A STUDY OF RAT MAMMARY PARENCHYMA: CELL SORTING AND CLONAL CHARACTERISATION OF LUMINAL AND MYOEPITHELIAL CELLS.

By

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ABSTRACT.

Rat mammary gland cell biology has been studied using a novel approach. Parenchymal cell types have been isolated and characterised by clonal behaviour in culture. Culture conditions with which normal mammary cells from adult virgin rats could be cloned with high efficiency were established. Several morphologically distinctive colony types were observed, and cells have been cloned from isolated ducts and alveoli. Luminal and myoepithelial cells were isolated from mammary gland using fluorescence activated cell sorting (FACS). Cells were flow sorted on the basis of the differential expression of two membrane antigens - a 70KD luminal-specific antigen , and neutral endopeptidase EC 3.4.24.11 (NEP/CALLA), which is expressed by myoepithelial cells in the intact gland. Sorted cells were then separately cloned so that the *in situ* origin of different clone types could be unequivocally determined. Monoclonal antibodies recognising cell-type specific cytokeratin polypeptides were used to confirm clonal phenotypes derived from sorted cells.

Primary clones have been studied with a panel of cell type-specific markers at various stages of growth, using indirect immunofluorescence and flow cytometry. In order to determine the number of different stable phenotypes and follow their epithelial differentiation status, primary clones have been re-cloned and the phenotypes of progeny colonies examined. These studies demonstrated the existence of distinct multipotent clone types capable of generating cells of both luminal and myoepithelial phenotypes in culture.

Responses of the different clones to various growth factors and hormones have been examined. Continuous cell lines have been established from both sorted luminal and myoepithelial cells by retroviral transduction of a recombinant construct encoding a temperature-sensitive non-DNA binding SV40 large T protein. The behaviour of these lines has been compared with sorted primary cultures, and the organotypic differentiation of both examined in fat pad transplants. The results are analysed in the context of the mechanisms and cellular interactions controlling growth and differentiation of the mammary parenchyma *in situ*.

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CHAPTER 1.

GENERAL INTRODUCTION.

Preface.

The mammary gland is a complex organ composed of different tissues and cell types and subject to many humoral influences. Understanding its development, growth and differentiation is therefore difficult, and various experimental approaches have been adopted. The rat mammary gland provides a useful experimental system with which to study mammary biology both *in vivo* and in culture. Although a great deal is known about the mechanisms which modulate overall growth and function of the mammary gland, many important questions remain unanswered at the cellular level . In particular, the precise mechanisms by which cellular and functional diversity of mammary cells is generated during histogenesis and maintained during cell renewal in the adult gland, and the factors which regulate specific cell types, are not fully understood. The aim of the work presented in this thesis was to isolate specific rat mammary cell types and then characterise them separately in culture. Dissecting the heterogeneous parenchyma at the cellular level and studying individual populations should aid in delineating lineage relationships between different cell types, and provide a basis for studying in greater detail factors influencing growth and differentiation of mammary gland parenchyma.

1.1 The development and structure of rat mammary gland.

1.1.1 Introduction.

This section briefly summarises the salient features of rat mammary development from the morphological and histological viewpoint. Detailed descriptions of developmental process will not be given here as many contemporary articles deal with this subject, Sakakura, 1987; Daniel and Silberstein, 1987; and additionally review the early literature, Russo et al., 1989. More detailed consideration will be given to markers of different cellular phenotypes, including a discussion of those utilised during this study.

1.1.2 Gross anatomy of rat mammary glands.

The rat has six pairs of glands and nipples; 1 thoracic, 2 axillary, 1 abdominal and 2 inguinal. Glands are located in two separated pairs of fat pads - the thoracic/ axillary and the abdominal/ axillary fat pads, Astwood et al., 1937, on either side of the ventral midline. Although nipples are situated ventrally and medially located, fat pads in adult animals extend laterally into dorsal regions, and thus have the capacity to accommodate parenchymal expansion during pregnancy.

1.1.3 Developmental morphogenesis and histogenesis of the mammary tree.

Progressive tissue histogenesis produces a compound tubulo-acinar epithelial structure which ramifies into adipose and fibroblastic connective tissue. Growth (that is quantitative changes in glandular mass) and development occur by cell division and differentiation, which are regulated by multiple factors. The gland remains functionally and structurally dynamic during life, reaching full differentiation during lactation when apocrine and merocrine secretory processes are initiated, Vorherr, 1974. On the basis of cytological profiles and structural alterations, 6 distinct stages broadly describe mammary gland development - prenatal, prepubertal, postpubertal, pregnancy, lactation and involution.

Prenatal: The rat mammary gland originates from an invagination of the embryonic integument. This process occurs between days 11-14 of intrauterine life at localised points on the 'milk lines', Raynaud, 1961. Two basic cellular structures are elaborated - glandular parenchyma developing from the ectodermal epithelium, and mesenchyma arising from mesoderm underlying the nipple primordium. Two distinct mesenchymes are distinguished; a dense fibroblastic mesenchyma which surrounds the epithelium, and fat pad precursor tissue,

Sakakura et al., 1982. The primordial parenchyma grows downward into the underlying mesenchyma, and morphogenesis depends on interactions between these tissues, Kratochwil, 1969. As development proceeds, both the epithelium and the stroma undergo characteristic morphogenesis and cytodifferentiation which are both controlled by systemic and local regulators, Topper and Freeman, 1980. Up until birth, which occurs after 22 days gestation, there is only rudimentary branching and canalisation of primary and secondary ducts, Myers, 1919.

Prepubertal: At birth and during the first weeks of postnatal development, individual glands consist of one or two main lactiferous ducts arising from the nipple which bifurcate into 3-5 straight secondary ducts, Ceriani, 1970b, ending in dilated terminal end buds, the sites of intense mitotic activity, Dulbecco et al., 1982. During the second week, ducts lengthen, undergo 4 or 5 generations of further branching, and small buds sprout laterally in a sympodial manner from them (the lateral buds). Mesenchyme regionally localises, Young and Hallowes, 1973, forming the fat pads by differentiation of precursor cells situated in perivascular regions, Bani-Sacchi et al., 1987. Gland development progresses with age by successive dichotomous branching and sprouting of lateral buds, and overall growth from

birth to puberty is approximately isometric. At 21 days, growth becomes allometric, Cowie, 1949. At this time the number of terminal end buds is maximal, and they begin to cleave into 3-5 smaller alveolar buds, the direct precursors of alveoli Russo et al., 1989, This structural differentiation is compartmentalised and occurs more in areas more distal from the nipple. In addition, differences in the degree of development are observed between individual glands such that this process is more common in thoracic glands, Russo et al., 1989.

Postpubertal: The onset of 4-5 day estrus cycles in female rats around 35 days of age, induces changes in glandular architecture with progressively more terminal end buds differentiating into alveolar buds in response to each cycle, Astwood et al., 1937. Concomitantly, lobules form by the further cleavage of existing alveolar buds. Some terminal end buds do not differentiate, but regress into atrophic terminal ducts which do not undergo any further morphologic changes in virgin animals. At around 60 days the mammary tree becomes structurally constant, and remains so until pregnancy.
Pregnancy and lactation: The hormones of pregnancy induce further morphological changes and differentiation which result in the full functional development of the mammary gland. Terminal end buds all but disappear

concomitant with their differentiation into alveolar buds. Lobules then form by increases in the number of individual alveoli, Russo et al., 1989. By the end of pregnancy, acini (alveoli exhibiting secretory activity) are composed of polarised alveolar epithelial cells which synthesise and secrete milk proteins, sugars and lipids (for reasons that are not known, most ductal epithelial cells do not respond in this way to the lactogenic hormones) Anderson, 1974. Myoepithelial cells (which do not secrete milk) contract in response to oxytocin, the secretion of which is regulated by a neuroendocrine reflex, aiding milk expulsion Cross, 1977.

Involution: The involution phase begins after weaning. Accumulation of milk within the lactogenic epithelial cells suppresses further milk synthesis, Vorherr, 1974. Alveolar structures collapse, Warburton et al., 1982b, and fat cells reappear in large numbers. Phagocytic cells infiltrate around the disintegrating lobules, Richards and Benson, 1971, and a combination of autolysis, phagocytosis and apoptosis are responsible for glandular regression, Richards and Benson, 1971b. Glands continue to regress over a 7-10 week period until a branched ductal system, similar in appearance to those in adult virgin animals remains, Vonderhaar, 1988.

Cycles of lobulo-alveolar differentiation, lactation and involution occur with each successive pregnancy.

1.1.4 Histology of the adult virgin female rat mammary gland.

Light level: Three general categories of parenchymal cells are typically discriminated on histological sections viz., cuboidal epithelial cells lining ducts; alveolar epithelial cells; and myoepithelial cells. Ducts are lined by one or two layers of columnar epithelium. A basally located layer of spindle-shaped myoepithelial cells, with their cytoplasmic processes orientated parallel to the long axis of the duct, lie between the secretory epithelium and the basement membrane, forming a continuous layer. Around alveoli, myoepithelial cells are stellate shaped and form discontinuous basket-like networks around single layers of cuboidal epithelium, Emerman and Vogl, 1986, some of which directly abut onto the basement membrane, Warburton et al., 1982b. Basement membrane, which is present at the earliest stages of development, surrounds the parenchyma separating it from the sub-adjacent stromal milieu. Outside the basement membrane, the loose connective tissue contains blood vessels and various wandering cells.

Ultrastructural level: The ultrastructure of rat mammary gland has been described during early development, Radnor, 1972; pregnancy, Murad, 1969; and lactation, Radnor, 1972b. Viewed in the electron microscope, three types of epithelial cells have been categorised on the basis of electron-density - "light", "dark" and "intermediate" cells, Russo et al., 1976. Additionally, large "pale clear" cells located between luminal and myoepithelial layers in both ducts and alveoli have been described Radnor, 1972. These cells, which are quite distinct from dentritic cells of lymphoid origin intercalating the parenchyma during phases of development, Joshi et al., 1985, are identified by the relative paucity of cytoplasmic organelles such as mitochondria and endoplasmic reticulum. Only at the ultrastructural level can these cells be unambiguously distinguished from wandering macrophage which can be seen within rat mammary parenchyma. Analogous large clear epithelial cells have been observed in all portions of the mammary tree during all stages of development of murine glands, Smith and Medina, 1988. These cells of undifferentiated appearance are possibly related to those situated around 21-day terminal end buds which show gradations in ultrastructure towards either myoepithelial cells or luminal cells. Transitional forms intermediate between stem cell progeny and fully differentiated phenotypes in these locations have been

postulated on the basis of electron microscopic observations, Ormerod and Rudland, 1984; Ormerod and Rudland, 1986.

Adjacent luminal epithelial cells are attached by apical tight junctions. Gap junctions, and desmosomes associated with tonofilaments, are located around the peripheries of cell-cell contact. Desmosomes connect the basal aspects of epithelial cells and the apical regions of myoepithelial cells, Radnor, 1972. Microvilli project from the apical surfaces of epithelial cells into the lumen. Occasional gap junctions connect adjacent myoepithelial cells which also elaborate hemidesmosome junctions at their basal surface above the basement membrane. Micropinocytotic vesicles are commonly observed at the basal surface of this cell type. Myoepithelial cells can be further discriminated from epithelial cells by the presence of abundant cytoplasmic myofilaments and dense peripherally located heterochromatin patterns in their nuclei, Pitelka, 1980.
The precise origins of luminal epithelial and myoepithelial cells during periods of renewal and rapid growth remains obscure. In particular, it is not known whether separate lineages exist for both luminal and myoepithelial cells, or whether a precursor population exists which is capable of giving rise to both these differentiated cell types. By studying the distribu-

tions of cell type-specific markers however, lineage connections between the different cell types can be probed, Dulbecco et al., 1983; Allen et al., 1984.

1.1.5 Markers of normal parenchymal phenotype.

Luminal epithelial and myoepithelial cells can be identified using histochemical or immunological techniques to detect differentially expressed proteins located in the membrane, cytoplasmic, cytoskeletal or basement membrane structures, Thus, alveolar epithelial cells and a few epithelial cells of minor ducts, Turkington, 1969; and Pitelka, 1980, are preferentially stained by antibodies recognising enzymes related to milk production, for example thioesterase II, Pasco et al., 1982; the casein phosphoproteins and α-lactalbumin milk proteins characteristic of the fully functional state of the parenchyma, Turkington, 1969. Epithelial cells are also specifically stained by antibodies recognising components of milk fat globule membrane, Warburton et al., 1982b; and the cytoplasmic calcium binding protein, calelectrin, Lozano et al., 1989. Other cellular markers which discriminate between luminal epithelial and myoepithelial cells include actin, myosin and certain lectins, the staining profiles of which have been recently reviewed, Gusterson et al., 1989.

Three classes of immunophenotypic markers identifying and discriminating between luminal and myoepithelial cells have been extensively used throughout this thesis, and will therefore be discussed in greater detail here. These are - 1. two membrane antigens; one a member of the ectoenzyme family of proteins, and the other an extra-cellular apically located 70KD glycoprotein; 2. the cytostructural actin, cytokeratin and vimentin filaments; and 3. different basement membrane proteins.

Endopeptidase EC. 3.4.24.11 - A membrane marker of myoepithelial cells in the mammary gland.

Monoclonal antibodies recognising the common acute lymphoblastic leukaemia antigen CD10, (CALLA) bind to myoepithelial cells but not luminal epithelial cells of intact rat mammary gland, Gusterson et al., 1986. The molecules bound by anti-CALLA antibodies on the myoepithelial cells are identical to authentic CALLA, obtained from lymphoid cells, Mahendran et al., 1989. First identified as a 100KD cell surface glycoprotein present on certain leukaemic cells, Greaves et al., 1975, recent data has shown that the predicted amino acid sequence for CALLA is identical to that for human membrane-associated neutral zinc metalloendopeptidase EC 3.4.24.11 (NEP; enkephalinase), Letarte et al., 1988. Subsequent studies have proved that CALLA is

indeed functional endopeptidase 3.4.24.11, Shipp et al., 1989. This molecule, which is also expressed by other rat tissues, Ronco et al., 1988, has been postulated to function in the regulation of growth and differentiation signals by modulating the activity of peptide factors, Kenny et al., 1989. Recent evidence suggests a possible role in extracellular matrix organisation by virtue of a role in enzyme cascades which ultimately activate pro-collagenases, Werb and Clark, 1989.

Several different monoclonal antibodies recognising endopeptidase-24.11 were used during this study. These cross-react with both the human and rat enzyme.

An apical membrane antigen specific for luminal epithelial cells.

Most if not all of the antibodies raised against human milk fat globule membrane antigens (for example, antibodies recognising the so-called Epithelial Membrane Antigen, Sloane and Ormerod, 1981) do not cross-react with rat cells. Recently, however, a monoclonal antibody (antibody 25.5) has been produced in this laboratory which binds to luminal epithelial cells at their apical surfaces, but not to myoepithelial cells of the intact rat mammary gland, when stained sections are viewed at the light microscope level, Mahendran et al., 1989. Although its epitopes are also distributed

in other rat tissues, this antibody binds mammary luminal epithelium at all stages of development. Monoclonal antibody 25.5 was raised against cell suspensions derived from adult female virgin rat mammary glands and binds two glycoproteins of approximately 70KD and 25KD relative molecular weight in these cells, with the 70KD molecule (glycoprotein-70KD; gp-70KD) the predominant reactive fraction. Antibody 25.5 was used as a rat luminal epithelial cell marker throughout this thesis. The cell surface distributions of the endopetidase ectoenzyme and 25.5-bound epitopes lent themselves to attempts at live cells sorting, for which antibody 25.5 was originally prepared.

An overview of cytoskeletal filaments.

The cytoskeletal fibre systems of all mammalian cells are composed of three different types of filaments: microfilaments (5-6nm diameter), intermediate filaments (7-11nm diameter) and microtubules (20-25nm diameter). The properties of filament types pertinent to this study are as follows:

Actin microfilaments: Six major actin isoforms have been characterised by 2-D electrophoresis and sequence analysis, Vandekerckhove and Weber, 1978. Four of these isotypes represent markers of muscle differentiation, skeletal muscle-α, smooth muscle-α, cardiac-α, smooth

muscle cell-τ, with the two other isoforms present in the vast majority of all other cells types.

Fluorescent phallotoxin conjugates are specific probes for total filamentous actin (F-actin) in cells, Barak et al., 1980. These stain both luminal epithelial and myoepithelial cells in intact mouse and human mammary glands, Emerman and Vogl, 1986. However, as with anti-actin antibodies, Franke et al., 1980, myoepithelial cells can be discriminated from luminal epithelial cells in intact glands on the basis of comparatively stronger staining with these reagents, Emerman and Vogl, 1986. This is consistent with the electron microscopic observations that myoepithelial cells contain large concentrations of myofilaments. More recently it has been demonstrated that an antibody which recognises only the smooth muscle α-isoform specifically bound myoepithelial cells of human mammary parenchyme, Skalli et al., 1986; and Gugliotta et al., 1988. The detection of microfilaments therefore provides a means of characterising myoepithelial cells and discriminating them from luminal epithelial cells, at least in the intact parenchyma.

Intermediate filaments: Intermediate filaments are complex set of proteins of more than 40 different members, which are coded for by a large multigene family (at least thirty genes in humans), reviewed by

Weber and Geisler, 1984; Steinert and Roop, 1988; and see Cooper et al., 1985. Intermediate filaments can be distinguished biochemically, immunologically and display quite specific patterns of tissue and cell type expression. Four groups of intermediate filament types are classified according sequence analysis of cDNA. These are - the 15 different type I 40-60KD acidic keratins; the 15 different type II 50-70KD neutral-basic keratins; single type III 53KD vimentin, 52KD desmin and 51KD glial acidic fibrillary proteins; and the type IV peripheral and central neuronal neurofilaments, the so-called 'neurofilament triplet' of 57-150, 60-200, 60-70KD, Steinert and Roop, 1988. The intermediate filaments display sequence homology with the nuclear lamin polypeptides which form the karyoskeleton, Franke, 1987, and which constitute a separate class of type V intermediate filament-like proteins.

The cytokeratins: In contrast to vimentin, desmin and glial filaments which are composed of one type of subunit protein, nineteen different 'epithelial' cytokeratins composed of multiple subunits have been extracted from human tissues and identified to date. These are catalogued on the basis of their specific coordinates on two-dimensional gel electrophoresis, Moll et al., 1982. Similar studies cataloguing both bovine and rodent cytokeratin subfamilies, Schiller et

al., 1982, indicates that although the patterns of expression of cytokeratin filaments from the same tissue broadly match between species, the corresponding polypeptides from different species do differ with respect to size and charge, Schiller et al., 1982.

Cytokeratin filaments are heteropolymers composed of tetrameric subunits of two chains each of type I and type II molecules, which complex in a size-rank fashion, Cooper et al., 1985; Sun et al., 1985; Steinert and Parry, 1985; Steinert and Roop, 1988. Different cis-regulatory elements control the differential transcription of cytokeratin genes, Blessing et al., 1989, resulting in remarkable tissue and cell type-specific expression of different cytokeratin filaments. Although evidence indicates that cytokeratin gene transcription may not be as stable has as been previously thought, Knapp and Franke, 1989; Knapp et al., 1989; and expression is modulated during growth, development and differentiation *in situ*, Cooper et al., 1985; Lane et al., 1985; Sun et al., 1985; and by growth factors and vitamin A compounds in culture, Sun et al., 1985; Schmid et al., 1983; Roop et al., 1987; Edmondson et al., 1990, detection of specific cytokeratin types generally provides hallmarks of the differentiation status of expressing cells and tissues. Thus, simple epithelium expresses cytokeratins 8, 18 with or without

cytokeratins 7 and 19; stratified and complex epithelia synthesis components 1-6 and 9-17. Monoclonal antibodies raised against cytokeratins may detect common antigenic determinants in all filament classes Pruss et al., 1981; determinants of either all of type I or type II filaments, Sun et al., 1985; or distinctive or identical determinants in different filament types Lane, 1982, and since the specificity of different antibodies can be defined by gel immunocytochemistry and immunofluorescence, they provide powerful tools with which to probe the differentiation status of cells. Combining analysis of tissue extractions using electrophoresis typing with antibody staining patterns reveals that the mammary gland can be considered as a mixed epithelium, since luminal epithelial cells express cytokeratins 7, 8, 18 and 19; and basal myoepithelial cells express cytokeratins 5 and 14, Moll et al., 1982; Taylor-Papadimitriou and Lane, 1987.

Although to date there is little experimental evidence which indicates any critical cellular requirements for cytokeratin filament networks Lane and Klymkowsky, 1982, these diverse molecules provide useful markers of cell type and differentiation status, and antibodies recognising different cytokeratin types have been used extensively throughout this thesis for this purpose.

Vimentin: The existence of vimentin filaments in intact mammary gland under normal circumstances is contentious. Two different polyclonal antiserum raised against vimentin have been reported to immunolocalise myoepithelial cells in rat mammary gland, Dulbecco et al., 1983; and Warburton et al., 1989, as well as an anti-vimentin monoclonal antibody, Warburton et al., 1989. However, other workers have reported that rat mammary parenchyma does not contain vimentin filaments on the basis of a failure to stain with several antibodies, Franke et al., 1980. With respect to human mammary gland a similar disparity exists, with several different monoclonal antibodies recognising vimentin having been reported to stain myoepithelial cells of normal tissue, Guelstein et al., 1988; Mork et al., 1990; whilst another monoclonal recognising this intermediate filament type does not stain any human mammary parenchymal cell type, Dairkee et al., 1985. Nevertheless, expression of epitopes recognised by at least some anti-vimentin antibodies provide a potential marker for myoepithelial cells, and these have been employed in this thesis.

The basement membrane proteins: Basement membrane is a specialised extracellular matrix which separates the glandular parenchyma from the stroma. A common set of proteins make up all basement membranes and include

laminin, the most abundant glycoprotein in these structures, Martin and Timpl, 1987, collagen type IV, various heparan sulphate proteoglycans and entactin. In addition, synthesis of the extracellular matrix glycoprotein tenascin is developmentally regulated and re-expressed during neoplasia in mammary gland, Chiquet-Ehrismann et al., 1986; Mackie et al., 1987; Inaguma et al., 1988; as in other tissues, (reviewed by Erickson and Bourdon, 1989). Recently tenascin was demonstrated in the basement membrane, as well as the adjacent layers of fibroblasts, surrounding the ductal parenchyma of normal human mammary gland. Tenascin expression in these structures was observed to be modulated by changes in ovarian steroids, Ferguson et al., 1990.

Basement membrane components possess both structural and biological activity. Thus, both laminin and tenascin are implicated in modulating branching morphogenesis in tissue systems, Schuger et al., 1990; Takeda et al., 1988, and tenascin is proposed to be essential during early morphogenetic processes in mouse mammary gland, Inaguma et al., 1988. Type IV collagen has been implicated in maintaining mammary ductal structures, Wicha et al., 1980. For the purposes of this study, two antisera recognising either laminin or type IV collagen were utilised in order to characterise rat mammary parenchymal cells. These antisera have been previously

shown to bind to the basement membrane of mature rat mammary gland, and to identify basal cells in terminal end buds by virtue of cytoplasmic staining, Warburton et al., 1982b, as well as reacting with cells derived from adult parenchyma in culture, Warburton et al., 1985.

1.2 Experimental advantages of the rodent mammary system.

1.2.1 Introduction.

In order to investigate the factors regulating mammary gland growth and differentiation, many experimental approaches have been adopted. This section briefly discusses methodologies which have been used in the past and the sorts of results these methods have yielded. Results of *in situ* and culture experiments have complemented each other and have aided in the identification of many of the complex endocrinological, paracrine and autocrine modes of control mechanisms operating in the mammary gland.

1.2.3 Experimental manipulation *in situ*.

Hormone ablation and implants: The influence of both steroid and polypeptide hormones on development of glandular parenchyma and lactogenesis have been inves-

tigated for many years by endocrine ablation and manipulation, see Lyons, 1958; Ceriani, 1974; Banerjee, 1976; Topper and Freeman, 1980; Haslam, 1987; Russo et al., 1989, for reviews.

Systemic injections of growth promoting agents have also been used to assess effects on mammary gland, and it has been observed that cholera toxin stimulates mammary parenchymal growth, Sheffield et al., 1985, when introduced in this manner. Recent experiments have been described which have used an alternative approach to assess the effects of hormones and soluble growth factors on murine parenchyma *in situ* in a localised manner. This approach utilises implants of a biologically inert material capable of slow, sustained release of low concentrations of growth promoting agents of interest into localised areas of the mammary gland, Silberstein and Daniel, 1982. This methodology reveals that EGF can promote or inhibit ductal growth depending on the status of the parenchyma at time of implant, Coleman et al., 1988; Coleman and Daniel, 1990; and stimulate lobulo-alveolar development in sub-adult mice, Vonderhaar, 1987. Similarly the influences of TGF-β, Silberstein and Daniel, 1987; Daniel et al., 1989, TGF-α, Vonderhaar, 1987; cholera toxin, Silberstein et al., 1984; and estradiol, Daniel et al., 1987 on the growth, morphogenesis and differentiation of

murine mammary parenchyma of various developmental stages have all been described.

Transplantation: A large amount of information regarding the biology of the mammary gland has been accumulated using transplantation techniques. White adipose tissue is essential for mammary parenchymal cell growth, Slavin, 1966, and syngeneic mammary or interscapular fat pads are favoured transplantation sites. Transplanted material has included whole mouse glands, Thompson, 1963; dissected ducts, alveoli or terminal end buds of mice or rats, Hoshino, 1962; Hoshino, 1964; Ormerod and Rudland, 1986; Smith and Medina, 1988; glandular disaggregates of rat origin, Gould et al., 1977; Sinha, 1981; cultures of murine, Daniel and DeOme, 1965; Ehmann et al., 1987; Miller and McInerney, 1988; or rat, Richards and Nandi, 1978, parenchyma. Lately, elaborate experiments investigating the effects of implants on transplanted material have been reported, Daniel et al., 1989. The importance of cell-cell and cell-matrix interactions in growth regulation have been demonstrated by transplantation experiments. Thus, it is clear that adipose tissue supports morphogenesis whilst the fibroblastic mesenchyme supports proliferation but does not control morphogenesis, Sakakura et al., 1982; furthermore, morphogenesis is due to a proliferation of parenchymal cells and not due

merely to shape changes of pre-existing parenchyma, Inaguma et al., 1987. Serial transplantation of putative preneoplastic lesions, or normal tissues in the mouse system have been used effectively to study neoplastic progression and cell aging, (reviewed by Medina, 1988; Daniel, 1975), respectively. Similar types of experiments show that there is little evidence that equivalent preneoplastic lesions occur in rat gland, Beuving et al., 1967.

The remarkable repopulation capacity of small amounts of transplanted parenchyma has been used to infer the existence of mammary stem cells, (see for example Smith and Medina, 1988). Individual segments of all types of mammary tree structure dissected from any age of donor animal at any developmental stage, including lactation, have the potential to regenerate mammary trees composed of cells, which at the ultrastructural level, resemble cells of other mammary locations, Hoshino, 1964; Ormerod and Rudland, 1986; Smith and Medina, 1988. These transplants can then be induced to undergo full functional differentiation, Smith and Medina, 1988.

The fact that cultured rodent cells retain morphogenetic ability after re-introduction into fat pads, Daniel and DeOme, 1965, enables the effects of manipulating

cells in culture to be assessed in the appropriate *in situ* environment. The effects of exposing rat mammary cells to carcinogens in culture has been examined *in situ* for example, Richards and Nandi, 1978. Another interesting application of the culture-transplant method is the formation of transgenic mammary glands. Transgenic organs can be made by introducing oncogenes genes into cultured cells by retroviral transduction, and subsequently re-introducing genetically altered cells back into mammary fat pads in order to examine effects on 3-dimensional growth and differentiation. This technique has been accomplished in murine glands with the oncogenes v-myc v-mil and v-Ha-ras. The expression of these genes can be correlated to alterations in growth and morphological differentiation, Edwards et al., 1988; Gunzberg et al., 1988; Strange et al., 1989.

Experimental carcinogenesis and transgenic animals: An obvious utility of rodent systems is that mammary tumours can be induced at will in order to study factors influencing the neoplastic process. Rat tumours are of particular importance in experimental oncology in view of their hormone-sensitive growth and pathological similarity to certain of those of the human breast, Young and Hallowes, 1973; Russo et al., 1989. Studies employing polycyclic hydrocarbons have revealed

that developmental status, diet and genetic background all influence susceptibility to experimental carcinogenesis, reviewed by Young and Hallowes, 1973; Russo and Russo, 1987; Rogers, 1989.

The advent of transgenic animals has enabled the effects of particular oncogenes on mammary gland development to be examined. Transgenic strains of mice carrying the myc, Stewart et al., 1984; ras and myc, Sinn et al., 1987; c-neu, Muller et al., 1988; and activated int-1, Tsukamoto et al., 1988, oncogenes have been reported. All of these genes appear to increase the probability of the development of mammary adenocarcinomas in time.

All of the experimental designs discussed above examine the heterogeneous populations of cells *in situ*. In addition to the experimental variables, multiple systemic factors may modulate the responses of cells, thus complicating the interpretations of the effects of the agent of interest. However, culture sytems provide methods of simplifying the analysis of mammary cell behaviour by enabling observations and manipulations of cells under conditions where experimental variables can be controlled or reduced.

1.2.4 Experimental manipulation in culture.

The main aim of tissue and cell culture is to be able to reproduce in an defined system the physiological and pathological phenomenon that occur *in vivo*. With regard to mammary biology, several different approaches of varying complexity have been pursued in the past in order to delineate the effects of hormones and growth factors on growth and functional differentiation.

Organ culture: Maintaining pieces of dissected adult mammary glands which retain the surrounding fatty stroma as whole fragment organ culture allows *in vivo* processes to be mimicked in culture. Cultured rat glands display limited cellular proliferation in this situation, but can be induced to undergo cycles of alveolar development, full functional differentiation and regression in response to hormonal, reviewed by Banerjee, 1976; and EGF stimulation, Tonelli and Sorof, 1980. In addition, it has been possible by maintaining fetal rat anlagen in chemically defined medium as organ cultures to define the hormones controlling the development of this structure, Ceriani, 1970; Ceriani, 1970b.

Although these studies examine cells in an appropriate structural environment, a distinct disadvantage of organ cultures is that they can only be maintained for short periods before the onset of necrosis, typically

10 to 14 days for rat tissue. Organ culture studies are therefore generally limited to short-term investigation. By contrast, primary culture techniques enable tissue fragments to be cultured for longer periods of time.

Explant culture: Fragments of mammary tree isolated by enzymatic digestion of mammary glands and stripped of subjacent stroma, termed organoids, Hallowes et al., 1977, provide the basic starting material for primary culture studies. Organoids can be plated directly into tissue culture plastic, and although 3-dimensional architecture is progressively lost in such cultures, some hormonal and growth factor responsiveness is maintained, Hallowes et al., 1977; Richards and Nandi, 1978b. Alternative methods of culturing organoids involve embedding them into reconstituted type I collagen hydrogels. Such cultured fragments generate morphogenetic patterns resembling those observed *in vivo*, Daniel et al., 1984; and separated alveolar, Pasco et al., 1982; duct and end bud structures, Richards et al., 1983; Daniel et al., 1984, have been cultured in this way. These types of cultures provide a system with which to investigate factors influencing cell growth, Pasco et al., 1982, and parenchyma from immature virgin rats can be induced to undergo secretory differentiation in response to the lactogenic hormones, as meas-

ured by casein synthesis and the acquisition of secretory ultrastructure, Richards et al., 1983.

Monolayer culture: A more commonly used culture method is to grow cells as monolayers derived from organoid suspensions. The selective reconstitution of mammary-specific influences on standardised cell cultures if this type has yielded significant results regarding specific cell-cell and cell-matrix regulation of growth. By exposing such cultures to conditioned medium it is apparent that both the mammary stromal mesenchyme, Taga et al., 1989; and fat pad adipose tissue, Beck et al., 1989, elaborate and secrete trophic factors which stimulate mitotsis of mammary parenchymal cells. The growth of murine mammary cells is also promoted by co-culture on layers of adipose-converting 3T3 L1 cells, Levine and Stockdale, 1984. This specific mammary cell-feeder cell interaction also maintains hormone dependent casein synthesis by cells cultured from mid-pregnant mice, Levine and Stockdale, 1985, and can induce cytodifferentiation in cells derived from non-pregnant mice, Wiens et al., 1987. Culturing cells on reconstituted basement membrane of floating collagen gels also provides models for studying cytodifferentiation and morphogenetic behaviour by virtue of the fact that cells reorganise into 3-dimensional structures, Emerman et al., 1979; Barcellos-Hoff et al., 1989.

However, although primary cultures are of proven value, the same drawbacks exist as with organ and explant cultures, in that it is difficult to interpret the effects of growth promoting agents on target cells because of the heterogeneity of the cellular populations. Different cell types may affect each other, respond to growth promoting agents in different ways, or modulate their phenotypes. To overcome these complications, several groups have developed clonal cell lines from both normal and neoplastic rat mammary parenchyma.

Use of mammary derived established cell lines: Rat mammary cell lines have been generated from neonatal parenchyma, Warburton et al., 1981; Ormerod and Rudland, 1985; and tumour-derived cells, Bennett et al., 1978; Dunnington et al., 1983; Dulbecco et al., 1981. Although the study of pure populations of cells is clearly advantageous, a major drawback of these types of lines is that the precise origins and initial phenotypes of the cloned cells are obscure. This is due to the fact that the starting populations were composed of heterogeneous mixtures of cells. In addition, because these lines are generally studied many passages after their establishment, it is quite possible that

changes in the differentiation status of cells may have occurred. Such lines may no longer be representative of the original cell types *in situ*.

1.5 Conclusions and objectives of the present study.

1.5.1 Conclusions

Although much has been elucidated about the factors which influence growth and differentiation of rat mammary gland using complementary *in vivo* and culture experiments, many interesting questions concerning the developmental capability of individual parenchymal cell types are as yet unanswered. Since cells in culture generally display a limited spectrum of differentiated properties characteristic of the parenchyma *in situ*, culture systems have been employed to investigate lineage relationships and differentiation of cell types. With regard to the rat mammary gland, such investigations have centred on the behaviour of cell lines, in particular the "RAMA" series of lines, reviewed by Rudland, 1987. However, as the precise origins of such cell lines are not known, this renders interpretation of their characteristics open to question. In addition, given the tendency for cultured parenchymal cells, particularly established cell lines, to alter or lose differentiated characteristics, it is

imperative that starting populations are pre-identified in order to eliminate any ambiguity. Such criteria of identity are particularly important when investigating the rodent mammary gland, given the postulated existence of stem cells and the possibility of various cellular conversions, Bennett et al., 1978; Dulbecco et al., 1981; Dunnington et al., 1983; Warburton et al., 1987.

A novel method of studying rodent mammary cell biology is, therefore, the use of populations of cells separated on the basis of cell type-specific surface markers prior to their culture. Such an approach using flow cytometry has been achieved with human mammary cells, O'Hare et al., 1989.

1.5.2 Objectives of the present study.

The primary objective of this thesis was the characterisation of cultured rat mammary luminal and myoepithelial cells isolated by fluorescence activated cell sorting. By defining the cell type-specificity of a panel of antibodies on the intact gland, the validity of these reagents for probing the differentiation status of sorted cells was examined. By combining clonal growth of sorted cells and marker analysis, an examination of lineage relationships between identified cell types was undertaken.

In order to generate established cells from flow sorted populations (so that the precise *in situ* origin of derived cell lines would be known), a recombinant retroviral construct capable of introducing mutant SV40 large T was employed. Epithelial cells of endodermal or ectodermal origin are susceptible to retroviral infection, and the life-span of several different cultured parenchymal cells of rodent and human tissues can be extended by the influences of the so-called acutely transforming retroviruses which transduce a variety of oncogenes, reviewed by Vecchio et al., 1986. The activity of these viral oncogenes stimulate incessant proliferation, and generally disrupt the normal differentiation program of the cells. The incompatibilty of the transformed state with the expression of differentiated properties is a therefore a major disadvantage. The use of temperature-sensitive mutants provides a way of overcoming this problem. By dissociating the growth promoting influence of the oncogene at the non-permissive temperature, the possibiltiy arises that cells will restore normal differentiated character. However the effects of the acutely transforming oncogenes are unpredictable and can either induce reversible, Fizman and Fuchs, 1975, or terminal, Colletta et al., 1983, blocks in the expression of differentiation markers at the non-permissive temperature, reviewed by Vecchio

et al., 1986. A potentially more useful system involves the use of temperature-sensitive large T, which is not acutely transforming gene, Jat and Sharp, 1986; Jat et al., 1986. Introducing SV40 large T into cells using retroviral vectors has been used to generate cell lines from particular cell types from mice, for example marrow stromal cells, Williams et al., 1988; and glio-neuronal precursor cells, Evrard et al., 1990. In this study, a recombinant retroviral vector coding for the tsA58-U19 sub-genomic construct was utilised which has previously shown to efficiently establish primary rat fibroblast cells in culture, Jat and Sharp, 1986. Cells expressing the foreign genes are growth restricted at the non-permissive temperature, Jat and Sharp, 1989.

By dissecting the rat mammary gland parenchyma at the cellular level using these novel techniques, and propa-gating cells with combinations of hormones and growth factors known to favour rat mammary parenchymal cell growth, (see Hallowes et al., 1977; Richards and Nandi, 1978b; Pasco et al., 1982; Richards et al., 1983), it was hoped to define more precisely the beha-viour of the individual cell populations in a culture environment.

CHAPTER 2.

GENERAL METHODS.

2.1 Cell culture.

Sources of all reagents and materials used for cell cultures are given in the appendix.

2.1.1 Cell Lines Used.

Table 1 details the nomenclature, properties and original references of previously published cell lines used during this study. All cell lines were proven mycoplasma free during periods of use (see 2.1.5).

CELL LINES USED IN THIS STUDY.

NAME	BIOLOGICAL PROPERTIES	REFERENCE
3T3 L1	ADIPOCYTIC DIFFERENTIATION IN RESPONSE TO ADDED GLUCOCORTICOIDS.	Green and Kehinde, (1974)
3T6	TRANSFORMED MOUSE FIBROBLAST CELL LINE.	Todaro and Green, (1963)
WI38-VA13	SV40 VIRUS-TRANSFORMED HUMAN EMBRYONIC LUNG FIBROBLASTS.	Girardi et al., (1966)
2.13-EP	HAT-SENSITIVE CLONAL DERIVATIVE OF RAMA 25 CELLS.	O'Hare (unpublished)
MAT-B	RAT MAMMARY ADENOCARCINOMA ASCITES DERIVED CELL LINE	Carraway et al., (1978)
NIH 3T3	CONTACT-INHIBITED MOUSE FIBROBLAST CELL LINE	Jainchill et al., (1969).

Table 1.

2.1.2 Culture media.

Different media types used are listed in Table 2. Media additives and the concentrations used throughout this study are given in Table 3.

TYPES OF MEDIA USED.

MEDIA COMBINATION	USAGE
DMEM:HAM'S F12(1:1)/ FCS/ PS	BASAL MEDIUM FOR MAMMARY CELL CULTURE.
BIORICH 2	SERUM-FREE MEDIUM FOR MAMMARY CELL CULTURE.
DMEM/ FCS/ PS	ROUTINE CULTURE MEDIUM FOR STOCK CELL LINES.
L15/ FCS/ PSKFM	HOLDING MEDIUM FOR DISSECTED TISSUE.
	COLLECTION OF SORTED CELLS FROM CYTOMETER.
L15/ FCS/ DMSO	'FREEZING' MEDIUM.
L15/ FCS/ COLLAGENASE / PSKFM	'DIGESTION' MEDIUM FOR ORGANOID PREPARATION.
DMEM:HAM'S F12/ FCS/ G418	BASAL 'SELECTION' MEDIUM.

Table 2.

MEDIA ADDITIVES AND CONCENTRATIONS USED.

ADDITIVE	CONCENTRATION
INSULIN	5ug/ml
HYDROCORTISONE	1ug/ml
CHOLERA TOXIN	10ng/ml
EPIDERMAL GROWTH FACTOR	10ng/ml
PROGESTERONE	100ng/ml
PENICILLIN\STREPTOMYCIN	100µg/ml
MINOCYCLIN	1µg/ml
KANAMYCIN	100µg/ml
FUNGIZONE	2.5µg/ml
G418	0.2-1mg/ml
FETAL CALF SERUM	10% v/v
DMSO	10% v/v
COLLAGENASE (TYPE 1A)	0.4% w/v

Table 3.

2.1.3 Culture conditions.

All cultures were propagated in the appropriate medium at 37^{o}C in a humidified, 5% v/v CO_2:air atmosphere. Cloning cultures were maintained in an incubator set aside solely for such experiments. Temperature shift experiments were performed in dedicated incubators set at 34^{o} or 39^{o}C.

2.1.4 Routine subculture.

All cell lines were routinely passaged at confluence in 25cm^2 plastic culture flasks with a 1:10 split ratio. The 3T3 L1 cell line was specifically replated at a density of 1x10^3 cells per mm^2 as recommended by Levine and Stockdale, 1984. Subculturing was carried out in a Class II laminar flow cabinet.

Fibroblastic lines: Cultures were briefly rinsed with versene (0.02% w/v in EDTA in calcium and magnesium-free Dulbecco's phosphate-buffered saline solution) and then incubated in 1mg/ml^{-1} trypsin (type III, porcine pancreatic) in versene at 37^{oC} for 3 minutes. Trypsinisation was stopped by the addition of 5 mls L15/ 10%v/v FCS. Cells were then harvested, pipette flushed and washed twice with L15/FCS by repeated centrifugation and resuspension in fresh medium.

Epithelial lines: Cultures were incubated in versene solution for 5 minutes. This was removed and replaced with 2mg/ml^{-1} trypsin/ versene. Cells were then incubated at 37^{o}C for a further 5 minutes, and then harvested as above.

2.1.5 Determining the mycoplasma status of cell lines.
Cells cultured on glass coverslips were rinsed twice with PBS then fixed in absolute methanol for 20 minutes at -20^{o}C. After removing the methanol and rinsing twice with distilled water, coverslips were incubated in a 2μg/ml^{-1} bisbenzimide (Hoechst 33258) solution for 10 minutes. Coverslips were finally rinsed three times with distilled water and mounted by standard procedure (see 2.5.1 below). Cells were screened under ultraviolet epi-illumination for extra-nuclear fluorescence indicative of mycoplasma DNA.

2.1.6 Freezing cells.
Cells trypsinised by standard protocol were finally resuspended in 1ml L15/ FCS containing 10% v/v dimethyl sulphoxide (DMSO) at a concentration of 10^{6}-10^{7} cells per ml, and frozen in 1ml aliquots. Vials were cooled slowly in the gas phase above liquid nitrogen, using a freezing head and the manufacturers recommended tim-

ings, and stored immersed in liquid nitrogen.

2.1.7 Thawing cells.

Vials containing frozen cells were thawed rapidly at 37°C in a water bath. The 'freezing' medium containing cells was transferred into a 25cm^2 flask and 1ml of fresh growth medium added dropwise. After slowly adding another 3 mls of fresh medium, cells were incubated overnight after which time the media was removed and cultures re-fed with the requisite fresh growth medium.

2.1.8 Preparation of feeder cells.

Cells were prepared as a cell suspension by standard trypsinisation procedure, and finally resuspended in 10 mls L15/ 10%FCS. The suspension was then exposed to a ^{60}Co source for 3 minutes. This resulted in a dose of approximately 20Gy. For all experiments, irradiated cells were plated in DMEM/ FCS at least one hour prior to the addition of epithelial cell suspensions, at a density of 50 cells per mm^2 area of tissue culture surface.

2.1.9 Preparation of conditioned medium.

Fresh medium exposed to 80-90% confluent cultures for 72 hours was collected, passed through a 0.45μm filter (FlowporeD) and hormones added at standard concentrations (see Table 3). Conditioned medium was used at 1:3 dilutions with the requisite fresh growth medium.

2.1.10 Growth Curves.

Cells were trypsinised by standard procedure and the suspension adjusted to 10^4 cells per ml in plating media. 1ml of this suspension was aliquoted into each well of a 24-well plate, which was then incubated under standard conditions. At selected time points, cells from three wells were trypsinised in 1ml trypsin for 10 minutes. The dispersed cells were then removed and the plates returned to the incubator. Trypsinised suspensions from each well were counted separately with a haemocytometer. Three separate counts were made for each well.

2.2 Preparation of organoids.

2.2.1 Dissection.

All procedures were performed under sterile conditions in a class III laminar flow cabinet. Rats were killed by cervical dislocation and pinned out dorsally then sprayed lightly with a 70% alcohol aerosol. An incision was made from the vulval region to the thoracic region taking care to ensure that the peritoneum remained intact. Incisions were then made from the vulval region to the knee on each lower leg. With forceps and scalpel, the pelt with mammary glands attached was reflected back off the peritoneal wall, and pinned out. Lymph nodes and the macroscopically visible major blood

vessel were dissected out of the abdominal/ inguinal mammary fat pad which was then gently pared off the pelt with a scalpel and placed in L15/ FCS containing all antibiotics at standard concentrations. Both fat pads were removed. 12 animals were used for cytometric analysis or sorting experiments, while 6 animals yielded sufficient material for several concurrent cloning experiments.

2.2.2 Organoid preparation.

The methodology used was a modification of previously published protocols by Hallowes et al., 1977, and Ehmann et al., 1984. The dissected fat pads were finely minced with a McIlwain tissue chopper and the homogenised tissue from three glands was suspended in 10mls collagenase containing 'digestion medium' (see Table 3) in a sterile 30ml 'universal' container and placed on ice until all dissected glands had been chopped. The tissue was subjected to a primary digestion period of 1 hour at 37^{o}C, after which time 40 mls of the digestion mixture was passed once through a 100µm nylon filter. The filter was washed with 20mls of sterile PBS, removed from the filter assembly with sterile forceps and placed in a 90mm^{2} petri-dish. 10mls of fresh 'digestion medium' was added, and the retained tissue gently resuspended using a cell scraper The resuspended tissue was then poured off into a fresh

universal and incubated for a further 2 hours. After this incubation period, the digestion mixture was centrifuged for 3 minutes at 1200 r.p.m. The pelleted organoids were taken up into a 2ml pipette, resuspended in L15/ FCS in a fresh universal and washed 5 times by repeated centrifugation and resuspension in fresh L15/ FCS medium to remove residual collagenase.

The parenchymal fragments were separated from fibroblastic/ stromal cells by plating the digested tissue in DMEM/ FCS for 2 hours in a $25cm^2$ plastic culture flask, during which time most the stromal cells attached while the organoids remained in suspension. The organoids were then harvested by collecting the 'preplating' supernatant. At this stage the organoid suspension could be left overnight at 4^oC in L15/ FCS containing all antibiotics.

2.2.3 Organoid Culture.

'Preplating' supernatant was collected, centrifuged at 1200 r.p.m. for 3 minutes and the purified organoids counted, resuspended and plated out in the appropriate growth medium. After 48 hours when the organoids had attached, the cultures were washed with fresh medium thus removing residual unattached debris, and re-fed with fresh growth medium. The cultures were fed with fresh growth medium every three days thereafter.

2.3 Cloning.

2.3.1 Primary cloning of parenchymal preparations.

Freshly prepared organoids were pelleted then washed twice in versene solution by successive pelleting and re-suspension. The pellet was then resuspended in Ca^{2+}-free/ serum-free DMEM and incubated at 37°C for 15 minutes. After centrifugation, the supernatant was removed and the pellet resuspended in 2mls 1mgml^{-1} trypsin/ versene and incubated at 37°C for 5 minutes, after which time an additional 2mls of fresh trypsin/ versene was added and the suspension gently pipette flushed, then incubated for a further 2 minutes. 5mls of serum-free L15 medium containing DNAase (Type I pancreatic) at a concentration of 5μgml^{-1} was added to the digestion solution, which was flushed repeatedly and then incubated for a further 5 minutes. The digestion was stopped by addition of 10mls L15/ FCS, and the preparation filtered through 30μm nylon mesh. The filtrate was washed twice with L15/ FCS by repeated pelleting and resuspension in fresh medium. After the final rinse single cells were suspended in 5 mls and counted. Cell suspensions were cloned at a ratio of 1:50 or 2:50 mammary cells: feeder cells per mm^2 in 6-well plates. Clone cultures were initiated in 7 mls of the requisite growth medium and were not re-fed during propagation.

2.3.2 Cloning of isolated duct and alveolar fragments. 500µl of freshly prepared organoid preparation was diluted into 10 mls L15/ FCS containing all antibiotics, and the suspension transferred to a 90mm petri-dish. Under phase-contrast at 6x objective magnification isolated organoids that were clearly identifiable as being either duct or alveolar fragments were separately taken up in a 2ml pipette and transferred into one of two centrifuge tubes. Care was taken to ensure that all media in the pipette tip was expelled. Ten fragments of each structure were separately collected , pooled and trypsinised by standard procedure. Trypsinisation was stopped by the addition of 5 mls of serum-containing medium which was pipette flushed five times. The suspension was then serially diluted 1:3 in 6-well plates in the requisite growth medium. Cultures were then propagated under standard cloning conditions.

2.3.3 Cloning organoid explant cultures.

Cultures were rinsed twice with versene solution, incubated for 15 minutes in Ca^{2+}-free/ serum-free DMEM then trypsinised by standard sub-culture technique. Cells were plated out at standard cloning densities as detailed above.

2.3.4 Ring cloning.

Cultures intended for ring cloning were plated in either petri-dishes or 6-well plates. Well-spaced colonies were outlined on the base of the dish. Culture media was pipetted off and the plates then rinsed with versene solution. Stainless steel cloning rings with 6mm internal diameter were sterilised in 70% ethanol then air dried in the flow of a sterile cabinet. Their bases were smeared with silicone high-vacuum grease and placed over marked clones and then firmly pressed down with forceps. Using a Pasteur pipette, one drop of $1mgml^{-1}$ trypsin/versene was added to each ring. After replacing the lid, plates were incubated for 5 minutes at $37^{o}C$. Cells were removed from the cloning ring by flushing several times with a Pasteur pipette containing 0.5mls of L15/ FCS, then spun down and resuspended in plating medium. Each ring clone was plated out into one well of a 24 well plate previously seeded with freshly irradiated feeder cells.

2.3.5 Dilution cloning of cell lines.

The culture to be cloned was grown to 60% confluence in $25cm^2$ flasks. Cell suspensions were prepared by standard protocol, cell numbers determined, and a suspension containing $2x10^3$cells ml^{-1} prepared. 100µl of the requisite plating medium was pipetted into each well of a 96-well plate, and the cell suspension inoculated

into each well in the first row of the plate and serially diluted down each row in 100µl aliquots using an octapipette. The final 100µl residue was discarded. During the subsequent 24 hours, the plates were examined and wells containing single cells marked. The cultures were incubated undisturbed for periods of up to 1 month, after which time the growing cells were harvested by standard protocol and replated individually into separate 24 well plates. Depending on the growth rate of the cells, cultures were expanded into $25cm^2$ flasks after a further 2 weeks. After confluence was attained during the first passage in flasks, cells were passaged 1:10 and the remainder banked down in liquid N_2 and thereafter banked down at every other passage.

2.3.6 Determining cloning efficiency and clone type frequency.

Cultures were fixed for 48 hours in a 0.25% v/v 2.5% stock solution of glutaraldehyde in PBS. Plates were then rinsed in water and stained for 48 hours with Erlich's hematoxylin. The dye was differentiated in running tap water and the plates air dried. Stained colonies were counted and the frequency of specific clone types scored on the basis of morphology.

2.4 Collagen gels.

2.4.1 Preparation of stock collagen.

Tails were collected from twelve 70-day rats and soaked in 70% alcohol overnight. The skins were peeled back and the collagen fibres stripped off, cut up into small fragments and thoroughly washed in PBS to remove blood cells. The collagen fibres were then air dried in the sterile air flow of a microbiological cabinet and their dry weight determined. A 0.6% w/v stock solution of collagen was made by dissolving the fibres in 0.01M acetic acid with continuous stirring at 4^{o} C for 4 days. The solution was then centrifuged at 15000 r.p.m. for 4 hours in sterile centrifuge buckets, to pellet undissolved fragments and adventitious particles. The supernatant was poured off and stored at 4^{o} C until required.

2.4.2 Preparation of gels.

An aliquot of collagen stock solution and a solution composed of 2 parts 10x DMEM concentrate, with added glutamine and pyruvate at standard concentrations mixed with one part 0.34N NaOH, were kept separately on ice. Keeping the solutions on ice to prevent gelation, 0.4mls of the neutralising solution were titrated into 2mls of collagen stock. 1ml of solution was then poured into wells of a 24 well plate, taking care to avoid

introducing air bubbles into the gel solution, and set at 37°C. The gels were then incubated with the requisite growth medium overnight in order to equilibrate the gel pH.

2.4.3 Cell culture on collagen gels.

The washing medium was removed and 10^4 cells per well were plated onto the gel surface and fed with 1ml of the requisite growth medium, and fed thereafter every three days.

2.5 Immunofluorescence.

Tables 4, 5, 6, 7 and 8 catalogue all primary antibodies, polyclonal antisera and secondary antibodies used during this study. Their antigens and working dilutions are also listed.

CATALOGUE OF PRIMARY ANTIBODIES:
MEMBRANE ANTIGENS.

ANTIBODY	ANTIGEN	DILUTION	REFERENCE/SOURCE
25.5 (IgG2a)	70KD extra-cellular membrane glycoprotein.	Neat	Mahendran et al., 1989
DAKOCALLA (IgG1)	Endopeptidase E.C 3.4.24.11. (CALLA).	Neat	Dakopatts
PHM6 (IgG2b)	Endopeptidase E.C. 3.4.24.11.	1:200	Pilkington et al., 1984
anti-MFGM polyclonal	Rat milk fat globule membrane.	1:50	Warburton et al., 1982.

Table 4.

CATALOGUE OF ANTI-CYTOKERATIN ANTIBODIES.

ANTIBODY	ANTIGEN RECOGNISED	DILUTION	REFERENCE/SOURCE
6B10	Human cytokeratin 4	1:5	Euro-Diagnostics
RCK 105	Human cytokeratin 7	1:5	Euro-Diagnostics
LE 41	Human cytokeratin 8	neat	Lane, 1982.
M20	Human cytokeratin 8	1:5	Euro-Diagnostics
RKSE 60	Human cytokeratin 10	1:5	Euro-Diagnostics
1C7	Human cytokeratin 13	1:5	Euro-Diagnostics
LL001	Human cytokeratin 14	neat	Leigh et al., 1988.
LE61	Human cytokeratin 18	neat	Lane, 1982.
C-04	Human cytokeratin 18	neat	J.Bartek
RGE 53	Human cytokeratin 18	1:5	Euro-Diagnostics
LP2K	Human cytokeratin 19	neat	Lane et al., 1985.
BA16	Human cytokeratin 19	neat	Bartek et al., 1985.
C11.40	Human cytokeratins 8,13	neat	J. Bartek
C46.2	Human cytokeratins 7,17	neat	J. Bartek
LP34	Human cytokeratins 4,5,6,10,3,14,18	neat	Taylor-Papadimitriou et al., 1983.

Table 5.

CATALOGUE OF PRIMARY ANTIBODIES: CYTOSTRUCTURAL ANTIGENS.

ANTIBODY	ANTIGEN	DILUTION	REFERENCE/SOURCE
MVI	Calf lens vimentin	1:5	Euro-Diagnostics
DAKO-vimentin	Swine lens vimentin	1:10	Dakopatts
1A4	Smooth muscle a-actin	1:100	Sigma
Rabbit antiserum	Laminin	1:50	M.J.Warburton
Rabbit antiserum	Type IV collagen	1:50	M.J.Warburton

Table 6.

CATALOGUE OF PRIMARY ANTIBODIES: NUCLEAR ANTIGENS.

ANTIBODY	ANTIGEN	DILUTION	REFERENCE/SOURCE
anti-BrdUrd	5-Iodo-uridine	1:10	Amersham
Pab 412	SV40 large T	neat	Harlow et al., (1981).
Pab 419	SV40 large T	neat	Harlow et al., (1981).

Table 7.

CATALOGUE OF SECONDARY ANTIBODIES.

SPECIFICITY	FLUOROCHROME	DILUTION	SOURCE
anti-mouse	FITC	1:40	Amersham
anti-mouse IgG1	FITC	1:40	Amersham
anti-mouse IgG1	TRITC	1:40	Southern Biotechnology Associates
anti-mouse IgG2a	FITC	1:40	Southern Biotechnology Associates
anti-mouse IgG2a	R-Phycoerythrin	1:40	Southern Biotechnology Associates
anti-rabbit	FITC	1:40	Amersham

Table 8.

2.5.1 Indirect immunofluorescence of membrane antigens. 13mm diameter coverslips were rinsed in acetone and sterilised by storage in 70% ethanol. Prior to use they were thoroughly rinsed several times with PBS followed by L15/ FCS. Cells were cultured on coverslips placed in 4-well plates. Staining was performed on viable cells and antibodies were diluted in L15 containing 10% FCS v/v in order to minimise non-specific staining. Prior to the initiation of staining plates were placed on ice and left for 3 minutes, then washed with two changes of ice cold L15/ FCS. All subsequent steps were performed over ice. Each coverslip was incubated in 200µl of primary antibody for 45 minutes. The primary antibody was then removed and cells rinsed with two changes of ice-cold L15/ FCS. 200µl of second antibody was then added and incubated for a further 45 minutes. After rinsing twice in ice-cold L15/ FCS, coverslips were removed from the 4-well plates with dissection forceps and mounted cell surface-down on a glass slide in a 1:1 mixture of Hydromount and the appropriate type of Citifluor anti-quenching mountant (see appendix). After the mountant had set, coverslips were ringed with clear nail varnish in order to permanently preserve the preparations.

2.5.2 Indirect immunofluorescence of basement membrane, cytostructural and nuclear antigens.

Frozen sections: Portions of mammary gland were dissected out, coated with cryomatrix embedding medium, and then immersed in liquid N_2. Tissue was further stored under N_2 until use. 6-10μm thick sections were cut using a temperature-regulated cryostat by standard methods. Sections were placed onto glass slides and briefly fixed for 3 minutes with acetone at -20°C. Sections were incubated for one hour with primary antibody, washed with PBS, incubated with fluorescent second antibody for one hour. After washing with PBS, sections were covered with a few drops of a 1:1 mixture of Citifluor and Hydromount and overlaid with a glass coverslip.

Cell cultures: Cells grown on coverslips were rinsed twice with PBS in order to remove cellular debris and serum proteins, then immersed in ice-cold absolute methanol for 20 minutes at -20°C. A subsequent permeabilisation step was performed in glass dishes in which coverslips were immersed in acetone at -20°C for 60 seconds. This enabled preparations to be air dried and stored at 4°C . Coverslips were replaced into 4-well plates for staining, and 200μl of appropriately diluted primary antibody was added per well and a small volume of water aliquoted into the base of the dishes to

prevent drying out. Plates were incubated overnight at 37°C. After this time, coverslips were rinsed 5 times with sterile distilled water and the appropriate second antibody incubated for 1 hour. After rinsing 7 times in distilled water, coverslips were removed from the dishes and mounted as described in section 2.5.1.

Control samples consisted of cultures fixed and processed as above stained with second antibody only.

Single-labelling studies were performed using either sheep anti-mouse or donkey anti-rabbit fluorescein isothiocyanate conjugated second antibodies as appropriate (see Table 8).

2.5.3 Detection of total F-Actin in cultured cells.
F-actin was visualised using either fluorescein or rhodamine isothiocyanate-phalloidin conjugate in a manner essentially identical to the published method of Verderame et al., 1980. Cells cultured on coverslips were fixed in 10% w/v formol saline for 20 minutes, rinsed with PBS, and extracted with 1% v/v Nonidet P40 in PBS for a further 20 minutes. After rinsing the coverslips twice with PBS, 200µl phalloidin-FITC or TRITC conjugate at a concentration of $1\mu g ml^{-1}$ in PBS was added and incubated at 37° C for 30 minutes. After rinsing twice with PBS, coverslips were mounted as

described in section 2.5.1. Control samples consisted of a 100 fold excess of unlabelled phalloidin mixed with labelled conjugate at standard concentration, and incubated as above.

2.5.4 Determining labelling indices of cell cultures.
Clone cultures set up on glass coverslips were incubated at various time points with fresh pre-warmed growth medium containing 40μM 5-bromo-2'-deoxyuridine for 2 hours. After fixation by standard methanol-acetone protocol (see 2.5.2), incorporated BrdUrd was visualised by indirect immunofluorescence using an anti-BrdUrd monoclonal antibody solution containing active nuclease, followed by sheep anti-mouse fluoresceinated second antibody. The preparations were then incubated in a $0.1 mg ml^{-1}$ w/v solution of propidium iodide in distilled H_2O for 5 minutes in order to stain all nuclei present. Coverslips were then mounted as described in section 2.5.1.

