 1993; Barraclough *et al*. 1990; Ke *et al*. 1993). Both cell lines produce amounts of bFGF (6–7 ng/10^6 cells) which are sufficient to stimulate the growth of myoepithelial-like and intermediate cell lines (Smith *et al*. 1984) and this result suggests the possibility of autocrine/paracrine control of mammary cell growth *in vivo*. It should be noted that between one-half and two-thirds of the HSPG low-affinity receptors for bFGF are associated with the basement membrane/extracellular matrix *in vitro* (Fernig *et al*. 1992) (Figure 6) and this has been confirmed by immunocytochemistry (Rudland *et al*. 1993). In resting mammary glands, bFGF is associated with the myoepithelial cells and the basement membrane, whereas in growing glands, bFGF is localized to individual cells, in particular, the intermediate stem cells of the end-bud (Figure 6). However, the presence of the bFGF HSPG receptors enhances the staining of the basement membrane/myoepithelial cell when bFGF antisera is preincubated with native bFGF, and then used for immunocytochemical staining of mammary tissue (Rudland *et al*. 1993).

Ectopic expression of FGFs in malignant tumours

Rodent and human mammary epithelial cell lines derived from malignant tumours possess receptors for bFGF. These are high-affinity, cell surface receptors which have a Kd in the range of those found on normal myoepithelial-like cells (Fernig *et al*. 1990, 1992, 1993; Ke *et al*. 1993). Neither rat nor human mammary epithelial cells derived from the normal mammary gland or benign tumours produce detectable levels of FGF receptors. Therefore

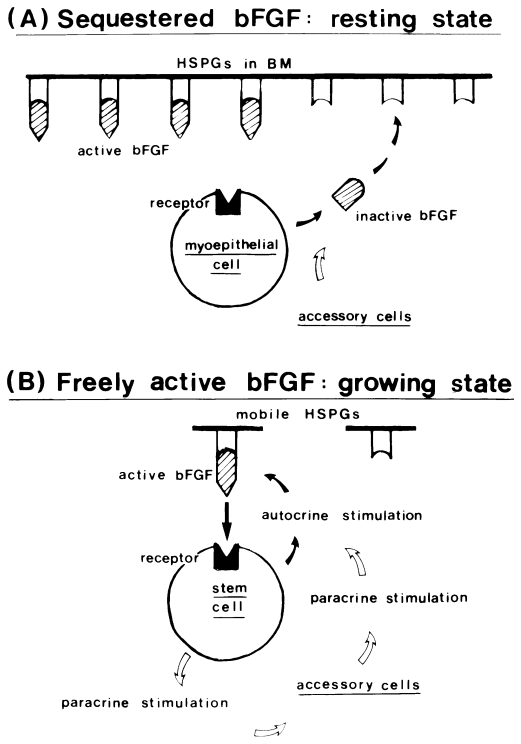

Figure 6. Location of bFGF in mammary tissue. A. In resting glands, bFGF is sequestered by heparan sulphate proteoglycans (HSPGs) which are mainly found in the basement membrane adjacent to the myoepithelial cells. Activated bFGF is therefore found in a separate compartment to its cell surface receptors. B. HSPGs in growing tissues, e.g. TEBS and carcinomas, are reduced in amount and not immobilized in the basement membrane. Thus the necessary activation of bFGF prior to binding of its high-affinity tyrosine kinase receptor can take place at the cell surface. The release of activated bFGF can cause autocrine effects by self-stimulation of growth and paracrine stimulation of adjacent cells, e.g. blood vessels in a carcinoma.

the expression of high-affinity receptors by malignant mammary epithelial cells *in vivo* is ectopic.

bFGF is a potent stimulator of malignant mammary epithelial cell proliferation (Peyrat *et al.* 1992; Karey & Sirbasku 1988; Stewart *et al.* 1990), despite the fact that only low levels of low-affinity HSPGs for bFGF are found on these cells. These results suggest that the low levels of HSPGs produced by the malignant cells, at least in the rodent, can activate bFGF (Chen *et al.* 1995). This would indicate that the dual receptor system for FGFs (Fernig & Gallagher 1994) is fully functional in malignant cells despite the apparent loss of most of the HSPG low-affinity receptors (Figure 6). These results have been confirmed on pilot studies of human breast specimens (Takahashi *et al.* 1989; Peyrat *et al.* 1992).

A bFGF growth-stimulatory activity is found in one in four human mammary carcinoma cell lines as well as in extracts of rat mammary carcinoma (Luqmani *et al.* 1992; Fernig *et al.* 1993; Anandappa *et al.* 1993). Expression of bFGF mRNA has also been detected in human breast carcinomas but the levels are lower compared to those found in normal and benign mammary tissues (Luqmani *et al.* 1992; Anandappa *et al.* 1993). These results correlate with the loss of myoepithelial cells in invasive carcinomas (Rudland 1993), since it is these cells which produce bFGF in cell lines derived from normal and benign mammary tissues. However, 25% of malignant tumours express bFGF mRNA at levels which are equivalent or higher than those in benign tissues (Anandappa *et al.* 1993). Isolation of bFGF from malignant breast tumours (Rowe *et al.* 1986) suggests that expression of bFGF mRNA by human carcinomas results in the synthesis of active bFGF polypeptides (Figure 6).