2.6 Cytometric analysis and fluorescence activated cell sorting.

2.6.1 Staining cells for antigen analysis and cell sorting.
Single-label sorts: Single cell suspensions prepared as

described in Section 2.3.3 were labelled separately with either with 25.5 or PHM6 (see Table 4) for 45 minutes over ice (400µl of antibody per 10^6 cells in a sterile plastic centrifuge tube). Cells were then washed four times in ice-cold L15/ FCS medium by repeated centrifugation at 1500 rpm for 5 minute periods and resuspension in fresh medium, and then incubated separately with 200µl sheep anti-mouse FITC second antibody (see Table 8) diluted in L15/ FCS medium for a further 45 minutes. Cells were then washed four times in ice-cold L15/ FCS medium by repeated centrifugation and resuspension in fresh medium, and held over ice in this medium until analysis.

Double-label sorts: Suspensions prepared as described above were simultaneously incubated with 200µl aliquots of both antibodies 25.5 and DAKOCALLA mixed together, for 45 minutes on ice. The DAKOCALLA antibody was used for double-label experiments because unlike PHM6 (subclass IgG2b), its IgG1 subclass was sufficiently different from that of 25.5 (an IgG2a antibody) to allow differential staining using class-specific second antibodies. Suspensions were then washed 5 times in ice-cold L15/ FCS medium, and then incubated for a further 45 minutes with a mixture of 200µl aliquots of both IgG2a-FITC and IgG1-PE class-specific second antibodies each diluted 1:20 in ice-cold L15/10%FCS

medium. These isotype-specific second antibodies bound 25.5 or DAKOCALLA, respectively. After this period, suspensions were washed 5 times in ice-cold L15/10%FCS medium, and held in this medium until analysis or sorting.

Controls: Control samples consisted of cell suspensions stained only with fluoresceinated second antibody, or in the case of double-label experiments, samples stained with one primary antibody were incubated with the reciprocal non-specific second antibody.

2.6.2 Staining cells for cell cycle analysis.
Two different methods of cell cycle analysis were employed depending on whether or not a simultaneous analysis of an independent marker was performed.

Simultaneous analysis of cytokeratins and cell cycle profiles: Cells suspension were prepared as described in Section 2.3.3, and then rinsed twice in PBS. Suspensions were centrifuged at 1500rpm for 5 minutes, and the PBS then aspirated off. Cells were then rapidly resuspended in absolute methanol at -20^{o}C. The vial containing cells suspended in absolute methanol was then placed in a -20^{o}C freezer for 20 minutes. Cells were then pelleted, washed twice in PBS and resuspended in PBS containing 10mM EDTA and 0.1% w/v BSA. 200µl of

the appropriate anti-cytokeratin primary antibody were added and incubated for 1 hour over ice. After this time cells were washed twice in PBS/EDTA/BSA, 200µl sheep anti-mouse-FITC added and suspensions incubated for a further hour. After washing twice in PBS/EDTA/BSA, cells were suspended in 800µl PBS and then 100µl of 100µgml^{-1} propidium iodide (PI) w/v in PBS/EDTA solution added followed by 100µl of 1mgml^{-1} RNAase solution in PBS/EDTA. Suspensions were then incubated at room temperature this solution for 15 minutes prior to analysis.

Analysis of cell nuclei: This procedure was employed for the analysis of cell cycle profiles only. Cell suspensions were prepared as described above, pelleted and then resuspended in 1ml of stain-detergent solution. This solution, which lysed cells thus releasing the nuclei, contained the following reagents- 1mgml^{-1} trisodium citrate; 0.56mgml^{-1} NaCl; 0.01mgml^{-1} ethidium bromide; 0.3µlml^{-1} Nonidet P-40 and 0.01mgml^{-1} RNAase - in distilled H_2O. Suspensions were briefly agitated using mechanically, then left on ice for 15 minutes prior to analysis.

2.6.3 Cytometric analysis and cell sorting.

Antibody analysis: Cells were analysed and sorted using an Ortho 50H Cytofluorograf linked to a 2150 computer

system. It was equipped with an argon-ion laser tuned to give 50mW at 488nm. Recorded parameters were light scattered orthogonally and at a narrow forward angle at 488nm; green fluorescence at 520nm (from FITC excited at 488nm) and red fluorescence at 576nm (from R-phycoerythrin also excited at 488nm) measured as appropriate in either single or double-labelled preparations. Scattered light was displayed by the computer in the form of a cytogram. Gates were then set within the displayed region to exclude cell clumps and debris. The profile of fluorescently labelled cells within this region was then displayed. Dead cells were excluded from analyses and sorting of single antibody labelled samples on the basis of PI ($10\mu gml^{-1}$) uptake. PI and FITC were excited simultaneously at 488nm, and red PI fluorescence was detected at 639nm. The overlap between PI and R-phycoerythrin emission wavelengths (639nm and 576nm emission maxima, respectively) meant that exclusion of dead cells from analysis of double-labelled (FITC/PE) samples was not possible.

Single label sorts: Cells were sorted from a flow stream of sterile PBS, and using anti-coincidence circuitry one drop was deflected for each sort command. Cells positively labelled (FITC-positive) and those not labelled (non-fluorescent) were simultaneously sorted and collected from separate streams. This was performed

separately for each primary antibody used.

Double-label sorts: Cells positive for either 25.5 staining (FITC-positive) or DAKOCALLA staining (R-PE-positive) were sorted simultaneously and collected from separate streams. Cells which did not stain with either antibody (FITC and R-PE negative) were sorted from regions delimited by re-adjusted gates in separate sorts.

Cell cycle analysis: Forward and orthogonal light scatter and red fluorescence (PI emission at 639nm excited at 488nm) were measured for single label preparations. Both the peak and area of the red fluorescent signal was measured. For samples double-labelled with PI and an antibody, green (antibody) fluorescence (520nm) was also measured. This enabled the DNA content (red fluorescence) of cells positively labelled by antibodies to be measured.

2.6.4 Culture of flow sorted cells.

Cells were collected in 4-well tissue cultures dishes containing L15/ FCS medium supplemented with hormone and antibiotic additives (see Table 2). Unstained (single-label sorts) or differentially stained cells (double-labelled sorts) were collected in adjacent wells in the same plate. After a brief phase-contrast

examination of the cells to confirm that only single cells had been acquired, plates were incubated at 37°C. When the sorted cells had settled (2-3 hours), the collecting medium was gently aspirated and replaced with 5% CO_2 buffered growth medium (see Table 2). Alternatively, if clonal growth cultures were to be initiated, the sorted cells were removed from the 4-well plates using a plastic pipette, and then serially diluted 1:6 into the wells of 6-well plates seeded 2 hours previously with irradiated feeder cells and containing growth medium.

2.7 Retrovirus infection of cell cultures.

2.7.1 Determining virus titre of producer cells.

All manipulations using ecotropic virus producing cells were performed under category 1 containment conditions as specified by the appropriate GMAG recommendations. Virus titre was determined for two different methods of infection, using either virus containing supernatant medium or irradiated virus-producing cells.

Culture supernatant: 70-80% confluent psi-2 helper cells were fed with fresh medium. After overnight incubation, medium was collected and filtered through 0.45μm filters. Polybrene was added to a final concen-

tration of 8μgml^{-1} and the medium then serially diluted in fresh DMEM/ FCS. Media was removed from cultures of NIH 3T3 cells seeded at concentrations of 10^2, 10^3, 10^4, 10^5 and 10^6 cells per 80cm^3 flask and replaced with 5mls of neat virus-containing supernatant or 1:10, 1:100 or 1:1000 dilutions of supernatant. After 2 hours exposure at 37^{o}C, supernatant was removed and 10mls of fresh medium added. After two days propagation at 37^{o}C, cultures were fed with selection medium containing 0.5mgml^{-1} G418 and grown on for a further 10 days at 34^{o}C. Cultures were fixed and stained as described in section 2.3.6 and the number of selection resistant colonies determined.

Irradiated virus-producing cells: NIH 3T3 cells were seeded at either 10^4or 10^5 cells per well in each well of a 6-well plate, and co-cultured with either 10^4, 10^5 or 10^6 irradiated virus-producing cells, (approximately 20Gys exposure). Cultures were grown for 2 days at 37^{o}C then for a further 5 days at 34^{o}C in selection medium. After this time, plates were fixed, stained and the number of selection resistant colonies determined.

2.7.2 Retrovirus infection of primary cultures.

Two infection protocols were used - Supernatants were harvested from 80% confluent virus-producing cells 16 hours after the addition of fresh medium. The virus-

containing medium was filtered through 0.45μm filters and used immediately. Primary explant and passaged stromal cultures were exposed to fresh virus containing supernatant with added polybrene at $8\mu gml^{-1}$ for two hours at $37^{o}C$, after which time fresh culture medium was added and the cultures propagated at $37^{o}C$. Cultures were challenged with selection medium containing G418, at $1mgml^{-1}$, Davies and Jimenez, 1980, after a further 48 hours growth at $37^{o}C$. Some cultures were passaged immediately after exposure to virus containing-medium then treated in the usual manner.

Standard clonal growth cultures propagated under different growth regimes were seeded with irradiated virus-producing cells. These cells were added at concentrations of either 10^{5}or 10^{6}cells per well. Cultures were incubated at $37^{o}C$ for 48 hours, then propagated for 72 hours at $34^{o}C$ before challenge with selection medium containing G418 at $0.2mgml^{-1}$ and the requisite hormones and growth factors at standard concentrations. At confluence, all infected cultures were passaged as bulk un-cloned cultures or re-cloned as described in sections 2.1.4 and 2.3.5.

2.8 Transplantation and xenografting.

2.8.1 Animals.

Animals of varying ages were used as recipients and donors for transplantation experiments. Wistar/ Furth/ CBI rats were used throughout this thesis, and when required Olac nu/nu mice were used for xenograft experiments. For cleared fat pad transplants, neonatal animals aged between 17 and 21 days were used as hosts and kept caged with mothers prior to and for 1 weak after the operation. Interscapular fat pad transplants were performed on both 21 day neonatal and 70 day mature virgin female animals.

2.8.2 Anaesthesia.

Animals were anaesthetised by an oxygen/ halothane mixture. Initial anaesthesia was induced by 6% halothane and animals maintained under 7% halothane, or 2% if young neonates, during the duration of surgery. Animals kept under anaesthetic for 20 minutes during experimental manipulations resuscitated in less than 5 minutes with no apparent side effects.

2.8.3 Preparation of cell cultures for transplantation.

Each culture was grown to 60-70% confluence in 80cm^2 flasks and trypsinised into single cell suspension by standard protocol, with the final rinse and suspension in serum-free L15 medium. Cell numbers were adjusted to

10^8cells ml^{-1} and 0.1mls of cell suspension was injected per transplant using a 23 gauge needle. Suspension were kept on ice until transplantation.

2.8.4 Dissection of glandular structures for transplantation.

21-day old neonatal animals were injected sub-cutaneously with 0.5mls of injection grade Methylene Blue into the abdominal/ inguinal fat pad, or intraperitoneally with 0.5mls of a 1% w/v solution of Trypan Blue in PBS the evening before dissection. The following morning animals were killed by cervical dislocation and the mammary fat pad dissected out. Under a dissecting microscope at 12x magnification, terminal end buds were clearly visible as blue stained structures, and were dissected out with attached stroma using spring scissors. Dissected structures were held in serum-free L15 medium until transplantation.

2.8.5 Cleared mammary fat pad transplants.

The procedures performed were a modification of those described by DeOme et al., 1958. All transplant manipulations were performed under aseptic conditions. Each 35-day neonatal animal was lightly anaesthetised under 2% halothane. It was then removed from the apparatus and its stomach shaved, then replaced in the anaesthetic mask dorsally, and maintained under 4-5% halothane.

The ventral surface was sprayed with a 70% ethanol aerosol, and an incision made along the abdominal midline. This incision was then continued laterally between the fifth and sixth nipples midway down each hind leg. The wound was then moistened with sterile PBS. The skin flap with fat pad attached was then carefully peeled back by blunt dissection to reveal the fifth inguinal fat pad and pinned out. The nipple area and major blood vessels infiltrating the fat pad in this area were cauterised. Using vital staining to delineate the general extent of mammary development in an other animal at this stage, a comparable portion of fat pad containing growing parenchyma was dissected out of recipient animals. Transplants of dissected glandular structures were then forced into the most distal portion of the cleared fat pad by using fine forceps, or 0.1ml of cell suspension was injected using a gauge 25 needle. Both sides of the animal received transplants. The flaps of skin were then re-positioned and the wounds sealed with surgical staples, and the animals placed in freshly prepared cages with their mother.

2.8.6 Interscapular fat pad transplants.

The transplantation procedures performed were a modification of those described by Ormerod and Rudland, 1986. Each recipient animal was lightly anaesthetised under

6% halothane flowing into a 'killing' box. It was then removed from the box, the area of skin between the shoulder blades shaved, and then placed ventrally into the anaesthetic mask and maintained under 6% halothane. The back of the animal was then lightly sprayed with a 70% ethanol aerosol. Holding the skin raised with forceps, a full thickness, 5 mm incision was cut just dorsal to the shoulder blades to reveal the underlying adipose tissue. The wound was moistened with sterile PBS. Using a fresh set of forceps, a portion of the fat pad was gently pulled out through the incision, held and then injected with transplant material. The fat pad was then re-positioned under the skin, the wound sealed with surgical staples. The animals were then placed in a freshly prepared cages and kept under normal husbandry conditions.

2.8.7 Preparation of wholemounts.

Fat pads were dissected out and stretched over one side of a glass microscope slide. The slide with adherent tissue was then immersed in formal saline for 48 hours, washed with distilled water several times, then immersed in Erlich's hematoxylin for a further 48 hours. The tissue was then soaked in several changes of acid water over three/ four days until all excess dye was leached out. The preparations were then soaked in alkaline water for 48 hours, dehydrated through a

series of 50-70-100% ethanol for 24 hours each, then cleared and stored in methyl salicylate.

2.9 Electron Microscopy.

Transmission electron microscopy: Cell cultures and organoid preparations were fixed in 2% phosphate buffered glutaraldehyde (0.05M, pH7.3, osmotic pressure made up to 350mML^{-1} with sucrose), post-fixed in osmium tetroxide, dehydrated in graded ethanol solutions and embedded in epoxy resin. Sections were cut on a Reichert Ultracut OMU4 ultramicrotome using a diamond knife, and then double stained with uranyl acetate and lead citrate in an LKB ultrastainer. Cells were examined in a Phillips CM10 electron miscroscope.

Scanning electron microscopy: Cell cultures were fixed as above, dehydrated, critical point dried and sputter coated with carbon-gold. Preparations were examined using an ISO-SS40 scanning electron microscope.

2.10 Photomicrography.

Phase-contrast photographs of living cultures were taken with a Wild-Heerbrugg M40 microscope-camera fitted with inverted optics, using Ilford Ortho-pan 6.5x9cm plates. Indirect immunofluorescence stained cultures were visualised using a Ziess vertical illuminator IV FL system fitted with pan-neofluar objec-

tives. Incident light was produced by a high pressure mercury lamp (HBO 50W/AC illuminator), and an exchangeable 2-filter system fitted with narrow-band pass interference filters was used for selective blue or green excitation. Green and red epi-fluorescence were recorded on Kodak T-Max or Tri-X black and white or Kodak EES colour films (which were 'push-processed' during development) using an 'in-line' Olympus 35mm C-35AD camera linked to an Olympus exposure control unit. Phase-contrast images were taken in green light.

CHAPTER 3.

CHARACTERISATION OF ADULT RAT MAMMARY GLAND PARENCHYMA *IN SITU* AND IN CULTURE.

3.1 *In situ* characterisation of mammary parenchyma.

3.1.1 Introduction.

The *in situ* structural and phenotypic characteristics of the mammary parenchyma of 70 day virgin female rats were examined. Intact glands were examined in whole-mount preparations and at the ultrastructural level by transmission electron microscopy. The profile of cytokeratin filament expression was examined by indirect immunofluorescence staining of cryostat sections. Sections were probed with the following monoclonal antibodies, the specificity of which are made with reference to the human keratin catalogue: LE41 (CK8), C46.2 (CKs 7, 17), LE61 (CK18), C-04 (CK18), LL001 (CK14), BA16 (CK19), LP2K (CK19), C11.04 (CKs 8, 13) and polyspecific monoclonal antibody LP34 (CKs 4, 5, 6, 10 13, 14, 18). The purpose of these studies was to define cellular phenotypes *in situ*, and set the basis for the characterisation of parenchymal populations in experimental situations.

3.1.2 Mammary arborescence in the adult virgin gland.

Figure 1 shows a wholemount preparation of an abdominal-inguinal fat pad from a 70 day rat. The densely basophilic parenchyma branched profusely into the mesenchymal tissue, with lateral extensions composed of alveolar bud and lobular structures. Terminal end buds

and terminal ducts, the outlines of which stained prominently, were usually located at the extremities of the mammary tree, in contrast to the more mature alveolar structures which were located in areas more proximal to the nipple.

Figure 1. Wholemount of hematoxylin stained 70 day virgin rat mammary gland.

The major structural units discriminated for the purposes of this study are - (1) ducts, and (2) alveoli. x80.

3.1.3 Electron microscopy of adult virgin mammary gland.

In order to obtain large amounts of parenchyma for electron microscopic analysis, portions of mammary gland located near the nipple were dissected out for processing. Cytological examination of toluidine blue stained thin resin sections at the light level revealed the presence of cells morphologically distinct from identifiable luminal or myoepithelial cells. Such cells were lightly stained by toluidine blue in comparison to other cell types, and possessed large spherical nuclei, relatively clear cytoplasm and a round smooth shape (Figure 2). Electron microscopic examination of a similar portion of gland to that displayed in Figure 2, enabled the cytology of cells to viewed with greater resolution. At low power, numerous large pale cells are again readily identifiable within the parenchyma. These cells are distinct from both large and small luminal epithelial cells and darker basally located myoepithelial cells. Some of the pale cells in this view possess a luminal surface, whilst others do not. Figure 3b is a high power view of the pale cell highlighted in Figure 3a. The epithelial nature of this cell is evident by the presence of junctional complexes between it and adjacent epithelial cells, and the fact that the apical surface extends into the lumen with characteristic microvilli. Mitochondria, Golgi, endoplasmic reticulum

and ribosomes can all be observed. The basal surface of this large pale cell abuts onto a thin myoepithelial process as well as the basement membrane (Figure 3b).

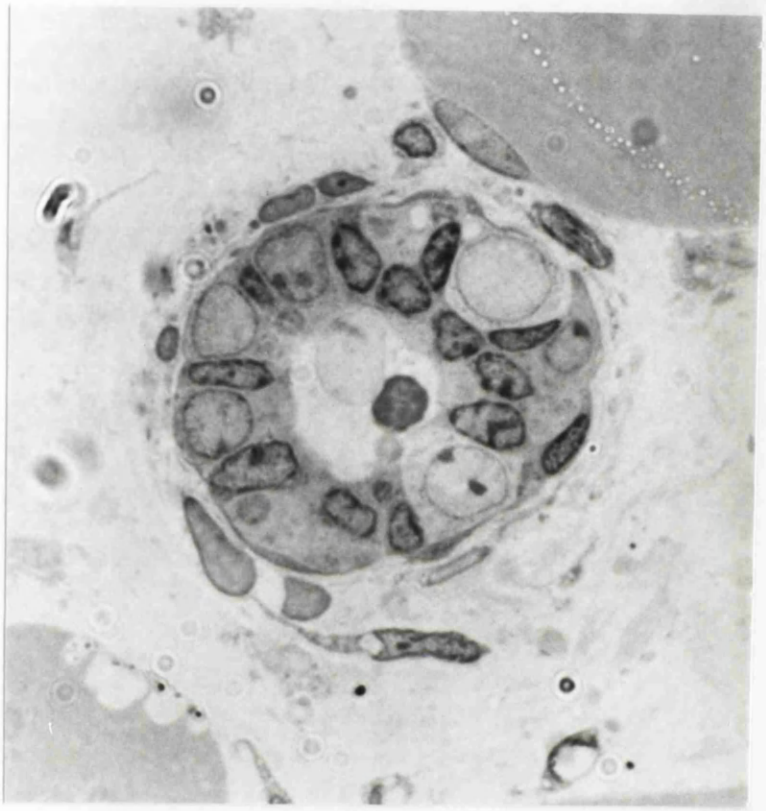


Figure 2. Toluidine blue stained resin section of 70 day virgin mammary parenchyma.

Note the presence of the large round pale-stained cells. x1260.

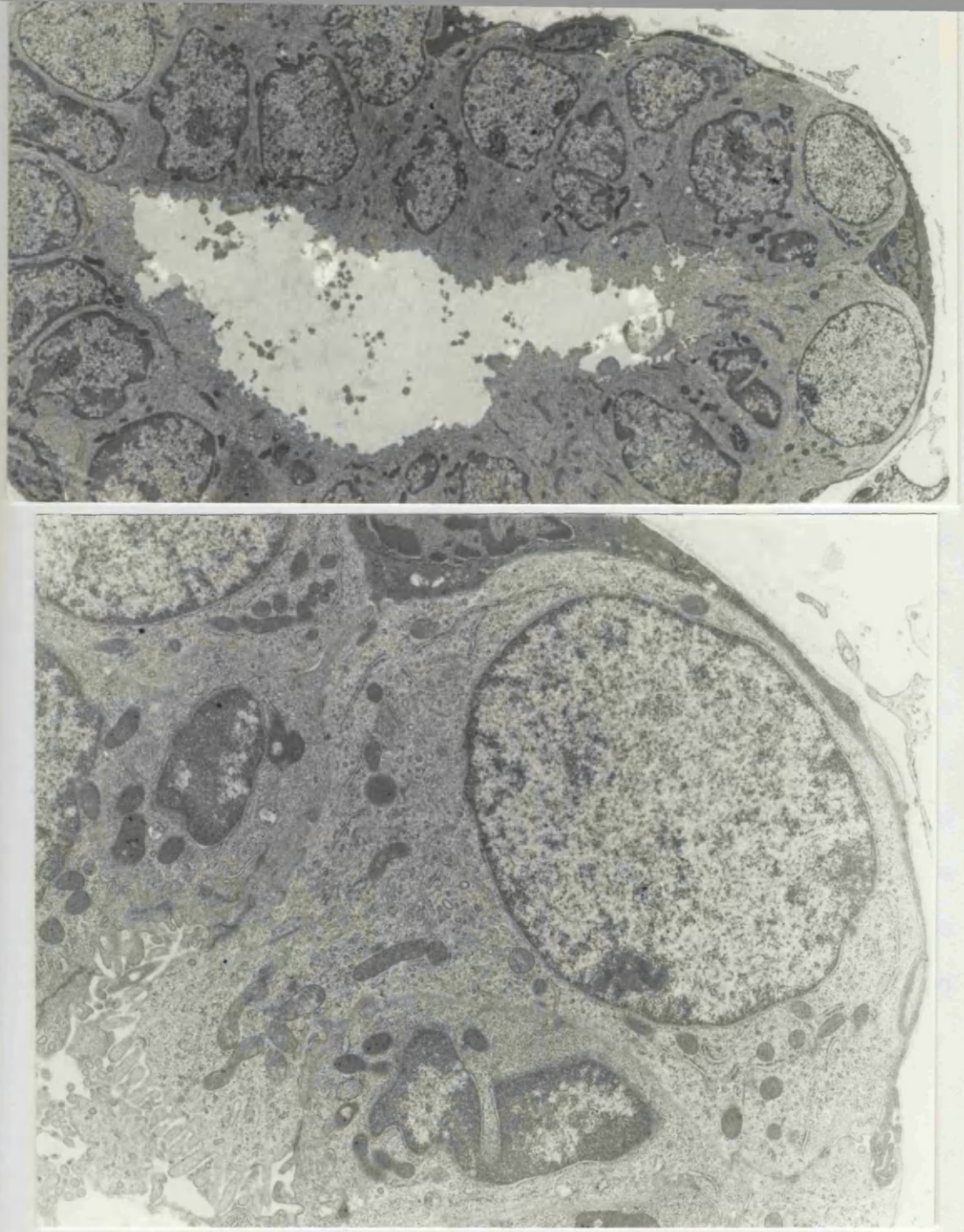


Figure 3. Transmission electron micrographs of 70 day virgin parenchyma.

(a) Cross-section of a duct. Note the presence of large pale cells (CC); luminal epithelial cells (LE); and myoepithelial cells (ME). x3300
(b) High power view of pale cell denoted in (a). Note (1) the presence of junctional complexes. The basal surface abuts both a myoepithelial cell and the basement membrane (2). Luminal microvilli are also evident. x10400

3.1.4 The cell type-specific expression of cytokeratin and vimentin filaments by luminal and myoepithelial cells.

A panel of specific anti-cytokeratin antibodies was applied to frozen sections of intact rat mammary gland. Despite poor morphology resulting from frozen sectioning, four cell types could be identified on the basis of their topography. These were - (1) luminal cells which did not contact the basement membrane, (2) cells with a luminal surface which did abut onto the basement membrane, (3) basally located cells without a luminal surface, and (4) cells interposed between luminal and basal layers and which appeared to have neither a luminal surface nor any contact with the basement membrane. The staining profiles of these different cells with cytokeratin-specific antibodies is detailed below.

Cytokeratin 7. Antibody RCK 105 stained the majority, but not all, of the luminal cells in the intact gland. Apical regions of positive cells were particularly strongly stained. Basal cells did not stain (Figure 4a).

Cytokeratin 8. Antibody LE41 stained all cells in both luminal and basal layers in all portions of the gland (Figure 4b). Interestingly, peripherally located lymph node cells also stained strongly with this antibody (

Figure 4b).

Staining of parenchymal cells by antibody C11.04 (a monoclonal bi-specific antibody recognising cytokeratins 8 and 13), was broadly similar to that produced by LE41, although not all basal cells stained positively.

Cytokeratin 14. Antibody LL001 produced uniformly strong staining of cells of the basal layer in both ducts and alveoli. Positive cells were arranged in a basket-like networks around alveoli or as continuous basal layers (Figure 4c). The latter pattern was clearly demonstrated in sagittal sections of duct (Figure 4c). Very rare LL001 positive cells were located on the innermost aspect large ducts (figure 4c). These cells may have been displaced by sectioning artifact. The great majority (>95%) of luminal cells did not stain with this antibody.

Cytokeratin 18. Antibody LE61 displayed marked heterogeneity in staining intensity. All cells in the luminal layer were positive, with approximately 1 in 4 cells in large ducts strongly stained. Such strongly stained cells in both ducts and alveoli did not always display a luminal aspect, but appeared interposed between luminal and basal layers (Figure 4d). Such cells rarely appeared to exhibit classical cuboidal/ columnar morphology, but were always more rounded, suggesting that they represented the mitotic compartment with

staining possibly intensified during the G2/ M phase of the cell cycle. Occasional epithelial cells located within the luminal layer, but which nevertheless did not appear to have a luminal surface and abutted onto the basement membrane, were also positive. Cells of the basal layer did not stain at all with this antibody. A similar staining distribution was produced by antibody C-04.

Cytokeratin 19. Variations of distribution and intensity of staining were observed with antibody LP2K. The majority of luminal cells were clearly positive but occasional cells interposed between the basal and luminal layers did not stain. Cells in the basal layer were either stained strongly or not at all in an apparently random fashion, with no apparent correlation between staining and location in either ducts or alveoli (Figure 4e). Antibody BA16 gave a similar staining pattern to LPK2.

Cytokeratins 4, 5, 6, 10, 13, 14, 18. Polyspecific monoclonal antibody LP34 uniformly stained cells of the basal layer. Cells in the luminal layer did not stain. Cells between the basal and luminal layers did not stain with this antibody.

Cytokeratins 7 and 17. No cells in any parenchymal structure appeared to stain with C46.2.

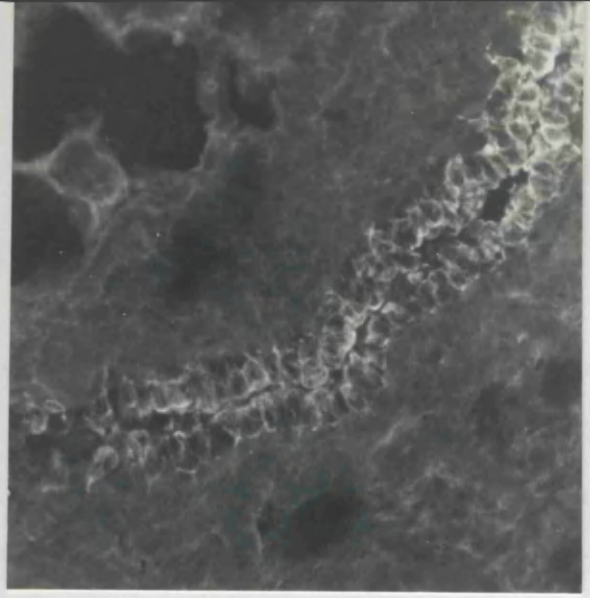
The differential expression of cytokeratins 14 and 18 by myoepithelial or luminal cells respectively was most

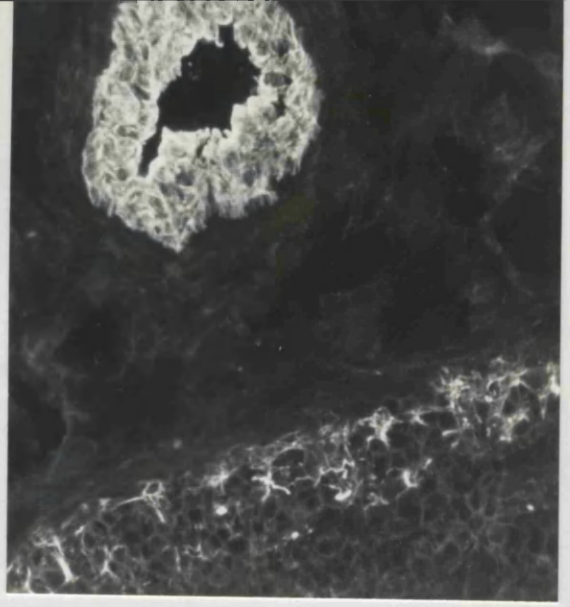
clearly illustrated by double labelling sections using antibodies LL001 and LE61 (Figure 4f). Cells co-expressing cytokeratins 18 and 14 could not be convincingly demonstrated in the section illustrated, although owing to the difficulties in interpretating frozen section morphology, the presence of cells of this phenotype could not be completely excluded. In general terms, however, antibodies LE61 and LL001 appeared to provide appropriate cell type-specific markers with which to follow the behaviour of luminal and myoepithelial cells in culture.

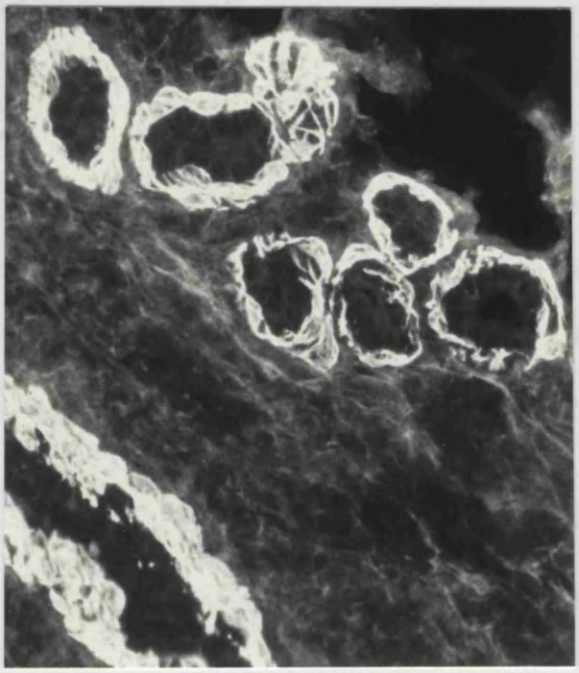
Cells of the basal layer stained positively with two anti-vimentin antibodies - MVI and DAKO-vimentin; with the DAKO-vimentin antibody decorating filaments very strongly compared to the staining obtained with antibody MVI (not shown). Basal cells also stained very strongly with antibody 1A4 recognising the smooth muscle α-actin isoform (not shown). By contrast, luminal epithelial cells were not stained at all with antibodies MVI, DAKO-vimentin or 1A4.

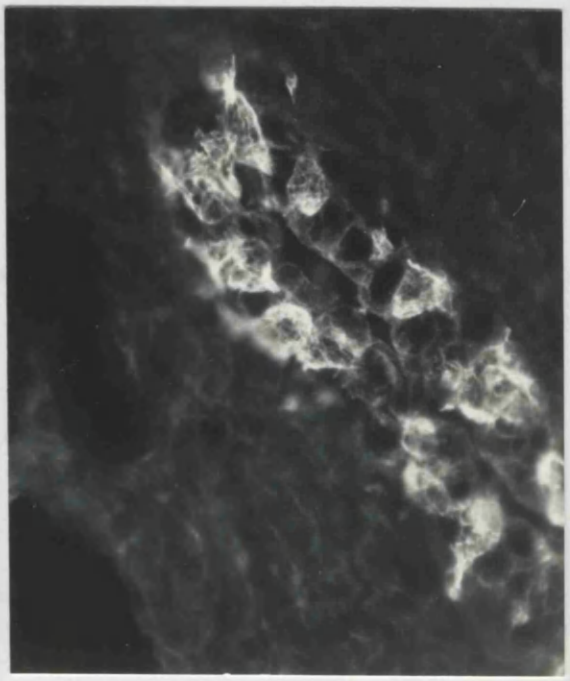
Figure 4 (Opposite). The distribution of different cytokeratin filament types detected by indirect immunofluorescence on frozen sections of 70 day virgin rat mammary gland.

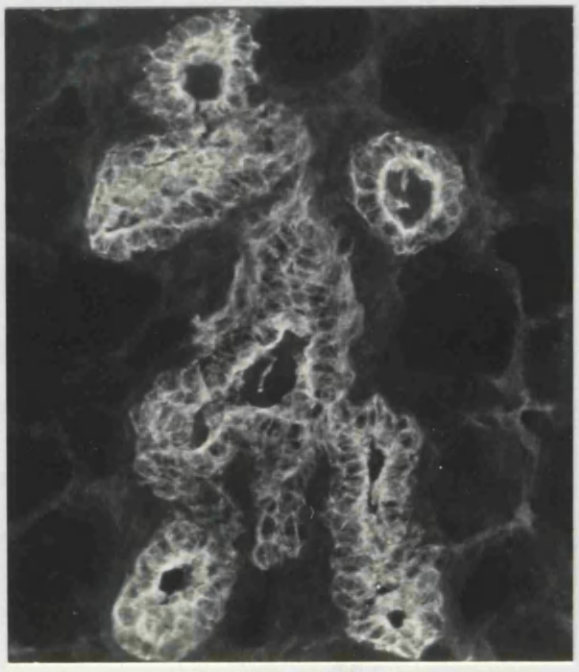
(a) Cytokeratin 7, sagittal section of duct; (b) Cytokeratin 8, cross section of duct. Note the positive staining of certain peripheral lymph node cells; (c) Cytokeratin 14. Note the basket-like arrangement of positive cells around alveoli, and the luminally located positive cell in the duct; (d) Cytokeratin 18, sagittal section of duct (e) Cytokeratin 19, cross-sections of duct and alveoli; (f) Double-labelling of cytokeratins 14 (i) and 18 (ii). x150

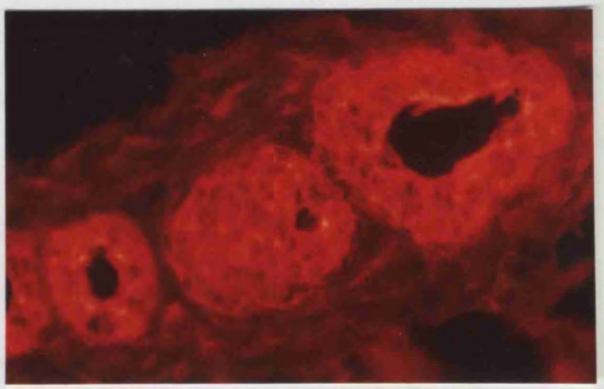


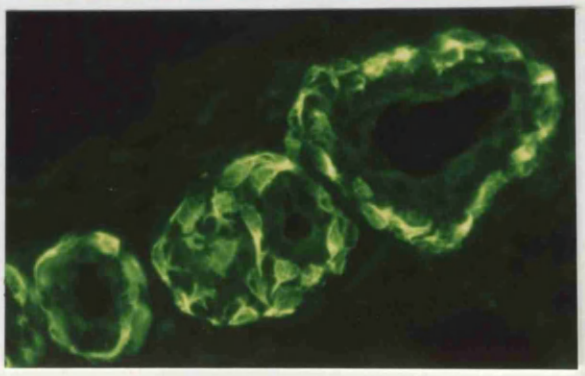












3.2 Characterisation of organoid preparations.

3.2.1 Introduction.

Parenchyma in the form of organoids constituted the basic resource for all further experimental investigations described in this thesis. Representative organoids prepared by the digestion procedure described in section 2.2.2 were therefore characterised prior to their culture. The identification of different types of parenchymal fragment is made with reference to the mammary structures observed in the wholemount illustrated in Figure 1. Organoid cell types were examined both ultrastructurally and by flow cytometric analysis of specific lineage markers.

3.2.2 Morphological characterisation of enzymatically purified duct and alveolar fragments.

The organoid preparation procedure yielded large amounts of adipose tissue, tubular blood vessel fragments, erythrocytes and single stromal cells as well as parenchymal fragments. After the final stage of preparation in which stromal elements were allowed to attached to a culture substratum during a brief culture period (see Section 2.2.2), fragments of duct, alveoli, alveolar buds and duct-alveolar bud structures were obtained in more purified form, and were readily identifiable in preparations viewed microscopically.

Representative examples of organoid structures are shown in Figure 5.

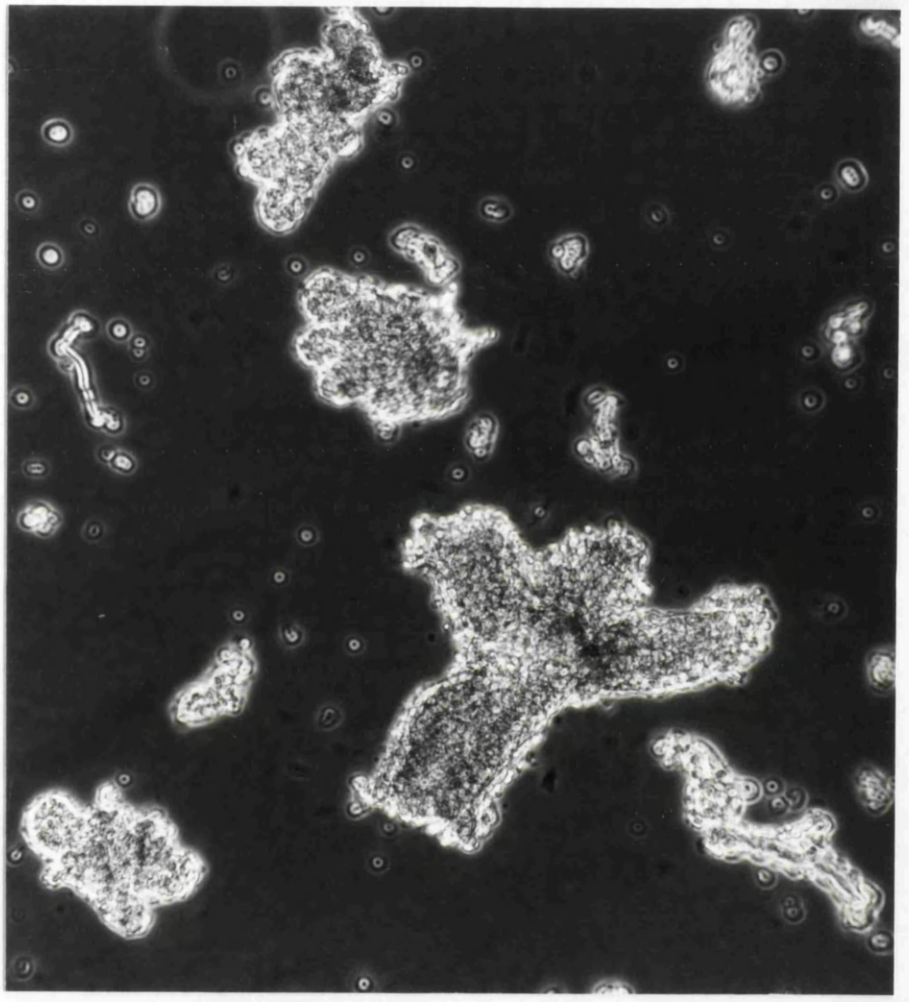


Figure 5. Examples of enzymatically prepared organoids.

Note the branching ductal unit, alveoli (c.f Figure 1), and small blood vessel fragments. x205

The edges of most organoids were clearly resolved by phase contrast, and indicated that the structures were substantially free of attached stromal elements. However, small blood vessels could be seen to protrude from certain large alveolar fragments. The majority of organoid fragments were either small alveolar buds or large alveolar fragments, some with attached duct. Fragments of isolated duct represented approximately 20% of organoid preparations, duct-alveolar units 30%, with the remainder comprised of alveolar structures.

3.2.3 Electron microscopic examination of organoids. Organoids maintained the histological topology characteristic of the parenchyma *in situ* (Figure 6). Myoepithelial cells surrounded the external 'basal' surface and were marked by dense heterochromatin in their nuclei. At higher magnification myofilaments were clearly observed. The occasional junctional complex could be identified between apical regions of myoepithelial cells and the basal aspect of large clear cells. Cuboidal epithelial cells lined the luminal surface and were characterised by microvilli and the presence of apical tight junctions. Large clear cells were also clearly visible in organoids, as were smaller darker epithelial cells apparently interposed

between the luminal and myoepithelial layers. These dark cells probably had luminal surfaces at other planes in the block, as (unlike the clear cells) they resembled luminal epithelial cells in all other respects. Collagenase action during preparation stripped off the majority of the basement membrane from the parenchymal fragments, and all stromal cells distal to the basement membrane.

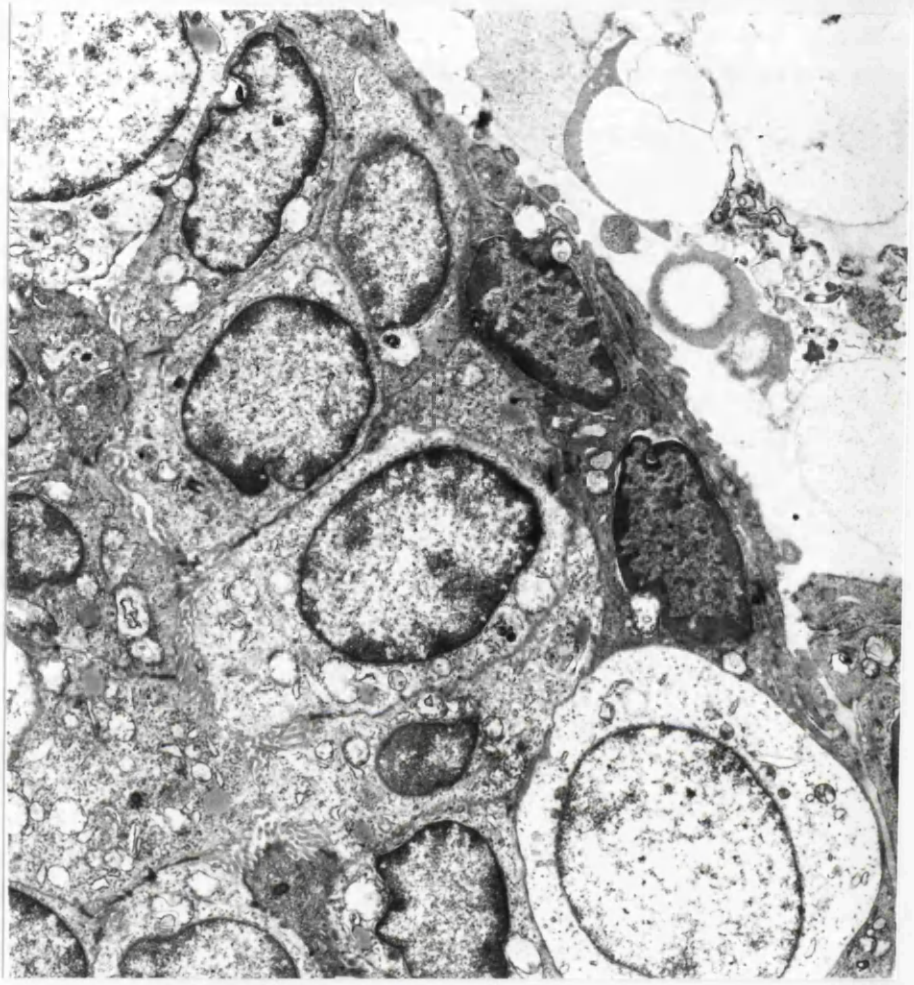


Figure 6. Electron micrograph of an enzymatically prepared organoid.

Note that prepared organoids retained a lumen (L) formed by epithelial cells (EP); and a basal surface surrounded by myoepithelial cells (ME). An epithelial pale clear cell (CC) is also prominent.
x6250

3.2.4 Flow cytometric analysis of luminal and myoepithelial cells identified by cell type-specific markers.

Organoid preparations could be disaggregated into viable cell suspensions using trypsin (see Section 2.3.1). This enabled flow cytometric analysis of parenchymal populations on the basis of lineage marker expression to be carried out.

Organoid cell suspensions were simultaneously labelled with the luminal marker 25.5 (IgG2a) and the myoepithelial marker DAKOCALLA (IgG1). Antibody binding was detected by class-specific second antibodies conjugated to either fluorescein or phycoerythrin, respectively (see Table 7). Using multiparametric analysis, cells were gated on the basis of light scatter to exclude debris and cell clumps, and were then analysed for positive fluorescence. Figure 7 illustrates a cytogram generated by the fluorescence profile of analysed luminal or myoepithelial cells (see section 4.2.4). Two separate populations of cells were detected, one exclusively 25.5-positive and the other exclusively stained with the anti-endopeptidase-24.11 antibody. Both populations showed considerable heterogeneity in the intensity of staining.

Since the emission wavelengths of R-phycoerythrin and propidium iodide overlap, dead cells could not be excluded from analysis on the basis of propidium iodide

uptake in double-labelled preparations. The small number (<3%) of double-labelled events that were detected were therefore considered to be dead, non-specifically labelled cells. By corollary, cells concurrently expressing both gp70KD and endopeptidase-24.11 did not constitute a numerically significant population of parenchymal cells, and thus the ratio of luminal:myoepithelial cells detected was approximately 5.5:1.

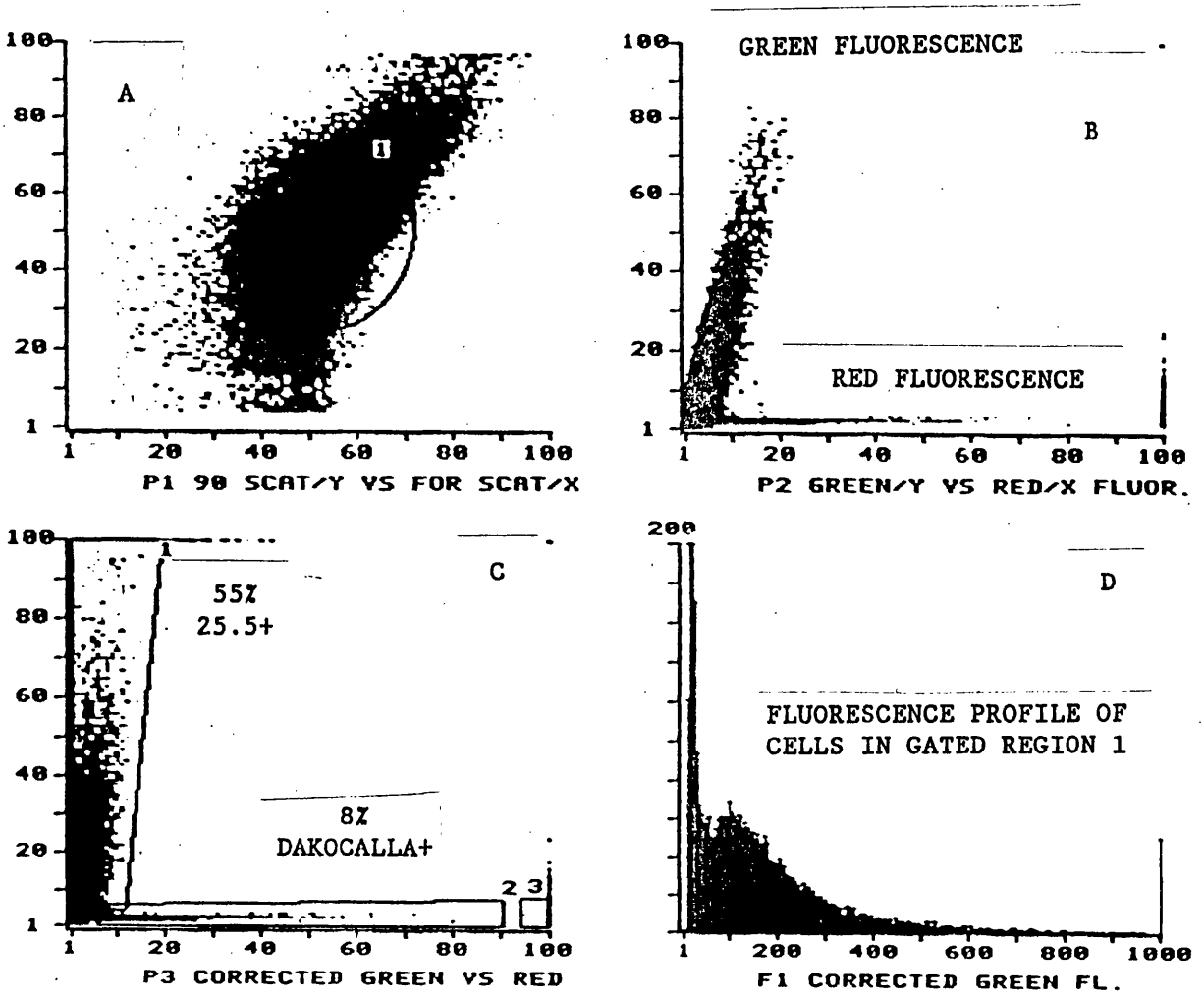


Figure 7. The form of data generated by flow cytometric analysis of a 25.5/DAKOCALLA double-labelled preparation.

A cell suspension derived from uncultured organoids was double-labelled as described in Section 2.6.3, and then analysed in the flow cytometer. Figure (A) - cytogram generated from measured light scatter. Dots represent individual cells. An elliptical region is set which excludes dead cells and debris; (B) differential fluorescence profile of cells within gated region 1; (C) cytogram electronically corrected to diminish spectral overlap of emitted fluorescence. Further gating delimits either 25.5 stained or DAKOCALLA stained cells (gate 1 or gates 2 and 3 respectively). The proportion of cells stained for either antibody arising in the gated regions are shown; (D) Histogram of detected 25.5/FITC fluorescence of cells in gated region 1. The profile of the curve indicates that the stained population has a broad and continuous distribution of fluorescence intensity. A similar profile is detected for DAKOCALLA positive cells.

3.3 Characterisation of organoid explant and monolayer cultures.

3.3.1 Introduction.

In order to observe the behaviour of constituent luminal and myoepithelial cells in culture, organoids were either plated out directly (explant cultures), or dissaggregated into single cell suspensions which were then plated out at high density to produce primary monolayer cultures. Morphological observations were made of viable cultures propagated on tissue culture plastic, using phase contrast optics. The integrity of lineage marker expression in culture was assessed by staining cultures grown on glass coverslips, propagated under otherwise identical conditions, using the indirect immunofluorescence technique, and viewing the preparations by epi-fluorescence. Cells were stained live with primary antibodies recognising extra-cellular membrane epitopes: antibodies 25.5, PHM6 and anti-MFGM antiserum. The expression of cytostructural proteins were probed using either antibodies recognising the intermediate (cytokeratin or vimentin) filaments, or fluorochrome conjugated NBD-phalloidin, which specifically decorates 6nm actin microfilaments. Basement membrane proteins were detected using polyclonal antisera recognising laminin or type IV collagen (see Table 6).

Culturing the organoids with a variety of growth promoting media additives (see Table 2) did not significantly alter the patterns of antigen expression detected with the above reagents. Furthermore, no quantitative changes in the levels of antigen expression attributable to the use of different media were detected using indirect immunofluorescence observation.

3.3.2 Morphology of organoid cultures.

Blood vessels and mesenchymal cells differentially attached within the initial two hour culture period. After collecting organoid containing supernatant, continued propagation of this "pre-plating" stromal fraction in DMEM/ 10%FCS medium resulted in cultures enriched with fibroblasts and endothelial cells. Areas of such cultures were observed to undergo adipocytic differentiation when propagated in media containing hydrocortisone and insulin. The cellular morphologies of these cultures were clearly distinguishable from those derived from organoids.

Serum-containing medium: Organoids plated in serum containing medium attached to tissue-culture plastic or glass with consistently high plating efficiencies of almost 100%. Within a three day culture period, the three-dimensional structure of the parenchymal fragment

degenerated as the cells migrated radially outwards and spread.

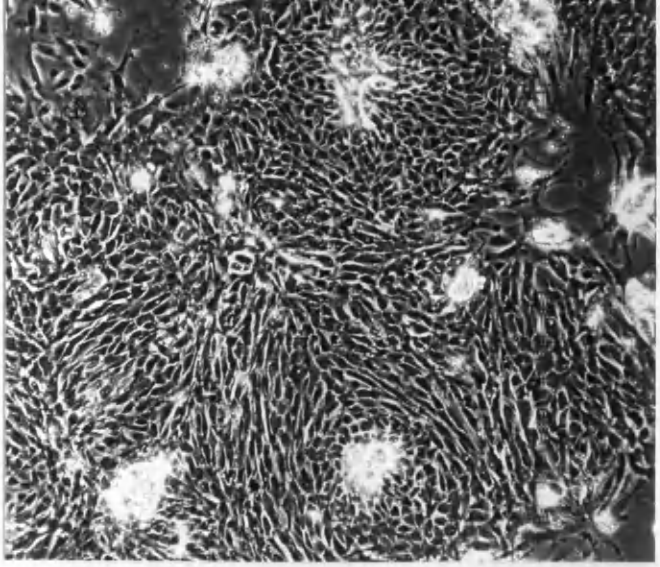
After 5 days culture in serum containing DMEM:F12 medium, the majority of small organoids had fully mobilised, irrespective of the combination of added hormones and growth factors. Remnants of organoid structure remained in the centre of extensive islands of cells derived from large alveoli and duct-alveoli fragments. The central zone of explants was composed of tightly packed, multilayered polygonal cells which spread and flattened as they moved outwards with characteristic epithelial lamellipodia at the monolayered periphery of the outgrowth. Occasional cells of flattened more elongated morphology were observed to spread around the margins of outgrowth (Figure 8a).

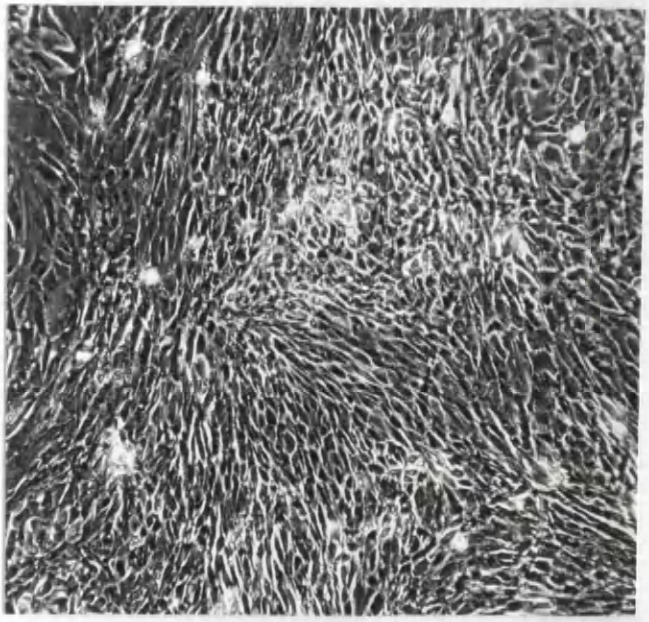
Although hormones and growth factors had little influence on the attachment or spreading behaviour of organoids, additives did significantly influence the behaviour of long-term cultures. After 16 days propagation in media containing either no additives or insulin and hydrocortisone in combination, fibroblastic overgrowth occurred. By contrast, cultures propagated in media containing hydrocortisone (HC), insulin (I), cholera toxin (CT) and epidermal growth factor (EGF) contained areas composed of flattened 'epithelioid'

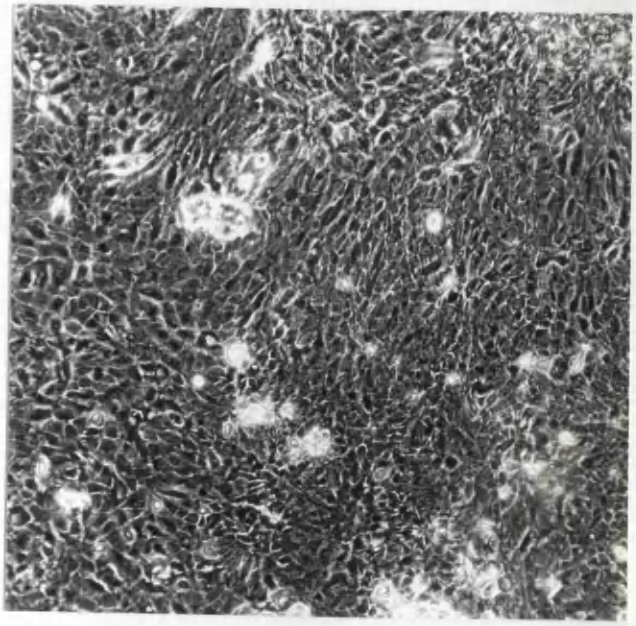
cells, and small islands of compact cells surrounded by larger, highly proliferative polygonal cells of distinctive morphology (Figure 8b). Mitotic cells could be seen both as densely packed fusiform cells, and also in a more flattened and spread form. These mitotic cells became the predominant population in HC:I:CT:EGF medium, and were morphologically distinguishable from fibroblasts growing in pre-plating stromal cultures. Furthermore, unlike fibroblasts, they did not grow on top of the islands of compact epithelial cells. Organoids plated at low density and maintained for 6 weeks in DMEM:F12 1:1/ 10%FCS media containing HC:I:CT:EGF resulted in cultures which consisted almost entirely of these cells.

Figure 8 (Opposite). Phase-contrast micrographs of organoid explant cultures grown in serum-containing medium.

(a) 3 days culture - Note cells stretching around the explant periphery (arrow); (b) 16 days culture; (c) 28 days culture.
All cultures grown in the presence of HC:I:CT:EGF. x270







The attachment, spreading and growth of organoids cultured on glass coverslips was similar to organoid behaviour on tissue culture plastic. However, when glass coverslip cultures were mounted face-down and viewed with x25 phase objective, cells of stretched morphology were observed randomly scattered underneath the explants, (see Figure 14a below). These cells were detectable due to their highly refractile lateral edges, but the extent of spreading was very difficult to discern by phase contrast. Such cells invariably appeared mitotically quiescent.

Separated ducts and alveoli in serum-containing medium: Explanting fragments of picked duct or alveolar structures in isolation revealed a distinctive mesenchymal population associated solely with alveolar fragments. Single bipolar cells could be observed on top of and underneath explants. These cells migrated from the margins of alveolar explants at a faster rate than the parenchymal cell types.

Serum-free medium: Both the initial spreading behaviour of explants and the morphology of migrating cells were significantly altered in the absence of serum in the growth medium. After 5 days culture on tissue culture plastic in serum-free Biorich2 medium containing added HC:I:CT, small organoids had fully mobilised resulting

in characteristic islands of cells. Larger fragments behaved in one of two ways- (1) Small compact polygonal cells at the centre of colonies migrated radially and spread outwards towards the explant periphery. At the margins of large explants and those arising from ducts, cells of distinctive flattened morphology occurred, appearing 'stretched' around the circumference of the mobilising organoid, (2) In some organoids this distinction between central polygonal and peripheral stretched cells was not seen, and the spreading margin of the explant consisted of large proliferating polygonal cells while the centres were composed of small tightly packed polygonal cells, as in the first type. Thus, the discrimination between several morphologically distinct cell types was much more obvious in serum-free conditions as compared with serum-containing medium, in which topographical segregation of different morphologies was difficult to discern using phase-contrast microscopy.