Protein markers of differentiation to myoepithelial cells

Changes in the expression of proteins mark discrete cellular stages of the differentiation pathway of our rat mammary epithelial stem cell lines to myoepithelial-like cells in culture (Paterson & Rudland 1985). A calcium-binding regulatory protein, p9Ka (Barraclough *et al.* 1987), also known as S100 A4 (Schafer *et al.* 1995) increases relatively early, keratin 14 increases in the middle stages and Thy-1 increases later on (Paterson & Rudland 1985). In contrast, the basement membrane proteins, laminin and Type IV collagen, increase relatively evenly throughout the differentiative pathway from an epithelial to a myoepithelial-like cell (Rudland *et al.* 1986). The stages of synthesis at which the production of the proteins are controlled varies with each protein. p9Ka and Thy-1 appears to be regulated at the mRNA level (Barraclough *et al.* 1984, 1987), whilst both laminin and Type IV collagen are regulated post-transcriptionally (Rudland *et al.* 1986). These changes in protein expression correlate with the levels of the marker proteins observed by immunocytochemistry in the end-buds of growing rats (Rudland 1987) and in human intermediate cell types *in vitro and in vivo* (Aung *et al.* 1993; Rudland *et al.* 1998). The protease, Cathepsin D, and the extracellular protein, hyaluronectin (HN), are also early marker proteins of myoepithelial cells which can be observed at lower levels in intermediate cells in end-buds but not in epithelial cells *in vivo* (Table 3).

The role of p9Ka has been investigated further since it was one of the first proteins detected during the differentiation of an epithelial to a myoepithelial cell (Paterson & Rudland 1985). When p9Ka genomic DNA was

Table 3. Summary of myoepithelial-asoociated proteins in TEBs and human carcinomas

Marker protein	Myoepithelial	TEB	Epithelial	Carcinoma
p9Ka	++f	+c	–	+c
Cathepsin D	++c	+c	–	+c
HN	++f	+c	–	+c
Keratin 14	++c	±c	–	±c
Laminin	++f	+c,f	–	+c,f
Type IV collagen	++f	+c,f	–	+c,f

The presence of myoepithelial-related proteins in other mammary cell types *in vivo* in either the cytoplasm (c) or in filaments (f) using immunocytochemical detection methods is shown. Key: ++, +, ± and -; present in 75–100%, 25–75%; 5–25% and < 5% of cells or carcinomas, respectively (Aung *et al.* 1993; Barraclough *et al.* 1998; Winstanley *et al.* 1993). Immunocytochemical data for hyaluronectin (HN) from P. S. Rudland and D. West (unpublished).

transfected into rat mammary epithelial cells, intermediate/myoepithelial cell colonies were produced at a higher frequency than in controls (Table 4). Antisera raised against rat p9Ka immunolocalized to the cellular cytoskeleton of cultured rat myoepithelial cells (Figure 7A,B) (Davies *et al.* 1993) and scrape-loading of recombinant p9Ka into epithelial cells caused similar changes in the cytoskeleton within 48 h (Figure 7C). These results suggest that p9Ka may play a role in the initiation of differentiation to a myoepithelial cell phenotype by binding to the cellular cytoskeleton. This proposed function correlates with the site of action on the cytoskeleton, however, the precise binding target of p9Ka has been controversial with reports suggesting binding of p9Ka to actin (Watanabe *et al.* 1993), tropomyosin (Takenaga *et al.* 1994) and nonmuscle myosin (Kriajeska *et al.* 1994; Ford & Zain 1995). Although p9Ka occurs cytoplasmically in intermediate cells within end-buds in growing glands (Figure 7D), the protein has been associated with filamentous material adjacent to the myoepithelial cells (Figure 7E). How the intracellular and extracellular forms of p9Ka are related is not known but variant forms of the protein detected in different subcellular fractions (Barraclough & Rudland 1994) may explain its unusual topographical distribution in mammary cells.

Table 4. Effect of DNA transfection of p9Ka on cultured rat epithelial cells

Transfecting DNA	Fraction of cell colonies (%)		
	Epithelial	Intermediate	Myoepithelial-like
None	244/250 (97%)	4/250 (2%)	2/250 (1%)
Vector alone	64/72 (89%)	5/72 (7%)	3/72 (4%)
Ha-*ras*-1 cDNA	99/120 (82%)	12/120 (10%)	9/120 (8%)
p9Ka cDNA	5/72 (7%)	20/72 (28%)	47/72 (65%)

Rat mammary epithelial cells were scored for epithelial, intermediate or more-elongated, myoepithelial-like cell colonies (Paterson & Rudland 1985) after DNA transfection with Ha-*ras*-1 cDNA or rat p9Ka cDNA coupled to the selectable vector pSV2*neo*, as described previously (Davies *et al.* 1993).

Appearance of intermediate markers of the myoepithelial pathway in carcinomas

The loss of the myoepithelial cell in advanced malignant carcinomas of rats and human should preclude the expression of protein markers of the myoepithelial pathway as has been observed for smooth-muscle actin and myosin (Gusterson *et al.* 1982; Dunnington *et al.* 1984). However, other myoepithelial markers are still present in tumours which may represent a limited ability of the carcinoma cells to differentiate along this pathway, such that 39/42% of breast carcinomas express laminin/Type IV collagen, 14% express keratin 14 and 60% of breast carcinomas express p9Ka (Figure 7F) (Barraclough *et al.* 1998). Cathepsin D and hyaluronectin, which occur early in the differentiation of the myoepithelial cell, are observed in 50–60% of breast carcinomas (Winstanley *et al.* 1993) (Table 3). Benign breast disease and benign tumours possess all marker proteins characteristic of the myoepithelial cell (Table 3) (Barraclough *et al.* 1998; Aung *et al.* 1993; Winstanley *et al.* 1993). These markers can also be detected in some human breast carcinoma cell lines (unpublished results) which suggests that malignant carcinoma cells retain some characteristics of myoepithelial cells.

Conclusion

The normal mammary gland consists of three parenchymal cell types which arise from a population of epithelial stem cells in end-buds of growing glands. These cells are intermediate in morphology and cytochemical staining between epithelial and myoepithelial cells, and correspond to the intermediate epithelial stem cell lines in culture (Rudland 1993). Stem cells and cells in the parenchyma and mesenchyme require systemic and local factors to proliferate during mammary growth. The majority of invasive carcinomas (70–75%) lose this requirement for systemic ovarian, adrenal and pituitary hormones. Such hormones may stimulate cell growth indirectly, via intermediary growth factors, e.g. transferrin, TGFα and/or IGFs (Westley *et al.* 1998). Overexpression of these growth factors and/or their receptors would confer autonomous growth to epithelial stem and malignant cells, and discharge the dependence upon hormones for proliferation. This may give rise to hormone-independent breast cancers and could