After 9 days culture under serum-free conditions, explants followed one of three distinctive patterns of growth, as illustrated in Figure 9. (1) In most explants the central cells remained tightly compacted, and although occasional mitotic polygonal cells were observed in this zone , their spreading appeared to be inhibited. These cells were circumscribed by extremely

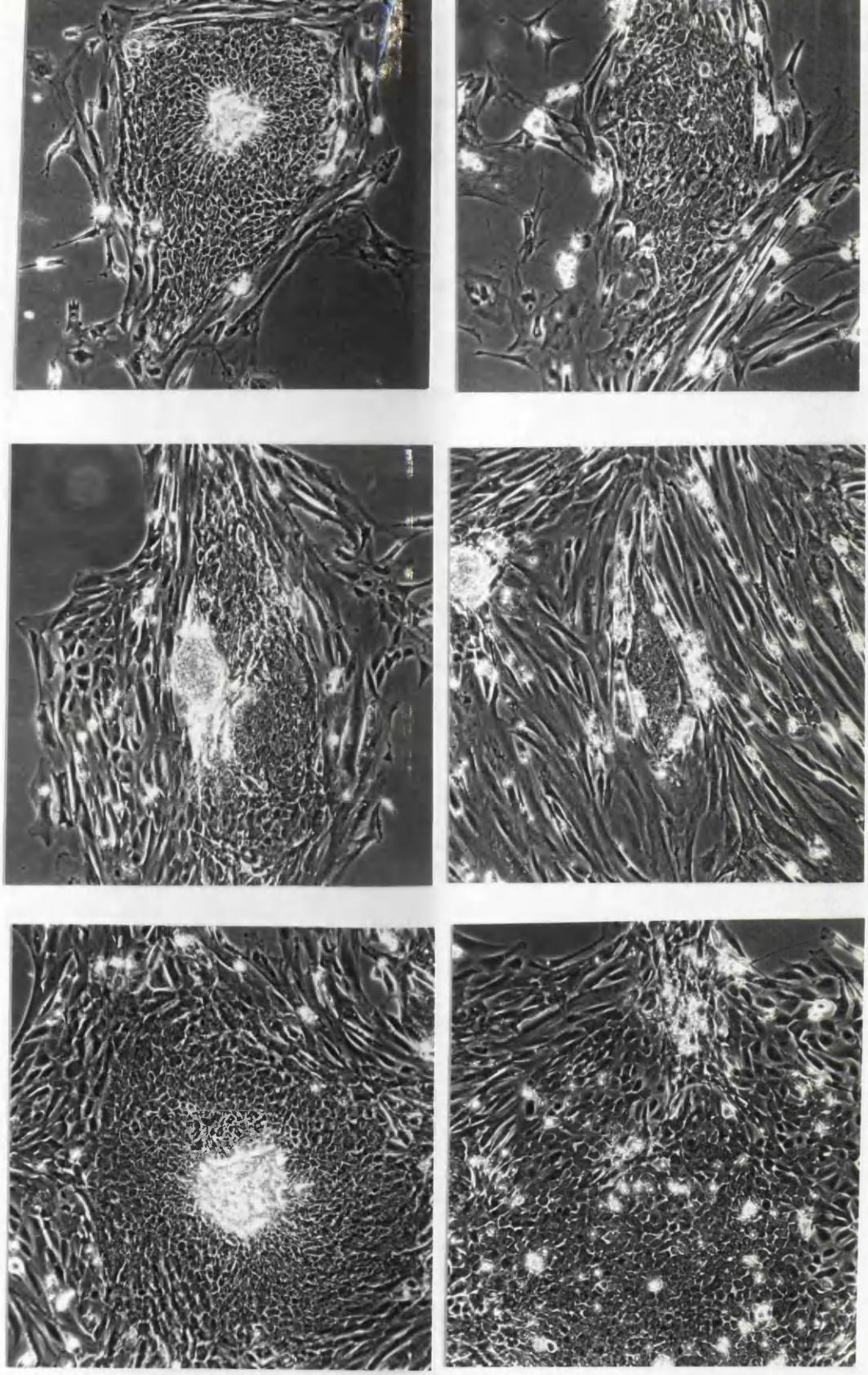
stretched quiescent cells at the edge of the explant (Figure 9a); (2) in other explants areas of the stretched marginal cells most distal to the centre of the explant contained numerous mitoses and generated cells of regular large polygonal morphology which spread and stretched around the explant (Figure 9c); (3) the organoid fragment did not fully mobilise, and two layers of cells were observed. Extremely compacted non-mitotic cells were situated on top of larger, polygonal cells which migrated out from underneath and proliferated. As can be seen in Figure 9e, many mitotic figures were observed among these migrating cells. These basally located cells maintained a proliferative state whilst maintaining regular polygonal morphology, or assumed a stretched morphology and became quiescent.

After 16 days culture the growth pattern of each type of explant growth was determined. The stretched cells surrounding type 1 explants developed a frankly mesenchymal appearance whilst the small polygonal cells spread slightly, becoming quiescent (Figure 9b). By this time peripheral cells of type 2 explant growths produced sheaves of stretched cells (Figure 9d). Type 3 explants generated areas of cells which consisted of both regular large highly mitotic polygonal and stretched quiescent cells (Figure 9f). At this stage type 3 explants gave rise to patches of densely packed

fusiform cells of similar morphology to those arising in long term cultures propagated in serum containing medium as described above (Figure 8b).

Figure 9 (Opposite). Phase-contrast micrographs illustrating the progression of organoid explant cultures grown in serum-free medium.

(a) Type 1 explant after 9 days culture - Note the peripheral stretched cells (arrow); (b) Type 1 explant after 16 days culture - Note that the peripheral stretched cells have become mesenchymal in appearance (arrow); (c) Type 2 explant after 9 days culture - note the mitosis in regions distal the explant centre (arrow); (d) Type 2 explant after 16 days culture; (e) Type 3 explant after 9 days culture - Note distinct layers of cells (arrow); (f) Type 3 explant after 16 days culture. x144



Unfortunately, the initial attachment and mobilisation of organoids in serum-free medium was severely restricted on glass coverslips. Although the organoid fragments were metabolically active as indicated by changes in media pH, the majority remained un-spread and grew as compact spheres, precluding morphological identification of cell types in this system.

3.3.3 Electron microscopic examination of organoid explant cultures.

Although basally-located cells with myofilaments and superficial microvilli-expressed cells were evident when explants were examined by EM, it was not generally possible to correlate specific cell morphologies defined under phase-contrast, with specific ultrastructural phenotypes.

3.3.4 Expression of membrane and cytostructural markers by organoid explant cultures.

Membrane markers.

Antibody 25.5: Cells positive with antibody 25.5 were readily detectable on organoids a few hours after attachment, and on cultures of all ages examined (up to six weeks). 25.5 positive cells existed in both explants of isolated ducts or alveoli, with no obvious differences in the levels of fluorescence intensity apparent. The great majority of cells on the surface of

mobilising explants in serum-containing medium were positive. Figure 10a illustrates 25.5 positivity of a singly labelled viable organoid explant stained after 4 days culture. The characteristic punctate staining may have been produced by the patching of unfixed membrane antigens occurring at 4°C. Cell boundaries (and perinuclear margins) appeared more strongly stained probably due to 'edge effect'. The intensity of 25.5 staining sometimes appeared brighter in dense cultures in HC:I:CT:EGF containing media. This was most likely due to the spreading of proliferating cells being restricted by the surface area of coverslips.

Cells exhibiting either polygonal or a more elongate morphology were both strongly positive. Although staining was uniform over the surface of any one cell, the positive population was heterogeneous in staining intensity; furthermore, there was no obvious correlation between morphology and fluorescence intensity. Positive cells, seen at several planes of focus on the explant surface, extended from the centre of the mobilising explant to the peripheral margins. The occasional polygonal cell on the surface of the explant did not stain, and both marginal stretched cells and bipolar cells migrating from the periphery of alveolar explants, were negative with antibody 25.5.

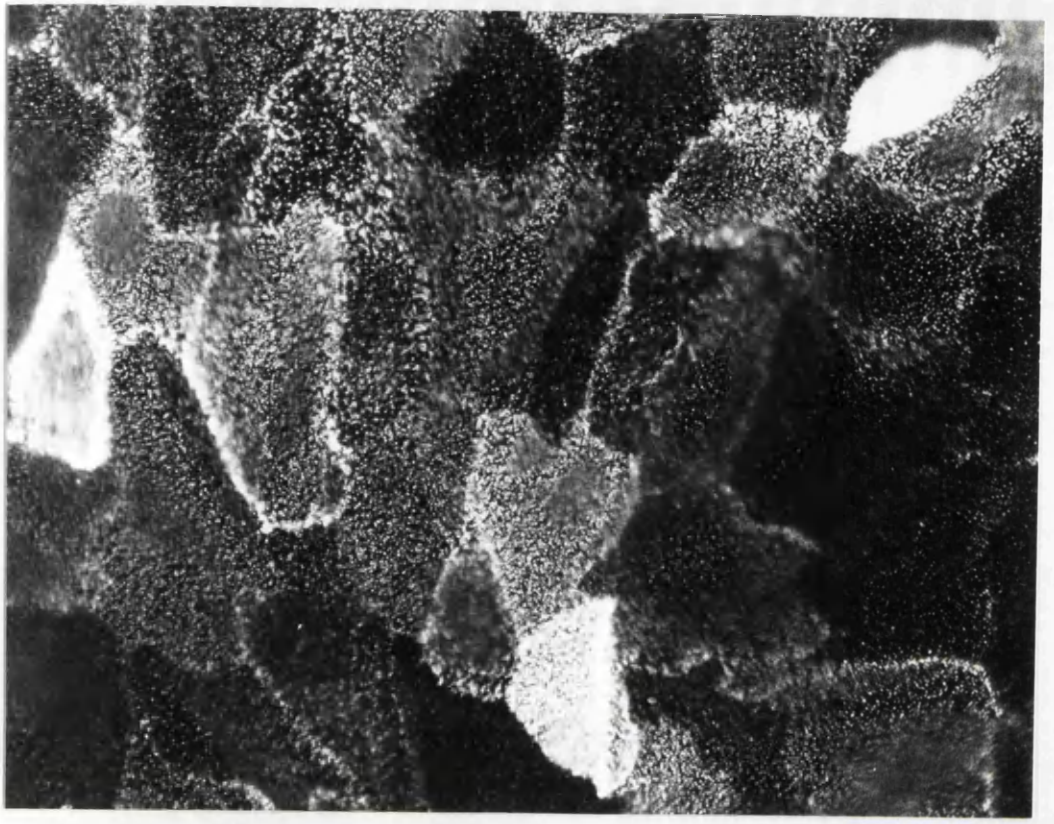
Occasionally the fluorescent image of 25.5 positive cells did not correspond to the field defined by phase-contrast. In such cases these cells were stained more strongly than the majority of neighbouring cells, and were above the plane of focus of the predominant phase-contrast image (Figure 10a).

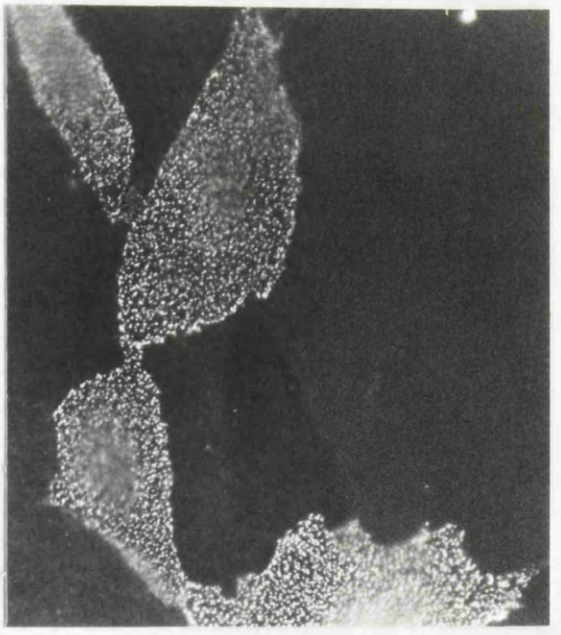
Anti-endopeptidase-24.11 antibodies: PHM6-positive cells were rarely observed when dense explants were stained as viable cultures. When isolated duct or alveoli fragments were propagated in isolation however, peripheral stretched cells were clearly positive (Figure 10b). Occasional patches of strongly stained cells were observed on the top of duct explants (Figure 10c), and the phase image of such cells was not clearly discernible. The nature of the staining of the boundaries of these cells (very little edge effect was seen) indicated that spreading over the surface of the explant was not limited by neighbouring cells. Similar staining patterns were obtained with DAKOCALLA antibody.

The antigens detected by antibody 25.5 and anti-endopeptidase-24.11 antibodies were expressed under all growth conditions used. However, no cells in explant cultures propagated under any of the growth promoting conditions used in this study stained with anti-MFGM antiserum.

Figure 10 (Opposite). Indirect immunofluorescence micrographs of membrane staining of 4 day organoid explant cultures.

(a) 25.5 positivity - Note the heterogeneous staining intensities, x460; (b) PHM6-positive cells located at the periphery of an explant. Arrow denotes the interface between the explant and the peripheral cells; (c) PHM6-positive patch cells. x390
All cultures grown in HC:I:CT containing medium.





Cytostructural markers.

Cytokeratin filaments: All the anti-cytokeratin antibodies described in section 3.1.4 which stained the rat mammary gland parenchyme in vivo gave positive staining of cells in culture. None of the antibodies used stained every cell in explant cultures. Cytokeratin expression by cultured cells also displayed antigenic heterogeneity, with cells of similar morphology stained to varying extents with the same antibody. As described in Section 3.3.2, cells within explant cultures of mammary organoids could be categorised into two major morphologies on the basis of phase-contrast observation of living cultures viz., stretched and polygonal. Using these categories enabled a simple interpretation of anti-cytokeratin antibody staining profiles of explant cultures.

Staining patterns described here relate to explants propagated in serum containing DMEM:F12 medium supplemented with HC:I:CT. Preliminary experiments showed that using different combinations of hormones and growth factors did not qualitatively influence cytokeratin expression. As described above, the restricted spreading of explants grown on glass in serum-free medium precluded the direct probing of cytokeratins in these cultures.

Immunostaining with anti-cytokeratin antibodies was entirely cytoplasmic, with the nature of the staining pattern varying depending on the specificity of the antibody used. Typically, in polygonal cells filaments were laterally distributed throughout the cytoplasmic area as a distinctive intricate meshwork, with a tendency for filaments to align at the edges of adjacent cell-cell contacts. Staining was more intense in perinuclear regions. Spreading polygonal cells contained thick bundles of lateral filaments concentrated around the perinuclear area which radiated into the leading edges of the cytoplasm as fine fibrillar structures. There was no intranuclear staining. Details of individual cytokeratin distributions were as follows.

Cytokeratin 7: Polygonal cells stained strongly with antibody RCK 105 (Figure 11a). Positive cells possessed a finely fibrillar network evenly distributed throughout the cytoplasm. Antigenic heterogeneity appeared less marked between adjacent cells than with antibodies to other cytokeratins. At cell-cell contacts and the margins of spreading cells, the staining pattern became significantly thicker and more obviously filamentous. Overall intensity of staining increased significantly in cells located in peripheral areas of the explant. Stretched cells did not stain with RCK 105.

Cytokeratin 8: The great majority (>95%) of polygonal cells were strongly stained by antibody LE41, although the occasional cell with this morphology did not stain. Filamentous staining was discernible only in localised areas in certain cells, being particularly visible in the leading edges of cells spreading at the margins of explants. Staining otherwise appeared either as a diffuse cytoplasmic 'smear' in centrally located cells or occasionally particulate and punctate (Figure 11b). Stretched cells occurring underneath the explant did not stain, but some located around the monolayered margins of explants were clearly positive.

Cytokeratin 14: Stretched cells invariably stained strongly with antibody LL001. Stained filaments were clearly resolved and were highly aligned and orientated along the longitudinal axis of cells. The edges of stretched cells, readily detected by phase contrast optics, were particularly strongly stained, indicating concentrations of bundles of filaments at cell margins (Figure 12b). Positive staining clearly revealed the extent to which individual cells spread, their overall shape being not as readily discernible under phase contrast. Cytokeratin 14 positive stretched cells in explant cultures generally appeared non-mitotic.
Although the majority of polygonal cells did not stain with LL001, a significant minority (~20%) of cells of

polygonal morphology stained with varying intensities. The positive staining filaments in these cells displayed different structural arrangements compared to filaments in cells of similar morphology stained with different antibodies (for example LE61). Some filaments were weakly stained and arranged around the nucleus, radiating into the cytoplasm as finely fibrillar structures, lacking the characteristic extensive network of filaments detected with antibody against cytokeratin 18 (see below). Some polygonal LL001-positive cells were mitotic, with cells undergoing cytokinesis staining strongly.

Cytokeratin 18: Almost all polygonal cells stained with antibody LE61 but displayed considerable antigenic heterogeneity. Staining appeared as a dense cytoplasmic network of discrete fibres (Figure 12a). The occasional polygonal cell did not stain, and stretched cells located either underneath or at the margins of explants, did not stain at all with this antibody. A similar staining pattern was observed with antibody RGE 53.

Significantly different staining patterns were observed with antibody C-04 which also reacts mono-specifically with cytokeratin 18. In this case ~70% of polygonal cells stained strongly, whilst ~30% did not stain at all. Stretched cells were not stained with C-04.

Cytokeratin 19: Using antibody LP2K, the majority of polygonal cells in explant cultures of mammary organoids stained strongly, but the occasional cell did not stain. Fluorescent fibres were clearly visible and formed obvious filamentous networks (Figure 11c). In general, stretched cells occurring throughout the explant did not stain with LP2K, but the occasional cell located at the explant periphery was clearly positive. Similar staining patterns were observed with antibody BA16.

Cytokeratins 8 and 13: When explants were observed at low power, the overall staining pattern with bi-specific antibody C11-04 was patchy. At high power this could be seen to be due to considerable differences in positivity between adjacent polygonal cells, some of which were apparently negative. This contrasts with the positive staining of almost all polygonal cells with mono-specific antibody LE41 recognising CK8 (see above). Stretched cells did not stain with C11.04.

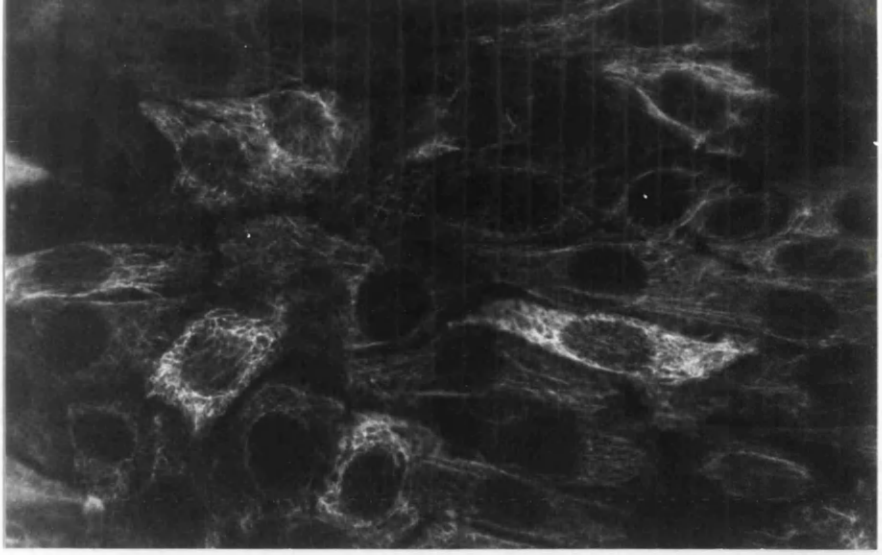
Cytokeratins 4, 5, 6, 10, 13, 14, 18: Cells staining with polyspecific antibody LP34 exhibited similar distributions and morphologies to those positive for LL001, as shown in Figure 12c.

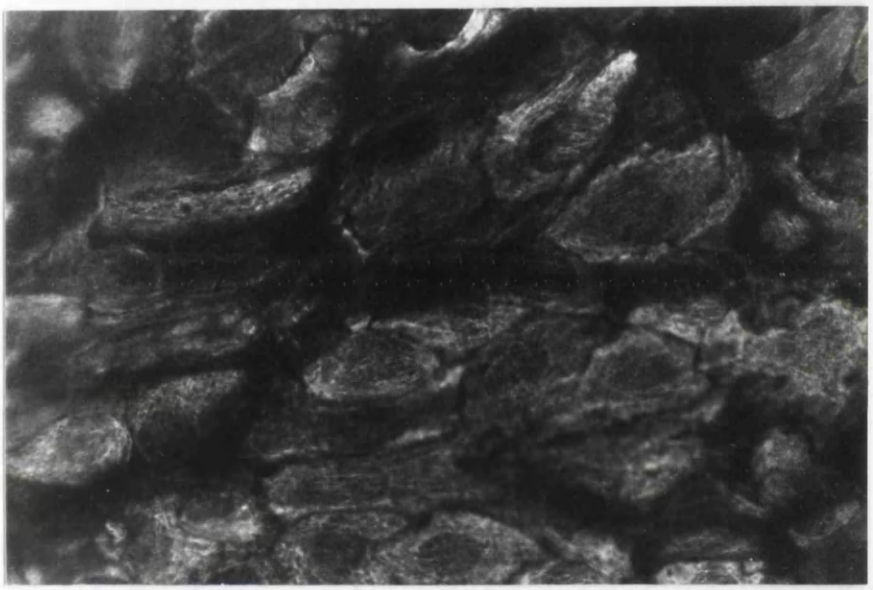
Other cytokeratins: No cells were observed to stain with antibody C46.2 (reacting with CKs 7 and 17) and no cells in explant cultures stained with the monoclonal antibodies recognising human cytokeratins 4 (CB10), 10 (RKSE 60) and 13 (1C7).

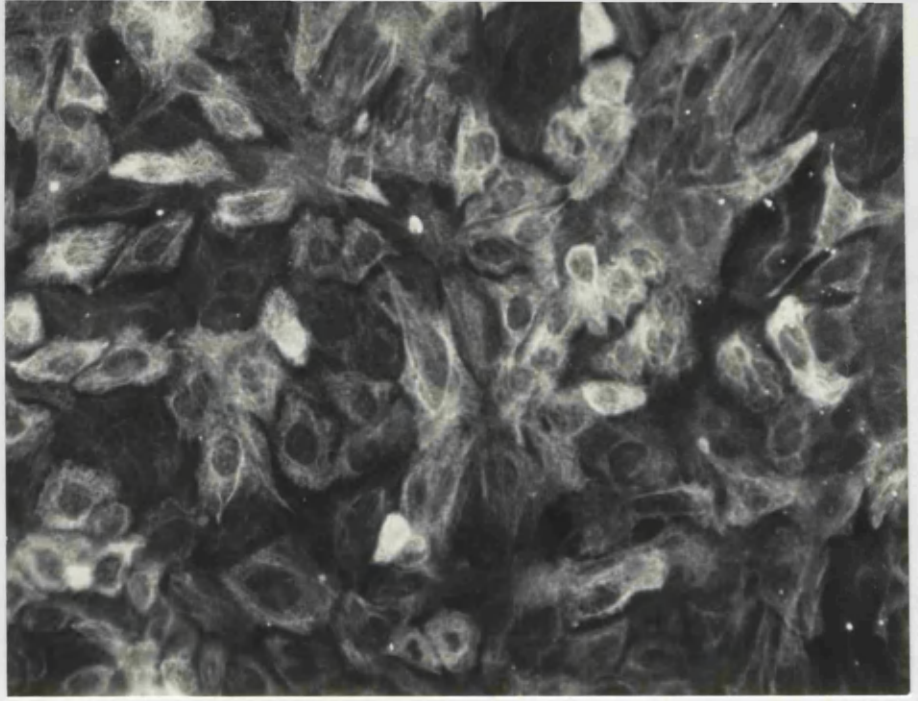
Double staining explants with LE61 and LL001: The distribution of LE61 and LL001 staining on intact gland (see Section 3.1.4) clearly discriminated between luminal and myoepithelial populations respectively. The different locations of luminal or myoepithelial cells, and their distinctive morphologies within explants were clearly revealed by double-labelling cultures with these two cell-type specific antibodies. Cytokeratin 14 was expressed by basally located stretched cells, whilst cytokeratin 18 expressing polygonal cells were superimposed over these myoepithelial cells. However, a proportion of polygonal cells double-labelled with the anti-14 and anti-18 antibodies. In contrast, stretched cells were never double-labelled LE61 and LL001.

Figure 11 (Opposite). Indirect immunofluorescence micrographs of cytokeratin filament expression by 3 day organoid explant cultures.

(a) Cytokeratin 7; (b) cytokeratin 8; (c) cytokeratin 19.
All cultures grown in HC:I:CT containing medium and stained as single labels using FITC-conjugated second antibody. x960







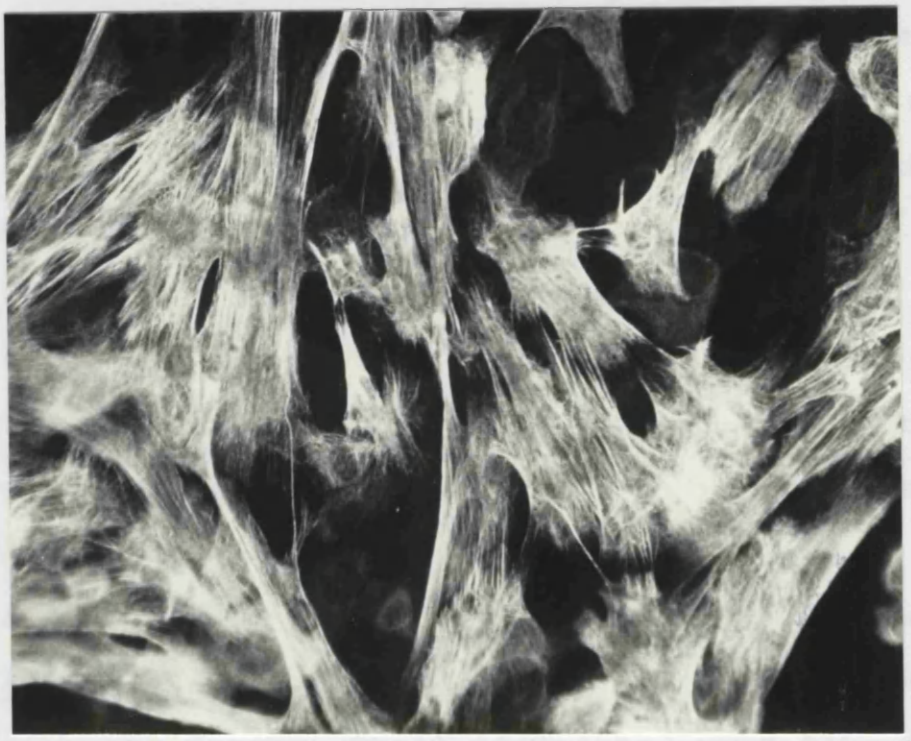


Figure 12. Explant culture double-labelled by indirect immunofluorescence for cytokeratins 14 and 18.

(a) Cytokeratin 18 (TRITC); (b) Cytokeratin 14 - identical field stained with FITC. Note that although there is a clear-cut morphological distinction between majority of 18+ cells which overlie the 14+ cells, some cells express both cytokeratins 18 and 14 (arrow). x400

Vimentin: There were considerable differences in the results of vimentin staining of explant cultures grown under identical conditions. Different anti-vimentin monoclonal antibodies gave different results.

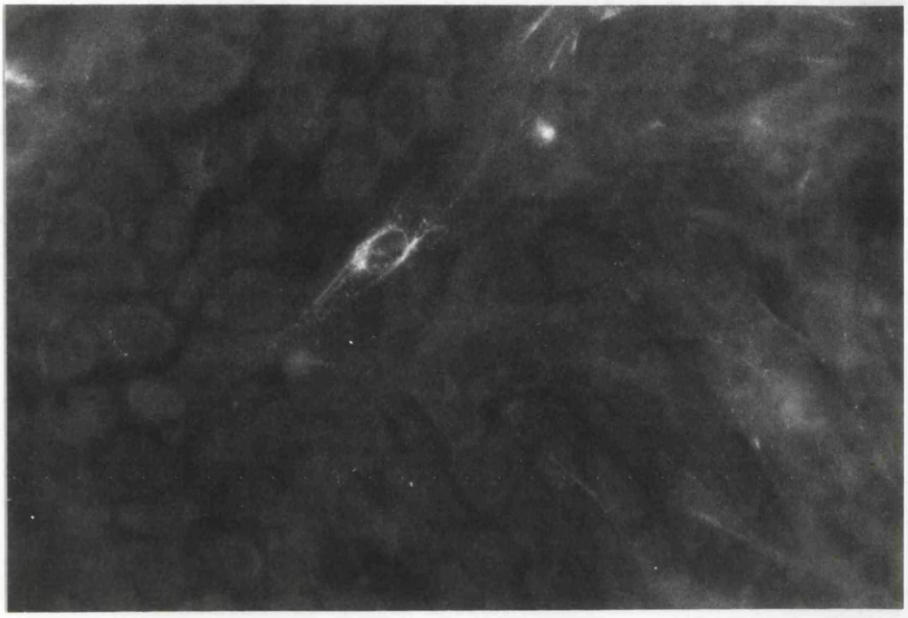
Using monoclonal antibody MVI, raised against calf lens antigen (see Table 6), relatively strong staining of occasional cells with bipolar mesenchymal morphology was observed (Figure 13a). These were probably stromal cells associated with the alveolar explants. Individual filaments were clearly discernible in these cells. Rarely, a cell of parenchymal morphology stained weakly. The majority of cells however, did not stain at all with this antibody.

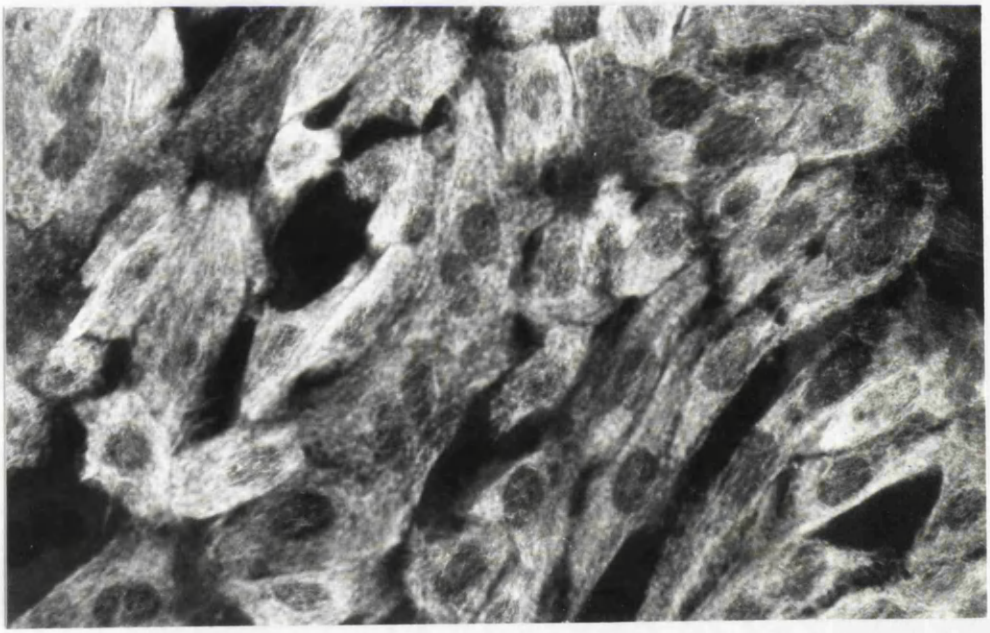
In contrast, the DAKO-vimentin antibody raised against porcine antigens strongly stained the majority of parenchymally-derived cells (Figure 13b), although negative cells of both polygonal and stretched morphology were seen. When present, the staining of polygonal cells was fine and wispy and not always in regular filamentous arrays. Small disjointed worm-like filaments were observed throughout the cytoplasm of spreading cells located at the margins of explants. Stained filaments of stretched cells were arranged in a more organised network. The overall intensity of fluorescence increased towards the peripheral areas of the ex-

plant. Bipolar stromal cells originating from alveolar explants stained very strongly this antibody. The filaments of these cells existed in highly organised arrays and the intensity of staining was always greater than that observed for cells of parenchymal morphology. Interestingly, the differences in overall staining intensity seen on frozen sections (that is weak staining with MVI compared with strong staining produced with DAKO-vimentin antibody) was reflected in cultured cells.

Figure 13 (Opposite). Indirect immunofluorescence micrographs of vimentin filament expression by 4 day organoid explant cultures.

(a) Staining produced by antibody MVI; (b) Staining produced by antibody DAKO-vimentin. Note the presence of cells not stained by this antibody. x400





Total F-actin: Cultures probed with NBD-phalloidin were brilliantly stained. All cells were positive. Stretched cells, corresponding in both morphology and location to those staining with antibody LL001, were particularly pronounced due to bundled, highly aligned arrays of intensely staining filaments which were orientated along the longitudinal axis of cells (Figure 14b). These actin filaments terminated at focal adhesions which were particularly evident in cells contacting the glass coverslip at the peripheral margins of explants.

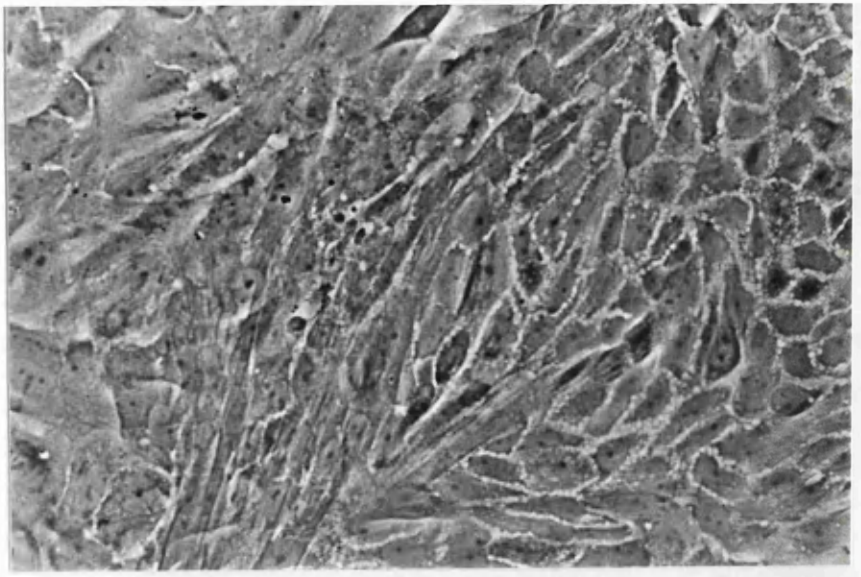
Three distinctive spatial arrangements of F-actin filaments were clearly discernible in polygonal cells. At the basal aspect of cells, filaments were arrayed laterally. At high magnification, the apical region of polygonal cells revealed dense localisations of actin along margins of cell-cell contact and punctate dots of positivity distributed under the entire apical surface. These staining patterns probably represent actin associated with terminal web-junctional complexes and microvilli respectively.

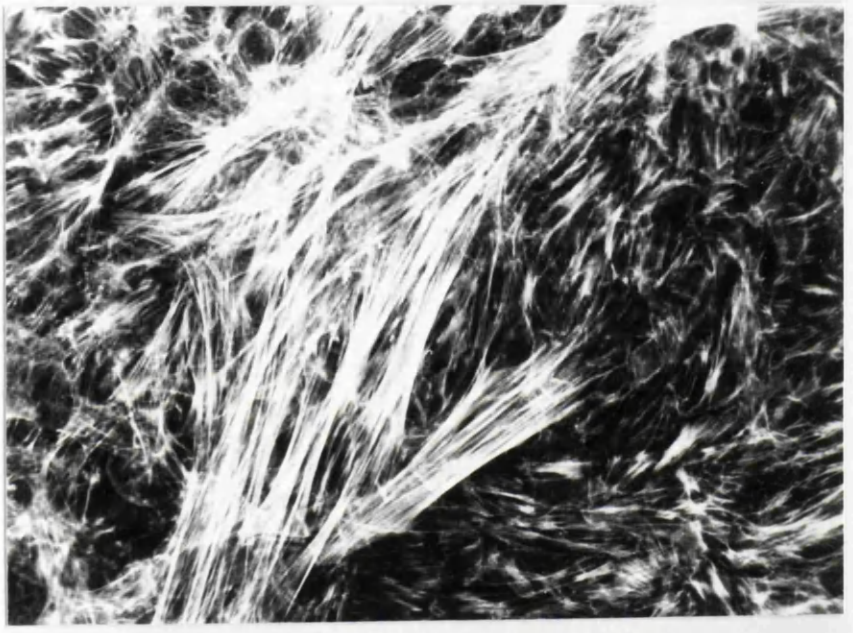
Smooth muscle α-actin isoform: Cells positively stained with a monoclonal recognising the smooth muscle α-actin isoform were detected in explant cultures (Figure 14c). The majority of polygonal cells did not

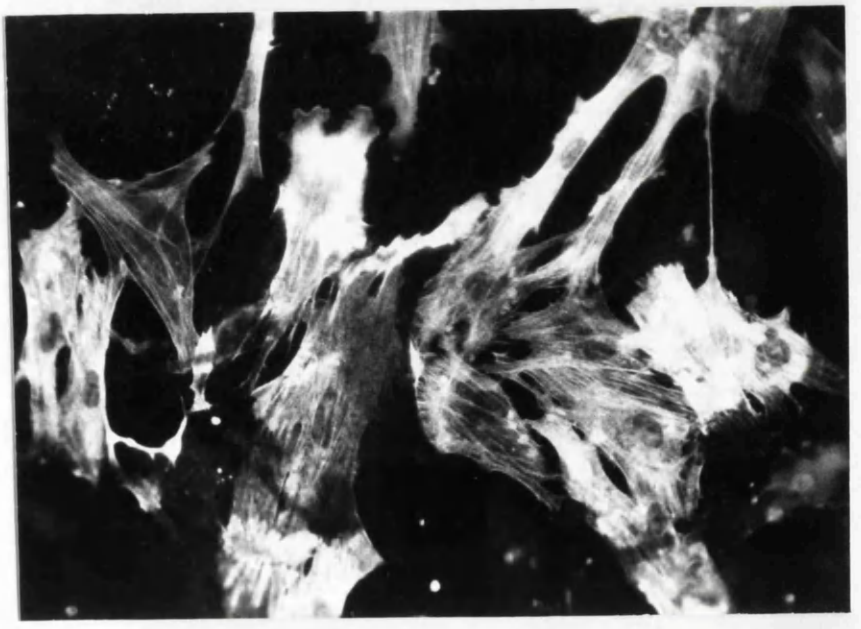
stain. Cells expressing the smooth-muscle α-actin isoform were generally of stretched morphology and located underneath the polygonal cells or at the peripheral margins of explant growths.

Figure 14 (Opposite). Indirect immunofluorescence micrographs of actin filament expression by 4 day organoid explant cultures.

(a) Phase-contrast image; (b) Actin filament distribution in
cells of the corresponding field detected using NBD-phalloidin. Note the intense staining of stretched cells which are not readily discernible with phase optics; (c) smooth-muscle isoform detected with an anti α-actin monoclonal antibody. x400







Basement membrane proteins: Explant cultures stained positively with polyclonal antisera recognising laminin and type IV collagen. Not all cells stained but proportionately more cells expressed laminin than type IV collagen. Staining patterns were punctate or 'smeared' depending on whether the proteins were localised intra- or extracellularly, respectively. Laminin was associated with both polygonal and stretched cells to varying degrees, and heterogeneous levels of laminin expressed by cells in the central region of explants. Cells more basally located (on the basis of focal plane differences), and stretched cells appeared more strongly stained than the majority of polygonal cells.
Type IV collagen expression was more strongly, but not exclusively, associated with stretched cells. Adventitious stromal cells stained strongly for both laminin and type IV collagen.

Double-labelling with membrane and cytokeratin markers: To determine whether the cytokeratin and the membrane markers were expressed in a coordinated manner in and on individual cells, explant cultures were double stained with LE61 (CK18) and 25.5 (luminal cell markers), or LE41 (CK8) and 25.5. The antigenic heterogeneity displayed by luminal cells with respect to both cytokeratins 8 and 18 and the luminal specific membrane glycoprotein recognised by 25.5 was dissociat-

ed. In both cases individual cells were positive for both markers. Cells strongly positive for 25.5 staining were not necessarily strongly stained by antibody LE61 (or LE41). Comparison of the two markers showed no obvious correlation in intensity of expression of the membrane and cytoplasmic luminal markers.

The existence of superficially located 25.5-positive cells with indistinct boundaries (see section 3.3.4) was also demonstrable with membrane-cytoplasmic double-labelling. Large flat cells strongly positive for the membrane antigen were superimposed over cells of different morphology which were delineated by the underlying cytokeratin 18 filament network. Thus these large spread cells located on the top of explants appeared not to express large amounts of cytokeratin 18.

3.3.5 Comparison of explant and primary monolayer cultures.

The growth patterns of luminal (CK 18-positive) and myoepithelial cells (exclusively CK 14-positive) altered when cells were cultured as primary monolayers. Seeded at densities of between 10^4 and 10^5 cells /cm^2, cellular morphology resembled that of explant cultures. At lower densities, cells assumed a more 'epithelioid' appearance. Stretched cells with refractile edges

occurred, which were broader and not stretched to the same extent as those observed in explant cultures. These cells were not always LL001 positive and some cells of stretched or elongated morphology solely labelled with antibody LE61. Areas of Ll001 positive stretched cells were observed underlying LE61 positive cells, although polygonal LL001-positive cells predominated. Cells co-expressing cytokeratins 14 and 18 were quite numerous. Double-labelling was not simply a manifestation of senescence or deterioration, as these cells were observed in mitosis.

Unlike explant cultures, therefore, the phase-contrast morphology of primary monolayer cells could not be easily related to constituent luminal or myoepithelial cells, making it difficult to follow the fate of different phenotypes in living cultures of this type.

3.4 DISCUSSION.

The results presented in section 3.1.4 demonstrate that the expression of certain cytokeratin polypeptides by 70 day rat mammary parenchyma is cell type-specific in so far as the great of majority of cells are concerned. The distribution of immunolocalised intermediate filaments matches that observed in human mammary gland

using the same panel of antibodies, Bartek et al., 1985%; and Taylor-Papadimitriou and Lane, 1987. Given that the antibodies used were monoclonal and monospecific, this identical co-distribution across species substantiates the assumption that it is the same cytokeratin filament determinants that are being identified in both rat and human mammary parenchyma. It therefore is appropriate to provisionally identify the keratin types in the rat mammary gland parenchyme using the numbering system based on immunoprecipitation of human cytokeratins, Moll et al., 1982, on the basis of the known binding characteristics of these antibodies, Lane, 1982; Bartek et al., 1985; Lane et al., 1985; Leigh et al., 1988; Taylor-Papadimitriou and Lane, 1987. This conclusion is further substantiated by the fact that when more than one monoclonal antibody recognising different epitopes of the same cytokeratins were used on the rat, the staining distributions were identical, for example antibodies LE61, C-04 and RGE 53 all recognise cytokeratin 18; antibodies LP2K, BA-16 and BA17 all recognise cytokeratin 19, and antibodies LE41, C11.40 and M20 all recognise cytokeratin 8. Therefore, it can be stated that luminally located cells in the rat are defined by the expression of cytokeratins 7, 18 and 19 and the absence of cytokeratin 14, whilst in the context of the mammary gland, myoepithelial cells are identified by the exclusive

expression of cytokeratin 14. Both luminal and myoepithelial cells express cytokeratin 8 and did not stain with antibodies recognising cytokeratins 4, 10, 13 and 17 used in this study. However lack of observed staining does not preclude the possibility that these cytokeratins are in fact expressed but not detected. A degree of epitope masking is suggested by the fact that cells in the intact gland stained positively with antibody RCK 105, recognising cytokeratin 7, but did not stain with a bi-specific monoclonal antibody recognising cytokeratins 7 and 17 (C46.2). Nevertheless, the spectrum of cytokeratin filaments demonstrated in the rat broadly reflects that detected in the human by biochemical analysis namely 5, 7, 8, 14, 15, 17, 18, 19, Moll et al., 1982,(although no antibodies recognising cytokeratins 5 and 15 were used during this study). In addition, the observed results are consistent with the cytokeratin profile of mouse mammary gland determined biochemically by Asch and Asch, 1985. Calibrating the molecular weights of the cytokeratins published by these authors with the murine catalogue, Schiller et al., 1982, and correlating them with the human cytokeratin catalogue, Moll et al., 1982, indicates that mouse mammary gland contains cytokeratins 8, 11, 17 and 22 which are probably homologous to human cytokeratins 5, 8, 14 and 19 respectively.

Alveolar luminal cells expressed cytokeratin 18. This

result corroborates the findings of Taylor-Papadimitriou and Lane, 1987, but contradicts those of Dulbecco et al., 1983. The observation that certain lymph node cells express cytokeratin 8 is consistent with the findings of Franke and Moll, 1987, who described the distribution of this cytokeratin in subpopulations of extrafollicular reticulum cells of human lymph node. At high magnification cells located between luminal and basal layers were observed on frozen sections which stained for cytokeratin 8 and 18, although not all cells in a similar location expressed cytokeratin 19. Because of the limitations of frozen sectioning, it is a matter for speculation at present whether these cells correspond to the large pale clear cells observed in the intact gland and in organoid fragments observed under transmission electron microscopy. The precise identity of the 'clear' cell population is of importance, since it has been postulated that cells of this type, which are present in all parts of the murine mammary parenchymal tree during all stages of development, represent a mammary stem cell population, Smith and Medina, 1988. These authors reported them as being cytokeratin positive, although no data was presented. Furthermore, it has been suggested that a subpopulation of cytokeratin 18 expressing luminal cells in small ducts and lobules of human mammary gland which do not express cytokeratin 19 represent cells might be the

cells which generate new growth at pregnancy, Bartek et al., 1985; Taylor-Papadimitriou and Lane, 1987.

The restricted specificity of the different members of the monoclonal antibody panel screened *in vivo* enabled the different cellular phenotypes in cultures of mammary organoids to be related to specific cell types. Organoid cultures generated diverse morphologies which changed with time and with different cultures conditions (for example serum-free versus serum-containing medium). Identifying a specific cell type (for example luminal epithelial versus myoepithelial) with a particular morphology in culture using solely phase-contrast appearance is in my opinion not possible as it involves unverifiable preconceptions as to the influence of tissue culture on the morphology of individual cell types. In the initial phase of these experiments an attempt was made to describe cells within such cultures using neutral terms which did not imply a specific identification. Two major cell morphologies were defined in this manner, namely polygonal and stretched cells. With specific membrane and intermediate-filament markers, however, an objective identification can be made. The use of the marker panel justified distinguishing cells on the basis of either polygonal or stretched morphologies . Antibodies 25.5 and LE61, LP2K and RCK 105 revealed that polygonal cells

expressed a luminal phenotype; these constituted the predominant population in short-term explant cultures propagated in serum containing medium. The stretched cells located basally and around explant peripheries displayed markers wholly consistent with a myoepithelial phenotype, specifically cytokeratin 14 expression and lack of detectable staining with antibodies LE61, LP2k and RCK 105. Unfixed cells in a basal location were presumably inaccessible to anti-endopeptidase antibodies, thus precluding their detection by this form of marker, although peripherally located stretched cells did express endopeptidase-24.11.

Thus the markers used in this study unequivocally establish that cells expressing markers characteristic of either differentiated luminal or myoepithelial cells *in situ*, survive in a tissue culture environment and the majority maintain the same pattern of differential expression. The results presented here contrast with previously published descriptions of cultured rat organoid cells, Warburton et al., 1985. These authors characterised cellular phase-contrast morphologies as being of either "small cuboidal", "large epithelioid" or "elongated morphology". Bearing in mind that culture conditions differed and that cellular morphologies are influenced by seeding density (Das et al., 1974), the "small cuboidal" and "large epithelioid cells" cells

described by Warburton et al., 1985, probably correspond broadly to the polygonal cells described here which express a luminal phenotype, on the one hand, and to the peripheral stretched cells which retain a myoepithelial phenotype, on the other. However, the relationship between the "elongated" cells described by Warburton et al., 1985, and cells found in explants described here is less obvious. According to these authors, "elongated" cells were positive with a guinea pig anti-serum raised against human callus keratin, Warburton et al., 1985; Warburton et al., 1989, and this was taken to indicate myoepithelial origin since the antibody stained basal cells *in situ*, Warburton et al., 1985; Warburton and Gusterson, 1989. However, since callus keratin does not contain cytokeratins 5 or 14, (i.e. myoepithelial specific cytokeratins), Taylor-Papadimitriou and Lane, 1987, the exact specificity of this antibody is open to question. Other isolated elongated cells in their cultures expressed vimentin, cytoplasmic laminin and type IV collagen. It seems probable that keratin positive "elongated" cells in the Warburton cultures correspond to basally-located stretched cells, which have here been shown to express cytokeratin 14 . The isolated elongated cells expressing vimentin, type IV collagen and laminin, on the other hand, may correspond to the bipolar spindle cells found here associated exclusively with alveolar ex-

plants, as these also expressed laminin, type IV collagen and vimentin, but never stained with any of the mono-specific anti-cytokeratin antibodies used. Furthermore, these bipolar cells were never associated with ductal explants, even when pieces of duct fragments were cultured in isolation. They probably represent cells derived from intra-alveolar stroma or vasculature. Hence it seems possible that there were two 'elongated' cell types in the Warburton cultures derived from different *in situ* lineages, namely myoepithelial and mesenchymal proper.

The patterns of actin filament distribution in cultured myoepithelial or luminal polygonal cells visualised with the fluorescent NBD-phalloidin is consistent with that seen in intact rodent gland using the same reagent. *In situ*, the myoepithelium is prominent and luminal cell apices and regions of junctional contact are highlighted, Emerman and Vogl, 1986. Thus although both cell types react with the phalloidin, the distribution of F-actin is distinctively different both *in situ* and in the cultured cells.

The staining patterns of vimentin antibodies seen on the intact gland, which showed that basal cells expressed this intermediate filament whilst luminally located cells did not appear to, agrees with the find-

ings of Dulbecco et al., 1983; Warburton et al., 1989, who used antibodies different to those employed here. The ability to detect vimentin filaments in cultured luminal epithelial cells (identified on the basis of cytokeratin expression and morphology), the counterparts of which do not stain with the same anti-vimentin antibodies in the intact gland, also occurs with human mammary tissue, Dairkee et al., 1985; Mork et al., 1990. Although it has been suggested that vimentin expression can be induced in cultured cells by alterations in cell shape, Ben-Ze'ev, 1984; Dairkee et al., 1985, evidence also strongly suggests that the elaboration of vimentin filaments is associated with rapid growth rate, Connell and Rheinwald, 1983; Domagala et al., 1990; Mork et al., 1990.

The results here show that cells with luminal phenotype predominate during the early stages of rat mammary explant culture. Differentiated myoepithelial cells were restricted to peripheral and isolated basal locations within the explant, and did not correspond to more than approximately 10% of the total parenchymal cell population after 7 days culture. Hence it seems that general organotypic growth characteristics are maintained in early explant cultures, with the myoepithelial cells undergoing proportionately less mitosis than luminal cells, as occurs *in situ*, Joshi et al.,

1986. However, a population shift clearly occurred in response to specific culture situations. In long term cultures for example (greater than three weeks propagation), grown with serum and the full complement of hormones and growth factors used during this study (HC:I;CT:EGF), myoepithelial cells gradually become the predominant phenotype, becoming tightly-packed and losing the characteristic 'stretched' shape as they proliferated. When explants were propagated in serum-free medium a similar morphological change occurred, and the majority of cells in late stage cultures of this type were exclusively cytokeratin 14 positive, indicating their myoepithelial derivation.

The three modes of growth displayed by the myoepithelial component of rat explant cultures - peripheral stretched cells which remained non-mitotic; stretched cells which assumed polygonal morphology associated with rapid proliferation; and proliferating polygonal cells which assumed quiescent stretched cells characteristics, resemble the described behaviour of human myoepithelial cells in culture, in which the switch from proliferating polygonal to non-mitotic spread morphology is accompanied by the emergence of certain markers characteristic of differentiated myoepithelium, Petersen and van Deurs, 1988.

Certain behavioural traits of luminal cells in explant culture appear common to both rat and human mammary systems. The so-called "patch" cells described by Edwards et al., 1984, in human cultures can be identified in those of rat by virtue of peanut lectin reactivity, Warburton et al., 1985, and morphological inconsistencies between phase image and 25.5 staining patterns, (Section 3.3.4 above). The comparative reduction in cytokeratin 18 staining of some of these cells double-labelled with 25.5 is similar to the observations of Rudland et al., 1989, that EMA or MFGM positive patch cells in human explant cultures do not stain significantly with antibody LE61. The tendency for human mammary epithelial cells to undergo squamous metaplasia during prolonged explant culture has been described, Hillman et al., 1983. This abnormal development of epithelial cells can be induced in mouse mammary organ cultures in response to agents elevating intracellular cAMP, Schaefer et al., 1980, and it has been suggested that patch cells represent the cellular products of this process, Rudland et al., 1989.

In summary, the results in this chapter have shown that certain lineage-specific markers can be used to unequivocally establish the identity of cells in explant cultures of rat mammary parenchyma. The two major cell types identified *in vivo*, that is luminal epithelial

and myoepithelial, both have counterparts in such cultures. Additionally, an important observation is the presence in these cultures of cells which although of a luminal phenotype morphologically, express cytokeratin 14 as well as cytokeratins 8 and 18. This raises questions as to the origins of such cells. Does the culture environment induce alterations in the phenotypes of a proportion of myoepithelial or luminal cells or do these cells with a dual-phenotype represent a third distinctive population which exists in situ? Unfortunately, in this study it was not possible to unambiguously identify double-labelled cells in frozen sections. However, it is interesting to note that cells simultaneously expressing cytokeratins typical of both luminal and myoepithelial cells can be distinguished from the two major cell types in the terminal ductal-lobular structures of human mammary gland during periods of proliferation, Jarasch et al., 1987.

The cellular heterogeneity of explant cultures meant that investigating lineage connections between luminal and myoepithelial cells, and determining whether phenotypic conversions generated cells of different differentiated status, could not be accurately approached using this system. In order to answer these questions and the examine the long-term behaviour of individual

cells of different initial phenotypes, populations of cells isolated both by cell sorting and cloning were studied in culture. The results of these experiments are presented in the next chapter.

CHAPTER 4.

CULTURE OF SEPARATED MAMMARY PARENCHYMAL CELL POPULATIONS.

4.1 Culture of flow sorted luminal and myoepithelial cells.

4.1.1 Introduction.

Viable luminal or myoepithelial cells derived from uncultured organoids were separated by flow sorting on the basis of the differential expression of the membrane antigens recognised by antibodies 25.5 and PHM6. Selectively sorted populations of cells were then plated separately at high density without or without feeder cell support. Primary cultures were observed by phase-contrast microscopy. The phenotypes of sorted cells were independently examined using the panel of cell-type specific anti-cytokeratin antibodies previously shown to stain luminal and myoepithelial cells in situ and in primary explant cultures (see Sections 3.1.4 and 3.3.4). Cells were sorted both from disaggregated organoid preparations, and cells harvested from short-term primary explant cultures (up to 1 week old). Since satisfactory yields of viable cells could be obtained using organoid material directly, the majority of experiments described here used cells sorted from organoid preparations. No qualitative differences were observed between the two sources of cells, and cultures initiated from populations of sorted luminal and myoepithelial cells were capable of independent growth.

4.1.2 Morphology of primary cultures of sorted luminal and myoepithelial cells.

Organoid preparations were disaggregated into cell suspensions and stained (by indirect fluorescence as single labels), with either antibodies 25.5 or PHM6, as described in Section 2.6.1. Using multiparametric analysis, cells were gated on the basis of light scatter to exclude clumps and debris. Propidium iodide staining was used to exclude cells which were dead at the time of sorting, and cells were sorted for positive antibody fluorescence. Two categories of cells were generated concurrently for each antibody: positively stained or negative cells. Thus, four groups of sorted cells could be separately collected and cultured - 25.5 positive/ 25.5 negative, or PHM6 positive/ PHM6 negative.

Immediately after collection from the flow cytometer in tissue culture medium, the majority of sorted cells were discrete single cells, although some cells in close proximity rapidly adhered to each other resulting in occasional doublets (Figures 15a and d). The observations of freshly sorted material confirmed that the settings of the light scatter gates were appropriate for collection of single cells.

Attachment and spreading of sorted cells occurred over a 48-72 hour culture period, with sorted luminal (25.5-positive) cells taking longer to attach than myoepithelial (PHM6-positive) cells. Generally about 10,000 cells were sorted into 1.5cm diameter wells, and plating efficiencies were generally between 20-40% depending on the cell type; this was increased to between 50-60% when cells were co-cultured with irradiated 3T3 L1 cells, and the rate of attachment was also significantly increased in the presence of feeder cells. Up to half the sorted cells could therefore be recovered from the cytometer in a long-term viable condition.

Sorted 25.5-positive and PHM6-positive populations differed significantly from each other with respect to phase-contrast morphology and growth rates. A series of photomicrographs detailing the progression of sorted cultures grown without feeder cell support are shown (Figure 15).

Luminal (25.5-positive) sorted cells: Differential rates of attachment and spreading were noticeable between individual sorted cells of this type. After 48 hours culture, a few isolated attached refractile cells could be observed, whilst in other areas of the culture mitoses were visible within patches of adjacent cells exhibiting an epithelial morphology.

After 48 hours culture, the epithelial patches had expanded by a combination of cell division and increased spreading of individual cells. Highly refractile rounded cells which were probably undergoing mitosis appeared in characteristic pairs. Other single rounded cells may have been entering the initial stages of mitosis, or could have been cells which had attached, but which had not yet fully spread on the culture surface (Figure 15b). After 12 days culture, cultures were approaching confluence and were composed of pleiomorphic cells. Flattened epithelioid cells were marked by refractile cellular boundaries; these were mixed with patches of other epithelial cells which possessed attenuated edges, the limits of which were not easily discernible with phase optics. At confluence, sorted 25.5-positive cells remained morphologically heterogeneous (Figure 15c).

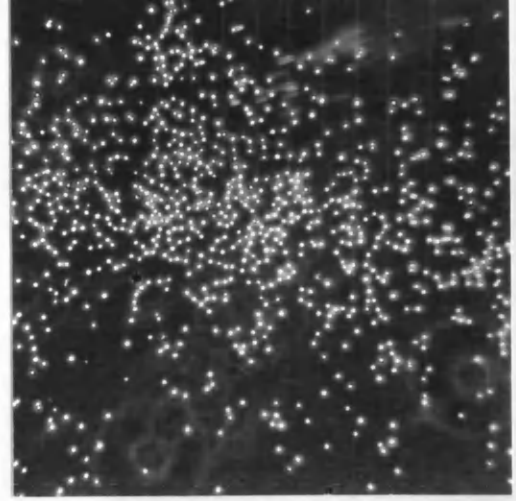
Contaminating cells of mesenchymal appearance eventually overgrew the epithelial component of luminal sorts despite representing less than 1% of sorted cells at the outset. After 2 weeks such cells represented a significant fraction (up to ~50%) of the culture, although this varied between sorts.

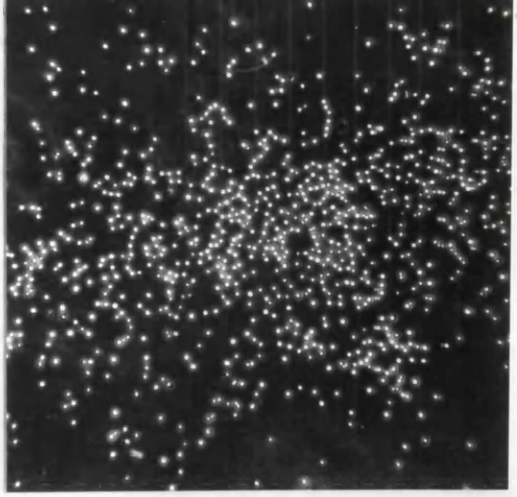
Myoepithelial (PHM6-positive) sorted cells: More cells attached from PHM6-positive sorts after 24 hours culture compared with luminal sorts of similar cell numbers. After 48 hours culture they could be seen to be distinctively different to sorted luminal cells. After 7 days culture, three morphologically distinguishable populations of cells were apparent in PHM6-positive sorted cultures, the frequency of which depended on the fluorescence gating parameters set during individual sorts. With reference to the cytometric histogram in section 3.2.4, two morphological variants arose in cultures sorted from region 1, which contained the less strongly stained cells. These cells displayed sheath-like or polygonal morphologies. Polygonal cells were notable for their considerable proliferative activity. Cells sorted from region 2 (very bright fluorescence) exhibited a mesenchymal appearance in culture.

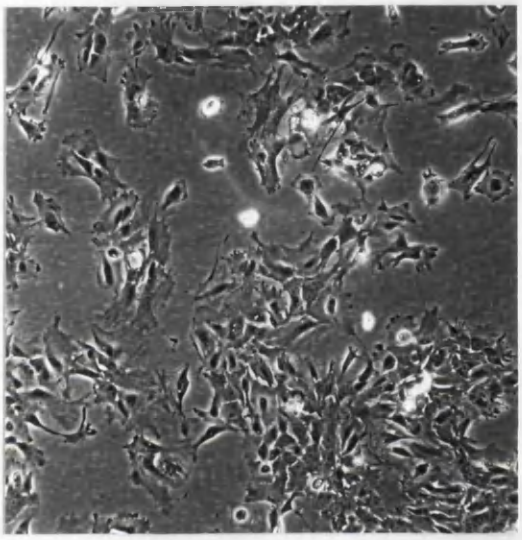
After 12 days in culture sheaths of stretched cells were observed in which mitotic figures were not obvious. Polygonal cells continued to divide, however, and produced layers of tightly-packed cells (Figure 15f). The morphology of the latter contrasted sharply with cultures of luminal origin at the same stage (compare Figures 15 b and f).