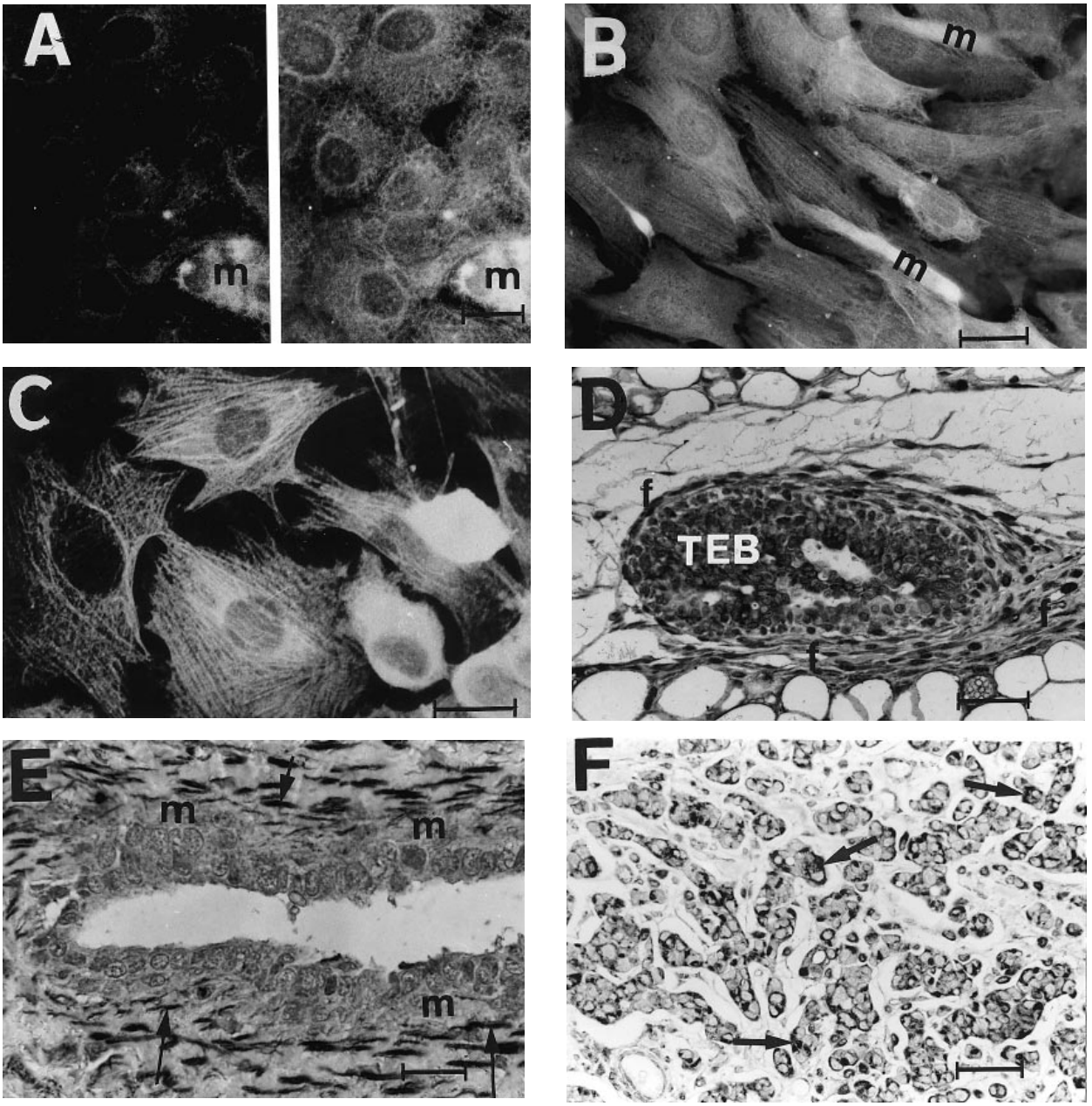

Figure 7. Immunolocalization of p9Ka in mammary cells and tissues. Rat epithelial cells are only weakly immunofluorescently stained by p9Ka antisera except when they are differentiating to intermediate/myoepithelial-like cells (m) in culture. Inset: overexposure of same image to show weak intracellular staining of circumferentially arranged filaments in epithelial cells. B. Immunofluorescent staining of rat myoepithelial-like cells in culture showing longitudinal arrays of filaments corresponding to the cellular cytoskeleton; mitotic cells (m) are stained more intensely. C. Immunofluorescent staining of scrape-loaded rat epithelial cells after 48 h. D. Immunocytochemical staining of a terminal end-bud (TEB) in a 21-day old rat showing cytoplasmic staining of parenchymal cells. Fibroblasts around the TEB are also stained (f). E. Extracellular filamentous arrays adjacent to the myoepithelial cells are immunocytochemically stained in the rat mammary gland (arrows). F. Human invasive ductal carcinoma showing cytoplasmic staining of the cells (arrows). Magnifications: $\times$520 for A, B and E; $\times$620 for C and $\times$210 for D and F. Bars = 20 μm for A, B, C and E and 50 μm for D and F, respectively.

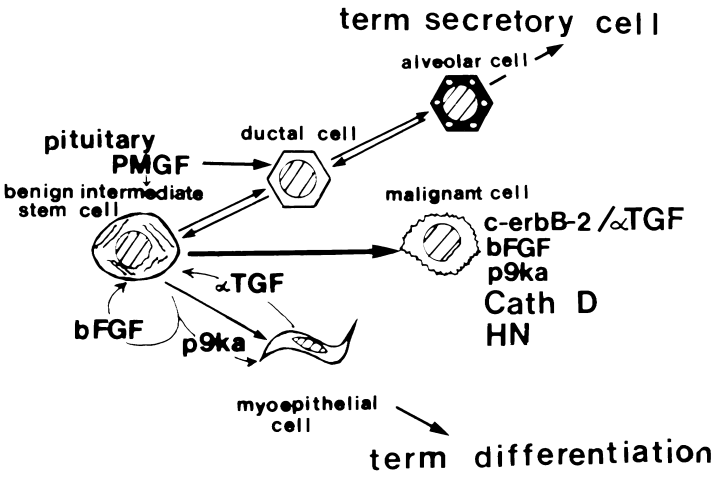

Figure 8. A potential molecular model for mammary development. Stem cells in the normal mammary gland and in benign lesions can differentiate to ductal epithelial and then to alveolar-like cells or to myoepithelial-like cells. Fully differentiated secretory alveolar and myoepithelial cells are terminally differentiated (term) being unable to proliferate. The differentiative capacity of the stem cells is largely lost in the malignant state, especially during the terminal phases. The absences of products of myoepithelial cells in malignant tumours is compensated for by their ectopic production and/or production of related molecules in some rat and human mammary carcinomas.

be caused by partial differentiation of the malignant cell along the myoepithelial pathway, at least for the TGFα/EGFR autocrine loop. Autonomous growth and hormonal independence can also be imparted by the overexpression of the EGFR-related c-*erb*B-2 which is true of 20% of breast carcinomas expressing this receptor (Rudland *et al*. 1998). Differentiation of epithelial cells along the myoepithelial pathway may also lead to a decline in the responsiveness to the growth stimulatory ability of PMGF and lead to pituitary-independent growth. Malignant cells which retain certain characteristics of this pathway may account for the inability of carcinoma cells to respond to hormonal growth stimuli (Figure 8).

Certain characteristics are shared by both intermediate stem cells and malignant cells such as the possession of FGF-Rs which enable stimulation of their growth by bFGF. Carcinoma and intermediate stem cells have a much lower number of HSPGs in the basement membrane/extracellular matrix in endbuds and in invasive carcinomas. bFGF produced by cells within these structures are free to act in an autocrine fashion by stimulating intermediate and carcinoma cells to grow or in a paracrine manner through neovascularization and the influx of circulating hormones and growth factors (Figure 6).