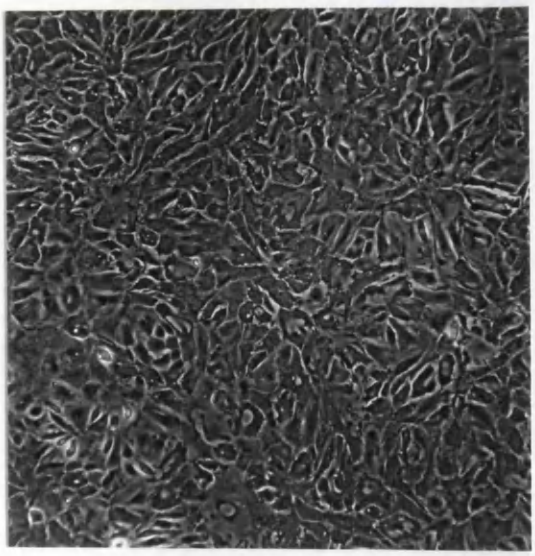
Negative sorts: Cells in cultures of reciprocal negative sorts were identical in morphology and growth rate to those generated by the corresponding positive sort, i.e. 25.5-negative sorts matched PHM6-positive ones, and PHM6-negative sorts resembled 25.5-positive cells. Cells of frankly fibroblastic morphology only appeared in significant numbers in either 25.5 or PHM6 negative sorts, when these were examine shortly after plating, although as described above, similar cells eventually overgrew some positive sorts.

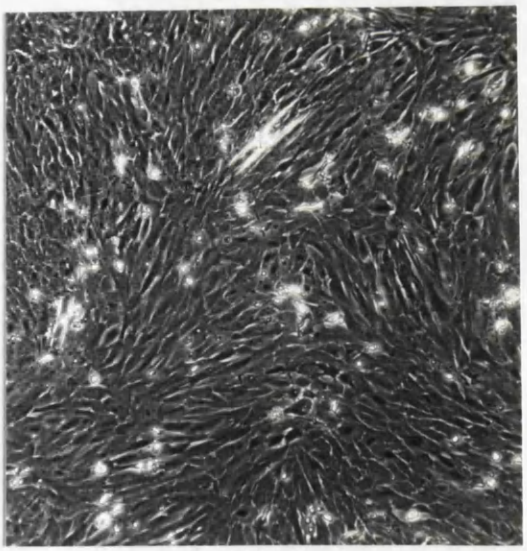
Figure 15 (Opposite). Phase-contrast micrographs of sorted luminal and myoepithelial cells and derived primary cultures.
(a) 25.5-positive cells immediately after sorting; (b) PHM6-positive cells immediately after sorting; (c) 25.5 sorted cells
after 48 hours culture; (d) PHM6 sorted cells after 48 hours culture; (e) 25.5 sorted cells after 12 days culture; (f) PHM6 sorted cells after 12 days culture.
Both cultures propagated in HC:I:CT containing DMEM:F12/FCS medium. x180











4.1.3 Cytokeratin expression by sorted cells in culture.

In order to verify that sorting on the basis of 25.5 or PHM6 staining did indeed separate luminal and myoepithelial populations, cells derived from short-term explant cultures were sorted, plated onto glass coverslips, fixed after 48 hours and then probed for the expression of cytokeratins 8, 14 or 18. The percentage of cells staining with individual markers was counted and the results are tabulated below.

PERCENTAGES OF SORTED CELLS EXPRESSING CELL TYPE-SPECIFIC CYTOKERATINS.

SORT MARKER	CYTOKERATIN MARKER		
	LE41+ (CK8)	LL001 (CK14)	LE61+ (CK18)
25.5+	89% +/-6	8% +/-2	92% +/-2
25.5-	1%	8% +/-2	1%
PHM6+	4% +/-1	6% +/-2	0%
PHM6-	38% +/-4	1%	41% +/-5

Table 9.

The expression of the appropriate cell-type specific cytokeratins demonstrate that the sorting procedure was very effective. Over 90% of cells in 25.5-positive sorts expressed the luminal phenotype as indicated by LE61 positivity. No luminal phenotypes were detected in PHM6 sorts. The presence of some CK14-positive cells in the 25.5 sort is consistent with the concurrent expression of CK14 and CK18 by some cells observed in explant cultures (see Section 3.3.4). The larger number of stromal cells observed in the negative sorts (see above) accounts for the substantially reduced numbers of cytokeratin-positive cells in both 25.5-negative and PHM6-negative populations. The fact that relatively few myoepithelial cells (CK14+/CK8+/CK18-) were detected in the PHM6-sort, was probably the result of using explant cultures (which yield low numbers of myoepithelial cells) as the starting material for these experiments .

The results presented in Table 9 were generated during preliminary experiments to determine the gating parameters enabling the separation of luminal and myoepithelial cells. Gating parameters thus differed from experiment to experiment and were further refined during successive sorting procedures. In addition, the use of uncultured organoid material substantially reduced the mesenchymal contamination reflected in this particular experiment.

Thus, sorting on the basis of 25.5 or PHM6 positivity results in the efficient separation of luminal and myoepithelial cells respectively. Cultures of sorted luminal or myoepithelial cells grow independently of each other, and retain the expression of cell-type specific cytokeratins.

4.2 Clonal growth of organoid preparations and cultures.

4.2.1 Introduction.

The heterogeneity of cell morphologies observed in cultures of sorted luminal and myoepithelial cells indicated that each population might not be composed of cells behaving in an identical fashion. This problem was addressed by cloning the sorted cells. However, before attempting to correlate the behaviour of isolated cells with their initial antigenic phenotype the number of recognisable discrete clonogenic phenotypes constituting normal rat mammary parenchyma was examined using unsorted preparations from both uncultured organoid preparations and explant cultures. The range of colonies was characterised by phase-contrast morphology over a period of time so that dynamic changes in appearance could be distinguished from intrinsic clonal differences persisting throughout all phases of culture. Clonal behaviour was further characterised by the

expression of membrane markers, basement membrane components, cytostructural cell-type specific markers, and by mitotic rates. The progressive morphological behaviour of individual clone types was monitored. Marked clones were followed under phase-contrast at various stages after initial establishment. The marker expression of equivalent clones in concurrent cultures was assessed. Finally, clonal growth of flow sorted populations enabled the unequivocal identification of clone types observed in unsorted cultures as being of either luminal or myoepithelial origin.

4.2.2 Morphology of parenchymal clones.

Propagating cell suspensions derived from freshly isolated organoids as clonal growth cultures consistently generated several distinctive colony types. After 3 days culture, small mitotic epithelial-like colonies were observable. These diverged into morphologically distinguishable clone types between 4 and 5 days culture. Individual marked clones were carefully followed and sequential observations were made to ensure that the different morphologies represented intrinsically different colony types and not merely different stages in the growth of similar clones. The clone types described below each progressed in a characteristic manner with time in culture, and each comprised a different proportion of the total clonal population.

Quantitative differences in clone yields, and their changes with time are described more fully in sections 4.5.3 and 4.3.4, respectively.

As the baseline for further work, clonal morphotypes were defined using 7 day cultures propagated with irradiated 3T3 L1 feeder cell support, (see section 4.4.2), in 1:1 DMEM:F12/ 10%FCS/ HC:I:CT containing medium. After this time all colonies arising in the same culture could be categorised into one of 6 different clone types. Each clone type could be discriminated on the basis of morphological criteria, both by the morphology of individual constituent cells and the overall colony morphology. Descriptions of each colony type, designated hereafter as types 1-6, will be adduced at this point. Since the morphology and chronological behaviour of unsorted and sorted clone types was essentially identical, representative examples of clones generated from sorted cells are illustrated (see section 4.2.3; Figures 18-24).

Clone type 1: These round colonies were composed of cells displaying a range of cell morphologies, depending on whether the cells were centrally or peripherally located and whether or not they were dividing. Most centrally located cells were spread with contiguous margins which were difficult to discern at low-power; in some areas these cells appeared necrotic as a result

of the collapse of cyst-like structures. Most dividing cells were polygonal in shape and smaller in diameter; those found centrally had characteristic eccentric nuclei and spread centrally, merging with the large spread cells in this zone. Dividing cells were also seen among tightly packed polygonal cells which formed a concentric zone, two to three cells wide, which sharply defined the edges of these colonies. The outermost cells formed a distinctive 'palisade-like' array, with which this clone type could be most easily recognised.

Type 1 clones were also distinctive in that they exhibited unique interaction with adventitious stromal cells present in cultures. Thus, with some such clones quite separate fusiform cells were observed to accumulate at their margins and to migrate over the clone surface. This apparently interactive behaviour between mesenchymal and parenchymal cells was not observed with any other clone type.

Clone type 2: These generally round colonies were composed of uniform polygonal cells with characteristic refractile edges seen throughout the clone. Colony edges were sharply defined by the refractile circumferential edges of peripherally located cells. Peripheral cells appeared to migrate outwards at different rates

as they formed projections composed of two or three closely apposed cells. This phenomenon gave a less regular margin to these clones compared with, for example, Type 1 clones.

Clone type 3: This type was composed of small, refractile cells which divided and progressively spread. Cells at clone peripheries possessed prominent lamellapodial leading edges which became attenuated and spread in a fan-like manner. Cell-cell boundaries were less prominent that Type 2 clones. In some of the more attenuated areas cell boundaries were difficult to discern with phase optics. Overall, one of the more easily recognisable characteristics of this clone type was the exaggerated scalloped appearance of the colony edge, more so that with Type 2 clones, and completely unlike the regular palisade-like edge of Type 1 clones.

Clone type 4: These large, round clones were composed of small, compact, mitotic isometric cells with refractile edges. Central areas of colonies appeared multilayered, and colony edges were sharply defined. Although similar in overall appearance to Type 2 clones at some stages in their development, sharing a cobblestone or pavement-like appearance, they differed in that they clearly grew more rapidly and for a longer period yielding the characteristic densely packed

refractile, multilayered central zones. By comparison, type 2 clones slowed down earlier and generated much less densely packed colonies.

Clone type 5: These clones were particularly distinctive in that they were small and composed of sheaves of markedly anisometric 'stretched' cells. These clones contained few obvious mitotic cells, and virtually ceased to grow by 7 days culture, whereas all other clone types were still actively proliferating at this stage.

Clone type 6: These large round clones were very distinctive in that they were composed of proliferating densely packed small round, dark cells. Numerous mitoses could be seen within Type 6 clones. The individual cell boundaries, were particularly visible towards the edges of the clones where cells were less densely packed. Compared with other clone types, the overall shape of Type 6 colonies appeared irregular.

Mixed clone type. Distinctive clones resembling both types 5 and 6 were observed. Areas similar in appearance to Type 5 clones could be seen to be adjacent to rapidly proliferating densely packed cells. As these clones progressively grew they came to resemble Type 6 clones (see section 4.2.3).

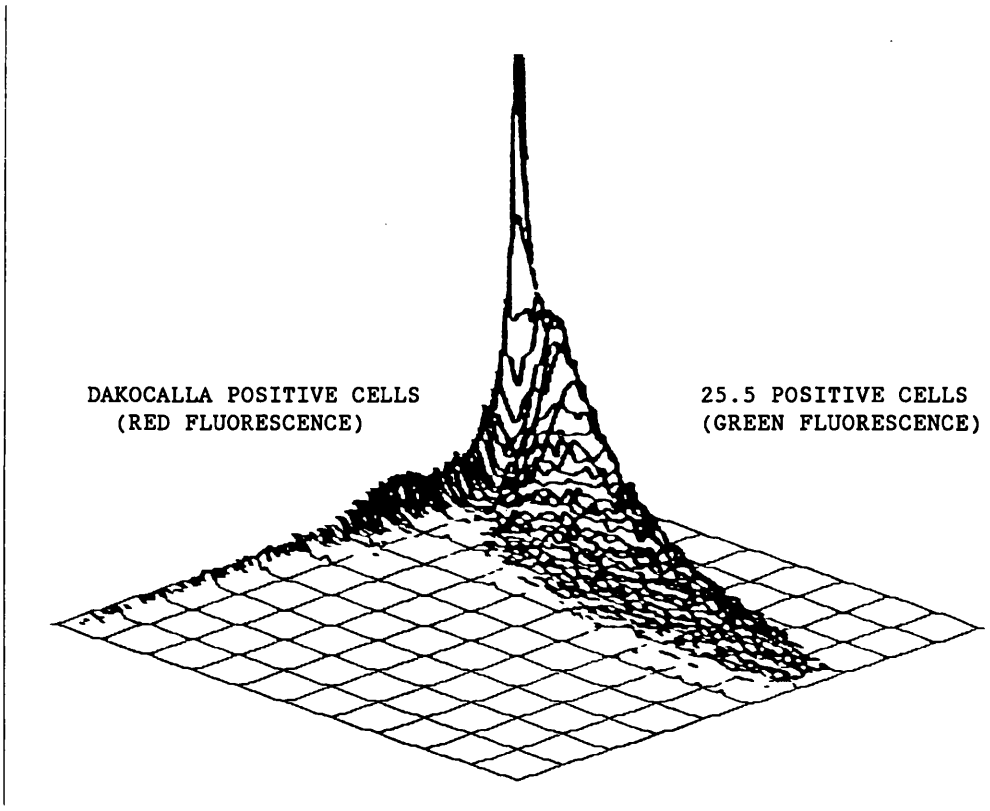
All of these different colony types were found in serum-containing medium supplemented with hormones, and all were generated from disaggregated uncultured organoids, explant cultures and primary monolayer cultures, albeit with differing frequencies.

4.2.3 Morphological progression of clones generated by sorted luminal or myoepithelial cells.

Having identified several colony types, the next objective was to determine which clone types arose from luminal or myoepithelial cells. This was achieved by propagating sorted cells at clonal densities. Dispersed organoids were double-labelled with 25.5 and DAKOCALLA and analysed cytometrically. Gates were set which delimited areas in either the 25.5 or DAKOCALLA positive profiles corresponding to cells expressing an average fluorescence and excluding very bright and very dim cells in each arm. The isometric display of the profiles of the sorted cells is shown in Figure 16. Cells positive for either antigen were sorted simultaneously. Due to the different proportion of each cell type in the preparation, approximately one DAKOCALLA positive cell was sorted for every four 25.5 positive cells. Collected cells were then seeded into separate dishes at clonal densities with irradiated feeder cells .

Figure 16 (Opposite). Isometric profile of cells sorted simultaneously on the basis of differential antibody staining from a 25.5/DAKOCALLA double-labelled organoid suspension.

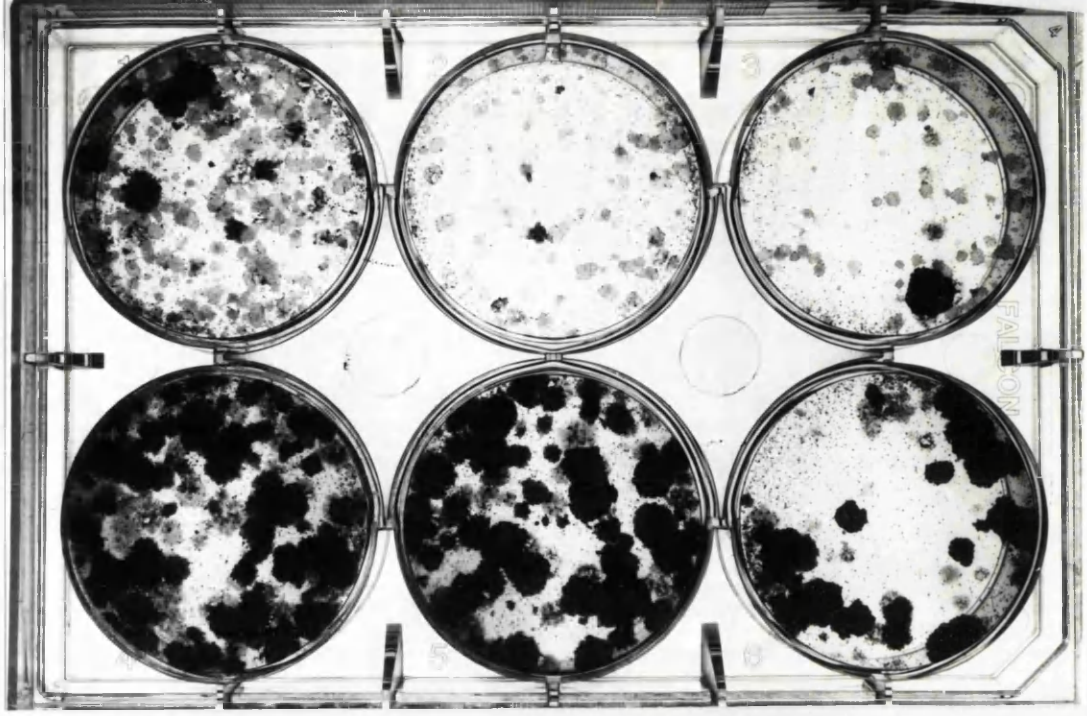
Cells occurring in gated regions similar to those shown in Figure 7 (c) were sorted simultaneously. The cytometric data was then plotted isometrically. Two distinct populations were clearly sorted - DAKOCALLA stained myoepithelial cells (sorted on the basis of red fluorescence); and 25.5 stained luminal epithelial cells (sorted on the basis of green fluorescence).

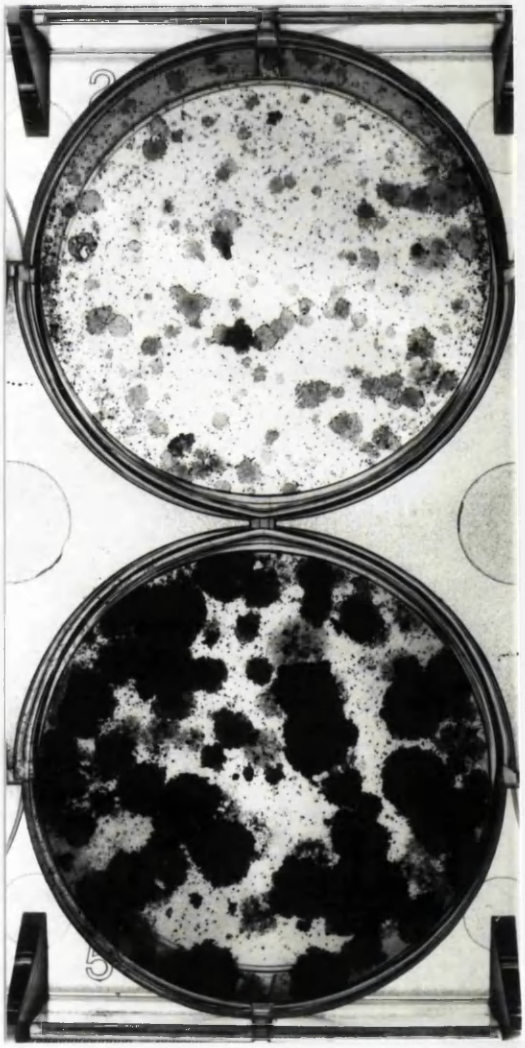

DAKOCALLA POSITIVE CELLS
(RED FLUORESCENCE)
25.5 POSITIVE CELLS
(GREEN FLUORESCENCE)

The resultant clonal cultures were fixed and stained after 7 days propagation. The clones arising from the sorted cells are shown in Figure 17. Clones in the top row of the plate were generated from luminal (25.5-positive) cells, and those in the bottom row from myoepithelial (DAKOCALLA-positive) cells. Even when examined macroscopically differences between the two populations are obvious. Myoepithelially derived clones are significantly larger and more basophilic than their luminal counterparts. A small number(<5%) of colonies in the 25.5 positive sorted culture resembled the majority myoepithelial-type clones. These are easily distinguished as large densely basophilic patches (Figure 18), as a result of considerably higher rates of proliferation, compared with the majority clone types. A significant number (~25%) of clones in the DAKOCALLA sort did not stain strongly like the majority. At higher power these were seen to be non-parenchymal cells of a stromal appearance. Un-cloned sorted myoepithelial cultures described previously indicated that there was a definite stromal component corresponding to endopeptidase-24.11-positive cells (Section 4.1.3). Clones of luminal morphology were never observed in cultures sorted for endopeptidase-24.11 positivity.

Figure 17 (Opposite). Photograph of fixed and stained clonal growth cultures initiated from cells sorted simultaneously from a 25.5/DAKOCALLA double-labelled preparation (see Figure 16).

(a) Top row: 25.5-positive sorted cells; bottom row: DAKOCALLA-positive sorted cells. x1.08; (b) higher magnification of wells 2 and 5. x1.6; Note the differences in staining intensities of luminally-derived clones. Small specks correspond to feeder cells.



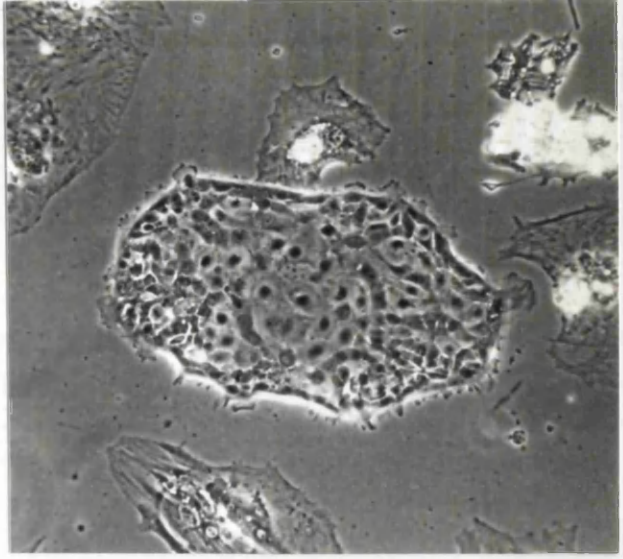


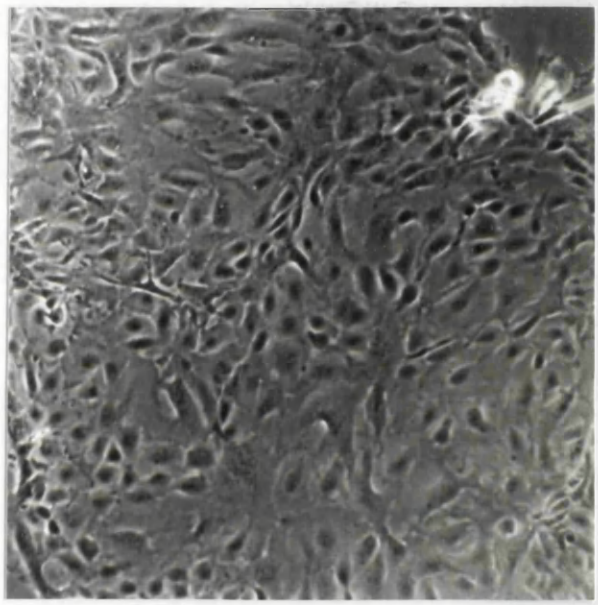
Phase-contrast observation provided very clear-cut results. All the clone types seen in unsorted cultures could be identified in one or other of the sorted clonal populations. The six clone types identified in unsorted cultures could be essentially grouped into two mutually exclusive sets. Clone types 1-4 were generated by luminal (25.5-positive or PHM6-negative sorted) cells, while clone types 5 and 6 arose from myoepithelial (PHM6-positive or 25.5-negative sorted) cells. Thus, as all clone types observed in unsorted cultures could be accounted for in either the sorted luminal or myoepithelial clonal cultures clearly indicating that no cells had been inadvertently excluded by the various gate settings used for flow sorting. Furthermore, the sorted clone types were the same when cells were sorted from single or double-labelled preparations showing that none of the parenchymal clone types observed in unsorted cultures corresponded to a double-negative (25.5-negative/ endopeptidase-24.11-negative) phenotype. No parenchymal clone type was exclusively found in both the 25.5-negative and the endopeptidase-24.11-negative arm of single sorts. In addition, when double-negative cells from a double-labelled sort were separately collected and cultured, they consisted predominantly of a mixture of stromal cells types, with no unique parenchymal clone type present.

The clones illustrated below were generated from the same organoid preparation which was disaggregated, stained and sorted as described above. Sorted cells were plated at clonal densities and co-cultured with irradiated 3T3 L1 cells in HC:I:CT containing medium. Each clone type generated from either luminal or myoepithelial populations progressed in a characteristic manner with time. This is illustrated in the series of sequential photomicrographs of individual sorted clones shown in Figures 18 to 24. The same clone of each type is shown at 5, 7 and then 11 days propagation.

Figure 18 (Opposite). Phase-contrast micrographs showing the morphological progression of a sorted 25.5-positive Type 1 luminal clone.

The same clone after (a) 5 days; (b) 7 days; (c) 11 days culture. x158





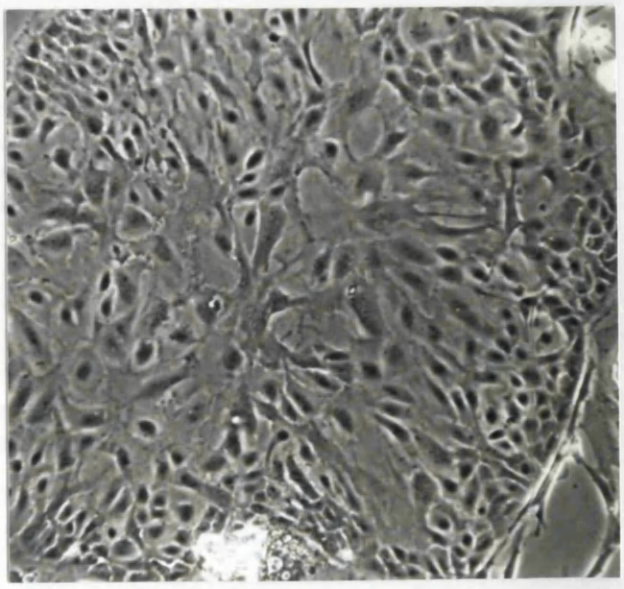
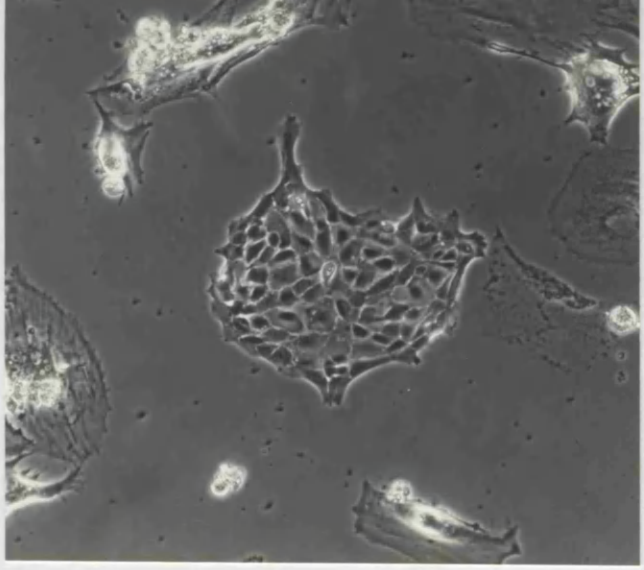
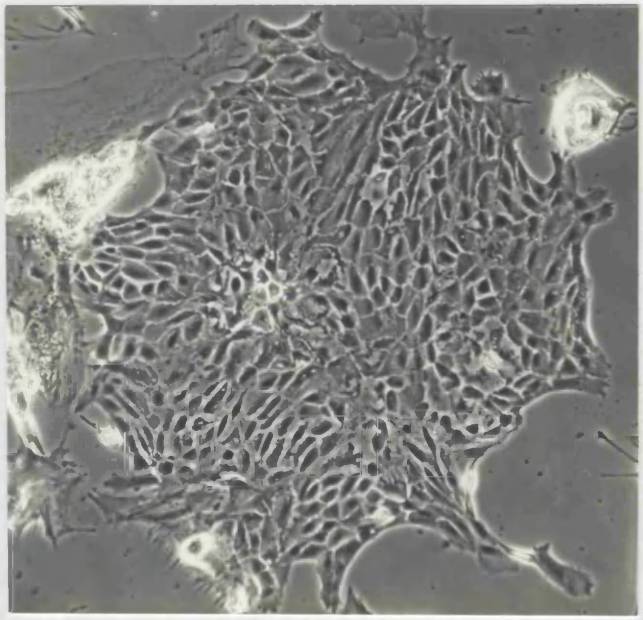


Figure 19 (Opposite). Phase-contrast micrographs showing the morphological progression of a sorted 25.5-positive Type 2 luminal clone.

The same clone after (a) 5 days; (b) 7 days; (c) 11 days culture. x158





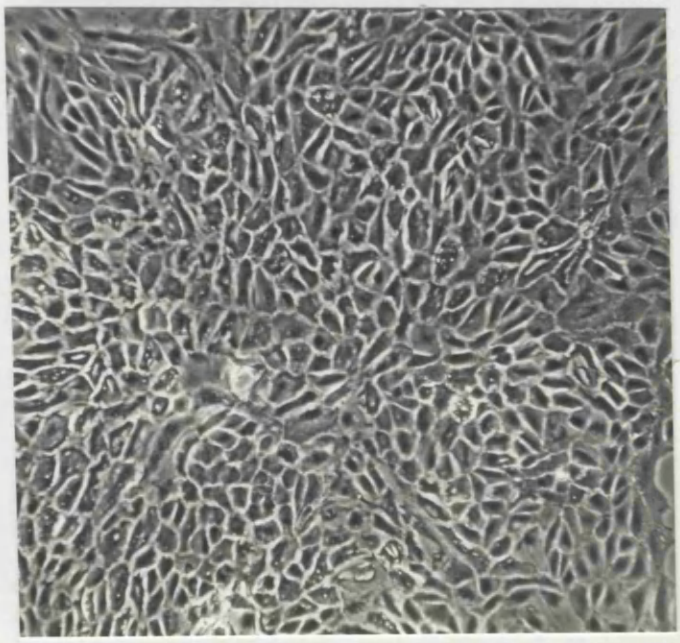
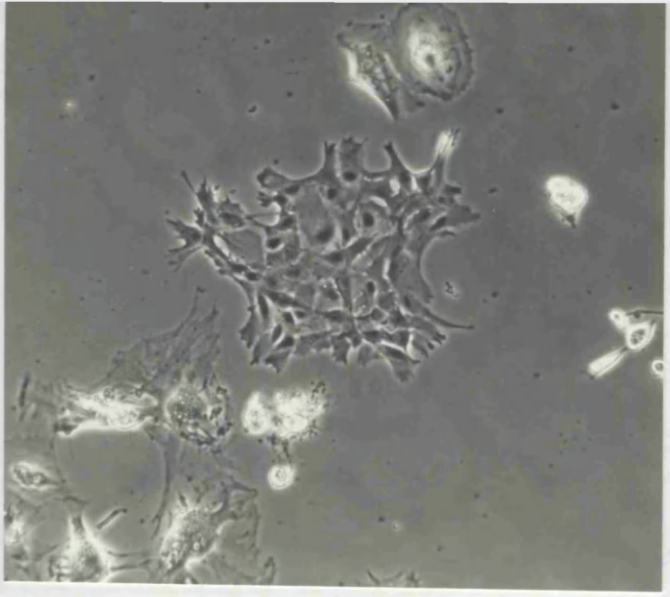
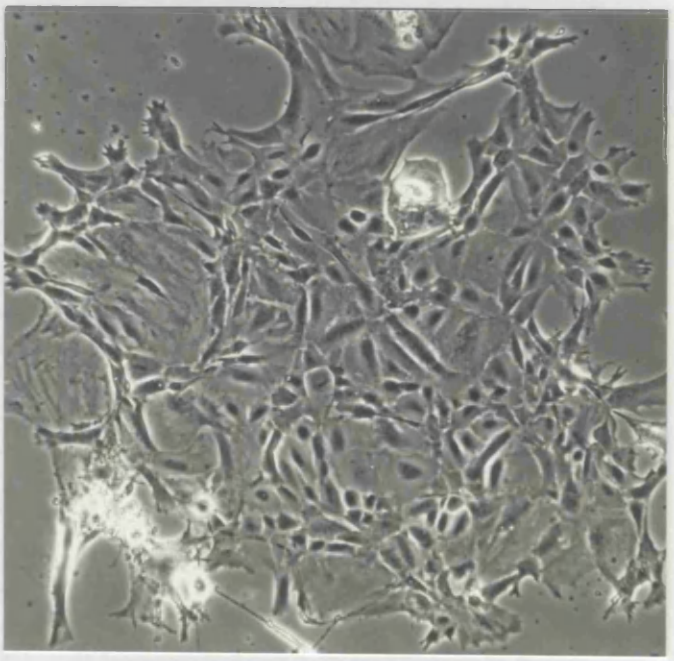


Figure 20 (Opposite). Phase-contrast micrographs showing the morphological progression of a sorted 25.5-positive Type 3 luminal clone.
The same clone after (a) 5 days; (b) 7 days; (c) 11 days culture. x158





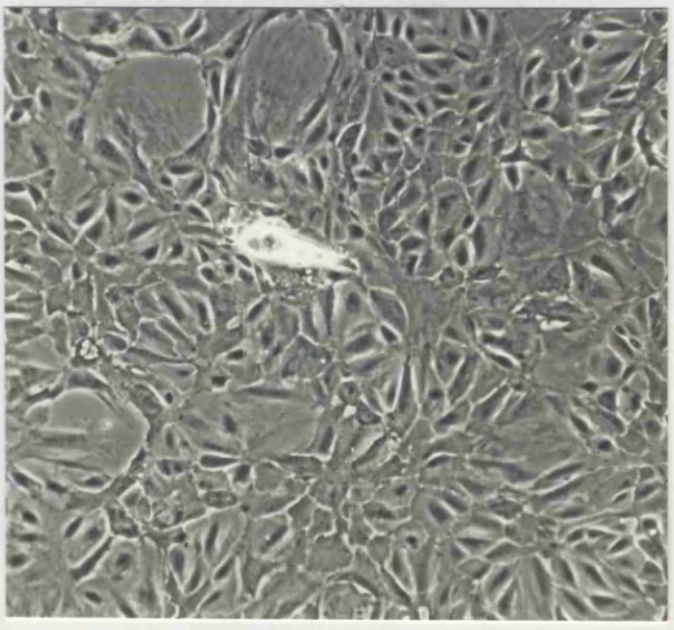
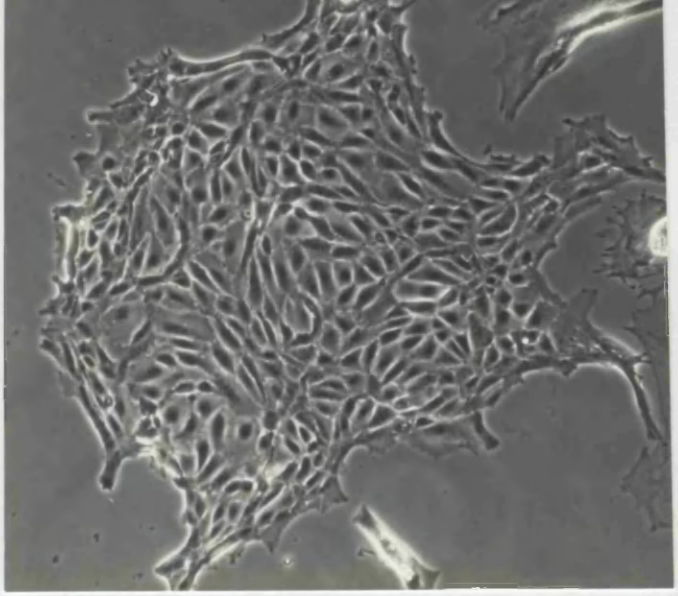
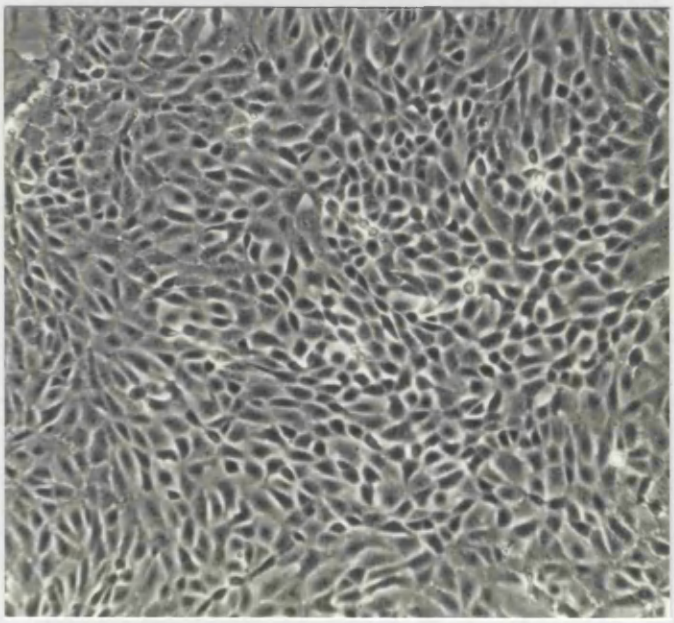


Figure 21 (Opposite). Phase-contrast micrographs showing the morphological progression of a sorted 25.5-positive Type 4 luminal clone.

The same clone after (a) 5 days; (b) 7 days; (c) 11 days culture.
x158





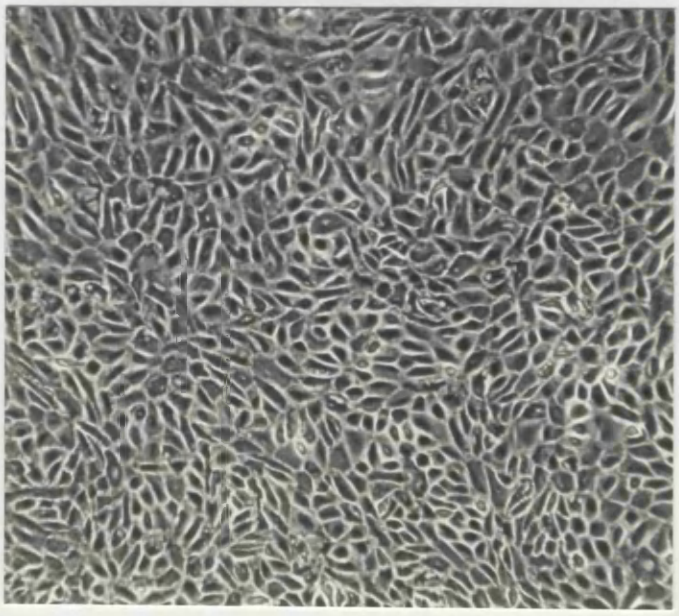
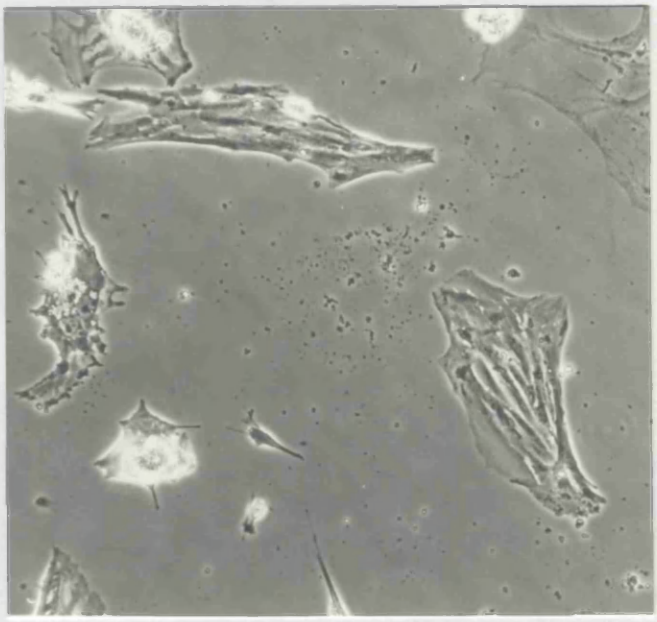


Figure 22 (Opposite). Phase-contrast micrographs showing the morphological progression of two adjacent sorted PHM6-positive Type 5 myoepithelial clones.

The same clones after (a) 7 days; (b) 11 days culture. x158



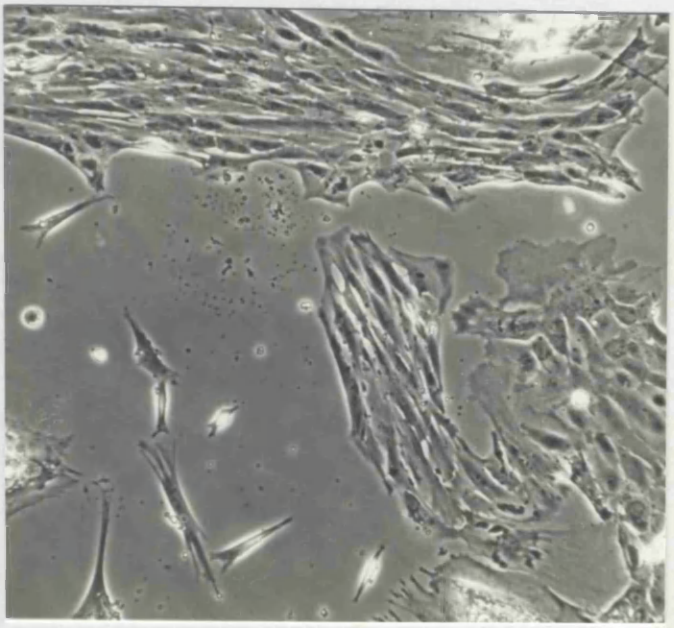
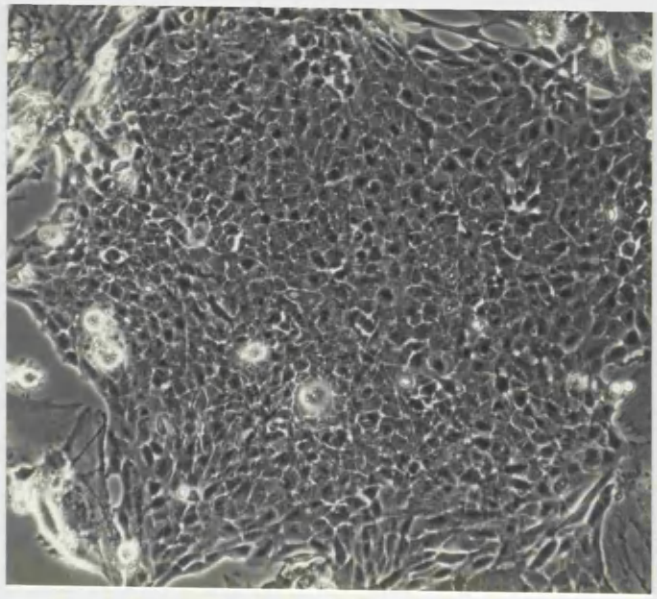
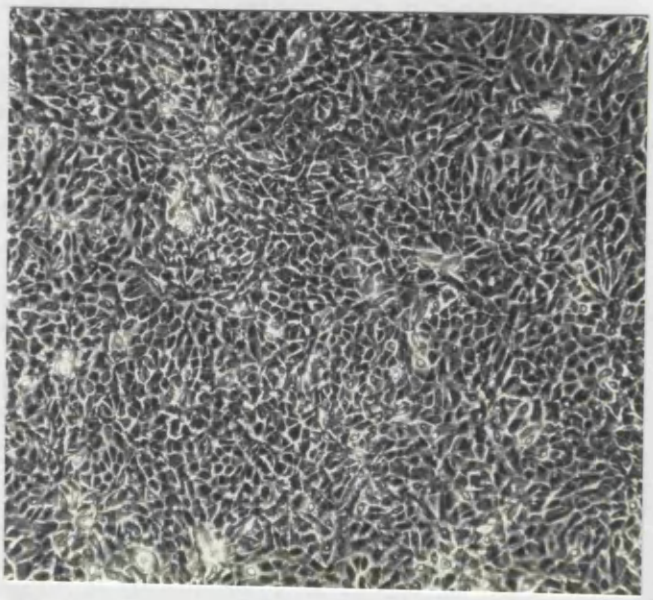


Figure 23 (Opposite). Phase-contrast micrographs showing the morphological progression of a sorted PHM6-positive Type 6 myoepithelial clone.

The same clone after (a) 5 days; (b) 7 days; (c) 11 days culture. x158





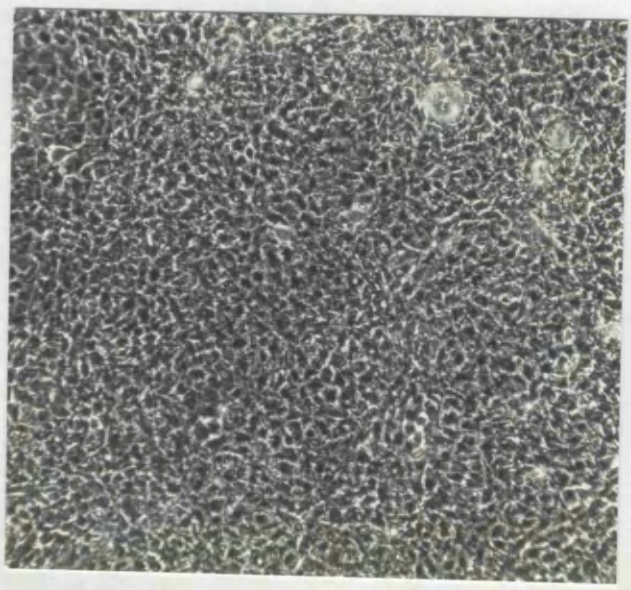
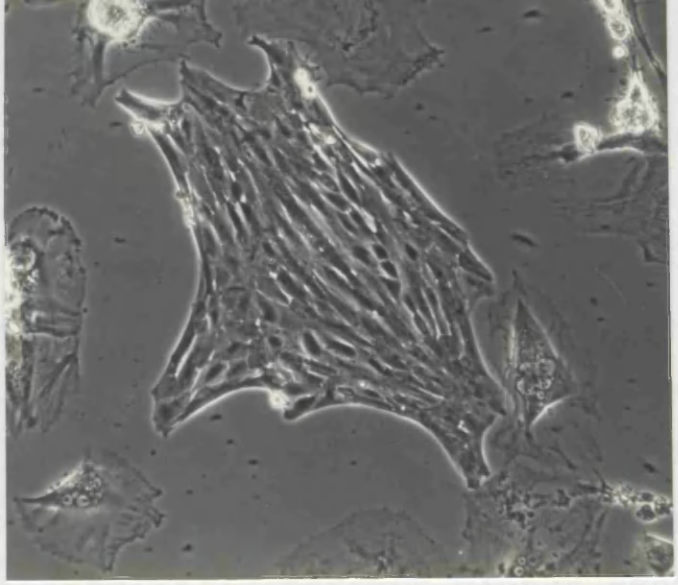
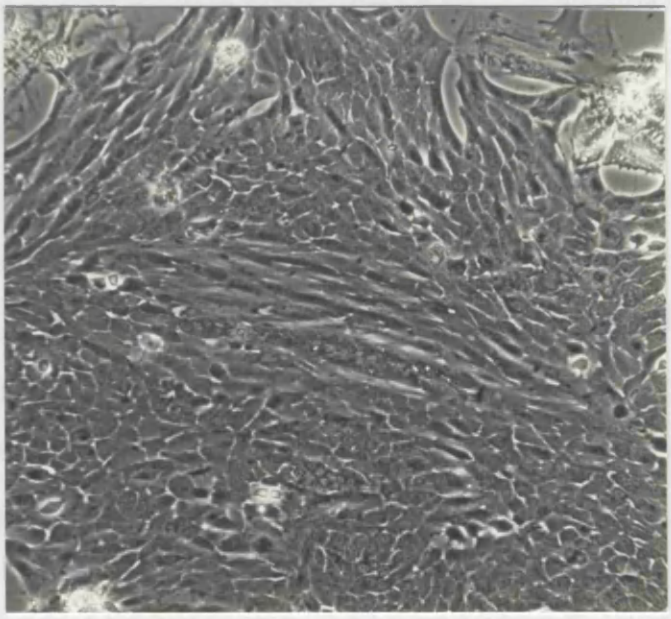
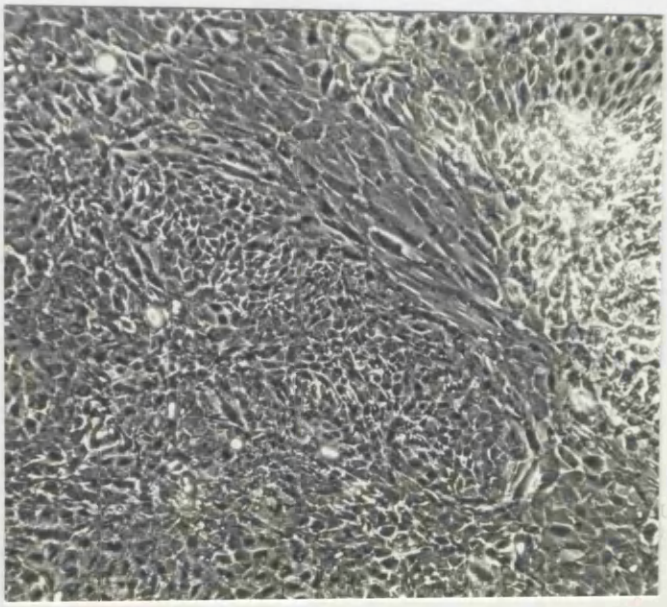


Figure 24 (Opposite). Phase-contrast micrographs showing the morphological progression of two adjacent sorted PHM6-positive myoepithelial 'mixed' clone.

The same clone after (a) 5 days; (b) 7 days; (c) 11 days culture.
Note the area of stretched cells which remain during this observation period. x158







4.2.4 Clonal expression of Gp70KD and endopeptidase-24.11.

When sorted clones were re-probed with 25.5 and antibodies to endopeptidase-24.11 after 7 days culture, some very weak staining of clones was detected with both markers. The general impression was that luminally-derived clones continued to express 25.5 and some cells in myoepithelially-derived clones expressed endopeptidase-24.11. Unfortunately, however, the overall lack of intensity detected using indirect immunofluorescence microscopy, precluded a definitive answer to the question of whether the two sorted populations invariably and completely retained their initially mutually exclusive staining patterns for membrane antigens. Flow cytometric analysis of clonal growth cultures indicated that colonies did however express the appropriate antigens (see Section 4.6).

4.2.5 Clonal expression of intermediate and actin filaments.

The parenchymal origin and differentiated status of sorted clones was verified by probing them with the panel of anti-cytokeratin and vimentin monoclonal antibodies, as well as reagents detecting total actin or cell-type specific actin filaments. This analysis had the added advantage that markers were completely independent criterion of identity, separate from those used for sorting cells. The marker profiles of the 7

day clones are detailed in tables 10 and 11. The results verify the in situ origin of each sorted clone type, with those in set I expressing luminal markers and those in set II myoepithelial markers. This analysis also demonstrated the heterogeneous nature of clone sets I and II, and confirmed the existence of different phenotypes in each set, which corresponded to different clone types originally identified by phase contrast morphology. The most striking result was the existence of clones within the luminal set which expressed myoepithelial-associated cytokeratins in addition to their expected luminal phenotype.

Cytokeratins: With respect to cytokeratins 14 (myoepithelial phenotype) and 18 (luminal phenotype), three different cell types could be seen within Type 4 clones, as indicated in Table 9 and Figure 25. The ability of type 4 clones to generate cytokeratin 14-containing cells was not affected by different hormone or growth factor combinations. One hypothesis to explain the existence of such a dual phenotype clone would be that a doublet of a myoepithelial plus a luminal cell was inadvertently sorted as part of the 25.5-positive stream. However, this is extremely unlikely for several reasons. Firstly, if both expressed their appropriate antigens as they passed through the sorter, then even if the light scatter gate failed to

exclude them, the doublet would register as a double-positive and be excluded from the sort (see Figure 17). Doublets are very rarely seen when cells are examined immediately after sorting (<1%) (see Section 4.1.3), and Type 4 clones were generated in large numbers exclusively from 25.5-positive sorted cells; they were never observed in endopeptidase-24.11-positive sorted populations. This would occur if the second cell in the hypothetical doublet expressed myoepithelial markers. Furthermore, when early sorted luminal clones (3 days culture) were double-labelled, cytokeratin 14-positive cells were already randomly distributed within certain clones, showing no evidence of a separate origin from two adjacently growing clones. This is in contrast to the appearance of occasional colonies arising in unsorted cultures (these unlike sorted cells do contain doublets) in which one half is composed of one phenotype and the other half remains recognisably distinct. This indicates that colonies which do arise from doublets do not coalesce.

Therefore, the only tenable hypothesis to explain the existence and attributes of type 4 clones is that a significant number of sorted luminal single cells, generate a heterogeneous clone type in which some cells express a myoepithelial marker in the form of cytokeratin 14. A similar phenomenon was observed in Type 2 clones, which resemble Type 4 in morphological beha-

viour, except that here a higher proportion of cells co-expressed cytokeratins 14 and 18.

Myoepithelial Type 6 clones strongly stained with antibody LL001 contained very rare cytokeratin 18 positive cells were (Figure 25b). However, such cells represented less than 0.001% of the total number of cytokeratin 14 expressing cells per clone and were not found in all such clones. This phenomenon was clearly different in magnitude to the expression of cytokeratin 14 by luminal Type 4 clones. Type 5 clones did not express cytokeratin 18.

Luminal cells also generate clones which breed true to type. Thus, as shown in Table 10, both Types 1 and 3 never expressed cytokeratin 14; their luminal phenotype was evident with staining for cytokeratins 7, 8, 18 and 19.

Vimentin: The detection of vimentin expression was dependent on the antibody used. With MVI antibody, all types of luminally-derived clones were negative, whilst both types of myoepithelial clones stained positively, albeit very weakly (Figure 26b). In contrast, DAKO-vimentin stained both luminal and myoepithelial clones very strongly (Figure 26c and d, respectively).

α-actin smooth muscle filaments: A monoclonal antibody recognising the smooth muscle α-actin isoform immunolocalised filaments in the majority of cells comprising myoepithelial type 5 clones. Patches of cells within large type 6 clones also stained positively, (Figure 27a). Luminal clones were invariably negative, however, as were adventitious stromal cells although irradiated feeder cells occasionally displayed very strong staining (Figure 27b). By contrast, NBD-phalloidin stained all clone types. However, it was noteworthy that highly aligned arrays of positive filaments in myoepithelial type 5 clones stained intensely.

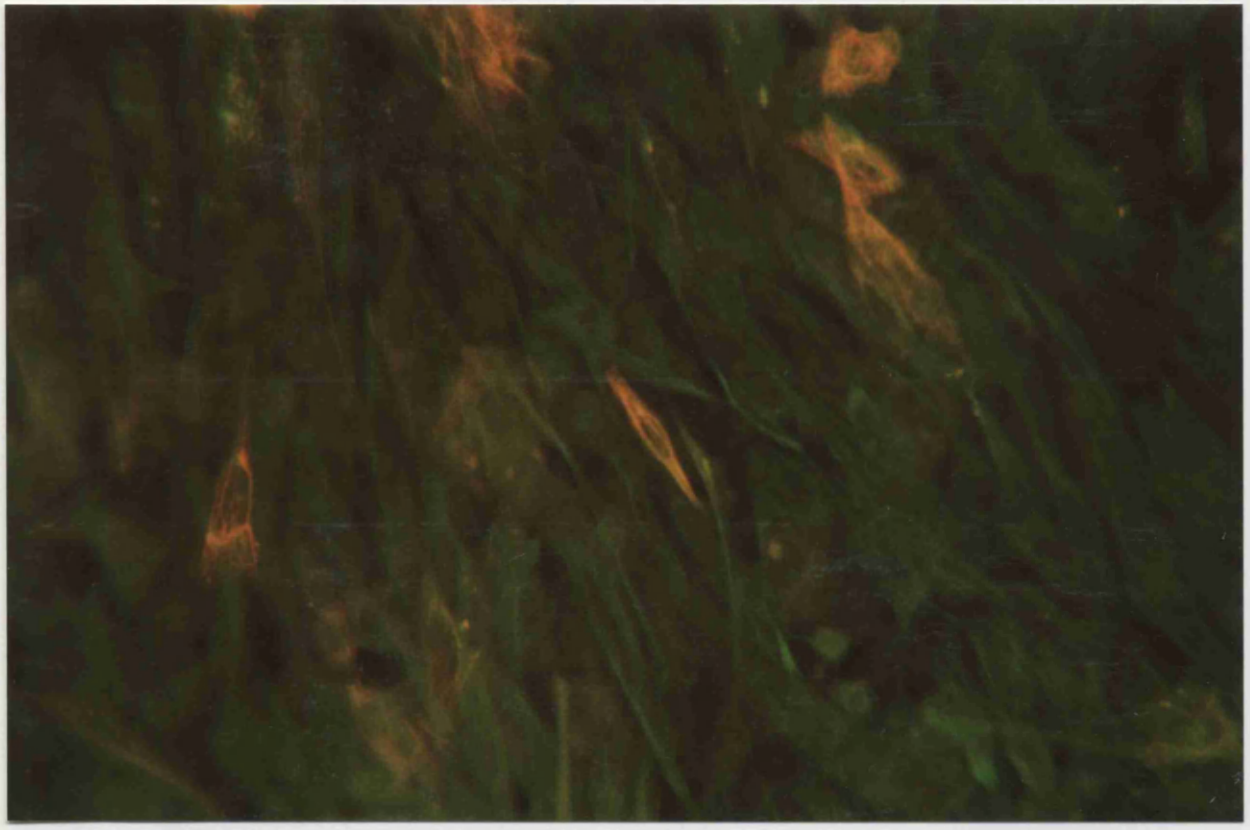
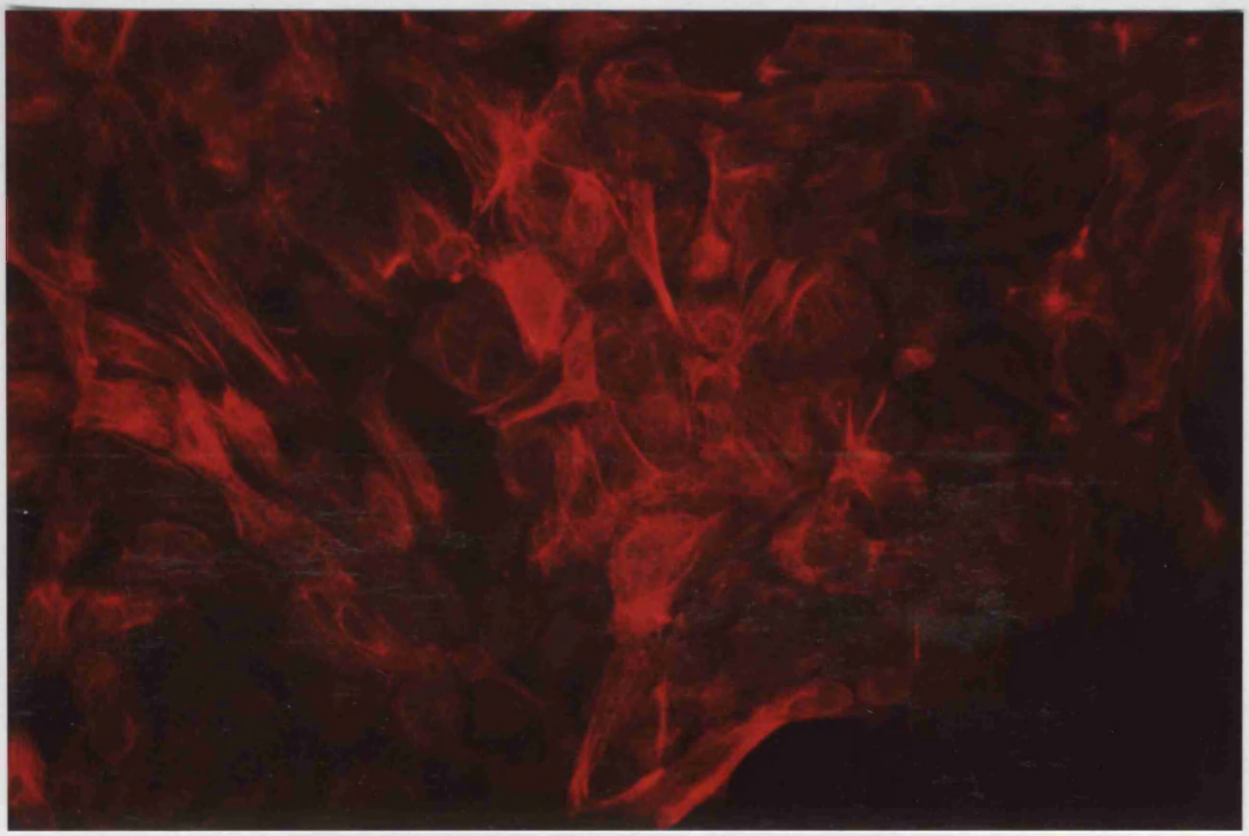
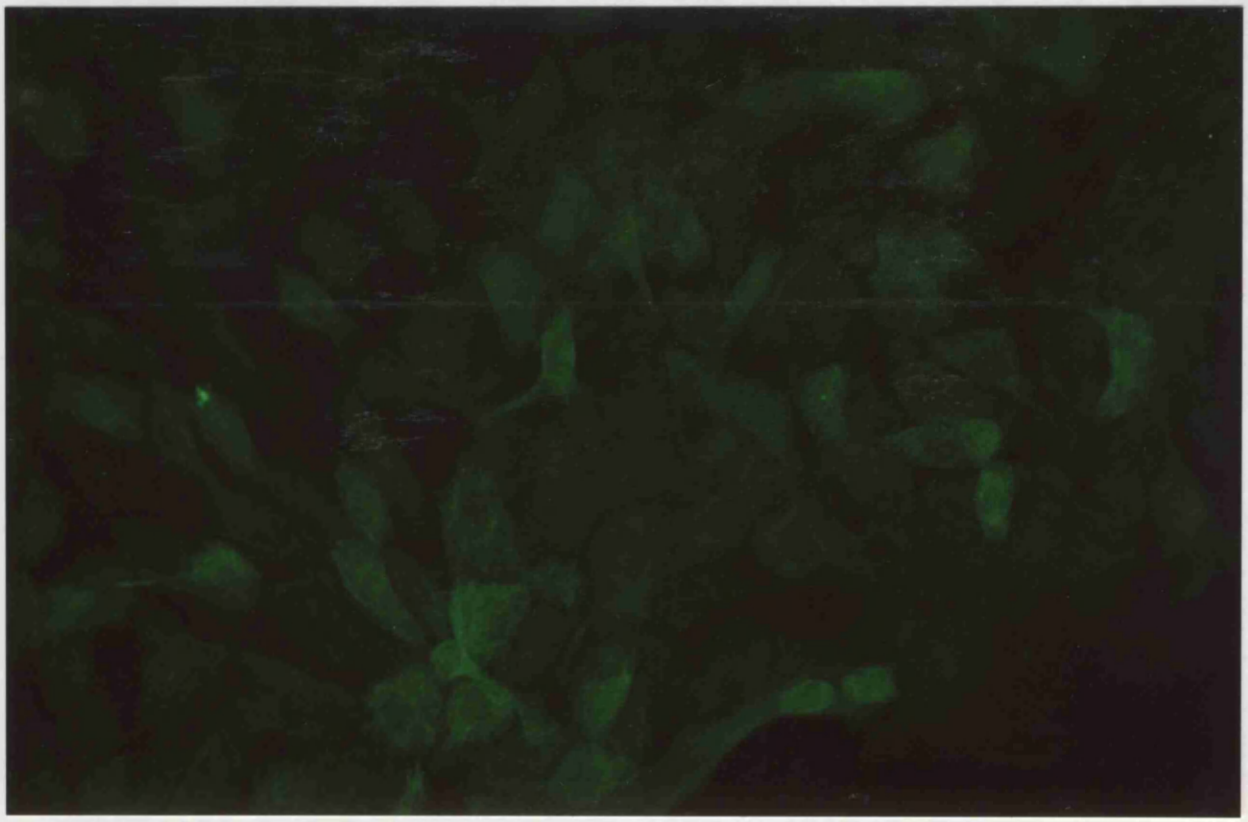


Figure 25. (This page and opposite). Primary clones double-labelled for cytokeratins 18 and 14 expression.