Autocrine and paracrine loops involving growth factors and their receptors can stimulate cell growth in the mammary gland. Downregulation of growth factor-mediated cell growth may be caused by the differentiation of the epithelial/stem cell to a secretory alveolar or myoepithelial cell both of which are nonproliferating in phenotype (Joshi *et al*. 1986), as well as by sequestration of certain growth factors through HSPGs (Fernig & Gallagher 1994). The inability of the malignant cell to produce a terminally differentiated secretory cell or myoepithelial cell may contribute to its tendency towards uncontrolled cell growth *in vivo* (Figure 8).

Acknowledgements

This work was supported by grants from the Cancer and Polio Research Fund, The North-West Cancer Research Fund, and The Cancer Research Campaign.

References

Anandappa S.Y., Winstanley J.H.R., Leinster S., Green B., Rudland P.S. & Barraclough R. (1993) Comparative expression of fibroblast growth factor messenger RNAs in benign and malignant breast disease. *Br. J. Cancer* **69,** 772–776.

Aung W., Zakhour H.D., Platt-Higgins A. & Rudland P.S. (1993) Immunohistochemical detection of cytokeratin 14, Type IV collagen and laminin in 100 cases of invasive ductal carcinoma; a correlation with histological grade. *The Breast* **2,** 165–170.

Barraclough R., Kimbell R. & Rudland P.S. (1984) Increased abundance of normal cell mRNA sequence accompanies the conversion of rat mammary cuboidal epithelial cells to elongated myoepithelial-like cells in culture. *Nucl. Acid Res.* **21,** 8097–8114.

Barraclough R., Kimbell R. & Rudland P.S. (1987) Differential control. of mRNA levels for Thy-1 antigen and laminin in rat mammary epithelial and myoepithelial-like cells. *J. Cell. Physiol.* **131,** 393–401.

Barraclough R., Fernig D.G., Rudland P.S. & Smith J.A. (1990) Synthesis of basic fibroblast growth factor upon differentiation of rat mammary epithelial to myoepithelial-like cells in culture. *J. Cell Physiol.* **144,** 333–344.

Barraclough R. & Rudland P.S. (1994) The S-100-related

calcium-binding protein, p9Ka, and metastasis in rodent and human mammary cells. *Eur. J. Cancer* **30A,** 1570–1576.

Barraclough R., Chen H.J., Davies B.R., Davies M.P.A., Ke Y., Lloyd B.H, & Oates A. (1998) The use of DNA transfer in the induction of metastasis in experimental mammary systems. In *Mammary Development and Cancer.* Eds. P. S. Rudland, D.G. Fernig & S. Leinster, S. Portland Press: London. pp. 273–294.

Chen H.-L., Rudland P.S., Smith J.A. & Ferme D.E. (1995) Activation of task fibroblast growth factor (bFGF) by heparin sulphate (HS) In: *Intercellular Signalling in the Mammary Gland.* Eds. C. J. Wilde, M. Peaker & C. H. Knight. New York: Plenum Press. pp. 73–74.

Cowley G., Gusterson B.A., Smith J.A., Headler F. & Ozanne B. (1984) In *Cancer Cells, the Transformed Phenotype.* New York: Cold Spring Harbor Labs. pp. 5–10.

Dao T.L. (1969) Mammary cancer induction by 7, 12-dimethylbenz[a]anthracene: Relation to age. *Science* **165,** 810–811.

Davies B.R., Davies M.P.A., Gibbs F.E.M., Barraclough R. & Rudland P.S. (1993) Induction of the metastatic phenotype by transfection of a benign rat mammary epithelial-cell line with the gene for p9Ka, a rat calcium-binding protein, but not with the oncogene EJ-RAS-1. *Oncogene* **8,** 999–1008.

Dawson E.K. (1934) A histological study of the normal mamma in relation to tumour growth. I. Early development to maturity. *Edinburgh Med. J.* **41,** 653–682.

DeOme K.B., Faulkin L.J., Bern H.A. & Blair P.B. (1959) Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of C3H mice. *Cancer Res.* **19,** 515–520.

Dunnington D.J., Kim U., Hughes C.M., Monaghan P. & Rudland P.S. (1984) Lack of production of myoepithelial variants by cloned epithelial cell lines derived from the TMT-081 metastasizing rat mammary tumour. *Cancer Res.* **44,** 5338–5346.

Fernig D.G., Smith J.A. & Rudland P.S. (1990) Appearance of basic fibroblast growth factor receptors upon differentiation of rat mammary epithelial to myoepithelial-like cells in culture. *J. Cell Physiol.* **142,** 108–116.

Fernig D.G., Rudland P.S. & Smith J.A. (1992) Modulation of bFGF action by low-affinity receptors in rat mammary myoepithelial-like cells in culture. *Growth Factors* **7,** 27–39.

Fernig D.G., Barraclough R., Ke Y.Q., Wilkinson M.C., Rudland P.S. & Smith J.A. (1993) Ectopic production of heparin-binding growth factors and receptors for basic fibroblast growth factor by rat mammary epithelial cell lines derived from malignant metastatic tumors. *Int. J. Cancer* **54,** 629–635.

Fernig D.G. & Gallagher J.T. (1994) Fibroblast growth factors and their receptors: an information network controlling tissue growth, morphogenesis and repair. *Prog. Growth Factor Res.* **5,** 353–377.

Folkman J., Watson K., Ingber D. & Hanahan D. (1989) Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature* **339,** 58–61.

Ford H. & Zain S. (1995) Interaction of metastasis associated mts-1 protein with non-muscle myosin. *Oncogene* **10,** 1597–1605.

Gullino P.M., Pettigrew H.W. & Grantham F.H. (1975) N-nitrosomethylurea as mammary gland carcinogen in rats. *J. Natl. Cancer Inst.* **54,** 401–411.

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

Hinton C.P., Bishop H.M., Holliday H.W., Doyle P.L. & Blamey R.W. (1986) A double-blind controlled trial of danozol and bromocriptine in the management of severe breast pain. *Br. J. Clin. Pract.* **40,** 326–330.

Huggins C., Grand L.C. & Brillantes F.P. (1961) Mammary cancer induced by a single feeding of polynuclear hydrocarbons and its suppression. *Nature* **189,** 204–207.

Joshi K., Smith J.A., Perusinghe N. & Monaghan P. (1986) Cell proliferation in the human mammary epithelium: Differential contribution by epithelial and myoepithelial cells. *Am. J. Pathol.* **124,** 199–206.

Karey K.P. & Sirbasku D.A. (1988) Differential responsiveness of human breast cancer cell lines MCF-7 and T47D to growth factors and 17β-estradiol. *Cancer Res.* **48,** 4083–4092.

Ke Y., Fernig D.G., Wilkinson M.C., Winstanley J.H.R., Smith J.A., Rudland P.S. & Barraclough R. (1993) The expression of basic fibroblast growth factor and its receptor in cell lines derived from normal human mammary gland and a benign mammary lesion. *J. Cell Sci.* **106,** 135–143.

Keon B.H. & Keenan T.W. (1993) Transferrin is a major cytosolic protein during early stages of involution in the rat mammary gland. *Protoplasma* **172,** 43–48.

Kim U. (1979) Factors influencing metastasis of breast cancer. In *Breast Cancer*, Vol. 3. Ed W. L. McGuire. New York: Plenum Press, pp. 1–49.

Kriajevska M., Cardenas M., Grigorian M., Ambartsumian N., Georgiev G. & Lukanidin E. (1994) Non-muscle myosin heavy chain as a possible target for protein encoded by metastasis-related mts-1 gene. *J. Biol. Chem.* **269,** 19679–19682.

Li P., Wilde C.J., Fernig D.G., Finch L.M.B. & Rudland P.S. (1998) Identification of cell types in the developing goat mammary gland: evidence for a stem cell type during lactation. *J. Histochem. Cytochem.* In press.

Lundy J.D., Schuss A., Stanick D., McCormack E.S., Kramer S. & Sorvillo J.M. (1991) Expression of neu protein, epidermal growth factor receptor and transforming growth factor-alpha in breast cancer – correlation with clinicopathological parameters. *Am. J. Pathol.* **138,** 1527–1534.

Luqmani Y.A., Graham M. & Coombes R.C. (1992) Expression of basic fibroblast growth factor, FGFR1 and FGFR2 in normal and malignant breast, and comparison with other normal tissues. *Br. J. Cancer* **66,** 273–280.

Lyons W.R. (1958) Hormonal synergism in mammary growth. *Proc. Royal Soc. London Series B.* **149,** 303–328.

Marte B.M., Jeschke M., Graus-Porta D., Taverna D., Hofer P., Groner B., Yarden Y. & Hynes N.E. (1995) Neu differentiation factor/heregulin modulates growth and differentiation of HC11 mammary epithelial cells. *Mol. Endo.* **9,** 14–25.

McAndrew J., Fernig D.G., Rudland P.S. & Smith J.A. (1994a) Secretion of transforming growth factor-alpha and expression of its receptor in human mammary cell lines. *Growth Factors* **10,** 281–287.

McAndrew J., Rudland P.S., Platt-Higgins A.M. & Smith J.A. (1994b) Immunolocalization of alpha-transforming growth factor in the developing rat mammary gland in vivo, rat mammary cells in vitro and in human breast diseases. *Histochem. J.* **26,** 355–366.

McCann A., Johnston P.A., Dervan P.A., Gullick W.J. & Carney

D.N. (1989) c-erbB-2 oncoprotein expression in malignant and nonmalignant breast tissue. *Ir. J. Med. Sci.* **158,** 137–140.

McGregor D.H., Land C.E., Choi K., Tokuoka S. & Liu P.I. (1977) Breast cancer incidence among atomic bomb survivors, Hiroshima and Nagasaki, 1950–69. *J. Natl. Cancer Inst.* **59,** 799–811.