(a) Staining of a Type 6 myoepithelial clone. Note that although most cells exclusively express cytokeratin 14 (green fluorescence), a few cell co-exprees cytokeratin 18 and fluoresce orange. (b) Cytokeratin 18 expression (red fluorescence) by a Type 4 luminal clone; (c) Cytokeratin 14 expression (green fluorescence) by cells shown in (b). Note that the clone is a mosaic of strongly and/or weakly expressing 18+/-/14+/- cells. x400





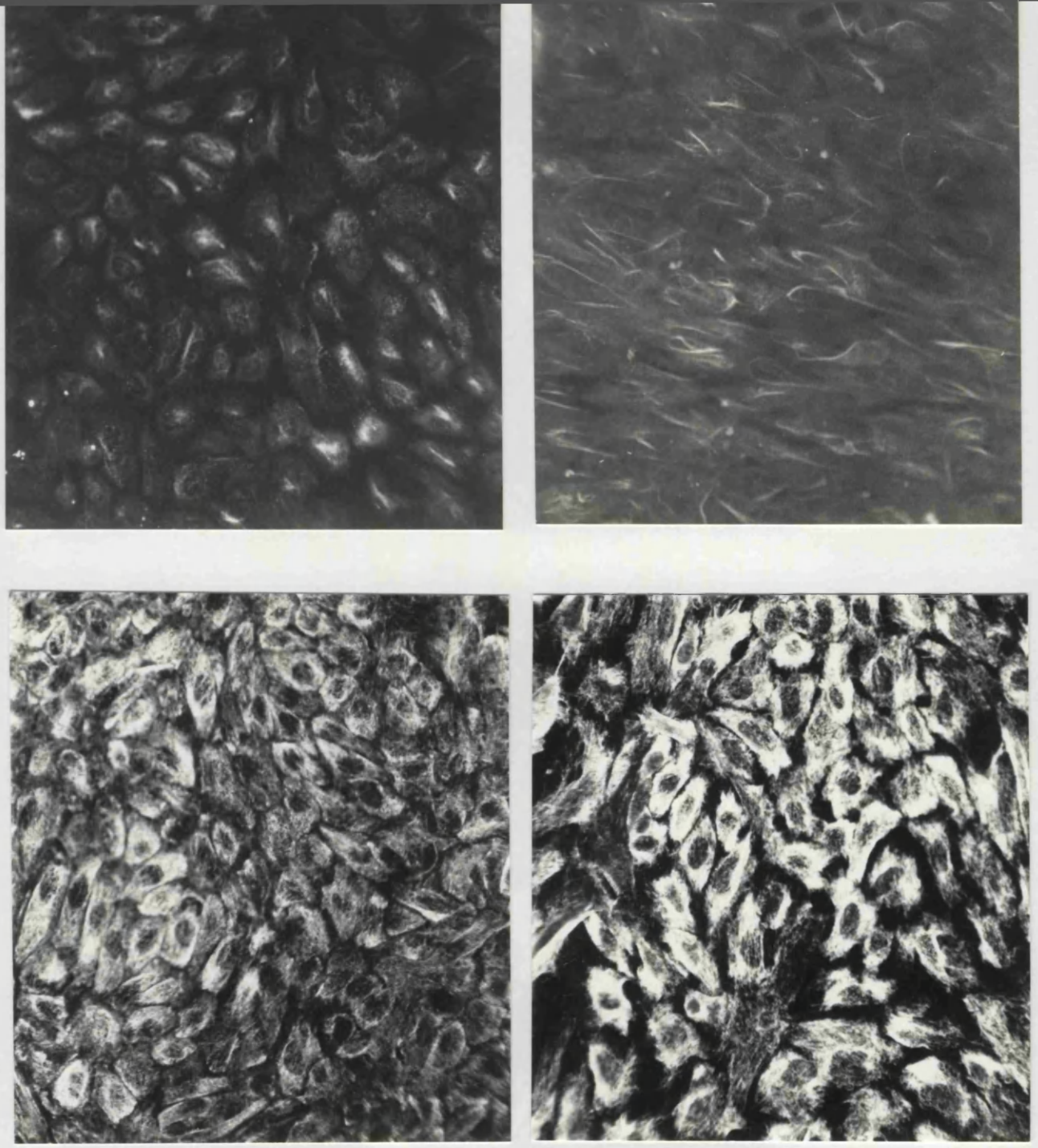


Figure 26. Micrographs of indirect immunofluorescence staining of primary clones with different anti-vimentin antibodies.

(a) Luminal clone stained with antibody MVI; (b) myoepithelial clone stained with antibody MVI; (c) Luminal clone stained with DAKO-vimentin antibody; (d) Myoepithelial clone stained with DAKO-vimentin antibody. x300

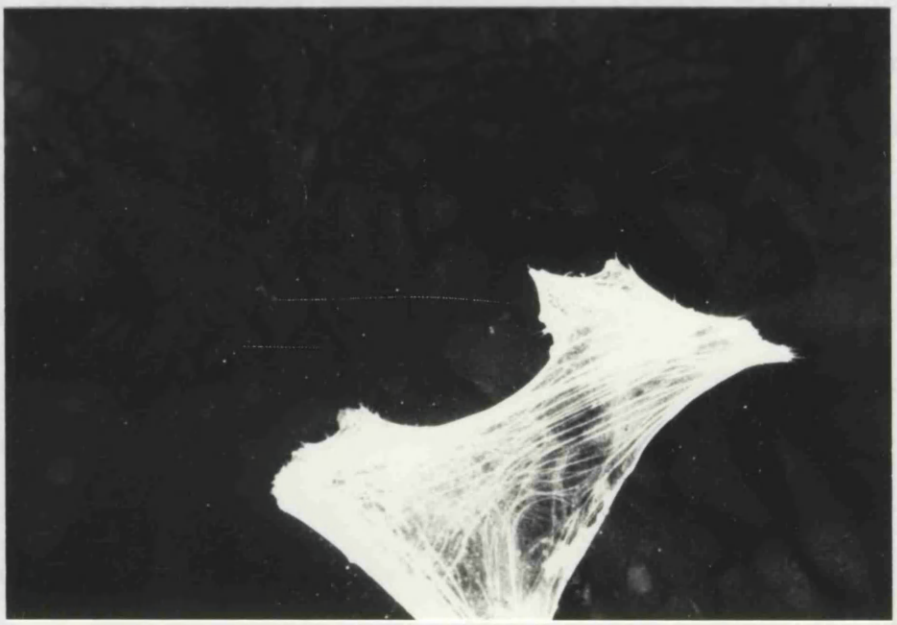


Figure 27. Micrographs of indirect immunofluorescence staining of smooth muscle α-actin filaments in primary clones.

(a) Staining of a Type 6 myoepithelial clone; (b) Staining of a Type 4 luminal clone. Note that the strongly stained cell is a basally located feeder cell. All luminal cells are negative. x350

4.2.6 Clonal expression of basement membrane markers. Myoepithelial clones showed strong positive staining with anti-type IV collagen anti-sera (Figures 28a and b). Luminal clones displayed little positivity (Figure 28a). Myoepithelial clones were also stained with anti-laminin antisera, with cells of Type 6 clones particularly strongly positive. Large myoepithelial Type 6 clones displayed heterogeneous levels of punctate cytoplasmic staining, resulting in a 'patch-work' pattern of positivity (Figure 28c). Occasional cells in type 4 clones stained positively with the anti-laminin antisera (Figure 28d), but the majority of cells in luminal clones fluoresced at background levels. Unlike any of the intermediate and thin filaments, the expression of laminin appeared to be influenced by hormonal additives. Levels of immunolocalised laminin were visibly lower in clones propagated in HC:I containing media compared with clones in HC:I:CT or HC:I:CT:EGF containing media indicating that CT may enhance laminin expression. This reduction in laminin expression was most obvious in large Type 6 myoepithelial clones. Adventitious stromal cells did not display reduced staining in HC:I media and were strongly positive under all growth conditions used, indicating that the CT effect may be myoepithelial cell-specific. The positive punctate staining appeared to be predominantly cytoplasmic in both myoepithelial and stromal cells.

The phenotypes of the different clones are summarised in Tables 10 and 11 opposite.

THE INTERMEDIATE FILAMENT PROFILE OF PRIMARY CLONES.

MARKER	CLONE TYPE 1	2	3	4	5	6
CYTOKERATIN 4	-	-	-	-	-	-
7	+	+	+	+	-	+/- (d)
8	+	+	+	+	+	+/- (a)
10	-	-	-	-	-	-
13	-	-	-	-	-	-
14	-	+(b)	-	+/-(c)	+	+
18	+	+(b)	+	+/-(c)	-	-/+(d)
19	+	+	+	+/-(c)	+(e)	-/+(e)
VIMENTIN (a)	+/-	+/-	+/-	+/-	+/-	+/-

(a) Clones contain mixtures of positive and negative cells; (b) Majority of cells composing this clone type co-express these cytokeratins; (c) Clones are mosaics of co-expressing and differentially expressing cells; (d) A few positive cells detected within some clones of this type; (e) Only a proportion of clones express this filament type.

ACTIN FILAMENT AND BASEMENT MEMBRANE PROFILES OF PRIMARY CLONES.

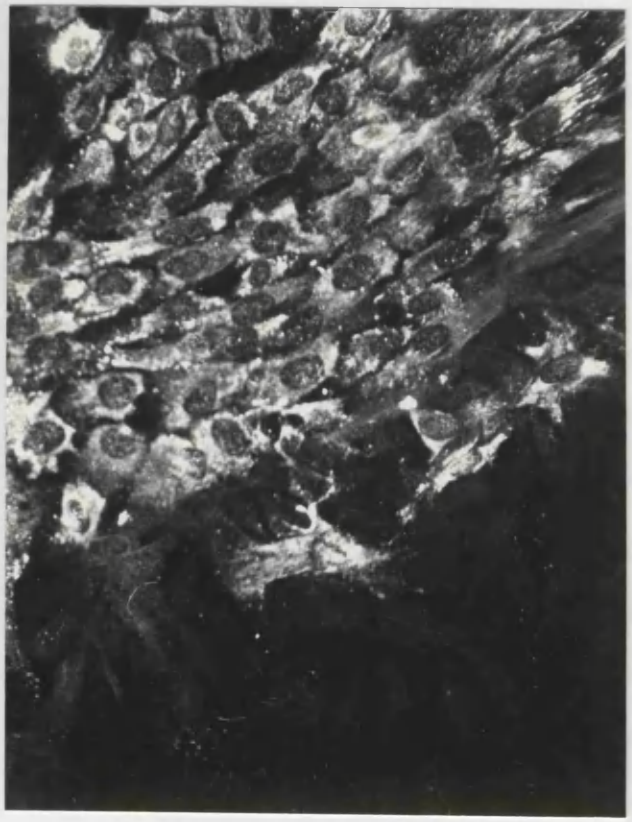
MARKER	CLONE TYPE 1	2	3	4	5	6
a-ACTIN	-	-	-	-	+	+/- (a)
Total F-ACTIN	+	+	+	+	+	+
LAMININ	-	-	-	-	+	+
TYPE IV COLLAGEN	-	-	-	+/-(a)	+	+

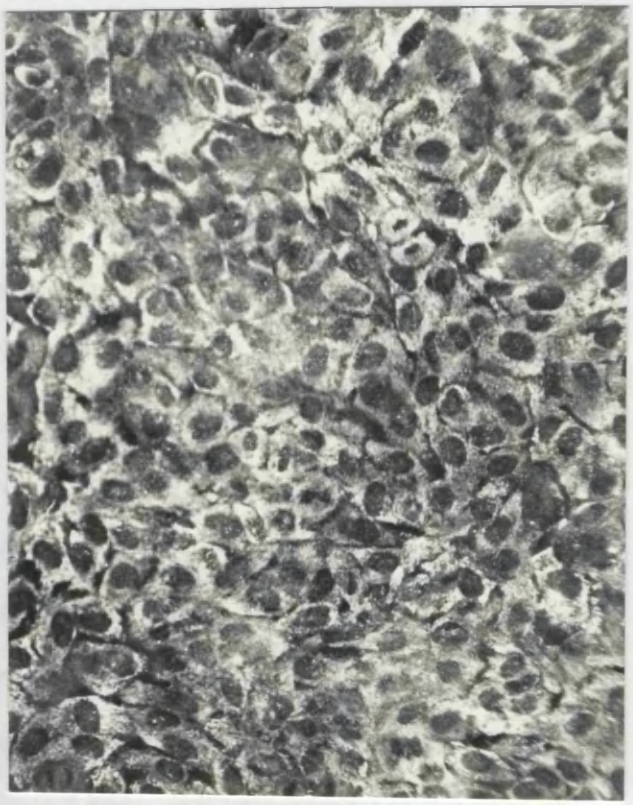
(a) Clone contains mixture of positive and negative cells.

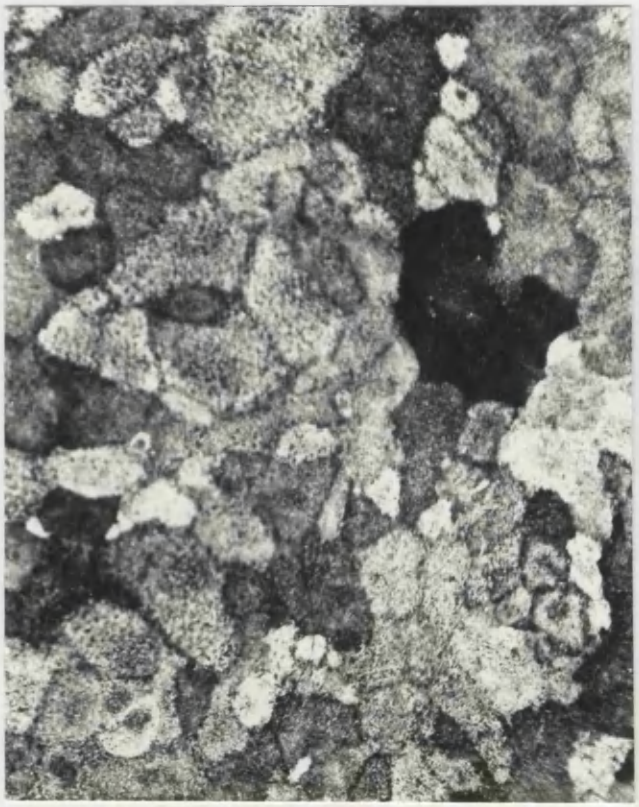
Table 11.

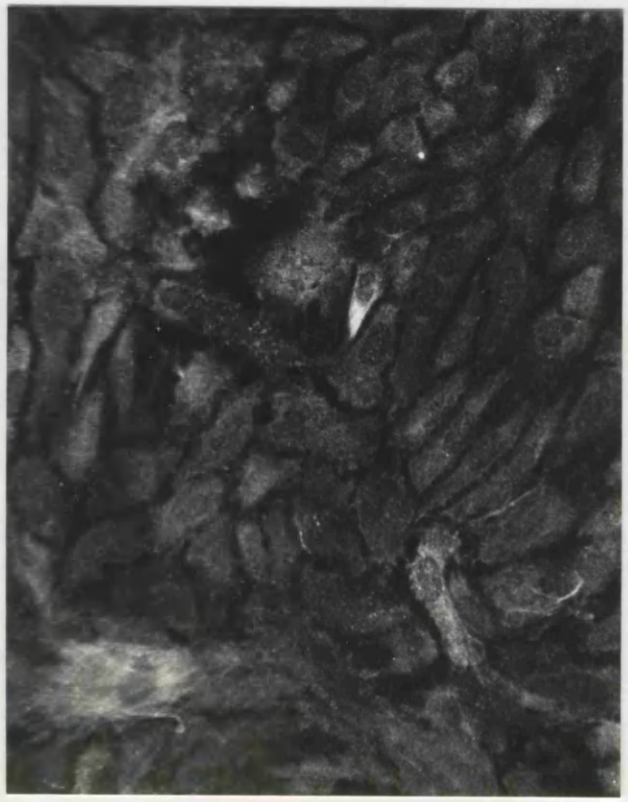
Figure 28 (Opposite). Micrographs of indirect immunofluorescence staining of primary clones for basement membrane components.

(a) Type IV collagen expression by a Type 5 myoepithelial clone. Note that an adjacent luminal clone is not stained; (b) Type IV collagen expression by a Type 6 myoepithelial clone; (c) Laminin expression by a Type 6 myoepithelial clone; (d) Laminin expression by a Type 4 luminal clone.
All clones grown in HC:I:CT containing medium. x400









4.3 Clonal growth of isolated duct or alveolar fragments.

4.3.1 Introduction.

Having characterised the range of both luminal and myoepithelial clone types morphologically and by the distribution of cell-type specific markers, the next question that was addressed was whether the cells giving rise to each clone type differed with respect to the topology and development of the mammary tree. Firstly, were different types of clone derived from cells located in different mammary structures *in situ*? To answer this question fragments of individually manually isolated duct or alveolar structures were separately cloned. Secondly, did certain types of clonogenic cell exist only during particular stages of mammary gland development? This was investigated by comparing clonal growth cultures initiated from 21 day neonatal glands, 70-day virgin as well as 21 day lactating parenchyma. As described below the results showed that the clone types arising from duct or alveolar structures were similar in type, but differed in frequency. Furthermore, both qualitative and quantitative differences in the type of clones arising from parenchyma were observed at different developmental stages.

4.3.2 Distribution of clonogenic precursors in ductal versus alveolar structures.

Clonal growth cultures were initiated from separated segments of duct or alveolus (see Figure 5) as well as from ductal or alveolar explant cultures initiated from single fragments. The frequencies of each clone type which arose from the different structures and explants are shown in Table 12.

FREQUENCY OF CLONE TYPES
ARISING FROM DUCT OR ALVEOLAR STRUCTURES.

ORGANOID TYPE.	MEAN % FREQUENCY					
	T1	T2	T3	T4	T5	T6
ALVEOLAR	10+/-3	8+/-3	19+/-3	56+/-5	6+/-2	1+/-1
DUCTAL	10+/-3	5+/-2	40+/-4	33+/-3	2+/1	10+/-2
EXPLANT TYPE.						
ALVEOLAR	22+/-2	11+/-2	21+/-3	39+/-4	7+/-2	0
DUCTAL	6+/-2	3+/-2	7+/-2	71+/-3	3+/-1	10+/-2

Table 12.

All clone types (1-6) could be found in cultures derived from both isolated duct as well as alveolar fragments, although in varying proportions. The most significant trend observed was that type 5 clones, composed of stretched cells, were the major myoepithelial population derived from both uncultured and cultured alveoli, whilst type 6 proliferative myoepithelial clones were predominantly associated with ductal fragments and were rarely generated from alveoli.

4.3.3. Distribution of clonogenic precursors at different developmental stages of the mammary tree.

Recognisable clone types were observed in 7 day cultures derived from either neonatal or lactating parenchyma. Proliferating luminal Type 4 clones represented the majority type (60-70%) in neonatal cultures, and significant proliferation of recognisable Type 6 myoepithelial clones was also evident. Clones could also be observed in such cultures which were not so easily identified on morphological grounds, but which nevertheless more closely resembled luminal-type clones than myoepithelial clones seen in cultures derived from 70 day parenchyma.
Rather surprisingly perhaps, myoepithelial Type 6 clones appeared to be the majority type in clonal cultures initiated from lactating parenchyma. However,

the occasional clone resembling Type 2/ Type 4 morphology could be observed, and several other distinctive clones of limited mitotic potential which did not resemble archetypal luminal or myoepithelial types were observed. The observed dearth of luminal clones in these cultures is probably explained by the fact that luminal cells at this stage of development contain large amounts of adipose material and would not, therefore, be readily isolated by the sedimentation protocol used during the preparation of organoids.

Thus, assuming that the organoid preparation procedure did not selectively affect neonatal tissue compared to adult tissue, the significant finding in these experiments was that clonogenic precursors giving rise to Type 4 clones were more numerous in neonatal glands than in 70 day parenchyma.

4.4 Effects of hormones, growth factors and feeder cells on luminal and myoepithelial clones.

4.4.1 Introduction.

The conditions used to propagate clones for the purposes of identification were based on a combination of additives that had been shown to be active in various different mammary culture systems (see chapter 1). These conditions were capable of sustaining the growth

of both types of rat mammary cell and enabled them to be classified by simple morphological criteria that were subsequently shown to correspond with their cell type of origin. The individual responsiveness of the different cell types remains an important question, since it may reveal individual mechanisms controlling luminal and myoepithelial growth, and possible interactions of different regulatory mechanisms.

The growth responses both luminal and myoepithelial clones were therefore assessed by evaluating plating efficiencies and clone sizes obtained from different types of cultures, as well as with a variety of hormones and growth factors added in various combinations. In addition, the effects of using different cell types as irradiated feeders and conditioned medium produced by these cells were studied. The objective was to determine whether any of these factors had a specific stimulatory or inhibitory effects on luminal or myoepithelial cells. The growth, differentiation behaviour and morphology of constituent cells of particular clonal populations was altered in response to mammogenic hormones, cholera toxin, epidermal growth factor and soluble factors secreted by established cell lines. The overall conclusion was that while both luminal and myoepithelial clones required the presence of mammotrophic hormones for growth, the proliferation of

luminal clones was predominantly influenced by paracrine acting factors elaborated by feeder cells, and that myoepithelially-derived clones were more effectively stimulated by growth factor additives.

4.4.2 Cloning efficiencies of parenchymal cells derived from organoids, explant cultures and primary monolayer cultures.

Cloning efficiencies of mammary parenchymal cells derived from explant cultures, monolayer cultures and uncultured organoids are illustrated in Figure 29. The greatest overall cloning efficiencies were obtained when cell suspensions were directly prepared from uncultured organoids. The relatively extended trypsinisation required to release all parenchymal cells from a plastic culture substratum may have resulted in the lower cloning efficiencies obtained with all types of pre-cultured cells. In all cases feeder cells greatly enhanced overall cloning by anything up to 10-15 times (Figure 29). Overall cloning efficiency also increased in response to both CT and EGF when these were added to HC:I containing medium. This occurred with cells cloned directly and from pre-cultured cells. The most significant effect seen under the conditions tested was the greater than two-fold increase in cloning efficiency produced by CT in cultures initiated from uncultured organoids (Figure 29, below).

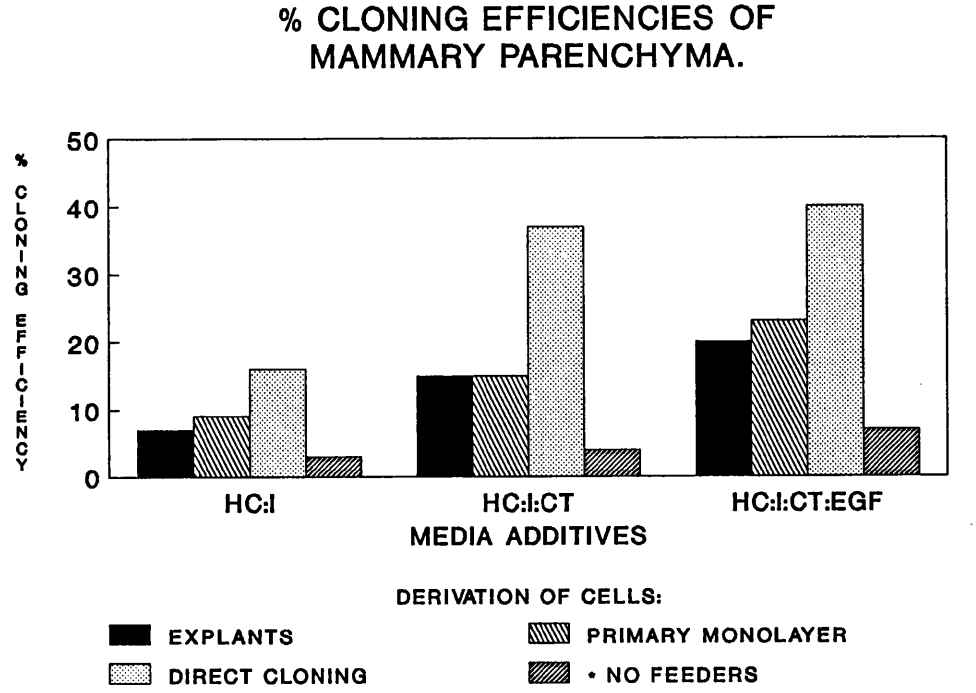


Figure 29.

4.4.3 Effects of hormones and growth factors on growth and morphology.

In the results presented above, overall cloning efficiencies were measured by counting clones. However, it was clear from examining the plates that significant differences in clone size could occur without any significant change in overall cloning efficiency. This effect is shown in Figure 30a. Furthermore, clones of approximately equal size could be composed of significantly different numbers of cells, reflecting different growth rates together with different cell densities. This is illustrated in Figure 30b. In this instance, clones propagated in HC:I:CT were composed of larger

numbers of cells as indicated by the intense basophilia of these clones compared to clones of equal size and equal frequency arising in 3T3L1 co-cultures in medium with only 10%FCS after the same period of time. It is difficult, therefore, to provide an exact comparison of growth rates by measuring clone size and frequency only. Therefore, both plating efficiency and the size of clones were determined for each experimental condition, and individual clones were assessed microscopically to attempt to take into account possible cell density differences. The objective was to screen for major effects and key differences between luminal and myoepithelial cells. Differences in growth rates between luminal and myoepithelial cells under standard conditions were confirmed by direct measurement of labelling indices using BrdUrd uptake (see Section 4.5.4).

Figure 30 (Opposite). Photographs of fixed and hematoxylin stained clonal growth cultures.

A cloned myoepithelial cell strain (see Section 4.8) was plated at clonal densities under the following conditions - (a) with 3T3 L1 feeders only (left well); or with 3T3 L1 feeders in HC:I:CT containing medium (right well). Note that although cloning efficiencies and clone sizes are similar, the intensity of hematoxylin staining of clones grown with additives is considerably stronger. (b) HC:I:CT:EGF additives without feeder cells (left well); or 3T3 L1 conditioned medium without additives. Note that the overall cloning efficiencies are similar but that the clone sizes differ considerably; x1.08

Effects of cholera toxin: Varying the concentration of CT had no statistically significant effect on total plating efficiency in 3T3 L1 co-cultures, (39% compared to 38%, with standard error overlap, for 10ngml^{-1} and 100ngml^{-1}, respectively). However, CT at 100ngml^{-1} induced morphological alterations in both luminal and myoepithelial clones. This was particularly noticeable with respect to Type 1 luminal clones, which were larger in response to increased CT concentration due to extensive cell spreading and proliferation, and Type 6 myoepithelial clones which were more basophilic in the presence of cholera toxin (probably because they contained more cells). Type 6 clones represented 36% of all clones in response to 100ngml^{-1} compared to 18.2% in standard HC:I:CT medium with 10ngml^{-1} (not shown).

Effects of epidermal growth factor: EGF at a standard concentration of 10ngml^{-1} primarily influenced myoepithelial clone types. Type 5 and type 6 clones responded reciprocally to EGF, with a 3-fold decrease in the proportions of type 5 clones and a 50% increase in the numbers of Type 6 clones occurring in primary clonal platings (see Table 13 below). As proliferation progressed, Type 6 clones became extremely compacted with very high cell densities. No discernible changes in luminal clones were observed in response to EGF as a single agent.

Effects of progesterone: Progesterone (5μgml^{-1}) caused subtle morphological alterations in all clone types. However, the behaviour of type 1 clones was most noticeably altered, such that the accumulation of adventitious mesenchymal cells occurring in the vicinity of these clones (see Section 4.2.2) was greatly enhanced. This phenomenon was observed in three different platings, and was not observed with other clone types.

Effects of feeder cell type: Figure 31 illustrates the cloning efficiencies of rat mammary parenchyma in response to different feeder cell types under varying growth conditions. Comparisons reported were obtained from experiments in which a single mammary parenchymal cell suspension was simultaneously cloned under a variety of conditions. General trends of note are - 1. Irradiated mesenchymal feeder cells significantly increased cloning efficiencies over those obtained with hormones and growth factors alone (compare Figure 29 with Figure 31), 2. The stimulatory effects of added hormones and growth factors are quantitatively similar with respect to each feeder cell type, although enhanced cloning efficiencies are obtained with HC:I and HC:I:CT combinations in the presence of 3T3 L1 cells compared to that produced by either primary mammary stromal cells or the 3T6 cell line (16% compared to 8% or 5% respectively). This is likely to be due to the

adipose conversion of 3T3L1 cells in response to HC:I, which further stimulates the growth of parenchymal cells (see Discussion).

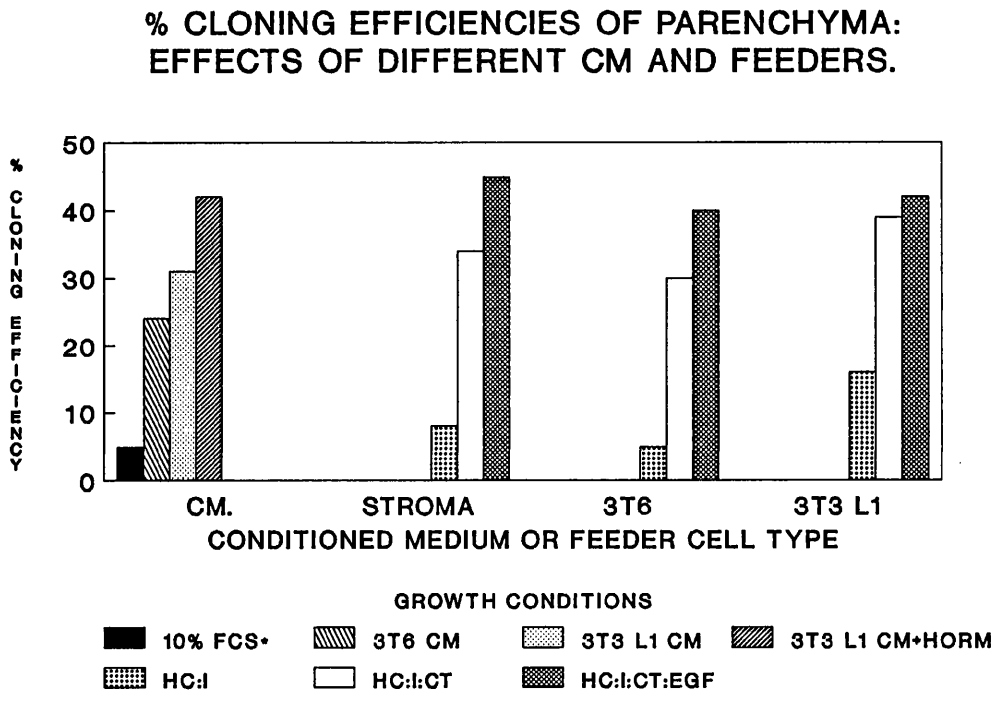


Figure 31.

On the basis of these results, the 3T3 L1 line was used as the feeder cell type for the majority of clonal growth experiments.

Effects of conditioned media: Clonal growth cultures were also initiated in conditioned media produced by

3T6 or undifferentiated 3T3 L1 cells. The resulting effects on cloning efficiencies are also illustrated in Figure 32. Conditioned medium from either source resulted in approximately four- and six-fold increases in plating efficiency over that produced by basal growth conditions, respectively. The cloning efficiency with conditioned medium (without additives) was invariably greater than that obtained by co-culture with irradiated feeder cells in the presence of HC:I, possibly because the pre-conditioned medium was obtained from viable cultures with much higher cell densities than used for co-culture. It is therefore possible to clone rat mammary cells without feeder cells, and without high concentrations of exogenous additives. This may be of considerable value in future studies of hormonal responsiveness in this system.

4.4.4 Analysis of differential growth responses of luminal and myoepithelially-derived clones.

As described above, different growth conditions could in some instances specifically influence the behaviour and/ or morphology of different clone types. However, the key question was the extent to which luminal and myoepithelial clones differed in their responses to individual agents. The difficulties in evaluating these responses accurately have also been described above.

Alternative approaches to assessing growth responses, such as enumerating overall increases in cell number, would necessarily prevent distinguishing between different morphological phenotypes. Therefore, as a way of overcoming these problems, overall cloning efficiencies have been collated as the individual frequencies of each clone type calculated as a percentage of the total parenchymal cloning efficiencies for each growth condition tested. This has yielded useful information on differential responses of luminal and myoepithelial populations. Differential growth responses were assessed in clone cultures derived both from organoids and pre-cultured cells. All clonal cultures for these experiments were initiated from the same preparation at the same time, with the culture conditions representing the only variable. A minimum of 50 individual clones of each type were enumerated for each culture condition so that its frequency could be accurately calculated. The experiment was repeated three times on separate occasions, and the mean percentage frequency for each clone type relative to the total number of scored clones calculated. The results are listed in Tables 13 and 14.

Effects of feeder cells: Myoepithelial clones were the predominant phenotype (~85%) when disaggregated organoids were grown without feeder cell support (Table

13). The ratio of luminal to myoepithelial clone types was about 1:5 and 1:6 in the absence of feeders in cultures grown in HC:I:CT and HC:I:CT:EGF containing media, compared with 3:1 and 2:1 when feeders were present under otherwise identical conditions (Table 13). Co-culture with irradiated 3T3 L1 cells in the presence of hormone and growth factor additives approximately reflected the proportions of luminal:myoepithelial cells present in freshly isolated uncultured organoids, (that is, 5.5:1, as determined by cytometric analysis of organoid single cell suspensions, see Section 3.2.4). These results indicated that luminal cells were dependent on feeder cell-related factors for growth whilst myoepithelial cells responded primarily to added hormones and growth factors.

Effects of hormones and growth factors: Some overall trends were noted in the relative frequency of luminal versus myoepithelial clones when different combinations of additives were use, although none produced the dramatic differences seen when feeder cells were included or omitted. Addition of cholera toxin tended to increase the luminal frequency but only by a few percent. Conversely, EGF influenced myoepithelial cells. When the effects on individual clone types were enumerated, however, some more significant changes were detected. Thus, EGF significantly decreased the rela-

tive frequency of type 5 clones (7% ±3 to 2% ±1), while the frequency of proliferating type 6 myoepithelial clones was increased (18% ±3 compared with 30% ±4), (Table 13, below).

THE MEAN % RELATIVE FREQUENCIES OF CLONES:
CULTURES PROPAGATED +/- FEEDER CELLS AND +/- EGF.

CLONE TYPE	MEDIA ADDITIVES			
	HC:I:CT		HC:I:CT:EGF	
	-3T3 L1	+3T3 L1	-3T3 L1	3T3 L1
TYPE 1	1.0+/-1	33.0+/-5	0.0	33.0+/-3
TYPE 2	0.0	3.0+/-2	0.0	2.0+/-1
TYPE 3	9.0+/-1	15.0+/-2	12.0+/-3	20.0+/-3
TYPE 4	7.0+/-3	23.0+/-4	2.0+/-1	13.0+/-2
TYPE 5	24.0+/-3	7.0+/-3	16.0+/-2	2.0+/-1
TYPE 6	59.0+/-3	18.0+/-3	70.0+/-5	30.0+/-4
Total % luminal =	17.0	74.0	14.0	68.0
Total % myoepithelial =	83.0	25.0	86.0	32.0

Organoid suspensions were plated at clonal densities under the conditions indicated. After 7 days culture, clones were identified as types 1-6 on the basis of phase-contrast morphology, and scored accordingly. The The frequency of each clone type was calculated as % of the total number of clones counted.

Table 13.

The diameter of clones was used as a first-order parameter to compare the relative growth rates of clone types. The results are presented as comparative scattergrams of the measured size distributions of myoepithelial clones (Types 5 and 6), (Figure 32a), and luminal clones (Types 1-4), (Figure 32b).

The population distributions are delimited by Tukey boxplots and the median of each population is indicated. For each clone type, the size distribution of clones, identified on the basis of morphology, is compared with that of equivalent clones grown under the different culture conditions. Although the proportion of myoepithelial clones was highest in the absence of feeders, the numbers and size of myoepithelial clones did increase significantly in response to feeder cells (for example, mean diameters of Type 6 clones grown in HC:I:CT containing medium increased from 0.7mm ±0.2 to 2mm ±0.5 in response to the presence of irradiated 3T3 L1 feeder cells, see Figure 32a). Thus, the myoepithelial clones either responded directly to the 3T3L1 cells or to the presence of luminally-derived clones which increased in number in response to feeder cells, or to both of these cell types.

In so far as luminally-derived clones (Types 1-4) in the presence of feeder cells were concerned, no significant trends in response to EGF were detected with any of them (Figure 32b), although clone Types 2 and 4 were significantly larger in response to CT and EGF in combination compared to those growing in HC:I medium (not shown).

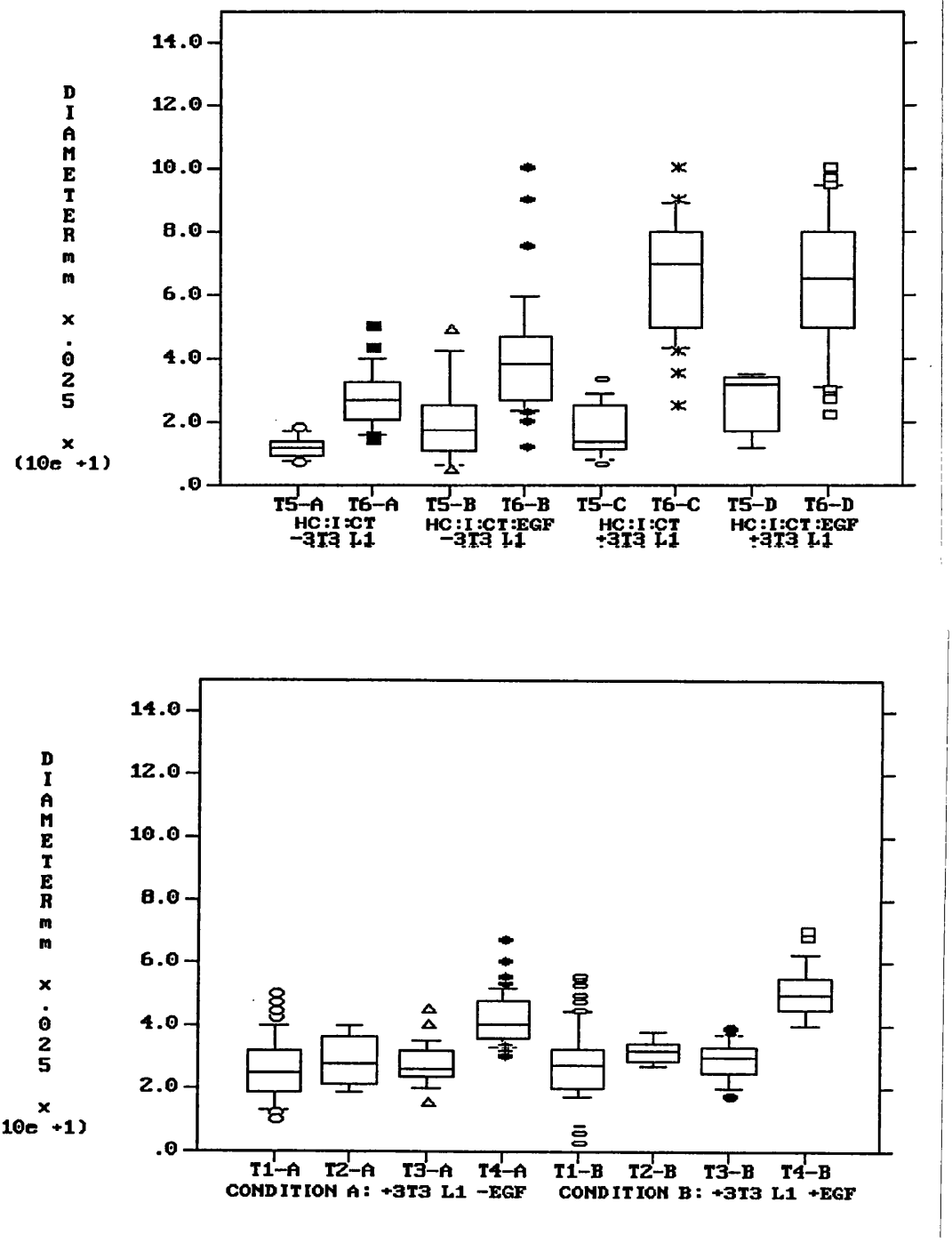


Figure 32. Scattergrams of the size distributions of primary clones grown under different conditions.

(a) Scattergram of the size distributions of myoepithelial clone types 5 and 6 grown with or without EGF/ with or without 3T3 L1 feeder cells. (b) Scattergram of the size distributions of luminal clones grown with or without EGF in the presence of 3T3 L1 feeders.

Note. The Tukey boxes delimit the 25th-75th percentiles of the population. The lines extending from the boxes indicate the 10th and 90th percentiles. Points outside these limits are plotted individually.

In the presence of feeder cells and various combinations of growth promoting additives, no great differences were noted when clones were derived from either uncultured organoid preparations, explant cultures or primary monolayer cultures. Luminal clones constituted the majority phenotype in all cases (data not shown).

Conditioned medium effects: Conditioned medium resulted most notably in the enhanced growth of luminal clone types, which proliferated to a significant extent (Figure 33). This was in marked contrast to the behaviour of luminal clones in cultures propagated without feeders. Significant differences in clone morphology were noted with conditioned medium when this was used without hormonal supplementation. All clones were flatter and more spread than clones arising with feeder cell support, and small vacuoles were observed in a number of cells (Figure 33). In particular the behaviour of myoepithelial clones differed from other types of culture. Thus, the proliferative Type 6 clones were not observed, and myoepithelial colonies were composed of atypical cells with prominent nuclei which ceased to grow after 5-7 days culture (Figure 33). After this time they became senescent, and a proportion of them showed marked cellular heterogeneity. This contrasted strongly with the behaviour in cultures with irradiated feeder cells. Cells composing myoepithelial clones were

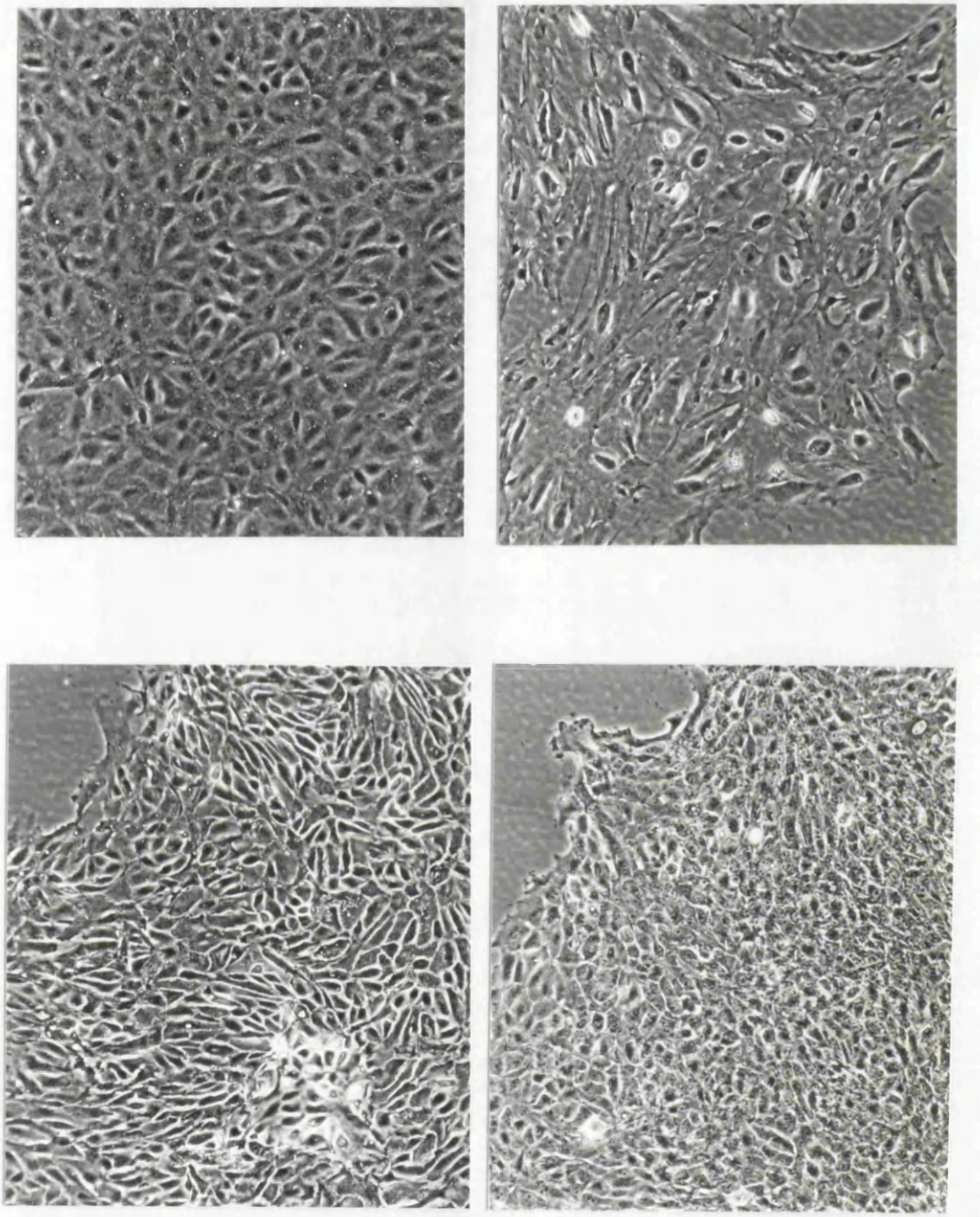
observed to change spatial relations and reorganise. Closely apposed, polygonal and non-overlapping cells could seen adjacent to cells with an elongated fusiform morphology which exhibited overlapping patterns of growth with non-apposing cell margins. While these disorganised cells resembled stromal cells seen in primary cultures, their epithelial derivation was evident from the fact that they continued to express cytokeratin 14, albeit a a reduced levels compared with typical Type 6 clone cells. Type 5 myoepithelial clones were more also more disorganised in conditioned medium only, with cells becoming detached from one another with non-apposing boundaries.

The addition of the full complement of trophic factors (HC:I:CT:EGF) to 3T3 L1 conditioned medium resulted in cloning efficiencies approximately equal to those observed with feeder cells (see Figure 31). The full range of the six clone types was generated, and growing clones were similar with respect to morphology and proliferative behaviour to those co-cultured with irradiated 3T3 L1 cells in HC:I:CT:EGF containing medium (Figure 33). Recognisable Type 5 clones were frequently observed and the frequency of Type 6 clones was considerably increased (8.0% ±3.5 to 27.0% ±5, Table 14). Considerable proliferation of Type 6 myoepithelial clones was induced. These large myoepithelial

clones remained intact and were composed of typical polygonal cells, and the disorganised areas of fusiform cells observed with conditioned medium alone were never observed (Figure 33). Therefore, provided that hormones and growth factors are present, irradiated feeder cells can be substituted for by conditioned medium produced by these cells.

Figure 33 (Opposite). Phase-contrast micrographs of 7 day primary clones arising in cultures grown in 3T3 L1 conditioned medium with or without added hormones and growth factors.

(a) Luminal Type 4 clone arising in culture grown in 3T3 L1 conditioned medium; (b) Myoepithelial Type 6 clone arising in culture grown in 3T3 L1 conditioned medium; (c) Luminal Type 4 clone arising in culture grown in 3T3 L1 conditioned medium supplemented with HC:I:CT:EGF at standard concentrations; (d) Myoepithelial Type 6 clone arising in culture propagated in 3T3 L1 conditioned medium supplemented with HC:I:CT:EGF at standard concentrations. x158



To ascertain if other types of feeder cells influenced the growth of both luminal and myoepithelial clones, the effect of conditioned medium from 3T6 transformed mouse fibroblasts (which do not undergo adipose conversion in response to HC and I) was tested. When these cells were used to prepare conditioned medium, cloning efficiencies were slightly lower than with 3T3L1 cells (Figure 31). Although 3T6 conditioned medium supported the growth of all clone types, the proportions of clone types were altered (Table 14, see over). In addition, the extent to which individual myoepithelial clones degenerated into stromal-like cells noted when conditioned medium was used without additives was more pronounced with 3T6 medium.

Conditioned medium from either the epithelially derived MAT-B rat carcinoma cell line, or the putatively epithelial rat cell line 2.13-EP (see Table 1) did not enhance the growth of any type of rat mammary parenchymal clones (data not shown).

THE MEAN % RELATIVE FREQUENCIES OF CLONES TYPES: CULTURES GROWN IN DIFFERENT CONDITIONED MEDIUM.

CLONE TYPE	CONDITIONED MEDIUM TYPE 3T6	3T3 L1	3T3 L1+HORMONES
TYPE 1	6.0	2.0	13.0
TYPE 2	10.0	5.0	9.0
TYPE 3	19.0	10.0	15.0
TYPE 4	34.0	48.0	31.0
TYPE 5	8.0	6.0	5.0
TYPE 6	4.0	8.0	27.0
TYPE 7*	19.0	20.0	0.0
Total luminal clones =	69.0	65.0	68.0
Total myoepithelial clones =	12.0	14.0	32.0

*Type 7 clones: Those clones exhibiting epithelial-mesenchymal transition (not counted in totals).

Table 14.

4.5 Growth characteristics of sorted luminal and myoepithelial clone types.

4.5.1 Introduction

The behaviour of clones generated by sorted cells was examined. This was done in order to determine whether interactions between luminal and myoepithelial cells influenced the growth of clones. Cloning efficiencies, the diameter of clones and labelling indices of clones arising from simultaneously sorted luminal or myoe-

pithelial cells were measured. In addition, the frequencies of each clone type arising in sorted cultures were determined. The results are compared for each clone type, and with those of unsorted clonal growth cultures initiated from the same cell suspensions as those used for sorting.

4.5.2 Cloning efficiencies of sorted cells.

Cloning efficiencies of sorted cells were greatly reduced compared to the parallel unsorted cultures, being 5-10% compared to 5-40%, respectively. When clones were prepared from cells sorted for 25.5-positivity, 96% of the clones were of luminal types (Types 1-4) while clones with myoepithelial morphology represented approximately 4% (±3%) of cells. In contrast, clones of non-parenchymal phenotype constituted a significant proportion, approximately 24% (+/-1%), of DAKOCALLA-positive sorted cells (see Section 4.2.3). Despite reduced cloning efficiencies, the overall range of clone types was identical to that observed in unsorted cultures, and each in proportions broadly corresponding to the frequency of specific clone types arising in unsorted clonal cultures (Table 15). These results substantiate the morphological findings (see Section 4.2.3) that no inadvertent selection or deletion of specific clones occurred as the consequence of the sorting procedure or the culture of sorted cells.

FREQUENCIES OF CLONE TYPES GENERATED FROM SORTED CELLS.

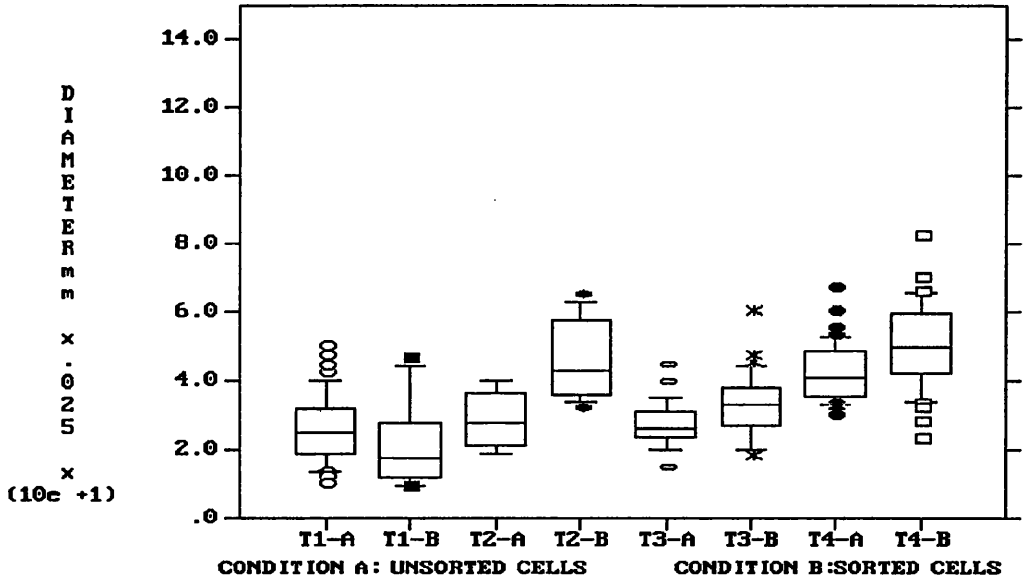
SORT LABEL	CLONE TYPE	% FREQUENCY
25.5+	TYPE 1	7.0
25.5+	TYPE 2	16.0
25.5+	TYPE 3	30.0
25.5+	TYPE 4	47.0
DAKOCALLA+	TYPE 5	8.0
DAKOCALLA+	TYPE 6	92.0

Clonal growth cultures were initiated separately from luminal or myoepithelial cells sorted simultaneously from a double-labelled cell suspension of uncultured organoids. Both culture types were propagated with irradiated 3T3 L1 feeder cells in HC:I:CT containing medium, and scored on the basis of phase-contrast morphology after 7 days growth.

Table 15.

4.5.3 Comparing growth rates of sorted with unsorted clones.

As in Section 4.4.4 above, the diameter of clones was used as a first order parameter, this time to compare the relative growth rates of unsorted clones, identified on the basis of morphology, with those of sorted equivalents identified by sort label and morphology. The results are presented as scattergrams of the measured size distributions of luminal clone Types 1-4, (Figure 34a), or myoepithelial clone Types 5 and 6, (Figure 34b).



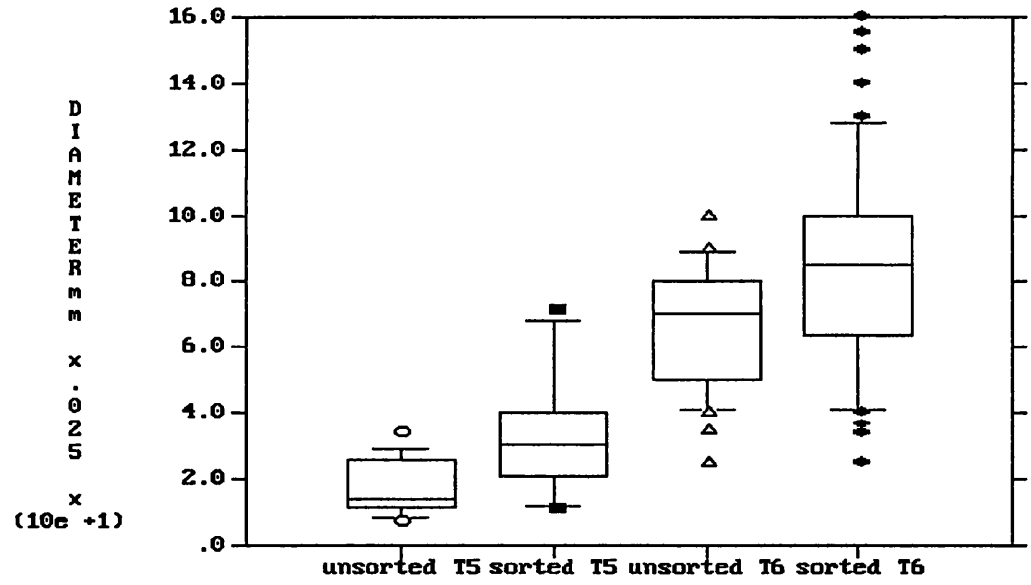


Figure 34. Scattergrams comparing the size distributions of clones generated by unsorted cells with the equivalent clone types generated by sorted cells.

(a) Scattergram of the size distributions of unsorted versus sorted luminal clones (Types 1-4; A versus B respectively); (b) Scattergram of the size distributions of unsorted versus sorted myoepithelial clone Types 5 and 6.

Both scattergrams illustrate a general trend indicating that sorted clones tended to be larger than unsorted equivalents. Statistical analyses were performed to compare the means of the paired independent samples generated by the unsorted and sorted distributions of each clone type. The Null Hypothesis is that the difference between the means of compared samples is 0, and that any difference is due to chance. Populations were distributed normally as indicated by the Mann-Whitney test, thus enabling the means to be compared using the Student's t test. The computed results are presented in Table 16 below.

COMPARED SAMPLES	TWO-TAILED p-VALUE
Luminal type 1	0.274
Luminal type 2	0.008
Luminal type 3	0.026
Luminal type 4	0.001
Myoepithelial type 5	0.118
Myoepithelial type 6	0.0003

Table 16. Statistical analysis of the differences in sizes of unsorted compared to sorted clones. Clone sizes were measured with an eye-piece graticule. The sample size distributions were analysed by "EPISTAT" computer software.

On the basis of these results it can be stated that the differences observed between unsorted and sorted clone sizes are significant with respect to luminal type 2 clones (significant at the 1% level) and type 4 clones (significant at the 0.1% level) and type 6 myoepithelial clones (significant at the 0.1% level). Furthermore, the data indicate that at 99% confidence limits for the differences, the average sorted luminal type 4 clones will be 20% larger than unsorted ones and that myoepithelial type 6 clones will be 29% larger than unsorted equivalents. In these experiments the overall total number of clones per well was no less than with unsorted clones. The differences cannot, therefore be attributed simply to steric hindrance between adjacent clones and/ or medium depletion.

These results therefore provide direct evidence of dynamic luminal-myoepithelial growth-regulating interactions.