Nandi S.J. (1958) Endocrine control. of mammary development and function in the C3H/He Crgl mouse. *J. Natl. Cancer Inst.* **21,** 1039–1055.

Ormerod E.J. & Rudland P.S. (1986) Regeneration of mammary gland in vivo from isolated ducts. *J. Embryo. Exp. Morph.* **96,** 229–243.

Ozzello L. (1971) Ultrastructure of the human mammary gland. *Pathol. Annu.* **6,** 1–39.

Paterson F.C. & Rudland P.S. (1985) Identification of novel stage-specific polypeptides associated with the differentiation of mammary epithelial stem cells to alveolar-like cells in culture. *J. Cell Physiol.* **124,** 525–538.

Peyrat J.P., Bonneterre J., Hondermarck H., Hecquet B., Adenis A., Louchez M.M., Lefebvre J., Boilly B. & Demaille A. (1992) Basic fibroblast growth factor (bFGF) – mitogenic activity and binding sites in human breast cancer. *J. Steroid Biochem. Mol. Biol.* **43,** 87–94.

Rowe J.M., Kasper S., Shiu R.C. & Friesen H.G. (1986) Purification and characterization of a human mammary tumor-derived growth-factor. *Cancer Res.* **46,** 1408–1412.

Rudland P.S. (1987) Stem cells and the development of mammary cancers in rats and humans. *Cancer Metastasis Rev.* **6,** 55–83.

Rudland P.S. (1991) Generation of lobuloalveolar development from isolated rat mammary ducts and endbuds. *J. Histochem. Cytochem.* **39,** 1257–1266.

Rudland P.S. (1993) Epithelial stem cells and their possible role in the development of the normal and diseased breast. *Histol. Histopathol.* **8,** 385–404.

Rudland P.S., Durbin H., Clingan D. & Jiminez de Asua L. (1977) Iron salts and transferrin are specifically required for cell division of cultured 3T6 cells. *Biochem.. Biophys. Res. Commun.* **75,** 556–562.

Rudland P.S., Paterson F.C., Monaghan P., Davies A.C. & Warburton M.J. (1986) Isolation and properties of rat cell lines morphologically intermediate between cultured mammary epithelial and myoepithelial cells. *Dev. Biol.* **113,** 388–405.

Rudland P.S., Platt-Higgins A.M., Wilkinson M.C. & Fernig D.G. (1993) Immunocytochemical identification of basic fibroblast growth factor in the developing rat mammary gland: variations in location are dependent upon glandular structure and differentiation. *J. Histochem. Cytochem.* **41,** 887–898.

Rudland P.S., Barraclough R., Fernig D.G. & Smith J.A. (1998) Growth and differentiation of the normal mammary gland and its tumours. In: *Mammary Development and Cancer.* Eds. P. S. Rudland, D. G. Fernig, S. Leinster & G. G. Lunt. London: Plenum Press. pp. 1–20.

Russo J., Saby J., Isenburg W.M. & Russo I.H. (1977) Pathogenesis of mammary carcinomas induced by 7, 12-dimethylbenz[a]anthracene. *J. Natl. Cancer Inst.* **59,** 435–455.

Russo J., Tay L.K. & Russo I.H. (1982) Differentiation of the mammary gland and susceptibility to carcinogenesis. *Breast Cancer Res. Treat.* **2,** 5–73.

Russo J. & Russo I.H. (1987) Development of the human mammary gland. In: *The Mammary Gland: Development, Regulation and Function.* Eds. M. C. Neville & C. W. Daniels. New York: Plenum Press. pp. 67–93.

Sandren E.P., Schroeder J.A., Qui T.H., Palmiter R.D., Brinster R.L. & Lee D.C. (1995) Inhibition of mammary gland involution is associated with transforming growth factor α but not c-myc-induced tumorigenesis in transgenic mice. *Cancer Res.* **55,** 3915–3927.

Schafer B.W., Wicki R., Engelkamp D., Mattei M.G. & Heizmann C.W. (1995) Isolation of a YAC clone covering a cluster of 9, S100 genes on human chromosome 1q21 – rationale for a new nomenclature of the S100 calcium-binding protein family. *Genomics* **25,** 638–643.

Segaloff A. (1978) Hormones and mammary carcinogenesis. In *Breast Cancer: Advances in Research and Treatment*, Vol. 2. Ed W. L. McGuire. New York: Plenum Press. pp. 1–22.

Sirbasku D.A. (1978) Estrogen induction of growth factors specific for hormone responsive mammary, pituitary and kidney tumour cells. *Proc. Natl. Acad. USA* **75,** 3786–3790.

Sirbasku D.A., Officer J.B., Leland F.E. & Lio M. (1982) Evidence of a new role for pituitary-derived hormones and growth factors in mammary tumor growth in vivo and in vitro. In *Growth of cells in hormonally defined media, Book B.* Eds G. H. Sato, Pardee, A. B. & Sirbasku, D. A. New York: Cold Spring Harbor Laboratory. pp. 765–788.