4.5.4 Labelling indices of sorted clones.

To provide direct measurement of proliferative activity, the labelling indices of sorted clones were determined using BrdUrd incorporation (see Section 2.5.5). Clones were distinguished according to sort label and gross morphology after 5 days culture. Five clones of

each type were analysed. On average, the number of cells per clone varied between 30 and 140 depending on whether clones were luminally- or myoepithelially-derived. The results are shown in table 17 below, and directly confirm the conclusion, based on clone morphology and size measurements, that myoepithelially-derived clones proliferate at a greater rate in culture than luminal counterparts.

CLONE TYPE		LABELLING INDEX
25.5+	Type 1	30.8% +/-3
	Types 2-4*	30.0% +/-9
PHM6+	Type 5	68.0% +/-5
	Type 6	79.0% +/-9

Table 17. Labelling indices of sorted clones. Types 2-4* were pooled because the route of clone progression cannot be reliably predicted at this stage of culture.

5 day clonal growth cultures propagated in HC:I:CT:EGF containing medium with 3T3L1 feeders were pulsed with BrdUrd for 2 hours, then fixed and stained as described in Section 2.5.4. Labelling indices were calculated as the ratio of FITC (DNA-synthesising):PI(total) labelled nuclei (see Figure 35).

Collating the data as a gross average, comparison between all luminal versus all myoepithelial phenotypes produced the following figures for luminal:myoepithelial labelling indices - 30.4% ±6: 74.0% ±7 (p value <0.0001).

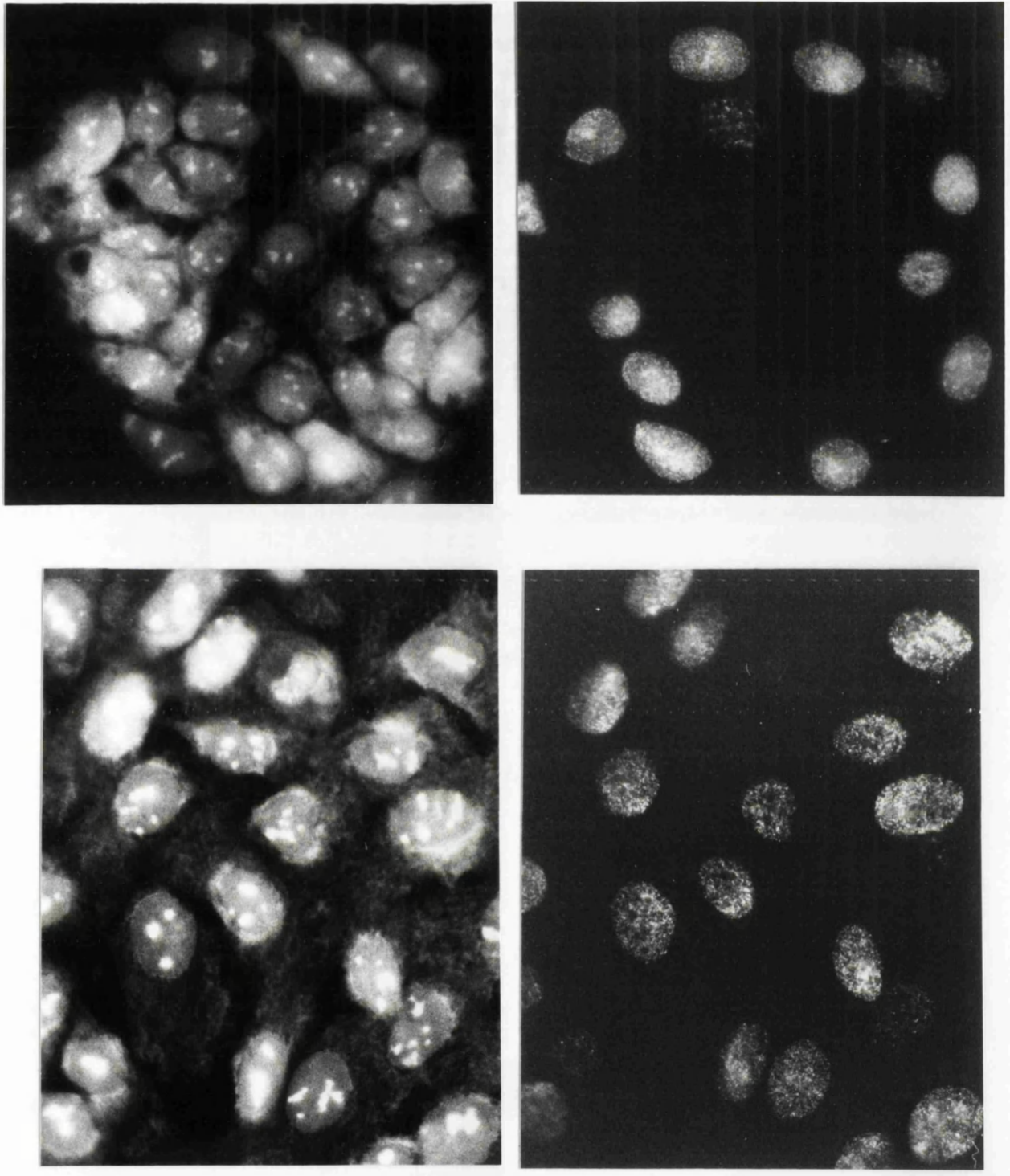


Figure 35. Micrographs of the immunofluorescence detection of BrdUrd uptake by primary clones generated by sorted cells.

(a) Staining of all the nuclei of a luminal Type 1 clone with PI; (b) BrdUrd incorporated by cells of the same clone; (c) staining of all the nuclei in a portion of a myoepithelial Type 6 clone; (d) BrdUrd incorporated by cells of the same clone. x960

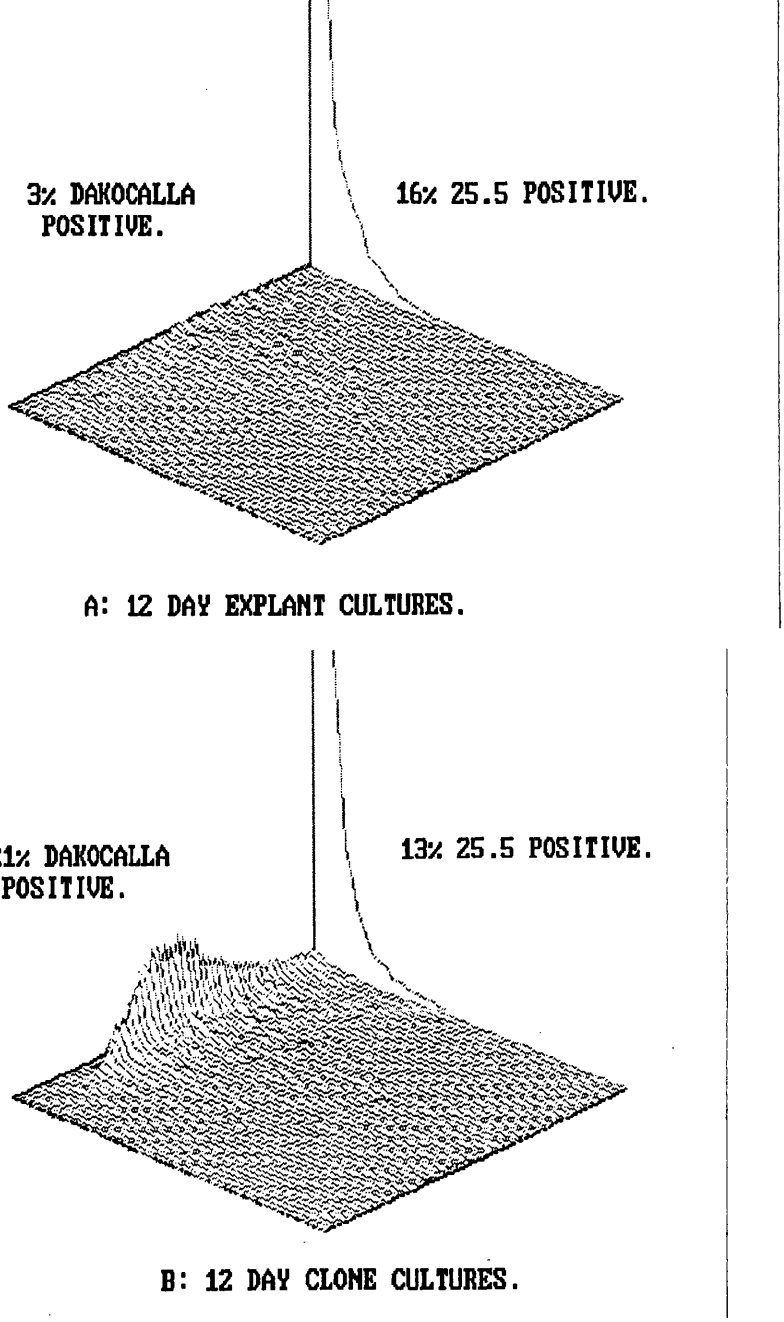


Figure 36. Isometric profiles of detected luminal and myoepithelial cells derived from different culture types.

Explant or clonal growth cultures propagated for 12 days in HC:I:CT containing medium were trypsinised and double-stained with antibodies 25.5 and DAKOCALLA. Each suspension was then analysed individually in the cytometer, with green (25.5 positivity) and red (DAKOCALLA positivity) fluorescence measured simultaneously. The proportions of cells of each phenotype arising in the different culture types are indicated.
(A) Isometric profile of cell types detected in explant cultures;
(B) isometric profile of cell types detected in clonal growth cultures.

As shown, two distinctive distributions of labelled cells within clones were observed in these cultures. In luminal Type 1 clones, labelled cells were arranged peripherally (Figure 35a and b), whilst in all other luminal or myoepithelial clones, labelled cells were distributed throughout the entire colony (Figure 35c and d).

4.6 Flow cytometric analysis of culture phenotypes.

4.6.1 Introduction.

Flow cytometric analysis on the basis of cell type-specific marker expression provides a precise means of analysing the constituent cells in various types of mammary cultures. It was clear that due to differential growth rates of myoepithelial cells in different culture types, cells present in a minority in explant cultures came to represent the majority cell type under clonal conditions. Flow cytometric analysis of both bulk cultures and clones was therefore carried out to give a precise numerical analysis of the relative numbers of luminal and myoepithelial cells present in such cultures. This enabled the behaviour of explant, monolayer or clone cultures to be correlated with the numbers of cells expressing either luminal or myoepithelial markers, or both, in response to propagation under different growth promoting conditions.

4.6.2 Analysis of luminal or myoepithelial phenotypes in explant, primary monolayer or clonal growth cultures.

The majority of detected cells expressed either luminal phenotype (25.5-positivity) or myoepithelial phenotype (endopeptidase-24.11 positivity). Thus in all type of culture cells remained as two distinct populations. Nevertheless, some double-labelled cells were detected, and a significant number of cells did not stain with either antibody. However, because the double-labelling method did not permit exclusion of dead cells (see Section 3.2.4), it was not possible to ascertain if these cells represented viable cell populations. Using data from cytograms such as those presented in Figure 36, it was possible to simultaneously analyse those cells still maintaining exclusive expression of luminal and myoepithelial markers in explant, monolayer and unsorted clonal growth cultures set up from the same material. Collating the cytometric data shows that the cellular composition of cultures is significantly altered by the type of culture, and by hormone and growth factor additives (Table 18). Thus, after 12 days of culture, luminal phenotypes are the predominant stained population in both explant and monolayer cultures, but when the same preparation was plated at clonal densities, the resultant clonal cul-

tures contained twice as many myoepithelial cells as luminal cells. Thus, proportionately more myoepithelial cells were detected in clonal cultures than monolayer or explant cultures.

Proportional differences between luminal and myoepithelial phenotypes in different culture types were seen with a variety of different medium additives (Table 18), but were most significantly influenced when EGF was included in the medium. In response to EGF, the ratio of luminal to myoepithelial cells was increased in favour of luminal phenotypes in both explant and monolayer cultures, but not clonal growth cultures.

CULTURE TYPE	MEDIA COMBINATION		
	HC:I	HC:I:CT	HC:I:CT:EGF
EXPLANT	1:5	1:5	1:12
MONOLAYER	1:2	1:2	1:9
CLONAL GROWTH	2:1	2:1	2:1

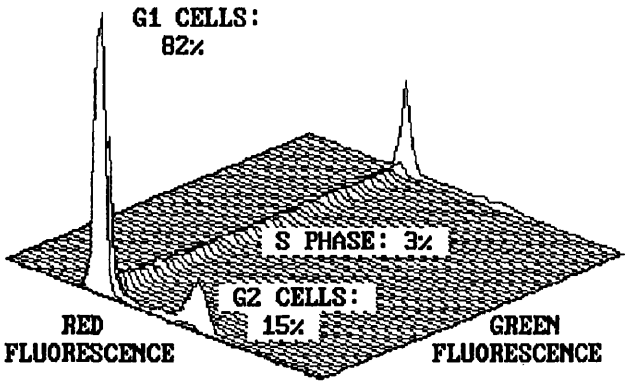
Table 18. The relative proportions of myoepithelial:luminal phenotypes in different culture types after 12 days, as determined by flow cytometric analysis with 25.5/DAKOCALLA double staining. Clonal growth cultures were propagated with 3T3 L1 feeder cells.

These results emphasise the distinction that has to be made between numerical analysis of clonal morphotypes and flow cytometric analysis of cellular phenotypes. Thus, although the total number of myoepithelial clones was less than luminal clones when feeder cells were used, (see Table 13), flow cytometric analysis revealed that the majority of stained cells in the clone cultures as a whole were of myoepithelial phenotype. These observations are consistent with both the visual appearance of the different clone types (Section 4.2.2), and their labelling indices (Table 17). The lesser proportion of myoepithelial cells in explant cultures is consistent with the distribution of the cytokeratin 14-positive cells directly visualised by indirect immunofluorescence (Figure 12).

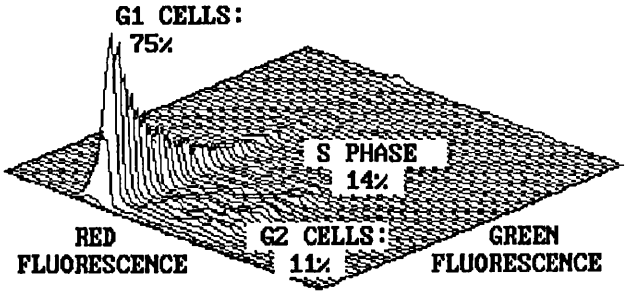
4.6.3 Cell cycle analysis and cytokeratin expression in clone cultures.

Using flow cytometry, the cell-cycle profiles of 12 day unsorted clonal growth cultures were analysed in conjunction with cell type-specific cytokeratin staining. The objective was to confirm the identity of the main proliferative cell type under these conditions using a criterion of identity other than the membrane markers. The cytometric data is presented in Figure 37 as plots

generated by the profiles of DNA fluorescence (abscissa) against either luminal cytokeratin 18 staining, or myoepithelial cytokeratin 14 staining (ordinate). The proportions of cells in each cycle phase are indicated. Cells expressing cytokeratin 14 were the only significant population in S-phase after 12 days culture. The majority of cytokeratin 18 expressing cells existed in either G0/G1 or G2/M phases. Neither cytokeratin-18 only cells (Types 1 and 3) or double-labelled cells such as are found in clone Types 2 and 4 are contributing significantly to the S-phase fraction at this stage. S-phase cells must therefore be represented predominantly by cytokeratin 14-only cells, that is clone Types 5 and 6. This result provides further evidence of the long-term proliferative potential of myoepithelial phenotypes from the basal layer, and the more limited growth obtained from all luminally-derived cells.



A: CYTOKERATIN 18 POSITIVE CELLS.



B: CYTOKERATIN 14 POSITIVE CELLS.

Figure 37. Isometric displays of flow cytograms of the cell cycle profiles of cytokeratin 14 or 18 expressing cells in clonal growth cultures.

Cell suspensions derived from 12 day clonal growth cultures propagated in HC:I:CT containing medium were double-labelled with PI and either antibodies LE61 (CK18) or LL001 (CK14). The isometric plots were generated from data collating the measured DNA content of cells (PI red fluorescence, x-axis) positively stained with either anti-cytokeratin antibody (FITC fluorescence, y-axis).

(a) the cell cycle profile of luminal cytokeratin 18 positive cells; (b) the cells cycle profiles of myoepithelial cytokeratin 14 positive cells. The proportions of cells in the G1, S or G2 phases of the cell cycle are indicated.

4.7 Determining the stability of differentiated clonal phenotypes.

4.7.1 Introduction.

Although the stable phenotype of primary clones derived from myoepithelial cells indicated that these cells breed true, the co-expression of cytokeratins 14 and 18 by luminal Type 4 clones suggested that such clones may not be lineage-restricted. The question of the long-term stability of the differentiated phenotypes of cells within clones was addressed by separately ring-cloning primary colonies. Morphologically identifiable clones generated from sorted cells were re-cloned, and each ring-clone replated separately at clonal densities. Progeny clones were classified both morphologically and by the expression of cell type-specific cytokeratins. The results showed that while some clone types did indeed breed true through a second cloning, luminal clone Type 4 displayed stem cell characteristics, and generated a range of different progeny clones.

4.7.2 Characterisation of re-cloned luminal sub-populations.

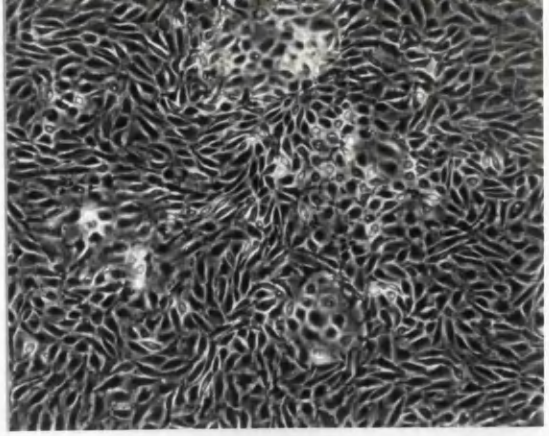
Colonies in daughter cultures generated from clone Types 1, 2 and 3 morphologically resembled the respective parent clone, although they appeared to be growing

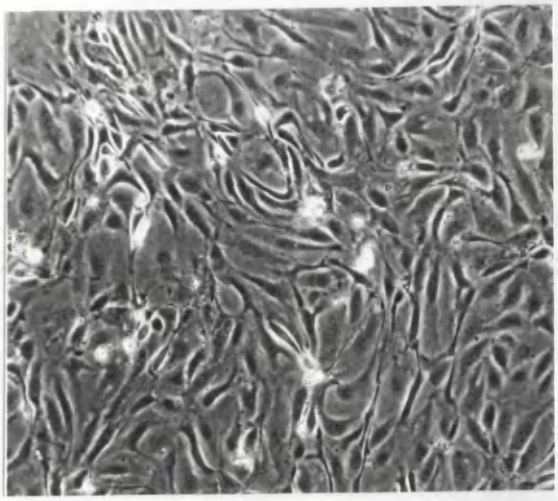
more slowly. Re-cloning efficiencies were approximately 43%, and altogether a total of 15 (5 of each type) primary clones were examined in this manner, without evidence of any diversity in their progeny.

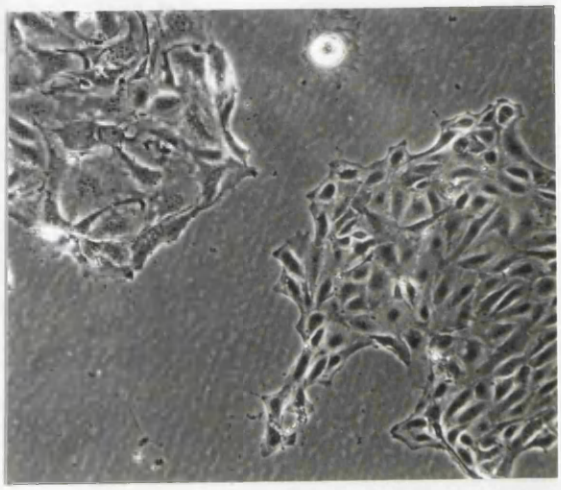
In marked contrast, 22 out of 22 re-cloned Type 4 clones generated a range of recognisably different daughter colonies. Although some atypical clones were seen , the majority of progeny clones in such cultures were identifiable as resembling specific primary clone types. Furthermore, collectively they included examples of all clone morphologies seen in primary clonings of unsorted mammary cells (Figure 38). Of note was the fact that clones of cytokeratin 14 expressing cells were generated which resembled type 6 primary clones. However, in these secondary clones the immunolocalised filaments were atypically distributed and differed from that observed in primary Type 6 clones. This indicated that such clones were not derived from contaminating cells derived from primary myoepithelial clones since re-cloned Type 6 clones generated clones with cytokeratin 14 filament arrangements characteristic of the parent colonies. The frequency with which different progeny clones were generated differed between individual parent Type 4 clones, with re-cloning efficiencies varying between 34% and 63%. Detailed analysis of results from six Type 4 re-clonings indicated that the

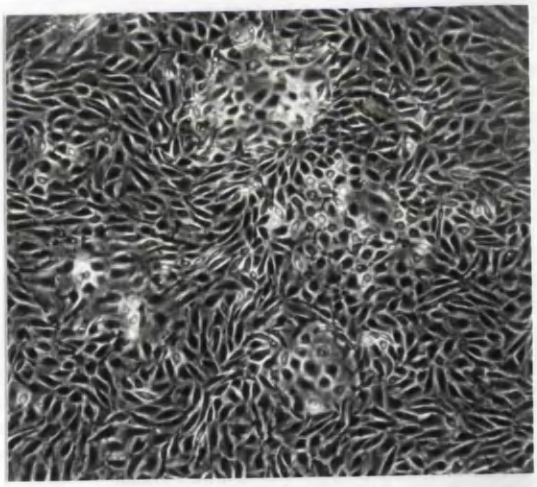
majority of classifiable progeny clones resembled the parental type. The frequency with which different luminal clone types were generated was greater than that with which recognisable myoepithelial-like clones were obtained, and not all Type 4 clones generated clone Types 5 and 6 when re-cloned (Table 19).

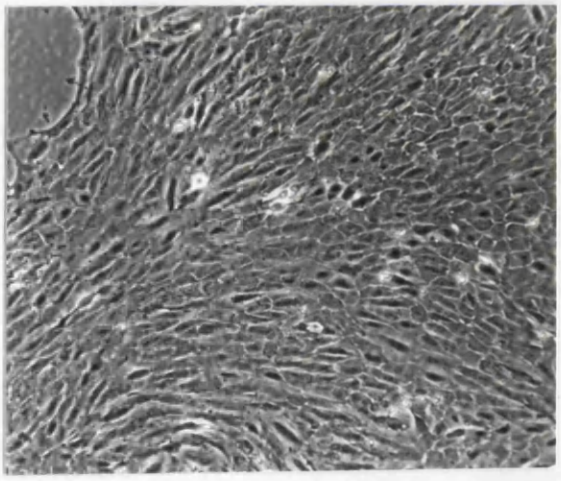
Figure 38 (Opposite). Phase-contrast micrographs of a parent Type 4 clone and representative progeny clones generated by ring-cloning. x158

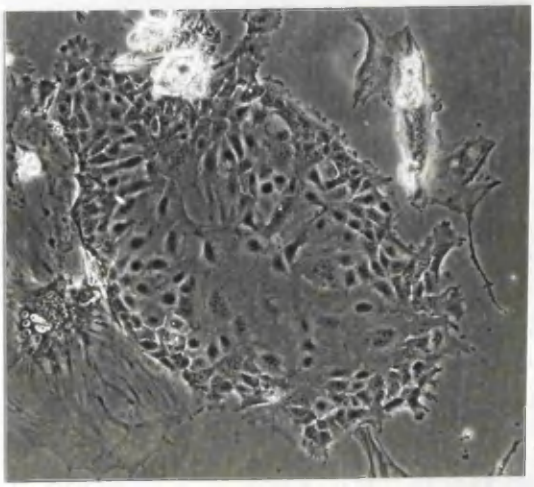












PARENT	PROGENY CLONE TYPE						
	T1	T2	T3	T4	T5	T6	T7*
Type 4	0	2	2	57.6	0	0	37.9
Type 4	3	10	5	46.0	0	5	30.0
Type 4	3	18	11	31.5	0	5	31.5
Type 4	4	2	11	42.0	0	0	40.0
Type 4	8	0	11	14.5	10**	13	43.5
Type 4	0	3	11	40.0	1.0	13.0	31.0
*** Mean relative frequency:							
(%)	4.8	9.0	14.0	62.0	<1	10	-

Table 19. The percentage frequency of progeny morphological phenotypes generated by ring-cloned Type 4 clones.

Primary clones generated by 25.5 positive sorted cells were propagated for 7 days in HC:I:CT containing medium with 3T3L1 feeder cell support. Morphologically identified colonies were then ring-cloned, and daughter cultures propagated under identical conditions for a further 7 days.

* Clones which could not be classified on the basis of primary morphologies.

** Denotes mixed T5/T6 clone type.

*** Calculated for identifiable clone morphologies.

4.7.3 Characterisation of re-cloned myoepithelial subpopulations.

The proliferative and morphological characteristics of Type 6 clones persisted after re-cloning. Vigorously proliferating Type 6 clones arose in daughter cultures, and Type 5 clones were rarely generated. By

contrast, re-cloned mixed colonies (composed of cells resembling those in both Type 5 and Type 6 clones) generated approximately equal numbers of both Type 5 stretched clones and proliferating Type 6 clones, and mixed clones in the same daughter culture. No identifiable luminal clones types were observed from a total of 10 individual myoepithelial primary re-clones. Re-cloning efficiencies of the myoepithelial primary clones were approximately 50-60%.

4.8 Derivation and characterisation of cloned myoepithelial cell strains.

4.8.1 Introduction.

The proliferative capacity of myoepithelial cells in culture, and the stability of their differentiated phenotype suggested that it might be possible to derive long-term cell strains with appropriate differentiated characteristics. To this end, single Type 6 clones identified morphologically in a primary clonal plating were isolated by ring-cloning and expanded by serial passage. Strains could be passaged for up to 6 months during which time they underwent an estimated 100 population doublings. Thus, myoepithelial cells displayed a substantial capability for continuous long-term growth in culture, and could be propagated as either clonal growth cultures with or without feeder

cell support, or as high density monolayer cultures without feeder cell support. As luminally-derived clones did not maintain vigorous proliferation after two rounds of cloning no attempts were made to establish strains from them.

4.8.2 Phenotypes of passaged myoepithelial cells.

Flow cytometric analysis demonstrated the myoepithelial origin of strains, and indicated that after 2 passages 24% of detected cells in double-labelled preparations expressed endopeptidase-24.11, whilst no cells stained with antibody 25.5. As concurrent experiments were being carried out with SV40-derived genes (see Chapter 5), passaged myoepithelial strains which had not been knowingly exposed to the virus were nevertheless tested for SV40 nuclear antigen. However, none of the passaged strains of myoepithelial cells described here stained with antibodies Pa412 and 419 which recognise SV40 large T protein.

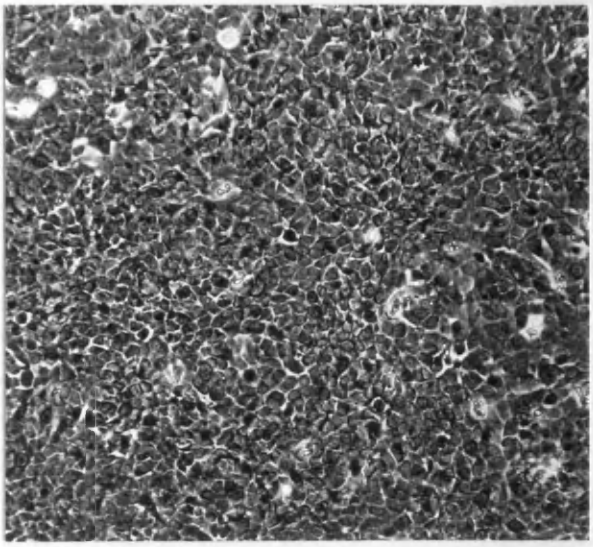
Morphological progression: During long-term culture sub-clones of the initially monomorphic myoepithelially-derived cells displayed progressive morphological alterations (Figure 39). Certain clones acquired feeder cell independent growth after 6 passages and displayed the morphologies shown in Figure 39b. By contrast, areas of other sub-confluent cultures and

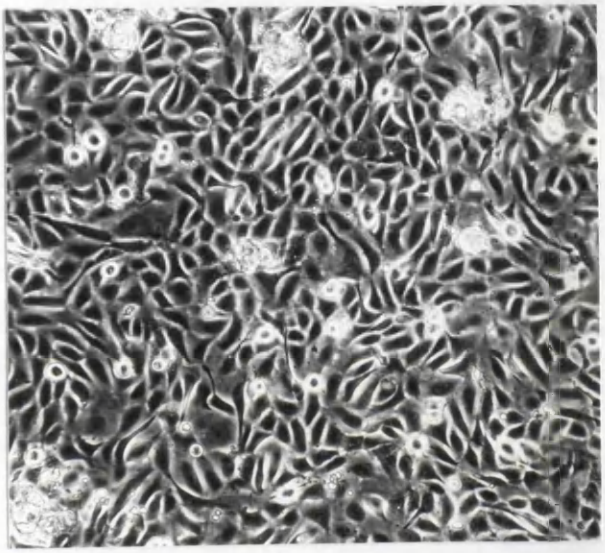
cells in the centres of slow growing clones exhibited squamous metaplasia. This was not observed at early passage numbers. Cells enlarged, forming multicellular flattened squames, slightly elevated above the plane of focus of the majority of cells (Figure 39c), and eventually shed off into the medium.

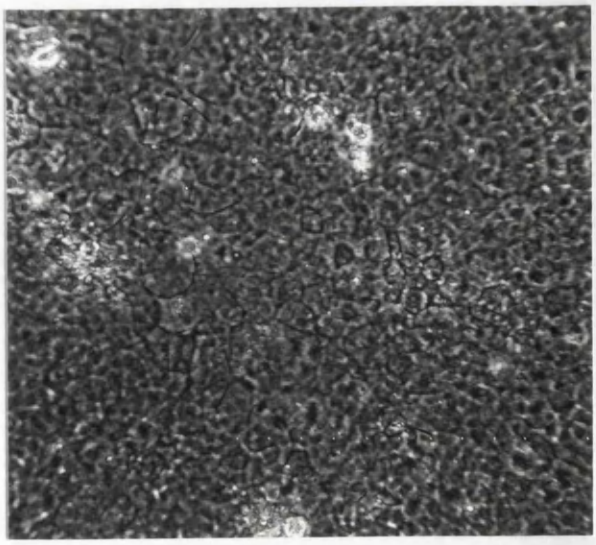
Cytokeratin expression: Squame-like cells described above contained cytokeratin 14 arranged in a diffuse punctate manner (Figure 40a), but they did not stain with antibody LE41 against cytokeratin 8 (Figure 40b). Cells at earlier passages expressed both cytokeratins 14 and 8. However, no cells in this particular sub-strain stained with antibody LE61 recognising the luminal cytokeratin 18 at any stage examined.

Figure 39 (Opposite). Phase-contrast micrographs of passaged clonal myoepithelial cell strains.

(a) Typical myoepithelial morphology; (b) atypical feeder cell-independent clone; (c) myoepithelial clone undergoing squamous metaplasia. x135







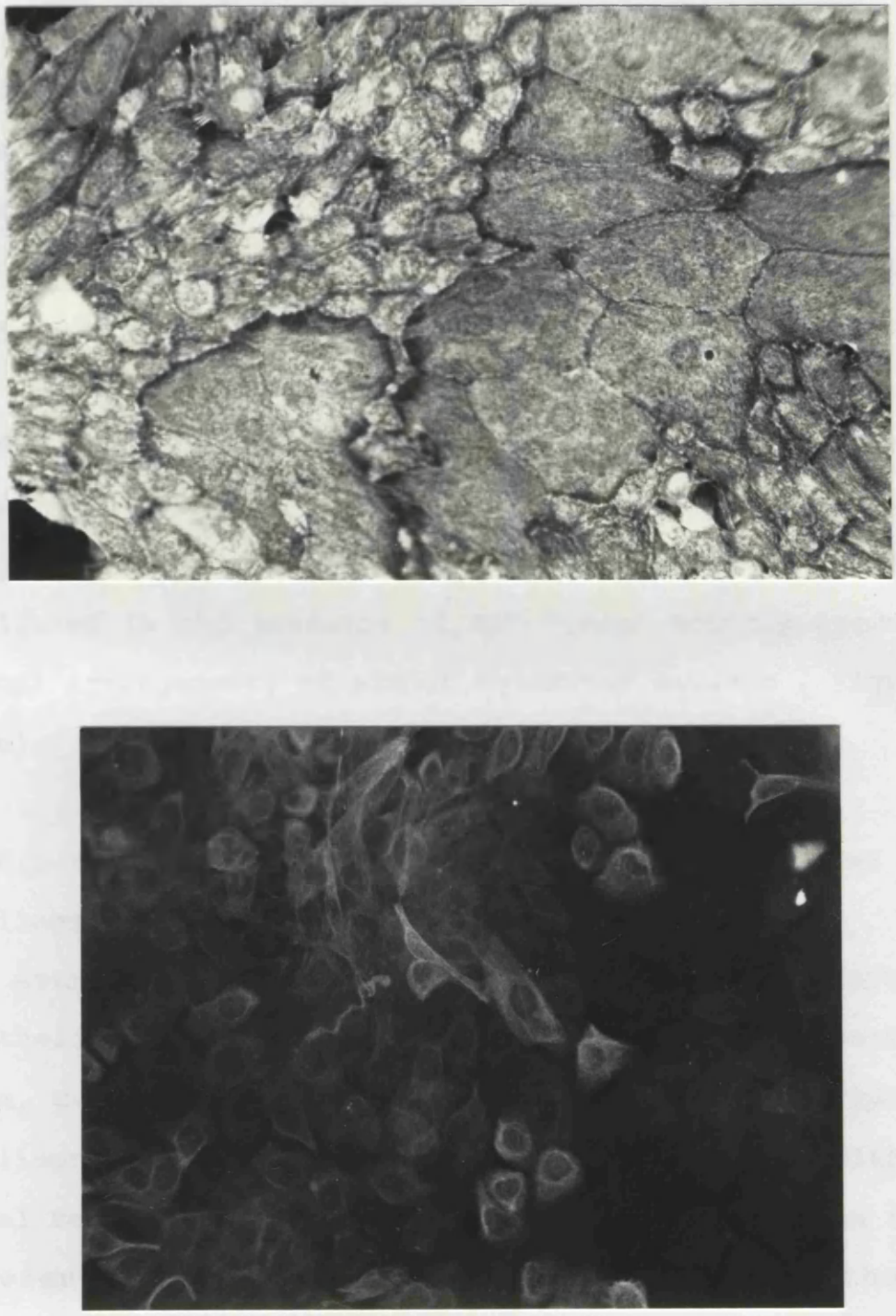


Figure 40. Micrographs of indirect immunofluorescence staining of myoepithelial squames.

(a) Cytokeratin 14 expression (LL001 staining); (b) cytokeratin 8 expression (LE41 staining). Note that an area of squamous cells does not stain. x400

Electron microscopy: Transmission electron microscopy revealed that at confluence, cells from the myoepithelial strains were multilayered on plastic substrata and elaborated both microvilli and desmosome junction complexes (see Figure 43). Scanning electron microscopy revealed differences in the surface architecture of the cells when they were maintained with different media. Cultures propagated in HC:I:CT displayed overlapping patterns of growth (Figure 41a) whereas cells cultured in the presence of EGF formed orderly geometrical arrangements of almost hexagonal squames (Figure 41b).

4.8.3 Behaviour of myoepithelial cell strains grown on collagen.

To examine the behaviour of the differentiated myoepithelial cell strains on a more appropriate substratum, cells were grown on top of reconstituted Type I collagen hydrogels. Attachment and growth of myoepithelial cells on such gels was critically dependent on the presence of both CT and EGF , and even when these additives were present, plating efficiencies and growth rates were considerably diminished compared to behaviour on tissue-culture plastic.

10^4 cells which had previously been passaged 5 times were seeded onto tissue culture plastic or unreleased collagen gels in wells with a $132mm^2$ surface area. They

were fed with HC:I, HC:I:CT or HC:I:CT:EGF containing medium. After 7 days, occasional elongated fibroblastoid cells and nodules of cells were observed on gels grown in HC:I media, (Figure 42a). In contrast, cells plated onto gels and grown in HC:I:CT medium generally formed distinctive ridges and multicellular nodules. The growth of cells and overall culture morphology differed significantly in HC:I:CT:EGF containing media. Here cells grew as multilayered sheets, the boundaries of which could be discerned with difficulty by phase contrast (Figure 42b). Cultures grown on tissue culture plastic had grown to confluence within this time and did not show nodules or ridges (see Figure 39a).

Electron microscopic examination of collagen gel cultures propagated with HC:I:CT:EGF, revealed that squamous metaplasia was exaggerated under these conditions to such an extent that cells formed a well-organised stratified structure resembling epidermal organisation (Figure 43b).

4.8.4 Growth response of a myoepithelial cell strain.
The considerable proliferative capacity of clonal cell strains enabled a variety of hormones and growth factors to be tested for their effects on proliferation, either by direct measurements of cell number, which is difficult to achieve with limited numbers of sorted

primary cells, or by cloning assays. The major question addressed was to what extent clonal strains were representative of primary myoepithelial cells.

As a first step to characterising strains, the population doubling time of a myoepithelial cells propagated in HC:I:CT:EGF containing medium was determined. The growth curve of a strain plated at low densities under these conditions indicated that the population doubling time for myoepithelially-derived cells during exponential growth was 14.0 hours.

Several facts emerged when myoepithelial strains were tested for responses to hormones and growth factors using a clonal growth assay. Cells showed small increases in cloning efficiency in response to feeder cells, and the responses to specific additives were smaller in magnitude than those observed with primary cells (data not shown). In addition to these differences, the morphology of the cell strain was altered by various additives in a manner which did not always correspond with the behaviour of primary myoepithelial clones. For example, when 3T3 L1 conditioned medium was used for cloning primary cells, type 6 myoepithelial colonies occasionally exhibited fibroblastoid cells at their edges when the hormonal additives were omitted from the medium (see Section 4.4.4). Under similar

conditions, a significantly greater number of these aberrant cells were seen in cultures derived from clonal strains, with 7% of clones formed exclusively of fibroblastoid cells (clones entirely composed of such cells were never seen in primary cultures). Furthermore, primary myoepithelial cells cloned in the presence of feeder cells did not generate fibroblastoid cells, but long-term strains did undergo this change in the presence of irradiated feeder cells(Figure 44). Thus, although the presence of HC:I could still prevented this transition, the frequency and conditions under which the shift from a recognisable myoepithelial morphology to a fibroblastic form occurred altered noticeably in passaged cells.

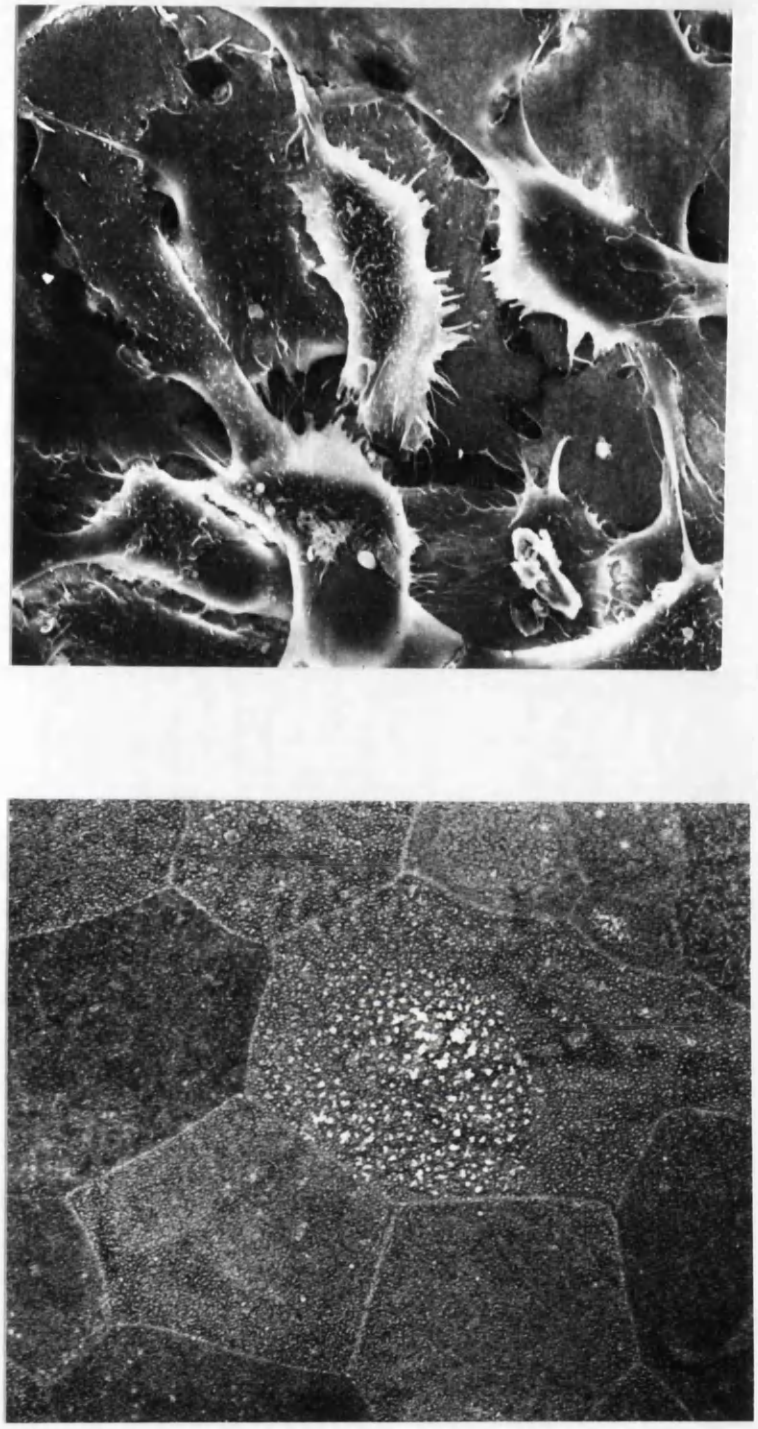


Figure 41. Scanning electron micrographs of a cloned myoepithelial cell strain grown under different conditions.

(a) Cells grown in HC:I:CT containing medium, x2008; (b) concurrent culture grown in HC:I:CT medium plus EGF at standard concentration, x1745.
Both cultures were fixed after the same 7 day culture period.

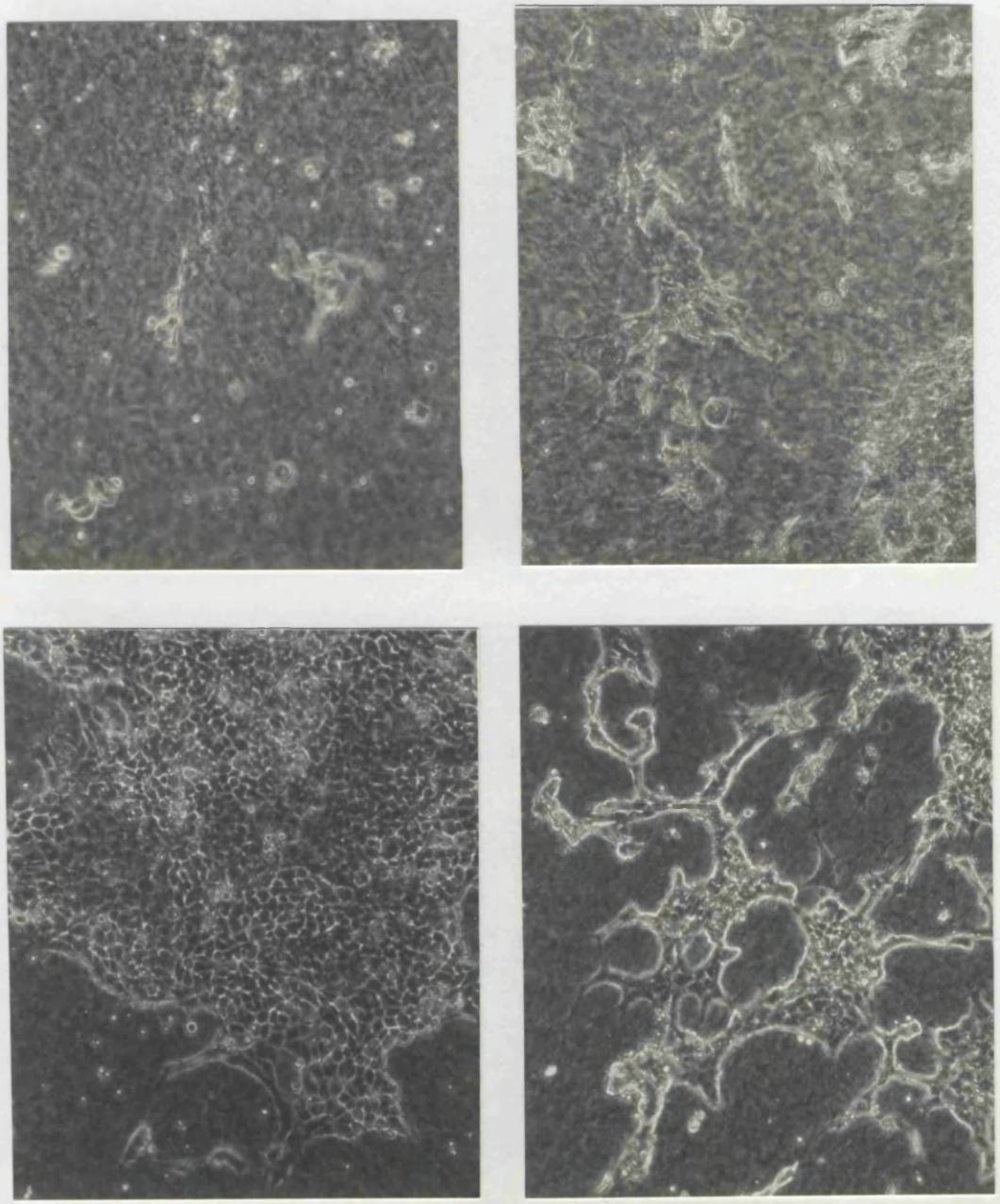


Figure 42. Phase-contrast micrographs of cells grown on top of collagen gels.

(a) Myoepithelial cell strain grown in HC:I containing medium; (b) Myoepithelial cell strain grown in HC:I:CT:EGF containing medium; (c) tsA-U19 luminally-derived cell line (see Section 5.2.5) (d) tsA58-U19 myoepithelially-derived cell line (see Section 5.2.5). x120

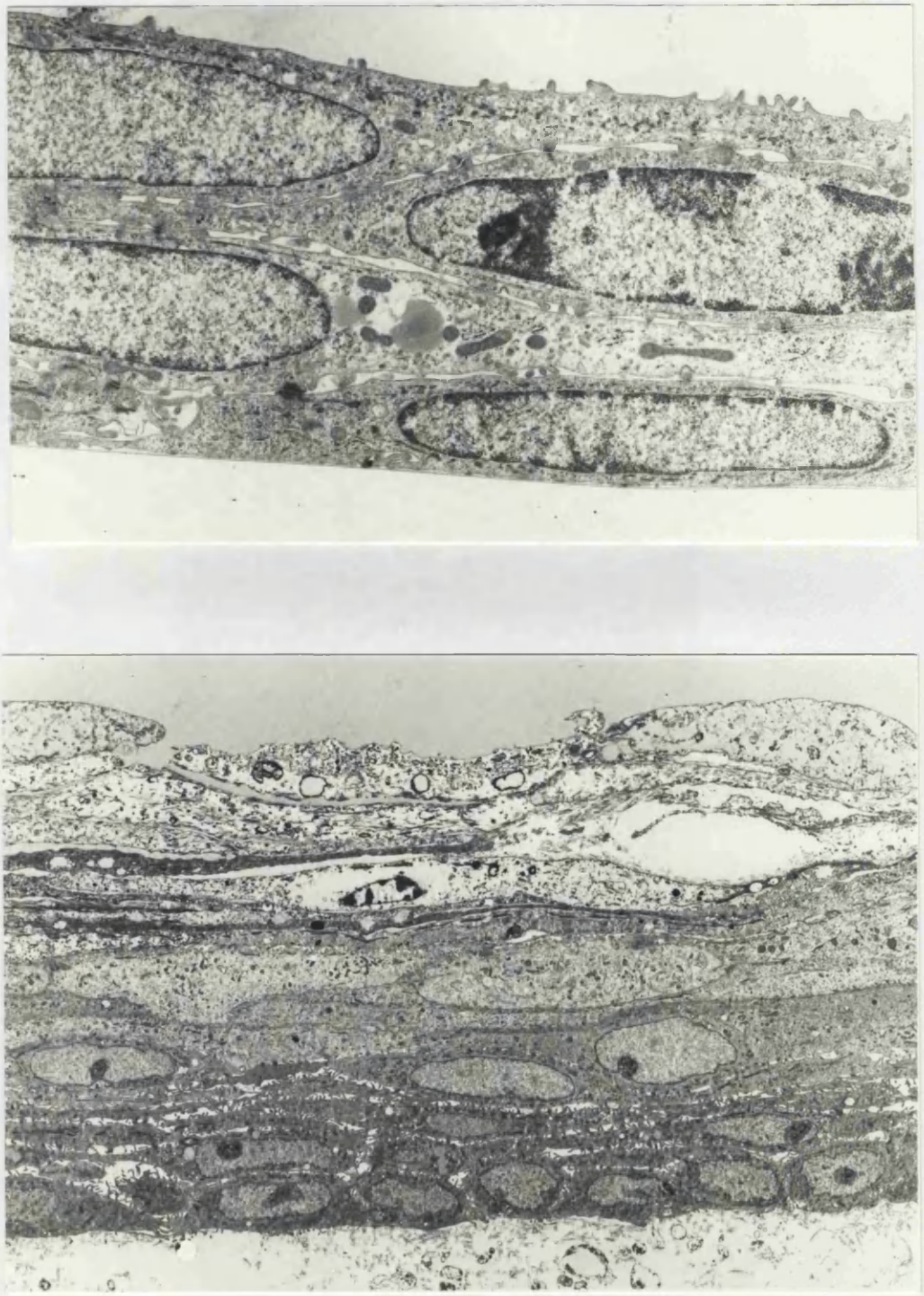


Figure 43. Transmission electron micrographs of a clonal myoepithelial cell strain.

(a) Cells at passage 5 grown on plastic. Note the presence of junctional complexes, x9100 . (b) Concurrent culture grown for 17 days on an unreleased gel, x1800.

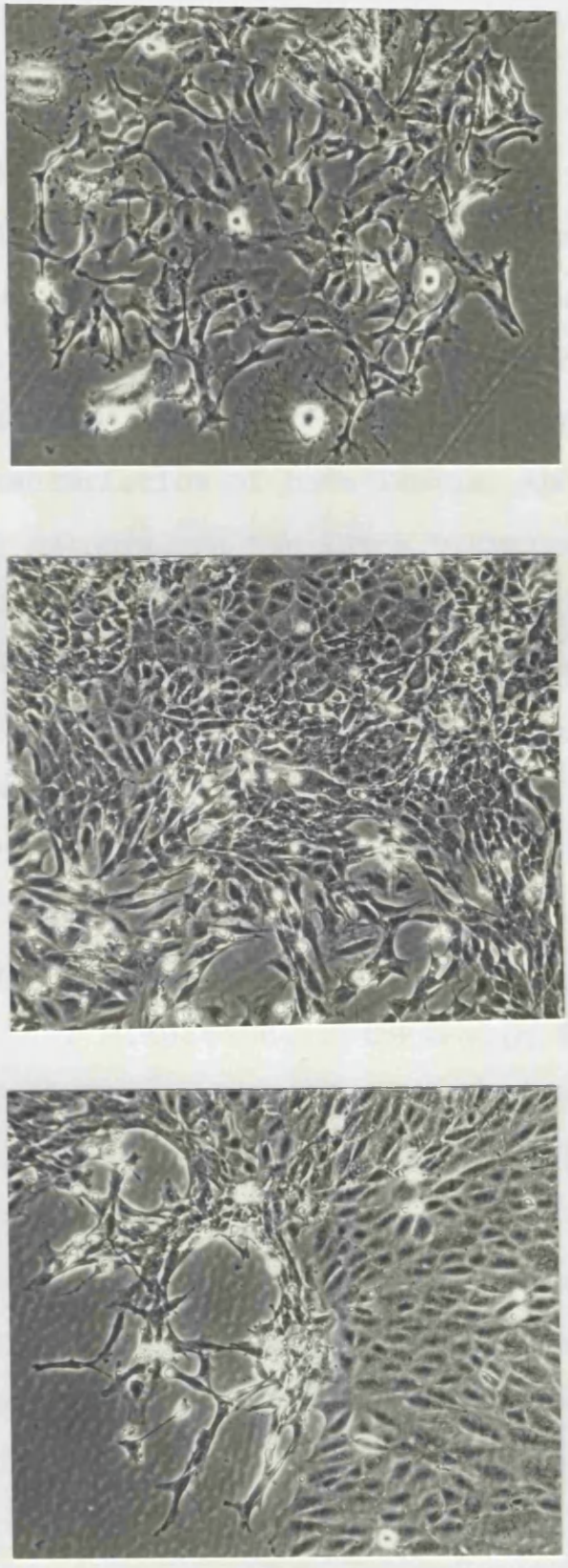


Figure 44. Phase-contrast micrographs illustrating epithelial-mesenchymal transitions.

(a) myoepithelial clone; (b) tsA58-U19 luminally-derived cell line; (c) tsA58-U19 myoepithelially-derived cell line. x180

4.9 **DISCUSSION.**

Applying the methodology of flow sorting and cytometric analysis, on the basis of cell-type specific marker expression, to rat mammary gland has provided a powerful tool for analysing the dynamic differentiation and growth characteristics of both luminal and myoepithelial cells in culture. In the first instance, it is clear that both luminal and myoepithelial cells are capable of independent growth, as clones or in isolation after cell sorting, whilst maintaining the expression of appropriate cytokeratins.

Cytometric analysis of different culture types revealed differences in the growth rates of myoepithelial cells. In the explant situation, cytokeratin 14 expressing cells generally appear non-mitotic and were the minority population, whilst monolayer cultures were conducive to the expansion of the myoepithelial population. Clones expressing cytokeratin 14 exhibited considerable proliferation, forming the largest colonies, a property which is potentiated by removing luminal cells by flow sorting. Collectively, these results suggests that the proliferation of myoepithelial cells is negatively regulated, and that progressive disruption of the parenchymal architecture (explants/ mixed monolayers/ mixed clonal/ sorted cultures, respectively) releases this powerful constraint. Growth regulation in this

system is likely to be multi-fold, and three possible mechanisms are suggested by the work presented here. (1) paracrine acting soluble factors, (2) cell-cell or (3) cell-matrix contact.

Since myoepithelial cells do not proliferate significantly in explants but do generate large clones under identical culture conditions, this suggests that the negative influence must be derived from the luminal component. This could be contact mediated (myoepithelial cells are invariably located basally in explant cultures), or the result of a paracrine factor secreted by luminal phenotypes acting within the localised microenvironment of the explant. Such a factor would be diluted in clonal cultures and absent from endopeptidase positive sorted cultures. Indeed, the fact that sorted type 6 myoepithelial clones grow at faster rate than those arising in unsorted cultures (as indicated by statistically significant differences in clone sizes), strongly suggests a functioning paracrine mechanism. In this regard the findings of Ethier and Van De Velde that normal rat mammary cells in culture secrete latent form TGF-β, and that the cells possess plasminogen activator-like proteases, Ethier and Van De Velde, 1990, are highly significant. Based on their findings, the authors proposed that TGF-β functioned in an autocrine manner, but since no attempt was made to

separate the parenchymal populations, the cell type (s) responsible for TGF-β secretion, or indeed the responsive cell type (s) in these cultures is a matter for speculation. However, the human breast cancer cell line MCF-7, which displays luminal characteristics in culture, secretes a TGF-β-like factor, Knabbe et al., 1987, as do many other cultured cells of epithelial origin, Roberts and Sporn, 1988. Normal human mammary cells in culture, Hosobuchi and Stampfer, 1989, and several human cell lines of putative mammary origin, Zugmaier et al., 1989, display pronounced growth inhibition in response to recombinant TGF-β1, and pure human TGF-β1 and -β2, respectively. These facts suggest that TGF-β1 may be a naturally occurring regulator of mammary parenchyma. This is supported by the fact that the peptide inhibits growth and morphogenesis of mouse mammary structures *in situ* in a localised and reversible fashion, Silberstein and Daniel, 1987, and does so in a highly specific manner, Daniel et al., 1989. Alternatively, growth inhibitory activities may be a result of rodent homologues of human mammastatin, Ervin et al., 1989; somatostatin analogues, Setyono-Han et al., 1987; or as yet unidentified polypeptides similar to those existing in bovine mammary gland, Bohmer et al., 1987.

The regulation of myoepithelial growth by cell-matrix

interactions is suggested by the behaviour of myoepithelial strains seeded onto type I collagen gel surfaces. A notable feature of this culture situation was that cell adhesion to collagen and subsequent growth was critically dependent on the presence of CT and EGF. These observations concur in part with the findings of Wicha et al., 1979, and Salomon et al., 1981, who noted that rat mammary cells preferentially attached to Type IV collagen, and that growth on Type I collagen was selectively inhibited by the omission of EGF and glucocorticoids, Nevertheless, with all hormones and growth factors present, the proliferation of cloned myoepithelial phenotypes was severely restricted compared to parallel cultures on standard tissue culture plastic. Such matrix influences probably pertain *in situ*.

Mesenchymal cells profoundly influenced the growth and differentiation behaviour of parenchymal clones. Although the influences of mesenchymal substrates or effects of feeder cells on parenchymal cell conformation cannot be discounted, it seems most probable that at the densities of feeder cells used in the studies presented, the effect was promoted by a paracrine acting soluble factor. This notion is reinforced by the observation that conditioned medium alone possessed potent luminal-specific growth promoting activity in

these experiments. In this respect it is interesting to note that Taga et al., 1989, have isolated and purified a mesenchyme-derived growth factor which promotes the proliferation of mouse mammary epithelium. Although no significant differences in the response of mammary cells were observed between conditioned medium from 3T6 or undifferentiated 3T3L1 cells, plating efficiency was greater in response to 3T3L1 feeder cells in the presence of hydrocortisone and insulin compared to those produced by either 3T6 or homologous mammary stromal feeder cells under identical conditions. This enhanced growth effect of differentiating 3T3L1 cells has been documented with respect to murine mammary cells, Levine and Stockdale, 1984. These authors suggested that qualitative and/ or quantitative differences were mediated by differentiating 3T3L1 cells compared to cells which did not differentiate. Since 3T3L1 cells undergo adipocytic differentiation in response to glucocorticoids, Green and Meuth, 1974; Green and Kehinde, 1975, it is not unreasonable to assume that adipose-specific molecules are active in the 3T3L1 co-culture system as well as fibroblastic-type factors. Evidence does suggest that mammary derived parenchymal cells respond to unsaturated fatty acids produced by mammary fat pads, Beck et al., 1989, and that mammary derived pre-adipocytes secrete active prostaglandin-E2, Rudland et al., 1984. If heterologous

stromal growth factors did indeed function in the 3T3L1 co-culture system, it would be consistent with the observation that two different mesenchymes modulate mammary development in situ, Sakakura et al., 1982.

In summary, it appears that the growth of myoepithelial cells is negatively regulated by a paracrine factor emanating from luminal cells, but they respond positively to added growth factors. By contrast, the mitosis of luminal cells seems to be selectively stimulated by mesenchymal secreted factors. This tissue type-specific interaction is displayed by homologous human cells, O'Hare et al., 1989. The failure of parenchymal feeder cells to promote mammary cell growth is a general observation reproduced between mouse, rat and human species, Ehmann et al., 1984; this work, and O'Hare et al., 1989, respectively, and is also independent of species-type combinations.

Clonal growth cultures facilitated the analysis of dynamic differentiation of parenchyma based on morphology and marker profiles. These are reliable criteria which have been effectively employed to examine differentiative behaviour in lung, Paine et al., 1988, and liver Germain et al., 1988, rat epithelial systems in culture. In the work presented here, the identity of clonogenic progenitor cells could be unequivocally

defined by sorting cells from uncultured preparations. This meant that any subsequent alterations in marker profile displayed by clones would reflect changes in the differentiation status of cells within these clones. This novel approach has revealed that both luminal and myoepithelial populations are composed of distinct clonal sub-populations. It is proposed that some of these sub-populations represent separate lineages, whilst certain other clone types represent stages in the same lineage pathway, as implied by marker and chronological relatedness. This contrasts with the behaviour of human mammary luminal and myoepithelial cells, which although indicating broad similarities with respect to differential antigenic behaviour, O'Hare et al., 1989, are relatively homogeneous populations after sorting. Thus, the spectrum of clonal phenotypes generated by rat parenchyma are not seen in human cultures, in which luminal and myoepithelial cells each generate a single recognisable clonal phenotype, O'Hare et al., unpublished work.

Since cytokeratins 14 and 18 provide a clear-cut distinction between the majority of luminal and myoepithelial cell types *in vivo*, emphasis was placed on the expression of these filaments for the purposes of delineating possible lineage pathways. The differential expression of these cytokeratins was maintained in

culture, with two notable exceptions - 1. The elaboration of cytokeratin 18 filaments by rare myoepithelial cells and 2. The expression of cytokeratin 14 by a significant proportion of cells within luminal Type 4 clones.

The expression of cytokeratin 18 by occasional myoepithelial cells did not occur in all clones, and is best understood in relation to the findings of Knapp and Franke, 1989. The fact that actual numbers of cytokeratin 18 expressing cells per clone were extremely small, (less than 0.001%), suggests that this is almost certainly an analogous phenomenon to the spurious elaboration of simple epithelial cytokeratins by mesenchymal cells at low frequency, Knapp and Franke, 1989; Knapp et al., 1989. Thus, certain clonogenic myoepithelial cells are more liable to adventitious cytokeratin expression due to spontaneous losses of cytokeratin gene control than others in culture. This does not, therefore, represent an archetypal differentiation process *per se*. Similarly, cytokeratin 19 expression is quite labile, Stasiak et al., 1989; Paine et al., 1988; Patton et al., 1990; and it has been postulated that this cytokeratin is a neutral keratin in terms of differentiation, Stasiak et al., 1989. Hence its expression by myoepithelial clones also does not indicate acquisition of luminal phenotype

by these cells. In support of these contentions, it must be emphasised that no progeny clones generated from myoepithelial re-clones ever displayed luminal morphotypes. In contradistinction, the expression of cytokeratin 14 and the potential to generate heterogeneous clonogenic luminal and myoepithelial clones displayed by Type 4 clones constitutes a clear demonstration of the plasticity of this clone type. This is consistent with the proposition that these clones derive from authentic stem cells which retain multipotent potential in culture, including a high capacity for self-renewal. In view of the hypothesis that large clear cells observed in intact gland possess stem character, Smith and Medina, 1988, it is tempting to speculate that such cells are the progenitors of the multipotent clones generated in culture, and indeed some evidence suggests that this may be the case. As demonstrated in the preceding chapter, clear cells can clearly have luminal aspects. Since no qualitative differences between 25.5 positive or PHM6 negative sorted clones was observed, this implies that clear cells stain with antibody 25.5 and arise in positive sorted populations. This is consistent with the supposed luminal origin of Type 4 progenitor cells, which are generated only from sorted luminal populations.

On the basis of the observed behaviour of Type 5 and Type 6 clones, the existence of two distinct clonal myoepithelial populations in these cultures is proposed. One is a precursor pool, normally growth restricted *in situ*, which gives rise to Type 6 clones and is self-perpetuating. Another more differentiated population of cells, mitotically restricted but less stable in culture, gives rise to Type 5 clones. This postulate is supported by several observations. (1) In contrast to Type 6 clones, the limited mitotic behaviour of Type 5 clones is refractory to the effects of sorting, (2) Type 5 clones strongly express the smooth muscle α-actin isoform diagnostic of true myoepithelial cells, but only rare cells occurring in small patches do so in Type 6 clones, (3) the predominantly alveolar location of Type 5 progenitor cells, (4) Type 6 clones elaborate junctional complexes and apical microvilli characteristic of epithelial cells, and (5) multipotent Type 4 clones were not observed to generate fully differentiated Type 5 clones on re-cloning. Points 1, 2, 3 and 4 are consistent with interpretation that fully differentiated myoepithelial cells give rise to type 5 clones, and as far as the myoepithelial population is concerned, acquisition of differentiated characteristics is associated with a loss of proliferative potential. This has been described for human mammary

myoepithelial cells in culture, Petersen and van Deurs, 1988, and other myogenic systems, Owens et al., 1986. Points 4 and 5 indicate a link between luminal cells and myoepithelial populations and suggest that myoepithelial differentiation involves a separate lineage ultimately derived from a luminal phenotype. The existence of 'transitional' clones composed of both stretched-quiescent and polygonal-proliferating cells reflects the observed behaviour of stretched cells in explant culture, which could assume proliferative status concomitant with morphological change. It was not determined during the course of the described experiments whether such clones represented the differentiation of Type 6 colonies, or were Type 5 clones undergoing the reverse process. However, it can be reasonably assumed that the progression of precursor cells to fully differentiated phenotypes is not favoured by the culture environment, and by corollary cells within Type 5 clones are capable of switching phenotype in culture by dedifferentiating into proliferative Type 6 cells, and that this process is induced by CT and EGF. Clearly, factors required for the complete differentiation of myoepithelial cells appear to be absent or non-functional in the culture environment. Despite the prime importance of basement membrane *in situ*, Radnor, 1972, laminin and Type IV collagen, which are both synthesised by cultured myoepithelial cells,

do not effectively induce or maintain the fully differentiated status of these cultured cell types.

During re-cloning experiments, the culture environment was nominally the same for each progenitor cell giving rise to Type 4 clones. Therefore, since daughter cultures contained different proportions of self-renewing and differentiating progeny, and additionally different types of progeny, this argues for a stochastic process, independent of environmental influences, generating the observed phenotypic diversity in the progeny of these multipotent clones. Similar conclusions have been proposed to explain the behaviour of hemopoietic blast cells after isolation as paired progenitors, Suda et al., 1984. Mechanistically, the pluripotential behaviour of 'luminal' cells in adult mammary tissue is the antithesis of stem cell features in other epithelial systems, for example skin and intestine, in which stem cells are preferentially compartmentalised in basal locations, (for a recent discussion of stem cell models see Cotsarelis et al., 1989). However, the location of 'clear' cells, which may be the *in situ* counterpart of Type 4 clone precursors, between the luminal and myoepithelial layers is consistent with the requirements of the mammary parenchyma. Two cell layers, each with completely different differentiated functions must be replenished, perhaps simultaneously,

as would be expected during pregnancy and lactation. If this proposed dual-vectorial system of differentiation actually occurs, it would be a novel pathway of stem cell differentiation behaviour, and have important implications for the derivation of mammary tumours, as will be discussed in Chapter 7. Clearly, rat mammary parenchyma should not be regarded as a simple stratifying system. A similar mechanism of both luminal and myoepithelial differentiation from 'clear' cell precursors, based on electron microscopic observations of cell migration, was proposed by Radnor, 1972. The present results provide dynamic evidence of this phenomenon.

The morphological differentiation of some clonal populations was not always stable in the longer term. Phenotype conversions were displayed by certain type 4 progeny clones and myoepithelial clones propagated in the absence of hormones and the presence of feeder cells. According to one school of thought, the generation of elongated cells by mammary derived cells represents a differentiation process, Bennett et al., 1978; Rudland et al., 1979; Warburton et al., 1981; Warburton et al., 1982; Ormerod and Rudland, 1985. However this proposal seems less likely in the light of the work presented herein and recently published literature examining epithelial-mesenchymal transitions.

Defined as the reversible/ irreversible conversion between epithelial and mesenchymal differentiation programs, Boyer et al., 1989, the process does not exclude either epithelial or mesenchymal character, Boyer et al., 1989, but results in the reorganisation of regular polygonal cells into disparate elongated cells with increased motility. The process can be induced in response to soluble factors, Greenburg et al., 1980; Boyer et al., 1989; Stoker et al., 1987; antibodies recognising extra-cellular cell-cell adhesion cadherin molecules, Shimoyama et al., 1989; Takeichi, 1987; and ovumorulin, Behrens et al., 1985, and 'hormone crisis', this work (Chapter 5). Fibronectin and other low molecular weight basement membrane components also induce epithelial-mesenchymal transition in chick atrioventricular endothelium in collagen gel culture, a process which correlates to that induced in vivo by the myocardium during development, Mjaatvedt and Markwald, 1989. The transition process is accompanied by disruption of junctional complexes, Stoker, 1984, as a result of modulation of desmosomal plaque proteins and a reorganisation of the cytokeratin and actin-fodrin filament systems, Boyer et al., 1989, and changes in gene expression, Barraclough et al., 1982; Barraclough et al., 1984.

The spontaneous production of elongated cells by

epithelial cultures has been described for many cell types in culture including thyroid cells, Hilfer, 1962; Lobach et al., 1987; cartilage cells, Holtzer et al., 1960; Coon and Cahn, 1966; mesothelium, Kim et al., 1987; bladder carcinoma cells, Boyer et al., 1989, and cells in amniotic fluids, Ochs et al., 1983. Furthermore it also appears to be a general property of mammary derived cell lines, Dulbecco et al., 1981; Chang et al., 1982; Dunnington et al., 1983; Schmid et al., 1983; Sonnenburg et al., 1986%; and this work (Chapter 5). Indeed, putative "myoepithelial-like" cells generated by tumour lines bear more resemblance to mesenchymal cells produced by such conversions, and are marked by the characteristic loss of cytokeratin expression and the acquisition of mesenchymal properties, Dulbecco et al., 1981; Barraclough et al., 1984 and Warburton et al., 1987. Thus, neither differentiation or dedifferentiation but various degrees of cellular metaplasia probably account for the spectrum of cell types produced by the "RAMA" cell line series and their derivatives, Paterson et al., 1985; Rudland et al., 1986; Jamieson et al., 1986; reviewed by Rudland, 1987.

Distinct from the mesenchymal transition phenomenon, cultured rat mammary cells also exhibited considerable squamous metaplasia. This has previously been observed

to occur in human mammary explant cultures, Hillman et al., 1983; Schaefer et al., 1983; Yang et al., 1987; and clonal cultures, Petersen and van Deurs, 1988; rat mammary end bud explants, Richards et al., 1983; and mouse mammary organ cultures, Schaefer et al., 1980. The work presented in this chapter demonstrates that isolated myoepithelial cells can switch differentiation programmes and undergo terminal squamous metaplasia, and that it is a clonal event. In view of the proposal that Type 6 clones are partially specialised in the myoepithelial differentiation lineage, it can be therefore be argued that myoepithelial squamous metaplasia represents a transdifferentiation event, as defined by Okada, 1980.

Since cell culture is biased towards the selection and maintenance of rapidly growing cells, and given the behaviour of myoepithelial cells described in this work, it is quite probable that several published "mammary epithelial" cell lines are in fact of myoepithelial origin. This surmise is supported by the fact that a population shift occurs in long-term passage of mixed, unsorted cultures resulting in the predominance of myoepithelial phenotypes. Additionally, it is readily apparent that commonly used phenotypic markers such as junctional complexes and microvilli (for example Butel et al., 1984 and Garcia et al., 1986), are not

reliable criteria since these structures are elaborated by myoepithelially-derived cells in long-term culture. Clearly, the capability to sort cells into pre-defined populations provides an ideal starting point for the generation of cell lines. The results of these manipulations will be presented in the following chapter.

CHAPTER 5.

GENERATION OF ESTABLISHED LUMINAL OR MYOEPITHELIAL CELLS BY RETROVIRAL TRANSDUCTION OF TEMPERATURE-SENSITIVE SV40 LARGE T PROTEIN.

5.1 Retroviral infection of primary cultures.

5.1.1 Introduction.

Cell strains such as those described in the last chapter clearly change with time in culture, and as a result the properties of long-term passaged cells cannot necessarily be considered representative of primary cells. An alternative approach to the generation of large numbers of luminal and myoepithelial cells necessary for experimental manipulation would be the establishment of cell lines using the SV 40 large T viral oncogene. As discussed in Chapter 1, temperature-sensitive mutants of the SV40 gene coding for large T protein permit, in principle, the reciprocal effects of T protein on cell proliferation and differentiation to be dissociated by switching cells from permissive to restrictive temperatures. The aim of the work described in this chapter was the generation of established cell lines using a recombinant retroviral vector system containing a construct coding for temperature-sensitive SV 40 large T. This was done so that cells could be grown up in numbers then induced to adopt characteristics more representative of luminal and myoepithelial cells *in situ*, by switching cultures to the temperature non-permissive for large T function.

Primary rat mammary cultures and sorted cell populations were successfully infected with the recombinant retroviral vector, using either virus-containing supernatant or irradiated virus-producing cells. The system was used to introduce a doubly mutant SV40 T-gene (temperature sensitive and non-DNA binding) into the cells. Integrated provirus was detected by the constitutive expression of the neomycin resistance gene, and cells expressing T were obtained from explant cultures and sorted luminal or myoepithelial cells. The behaviour and phenotypes of cells established by this method were characterised morphologically and by expression of cell-type specific differentiation markers.

5.1.2 The recombinant tsA58-U19 construct.

The recombinant construct used was derived from the shuttle vector pZipNeoSV(X)1 which contains the transposon Tn5 phosphotransferase gene (neo-r), Cepko et al., 1984. Two mutant SV40 inserted sequences replaced sequences in the wild-type SV40 plasmid pZipSV40 which coded for regions of the temperature-sensitive tsA58 gene and the SV-U19 mutation. This construct was then inserted into the shuttle vector at the BamHI restriction site. Thus, the recombinant DNA coded for an early region thermolabile large T protein which did not bind the SV40 origin of replication, Tegtmeyer,

1975, and Paucha et al., 1986, and infected cells could be selected for by growth in the presence of the antibiotic G418, Davies and Jimenez, 1980.

The construct was packaged in the Moloney murine leukemia virus capsid produced by the 3T3 psi-2 cell line, which was originally transfected with viral protein coding sequences in a non-packable form, Mann et al., 1983. The F4 ecotropic virus-producing line used in this study was derived from psi-2 by transfection with the packageable recombinant construct described above. Thus, the cell line packages replication-incompetent retroviral RNA into an infective virion, which after infection of the target cells releases viral RNA containing the construct. This in turn is converted into proviral DNA by the action of the reverse transcriptase which is also coded for by the viral genome. In theory, the proviral DNA is integrated into the host genome with great efficiency, usually at a low copy number and at transcriptionally active sites.

All retroviral construction procedures and the preparation of the F4 line were performed by P. Jat, Jat et al., 1986; Jat and Sharp, 1986; Jat and Sharp, 1989.
A schematic representation of the structure of the provirus is illustrated below.

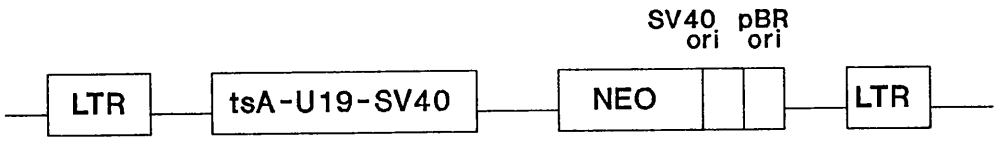


Figure 45. Diagram of the structure of the provirus transduced by the recombinant retrovirus particles produced by F4 cells.

5.1.3 Viral titres produced by F4 cells.

Before infecting primary cells, viral titres were determined using wild-type NIH 3T3 cells, with two different infection strategies - infection with cell-free virus-containing supernatants in the presence of polybrene; and the use of irradiated F4 cells as the source of virus in co-culture with target cells in a 50:1 ratio. Infected cells were detected using G418 at 1 mg/ml. The results indicated that virus titres produced by neat F4 supernatant were $0.2x10^4$ $cfuml^{-1}$, and that 150 cfu were produced by 10^5 irradiated F4 cells. Thus, effective infection of wild-type 3T3 cells could be obtained using either protocol.

5.1.4 tsA58-U19 infections of explant or stromal cultures.

3-day old organoid explant cultures were exposed to retrovirus-containing F4 supernatant in the presence of polybrene for 3 hours. All infections and selections were carried out at 37^oC. After 48 hours cultures were

selected with 0.5mgml^{-1} G418. Selection-resistant cells were detected after 1 week. However, cellular morphology suggested that growing cells were mesenchymal types. Infection of 'pre-plating' stromally enriched cultures produced cells with a similar fibroblastic morphology. The nuclear staining patterns produced by Hoescht dye (lack of punctate staining of mouse nuclei) indicated that cells derived from both types of culture were not contaminating murine F4 cells.

The preferential generation of selection-resistant mesenchymal cells from organoid explant cultures was observed with two separate sets of experiments. Examination of the plated organoids used here indicated a contaminating mesenchymal component, perhaps representing between 1-5% of the total cells in the culture. Nevertheless, the only selection resistant cell type obtained was fibroblastic, despite the presence of hormones and growth factors favouring parenchymal cell proliferation. It was concluded that either infection, integration or both processes were unsuccessful in parenchymal cells growing in explant cultures.

Three G418-resistant stromal clones were chosen at random and isolated by ring cloning. After expanding them further, each original clone (one arising from explant culture, and two from enriched stromal cul-

tures), was single cell cloned twice and three single cell clones derived from each parent clone expanded in selection medium. All three of the original ring-clones readily generated cell lines without obvious crisis after infection with tsA58-U19.

5.1.5 tsA58-U19 infections of sorted luminal or myoepithelial cells.

Since successful integration of retrovirus-derived proviral DNA generally requires infected cells to be actively proliferating, the failure to generate selection resistant parenchymal cells from explant cultures could have been due to differences in the rates of stromal versus parenchymal proliferation in the explants. In an attempt to successfully infect parenchymal cells, clones were grown under conditions optimising proliferation and were then exposed to virions by co-culture with F4 cells.

Clonal growth cultures were initiated from flow sorted luminal and myoepithelial populations propagated separately with irradiated 3T3L1 cells. After 5 days growth in either HC:I, HC:I:CT or HC:I:CT:EGF containing medium when actively proliferating clones were composed of between 30-100 cells, the cultures were seeded with 10^5 irradiated virion-producing F4 cells.

Using this protocol selection-resistant cells were generated from both luminal and myoepithelial arms of the sort with varying degrees of efficiency. Taking into account the plating efficiencies of sorted cells under normal conditions, approximately 5% of sorted luminal clones grown in HC:I:CT containing medium became selection resistant colonies. Thus passaged un-cloned cultures were typically the product of between 20-50 individual resistant luminal colonies obtained at the outset. By contrast, the efficiency of infection was at least 10-fold higher for myoepithelial derived clones. Because of this and the higher cloning efficiencies of sorted myoepithelial cells, un-cloned myoepithelial tsA58-U19 cultures were probably derived from several hundred individual resistant colonies.
Once selection-resistant cells had been identified cultures were propagated at 34^{o}C.

In contrast to the observations made with stromal cells, successful generation of established parenchymal cells was critically dependent on the concentration of G418 in the selection medium. A concentration of $0.2mgml^{-1}$ was optimal for the selection of infected cells, enabling the continued growth of certain clones, whilst being sufficiently toxic to kill presumptively uninfected cells. However, although G418 at 0.5 or $1mgml^{-1}$ was too high a concentration initially,

after several passages cells selected with $0.2mgml^{-1}$ G418 became resistant to the antibiotic at these higher concentrations.

G418-resistant cells arising from luminal or myoepithelial populations behaved in contrasting ways during selection. Luminally-derived cells underwent a 'pseudo-crisis' stage, in which G418-resistant colonies in $0.2mgml^{-1}$ displayed a long latent period before the onset of proliferation, and the subsequent ability to be serially passaged. This phenomenon was at least partly due to the influence of media additives, since CT had a detrimental influence on the viability of luminally derived cells during establishment. The luminally-derived cells were also still dependent on the growth factors elaborated by 3T3 L1 feeder cells. Even in the absence of CT , epithelial clones became quiescent and the cells tended to become dispersed with transition of some epithelial cells into elongated cell types. Cultures which had been in the crisis state for two weeks could be rescued by replenishing with fresh irradiated 3T3 L1 feeders or with conditioned medium produced by myoepithelially derived cultures. Both caused the reorganisation of epithelial colonies within 48 hours into a more typical luminal phenotype, and mitosis was again observed, although overall growth rate remained slow. Nevertheless the cultures could be

propagated by serial passage of all the resistant cells at high plating densities. After 5 passages the growth of these same cultures became independent of feeder cells, but retained a requirement for HC and I in combination. By contrast, growth of G418-resistant myoepithelially derived cultures was rapid and feeder cell-independent from the outset, and no overt crisis was observed at any stage.

5.2 General characteristics of tsA58-U19 infected mammary cells.

5.2.1 Introduction.

The initial questions to be addressed with the retrovirally infected cultures were whether cells expressed SV40 large T antigen, and whether the T protein was temperature-sensitive. To this end, retrovirally infected, selection resistant stromal, luminal or myoepithelial cells were ring cloned, single cell cloned three times or passaged as un-cloned 'bulk' cultures at 34^{o}C. Cell lines and un-cloned cultures were examined by indirect immunofluorescence for the expression of SV40 large T at different temperatures. Alterations in growth rates as a result of temperature shifting were determined by cell counts and by cell cycle analysis using the flow cytometer.

5.2.2 Immunofluorescence detection of SV40 large T protein at 34°C and 39°C.

Since it was possible that re-cloned cell lines may not have been representative of the total range of infected phenotypes originally present in the selected cultures, both clonal cell lines and bulk un-cloned cultures from which they were derived were examined. At 34°C, large T protein was detected in the nuclei of 100% of cells using antibodies Pab412 and 419. Un-cloned cultures displayed heterogeneous levels of large T expression. Different clonal lines varied in overall intensity of T fluorescence. There were also differences in the magnitude of the response to elevated temperature displayed by individual cell lines, although in all instances there was a perceptible reduction of staining intensity. When the heterogeneously staining bulk cultures were temperature shifted, their overall intensity was reduced but they remained heterogeneous for nuclear T expression. Differences between high and low temperature cultures could be detected within 48 hours, although some weak staining remained visible even after 5 days at 39°C (see Figure 49).

Additionally, differences in the intensity and pattern of nuclear staining between each cell type were apparent, with luminal cells showing a weaker more diffuse pattern of nuclear staining, while myoepithelial cells

showed prominent unstained nucleolar regions. Stromal cells stained more weakly than either luminal or myoepithelially-derived cells. Membrane-bound large T was not detected on live or methanol-fixed cells using antibody Pab412 at either the permissive or non-permissive temperatures.

5.2.3 Growth curves of large T expressing cells grown at either 34^{o} or 39^{o}C.

To check that the integrated tsA58-U19 construct was acting on the cells in a temperature-dependent manner, growth curves were determined for two tsA58-U19 stromal cell lines and WI38-VA13 cells. This cell line, which was transformed with wild-type SV40, was used as a control. Cells were grown at 34^{o} and parallel cultures were temperature shifted to 39^{o}C. The results are shown in Figure 46. In the case of the control wild-type line, cells grew at a greater rate at 39^{o}C, whereas both of the tsA58 infected cells showed markedly reduced growth at 39^{o}C. In one case slow growth recommenced after 5-6 days at the restrictive temperature. At 34^{o} the growth rates of both tsA58-U19 lines were greater than that of cells transformed by wild-type SV40. Thus, the effect of elevated temperature on the growth of tsA58-U19 expressing cells was due to the thermolability of large T protein and not the effects of high temperature *per se*.

Detailed growth curves were not obtained for parenchymal derived cells, but direct observation showed that, like the stromal lines, they were growth restricted at non-permissive temperatures. Of 4 separate un-cloned cultures (two luminally- and 2 myoepithelially-derived), 4 multiply-cloned luminal lines and 14 multiply-cloned myoepithelial lines, all were growth restricted at the non-permissive temperature.

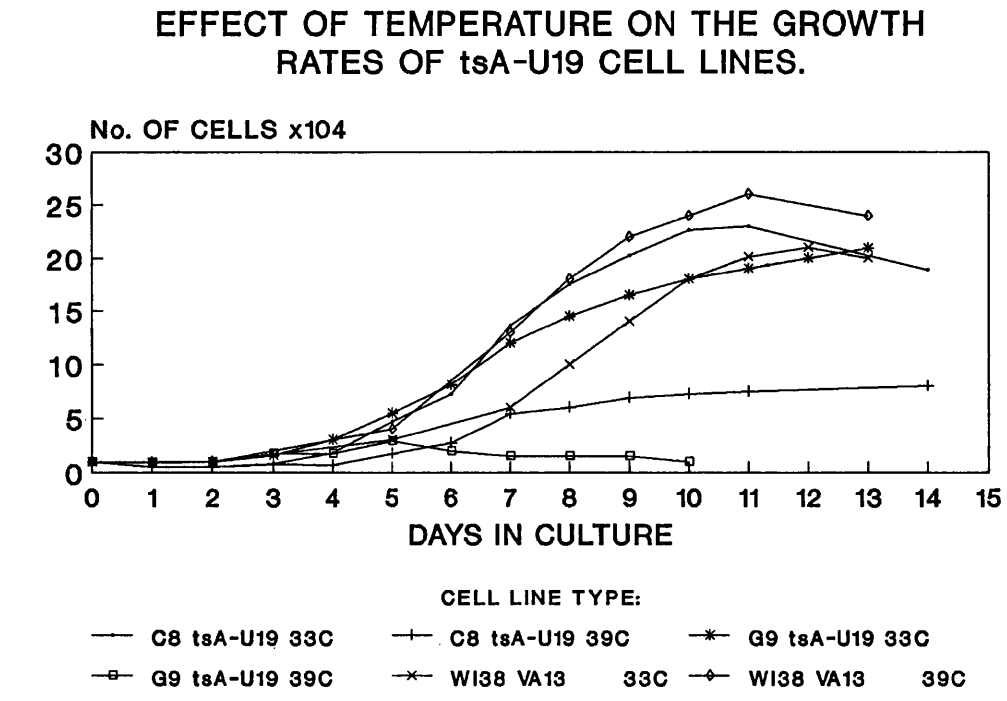


Figure 46.

5.2.4 Cytometric analysis of DNA content of tsA58-U19 cells.

The cell cycle kinetics of the temperature-dependent lines were examined by flow cytometry in order to - 1: confirm that the reduced cell numbers at restrictive temperature were caused by inhibition of DNA synthesis (and not by some secondary phenomenon such as reduced adherence), and 2: To determine whether growth inhibited cells accumulated in G1, G2 or both. Luminal and myoepithelial lines were grown at 34^{o} or temperature shifted to 39^{o}C for five days and then analysed by cytometric analysis of DNA content using propidium iodide staining of fixed cells. The percentages of cells in the G1:S:G2 cycle phases at the two temperatures were approximately: at 34^{o}C - 60%:30%:10%; at 39^{o}C - 75%:5%:20%. The results therefore showed a clear reduction in S-phase fraction at restrictive temperature and accumulation of cells in both G1 and G2 for both types of lines.

5.2.5 General growth characteristics of parenchymal lines.

As mentioned in section 5.1.5, SV40 T-expressing cells assumed feeder cell-independent growth, progressively in case of the luminal cells, but rapidly in the case of myoepithelially-derived cell lines. The results of clonal growth experiments also indicated that cloning

efficiencies increased progressively with serial passage, and that the magnitude of increase was greatest in luminally-derived lines. Thus, the cloning efficiency of luminally-derived lines in HC:I:CT:EGF containing medium increased from 7 to 40% between passages 8 and 34, whilst myoepithelially-derived cells increased from 16 to 24% over this same time period. Interestingly, unlike primary cells, myoepithelially-derived and luminally-derived lines grew well on reconstituted type I collagen gels, (see Figure 42). Myoepithelially-derived lines did not undergo obvious squamous metaplasia when cultured at 34°C, but did display some squamous behaviour after culture at 39°C.

Both luminal and myoepithelially-derived clonal lines displayed epithelial-mesenchymal transitions similar to those seen in some long-term passaged or re-cloned normal cells (Figure 44). This phenotypic instability also showed clonal variation, in that clonal lines generated from the same original parent clone underwent transitions at different rates. The behaviour of unstable lines plated at clonal densities indicated that the generation of fibroblast-like morphological variants was a stochastic process, in that well-organised parenchymal colonies were observed directly adjacent to clones of 'dedifferentiating' cells in an apparently random distribution. In addition to spontaneous switch-

ing, the mesenchymal transition could be rapidly induced in certain myoepithelially-derived lines which had been grown for many passages with hydrocortisone and insulin by removing the hormones (Figure 44). When such cultures were then passaged continuously in basal medium, cells assumed frankly fibroblastic appearance.

5.3 Characterising the differentiated status of SV-40 large T expressing luminal and myoepithelial cells.

5.3.1 Introduction.

Cells established from flow sorted luminal myoepithelial cells were characterised morphologically, and the expression of cell-type specific markers was probed with appropriate antibodies. The influences of temperature and continued passage on these parameters were also examined. These analyses were performed with a view to determining whether the effects of the temperature-sensitive T gene and the differentiated status of established cells were dissociated when cells were maintained at the non-permissive temperature.

5.3.2 Morphology of established cells at permissive and non-permissive temperatures.

Culture at 34^{o}C: During establishment cultures displayed pronounced progressive morphological changes (

Figure 47). This was particularly rapid with respect to myoepithelially derived cultures, such that between passages 4 and 7, cultures initially composed of cells displaying recognisable Type 6 primary morphology adopted morphologies not resembling any primary culture type (Figure 47a and c). This may have been due to progressive overgrowth by morphologically variant large T expressing cells with some residual uninfected cells remaining viable for a period of time as relatively low G418 concentrations were used for selection. Alternatively, fast growing morphological variants could have emerged from an initially heterogeneous infected pool of cells, or tsA-U19 cells could progressively assume an altered morphology *en masse*.

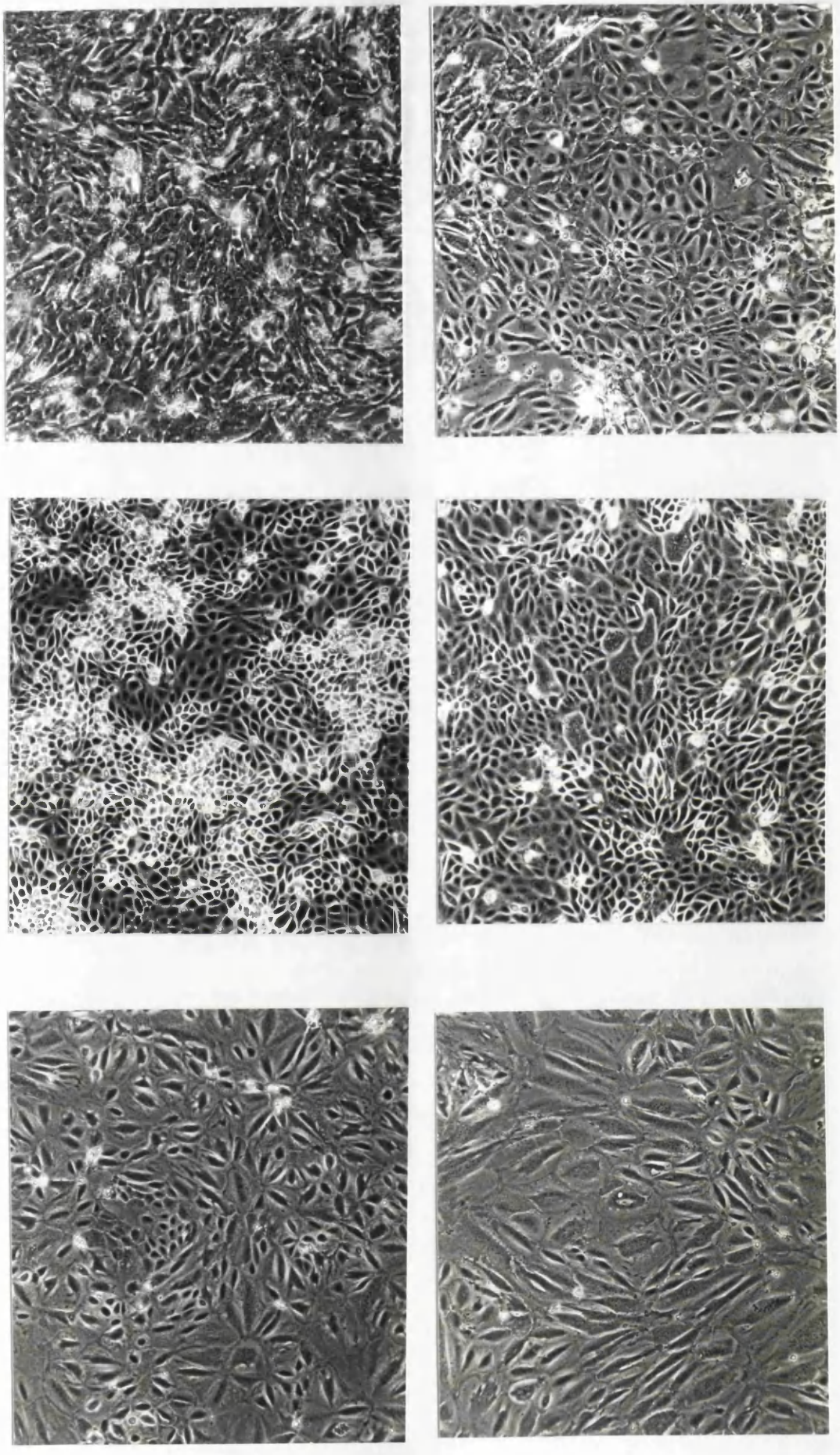
Luminally-derived cells also showed altered morphology on continued passage, and ultimately came to resemble the myoepithelial lines (Figures 47d). In addition, the cellular morphologies of both luminal and myoepithelial cloned lines were not uniform but were significantly altered by cell density between the two extremes of clonal growth and saturation density (Figure 48). Certain early passage (p13) luminally derived clonal cells lines plated at clonal densities formed a range of clonal morphotypes resembling those arising in primary platings (Figure 49). The colonies in such cultures also differed in size, indicating that these clonal lines generated cells of significantly

different intrinsic mitotic rates as well as different morphological phenotypes.

Culture at 39°C: Cell lines of both luminal and myoepithelial derivation showed an altered morphology after 3 days culture at the elevated temperature. With time, cells spread and flattened until after 7 days the cultures were composed of quiescent but viable cells (see Figure 47). When parenchymal cultures grown at 39°C were double stained for both large T expression and total F-actin, the results demonstrated that spread morphology directly correlated to decreased levels of large T protein, and was linked to the organisation of the actin filament system (see Figure 50).

Figure 47 (Opposite). Phase-contrast micrographs illustrating progressive and temperature induced alterations of tsA58-U19 cell line morphology.

(a) Myoepithelial cell line at p3; (b) luminal cell line at p2; (c) myoepithelial cell line shown in (a) at p27; (d) luminal cell line shown in (b) at p22; (e) myoepithelial cell line shown in (c) after 5 days culture at 39°C; (f) luminal cell line shown in (d) after 5 days culture at 39°C. x150



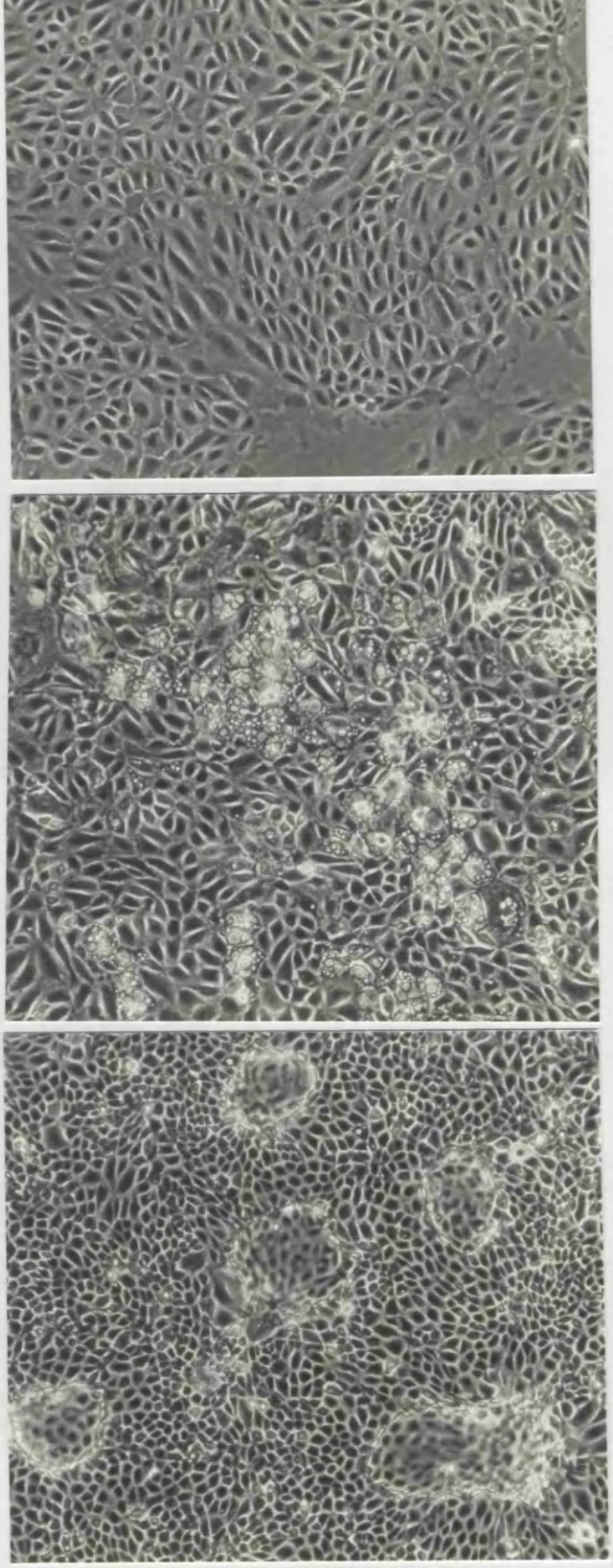
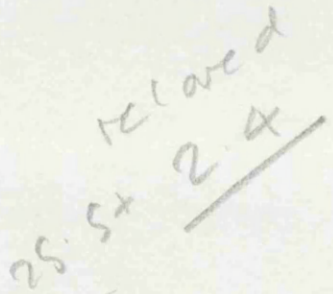


Figure 48. Phase-contrast micrographs illustrating density-dependent morphological alterations of a tsA58-U19 myoepithelially-derived cell line.

(a) 48 hours after plating; (b) confluence; (c) saturation density. x180


Relaxed
25.5+ 2.4

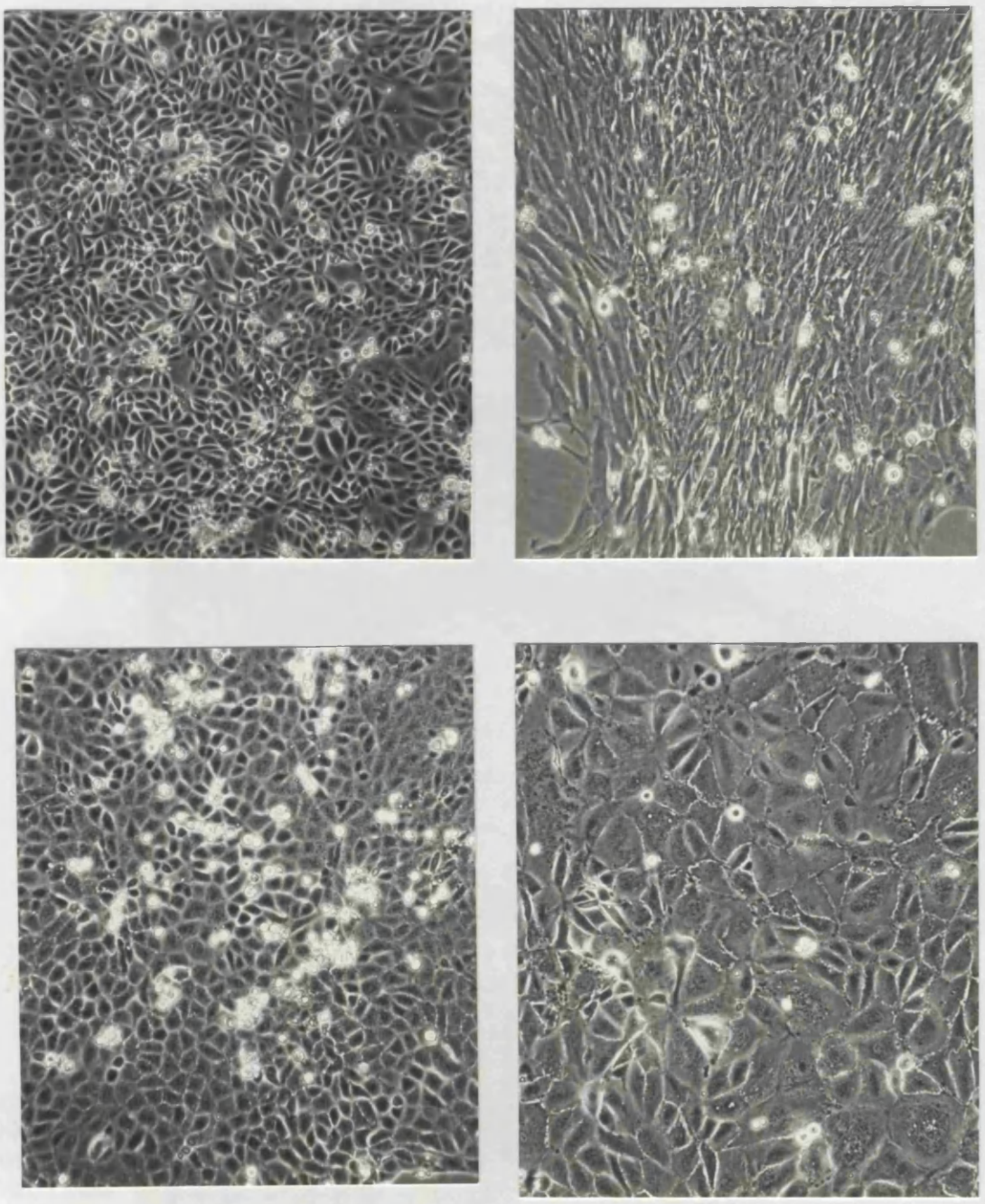


Figure 49. Phase-contrast micrographs illustrating colonies generated by a luminally-derived clonal cell line plated at clonal densities.

(a) Clone representing morphology of parent cell line. x158

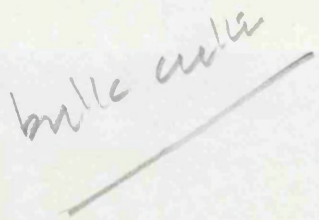
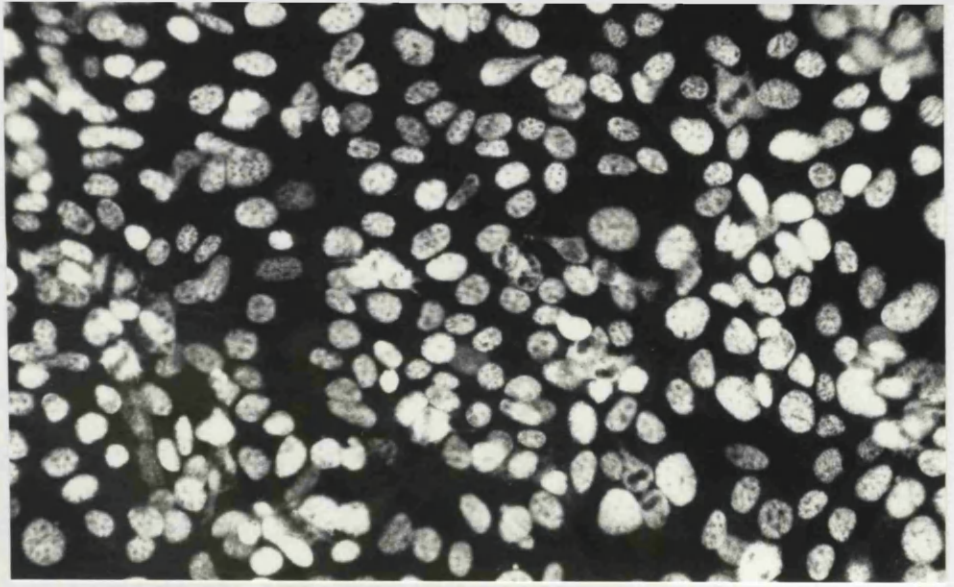
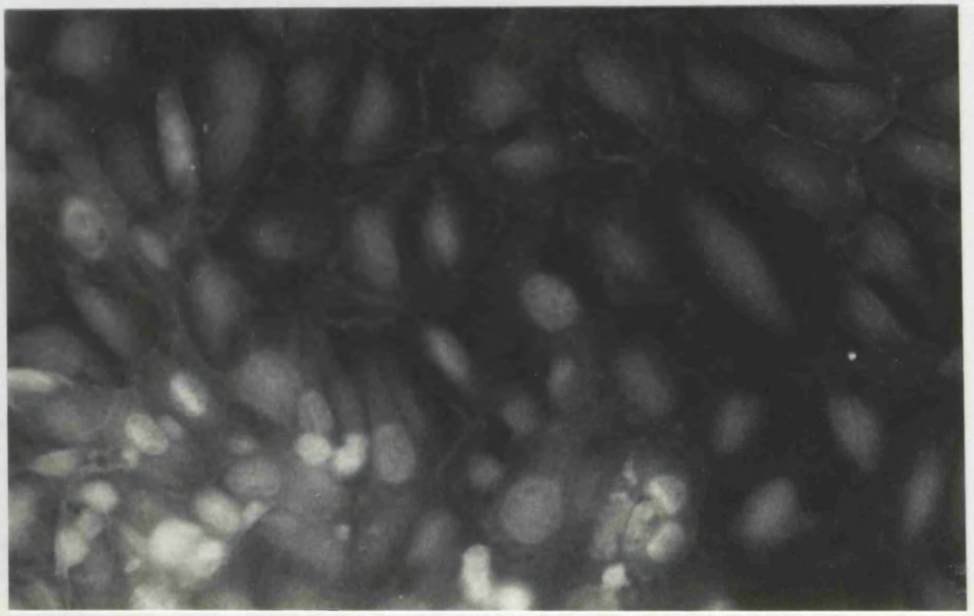
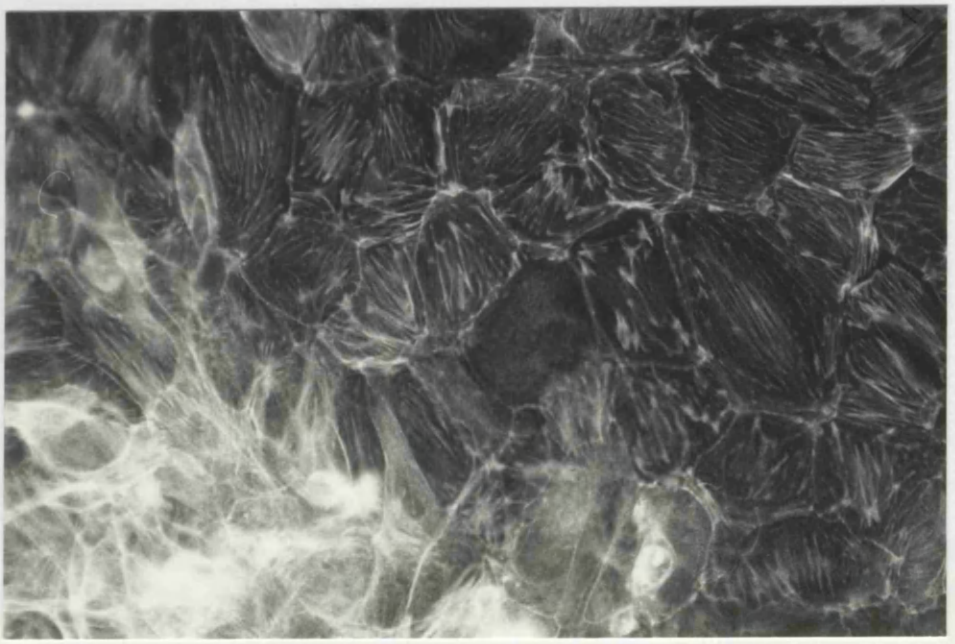


Figure 50 (Opposite). Indirect immunofluorescence micrographs illustrating the relationship between SV40 large T protein expression and actin filament distribution in an un-cloned tsA58-U19 myoepithelially derived culture.

(a) Large T expression at 34°C; (b) large T expression after 5 days culture at 39°C. Note the heterogeneous responses to elevated temperature; (c) double-label exposure of (b) showing actin detected by NBD-phalloidin. Note the different distribution of filaments by the cell expressing detectable levels of large T (arrow) and the formation of adhesion structures by surrounding cells. x400







5.3.3 Expression of cell type-specific markers at both permissive and non-permissive temperatures by established cells and clonal cell lines.

Membrane markers: Expression of the antigens recognised by luminal-specific antibody 25.5 and myoepithelial-specific anti-endopeptidase-24.11 antibodies were assessed by indirect immunofluorescence and analysed using the flow cytometer. Broadly speaking, at 34^{o}C both luminal and myoepithelially-derived bulk cultures contained cells which stained with antibodies 25.5 and PHM6. In both cases single and double-labelled cells were seen in the cultures. At the earliest stages, passages 1-5, myoepithelial lines stained strongly with PHM6, and the luminal lines strongly with 25.5, but this differential staining pattern altered with continued culture; the loss of 25.5-positivity of luminally-derived cell lines being particularly noticeable. A similar lack of specificity was noted in the re-cloned lines, which additionally exhibited significant differences in membrane antigen expression one from the other, even when derived from the same parental line. Growth at the non-permissive temperature resulted in quantitative but not qualitative differences in membrane antigen expression with respect to the population as a whole. Thus, the range of fluorescent intensities for both 25.5 and endopeptidase-24.11 increased at

39°C, but neither luminally or myoepithelially-derived cells reverted to the exclusive expression of cell-type specific membrane markers.

Cytokeratins: In contrast to the membrane markers, the expression of cytokeratins remained cell-type specific at both low and high passage numbers, although notable differences in the intensities of staining were apparent between different lines derived from the same parental stock. Interestingly, both luminal and myoepithelial cell lines expressed cytokeratin 8 at the permissive temperature (Figures 51a and b; 52a and b, respectively). The expression of differentiation-specific cytokeratins by luminal lines was critically temperature-dependent. Thus, both cytokeratins 18 and 19 were detected only at very low levels in a few cells in cultures grown at 34°C (Figure 51c and e), but high levels of both cytokeratin types were expressed by cells cultured at 39°C (Figure 51d and f). Some cells in luminally-derived lines also expressed cytokeratin 14, which could be detected at both permissive and non-permissive temperatures. The existence of such cells is consistent with the behaviour of certain primary clone types which express both luminal and myoepithelial associated cytokeratins (see Section 4.2.5).

Myoepithelially-derived lines also expressed high levels of cytokeratin 14 at the permissive temperature (Figure 52c), although more cells expressed this differentiation-specific cytokeratin after being maintained at 39^{o}C (Figure 52d). Clonal lines derived from myoepithelial cells consistently maintained the expression of cytokeratin 14 when probed at various stages throughout the period of study, and did not express cytokeratin 18 at either temperature at any passage number. By contrast, certain of the luminally-derived tsA58-U19 clonal lines tended to lose the expression of the cytokeratins 18 and 19 around passages 16-20, although this was not a general observation. However, even when these cytokeratin types could not be detected at all, the same cells still expressed cytokeratin 8.

Apart from temperature, the ability of luminally-derived cells to form intact filament networks was influenced by the two factors - the 'culture age' of cells, and cell density. Thus, although un-cloned cultures expressed appropriate cytokeratin types, multiply-cloned cultures of the same passage number grown under identical conditions did not always do so. The only variable accounting for this discrepancy is the fact that dilution-cloned cultures would necessarily have undergone a greater number of population doublings than un-cloned equivalents. In addition, unlike primary

cells, the elaboration of filaments depended on the density of cells such that dense cultures of cells which expressed filaments would not necessarily do so if plated at clonal densities.

In contrast to the behaviour of membrane antigens, temperature shifting resulted in individual cells expressing higher levels of cytokeratin filaments (as determined by indirect immunofluorescence). This effect was particularly pronounced when cultures with relatively low levels of expression at 34^{o}C were shifted to 39^{o}C. It would of interest to double-label cells for cytokeratins and large T protein in order to establish whether a direct reciprocal relationship existed between levels of these differentiation-associated markers and levels of the nuclear viral antigen.

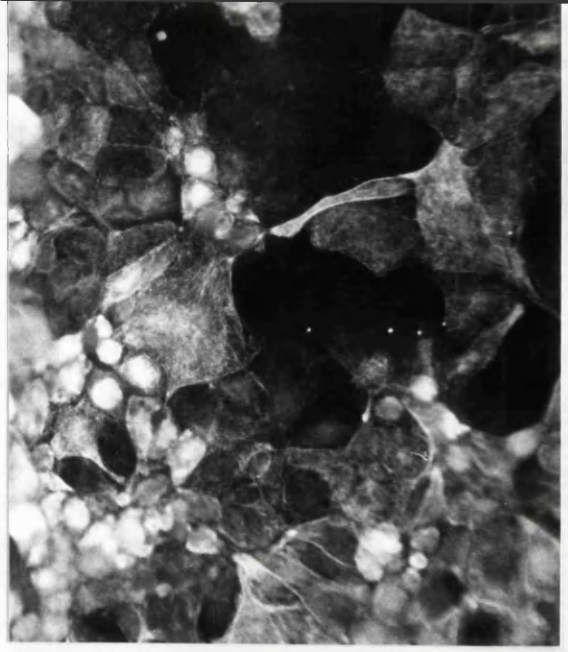
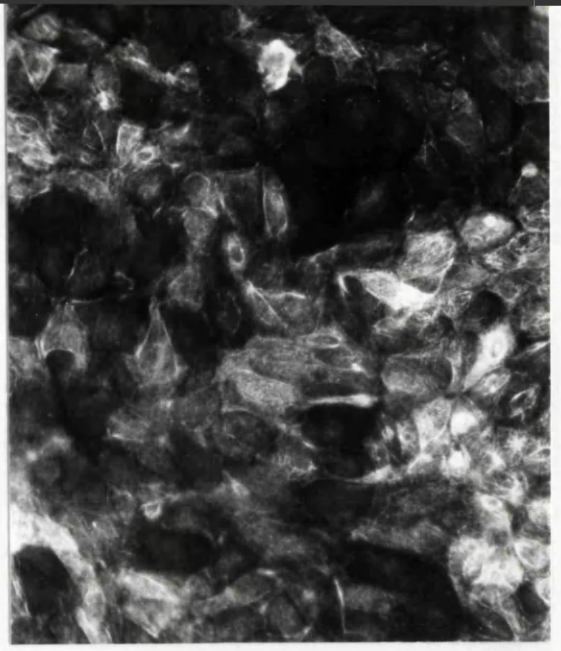
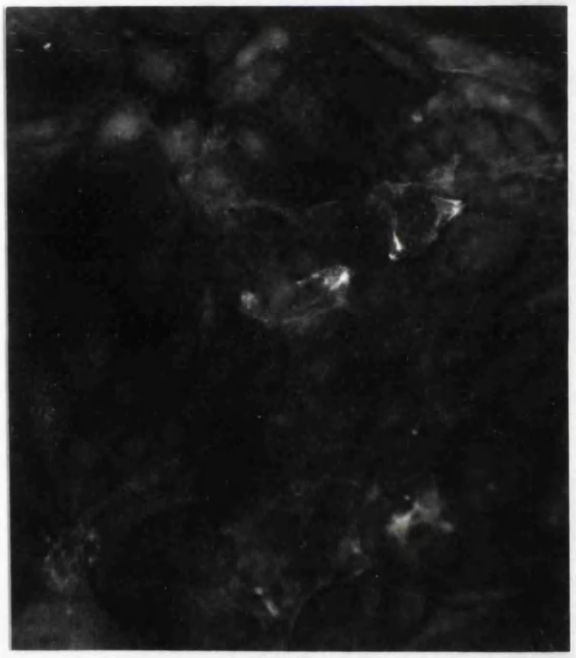
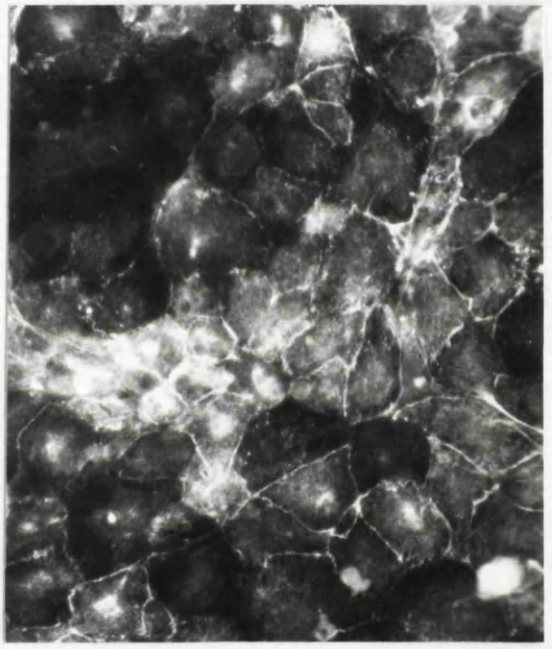
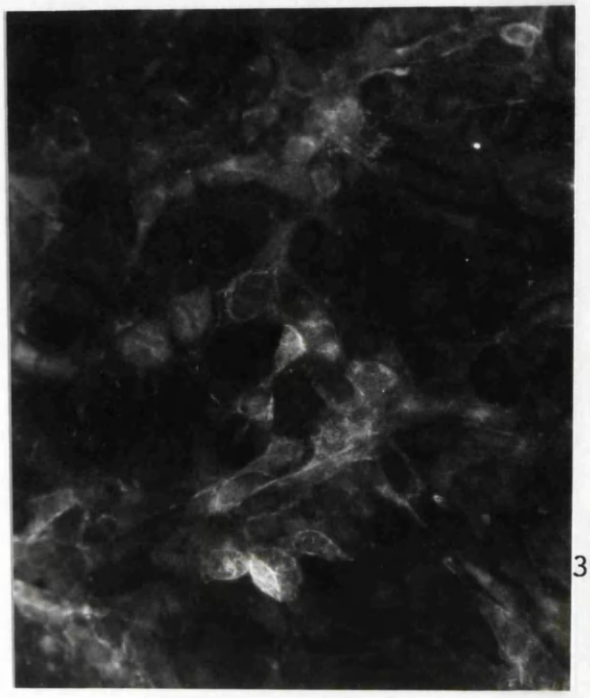
Three distinctive cytokeratin staining patterns were consistently observed. Immunolocalised cytokeratins were detected as perinuclear 'caps' and/ or as lines of positive staining detected at peripheral margins of cell-cell contacts (presumably indicating filaments associated with desmosomal junctions), or more typical diffuse filament networks (see Figures 51 and 52).

Basement membrane proteins: Laminin or collagen type IV were never detected in cultures of any type grown at 34°C. However, high levels of both these basement membrane components were strongly expressed by myoepithelially-derived lines after they were maintained for 5 days at 39°C (Figure 53).

? Buds cells ?

Figure 51 (Opposite). Indirect immunofluorescence micrographs illustrating the temperature-dependent expression of cytokeratins by a tsA-U19 luminally-derived cell line.

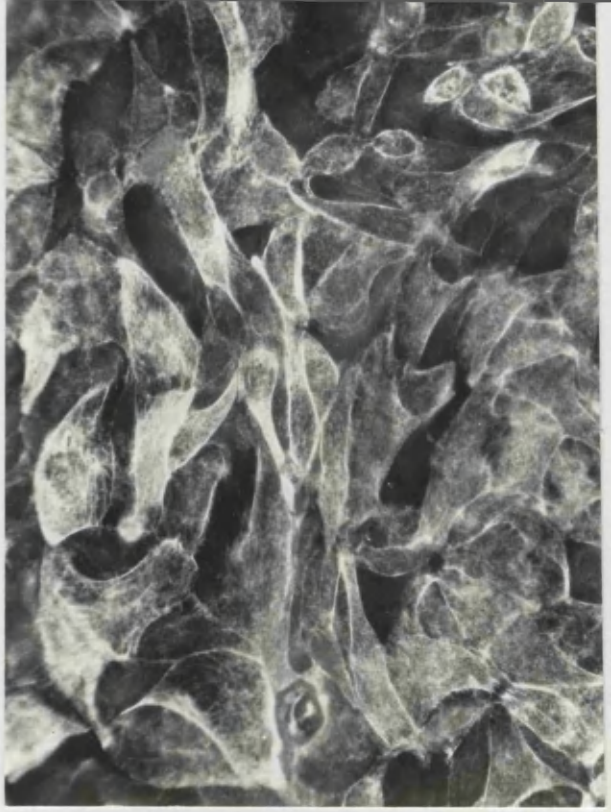
(a) Cytokeratin 8 expression at 34^{o}C; (b) cytokeratin 8 expression at 39^{o}C; (c) Cytokeratin 18 expression at 34^{o}C; (d) Cytokeratin 18 expression at 39^{o}C; (d) Cytokeratin 19 expression at 34^{o}C; (f) Cytokeratin 19 expression at 39^{o}C. x600

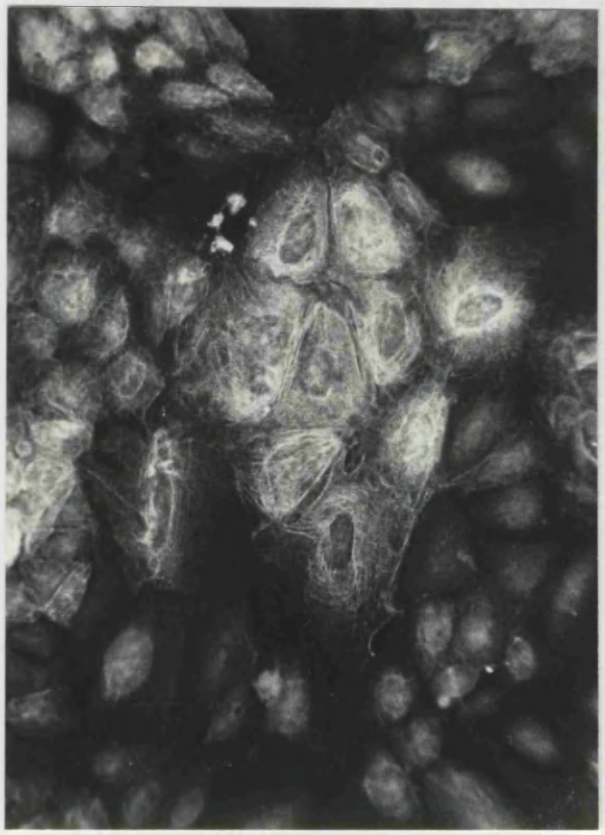







Basic actin ✓

Figure 52 (Opposite). Indirect immunofluorescence micrographs illustrating the temperature-dependent expression of cytokeratins by a tsA-U19 myoepithelially-derived cell line.