Slamon D.J., Clark G.M., Wong S.G., Levin W.J., Ullrich A. & Mcguire W.L. (1987) human-breast cancer – correlation of relapse and survival with amplification of the her-2 neu oncogene. *Science* **235,** 177–182.

Smith G.H. & Medina D. (1988) A morphologically distinct candidate for an epithelial stem cell in mouse mammary gland. *J. Cell Sci.* **89,** 173–183.

Smith J.A., Barraclough R., Fernig D.G. & Rudland P.S. (1989) Identification of alpha transforming growth factor (αTGF) as a possible local trophic agent for the mammary gland. *J. Cell Physiol.* **141,** 363–370.

Smith J.A., Winslow D.P. & Rudland P.S. (1984) Different growth factors stimulate cell division of rat mammary epithelial, myoepithelial and stromal cell lines in culture. *J. Cell Physiol.* **119,** 120–126.

Stewart A.J., Johnson M.D., May F.E. & Westley B.R. (1990) Role of insulin-like growth factors and the type I insulin-like growth factor receptor in estrogen-stimulated proliferation. *J. Biol. Chem.* **265,** 21171–21178.

Takahashi K., Suzuki K., Kawahara S. & Ono T. (1989) Growth stimulation of human breast epithelial cells by basic fibroblast growth factor in serum-free medium. *Int. J. Cancer* **43,** 870–874.

Takenaga K., Nakamura Y., Endo H. & Sakiyama S. (1994) Involvement of S100-related calcium-binding protein pEL98 in all motility and tumour cell invasion. *Jpn. J. Cancer Res.* **85,** 831–839.

Topper Y.J. & Freeman C.S. (1980) Multiple hormone interactions in the development of the mammary gland. *Physiol., Rev.* **60,** 1049–1105.

Umekita Y., Enokizono N., Sagara Y., Kuriwaki K., Takasaki T., Yoshida A. & Yoshida H. (1992) Immunohistochemical studies on oncogene products (EGF-R, c-erbB-2) and growth factors (EGF, TGF-alpha) in human breast cancer – their relationship to estrogen receptor status, histological grade, mitotic index and nodal status. *Virchows Arch. A. Pathol. Anat.* **40,** 345–351.

VANDEWALLE B., HORNEZ L., REVILLION F. & LEFEBVRE J. (1989) Secretion of transferrin by human breast cancer cells. *Biochem. Biophys. Res. Commun.* **163,** 149–154.

VORHERR H. (1980) Treatment of primary and recurrent breast cancer. In *Breast Cancer: Epidemiology, Endocrinology, Biochemistry and Pathobiology*. Baltimore: Urban and Schwarzenberg. pp. 374–408.

WALKER R.A. & DAY S.T. (1986) Transferrin receptor expression in non-malignant and malignant human-breast tissue. *J. Pathol.* **148,** 217–224.

WARBURTON M.J., HEAD L.P., FERNS S.A. & RUDLAND P.S. (1983) Induction of differentiation in a rat mammary epithelial stem cell line by dimethylsuphoxide and mammatrophic hormones. *Eur. J. Biochem.* **133,** 707–715.

WATANABE Y., USADA N., MINAMI H., MORITA T., TSUGANE S.-I., ISHIKAWA R., KOHAMA K., TOMIDA Y. & HIDAKA H. (1993) Calvasculin as a factor affecting the microfilament assemblies in rat fibroblasts transfected by *src* gene. *FEBS Lett.* **324,** 51–55.

WESTLEY B.R., CLAYTON S.J., DAWS M.R., MOLLOY, C.A. & MAY F.E.B. (1997) Interactions between the oestrogen and insulin-like growth factor signalling pathways in the control of breast epithelial cell proliferation. In *Mammary Development and Cancer.* Eds. P. S. Rudland, D. G. Fernig, S. Leinster & G. G. Lunt. London: Portland Press. pp. 35–44.

WILKINSON M.C., NUNEZ DE CROKER C.A., FERNIG D.G., RUDLAND P.S. & SMITH J.A. (1998) Ferritin and haemaglobin in bovine pituitary extracts are mitogenic for rat mammary epithelial cells in culture. *In Vitro*, in press.

WILLIAMS J.C., GUSTERSON B.A., MONAGHAN P., COOMBES R.C. & RUDLAND P.S. (1985) Isolation and characterisation of clonal cell-lines from a transplantable metastasizing rat mammary-tumor, TR2CL. *J. Natl. Cancer Inst.* **74,** 415–428.

WINSTANLEY J.H.R., COOKE T., MURRAY D.G., PLATT-HIGGINS A., GEORGE W.D., HOLT S., MYSKOV M., SPEDDING A., BARRACLOUGH B.R. & RUDLAND P.S. (1991) The long-term prognostic significance of c-erbB-2 in primary breast cancer. *Br. J. Cancer* **63,** 447–450.

WINSTANLEY J.H.R., LEINSTER S.J., COOKE T.G., WESTLEY B.R., PLATT-HIGGINS A.M. & RUDLAND P.S. (1993) Prognostic significance of cathepsin D in patients with breast cancer. *Br. J. Cancer* **67,** 767–772.