(a) Cytokeratin 8 expression at 34°C; (b) cytokeratin 8 expression at 39°C; (c) cytokeratin 14 expression at 34°C; (d) cytokeratin 14 expression at 39°C. x600





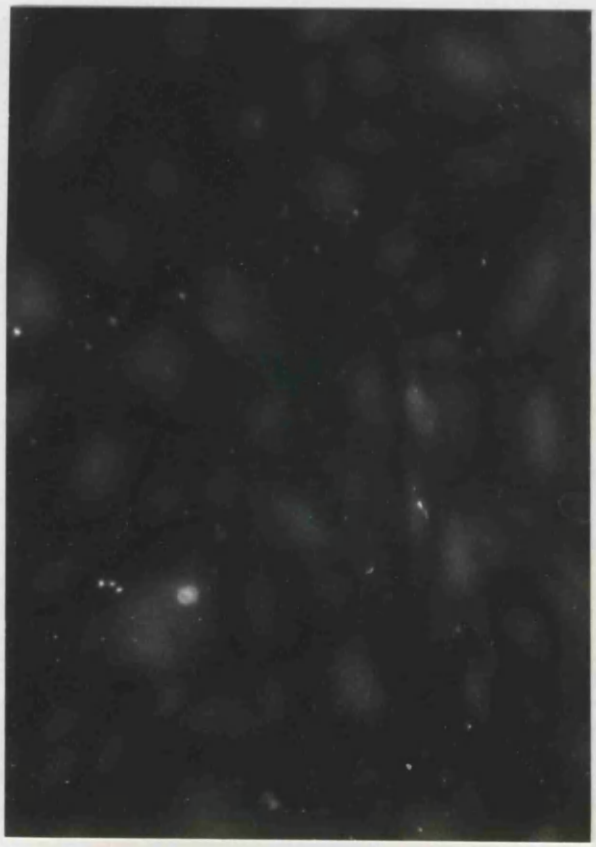
C

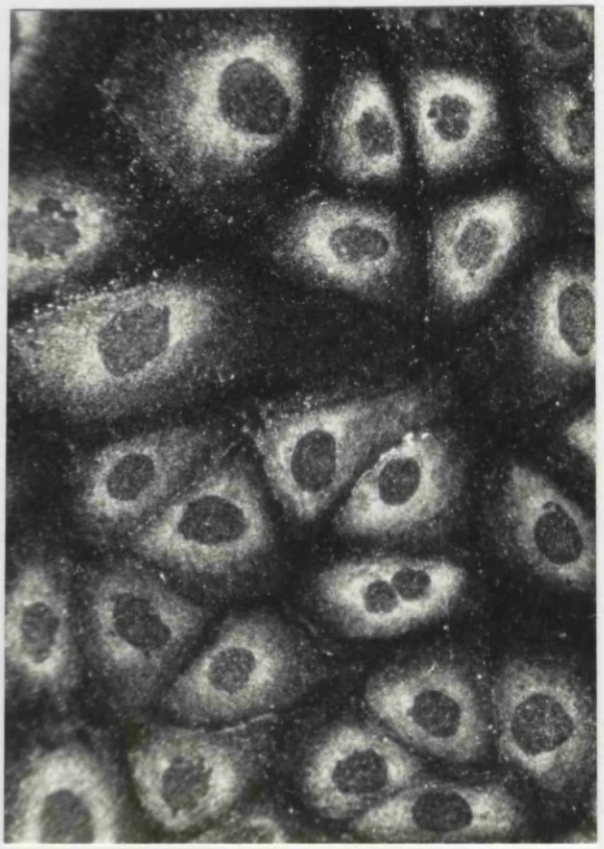
D

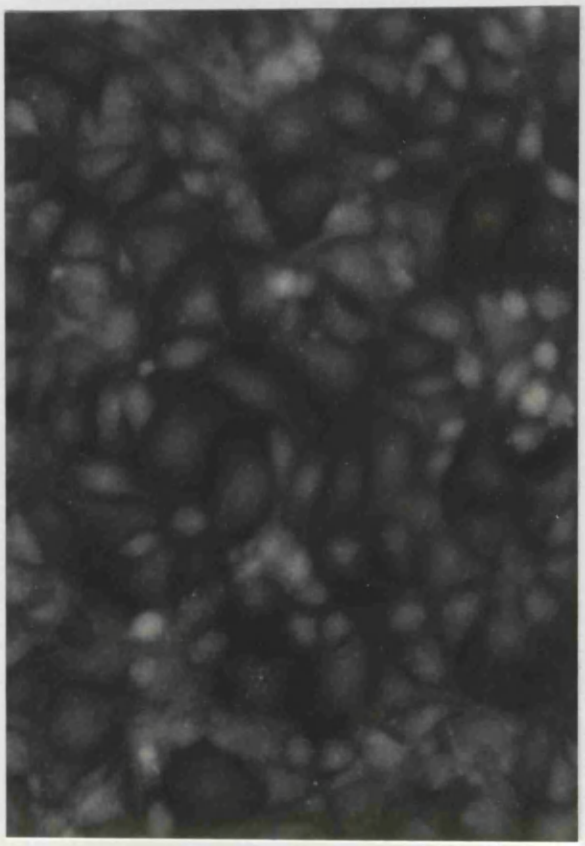
25.5- 7F 2 →

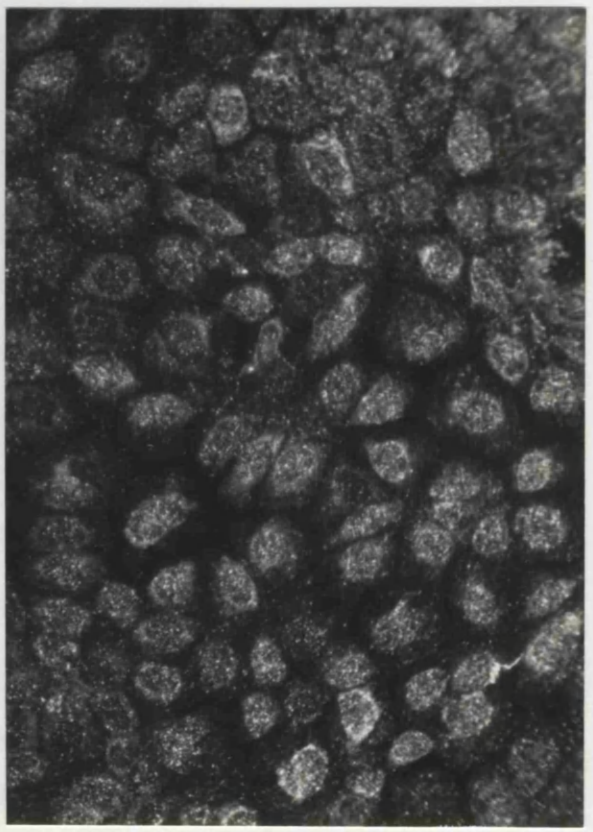
Figure 53 (Opposite). Indirect immunofluorescence micrographs illustrating the temperature-dependent expression of basement membrane components by a tsA-U19 myoepithelially-derived cell line.

(a) Typical staining pattern for laminin at 34°C; (b) laminin expression at 39°C; (c) typical staining pattern for Type IV collagen at 34°C; (d) Type IV collagen expression at 39 °C. x600









5.4 DISCUSSION.

Published reports of SV40 resulting in the 'transformation' of mammary parenchyma differ according to whether primary cells or established lines were used as target cells, and by the methodology used to transduce wild-type large T protein. Studies have employed wild-type virus infections of spontaneously established mouse mammary cell lines, Butel et al., 1984; human mammary milk cultures, Chang et al., 1982; primary cultures of human organoids, Rudland et al., 1989b; carcinogen treated human mammary cells infected with recombinant retroviral constructs, Clark et al., 1988; and microinjection of SV40 DNA plasmids into primary cultures of rabbit mammary cells, Garcia et al., 1986. The work presented in this chapter presents significant advantages over other systems for four main reasons.

1. Starting populations were pre-identified and phenotypically defined. Uncultured luminal or myoepithelial cells, separated by flow sorting on the basis of reliable criteria, were separately manipulated. This meant that probing the profiles of differentiation markers expressed by these cells was not required as a retrospective attempt at phenotypic identification, but could be used to ascertain the influence of establishment and growth on subsequent differentiated status.

2. The candidate gene was introduced into recipient cells via retroviral infection. Due to the comparative-

ly high efficiencies of infection and integration mediated by retroviruses, Hwang and Gilboa, 1984, the construct was introduced into a large proportion of cells. In addition, the cellular genome of infected cells should have contained only one whole integrated provirus insert, Hwang and Gilboa, 1984, thus precluding unwanted gene dosage effects.

3. A selectable marker was utilised. This allowed the unequivocal identification of infected cells, and their isolation without biased pre-selection. This contrasts favourably with selection based on specific transformation properties, for example morphological alterations, Fusco et al., 1982; Reddel et al., 1988, or abrogation of growth factor requirements, Weissman and Aaronson, 1983; Clark et al., 1988, used in other epithelial systems, which could potentially isolate atypical cell types.

4. The influence of the large T protein was temperature sensitive. This provided the opportunity to reciprocally dissociate growth and differentiation by temperature shifting cultures. It was hoped that this would enable propagation and cloning of cells, particularly luminally-derived populations, and then after culture for an appropriate time at the non-permissive temperature, cells could be examined in a state more typical of the parenchyma *in situ* as a result of quiescence and re-expression of normal phenotypes.

In the first instance, the results presented demonstrate that both luminal and myoepithelial lineages are susceptible to retroviral infection (and therefore by corollary both express the appropriate receptor molecule), and that functional large T protein alone is capable of extending the *in vitro* life span of both luminally and myoepithelially-derived cells.

Increased longevity of primary mammary cultures influenced by wild-type SV40 virus has been observed with human, Chang et al., 1982; murine, Butel et al., 1984; and rabbit, Garcia et al., 1986, cells. Direct transformation of primary mammary epithelial cells mediated by large T, if it occurs at all, is an infrequent event, Clark et al., 1988; Garcia et al., 1986, and this reflects the lack of transformation of primary fibroblastic cells by SV40 large T, Jat and Sharp, 1986; Jat et al., 1986. It is generally accepted that large T protein is best considered as an establishment factor, Jat and Sharp, 1986; Garcia et al., 1986; Jat and Sharp, 1989; Jat et al., 1986, rather than a transforming agent *per se*. The behaviour of sorted rat mammary luminal and myoepithelial cells expressing large T protein is in good agreement with this hypothesis, and the finding that functional large T protein was required for the continued proliferation of murine, Tegtmeyer, 1975; Brockman, 1978; rat, Jat and

Sharp, 1989; Petit et al., 1983; and human, Radna et al., 1989; Wright et al., 1989, fibroblast systems, as well as human placental, Chou, 1978, and keratinocyte cells, Banks-Schlegel and Howley, 1983. Although wild-type SV40 infections and transfections of wild-type DNA induce chromosomal aberrations, Sack, 1981; Nichols et al., 1985; Reddel et al., 1988, such abnormalities tent not to be produced by transduction of sub-genomic DNA in the form of the large T gene using retroviral infection, Clark et al., 1988. It has been suggested that cell replication of construct carrying cells is influenced directly by active large T protein, and not by any secondary genomic alterations, Clark et al., 1988. While this is probably true in the earliest stages of culture, the DNA profiles of un-cloned cultures indicated that ploidy is disrupted with passage, and since temperature-shifting altered culture ploidy, this suggests that certain genomic alterations may provide selective growth advantage.

Epithelial cells proliferating under the influence of SV40 large T can maintain tissue-specific differentiated functions, at least during the earliest stages of their growth, Schlegel-Haueter et al., 1980; Scott et al., 1986; Woodworth et al., 1986; Woodworth and Isom, 1987; and the ability to undergo induced differentiation, Ke et al., 1988. In addition, the large T ex-

pressing cells can maintain the expression of specific markers, Clark et al., 1988; and cytokeratins, Chang et al., 1982; Banks-Schlegel and Howley, 1983%; Garcia et al., 1986; Reddel et al., 1988. Alternatively, specific cytokeratins can be lost, Hronis et al., 1984; Taylor-Papadimitriou et al., 1982, or aberrantly expressed Bernard et al., 1985, as tends to be the case with 'post-crisis' human cells. Reports describing effects of temperature-sensitive SV40 large T include the restoration of certain differentiated functions, including increased expression of specific cytokeratins by human epidermal cells, Banks-Schlegel and Howley, 1983; the re-expression of chorionic gonadotropin and increased alkaline phosphatase activity by human placental lines, Chou, 1978; increased levels of albumin, transferrin and glucagon receptors by adult rat liver cells, Chou, 1983; increased levels of albumin, transferrin and α-fetoprotein in fetal rat liver cells, Schlegel-Haueter et al., 1980; Chou and Schlegel-Haueter, 1981. These effects are generally concomitant with the loss of "transformed phenotypes" at non-permissive temperatures. At first sight these reported influences seem contradictory, however a close examination of the methodology used by individual laboratories provides an explanation. Broadly speaking, it appears as though cells subjected to wild-type infections are more prone to aberrations in marker expression, Steinberg and

Defendi, 1979; Hronis et al., 1984; Bernard et al., 1985; whilst the transfection of SV40 DNA apparently results in more stable phenotypes, Woodworth et al., 1986; Woodworth and Isom, 1987; Clark et al., 1988; Ke et al., 1988. By contrast, the effects of non-permissive temperature are independent of whether large T is introduced by wild-type infection, Chou, 1978, or transfection, Banks-Schlegel and Howley, 1983. The behaviour of tsA58-U19 lines encompasses both general trends, in that tsA58-U19 activity at the permissive temperature drives the continuous proliferation of cells and is concomitant with reduced marker expression, while differentiated status is partly restored on shift to the non-permissive temperature.

The following observations strongly suggest that a hierarchal control of differentiation-related marker expression existed in these cultures - 1. T function was not conducive to the synthesis of basement membrane proteins, although cells remained competent to do so at the non-permissive temperature; 2. The integrity of membrane markers was rapidly lost independently of T function and 3. Cytokeratin expression was maintained and remained cell-type specific. These points indicate that expression of different sets of genes are more rigorously regulated than others and that SV40 large T interferes in a selective manner. In addition, it can

be concluded that control of cytokeratin gene expression operates at different levels in this system, the restriction of which differs between cell types. This interpretation is based on the observations that, although some luminally-derived lines failed to express cytokeratins 18 and 19, they maintained expression of cytokeratin 8 , indicating that tissue-type (parenchymal versus mesenchymal) expression is maintained more rigorously that cell-type expression (luminal versus myoepithelial). The fact that myoepithelially-derived cell lines maintained expression of cytokeratin 14 as well as 8 indicates that different differentiation-related cytokeratin types were also differentially regulated. The fact that these patterns of expression also occur at the non-permissive temperature indicates that such controls probably operate under normal circumstances. These results are in good agreement with findings that the gene encoding cytokeratin 8 is differentially regulated at the transcriptional level, Knapp et al., 1989, and that differentiation-specific keratins are not necessarily expressed coordinately in differentiating cells, Roop et al., 1987.

Many established cell types differentiate in culture when exposed to appropriate stimuli, Marks et al., 1987, although as discussed above, the differentiation pathways operative in established cells may not neces-

sarily be typical of those mechanisms in normal cells. Another example of this is provided by the influence of large T in derived myoepithelial lines. Squamous metaplasia did not occur in these cells at the permissive temperature, despite the fact that long-term passaged myoepithelial phenotypes underwent this process spontaneously. In view of the fact that human bronchial epithelial cells transfected with SV40 can undergo serum-induced differentiation (although some sub-clones are resistant), it has been suggested that changes modulated by SV40 are not closely linked to pathways controlling squamous differentiation, Ke et al., 1988. Taken together, these results suggest that distinct pathways govern squamous metaplasia and differentiation *per se*., and in this regard it is interesting to note that two distinct pathways are associated with squamous differentiation, Ke et al., 1989.

Other alterations in cell behaviour peculiar to large T function included modulations in intermediate filament organisation. The association between functional large T and actin filament distribution has been documented before using immunofluorescence and electron microscopic examination of fibroblast cells, Pollack et al., 1975; Vollet et al., 1977. This work establishes that profound, T protein-modulated rearrangements also occur

in both epithelial and myoepithelially-derived cells. Whether the accompanying changes in morphology represent a cause-or-effect phenomenon is a matter for conjecture. The variable levels and types of individual cytokeratin filaments expressed indicate that these filaments are dynamically regulated, (Lane and Klymkowsky, 1982), by amongst other factors cell density, (this work, and Ben-ze'ev, 1984). From the observations of the topology of immunolocalised filaments in temperature shifted cultures, it appears that cytokeratins can be disrupted in more than one way. Thus, although patterns of filaments seen here resemble those described for human epithelial cells expressing SV40 large T, Chang et al., 1982, they are quite distinct from disrupted filament arrangements produced by microinjection of anti-keratin antibodies, Lane and Klymkowsky, 1982.

Gradual deterioration in the expression of differentiation markers with continued passage, exemplified by diminishing 25.5 staining and cytokeratin loss displayed by certain lines, is in keeping with variations in gene expression found in hepatocyte lines, Woodworth et al., 1988. Conceivably such changes could arise as a result of secondary genomic alterations produced by long-term culture. In any case, the fact that alterations occur at all proves that specific markers of

differentiated status may no longer provide reliable phenotypic identification of mammary derived cells after long-term passage or establishment in culture.

Genomic alterations may account for the generation of elongated cells by cell lines. As discussed in the previous chapter, this is probably a general parenchymal phenomenon. Since mesenchymal transitions were displayed both by luminal and myoepithelially-derived cell lines this behaviour is not consistent with the differentiation of myoepithelial cells from stem precursor. Although transitions could be rapidly induced by removing hormones from cultures, it was not convincingly demonstrated during these studies that the process could be reversed, as has been shown in other epithelial systems, Boyer et al., 1989. It was interesting to note that luminal epithelial cells 'crisised' during establishment. This perhaps suggests that crisis may be a phenomenon shared by all cells displaying extremely limited *in vitro* lifespan and not just by human cells, as is commonly believed. Similarly, epithelial-mesenchymal-transitions may not be generally displayed by luminal cells in primary culture simply because they do not proliferate enough to reach this critical point in differentiation. Interestingly, the activity of large T protein appeared to influence luminally-derived cells to a greater extent than it did

myoepithelially-derived cells. This was reflected in greater magnitudes of increased cloning efficiencies and loss of marker expression with passage.

In summary, established temperature-sensitive luminal and myoepithelial cells can be generated by tsA58-U19 expression. It is most likely that the established luminal and myoepithelial phenotypes described derive from Type 4 and Type 6 clones, respectively. Furthermore, established lines from the two sources remain as distinctive phenotypes during culture. Having generated such lines, the next step was to examine their organotypic differentiation and growth in syngeneic fat pads and nude mice, comparing it to normal cell behaviour in these situations. The results of these studies are presented in the following chapter.

CHAPTER 6.

TRANSPLANTATION AND XENOGRAFTING OF PARENCHYMAL POPULATIONS INTO SYNGENEIC FAT PADS AND NUDE MICE.

6.1 Transplantation of normal parenchymal cell populations.

6.1.1 Introduction.

In order to assess the morphogenetic capability of unsorted and sorted parenchymal populations, the behaviour of cells was examined *in vivo* by implantation of cells into two different sites - interscapular fat pads of syngeneic rats or mammary fat pads of nude mice. Preliminary experiments were made to assess the suitability of cleared fat pads in sygeneic rats as a transplant site, as distinct from the more commonly used cleared mouse fat pad. The growth of dissected terminal end buds, ductal and alveolar organoids, primary monolayer cultures and sorted cells was then examined at various stages after transplantation in whole-mount preparations and histological sections. Implants that were detected were then scored with respect to the extent to which they had grown and the normality or otherwise of the pattern of the outgrowth. The results indicate that specific populations of mammary parenchymal cells are required for an arborescent growth pattern to occur *in vivo*.

6.1.2 Cleared rat mammary fat pads.

The 5th inguinal fat pads of immediate post-weaning (21 day) neonatal rats were 'cleared' by the operating

procedure described in Section 2.8.5. Examination of wholemounted 'cleared' fat pads dissected from the animal immediately after the operation indicated that in general, not all endogenous parenchyma was removed and usually several terminal end buds were left behind after resection. At this stage of development, the parenchyma has already ramified distally into the fat pad to such an extent that its encroaches major blood vessels and lymph nodes. Wholemounted glands from control animals (cleared but no transplant) examined after varying periods of growth revealed that fat pads developed normally, but were substantially repopulated by growth of the residual parenchyma.

In addition to the difficulty of ensuring that all glandular tissue elements were accurately removed, inter-animal variation in the patterns of vasculature meant that it was difficult to cauterise all the vessels. Since the procedure was time-consuming and preliminary experiments were of limited success, this technique not used further.

6.1.3 Interscapular fat pads.

Although abdominal fat pads extend into dorsal areas in the rat, the extremities of the mammary fat pads and the boundaries of interscapular fat pads are clearly demarcated. Interscapular pads consist predominantly of

white fat composed of typical adipose cells, although brown adipose tissue masses were located in the most caudal regions. Vascular networks traversed throughout the pads. No endogenous mammary parenchyma was ever observed in interscapular fat pads.

6.1.4 Morphogenetic capability of transplanted terminal end buds, organoids and primary monolayer cultures *in vivo*.

Samples of both un-disaggregated organoids as well as monolayer cultures prepared from them were implanted into the white fat of interscapular fat pads of 70 day virgin females. Terminal end buds directly dissected from 21-day old rats were used as a control, and were implanted singly.

Terminal end buds: Terminal end buds successfully implanted into interscapular fat pads reproducibly regenerated mammary trees with typical structure and spacing. Primary, secondary and tertiary branching ducts extended radially into the fat pad from the implantation site (Figure 54a). Lateral buds, terminal ducts, alveolar buds and terminal end buds were all conspicuous.

Organoids: Isolated fragments of duct or alveolar organoids produced arborescent outgrowths, although branching was less extensive than that produced by terminal end buds. Nevertheless, primary, secondary and tertiary branches were observed as were lateral buds, terminal ducts, terminal and buds and alveolar buds Arborescent growths were produced by fragments, with ducts extending from the probable points of implant showing a structure typical of normal mammary trees in situ (Figure 54b).

Monolayer cultures: Single cell suspensions prepared from 7 day primary monolayer cultures could also generate arborised glandular configurations in fat pads. Foci of rudimentary primary and secondary ductal branching structures extended into the fat pad. Some growths existed as simple blind-ending structures, whilst within the same time period others grew more elaborately, displaying lateral and terminal end buds (Figure 55a). Thus, although the extent of morphogenesis was not as great as that seen with un-disaggregated tissues, elements of glandular organisation could be regenerated from cultured cells.

6.1.5 Morphogenetic capability of flow sorted cells *in vivo*.

Sorted myoepithelial cells were propagated in HC:I:CT:EGF containing medium for between 3 and 8 passages in order to generate approximately 10^7 cells. Such cultures, composed of Type 6 cells, were harvested by trypsinisation and injected as dense single cell suspensions into the interscapular region of syngeneic newborn pups and nude mice, and the interscapular fat pads of 70 day virgin syngeneic rats. The objectives were 1) to compare the morphogenetic capability of such purified cell population with that of cultured unsorted cells, 2) to determine whether there were any differences in the behaviour of these cells transplanted into animals of different developmental stages, and 3) to determine whether such rapidly proliferating cultured cells were tumourigenic.

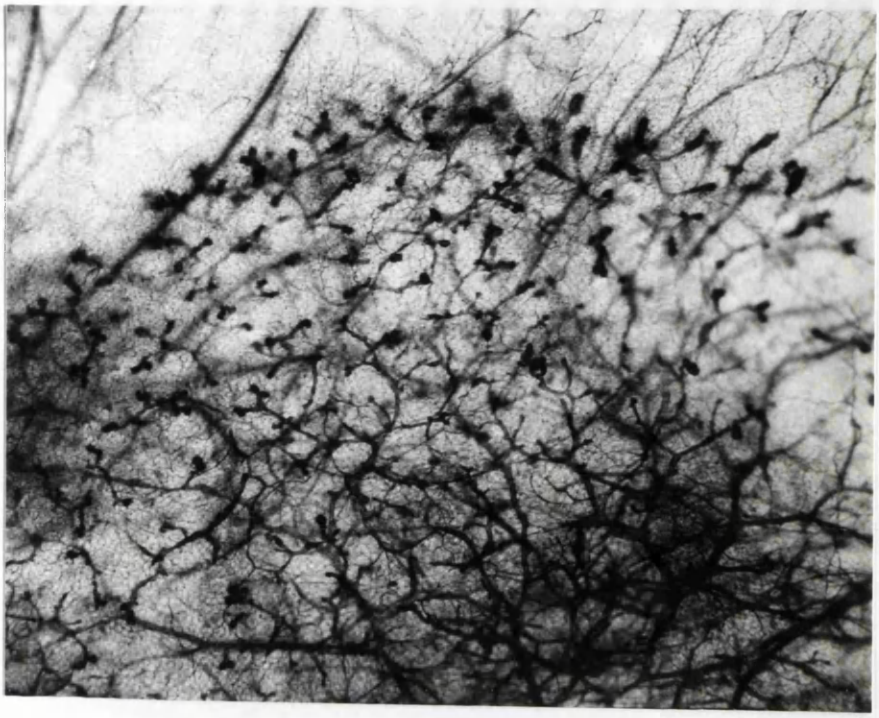
No organised mammary structures were observed when myoepithelial cells were transplanted into any of the sites described above, when animals were killed between 2 and 8 weeks after inoculation. The wholemount illustrated in Figure 55b shows a 23-day myoepithelial transplant in an adult interscapular fat-pad. In addition to the foci of basophilic staining, large numbers of single cells could be observed located between

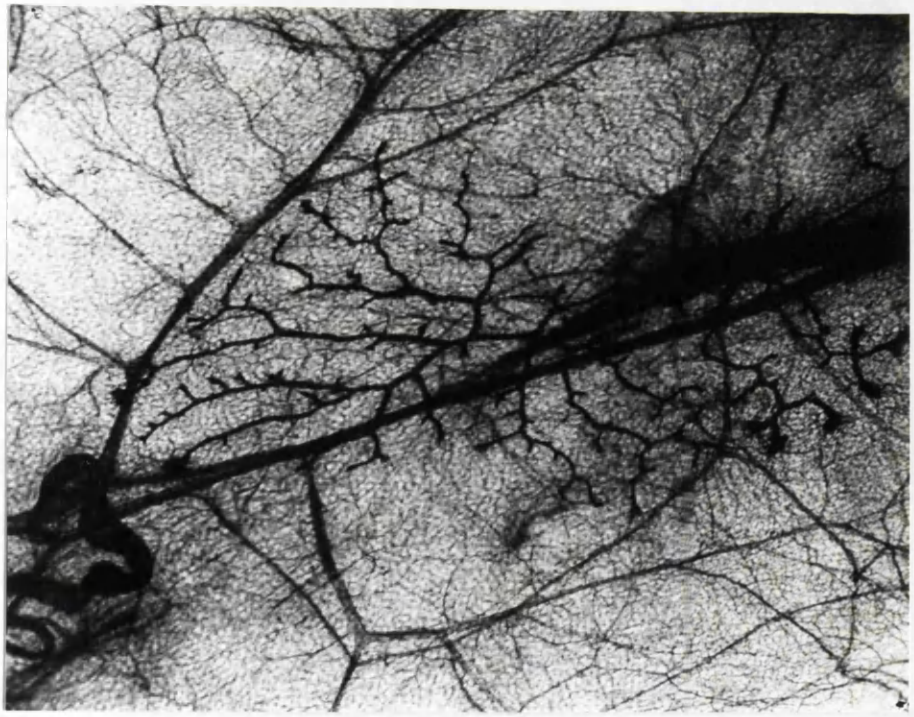
adjacent adipose cells and along the axis of blood vessels in the vicinity of the injection site. Several transplanted cultures, between passage numbers 3-8, reproduced these patterns of growth.

Unfortunately, due to the limited proliferative potential of luminal cells after sorting, and the practical difficulties associated with sorting large numbers of cells, transplantation of purified populations of luminal phenotypes could not be performed.

Figure 54 (Opposite). Photographs of wholemounted hematoxylin stained interscapular fat pad transplants.

Growths produced by (a) one dissected terminal end bud after 8 weeks, x16; (b) enzymatically prepared organoid fragments after 12 weeks. x30





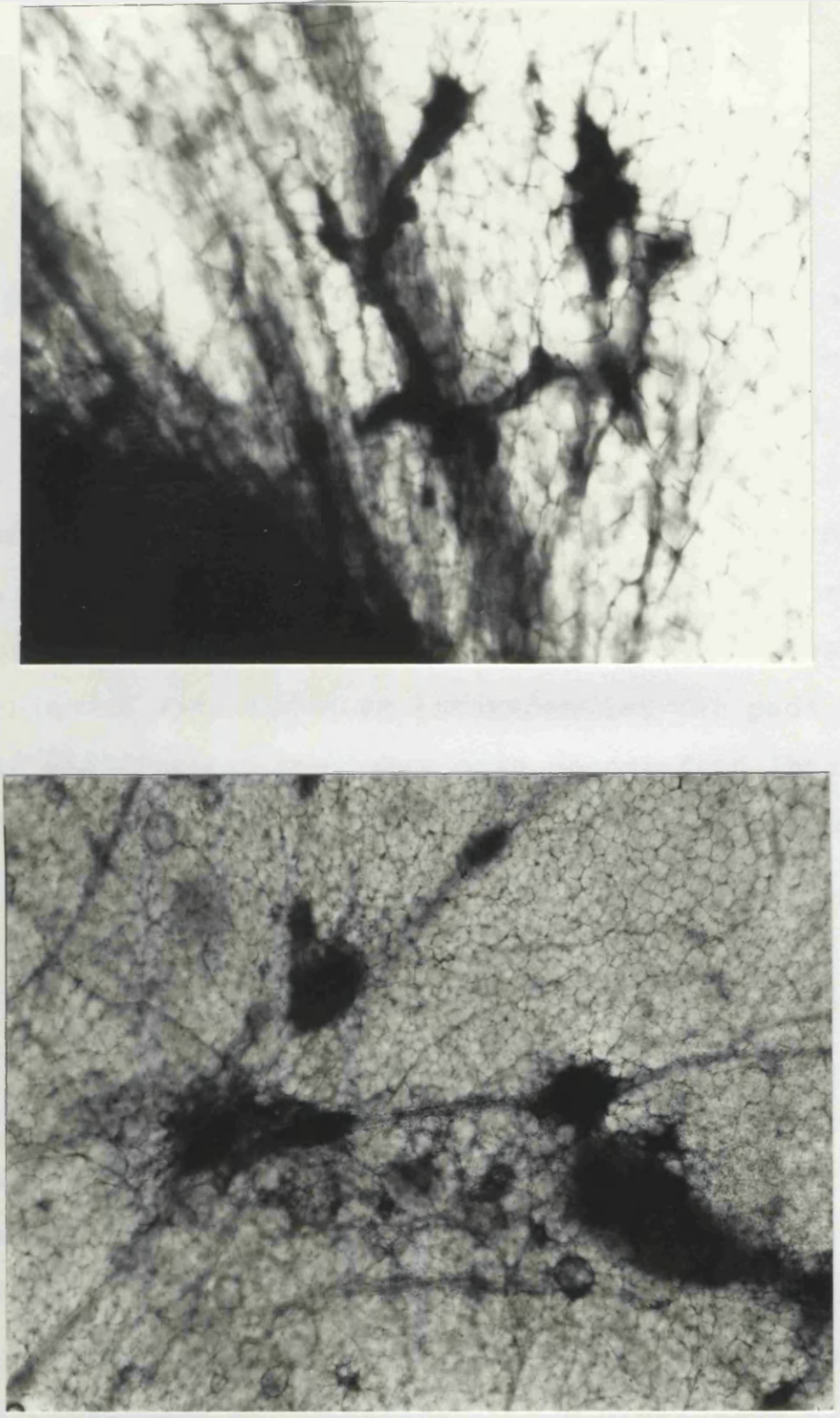


Figure 55. Photographs of hematoxylin stained interscapular fat pad transplants.

(a) Growth produced by a primary monolayer culture after 12 weeks, x70; (b) typical myoepithelial transplant after 3 weeks. x30

6.2 Transplantation of tsA58-U19 cell lines.

6.2.1 Introduction.

As a direct result of the extended proliferative potential of large T expressing cells, large numbers of both luminally and myoepithelially-derived cells could be grown and harvested. In order to assess the behaviour of tsA58-U19 expressing cells in an *in vivo* environment, both luminally and myoepithelially derived cloned and un-cloned lines of various passage numbers were transplanted into sygeneic interscapular fat pads of 70-day old females. They were also xenografted interscapularly and into the mammary fat pads of 5-week-old female nude mice.

6.2.2 Transplants and xenografts of tsA58-U19 cell lines.

11 separate transplantation and xenografting experiments were performed, each using 10 nude mice and 10 syngeneic adult and newborn rats. Animals were sacrificed between 4 days and 4 months after transplantation. Unlike the normal tissues and cells, no growth or morphogenesis was observed at the graft site with the tsA58-U19 derived cell lines. This result was obtained with all cell lines tested and at all transplant sites.

Syngeneic transplants: Established cells transplanted into syngeneic fat pads were apparently rejected by an immunological and/or inflammatory mechanism. Diffuse staining around the periphery of the nodule formed by injected cells indicated massive influx of lymphoid cells. Within 30 days the nodules of transplanted cells has disappeared, the rejection reaction subsided and fat pad structure appeared to return to normal. This result was obtained with transplants of both luminally-derived or myoepithelially-derived cell lines. Similar cells injected subcutaneously into newborn rats also failed to grow.

Nude mouse xenografts: Luminally- or myoepithelially-derived lines between passages 5 and 30 were xenografted into mammary fat pads and subcutaneously injected into dorsal neck regions of nude mice. After 5 days palpable nodules appeared on the flanks of animals and at the sites of injection of tsA58-U19-derived cells. Within 14 days however nodules regressed, and did not reappear in animals maintained for 12 weeks after injection. Histological examination after 21 days revealed that nodules were composed of a dense fibrotic capsule composed of host tissue, surrounding both host and xenografted cells. The graft was massively infiltrated by host lymphoid cells and necrosis of implanted cells was evident.

6.3 DISCUSSION.

Transplants and xenografts of tsA-U19 cells were apparently rejected immunologically. Although disappointing, these results were not entirely surprising. The literature concerning the tumourigenicity (that is, not the formation of transient nodules but of ongrowing tumours, Stiles et al., 1975) of SV40 expressing cells is conflicting. This is due, most probably, to differences in the behaviour of transformed primary cells and established cell lines, and differences between different established cell lines. The evidence strongly suggests that expression of SV40 large T by primary epithelial cells of both human and rodent origin does not confer the ability to grow in nude mice, Banks-Schlegel and Howley, 1983; Ke et al., 1988; Reddel et al., 1988; Garcia et al., 1986. Pre-established lines induced to express large T via retroviral transduction also fail to grow, Clark et al., 1988. While fibroblastic cells retrovirally infected with the tsA58-U19 construct do not grow in irradiated nude mice lacking natural killer cells, Jat et al., 1986, NIH 3T3 cells expressing the same construct do form tumours, Jat et al., 1986. SV40 infected hepatocytes are capable of forming tumours in irradiated, Isom et al., 1981, and newborn sygeneic rats, Woodworth et al., 1988. Interestingly, the latter authors determined that prolonged *in vitro* passage conferred tumourigenicity to cells

previously non-tumourigenic at low passage numbers, and that tumourigenicity increased steadily with time in culture. This behaviour may be similar to the tumourigenicity of pre-established late passage fibroblast cell lines. Thus, long term passage in culture may independently change the properties of SV40 large T-expressing cells.

The failure of cells expressing SV40 large T to grow or form tumours in nude mice and syngeneic animals, does not necessarily indicate an absence of tumourigenic potential but may be due to the expression of membrane-bound transplantation antigens, Choi et al., 1983. A large amount of biochemical and serological evidence indicates that SV40 large T with similar biochemical characteristics to nuclear protein, is found specifically orientated on the extra-cellular membrane of transformed cells, (reviewed by Butel, 1986; Tevethia and Butel, 1987). Approximately 1-2% of total cellular large T protein is detected in this location. Regions of the amino-terminal, Tevethia and Tevethia, 1984, and carboxy-terminal ends, Jay et al., 1978, of surface-bound T molecule correspond to the classically defined tumour-specific transplantation antigens and can independently induce transplantation rejection responses mediated by cytotoxic lymphocytes, (reviewed by Tevethia and Butel, 1987). Such cellular responses can be mounted by nude mice, Holub, 1989.

Although membrane-bound T antigen was not detected when live cells were stained with Pab419, the epitope to which this antibody binds (~amino acid 100, Gooding et al., 1984) does not correspond to regions responsible for the transplantation response (amino acids 189-211; 220-223 and 224-228, see Tevethia and Butel, 1987). Another variable is the body temperature of recipient mice and rats. While this is difficult to determine accurately at the transplantation site, it might not be high enough (>38^{o}C) to prevent expression of T as a transplantation antigen. Furthermore, the transplanted cells may not have been of sufficiently advanced culture age to be independently tumourigenic.

Whatever the reasons for their failure to grow as transplants, luminally- and myoepithelially-derived tsA58-U19 cells were unfortunately of little use in examining the growth and differentiation behaviour of populations of cells *in vivo*. By contrast, the results of the transplantation experiments involving normal parenchymal populations were of greater significance.

The studies of normal parenchyma presented in this chapter are novel in several respects - 1. Transplanted material consisted of fragments of glandular structures, the cellular composition of which was defined by

flow cytometric analysis and which were enzymatically stripped of basement membrane and stromal tissue, 2. the behaviour of phenotypically defined pre-cultured rat parenchyma transplants has been described, and 3. populations of cells of a defined phenotype originally isolated by flow sorting from intact gland were examined in fat pads.

The fact that dissected terminal end buds regenerated organised mammary trees confirms the report by Ormerod and Rudland, 1986. In addition, it is clear that enzymatically prepared organoids from adult rats are capable of considerable regenerative growth and that primary monolayer cultures initiated from dispersed organoids can form organised outgrowths in adult virgin fat pads. Interestingly, although pre-culture can induce abnormal growth patterns in mouse transplants, Daniel and DeOme, 1965, and a predisposition to the formation of hyperplastic alveolar nodules and tumours, DeOme et al., 1978; Ethier et al., 1984, no grossly atypical structures were observed in pre-cultured rat mammary trees during the propagation periods of this study. Thus, primary cultures retained normal phenotypes and were marked only by extremely slow growth and relative inefficiency at reconstituting normal architecture, a common abnormality in the mouse system, Daniel and DeOme, 1965; Miller and McInerney, 1988.

The remarkable "repopulation capacity" of transplanted parenchyma has been used to imply the existence of mammary stem cells, see for example, Medina et al., 1986 and Smith and Medina, 1988. However, the starting material for previously published transplant experiments have been portions of intact mammary tree or cultures initiated from whole glands, both consisting of variable mixtures of various cell types. This means that the results of such studies do not prove the existence of multipotent stem cells. Clearly, mitosis of pre-existing heterogeneous luminal and myoepithelial populations could conceivably re-generate glandular architecture and retain the capacity for differentiation. The results of transplantation of 'normal' cell lines *in vivo* have been at best, ambiguous. For example the murine line COMMA-1D, originally reported to exhibit normal morphogenesis *in vivo*, was not cloned, Danielson et al., 1984. Clonal lines derived therefrom showed a dramatic reduction in morphogenetic potential, Medina et al., 1986.

The behaviour of passaged myoepithelial cells in fat pads (that is their failure to form organised structures) has implications for organogenetic development *in vivo*, and can be interpreted in several ways -

1. Myoepithelial cells may possess the capacity to form

organised glandular structures, but that this capability is lost as a result of growth in culture prior to transplant. It is well documented that continued passage of mouse parenchyma in culture diminishes subsequent growth potential *in vivo*, Daniel and DeOme, 1965; Medina et al., 1986; Ehmann et al., 1987. However, it is unlikely that this phenomenon explains the observed *in vivo* growth patterns of sorted myoepithelial cells, since early and late passaged cultures behaved similarly. Furthermore, the characteristics of clonal growth cultures show that myoepithelial phenotypes are a self-perpetuating population and do not generate luminal types. Thus the *in vivo* results emphasise that mammary basal cells in the adult parenchyma are not a stem cell population, and therefore cannot reconstruct a normal mammary tree.

2. Self-perpetuating disparate luminal and myoepithelial phenotypes may be required to interact by "sorting out" in order to generate organised glandular structures. Sorting out mediated by cell adhesion molecules has been implicated in many diverse developmental systems, Friedlander et al., 1989,

3. Myoepithelial cells may be growth restricted in the fat pad environment and therefore intrinsically devoid of morphogenetic capability. Organogenesis would then be dependent on the *de novo* production of luminal and

myoepithelial cells by differentiating progeny of multipotent cells of luminal phenotype.

It can be stated that, on the basis of sorted myoepithelial cell behaviour *in vivo*, the formation of arborised glandular growths is dependent on the presence of luminal populations. This is consistent with hypotheses 2 or 3. The observed multipotence of luminal Type 4 clones, which are capable of generating other luminal cell types and myoepithelial phenotypes in culture, could generate the requisite cellular diversity if the differentiation patterns observed in culture are representative of *in situ* processes. This would be consistent with hypothesis number 3. There is evidence that clonal proliferation accounts for the formation of a proportion of alveoli in situ (at least in lactating mice), Thomas et al., 1988.

Experimental discrimination between the possible modes of mammary regeneration exhibited by different transplants is imperative to an understanding of differentiation pathways active during glandular development and growth. Such investigations are clearly feasible and hinge tantalisingly on the ability to manipulate large numbers of purified luminal populations. Methods of approaching this problem, and the potential of such a system will be discussed in the final chapter.

CHAPTER 7.

FINAL DISCUSSION AND PROSPECTS.

7.1 Discussion.

The major conclusions that can be drawn from the results presented in this thesis can be summarised as follows -

1. Luminal epithelial and myoepithelial cells derived from the mammary parenchyme of adult virgin female rats are capable of growing in culture independently of each other, whilst maintaining certain differentiated phenotypic characteristics of the corresponding cells *in situ*.

2. Both the luminal and myoepithelial components of rat mammary parenchyme are composed of heterogeneous clonogenic populations of cells which behave differently in culture, with six different clonal phenotypes identifiable in clonal growth cultures. Four types are derived from luminal cells, and two from myoepithelial cells.

3. A distinct population of cells exists in both duct and alveolar structures, which expresses a luminal-specific membrane marker, and which generates clones (Type 4 clones) possessing multipotent potential under the growth conditions described; both luminal and myoepithelial type clones are produced when clones of this type are re-cloned. These clones are different from any previously described cultured rat mammary cells.

4. Paracrine growth interactions exist between the

different components of rat mammary gland. Factors produced by luminal epithelial cells negatively regulate the growth of myoepithelial cells, while stromal cells stimulate the growth of the luminal epithelial cells and to a lesser extent myoepithelial cells.
5. Cells derived from the basal layer which are endopeptidase-24.11 positive, Gusterson et al., 1986 and cytokeratin 14 positive, cytokeratin 18 negative (this work, see Chapter 3) do not possess any multipotent potential in culture or after transplantation *in vivo*. They breed true and do not generate luminal cell types.
6. Established cell lines generated from sorted luminal epithelial and myoepithelial cells by retroviral transduction of the tsA58-U19 recombinant construct express markers characteristic of the corresponding cell *in situ* when maintained at the non-permissive temperature for large T activity.

A unifying hypothesis is proposed to relate the behaviour of clonal phenotypes characterised in culture in this work and previously published observations of the origins of differentiated phenotypes during parenchymal growth and renewal, Radnor, 1972; Dulbecco et al., 1982. This is that luminal and myoepithelial populations in the adult rat mammary gland have a common progenitor - the cell type which gives rise to Type 4 clones, and differentiated phenotypes are therefore

generated by diversification of cells along distinct lineage pathways. The cells which give rise to multipotent Type 4 clones may be related to those precursor cells located in the end buds of adult rats, Dulbecco et al., 1983; Allen et al., 1984, with migration into subtending ducts, Dulbecco et al., 1982; Daniel and Silberstein, 1987, responsible for their presence in this location. The proposal that the stem cells responsible for mammary development are located in the basal layer of ducts, Dulbecco et al., 1986, is not at all consistent with the results presented in this thesis.

7.2 Prospects.

The cells which give rise to multipotent Type 4 clones may arise from epithelial clear cells which have been previously described in the rat and murine mammary gland, and which have been postulated as having stem cell character, Radnor, 1972, and Smith and Medina, 1988, respectively. Cell sorting using an antibody selectively recognising rat mammary clear cells followed by transplantation of the sorted populations into fat pads *in situ* would prove whether or not these cells are indeed the stem cells of the rodent mammary parenchyma. Unfortunately, this definitive experiment is confronted by two major problems. Firstly, a specific anti-clear cell antibody has not been reported; and secondly, as discussed in Chapter 6, transplanting

sorted cells directly is not practicable. However, several other approaches to investigate the morphogenetic capability of luminal populations using the culture-transplant technique may be possible if large numbers of purified luminal epithelial cells could be obtained. Different ways of attempting this are discussed below.

As shown in Chapter 6, transplanted monolayer cultures composed of both luminal epithelial and myoepithelial cells can re-generate mammary trees. Selectively eliminating myoepithelial cells from such cultures by cytotoxic anti-endopeptidase-24.11 antibody-ricin conjugates may serve as a way of obtaining purified luminal populations. Selective killing of leukaemic cells bearing this antigen has been reported using such a technique, Raso and Lawrence, 1984. Alternatively, the selective growth of luminal cells may be accomplished by complement-mediated killing of myoepithelial cells utilising anti-endopeptidase antibodies. Selective complement-mediated lysis has been used successfully to kill contaminating fibroblasts in at least two different cell culture systems, Edwards et al., 1980, and Gusterson et al., 1981. This technique appears quite promising in theory given the large number of different commercially available monoclonal antibodies recognising the endopeptidase-24.11 antigen, since killing of

cells is potentiated if two or more antibodies recognising different epitopes on the same molecule bind complement, Howard et al., 1979.

Another approach would be to generate new cell lines from specific clonal populations, the growth of which may be tolerated in syngeneic transplants. Lines from primary clones could possibly be generated either by retroviral transduction of other genes capable of inducing continuous growth of primary cells, for example the gene coding mutant p53, Jenkins et al., 1984, which has been shown to establish rat chondrocytes; or the v-ras gene, which can establish primary murine mammary cells in culture, Redmond et al., 1988. This approach would enable the formation of transgenic mammary glands. Alternatively, neoplastic transformation of clonal growth cultures could be induced by chemical carcinogens, as has been achieved with heterogeneous cultures of rat, Richards and Nandi, 1978; Greiner et al., 1983; mouse, Guzman et al., 1987; and human mammary cells, Stampfer and Bartley, 1985. Such carcinogen transformed lines might not express strong transplantation antigens, and therefore such lines could be a practicable alternative to tsA58-U19 lines which are rejected in syngeneic transplants.
An advantage of continuous lines (or indeed passaged myoepithelial cell strains) is that they could be

further manipulated by retroviral insertion of the lacZ (β-galactosidase) 'reporter' gene. Histochemical detection of this marker gene would then facilitate analysis of lineage behaviour of defined clonal populations in fat pad transplants. This technique has been successively employed to follow clonal lineages in the embryonic chick brain, Price et al., 1987; Galileo et al., 1990, proving that neurons and glia arise from a common progenitor in the optic tectum, Galileo et al., 1990, and in determining the fibroblastic origin of rat perineurium, Bunge et al., 1989. This methodology could be used to determine whether lines derived from Type 4 clones generated mammary trees with normal histogenesis *in vivo*.

Lineage analysis would be complemented by studying the *in vivo* patterns of specific cytokeratin filament expression at different developmental stages, especially exploiting the double-labelling of cytokeratins 14 and 18 as illustrated in Chapter 3. Although Asch and Asch, 1985, reported that the qualitative expression of different cytokeratins does not alter significantly during the major stages of adult mammary gland development, that is resting, pregnant, lactating and involuting gland, their studies were performed using 2-D gel electrophoresis, a process which reveals no information regarding the actual cell type or pattern of expression

in situ.

The fact that four distinctive luminally-derived clonal phenotypes are seen in culture raises interesting questions as to the *in situ* function of their precursor cells. For example, do the different luminal clone types represent distinct stages in a sequential differentiation pathway, or does each different clone arise from cells which have completely different functions *in situ* and which traverse distinct differentiation pathways? Given the morphological and immunophenotypic profiles, it seems likely that Type 2 and Type 4 clones are related stages in the same pathway. It is probable that the precursors of clone Types 1 and 3 are more terminally differentiated, as indicated by their phenotypic stability on re-cloning and limited proliferative potential. One method of investigating the function of the cells giving rise to different luminal clones would be to examine at the clonal level which population (s) respond to lactogenic hormones or growth factors. Although functional differentiation of mammary cells grown on plastic is very limited, Emerman and Pitelka, 1977, it may be possible using the polymerase chain reaction to amplify different casein mRNA species (rat casein genes have been cloned and sequenced, Jones et al., 1985) in order to determine which clone types synthesise this molecule in response to hormone stimulation.

With respect to the two different primary myoepithelial clones, it would be interesting to examine factors which may regulate the differentiation of proliferative Type 6 clones, for example to determine whether the cells are influenced by either basic and acidic Fibroblast Growth Factor, which have both been shown to modulate myogenic differentiation in other systems, Clegg et al., 1987.

A potential use of the clonal growth system described in this thesis would be to identify more precisely the cell or cell types responsible for neoplastic growth *in situ*. This investigation could be complemented by the generation of different cell lines by chemical carcinogenesis, as discussed above. Possible differences between luminal and myoepithelial cells and between sub-populations within each cell type could be examined in this manner with respect to tumourigenic potential and pathology of derived tumours *in situ*. On the basis of morphological criteria, primary carcinogen-induced rat mammary adenocarcinomas exhibit various patterns of growth, Murad and von Haam, 1972; see also Young and Hallowes, 1973; Russo et al., 1989. The relative role of luminal epithelial and myoepithelial cells in such lesions remains contentious. Lesions may be broadly categorised as those in which epithelial and myoepithelial cells are admixed; those which are composed pre-

dominantly of epithelial cells; those composed predominantly of myoepithelial cells. The myoepithelial component of tumours may be normal cells in typical basal locations circumscribing epithelial tumours, or cells, usually identified on the basis of differentiated ultrastructure, occurring in 'mixed' tumours. Cells in the latter category may be a neoplastic component, or represent remnants of normal architecture passively disrupted by neoplastic proliferation. By contrast, hyperproliferation of differentiated myoepithelial cells leads to recognisable adenomyoepitheliomas, Rhem, 1990. However, recent evidence indicates that a proportion of infiltrating ductal carcinomas (pure epithelial proliferations) of the human mammary gland are composed of cells which strongly and homogeneously stain with different anti-cytokeratin 14 antibodies, Dairkee et al., 1987; Wetzels et al., 1989; Dairkee et al., 1988. Serial sections of some of these cytokeratin 14 expressing tumours (which were morphologically indistinguishable from the majority of other carcinomas, did not stain with antibody LE61 which recognises cytokeratin 18 (a luminal cell specific cytokeratin), Dairkee et al., 1988. The latter authors speculate that such tumours may arise from undifferentiated basally located precursor cells existing in the human mammary gland. Although infiltrating tumours with this phenotype have not been described in the rat system (no

study detailing the specific cytokeratin profiles of rat mammary tumours has been published), it is interesting to speculate that cells giving rise to Type 6 clones may form tumours resembling those described by Dairkee et al., 1988, and that the progenitors of clone Type 5 are involved in myoepithelial hyperplasias.

Another question to address is whether stem cells in the rat mammary gland are liable to tumourigenic conversion, and subsequently retain the capacity differentiate. If this is the case, then mosaic tumours composed of epithelial and myoepithelial phenotypes might be expected. On the basis of immunocytochemical staining, cells expressing markers characteristic of either luminal epithelial or myoepithelial cells can be detected in carcinogen-induced rat tumours around, although fully differentiated myoepithelial cells were not seen in such lesions, Ormerod et al., 1985. However, the histogenetic origin of these cells is debatable, Warburton and Gusterson, 1989. An intriguing example of the production of two differentiated phenotypes by a neoplasm is provided by the transplantable rat mammary tumour R3230AC. This tumour forms adenocarcinomas composed of well organised glandular structures with MFGM-positive columnar epithelial cells and basally-located myoepithelial cells which express smooth muscle α-actin. Both components are neoplastic,

Papotti et al., 1988. Interestingly, this dual-differentiation capacity is retained in culture, Papotti et al., 1988. However, neither this sub-line, Hilf et al., 1965, nor the original tumour, Hilf et al., 1964, were single-cell cloned. The transplantable tumour may therefore be composed of tumour elements derived from both the major parenchymal cell types. Clearly, it would be of interest to determine whether neoplastically transformed Type 4 clones generated tumours of this type.

Employing the culture-transplant technique to grow neoplastically transformed cell lines derived from pre-identified, phenotypically defined clone types would be ideally suited to investigating whether different subclasses of cells give rise to different types of mammary tumour. Such an approach would lend itself to clarifying the involvement of luminal and myoepithelial cells in the histogenesis of rat mammary tumours. In addition, the influence of the Epithelial-Mesenchymal-Transition phenomenon in the generation of tumour spindle-cell elements, (like those of the formed by the 'RAMA' series of lines *in situ*, Rudland et al., 1982; Dunnington et al., 1983; Dunnington et al., 1984; Warburton et al., 1987), and in tumour progression could be assessed by comparing the behaviour of lines displaying this behaviour with those which do not when

transplanted *in vivo*.

The potential of the novel tsA58-U19 cell lines generated from sorted luminal and myoepithelial cells, (see Chapter 5) to answer some of the questions raised above makes it important to develop a technique that enables them to be grown *in vivo*. This could be achieved by 'immunising' animals to large T by repeated injections of large T expressing cell suspensions, Choi et al., 1983, or by using immunosuppressed animals. Culturing these cell lines in a more physiological environment may be accomplished by using fat pad organ cultures as transplant sites, as has been done for human organoids, Stewart et al., 1987. Interestingly, the expression of SV40 large T and/or small t under the control of the MMTV LTR promoter in transgenic mice fails to induce mammary tumours in resting glands, even although the ductal luminal cells express high levels of the transgenes (ductal epithelial cells of lung and kidney do form tumours in the same animals), Choi et al., 1988. The tsA58-U19 lines are therefore ideal tools with which to analyse the apparent resistance to SV40-induced tumourigenesis of mammary cells. In addition, comparing the properties of tsA58-U19 expressing cells with non-established counterparts also provides a system with which to examine at the molecular level the influence of SV40 large T activity on the expression of various differentiation-specific markers.

In conclusion, studying sub-populations of the rat mammary parenchyme at the clonal level provides a means of elucidating the origins of the separate luminal and myoepithelial lineages in the mammary gland; of identifying factors which regulate the growth of specific populations, and for defining more precisely the cell type (s) involved in tumourigenesis.

APENDIX I: ABBREVIATIONS USED.

BrdUrd	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin.
CT	Cholera toxin.
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethylsulphoxide.
EGF	Epidermal growth factor.
EMA	Epithelial membrane antigen.
F	Fungizone.
FCS	Heat inactivated fetal calf serum.
FITC	Fluorescein isothiocyate.
F12	Ham's F12 medium.
GMAG	ICR. Genetic manipulation guidlines.
Gy	Gray (SI unit of absorbed dose of ionising radiation). 1Gy = 100rem.
G418	Geneticin.
HC	Hydrocortisone.
I	Insulin.
K	Kanamycin Acid Sulphate.
LTR	Long terminal repeats.
L15	Leibovitz L15 medium.
M	Minocyclin.
MFGM	Milk fat globule membrane.
MMTV	Mouse mammary tumour virus.
NBD	7-nitrobenz-2-oxa-1,3-diazole.
NP40	Nonidet-P40.
PBS	Phosphate buffered saline (pH 7.2).

PCR	Polymerase chain reaction.
PS	Penicillin and Streptomycin.
PROG	Progesterone.
PI	Propidium iodide.
TRITC	Tetramethylrhodamine isothiocyanate.
R-PE	Phycoerythrin.

APPENDIX II: REAGENT APPENDIX.

Culture media:

Biorich2 Serum-free Medium	Flow Laboratories Irvine, Scotland.
Dulbeccos' Modified Eagles' medium	Gibco Ltd. Paisley, Scotland.
Hams F12 medium	Gibco Ltd,
Leibovitz (L15) medium	Flow Laboratories.

Medium additives:

Cholera Toxin	Sigma Chemical Company, Poole, Dorset, England.
Epidermal Growth Factor (Mouse submaxillary gland)	Sigma Chemical Company.
Fetal Calf Serum	Gibco Laboratories or Imperial Laboratories.
Fungizone	E. R. Squibb, Wirral, Cheshire.
Geneticin (G-418)	Gibco Ltd.
Hydrocortisone	Sigma Chemical Company.
Insulin	Sigma Chemical Company.
Kanamycin	E. R. Squibb.

L-Glutamine concentrate	Gibco Ltd.
Minocyclin	Lederle, Gosport, Hants.
Penicillin and Streptomycin stock solution.	Gibco Ltd..
Progesterone	Sigma Chemical Company,
Pyruvate	Gibco Ltd.
Sodium Bicarbonate stock solution.	Gibco Ltd.

Enzymes:

Deoxyribonuclease I	Sigma Chemical Company.
Collagenase Type IA	Sigma Chemical Company.
Pepsin	Sigma Chemical Company,
Ribonuclease A	Sigma Chemical Company,
Trypsin Type III	Sigma Chemical Company,

Miscellaneous.

Autoclips- 9mm wound clips	Clay Adams, c/o Beckton Dickenson and Company.
BrdUrd	Sigma Chemical Company.
Bisbenzimide (Hoechst 33258)	Sigma Chemical Company.
Cell Scrapers	Costar Corporation Cam. MA., USA.
Citifluor AF-1 (TRITC) or AF-2(FITC)	Citifluor Ltd, Connaught Building London University.
Cryomatrix	Shandon Southern Products Ltd.
Culture flasks	Nunc. Roskilde, Denmark.
Coverslips	Chance Propper Ltd, Smethwick, Warley, England.
DMSO	Sigma Chemical Company.

FlowporeD filters	Flow Laboratories.
Glutaraldehyde Solution	BDH Chemicals Ltd, Poole, England.
High vacuum grease	Dow-Corning.
Hydromount	National Diagnostics, New Jersey.
Kodak Unifix T.M.	Kodak Ltd. Hemel Hempstead, UK.
Methyl Salicylate	Sigma Chemical Company.
Nonidet P-40	Sigma Chemical Company.
Nylon mesh	H. Simon, Stockport, UK.
Ortho Technical Pan Film 6.5x9cm	Ilford Ltd Mobberly, Cheshire.
Phalloidin	Sigma Chemical Company.
Phalloidin-FITC	Sigma Chemical Company.
Polybrene	Aldrich Chemical Co. Milwaukee, USA.
Propidium iodide	Sigma Chemical Company.
PQ Universal paper developer	Ilford Ltd Mibberly, Cheshire.
Sodium Hydroxide	BDH Chemicals Ltd.
Thermanox coverslips	Lux Scientific Corporation, California, USA.
Tissue chopper	Mickle Laboratory Engineering Co.
Trypan Blue	BDH Chemicals Ltd.
'Universal' containers	Sterilin Ltd. Hounslow, UK.
Versene solution	Imperial Laboratories.
Halothane	Veterinary Drug Chemical Co., Colnbrook, Slough.

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